Factors affecting the response to tyrosine kinase inhibitors in chronic myeloid leukaemia

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By

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Abstract

Chronic myeloid leukaemia (CML) is a clonal stem cell disorder characterised by the Philadelphia chromosome. The treatment and outcomes of CML patients have improved with the introduction of tyrosine kinase inhibitors (TKI). Imatinib is associated with complete cytogenetic response (CCR) rate of 71% at 12 months, as documented by large phase 3 clinical trials. I carried out a large population study in the Merseyside, Cheshire and North Wales area, which showed a maximal CCR rate of 65% over 5 years of observation. This suggests there is a higher rate of imatinib failure in a general unselected CML population compared to large clinical studies which have strict exclusion criteria. My population study also confirmed that second generation TKIs can produce high CCR rates in imatinib intolerant/resistant patients.

There are a number of mechanisms of imatinib resistance. The hOCT1 transporter has been shown to be an important predictor of response to imatinib treatment. However, it has been suggested there are other drug transporters involved in imatinib transport which may have prognostic significance. This thesis examined the role of SLCO1A2, OCTN1 and OCTN2 in the transport of TKIs. Transfected cell lines expressing high levels of the respective drug transporter were made using the AMAXA nucleofection process. The cell lines with the highest gene expression, as quantified by TaqMan PCR, were then selected and used in radioactive uptake experiments. Imatinib was confirmed to be a substrate for SLCO1A2. However, the mRNA expressions levels of SLCO1A2 did not have any prognostic correlation to outcome. A review of patient co-medication also showed inhibitors of SLCO1A2 had no effect on CCR and major molecular response (MMR) rates in imatinib treated patients. OCTN1 and OCTN2 did not
transport imatinib. Nilotinib and dasatinib are not substrates for SLCO1A2, OCTN1 or OCTN2.

Drug drug interactions have also been implicated in drug resistance. Imatinib and metformin are actively transported by hOCT1. It was postulated that varying concentrations of metformin could potentially affect the uptake of imatinib by competitive inhibition. A metformin concentration of 768µM was required reduce imatinib uptake by 50%. However, this concentration is much higher than therapeutic metformin levels, therefore these drugs do not interact at therapeutic concentrations.

This thesis shows imatinib is an effective treatment in CML but the CCR rate is lower than in published phase 3 trials. SLCO1A2 transports imatinib but RNA levels have no prognostic significance. Imatinib and metformin do not interact at normal therapeutic concentrations.
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<td>ABC</td>
<td>ATP Binding Cassette</td>
</tr>
<tr>
<td>AP</td>
<td>Accelerated Phase</td>
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<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BC</td>
<td>Blast Crisis</td>
</tr>
<tr>
<td>BCR-ABL</td>
<td>Breakpoint Cluster Region - Abelson Fusion Protein</td>
</tr>
<tr>
<td>CCR</td>
<td>Complete Cytogenetic Response</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic Myeloid Leukaemia</td>
</tr>
<tr>
<td>CP</td>
<td>Chronic Phase</td>
</tr>
<tr>
<td>E-3-S</td>
<td>Estrone-3-sulphate</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>hOCT1</td>
<td>Human Organic Cation Transporter 1</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus Kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
</tr>
<tr>
<td>MMR</td>
<td>Major Molecular Response</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>OATP</td>
<td>Organic Anion Transporter Protein</td>
</tr>
<tr>
<td>OCTN</td>
<td>Organic Cation/Carnitine Transporter</td>
</tr>
<tr>
<td>OS</td>
<td>Overall Survival</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PFS</td>
<td>Progression Free Survival</td>
</tr>
<tr>
<td>Pgp</td>
<td>P-Glycoprotein (ABCB1)</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-Kinase</td>
</tr>
<tr>
<td>SLC</td>
<td>Solute Carrier</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>STAT</td>
<td>SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>TEA</td>
<td>TETRAETHYLAMMONIUM</td>
</tr>
<tr>
<td>TKI</td>
<td>TYROSINE KINASE INHIBITOR</td>
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1. Introduction

1.1 Chronic Myeloid Leukaemia (CML)

1.1.1 Historical

Chronic myeloid leukaemia (CML) is a rare clonal disorder of haematopoietic stem cells. Numerous descriptions of this disease have been documented in the past. In 1825, Velpeau described the autopsy findings of a 63 year old lady with massive liver and spleen and blood resembling pus. Donne recorded, in 1839, a 44 year old lady whose death was associated with features of splenomegaly and blood containing predominately “white globules” (Donne, 1844).

In 1844, David Craigie also noted two patients who had splenic enlargement and an unusual blood consistency. John Hughes Bennett investigated these two patients further and published his finding of an enlarged spleen weighing 124 ounces, lymphadenopathy and pus like material from the veins (Bennett, 1845). Bennett assumed the cause for the enlarged spleen and blood appearance was due to an infective process. However, the same year Rudolf Virchow was referred a patient with tiredness and lethargy, nosebleeds and swollen abdomen and legs. An autopsy showed an enlarged spleen and blood containing pus material. On microscopic examination of the blood, he noted a reversal of the normal red cell to white cell ratio, with cells having nuclear variation (Virchow, 1845). He suggested the disorder be named “leukaemie”. Virchow proposed the cause of the disease was associated with the tissue primarily responsible for producing white cells and not an infective aetiology (Virchow, 1856).
Staining methods for peripheral blood cells were established by Paul Ehrlich in 1879. This allowed the characteristic finding of numerous polymorphonuclear and mononuclear containing cells to be identified on the blood film (Ehrlich, 1891). In the early twentieth century, peroxidase stains revealed the predominance of neutrophils with smaller proportions of myelocytes and basophils, and thrombocytosis (Stillman, 1912).

There had been speculation, in the mid twentieth century, cytogenetic abnormalities were associated with leukaemia and cancer in general. In 1960, Nowell and Hungerford discovered an acrocentric chromosome in CML patients. This chromosomal abnormality was termed the Philadelphia chromosome (Nowell and Hungerford, 1960). This finding was confirmed by other groups, and noted to be present in erythroid and megakaryocytic precursors. It was later shown there was a translocation between the long arm of chromosome 22 and chromosome 9, resulting in a 9q+ chromosome (Rowley, 1973). Later studies showed the ABL proto-oncogene located on chromosome 9 is translocated to chromosome 22. Further studies showed the new fusion protein (BCR-ABL1) behaved as a cytoplasmic tyrosine kinase with autonomous phosphorylating properties (Sawyers, 1999).

The identification of the Philadelphia chromosome and its association with CML has improved the understanding of the pathobiology of CML. It proved CML was a clonal disorder and helped differentiate it from other myeloproliferative disorders.
1.1.2 Epidemiology of CML

The epidemiology of CML has been studied by using sources such as the Swedish cancer registries (Swedish cancer registry, 1998-2006), mortality statistics (Parkin et al, 2006) and Surveillance, Epidemiology and End Results (SEER) Program of the United States (Ries et al, 2005). However, there is marked variability in CML incidence rates ranging between 0.6 – 2.0 per 100000 people (Swedish cancer registry, Scotland leukaemia registry and cancer registry of Saarland). The differences in incidence rates can be explained by differences in the way CML cases are recorded, as per the 2nd Edition of International Classification of Diseases for Oncology (ICD-O) (WHO Geneva 1990), which included chronic myelomonocytic leukaemia and atypical (Philadelphia chromosome and BCR-ABL1 negative) CML. This may thus lead to an overestimation of CML incidence rates. Variation in CML incidence may also be influenced by ethnicity. This is of great relevance as reports show there is a difference in CML incidence between Northern and Southern European populations. There was an incidence of 1.34 per 100000/yr in Italy compared to only 0.69 per 100000/yr in Poland (Hoffman et al, 2015). The Scotland Leukaemia registry (Harrison et al, 2004) recorded a CML incidence of 0.6 per 100000 people, though the data need to be interpreted with caution as only 18 out of the 26 haematology units responded to the survey. A European cancer registry (HAEMACARE working group) initiative funded by the European commission recorded an incidence of 1.16 per 100000 in Italy (Sant M et al, 2010). Large scale population studies are required to determine variations in incidence in the world population.
The average age at diagnosis of CML ranges between 56 to 66.5 years old. The disease incidence increases with age with the highest incidence in patients greater than 85 years old (Hoffmann et al, 2015). There is an increased incidence of males compared to females, with a male: female ratio ranging between 1.3 – 1.8 (phekoo et al, 2006; Mcnally et al, 1997; Rohrbacher et al, 2009). However, very few of these studies are truly population studies, where a major effort has been made to identify all cases in a given geographical area. Furthermore, some of these studies predate the introduction of tyrosine kinase inhibitors (TKIs).

### 1.1.3 Pathogenesis of CML

The *ABL* gene codes for a non receptor kinase. It has 2 isoforms from alternative splicing of the first exon (Laneuville P, 1995). The protein has 3 SRC homology domains (SH1-3), with SH1 carrying the tyrosine kinase function SH2 and SH3 interacting with other proteins (Cohen et al, 1995). In normal physiology, ABL is thought to be involved in cell cycle regulation (Kipreos et al, 1990 and Sawyers et al, 1994), response to DNA damage (Yuan et al, 1999) and relaying of cellular environment status via integrin signalling (Lewis and Schwatz 1998).

The BCR protein has several motifs including the dimerisation domain and two cyclic adenosine monophosphate kinase domains at the N terminal end. The kinase domains can be autophosphorylated and can bind Grb-2, which has a role in the Ras pathway (Reuther et al, 1994 and Wu et al, 1998 and Ma et al, 1997). The centre of the BCR protein is named the RHO-GEF region, and augments the exchange of guanidine triphosphate (GTP) for guanidine diphosphate (GDP) on Rho guanidine factors (Denhardt et al, 1996) at the C-terminus, the domain for Rac-
GTPase. This is involved in NADPH oxidase activity and actin polymerisation (Diekkmann et al, 1995). It has been suggested that BCR is involved in signal transduction, though this is still disputed.

The Philadelphia chromosome is the result of a t(9;22) reciprocal translocation. The ABL gene is located at 9q34, and breakpoints can occur in a large area (> 300 kb) at the 5’ end, most frequently between exon 1a and 1b, although studies have shown breakpoints occurring upstream and downstream of exon 1b and exon 1a respectively (Melo, 1994). However, after the hybrid transcript has been spliced and introns removed, regardless of the breakpoints, the resulting mRNA has BCR sequences fused to ABL exon a2. The breakpoints in the BCR gene occur in three defined areas termed breakpoint cluster regions (bcr). In the majority of patients with CML, the breakpoint occurs between BCR exon 12-16 (previously referred to as b1-5), known as the major breakpoint cluster (M-bcr). Due to alternative splicing, with removal of certain exons, b2a2 or b3a2 fusion transcripts are produced leading to a 210-kd chimeric protein (Melo et al, 1994). In a minority of patients with CML, the breakpoint can occur in the minor breakpoint cluster between exon e2’ and 2, resulting in e1a2 mRNA. This is translated into a 190kd protein. The third breakpoint, termed the μ-bcr, gives rise to a 230kd protein associated with chronic neutrophilic leukaemia (Pane et al, 1996).

1.1.4 Effects of BCR-ABL fusion protein

The ABL tyrosine kinase is under strict physiological regulation and the SH3 domain appears to have an inhibitory effect on ABL and prevent unregulated kinase activity. Deletions or positional alterations of SH3 have been associated with kinase activity...
(Van Etten et al, 1989 and Mayer BJ et al, 1994). Other proteins noted to interact with SH3 and inhibit the kinase activity of Abl are Abi-1 and Abi-2 (Abl interactor proteins 1 and 2). When ABL proteins are activated, they degrade Abi-1 and Abi-2 via a proteosome complex (Dai et al, 1998). Another possible inhibitor of ABL is Pag/Msp23; and when cells are under oxidative stress or exposed to ionising radiation Pag/Msp23 dissociates from ABL, leading to unopposed kinase activity (Wen et al, 1997).

BCR-ABL1 tyrosine phosphorylates a large number of substrates. There are an increased number of phosphotyrosine residues on the BCR-ABL1 protein, thus promoting autophosphorylation and allowing other proteins to bind via their SH2 domains. The substrates of BCR-ABL1 include adaptor proteins, such as Crkl, and cytoskeletal proteins such as talin and paxillin (Carpino et al, 1997 and Skorski et al, 1995). In normal physiology, tyrosine phosphatases regulate tyrosine kinases by reducing the amount of cellular phosphotyrosine available. SYP (Tauchi et al, 1994) and PTP1B (LaMontagne et al, 1998), are two tyrosine phosphatases, which can complex with BCR-ABL1 and cause dephosphorylation. Studies have shown increased expression of PTP1B can impair the function of BCR-ABL1 and can promote normal haematopoiesis (LaMontagne et al, 1998).

1.1.5 Signalling pathways involved in BCR-ABL positive cells

BCR-ABL promotes malignant transformation by 3 different mechanisms:
- Decreased adhesion to bone marrow stromal cells
- Activation of signalling pathways
- Reduced apoptosis.
1.1.6 Altered adhesion properties

CML cells have reduced adhesion to bone marrow stromal cells, and this allows them to proliferate and expand. Adhesion to stromal cells normally inhibits rapid proliferation (Gordon et al, 1987); this process is governed by β integrins which relay information between stromal cells and progenitor cells. Studies have identified a variant β integrin (β1B integrin) found in CML cells impairs adhesion. Alpha5β1 integrin is expressed on CML progenitors, and the addition of an activating antibody 8A2 led to increased affinity to fibronectin and a poor proliferative potential (Lundell et al, 1996). Integrins can also allow signal transduction from outside to inside the cell (Lewis et al, 1996). Crkl, a tyrosine phosphate protein, is found in high concentrations in CML neutrophils and is involved in cellular motility (Uemura et al, 1999) and also involved in integrin mediated cell adhesion (Sattler et al, 1996) via focal adhesion proteins such as paxillin and Hefl (Sattler et al, 1997). Hence there are multiple mechanisms whereby CML progenitor cells can migrate from the bone marrow microenvironment.

1.1.7 Activation of signalling pathways

1.1.7.1 Ras and MAP kinase pathways

There are several potential pathways linking BCR-ABL and Ras. Grb-2 is an adapter molecule which binds to the tyrosine 177 site on BCR-ABL near the N terminus (Pendergast et al, 1993). Grb-2 can then bind to Sos protein. The resultant complex can stabilise Ras in its active GTP bound form. Shc and crkl can also
activate Ras, by binding to the SH2 and SH3 domains on the BCR-ABL1 protein (Oda et al, 1994 and Pelicci G et al, 1995).

The mitogen activated protein kinase pathways downstream of Ras are still a controversial area in CML. IL-3 stimulation leads to activation of Ras, which in turn leads to Raf recruitment, a serine-threonine kinase, to the cell surface (Marais et al, 1995). RAF can then start the signal cascade involving MEK1/MEK2 and ERK, finally leading to gene transcription (Cahill et al, 1996). BCR-ABL1 can also activate the JNK/SAPK pathway and this has been shown to be important in malignant transformation (Raitano et al, 1995). Therefore, Ras may potentially activate Rac (Skorski et al, 1998), a GTP-GDP exchange factor, and Gckr (germinal centre kinase pathway) and then finally Jnk/Sapk (Shi et al, 1999).

However, other studies have shown N-ras mutations are rare in both blast and chronic phase of CML. This therefore suggests progression to blastic phase of CML is not governed by N-ras activation. This is in contrast to the high incidence of N-ras mutations in acute myeloid leukaemia (Watzinger et al, 1994).

**1.1.7.2 Jak-Stat pathway**

BCR-ABL1 has been shown to activate the Jak-Stat pathway directly, without prior tyrosine phosphorylation of Janus kinase proteins. In murine B lymphocytes transformed with the Abelson murine leukaemia virus, Janus kinase 1 and Janus kinase 3 have constitutive tyrosine kinase activity, and STAT proteins were tyrosine phosphorylated without prior activation from IL-4 and IL-7 (Danial et al, 1995). Autophosphorylation of STAT1 and STAT5 has been demonstrated in primary CML cells (Chai et al, 1997), and STAT5 activation is a contributing factor to malignant
transformation (de Groot et al, 1999). STAT5 appears to have an anti-apoptotic effect on BCR-ABL positive cells, and this may be due to transcriptional activation of Bcl-xL (Horita et al, 2000 and Sillaber et al, 2000).

The activation of the Ras and Jak/Stat pathways can lead to BCR-ABL1 cell lines becoming independent of growth factor reliance (Kabarowski et al, 1994 and Daley et al, 1988). This explains why BCR-ABL1 cell lines may be proliferative with minimal external stimulation. BCR-ABL1 cell lines have also been shown to proliferate by using an autocrine loop, whereby the fusion protein stimulates autocrine secretion of IL-3 and G-CSF (Sirard et al, 1994).

1.1.7.3 PI3 kinase pathway

BCR-ABL1 positive cells require PI3 kinase activity to promote proliferation (Skorski et al, 1996). BCR-ABL1 forms complexes with Cbl, Crk and Crkl which are then able to activate the PI3 kinase (Sattler et al, 1996). Activated Cbl can then activate Akt (Skorski et al, 1997) which is an important anti-apoptotic signalling protein which can confer survival advantage to a malignant clone (Franke et al, 1997). Bad is a pro-apoptotic protein and a known substrate for Akt (del Peso et al, 1992); however when Bad is phosphorylated it becomes inactive. When Bad is inactive, it is unable to bind to Bcl-xL and other anti-apoptotic proteins and is localised to the cytoplasm by cytoplasmic 14-3-3 proteins. This may point to BCR-ABL1 mimicking IL-3 activation and inhibiting apoptosis.
1.1.7.4 Myc pathway

High expression of Myc, a known transcription factor, is a feature of many human malignancies. The SH2 domain in BCR-ABL is important in Myc activation. It has been shown that dominant negative mutations of c-Myc, such as D106-143, can inhibit transformation by BCR-ABL (Sawyers et al, 1992). It is thought signal transduction from BCR-ABL to Myc occurs via Ras/Raf activation followed by activation of cyclin-dependent kinases and E2F transcription factors (Zou et al, 1997). This has been confirmed in murine myeloid cells (Stewart et al, 1995). However, these interactions have not been universally accepted with different groups unable to show their function in human models.

1.1.8 Inhibition of apoptosis

BCR-ABL can prevent apoptosis in human cell lines even after growth factor withdrawal (Sirard et al, 1994). This is dependent on tyrosine kinase activity and associated Ras activation (Puil et al, 1994 and Cortez et al, 1995). Even after DNA damage BCR-ABL cell lines are resistant to apoptosis (Bedi et al, 1994 and Bedi et al, 1995). It has been shown that BCR-ABL positive cell lines impair cytochrome c release into the cytoplasm and also prevent the activation of caspase pathways compared to BCR-ABL negative cell lines after etoposide induced DNA damage (Dubrez et al, 1998 and Amarante-mendes et al, 1998). The activation Bcl-2, by BCR-ABL cells is thought to be be mediated by Ras signalling (Sanchez Garcia et al, 1997).
As described earlier, the activation of Akt and Raf1 by BCR-ABL, and the resulting phosphorylation of Bad, can also lead to an anti-apoptotic signal (Wang et al, 1996 and Zha et al, 1996). BCR-ABL can also down regulate interferon consensus sequence binding protein (ICSBP) (Gabriele et al, 1999 and Hao et al, 2000). This is of great significance because ICSBP knockout mice exhibit the signs and symptoms of a myeloproliferative disorder (Holtschke et al, 1996). Interestingly, ICSBP -/- mice progenitor cells showed reduced cell adhesion to the bone marrow microenvironment, long-term reconstitutive capability and increased myeloid expansion (Scheller et al, 1999). This could well explain the mechanism of action of interferon alpha.

1.1.9 Clinical features

The natural history of CML is characterised by three different phases, chronic (CP), accelerated (AP) and blastic phase (BP). The initial phase of CML can be used to predict complete cytogenetic response and overall survival. Patients in CP, AP and BP achieved complete cytogenetic response, following imatinib treatment, in 77%, 39% and 11% cases respectively, with a follow up time of 48 months. The 3 year overall survival for the patients divided into CP, AP and blast phases were 95%, 63% and 11% respectively (Cortes et al, 2006).

In the chronic phase of CML the leukaemic cells are mainly found in blood, bone marrow and spleen. There can be infiltration of the liver, though this is not common. However, in the blastic phase of CML leukaemic cells can infiltrate the lymph nodes, soft tissues and skin (Cotta et al, 2007 and Jacknow et al, 1985 and Muehleck et al, 1984).
Many patients (20-50%) are asymptomatic at presentation, and the diagnosis comes to light following a routine full blood count showing an elevated white cell count with the differential mainly consisting of neutrophils, eosinophils and basophils (Cotta et al, 2007 and Garcia-Manero et al, 2003 and Savage et al, 1997). Fatigue, night sweats, weight loss, anaemia and splenomegaly are also frequently noted. Some patients can present with isolated thrombocytosis, or present in blast crisis with no recognisable chronic phase (Anastasi et al, 1996 and Cotta et al, 2007 Peterson et al, 1976). With no treatment or treatment failure patients will progress from chronic phase to accelerated phase and then blast crisis (Garcia-Manero et al, 2003).

1.1.10 Chronic phase

The white cell count varies between 12-1000x10^9/L, with the differential mainly comprising mature neutrophils, metamyelocytes and myelocytes (Cotta et al, 2007 and Garcia-Manero et al, 2003 and Spiers et al, 1977). Eosinophilia and basophilia are also present. Monocytosis can also be present but is normally less than 3%, unless the p190 BCR-ABL isoform is present (Melo et al, 1994). The bone marrow aspirate is hypercellular with myeloid series expansion showing all forms of myeloid maturation. The blast count is <5% (Muehleck et al, 1984). The trephine biopsy shows hypercellular marrow with the paratrabecular areas composed of immature granulopoiesis 5-10 cells thick (Cortes et al, 2006).
1.1.11 Accelerated phase

The World Health Organisation classifies accelerated phase as any of the following:

1. Persistent or increasing white cell count (>10x10^9/L) and increasing splenomegaly unresponsive to treatment
2. Persistent thrombocytosis >1000x10^9/L uncontrolled by therapy
3. Persistent thrombocytopenia unrelated to therapy
4. Clonal cytogenetic evolution developing after diagnostic karyotype
5. >20% basophils in peripheral blood
6. 10-19% blasts in blood or bone marrow

[3rd Edition of the WHO Classification]

1.1.12 Blast phase

Blast crisis is diagnosed when blasts are >20% in the peripheral blood or marrow or when there is extramedullary proliferation. Most cases of blast crisis are myeloid in origin, while 20-30% of cases are lymphoid (Derderian et al, 1993 and Saikia et al, 1988).

[3rd edition of WHO Classification]
1.1.13 Risks stratification scores

All patients diagnosed with CML can be risk stratified. The Sokal score is a widely used scoring system for CML based on patients predominately treated with busulphan single agent chemotherapy. It is based on age, spleen size, platelet count and peripheral blood (PB) blast percentage (Sokal et al, 1984). The Sokal score has been shown to be important in the imatinib era, with high risk CML patients achieving only 38% complete cytogenetic response (CCR; defined as no evidence of the Philadelphia chromosome among at least 20 metaphases) at 12 months, while low and intermediate Sokal score patients achieve CCR rates of 76% (Forrest et al, 2009). Recently, the EUTOS score has been used to stratify CML patients. This uses the spleen size and PB basophil count. The EUTOS score separates patients into high and low risk groups. Patients with high EUTOS score were noted to have a higher rate of CCR, improved progression free survival (PFS) and overall survival (OS) (Hasford et al, 2011). However the reliability of the EUTOS score to predict outcome has been disputed (Jabbour et al, 2012).
1.1.14 Treatment options

1.1.14.1 Interferon

In 1986, it was noted that interferon alpha could induce haematological remission and reduce spleen size in the majority of patients. Thirty-five percent of patients showed no evidence of the Philadelphia chromosome (CCR) after 12-18 months on at least one occasion although very few patient maintained this response. This minority group achieving CCR on interferon had an improved overall survival (Talpaz et al, 1986). However, in real life practice interferon has not been shown to be as efficacious. In a similar trial interferon was shown to be superior to hydroxycarbamide and busulphan, with a higher rate of minor cytogenetic response (defined as >33% of metaphases negative for Philadelphia chromosome), increased time from CP to AP/Blast crisis and improved overall survival (The Italian Cooperative Study Group on Chronic Myeloid Leukemia, 1994). Subsequent clinical trials compared interferon and cytarabine against single agent interferon and showed CCR rates of 15% and 9% respectively. The survival rates at 3 years were 85.7 % for the interferon and cytarabine group and 79.1% in the interferon alone group. The improved rates of CCR and overall survival were both statistically significantly better in the interferon and cytarabine group (Guilhot et al, 1997). Up until 2003, the standard of care for non transplant CML patients was interferon with or without cytarabine.
Tyrosine kinases, such as BCR-ABL, transfer the phosphate moiety from ATP to tyrosine residues on a protein. These phosphorylated proteins, as described earlier, lead to the phenotypic changes associated with CML. All protein kinases utilise ATP as a phosphate donor, hence the domains of kinases are usually well conserved (Hanks et al, 1988). High output screens from chemical libraries with inhibitory effects on kinase activity revealed a compound with low potency and efficacy, identified as a 2-phenylaminopyrimidine. This chemical was modified in order to optimise its ability to bind to ABL, and compete with ATP for the ATP binding site. Imatinib, an inhibitor of ABL kinase and platelet derived growth factor (PDGF), was one such compound which emerged and was promoted due to its favourable pharmacokinetics and in vitro selectivity against CML cells (Druker et al, 2000). Imatinib has been shown to be a selective and potent inhibitor of BCR-ABL (Druker et al, 1996) and c-kit (Heinrich et al, 2000). Studies have confirmed imatinib at 1µM can reduce BCR-ABL colonies, with no effect on normal colonies (Deininger et al, 1997), and also have an inhibitory effect on CML progenitors (Kasper et al, 1999). In animals, tumour reduction was positively correlated to dose, though total eradication of the tumour was not achieved (Druker et al, 1996).

The IRIS (International Randomised study of Interferon and STI571) study was a phase 3 randomised controlled clinical trial, comparing imatinib against interferon and cytarabine. 1106 patients were randomised to receive either imatinib or interferon and cytarabine in a 1:1 ratio. The imatinib treated patients had a cumulative CCR rate of 69% by 12 months, and 87% by 60 months. As previously, CCR was defined as no evidence of Philadelphia chromosomes detected in at least 20 metaphases. On imatinib 96% attained a complete haematological
response by 12 months. The overall survival was 86% at 7 years, and 94% if deaths unrelated to CML were excluded. The Sokal score also had clinical relevance in the imatinib era, with low Sokal scores having 89% CCR; this is contrasted with high Sokal score patients having a 69% CCR. The importance of achieving CCR at 12 months was demonstrated by the fact only 3% progressed to AP/Blast crisis. However, of patients failing to achieve a major cytogenetic response (<35% Philadelphia chromosome positive metaphases) within 12 months 19% progressed to AP/Blast crisis. Imatinib had clearly revolutionised the treatment of CML and improved patient expectations (Druker et al, 2005 and Hochhaus et al, 2008).

Imatinib is usually prescribed at 400mg daily. However, the optimal starting dose was not known and investigators used varying starting doses ranging from 400 - 800mg. The rationale for this assumption was higher imatinib doses would produce better long-term results. A single arm study of imatinib 800mg as initial starting dose produced CCR rates of 90% at 15 months, with 82% achieving CCR at 6 months. This is superior to outcomes when compared to imatinib 400mg treated patients. The speed to CCR was also substantially faster at higher imatinib doses. The adverse effects of high dose imatinib were similar to standard dose imatinib, but the frequency of myelosuppression was 16% in patients treated with 800mg. 66% of patients were still on imatinib 800mg at 12 months (Kantarjian et al, 2004). At present the initial starting dose of imatinib is 400mg daily.

Imatinib has been shown to be effective in the treatment of CML, but it is not without side effects. The 6 year follow up of imatinib treated patients with IRIS revealed that 56% of patients had discontinued imatinib. The causes were unsatisfactory therapeutic effect 29%, adverse events 8%, allogeneic transplantation 1% and death 4%. 14% withdraw consent. The incidence of grade 3 or 4 non haematological toxicity was less than 5%. The main side effects were fluid retention, nausea, muscle cramps, diarrhoea, fatigue weight gain and abdominal
pain. In terms of grade 3 or 4 haematological toxicity, 33% developed neutropenia, 18% thrombocytopenia and 6% anaemia in the first 12 months. After the first year the side effects of imatinib reduced (Hochhaus et al, 2008).

1.1.14.3 Nilotinib

Nilotinib is another tyrosine kinase inhibitor with potent activity against BCR-ABL. It was designed by incorporating alternative binding sites for the N-piperazine group, but still retaining an amide group to maintain the H-bond interactions with Glu286 and Asp381 (Manley et al, 2004). This allowed nilotinib to have a greater affinity for the ATP binding site on ABL kinase, and less cross reactivity with c-kit and PDGFR. This compound has activity against BCR-ABL mutants such as G250E, F317L, M351T, F486S, M244V, L248R, Q252H, Y253H, E255K, E279K, E282D and V289S, which are resistant to imatinib (Weisberg E et al, 2005).

A phase 3 randomised controlled trial, ENESTnd, compared nilotinib at 300mg and 400mg twice daily against imatinib 400mg daily, in a 1:1:1 ratio. There was a superior 12 month major molecular response (MMR) rate for the nilotinib 300mg (44%) and nilotinib 400mg group (43%) compared to the imatinib group (22%). MMR was defined as a BCR-ABL to ABL transcript ratio of less than 0.1% by RQ-PCR on peripheral blood, as per the International scale (Hughes et al, 2008). The median time to reach MMR was faster on nilotinib than imatinib; with the median time for nilotinib 300mg, nilotinib 400mg and imatinib arms to achieve MMR at 8.6 months, 11.0 months and 11.1 months respectively. The CCR rates at 12 months were also superior in the nilotinib treated patients (Saglio et al, 2010).
At 3 year follow up for the ENESTnd trial, there were noticeable differences in progression to AP/Blast crisis in the imatinib group with 4.2% progressing; this is contrasted with only 0.7% and 1.15% in the nilotinib 300mg and nilotinib 400mg group respectively. Interestingly, at the 3 year point 29.1%, 26.3% and 38.2% had discontinued nilotinib 300mg, nilotinib 400mg and imatinib respectively. These discontinuations were due to adverse effects in 9.9%, 14.2% and 11.0% in the respective groups. Nilotinib was more frequently associated with Grade 3 or 4 thrombocytopenia, while imatinib was associated with grade 3 or 4 anaemia and neutropenia. In total 7 patients on nilotinib developed peripheral arterial occlusive disease, no imatinib treated patient developed this complication. Nilotinib was also associated with a higher incidence of hyperglycaemia and hyperbilirubinaemia (Larson et al, 2012). There was an increased frequency of arterial cardiovascular events in the nilotinib treated patients compared to the imatinib treated patients. There were only 7 patients (2.5%) in the imatinib treated group who subsequently developed cardiovascular events, compared to 28 patients (10%) in the nilotinib 300mg bd group and 44 patients (16%) in the nilotinib 400mg bd group (Larson 2014 ASH abstract 4541).

1.1.14.4 Dasatinib

Dasatinib is a multi-targeted tyrosine kinase inhibitor which has activity against BCR-ABL and SRC family kinases. It is more than 300 times more potent than imatinib. Resistance to imatinib can be mediated by mutations in the P-loop of the ABL protein structure, which distorts the ABL structure in the inactive form, thus preventing imatinib from binding. Dasatinib binding is not dependent on P-loop binding, and can bind both active and inactive BCR-ABL protein and prevent autophosphorylation (O'Hara T et al, 2005).
A phase 3 randomised controlled trial, DASISION, compared imatinib 400mg against dasatinib 100mg daily in patients with newly diagnosed chronic phase CML. At 1 yr follow up, dasatinib treated patients had a superior CCR and MMR rate compared to the imatinib treated patients (CCR 83% vs 72%) and (MMR 46% vs 28%). The median time to CCR was 3.2 months and 6.0 months for dasatinib and imatinib respectively. However, there was no overall survival advantage between the two arms (97% vs 99%) (Kantarjian et al, 2010). At 2 yr follow up, the cumulative CCR rates were 86% versus 82% in the dasatinib and imatinib arms. The cumulative MMR rate continued to be superior in the dasatinib arm with 64% achieving MMR at 24 months compared to 46% in the imatinib arm. The response at the MR 4.5 level (BCR-ABL/ABL ratio < 0.0032%) was also superior in the dasatinib arm compared to the imatinib arm (17% vs 8%). Six out of 259 (2.3%) patients progressed to AP/blast crisis in the dasatinib arm, while 13 out of 260 (5%) patients progressed in the imatinib arm (Kantarjian et al, 2012).

At 2 years, 59 patients (23%) had discontinued dasatinib, of which 18 (7%) discontinued due to adverse drug reactions. Sixty four (25%) patients discontinued imatinib, of which 12 (5%) discontinued due to adverse effects. Grade 3-4 thrombocytopenia occurred more frequently in dasatinib treated patients. Grade 3-4 neutropenia occurred in equal frequency in both arms. Imatinib was associated higher rates of fluid retention, oedema and myalgia. Fourteen percent of patients developed pleural effusions on dasatinib, while none occurred in the imatinib arm (Kantarjian et al, 2012). The SPIRIT 2 study is a British randomised controlled prospective study also comparing imatinib 400mg daily against dasatinib 100mg daily. Patients were recruited in the UK from August 2008 to March 2013 with the results presented at ASH 2014 in abstract form. At a median follow up of 34 months, 11.6% of patients had discontinued imatinib, of which 9.1% stopped due to suboptimal response. In the dasatinib arm, 17.5% of patient discontinued dasatinib
mainly due to side effects. There was a superior MMR rate at 1 year for the
dasatinib group though no difference on disease progression or overall survival
(O’Brien et al, 2014 ASH).

1.1.15 Responses to imatinib

The IRIS study reports that 69% of imatinib treated patients achieved a CCR by 12
months, and at 5 years 87% had achieved this landmark. Thus, 31% failed first line
imatinib and needed either second generation tyrosine kinase inhibitors or an
increase in the imatinib dose. Also, 28% patients were not on imatinib at 5 years of
follow up (Druker et al, 2006). Failure to achieve CCR at 12 months or complete
haematological response at 3 months is termed primary resistance. The
mechanisms underlying imatinib resistance are due to a combination of BCR-ABL
overexpression, dysregulated BCR-ABL signalling and genomic instability (Perrotti
et al, 2010). Primary resistance to imatinib can be treated with imatinib dose
increase to 600-800mg/d or by switching to second generation tyrosine kinase
inhibitors (Baccarani M et al, 2006). Secondary resistance to imatinib occurs when
CCR has been achieved by 12 months, but is subsequently lost at a later date. The
most common cause for this is mutations in the BCR-ABL kinase domain.

The European LeukaemiaNet recommendations were published in 2013 and
recommend response to imatinib in CML patients is assessed at 3, 6, and 12
months, since the response to imatinib at these times has been shown to have
prognostic significance. “Failure” necessitates change of treatment and is defined
as non complete haematological response or Philadelphia chromosome>95% at 3
months, BCR-ABL1>10% or Philadelphia chromosome>35% at 6 months and BCR-
ABL>1% or Philadelphia chromosome>0 at 12 months. Optimal response is
associated with life expectancy similar to the general population. “Optimal response” is defined as BCR-ABL <10% or Philadelphia chromosome <35% at 3 months, BCR-ABL <1% or Philadelphia chromosome 0% at 6 months and BCR-ABL <0.1% at 12 months. Patients are classified into the “warning” category if at baseline they have certain clonal chromosome abnormalities in the Philadelphia chromosome positive cells, BCR-ABL >10% or Philadelphia chromosome positive cells 36-95% at 3 months, BCR-ABL 1-10% or Philadelphia chromosome positive cells 1-35% at 6 months and BCR-ABL >0.1-1% at 12 months. Patients in the warning category need to be monitored closely with frequent BCR-ABL monitoring (Baccarani et al, 2013).

1.2 Mechanisms of resistance to imatinib

1.2.1 Compliance

The World Health Organisation defines adherence to prescribed medication as being the extent to which a person's behaviour corresponds with the agreed recommendations of a healthcare provider (Sabate et al, 2003). Regular imatinib adherence is important for optimal outcomes in CML, but is often overlooked by physicians. A prospective, observational study of imatinib adherence in 169 patients showed only 14.2% patients were taking imatinib as prescribed by their physicians. Adherence was measured as a pill count, and expressed as the proportion of imatinib taken against the amount of imatinib prescribed. Patients with suboptimal responses had the highest number of imatinib pills not taken at 90 days (23.2%) compared to patients with optimal responses (7.3%). This study also showed patients who had achieved a CCR had better rates of adherence to imatinib (on average 9% of medication not taken) than patients without achievement of CCR.
(26% of medication not taken). However, imatinib adherence did not affect achievement of complete haematological response or MMR (Noens et al, 2009).

In another cohort of 87 CML patients who had already achieved CCR, patient adherence to medication was monitored by a microelectric monitoring system which was attached to the bottle cap containing the medication. Adherence was also monitored by pill counts, frequency of drug prescriptions and imatinib plasma levels. This study showed patients who had >90% adherence to imatinib had a significantly greater rate of achieving MMR (94.5% vs 28.4%) and “continued molecular response” (43.8% vs 0%). Continued molecular response (MR4) is defined as BCR-ABL <0.01%. Patients with imatinib adherence <80% did not achieve MMR (Marin et al, 2010).

1.2.2 BCR-ABL duplication and over expression

One potential mechanism for imatinib resistance is BCR-ABL amplification. BCR-ABL cell lines partially resistant to imatinib, through gradual culturing in media containing increasing concentration of imatinib over time, showed increased BCR-ABL mRNA transcript levels and an increase in BCR-ABL protein (Weisberg et al, 2000). This had the effect of increasing the IC50 for the resistant cell line compared to parental cell line (Le Coutre et al, 2000). The clinical significance of BCR-ABL over amplification is debatable. In a cohort of 66 imatinib resistant patients, only 12% had a >10 fold increase in BCR-ABL transcripts and genomic amplification of BCR-ABL was found in only 6% (Hochhaus et al, 2002).

The association between BCR-ABL over expression and mutations affecting the ABL kinase ATP binding site have also been explored. After BCR-ABL cell lines
were cultured in increasing concentrations of dasatinib up to 200nM, 4/10 cell lines developed BCR-ABL amplification which progressed to kinase domain mutations. This would suggest BCR-ABL over amplification as the initiating step to kinase domain mutations. However, it is also known high expression of BCR-ABL is toxic to the cell, and hence cells with lower levels of BCR-ABL amplification and kinase domain mutations would have a survival advantage (Tang et al, 2011).

BCR-ABL over expression can also be associated with in-frame splice deletions of the GSK glycogen synthetase 3 beta kinase (GSK) domain in granulocyte-macrophage progenitors of blast crisis patients. This particular splice deletion may not be present in blasts or normal haematopoietic stem cell progenitors. Blast crisis CML progenitors, with missspliced GSK3beta have high expression of beta-catenin and high engraftment potential. If the full length GSK3beta is reintroduced, there is a reduction of leukaemia engraftment and in vitro replating efficiency (Abrahamsson et al, 2009).

1.2.3 BCR-ABL mutation

Imatinib binds to the highly conserved central catalytic nucleotide binding pocket of ABL, in the inactive conformation. Imatinib occupies the site where the adenosine part of the ATP molecule would reside, and protrudes deep into the catalytic domain. It forms a complementary structure with the NH2 terminal region of the activation loop (Schindler et al, 2000). Changes in the ABL kinase conformation due to mutations can therefore lead to imatinib resistance. Mutations within the kinase domain are the most frequent cause of secondary imatinib resistance. There are 3 general mutations affecting the kinase domain that can lead to imatinib resistance. The first group of mutations involves contact
residues, such as T315I, which will hinder imatinib access. The second group of mutations affects the ATP binding loop, hence preventing ABL from attaining the specific complementary shape to bind to imatinib. The last group of mutations occurs in the regulatory motifs, such as in the activation loop, which stabilises ABL in the active conformation thus preventing imatinib from binding (O'Hare et al, 2007). Kinase domain mutations need to be tested by cell proliferation assays and purified kinase assays before they are deemed to cause imatinib resistance. The T315I mutation is known to confer resistance to imatinib, but other mutations such as F317L, also affecting the hydrogen bonding to imatinib, may not be sufficient to cause imatinib resistance (Corbin et al, 2003). Some kinase domain mutations may be associated with other mechanisms of resistance and hence behave as a bystander (Hochhaus et al, 2002).

Several studies suggest kinase domain mutations are part of the natural history of CML. These mutations are more prevalent in patients with accelerated disease or blast crisis (Branford et al, 2003 and Soverini et al, 2006). Some ABL mutations are present pre therapy and increase in clone size after time leading to imatinib failure, suggesting the particular clone has been positively selected during treatment (Roche-Lestienne et al, 2002). If the clone is dominant pre imatinib therapy, these patients will have primary resistance to imatinib (Willis et al, 2005; Shah et al, 2002).

Ordinarily, mutations with a high degree of resistance to imatinib are preferentially selected, and occur more frequently in clinical samples. However, there are exceptions to the rule with the weakly resistant T351M occurring more frequently than the more resistant E255V. The dominance of certain mutations in clinical practice is due to drug resistance leading to clonal selection advantage and also an increased chance of acquiring certain mutations due to favourable nucleotide substitutions. Nucleotide substitutions are frequently transitions as opposed to
transversions, with C↔T transitions present in 40% of clinically relevant mutations (Hughes et al, 2006).

A large number of kinase domain mutations have now been documented in the literature. Clinical studies have attempted to demonstrate the clinical relevance of these mutations. In an 89 patient retrospective study of imatinib resistant patients, 94 BCR-ABL mutations were detected. Six patients had 2 mutations. The majority of patients were in chronic phase (57/89), while 21 patients were in accelerated phase and 11 patients in blast crisis. Mutations were most frequently found in the P-loop (28%) and T315I (20%). These mutations were more frequently found in accelerated phase than any other phase of disease (p=0.003). Typically, P-loop mutations and T315I occurred after a shorter period of time on imatinib therapy with mutations detected at 25 months (range 2.4-44) and 20 months (range 2.8-44.2) respectively. Other mutations were present at 37 months (range 6.7-53.9 months); this finding was statistically significant (p<0.05). Patients in chronic phase with P-loop mutations and T315I failed to achieve a major cytogenetic response on imatinib (p<0.05), consistent with aggressive disease. The overall survival for P-loop mutations and T315I was also reduced with median survival times of 28.3 months and 12.6 months respectively. The median survival time for patients without either P-loop mutations or T315I was not reached at 39 months (p<0.05). Progression free survival was also reduced in the presence of either P-loop mutations or T315I mutations (Nicolini et al, 2006).

The significance of P-loop mutations has been disputed. The study described above suggests P-loop mutations are associated with poor prognosis. However, another study of 171 CML patients reported mutations in 44 out of 134 (32.8%) patients in chronic phase CML. There was no difference in survival between patients with or without BCR-ABL mutations. Also P-loop mutations, though the most frequent
mutation, were not associated with poor prognosis, and surprisingly patients with non-P loop mutations had significantly reduced overall survival. The T315I mutation was noted in only 2% of patients (Jabbour et al, 2006). The importance and frequency of kinase domain mutations vary between studies due to technical methods used to detect mutations, patients in different phases of CML and also the inclusion of patients with either primary or secondary resistance (Corbin et al, 2003 and Branford et al, 2003 and Hochhaus et al, 2002).

1.3 BCR-ABL independent mechanisms of resistance

1.3.1 Imatinib plasma levels

Studies have shown there is variability in imatinib plasma levels in CML patients. The cause of imatinib trough variability could be due to differences in plasma binding, drug-drug interactions, patient’s demographics and also P450 cytochrome enzymes. This could be a factor in suboptimal responses to imatinib. The imatinib trough levels can vary between 181-2947 ng ml⁻¹, in patients taking 400-600mg imatinib doses (Picard et al, 2007). A subanalysis of the IRIS study showed imatinib trough plasma concentrations of 979±530 ng ml⁻¹ (Larson et al, 2008).

After treating CML patients for at least 12 months, imatinib plasma levels correlated to clinical outcome. Patients who achieved CCR had a higher imatinib plasma level (1452±649 ng ml⁻¹) when compared to patients without CCR (1123±617 ng ml⁻¹). MMR was also positively correlated to imatinib trough levels, with patients in MMR (1452±649 ng ml⁻¹) having higher imatinib plasma concentrations than patients not in MMR (869±428 ng ml⁻¹) (Picard et al, 2007). The IRIS subanalysis showed if the day 29 imatinib plasma level >647 ng ml⁻¹, then there was a higher rate of CCR (p=0.005) and MMR (P=0.008). Side effects such as fluid retention, rash, myalgia
and anaemia are more likely to occur in patients with high imatinib levels (Larson et al, 2008). Both studies suggest imatinib trough levels >1000 ng ml⁻¹, are associated with improved rates of CCR and MMR. However, one study has shown that there is no correlation between imatinib levels and outcome in CML, but this was limited by small sample size and heterogeneous population and sampling times (Forrest et al, 2009).

1.3.2 Drug binding

Alpha1-acid glycoprotein (AGP) is a plasma protein which has been implicated in imatinib resistance. AGP, an acute phase protein, can bind to plasma imatinib and reduce its effect on BCR-ABL in murine models, with increasing concentrations. High AGP levels were positively correlated to CML tumour load. Mice treated with imatinib and erythromycin demonstrated improved tumour free survival and greater tumour mass reductions. Erythromycin and imatinib have broadly similar binding affinities for AGP and high erythromycin concentrations can interfere with imatinib-AGP binding (Gambacorti-Passerini et al, 2000). The role of AGP has been disputed in the literature, with groups suggesting AGP does not cause in vitro imatinib resistance and is not important in binding imatinib (Jorgensen et al, 2002). This difference can be explained by experimental and technical differences used to isolate AGP and possible desialylation of the AGP, which may affect drug binding. In CML patients, there was marked variation in AGP levels ranging from 0.4 -3.2g l⁻¹. AGP plasma concentrations had an effect on imatinib pharmacokinetics and explained some of the variation in imatinib levels. As imatinib has limited hepatic extraction, changes in plasma levels of AGP should not greatly affect imatinib. No clinical study has addressed the role of AGP levels on patient clinical outcomes (Widmer et al, 2006).
1.3.3 Imatinib metabolism and the cytochrome P450 enzymes

The terminal half-life of imatinib is 18 hours and it is mainly eliminated by the faecal route (68%) and urine (13%) (Cohen et al, 2002). After metabolism in the liver, imatinib represents 70-80% of the final drug concentration with 20-30% made up of N-desmethylimatinib. Imatinib is mainly metabolised by CYP3A4, CYP3A5 and CYP2C8 enzymes (Peng et al, 2005 and Filppula et al, 2013). The varying contributions of these metabolising enzymes are still not clear. The activity of CYP enzymes is known to affect imatinib metabolism and shows interpatient variability (Rochat et al, 2005).

A small study of 14 patients positively correlated high levels of CYP3A4 with continued molecular response. However, it was not known whether imatinib altered the activity of CYP3A4 in the long-term. This may suggest the metabolite of imatinib, N-desmethylinatinib, also contributes to inactivation of BCR-ABL (Green et al, 2010). Other studies have shown polymorphisms of the major allele in CYP3A4 (AA rs2740574) was correlated to reduced MMR rates (Angelini et al, 2012).

1.3.4 Alternative signalling pathways

BCR-ABL can directly activate the Src family of kinases, and may allow the Abl kinase to be stabilised in an active conformation. This will affect the kinetics and regulation of the kinase enzyme (Dan hauser-riedl et al, 1996, Stangmaier et al, 2003). Lyn, Fyn and Hck (myeloid Src family kinases) have been shown to
phosphorylate the SH3 and SH2 domains of BCR-ABL. The phosphorylation site Tyr 89 on the SH3 domain was consistently phosphorylated in CML patients. This Src family kinase phosphorylation of BCR-ABL may change the conformation and enhance intracellular signalling, leading to reduced imatinib sensitivity (Meyn et al, 2006). Interestingly Lyn, a Src family kinase, has been shown to be overexpressed in K562 cells resistant to imatinib, and inhibition of Lyn reduced proliferation of resistant K562 cells. In patients with CML, Lyn overexpression has been shown to be present in patients resistant to imatinib. Thus imatinib resistance may be driven by upregulation of Lyn, which is independent of BCR-ABL (Donato et al, 2003).

Using siRNA to silence Lyn overexpression in imatinib resistant cell lines, within 48 hours abnormal BCR-ABL cells underwent apoptosis while normal haematopoietic cells remained viable (Ptasznik et al, 2004). However, in clinical practice if Src family mutations caused imatinib resistance, patients with T315I mutation would respond to dasatinib. Unfortunately this is not the case hence other intracellular pathways are involved in imatinib resistance.

As described previously, the mitogen-activated protein kinases (MAPK) which are activated by Ras have also been implicated in imatinib resistance. Raf can be recruited to the cell membrane, which can then lead to activation of Mek1/Mek2 and the extracellular receptor regulated kinase (Erk) pathway. In CML cell lines resistant to imatinib up regulation of Erk1/2 was noted and also localisation of the Erk1/2 in the nucleus. This did not occur in imatinib sensitive cell lines. Also, in patient samples resistant to imatinib, high levels of Erk1/2 were detected in the presence of high BCR-ABL levels, while CML patients who were responding to imatinib had low BCR-ABL and low Erk1/2 levels (Aceves-Luquero et al, 2009).

The protein phosphatase 2 (PP2A) is a tumour suppressor gene, which down regulates MAPK and PI3K pathways (Janssens et al, 2001). PP2A is highly expressed in mammalian cells and is the main source of phosphatase activity. It is
composed of a core dimer formed from the interaction between the subunit A and a catalytic subunit. This core dimer then interacts with a regulatory subunit B which then determines the function of the specific PP2A (Mumby et al, 2007). In CML, the BCR-ABL oncogene maintains PP2A in a phosphorylated state at tyrosine 307, thus inactivating it (Perrotti et al, 2006 and Neviani P et al, 2005). Imatinib treatment can reactivate PP2A, and allow apoptosis and reduced proliferation (Neviani P et al, 2005). Proteins implicated in PP2A inhibition are a) SET, which is elevated in BCR-ABL1 over expression, via a Jak2 mediated process (Samanta et al, 2009). b) SET binding protein 1 (SETBP1), which can stop the degradation of SET and thus maintain PP2A inhibition. However, SETBP1 does not predict clinical outcome in CML so the significance of this protein is doubtful (Lucas et al, 2010). c) Cancerous inhibitor of PP2A (CIP2A) is a protein which can stabilise c-Myc in its phosphorylated state and promote proliferation (Junttila et al, 2007).

CIP2A levels have been shown to correlate with progression to blast crisis in patient samples, with high levels at diagnosis leading to an actuarial progression rate blast crisis of 100% at 21 months. CIP2A reduction by siRNA in CML cell lines was associated with a fall in PIM1 and c-Myc levels. PIM1 can phosphorylate c-Myc and prevent degradation of this protein causing cell proliferation. The lesser importance of SET protein in CML was also confirmed by the observation that patients with achievement of CCR had relatively high SET levels, while blast crisis patients had low levels (Lucas et al, 2011).
1.4 Drug transporters

1.4.1 Drug transporters general overview

Membrane transporters are important for regulating the influx and efflux of molecules, thereby maintaining the optimal intracellular physiological pH and solute concentrations. They also have important roles in the liver for facilitating influx of molecules for metabolism and in the kidney they are vital for elimination of xenobiotic drugs. The normal physiological roles of these transporters are to transport a diverse group of endogenous substances such as steroids, bile salts, hormones and sugars (Ho et al, 2005).

1.4.2 Drug transporters and their role in normal physiology

Drug transporters are generally classified as either influx or efflux pumps. Efflux pumps mainly belong to the adenosine triphosphate (ATP)-binding cassette (ABC) family, while influx pumps are mainly comprised of the uptake transporters of the solute carrier (SLC) family (Li et al, 2011). The main clinically relevant influx pumps of the SLC family are the organic anion transporting polypeptides (OATP, SLCO), organic cation transporters (OCT, SLC22A), organic cation/carnitine transporters (OCTN, SLC22A), organic anion transporters (OAT, SLC22A) and peptide transporters (PEPT, SLC15A). The ATP-binding cassette (ABC) family of efflux transmembrane transporters use ATP hydrolysis to facilitate the movement of drugs across the cell membrane. The most prominent members of this family are P-glycoprotein (MDR1, ABCB1), multi drug resistance protein 1 (MRP1, ABCC1) and
the breast cancer resistance protein (BCRP, ABCG2). The table below shows the clinically relevant drug transporters known to date, with their substrates (Li et al, 2011).

Table 1.1: Clinically relevant drug transporters and their tissue distribution, substrates and polarity (Li et al, 2011).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Tissue distribution</th>
<th>Polarity</th>
<th>Representative drug substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCB1</td>
<td>MDR1</td>
<td>Liver, intestine, kidney, blood—brain barrier, lymphocytes, placenta</td>
<td>AP</td>
<td>Anthracyclines, taxanes, vinca alkaloids, imatinib, etoposide, levofloxacin, erythromycin, cyclosporine, tacrolimus, digoxin, quinidine, verapamil, diltiazem, ritonavir, saquinavir, aminophylline, furosemide, phenytoin, cimetidine, simvastatin, morphine, hydrocortisone, doxorubicin, methotrexate, cyclosporin, amphotericin B, lamivudine, 5-fluorouracil, vincristine, vinblastine, vinorelbine, doxorubicin, cisplatin, paclitaxel, fluorouracil, irinotecan, SN-38, methotrexate, camptothecins, saquinavir, ritonavir, diltiazem, drug-Glcuronide-glutathione-sulfate conjugates</td>
</tr>
<tr>
<td>ABCG2</td>
<td>BCRP</td>
<td>Liver, intestine, placenta, breast</td>
<td>AP</td>
<td>Anthracyclines, vinca alkaloids, methotrexate, camptothecins, ritinpin, pravastatin, and drug-Glcuronide-glutathione-sulfate conjugates</td>
</tr>
<tr>
<td>OATP family</td>
<td>OATP1A2</td>
<td>Ubiquitous, with highest expression in brain and testes</td>
<td>BL</td>
<td>Rosuvastatin, methotrexate, ouabain, D-penicillamine</td>
</tr>
<tr>
<td>OCT family</td>
<td>OCT1</td>
<td>Liver</td>
<td>BL</td>
<td>Metformin, ciproflaxacin, omacetram, procainamide, citalopram, cimetidine, quinidine, verapamil, acyclovir</td>
</tr>
<tr>
<td>OAT family</td>
<td>OAT1</td>
<td>Kidney, brain</td>
<td>BL</td>
<td>Methotrexate, salicylate, antiviral agents (e.g., acyclovir)</td>
</tr>
<tr>
<td></td>
<td>OAT2</td>
<td>Liver, kidney</td>
<td>BL</td>
<td>Methotrexate, cimetidine, pravastatin, salicylate</td>
</tr>
<tr>
<td></td>
<td>OAT3</td>
<td>Kidney, brain, muscle</td>
<td>BL</td>
<td>Methotrexate, antiviral agents (e.g., acyclovir), cimetidine, pravastatin, salicylate</td>
</tr>
<tr>
<td></td>
<td>OAT4</td>
<td>Kidney, placenta</td>
<td>AP</td>
<td>Methotrexate, cimetidine, salicylate, tacrolimus</td>
</tr>
</tbody>
</table>

Li et al, 2011.
The expression of influx and efflux pumps at the apical or basolateral surface of any epithelial cell will determine the movement of a specific drug into or out of the cell, and thus from one body compartment to another. This has great clinical significance and will determine whether a drug is primarily taken up by hepatocytes and thereby potentially undergo phase I/II metabolism, or be eliminated by the epithelial cells of the proximal tubular cells in the kidney. Many drug transporters have substrate specificities which overlap and this evolutionary adaptation allows xenobiotic drugs to be metabolised or excreted to reduce their harmful effects. The highest expression of drug transporters occurs in the liver, kidney, blood brain barrier and small bowel.

The expression and localisation of certain drug transporters at the small bowel luminal surface dramatically affects drug bioavailability. MDR1 (P-glycoprotein) is expressed in enterocytes and has been shown to efflux certain drugs back into the small bowel and prevent systemic absorption. Mice with mdr1a knockout have markedly reduced uptake of digoxin, cyclosporin and to a lesser extent dexamethasone (Schinkel et al, 1995). In humans MDR1 expression is negatively correlated to cyclosporin (Lown et al, 1997) and digoxin (Drescher et al, 2003) concentrations.

The expression of OCT and OATP is high in the liver and determines the hepatic clearance of certain drugs such as pravastatin which is a substrate for OATP1B1 (Nakai D et al., 2001). Other efflux transporters such as MRP2, which are also expressed in the liver, have an important role in biliary excretion of drugs such as methotrexate (Masuda et al, 1997). In the kidney, excretion of drugs relies on the dynamic interplay between influx and efflux pumps present at the proximal tubular cells. The OAT family is highly expressed in the kidney and determines the
movement of drugs such as nonsteroidal anti-inflammatory drugs and diuretics (You et al, 2002). The expression of drug transporters, such as MDR1, in addition to tight junctions at the blood brain barrier prevents harmful substances from entering the central nervous system. High expression of MDR1 at the blood brain barrier limits entry of drugs such as HIV-1 protease inhibitors (Kim et al, 1998).

1.4.3 The OATP family

The organic anion transporting polypeptide (OATP) family of drug transporters are divided into 6 families as determined by their amino acid composition. In total 11 OATPs have been discovered and they are encoded by the SLCO genes. OATPs transport a large number of structurally diverse substrates including bile salts and steroid hormones (Roth et al, 2012).

1.4.3.1 Tissue expression

There is ubiquitous expression of OATP1A2 mRNA in the brain, liver, lung and kidney. OATP1A2 protein is highly expressed in liver cholangiocytes (Lee et al, 2005), the apical membrane of the distal nephrons (Lee et al, 2005) and the luminal surface of blood brain barrier (Bronger et al, 2005). This particular protein expression is highly suggestive of OATP1A2 having a significant role in excretion of xenobiotic drugs into the bile and also excretion of drugs into the urine and maintenance of the blood brain barrier. OATP1B1 and OATP1B3 are also expressed in the liver, but their pattern of distribution varies, with OATP1B1 expressed throughout the lobule while OATP1B3 is mainly located around the central vein (Konig et al, 2000). OATP1C1 protein expression is found in the testes (Pizzagalli et al, 2002) and the choroid plexus (Roberts et al, 2008). OATP2A1 is a
1.4.3.2 Substrate specificity

The method of OATP transport of substrates is still unclear. It is accepted that OATP transport is ATP and sodium independent. Individual members of the OATP family may use different exchangers such as bicarbonate (Satlin et al., 1997), glutathione (Li et al., 1998) and glutathione conjugates (Li et al., 2000) to facilitate movement of their substrates. Glutathione exchange is not required for OATP1B1 and OATP1B3 transport (Mahagita et al., 2007). The net exchange is always electro-neutral. It has been proposed that substrates translocate down a positively charged pore by a rocker switch process (Meier-Abit et al., 2005). OATPs are able to function as bidirectional transporters and transport efficiency is affected by pH (Roth et al., 2012). Acidic conditions are associated with increased OATP1A2 transport activity (Yarma et al., 2011) by potentially protonating the histidine residue on the extracellular transmembrane domain 3 (Mahagita et al., 2007). This may influence intestinal absorption as this transporter is expressed in the stomach.

Acidic conditions are associated with increased OATP1A2 transport activity (Yarma et al., 2011) by potentially protonating the histidine residue on the extracellular transmembrane domain 3 (Mahagita et al., 2007). This may influence intestinal absorption as this transporter is expressed in the stomach. (Mandery et al., 2010). OATP2A1 protein is predominately expressed in skeletal muscle (Knauer et al., 2010) and basolateral surface of the syncytiotrophoblasts (St-Pierre et al., 2002), nephrons and hepatocytes (Roth et al., 2012). mRNA expression of OATP3A1 is found in the testis, heart and brain (Adachi et al., 2003). OATP4A1 and OATP4C1 protein expression is found in placenta (Sato et al., 2003) and kidney (Mikkaichi et al., 2004) respectively. OATP5A1 mRNA expression is present in the prostate, skeletal muscle and thymus, while OATP6A1 mRNA is found in the testes (Suzuki et al., 2003; Mikkaichi et al., 2004).
Substrates transported by the OATP family are generally anions with amphipathic properties and a molecular weight greater than 350mg. However, some OATPs are capable of transporting cationic drugs (Bossuyt et al., 1996). The known endogenous substrates transported by the OATP family are bile salts, steroids and thyroid hormones. Estrone-3-sulphate (E-3-S) is a substrate which is transported by most OATP transporters and along with bromosulphophthalein is considered a model substrate. Substrates share in common a hydrogen bond donor, two hydrophobic regions and two hydrogen bond acceptors (Chang et al, 2005). Substrates can bind to numerous sites on the OATP protein which correspond to either high or low affinity binding site interactions and hence differential transport kinetics. E-3-S has biphasic kinetics when transported by OATP1B1 supporting this concept of varying strengths of substrate binding potential to a transporter (Noe et al, 2007).
1.4.3.3 Regulation of expression

OATP expression is predominately governed by a diverse family of transcriptional factors belonging to the Hepatocyte nuclear factor (HNF) family. OATP1B1 expression in hepatocytes is determined by a combination of factors including HNF1α (Furihata T et al, 2007), STAT5 (Wood et al, 2005) and IL-1 (Le Vee et al, 2008). OATP1B3 expression is decreased by increasing levels of HNF3β, possibly by the transcription factor binding to the promoter site for the OATP1B3 and impairing RNA polymerase activity (Vavricka SR et al, 2004). OATP1A2 expression in the liver is influenced by an increase in bile acid concentrations, affecting expression in the liver and small bowel (Kullak-Ublick et al, 1997).

OATP protein expression is also regulated by PDZ proteins. PDZ proteins have a role in attaching transmembrane proteins, such as OATP, to the cytoskeleton (Ranganathan et al, 1997). The majority of OATP proteins have PDZ consensus sequences (Wang et al, 2005) which can interact with PDZ proteins and affect OATP protein stability at the membrane surface (Kato et al, 2004).

1.4.3.4 Transporter structure

The general size of the OATP proteins varies between 643-724 amino acids in length. They have 12 transmembrane domains (Wang et al, 2008) and both the amine and carboxyl group termini are located intracellularly. N-glycosylation sites are relatively conserved at the 2nd and 5th extracellular domains, but the sites vary between proteins. The crystal structure for the OATP proteins has yet to be determined hence the amino acid interactions important for substrate translocation
are yet to be confirmed. However, chimera studies have shown extracellular domains 8, 9 and 10 are important for substrate transfer in OATP1B1 (Gui et al, 2009) while extracellular domain 10 is important for substrate passage in OATP1B3 (Giu et al, 2008). Crystallography and nuclear magnetic resonance studies will help determine the exact amino acids involved in substrate binding and translocation.

1.4.3.5 Pathology and significance

Certain clinical conditions have been associated with an altered expression of OATP mRNA and protein expression. Inflammatory bowel disease is known to be associated with increased OATP2B1 and OATP4A1 mRNA expression levels (Wojtal et al, 2009). Cholestasis due to any aetiology can lead to a reduction in mRNA levels of OATP1A2, OATP1B1 and OATP1B3 in the liver (Kietal et al, 2005; Chen et al, 2008; Congiu et al, 2009).

The expression of OATPs also changes with different types of cancer. Gastric, pancreatic and colonic cancers have been associated with OATP1B3, which is normally localised to the liver only (Abe et al, 2001; Lee et al, 2008). However, in hepatocellular carcinoma, the expression of OATP1B3 is decreased (Vavricka et al, 2004). The high expression of OATP in some cancers may provide a survival advantage, as some cancers are known to proliferate in response to certain hormones, and hormones are the natural substrates for the OATP family.
1.4.4 The OCT family

The SLC22A family of genes encodes the OCT (Organic cation transporter) family of proteins, which include OCT1-3 (SLC22A1-3), and OCTN1 (SLC22A4) and OCTN2 (SLC22A5).

1.4.4.1 Tissue distribution

The OCT1 (SLC22A1) protein is predominately expressed at the basolateral surface of hepatocytes (Nies at al, 2008) and the apical surface of lung epithelia (Lips et al, 2005). In situ hybridisation techniques failed to identify expression in the kidney. Low levels of OCT1 mRNA expression have been detected in the heart, brain and placenta (Gorboulev et al, 1997). OCT2 protein expression is mainly localised to the convoluted tubules of the kidney (Gorboulev et al, 1997). The spleen and small intestine have been shown to express low levels of OCT2 (SLC22A2) expression. OCT3 is the most ubiquitously expressed OCT family drug transporter, and is expressed at protein level in hepatocytes (Nies et al, 2009), at the luminal surface of the lungs (Lips et al, 2005) and also in trophoblasts (Sata et al, 2005).

OCTN1 mRNA expression is found in the bone marrow, kidney and trachea. Protein expression has been confirmed on the apical surface of the enterocytes and epithelial cells lining the proximal tubule (Tamai et al, 1997). OCTN2 protein expression has been confirmed at the apical borders of the proximal tubules (Masuda et al, 2006) and syncytiotrophoblasts (Grube et al, 2005), while OCTN2
mRNA expression is strongly expressed in the heart, placenta, kidney and pancreas (Wu et al., 1998).

1.4.4.2 Substrate specificity

OCT family proteins translocate organic cations by facilitated diffusion along an electrochemical gradient. This transport is not mediated by sodium exchange but can be influenced by pH. The pH can affect the ionisation of a substrate and hence change its affinity for a specific OCT binding site. The OCT family of drug transporters transport monoamine neurotransmitters, catecholamines and steroid hormones as endogenous substrates. 1-methyl-4-phenylpyridinium (MPP) is a common substrate for OCT1-3, while Tetra ethyl ammonium (TEA) is transported by OCT1 and OCT2, but not by OCT3 (Grundemann et al., 1998). Studies have shown OCT1 binding to substrates is stereoselective, and a pharmacophore model based on 22 diverse substrates revealed the importance of a positive ionisable site, a hydrophobic site and two hydrogen acceptor sites for substrate interaction. However, this model does not fit all substrates, and dopamine and thiamine only have two interactive binding sites with the pharmacophore model (Moaddel R et al., 2007).

OCTN1 and OCTN2 transport both cations and carnitine. The endogenous substrate for OCTN1 is ergothioneine, although TEA and L-carnitine are also transported by OCTN1 but with less efficiency. Ergothioneine is an antioxidant though its role in the body is not clear (Gründemann D et al., 2005). Carnitine and TEA are also transported by OCTN2, but the substrate binding sites vary as certain mutations affecting OCTN2 inhibit carnitine transport but have no effect on TEA transport. Ergothioneine was poorly transported by OCTN2 (Seth et al., 1999).
**1.4.4.3 Regulation of OCT expression**

The regulation of OCT occurs both at a transcriptional and protein level, and varies with respect to tissue distribution and the specific drug transporter. In the promoter region of hOCT1, there are two response elements which interact with HNF4α. Both of these elements are vital for OCT1 transcription, as site mutagenesis reduced the expression of hOCT1. Small heterodimer partner (SHP), which is a bile acid inducible transcriptional repressor, can also inhibit hOCT1 transcription (Saborowski et al, 2006). OCT2 mRNA expression level was noted to increase with dexamethasone and hydrocortisone, but reduced with oestradiol. This would suggest the OCT2 promoter regions may contain androgen receptor elements (Shu et al, 2001). OCTN1 (SLC22A4) regulation is affected by RUNX1, a transcriptional repressor, which can bind to the intronic SNP of OCTN1 (SLC22A4) and reduce transcription of the SLC22A4 gene (Tokuhiro S et al, 2003). The PDZ proteins are known to interact with both OCTN1 and OCTN2, and help with protein stabilisation at the luminal surface of enterocytes. One such PDZ protein, PDZK1, has been shown to enhance substrate translocation when interacting with OCTN2 (Kato et al, 2007).

**1.4.4.4 OCT transporter structure**

The OCT family of drug transporter proteins vary in size from 543-557 amino acids in length. They have 12 transmembrane domains with the carboxyl and amine termini groups located intracellularly. The N-glycosylated sites are predominately located between the first and second transmembrane domains, while the phosphorylation sites are located in a large intracellular loop between the sixth and
seventh domains. The amino acid sequences of OCT1 and OCT2 are 70% homologous, while there is 50% homology between OCT3 and OCT1/OCT2 (Gorboulev et al., 1997). OCTN1 and OCTN2 also share similar amino acid sequences with 77% homology.

The predicted 3 dimensional structure for OCT1 has given rise to the theory that substrates interact with OCT1 within a certain region as opposed to a single binding site. During substrate electrogenic facilitated transfer, the transporter undergoes 3 distinct conformational changes which include an outward facing conformation, followed by an occluded state and then finally an inward facing state (Koepsell et al., 2011).

1.4.4.5 Pathology and significance

OCT 1-3 polymorphisms have not been associated with any human diseases to date, and OCT knockout mice do not develop a distinct phenotype. Single nucleotide polymorphisms (SNPs) of hOCT1 alter functional characteristics of the protein as demonstrated by different binding affinities to known substrates when compared to the wild type (Kerb et al, 2002). However, OCTN1 and OCTN2 have been associated with familial inflammatory bowel disease (Lin Z et al., 2010) and systemic carnitine deficiency (Lahjouji et al, 2001) respectively. Mutations affecting OCTN2 impair the nephrons from reabsorbing carnitine. Carnitine is an amine which is vital for mitochondrial β-oxidation. Systemic carnitine deficiency manifests with skeletal myopathy, cardiomyopathy and hypoglycaemia. This disorder is inherited in an autosomal recessive pattern (Lahjouji et al, 2001).
1.5 The ABC family of drug transporters

The ATP-binding cassette (ABC) family of drug transporters are the main efflux pumps in the human body. These drug transporter proteins transport a diverse group of endogenous and xenobiotic substrates including bile salts, lipids, sterols, amino acids and sugars. There are currently 48 ABC proteins which are classified into seven ABC subfamilies (A→G) (Dean et al, 2001).

All ABC drug transporters have a similar basic functional structure. This includes two membrane bound domains which allow translocation of the substrate from the cytosol to the extracellular space, and two nucleotide binding domains (NBDs) which allow the hydrolysis of the phosphate groups in ATP. The four domains create a single membrane bound protein with internal duplication. The ABCC family of efflux pumps differ from other subfamilies by having an extra domain located near the N-terminal. This domain has 5 transmembrane segments. All substrates transported by the ABC proteins are effluxed against their concentration gradient, from energy provided by ATP hydrolysis (Sharom et al, 2008).

1.5.1 ABC drug transporter substrate and expression

Three specific ABC efflux transporters have consistently been associated with human multidrug resistance. They are P-glycoprotein PgP (ABCB1), MRP1 (ABCC1) and BCRP (ABCG2) (Litman et al, 2001). P-glycoprotein and MRP1 are 170-190 kDa in size, while the half transporter ABCG2 is 72kDa in size and forms homodimers (Ozvegy C et al., 2001). Their main physiological role is to efflux toxic
substances from the intracellular compartment to the extracellular compartment. These drug transporters are expressed in many tissues and hence have a significant impact on pharmacokinetics and dynamics of xenobiotic drugs.

PgP is capable of transporting a wide group of substrates, though all are amphipathic with a molecular weight ranging between 300-1000. At physiological pH the substrates are generally protonated. Phospholipids (Eckford et al, 2005), glycopospholipids and steroid hormones such as aldosterone (Bello-Ruess et al, 2000) and β-oestradiol (Lui et al, 1996) are all endogenous substrates for PgP. This drug transporter also transports xenobiotic drugs such as mitoxantrone, etoposide, anthracyclines and methotrexate (Sharom FJ et al., 2008). PgP knockout mice have a dysfunctional blood brain barrier, with signs of neurotoxicity. This suggests an important physiological role in blood brain barrier maintenance.

The expression of PgP is highest at the luminal surface of the small intestine, pancreas, colon and the proximal tubules of the kidney. It is also present at the blood brain barrier and testis, which are immuno-privileged sites. There is moderate expression in the endometrium, placenta and bone marrow (Sharom et al, 2008).

MRP1 transports substrates which are generally anionic. It has a similar spectrum of substrates to PgP, but in addition can efflux sulphate conjugates, glucuronates and drugs conjugated with glutathione. It is proposed that MRP1 could be important in leukotriene mediated inflammation and lipid signalling, as it effluxes leukotriene C4. Glutathione cooperates with MRP1 by transporting chemotherapy drugs and negatively charged conjugates, but the precise role is not known. The transport of vincristine by MRP1 is significantly enhanced by glutathione. MRP1 has been postulated to regulate glutathione homeostasis (Loe et al, 1998).
Protein expression of MRP1 has been localised to the basolateral surface of renal epithelial cells, bone marrow and enterocytes. Drug transporters localised to the basolateral surface efflux substrates into the lumen for excretion (Leslie et al, 2005). ABCG2 can transport a large group of substrates which can be either positively or negatively charged. The endogenous substrates for ABCG2 are porphyrin/haem (Krishnamurthy et al, 2004), glutamates of folate (Lomos et al, 2009) and uric acid (Woodward et al, 2009). ABCG2 is thought to have a physiological role in porphyrin/haem homeostasis. The intracellular levels of porphyrin increase in hypoxic conditions, and ABCG2 has been shown to actively efflux this into the extracellular environment. Reduced levels of folate can upregulate ABCG2 expression, but this is strictly confined to cytoplasm only.

ABCG2 is expressed in the brain, placenta, kidney and intestine. The hormonal changes of pregnancy have been associated with increased BCRP expression in the mammary glands. Interestingly, riboflavin has been shown to be a substrate for ABCG2, and riboflavin is actively secreted into breast milk (van Herwaarden et al, 2007).

1.5.2 ATP binding and hydrolysis

All the ABC drug transporters have nucleotide binding domains (NBD) as part of their structure. Structural studies suggest Pgp and MRP1 have two NBDs while the half transporter ABCG2 has only one NBD. The NBDs are important for ATP hydrolysis and therefore have conserved sequence motifs. The three sequence motifs which are common to all ABC transporters are the Walker A and Walker B motifs and the signature C motifs. The signature C motif is present only in ABC drug transporter proteins while the Walker A and B motifs are also present in other proteins which interact with ATP. The ATP molecule interacts with the Walker A and
B motifs of the first NBD and the signature C motif of the adjacent NBD. At a particular time, two ATP molecules bind at the dimer sandwich interface flanked by the Walker A and B unit at one end and the adjacent signature C motif at the other end (Smith et al, 2002).

1.5.3 Drug binding

PgP is a large polypeptide containing 12 trans membrane domains and two NBDs which are located in close proximity. Both the amino and hydroxyl terminals are located within the cytoplasm. The NBDs are located at the at the carboxyl end of the protein relative to the transmembrane domains. The drug binding site for PgP is located intracellularly and is derived from the transmembrane helices. There are multiple binding sites on PgP, and different substrates have been shown to bind to different parts of the drug binding pocket (Loo et al, 2005). The drug binding pocket is large, conical and flexible and these characteristics allow transport of two substrates at a time (Loo et al, 2003). Substrates gain access to the drug binding pocket by entering through portals flanked by the transmembrane domains of 2/11 and 5/8, which are sterically in close proximity (Loo et al, 2005). The substrate is able to interact with the binding pocket via hydrogen bonds, van der Waal forces and hydrophobic interactions. This "induced fit type model" enables a diverse group of substrates to interact with different residues contained in the drug binding pocket (Loo TW et al., 2003). P-glycoprotein substrates in general require hydrophobic groups and hydrogen bond receptors to interact effectively with the drug binding pocket (Pajeva et al, 2002).

ABCC1 has twelve transmembrane domains and two NBDs, which are located at the amino terminal in relation to the trans membrane domains. ABCC1 also has five
extra transmembrane domains, the function of which is unknown (Sharom et al, 2008). The nature of the ABCC1 binding site pocket is speculated to be in two parts, in order to permit the binding of both a hydrophobic substrate and an anion such as glutathione. The important substrate drug binding pocket interactions are likely to involve the transmembrane domains 10, 11, 16 and 17 (Campbell et al, 2004).

ABCG2 contains only 6 transmembrane domains with a solitary NBD. It is likely the half transporter forms homodimers to form a complete structure at the membrane in order to allow substrate translocation. The ABCG2 drug binding pocket mechanism is similar to PgP, however in contrast to PgP there are two symmetrical drug binding sites which can facilitate simultaneous substrate translocation (Clark R et al, 2006).

1.5.4 Transport mechanism

The movement of a substrate from the cytoplasm to the extracellular side involves two related processes. The first process is energy production derived from the hydrolysis of ATP into ADP and Pi; this is termed the catalytic cycle. The second process is the movement of the substrate to the extracellular side, termed the substrate transport cycle. There is still controversy over the precise function of the NBD. It is accepted NBDs are important in binding the ATP molecule and initiating hydrolysis, but they may be involved in the transport cycle and there are suggestions they are not functionally identical (Gao et al, 2000).
The binding of ATP to the NBD structures is of low affinity, thus allowing the interaction of two NBDs with one ATP molecule and ultimately leading to nucleotide sandwich. There is preferential binding of a particular ATP to the NBD, this ATP molecule is then hydrolysed and the ADP and Pi are released from the NBD. This same NBD then binds to another ATP, and hydrolysis of the partner ATP commences to produce energy. It is not understood how the two NBDs communicate with each other (Senior et al, 1995).

The transport of a substrate involves initial high affinity binding to the drug binding pocket. This high affinity binding then triggers conformation changes in the protein structure which is driven by ATP hydrolysis. In this way, the substrate is progressively moved to a lower affinity binding site and eventually effluxed into the extracellular side. This process has been modelled on changes observed by fluorescence spectroscopy (Liu et al, 1996). This has been confirmed only for PgP.

### 1.5.5 Clinical significance

ABC drug transporters have been implicated in multidrug resistance in cancer. Numerous cancers express increased levels of PgP and ABCG2, and this has been associated with poor responses to chemotherapy. Certain SNPs in the PgP protein are associated with inflammatory bowel disease and colon cancer (Potocnik et al, 2004; Osswald et al, 2007).
1.6 The role of drug transporters in CML

1.6.1 Imatinib

As mentioned previously, there are a number of factors which have been shown to cause imatinib resistance in CML patients. Classical human drug transporters control the influx and efflux of xenobiotic drugs into CML cells. This dynamic interplay between influx and efflux pumps can have important clinical effects on the absorption, distribution and elimination of tyrosine kinase inhibitors in the small bowel, liver and kidney.

Imatinib is actively influxed into cells by hOCT1. This was demonstrated by reduced imatinib uptake with increasing concentrations of prazosin (inhibitor of OCT1 and OCT3), and procainamide and amantadine (which are known inhibitors of OCT1 and OCT2) in CEM cells. Imatinib uptake is an active process as suggested by increased imatinib influx at 37°C as compared to 4°C (Thomas et al, 2004). Studies by other groups have been able to reproduce the importance of hOCT1 and have also revealed that the variation in clinical IC50 imatinib patient samples is correlated to imatinib influx and retention. Higher intracellular concentrations of imatinib result in decreased phosphorylation of Crkl (White et al, 2006). KCL22 cells transfected with hOCT1 also had increased uptake of imatinib compared to cells transfected with an empty vector (Wang et al, 2008). All three studies have identified the key role hOCT1 plays in imatinib uptake.

Numerous investigators have studied whether variable expression of hOCT mRNA affects clinical outcome in CML patients. In a 30 patient CML study, the hOCT1 levels at baseline showed variability similar to normal samples. However, there was
a marked difference in baseline hOCT1 levels between patients achieving CCR at 12 months and those failing to achieve CCR at 12 months (Crossman et al, 2005). In a larger study of 70 CP-CML patients, those with a high baseline hOCT1 had a superior progression free and overall survival compared to patients with low baseline hOCT1 levels. Univariate analysis showed hOCT1 expression at diagnosis was the only factor of importance in achieving CCR (Wang et al, 2008). The achievement of MMR has also been associated with baseline hOCT1 levels and patient adherence to imatinib (Marin et al, 2010).

Unfortunately, some investigators have been unable to correlate hOCT1 expression to treatment outcome. In a sample of 46 patients in CP-CML, patients with high and low OCT1 expression levels at diagnosis had no difference in MMR rate, EFS or BCR-ABL mutations. Patients in the lowest quartile for hOCT1 mRNA expression did not have an inferior overall or progression free survival (White et al, 2010). The explanation for this could be due to post transcriptional modifications, change in transcriptional activity due to bile acids and aberrant membrane localisation for hOCT1. The primers used to detect hOCT1 mRNA levels can also influence results as some primers do not cover the common OCT1 SNP M420del. OCT1 activity is a functional assay which is based upon the uptake of imatinib intracellularly with and without prazosin (White et al, 2006). OCT1 functional activity is associated with achievement of MMR, CCR, and overall and progression free survival at 5 years. Patients in the lowest quartile for OCT1 activity had reduced overall survival and were more likely to progress to blast crisis. At 5 years only 43% in the lowest quartile group achieved MMR (White et al, 2010).

Numerous single nucleotide polymorphisms (SNPs) have been associated with OCT1 and have an effect on substrate translocation. In a large 336 patient study of newly diagnosed CP-CML patients, the effects of OCT1 SNPs were evaluated. The
SNP M420del was associated with increased risk of treatment failure. However, M420del had no effect on PFS or EFS. Functionally, there was reduced imatinib uptake in M420del cell lines, but this was negated if M408V was also present (Giannoudis et al, 2013). In a 229 patient study, the SNP L160F was correlated to treatment failure and loss or response (Kim et al, 2009). Other studies have shown the SNP G401S, with a frequency of 4.5%, is associated with molecular response in imatinib treated patients (Bazeos et al, 2010). In a 136 patient study, there was no association between OCT1 SNPs and clinical outcome (White et al, 2010).

Several studies have confirmed imatinib is a substrate for ABCB1. In an ABCB1 over expressing MDCK cell line, there was increased imatinib transport from the basal to the apical membrane as opposed to the apical-basal direction, but this was reduced on addition of the PgP inhibitor PSC-833 (Thomas et al, 2004). Other groups have also confirmed this finding (Giannoudis et al, 2008; Mahon FX 2003). However, whether imatinib is a substrate for ABCG2 is controversial. Saos2 cells with overexpression of ABCG2 had no significant effect on intracellular retention or efflux, suggesting imatinib is not transported by ABCG2 (Houghton et al., 2004). Imatinib has also been demonstrated to be an inhibitor of ABCG2, with imatinib causing intracellular accumulation of mitoxantrone. In CD34+ stem cells ABCG2 inhibition did not alter imatinib intracellular retention. This would again suggest ABCG2 is not involved in imatinib transport (Jordanides et al, 2006). However, another group of investigators demonstrated ABCG2 transduced K562 cells had reduced imatinib intracellular accumulation and were viable in imatinib containing media. However, when PSC833 was added to media the cells were sensitive to imatinib and underwent apoptosis. This study suggests imatinib is a substrate for ABCG2, but is concentration dependent (Brendel et al, 2007). The baseline mRNA expression levels of ABCB1, ABCG2 and ABCC1 have no effect on progression free or overall survival (Wang et al, 2008).
1.6.2 Nilotinib

Nilotinib is a lipophilic drug and hOCT1 inhibitors have no impact on nilotinib uptake, suggesting that it does not require hOCT1 to enter the cell. Indeed, nilotinib uptake does not vary with different temperatures suggesting nilotinib uptake is not actively influxed. However, studies have suggested nilotinib can alter the hOCT1 drug transporter. This was evidenced by reduced imatinib and TEA uptake in the presence of nilotinib. This group also showed nilotinib is not a substrate for ABCB1, ABCC1 and ABCG2. However, nilotinib can reduce the efflux efficiency of ABCB1 and ABCG2 (Davies et al, 2009). Other groups have demonstrated nilotinib to be a substrate for ABCB1, with overexpressed cell lines having lower nilotinib concentrations intracellularly. Nilotinib transport was reduced on addition of verapamil, an ABCB1 inhibitor. Nilotinib was not a substrate for ABCG2 (Eadie et al, 2013). Other studies have shown nilotinib interacts with ABCG2 and is transported at subtherapeutic concentrations in the 10-25nM range (Brendel et al, 2007). However, higher nilotinib concentrations did not interact with ABCG2.

1.6.3 Dasatinib

Dasatinib is a lipophilic drug and is a substrate for a number of drug transporters. Studies have shown higher dasatinib uptake in cells overexpressing hOCT1. However, inhibitors of hOCT1 had no effect on dasatinib uptake and did not reduce intracellular dasatinib concentrations. This would suggest dasatinib uptake is independent of hOCT1 (Giannoudis et al, 2008). This result has been confirmed by other groups, with dasatinib uptake having no correlation to OCT1 functional activity.
Dasatinib appears to be a substrate for ABCB1, with MDCK overexpressing ABCB1 showing increased basal to apical transport of dasatinib. This was significantly reduced with the addition of PSC833, an inhibitor of ABCB1 (Giannoudis et al., 2008). Other groups have used K562-DOX cell lines, which overexpress ABCB1. The IC50 for dasatinib was significantly increased in the K562-DOX cell line compared to the parental K562 cell line, but on addition of PSC833 the IC50 of dasatinib for K562-DOX was significantly reduced. This is further evidence that ABCB1 transports dasatinib. This group has also demonstrated dasatinib is a substrate for ABCG2 (Hiwase et al., 2008).
1.7 Aims

Studies using a Xenopus oocyte model suggest OCT1 may be a surrogate marker of expression for other clinically relevant drug influx and efflux pumps, in particular OATP1A2, OCTN1 and OCTN2. Moreover, not all patients with low hOCT1 are destined to fail imatinib. This might suggest other drug transporters, such as OATP1A2, OCTN1 and OCTN2 may have an important clinical role in imatinib uptake (Hu et al, 2008). This needs to be further investigated. This thesis will:

1. Define the real life magnitude of imatinib resistance as a clinical problem, by assessing the response to imatinib in CML patients in a general population, and the population impact of second generation TKIs on imatinib intolerant/failure patients

2. Assess the role of OATP1A2 in imatinib uptake, and identify whether baseline OATP1A2 levels predict clinical outcome in CML.

3. Assess the role of OCTN1 and OCTN2 in imatinib uptake.

4. Assess whether coadministration of hOCT1 substrates can lead to reduced imatinib uptake at pharmacologically relevant concentrations.
2. A population study showing that the advent of second generation tyrosine kinase inhibitors has improved progression-free survival in CML

2.1 Introduction

The IRIS study was a phase 3 trial demonstrating high complete cytogenetic response (CCR) rates with imatinib treated patients. However, subsequent studies have suggested the responses to imatinib are less impressive. Lucas et al showed only 41% of imatinib treated patients achieved a CCR equivalent (CCR equivalence, defined either conventionally by metaphase analysis or by BCR-ABL/ABL transcript ratio less than 1%) at 12 months (Lucas et al, 2008). Recent studies have suggested patients treated with imatinib have high discontinuation rates. The six year follow up of imatinib treated patients in the IRIS study showed that only 66% of patients randomised to the imatinib arm were still receiving the drug. Patients discontinued imatinib principally due to inadequate response to imatinib (12%), withdrawal of consent (6%) or adverse events (4%) (Deininger et al, 2009). In a large single centre study the 5 year probability of being in cytogenetic remission while remaining on imatinib was 62.7% (de Lavallade et al, 2008).

In imatinib failure, the second generation TKI dasatinib and nilotinib are associated with durable haematological, cytogenetic and molecular responses (Talpaz et al, 2006 and Kantarjian et al, 2007). With dasatinib treatment in 1067 chronic phase patients with resistance or intolerance to prior imatinib, the 24-month rate of major molecular response (MMR) was 34% in patients with resistance or suboptimal response and 63% in imatinib-intolerant patients (Hochhaus et al, 2009). In a randomised study in 150 patients failing standard dose imatinib (400 mg daily),
dasatinib produced higher CCR and MMR rates than high dose (800mg daily) imatinib (Kantarjian et al, 2007). Similarly for nilotinib, 44% of patients with imatinib resistance or intolerance achieved CCR with nilotinib, and 84% of these were maintained at 24 months of treatment (Talpaz et al, 2006).

However, these and other studies have focused on patients who enter clinical trials or who are referred to specialised institutions. Such patients tend to be younger than the general population and have fewer intercurrent illnesses that may modulate treatment decisions, and in addition some clinical trials may specifically exclude patients with, for example, cardiac, renal or hepatic dysfunction, even if mild. Clinical trial results may therefore overestimate the benefit of a treatment in the general population.

Many of the patients in these studies failed imatinib at a time when second generation TKI were not generally available. It would therefore be helpful to know the impact of second generation TKI on CML in a more recent general population, for whom these drugs were potentially available. Here I report on the impact of second generation TKI by updating our earlier regional population study to include the era when these drugs have been readily available in our geographical region.

2.1.1 Aims

1. Determine the CCR and MMR rates for patients treated with first line imatinib
2. Determine the outcome of patients switched from imatinib to second generation TKIs for intolerance/failure
3. Determine the prognostic value of the Sokal score in the TKI era
4. Assess the progression free and overall survival in first line imatinib treated patients pre and post 2005 (the time when second generation TKIs became available in our geographical area)

2.1.2 Patients and methods

The study was undertaken in the Merseyside, Cheshire, and North Wales areas of the UK and the Isle of Man. This is a geographically adjacent area, comprising a population of 2.3 million people, who receive their haematological care at 12 National Health Service hospitals that work together as a network. The Liverpool Research Ethics Committee took the view that the study was an audit of clinical practice rather than a research investigation; approval was also given for the study on behalf of the network by the Royal Liverpool University Hospital Audit Committee.

All CML patients that were newly diagnosed between 1st January 2003 and 31st December 2009 were included in this study, enabling at least 18 months of follow up on all patients. Considerable effort was made to include all cases of CML diagnosed in the 7 year period of study. This was achieved by obtaining a list of patient names from consultants at each hospital, contacting the 3 regional cytogenetic laboratories serving the 12 hospitals at the time (in Liverpool, Manchester and Cardiff) and also checking records at the BCR-ABL1 transcript monitoring centre at the Royal Liverpool University Hospital. During this time period, imatinib 400mg daily has been generally available as first line treatment throughout the area of the study. Second generation TKI became available for second line treatment (i.e. imatinib resistance or intolerance) from late December 2005. First line treatment with second generation TKI has also been available since September
2007, either nilotinib within the ENESTnd trial (Saglio et al, 2010) from September 2007 to September 2008, or dasatinib since then, within the recently completed UK SPIRIT2 study. The study cut off of January 2005 was arbitrarily used to compare the effect of second generation TKIs pre and post the free availability of second generation TKIs.

2.1.3 Definitions of response and progression free survival

The response to treatment was defined according to the European LeukemiaNet recommendations (Baccarani et al, 2009), using conventional definitions of CCR (no Philadelphia positive metaphases amongst at least 20 G-banded metaphases) and MMR (BCR-ABL1/ABL1 transcript ratio of \( \leq 0.1\% \)). This was done with cytogenetic and molecular data at 12 months and with molecular data at 18 months for all patients, and at subsequent 6 monthly intervals for those with longer follow up. In some cases, serial cytogenetic data were not available. When this arose, the cytogenetic remission status was determined indirectly by a BCR-ABL 1 transcript ratio of <1%. This has been previously shown to correlate with CCR defined by cytogenetics in a 43 patient study by Wang et al (2002), where all patients achieving a cytogenetic CCR had BCR-ABL/ABL ratio \( \leq 1\% \). This confirms the BCR-ABL1/ABL1 ratio can be used as a surrogate marker of marrow cytogenetic responses, allowing distinction between MMR, CCR and no CCR (Wang et al, 2002). Where the molecular ratio of <1% has been used to define CCR equivalence, this is denoted by CCR\textsubscript{e}.

For the calculation of progression-free survival, death from any cause and the development of accelerated phase or blast crisis were regarded as events; loss of cytogenetic, molecular or haematological response were not considered as events.
provided the patient remained in chronic phase. SPSS 18.0 was used to calculate overall survival and progression free survival, by the Kaplan-Meier method.

2.2 Results

2.2.1 Epidemiology

Over the 7-year recruitment period, 192 patients (93 male; 99 female) were diagnosed with CML. This gives an annual incidence of 1.34 cases per 100,000 population. Their mean age was 55.0 years (range 18.7 – 94.3). The mean time from diagnosis to commencement of imatinib was 20.3 days (range 1-131 days). Almost all patients received 400mg daily; only three patients received 800mg. Sokal scores were available for 174 patients, with 51, 69 and 54 patients having low, intermediate and high Sokal scores respectively.

2.2.2 Patient outcome between diagnosis and 24 months

There were 192 new cases of CML diagnosed between January 2003 and December 2009. Of the initial 192 patients, 8 patients presented in blast crisis at diagnosis (5 myeloid blast crisis and 3 lymphoid blast crisis) and they were treated with acute leukaemia-style therapy, of whom 2 died during chemotherapy induction and the other 6 proceeded to allogeneic stem cell transplantation (3 of whom died of CML relapse and 3 patients who are still under regular follow up). One additional presented in accelerated phase and progressed rapidly. A total of 183 patients presented in chronic phase CML. Three patients, all aged over 77 years old, were treated with hydroxycarbamide, though 1 patient was eventually switched to
imatinib 3 years later; 2 patients died of blast crisis and 1 patient died of heart failure. A further 20 patients received either nilotinib or dasatinib as first line therapy, after entry into ENESTnd or SPIRIT2 respectively. The disposition of the study population up until 24 months is given in Figure 2.1.

Figure 2.1 Outcome of all patients, from presentation until 24 months of treatment.
2.2.3 Outcome of first line imatinib treated between diagnosis and 60 months

A total of 160 patients were therefore started on imatinib. Their serial outcome is given in Figures 2.1 and 2.2 (BC= blast crisis; HC= hydroxycarbamide; FU= follow up. Other abbreviations are as defined in the text). During the first 12 months, 20 patients had to discontinue imatinib. Five patients progressed to blast crisis, 1 patient developed and died of a biopsy- proven cerebral glioma at 5 months of imatinib treatment, and 1 patient discontinued imatinib due to dementia. Eight patients stopped because of intolerance and 5 because of resistance with rising BCR-ABL1 transcript levels; all 13 of these switched to a second generation TKI.

One hundred and forty patients were therefore assessable for response after 12 months of imatinib treatment. At this time, 94 (59%) patients achieved at least CCR, of whom 51 (32%) patients had attained an MMR and 43 (27%) had achieved CCR (but not MMR). Forty-six (29%) patients failed to achieve a CCR at 12 months (the remaining 20 of the original 160 cases failed for the reasons given above).

Of the 94 patients in at least CCR at 12 months, by 18 months 64 of these achieved MMR, 19 remained in CCR but not MMR, 3 lost their CCR and 2 had developed imatinib intolerance and were switched to a second generation TKI. Six patients were under ongoing follow up. Of the 46 patients who were not in CCR at 12 months, at 18 months 5 patients had switched to a second generation TKI, 3 underwent SCT, and 1 progressed to accelerated phase and died of pseudomonas septicaemia. Seven patients had achieved MMR, 7 achieved CCR (but not MMR) and 19 patients had not achieved CCR. Four patients had ongoing follow up.

Similarly, between 18 and 24 months of imatinib treatment, 4 patients switched to a second generation TKI, 1 progressed to blast crisis and another died of heart
failure. Between 24 and 36 months, 7 patients required a second generation TKI for failure or intolerance, 1 progressed to blast crisis, 1 lost haematological response and required SCT and 2 elected to stop treatment, though the imatinib was later restarted as the BCR-ABL1 transcript level increased. Between 36 and 48 months, 2 patients switched to a second generation TKI, and 1 died of metastatic breast cancer. Between 48 and 60 months, 2 patients switched to a second generation TKI and 1 died of pneumonia. These later patient outcomes are summarised in Figure 2.2.
Figure 2.2 Continuation from Figure 1, showing outcome from 24 until 60 months of treatment.
2.2.4 CCR and MMR rates for imatinib treated patients

The overall rates of MMR from 12 to 60 months are given in Table 2.1. These were 49%, 50%, 44% and 38% at 24, 36, 48 and 60 months respectively. Table 2.1 also gives the rates of CCR over these time intervals. At 12 months this was 59% (94 of 160), and during the entire 60 months of study it was maximal at 65% (93 of 144) at 24 months.

Table 2.1. Rates of MMR, CCR but without MMR (labelled CCR) and absence of complete cytogenetic remission (labelled no CCR) by time, for all 160 chronic phase patients who commenced on imatinib. Figures in parentheses denote percentages of patients achieving each response.

<table>
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<tr>
<th>Months from diagnosis</th>
<th>12</th>
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2.2.5 Comparison of CCR and MMR rate between first line imatinib and first line dasatinib or nilotinib

As previously described above, 160 chronic phase CML patients were treated with first line imatinib. One hundred and four (65%) patients treated with imatinib first line achieved a CCR by 60 months. At 60 months 81 (50.6%) patients achieved a MMR while on imatinib.

Twenty patients were treated with either dasatinib or nilotinib as first line treatment for chronic phase CML. Seventeen patients (85%) achieved a CCR at 12 months, while the 3 patients failing to achieve CCR were switched to other TKIs. Twelve patients achieved a MMR at 18 months. At 60 months 13 patients (65%) had achieved a MMR. The figures below compare the cumulative incidence of CCR and MMR between patients treated with either imatinib as first line treatment or a second generation TKI as first line treatment for chronic phase CML. Figure 2.3 shows the CCR rates are higher in the group treated with second generation TKI as first line (p<0.05). Figure 2.4 shows the MMR rates showed a trend towards superior MMR rates with second generation TKIs.
Figure 2.3. The cumulative CCR rates for first line imatinib treated patients compared to first line second generation TKIs in chronic phase CML.
Figure 2.4 The cumulative MMR rates for first line imatinib treated patients compared to first line second generation TKIs in chronic phase CML.
2.2.6 Second generation TKIs

During the period of study, 33 patients changed from imatinib to a second generation TKI. This was either dasatinib or nilotinib; no patients in the study received bosutinib or ponatinib at any time. The reason for switching was either imatinib intolerance, defined as grade 3 or 4 toxicity requiring discontinuation of drug for more than 14 days (15 patients) or failure to achieve cytogenetic targets within the ELN recommendations (18 patients). The average length of time on imatinib prior to switching to a second generation TKI was 20.9 months (range 2.4 – 47.9). Figure 2.5 gives the serial outcomes in these 33 patients. At 12 months after switching treatment, 21 (70%) of the 30 assessable had achieved MMR, 4 reached CCR or but not MMR (3 of whom achieved MMR by 18-36 months) and 5 had not reached CCR (1 of whom later achieved CCR, 1 remained unchanged, and the remaining 3 progressed to blast crisis). The serial MMR and CCR-only rates with time are given in Table 2.2. Overall, second generation TKI were able to produce MMR in 73% (24/33) of patients requiring them, and no patient who reached MMR has lost this at latest follow up.
Figure 2.5 Outcome of the 33 patients who were switched from imatinib to a second generation TKI.
Table 2.2. Rates of MMR, CCR without MMR and absence of CCR by time, for the 33 patients who switched from imatinib to a second generation TKI. Figures in parentheses denote percentages.

<table>
<thead>
<tr>
<th>Months from diagnosis</th>
<th>12</th>
<th>18</th>
<th>24</th>
<th>30</th>
<th>36</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>30</td>
<td>24</td>
<td>20</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>MMR</td>
<td>21 (70)</td>
<td>17 (71)</td>
<td>13 (65)</td>
<td>11 (69)</td>
<td>10 (67)</td>
</tr>
<tr>
<td>CCR only</td>
<td>4 (13)</td>
<td>3 (13)</td>
<td>3 (15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No CCR</td>
<td>5 (16)</td>
<td>2 (8)</td>
<td>1 (5)</td>
<td>2 (13)</td>
<td>1 (7)</td>
</tr>
</tbody>
</table>

2.2.7 Outcomes according to Sokal score

A total of 183 patients presented in chronic phase. They were treated with either first line imatinib (n=160), first line second generation TKI (n=20), or hydroxycarbamide alone (n=3). In this group 52 patients, 68 patients and 45 patients had high, intermediate and low Sokal scores respectively. Eighteen Sokal scores were unavailable, and hence these patients were excluded, as were patients presenting in blast crisis. Figure 2.6 panel A shows the progression-free survival of the 183 patients presenting in chronic phase according to Sokal score. Twenty five patients progressed to blast crisis/accelerated phase or death (one patient had no Sokal score). None of the patients treated with second generation TKI as first line treatment progressed. There is a significant difference between outcome for low vs. intermediate vs. high risk score using the Mantel-Cox log rank test (p =0.022), and
superior outcome for low versus high scoring patients (p = 0.008). Of the 183 patients 22 patients died. Figure 2.6 panel B shows similar findings for the effect of Sokal score on overall survival (p = 0.046 overall; low vs. high scoring patients = 0.016).

Figure 2.6 Progression free and overall survival according to Sokal score for all 183 patients in chronic phase. Panel A gives progression free survival, demonstrating significant differences according to Sokal score (p=0.022). Panel B shows significant differences in overall survival according to Sokal score (p=0.046).
2.2.8 The impact of second generation TKIs

Second generation TKI became available in our geographical area in late December 2005. Therefore, a patient diagnosed on or after January 1st 2005 who was not in CCR at 12 months was able to receive a second generation drug promptly, whereas someone diagnosed before this date was not. In order to examine the clinical impact of the availability of second generation drugs, the outcome of treatment was stratified according to the date of diagnosis. There were 160 patients treated with imatinib in chronic phase, with 45 patients diagnosed before January 2005 and 115 patients after January 2005. The pre and post 2005 groups are comparable for age, sex and Sokal score distribution (see Table 2.3). Of the 45 patients diagnosed before 2005, 12 patients (26.7%) had disease progression, while only 10 patients (8.7%) diagnosed after 2005 progressed. Figure 2.7 gives the progression-free survival, stratified according to diagnosis before or after 1st January 2005. This is significantly superior in the cohort diagnosed after 1st January 2005 compared to those diagnosed before this date (p = 0.022; log rank test). It is acknowledged that this cut-off date is rather arbitrary; however, moving this date forward or backward by 3 months makes no difference to the findings. The overall survival was shown not to be significant between the patients diagnosed before and after January 2005.
Table 2.3. Characteristics of the 160 patients in chronic phase CML treated with imatinib as first line therapy

<table>
<thead>
<tr>
<th>Variables</th>
<th>Pre 2005</th>
<th>Post 2005</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Mean, Standard deviation)</td>
<td>53.2(15.0)</td>
<td>55.5(16.2)</td>
<td>p=0.41</td>
</tr>
<tr>
<td>Sokal scores n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>16 (35.6%)</td>
<td>23 (20%)</td>
<td>P=0.21</td>
</tr>
<tr>
<td>Intermediate</td>
<td>16 (35.6%)</td>
<td>42 (36.5%)</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>11 (24.4%)</td>
<td>34 (29.6%)</td>
<td></td>
</tr>
<tr>
<td>Missing</td>
<td>2 (4.5%)</td>
<td>16 (13.9%)</td>
<td></td>
</tr>
<tr>
<td>Sex n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>29 (64.4%)</td>
<td>56 (48.7%)</td>
<td>p=0.08</td>
</tr>
<tr>
<td>Males</td>
<td>16 (35.6%)</td>
<td>59 (51.3%)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.7 Progression-free survival according to diagnosis date. Patients diagnosed from 2005 onward had an opportunity to receive second generation TKI from 12 months onward if failing imatinib, and show superior progression-free survival ($p = 0.022$).
2.3 Discussion

The CCR rate at 12 months in patients treated with imatinib was 59%, with a maximal CCR rate, during the 5 year observation period, of 65%. The maximal MMR rate was 50% at 36 months. These results are lower than the results published in phase 3 clinical trials such as the IRIS study. Patients intolerant or resistant to imatinib first line had an 83% CCR rate at 12 months. This suggests that second generation TKIs can improve outcomes in imatinib intolerant/resistant patients. This is supported by improved progression free survival and significantly faster time to CCR in patients diagnosed in or after 2005, who could be treated with either nilotinib or dasatinib if intolerant/resistant to imatinib.

The Sokal score which was initially used as a scoring system in the pre TKI era still has an important prognostic value in the TKI era. High Sokal scores are associated with inferior progression free survivals.
3. SLCO1A2 facilitates the influx of imatinib but levels do not predict outcome

3.1 Introduction

The organic anion transporting polypeptide (OATP) family of influx drug transporters actively transports numerous clinically relevant drugs and endogenous substrates (Niemi, 2007). The human OATP family has twelve transmembrane domains, and hydropathy analysis has suggested these drug transporters have a large fifth extracellular loop (Hagenbuch et al, 2003). The OATP1 family of proteins is encoded by the SLCO family of genes. These genes are located on the short arm of chromosome 1. There have been a number of single nucleotide polymorphisms identified in the SLCO family of genes (Niemi, 2007). These SNPs may have a significant role in drug transport, potentially leading to altered pharmacokinetics and reduced drug transport (Konig et al, 2006).

OATP1A2 has a wide tissue distribution and is predominately found in the liver, intestines, kidney and brain (Kullak-Ublick et al, 1995; Glaeser et al, 2007). Steroid hormones, bile salts and thyroid hormones have been shown to be substrates for OATP1A2 (Bossuyt et al, 1996; Fujiwara et al, 2001). OATP1A2 has also been implicated in the transport of xenobiotic drugs and has been shown to transport imatinib into cells. OATP1A2 mRNA expression levels have been shown to be positively correlated with hOCT1 level (Hu et al, 2008).

Resistance to imatinib is potentially mediated by altered pharmacokinetics leading to reduced drug exposure. There is variation in imatinib plasma exposure between
individuals, and this may cause low intracellular imatinib levels in non-responding patients (Nikolova et al, 2004). This has implicated drug transporters as potential variables between individuals accounting for these variations in imatinib trough levels.

### 3.1.1 Aims

The aim of the chapter was to

1. Generate stable cell lines expressing high levels of OATP1A2
2. Determine whether imatinib is a substrate for OATP1A2
3. Assess the mRNA expression levels of OATP1A2 at diagnosis (i.e. prior to TKI treatment) in patients with CML and determine whether this affected outcome.

### 3.2 Methods

#### 3.2.1 Transfection of KCL22 cell line with SLCO1A2

##### 3.2.1.1 Generation of pcDNA3.1-SLCO1A2 and plasmid purification

The SLCO1A2 coding sequence (NM_134431.3) was cloned from human brain cDNA (Human Total RNA Master Panel II, Takara Bio Europe/Clonatech, Saint-Germain-en-Laye, France) and inserted into the pcDNA3.1 vector (Invitrogen GmbH, Karlsruhe, Germany). Human embryonic kidney cells (HEK 293) were then
transfected with pcDNA3.1-SLCO1A2. The HEK pcDNA3.1-SLCO1A2 clone was a kind gift from Prof M Fromm (Erlangen, Germany).

3.2.1.2 Plasmid purification

The PureYield Plasmid Miniprep System (Promega UK, Southampton) was used to obtain purified plasmid pcDNA3.1-SLCO1A2. The product components included Cell lysis buffer, Neutralization solution, Column wash solution, Endotoxin removal wash, Elution buffer and PureYield minicolumns and collection tubes. The manufacturer's protocol was followed, as outlined briefly below.

100µl of cell lysis buffer was added to 600µl of HEK pcDNA3.1-SLCO1A2 in a 1.5ml microcentrifuge tube. 350µl of Neutralisation solution was added and vigorously mixed. The solution was centrifuged at 300g for 3 minutes. The supernatant (900µl) was then placed in a PureYield Minicolumn, and centrifuged at 300g for 3 minutes. Endotoxin removal wash was then added to the column and centrifuged at 300g for 15 seconds. 400µl column wash solution was then added to the minicolumn and centrifuged for 30 seconds. Elution buffer (30µl) was added to the minicolumn and centrifuged for 15 minutes. The eluted plasmid DNA was stored at -20°C. The plasmid DNA 3.1 was sequence verified to contain the cDNA of SLCO1A2 gene (gene of interest). The sequence was verified by Source Bioscience (Nottingham, UK) using forward 5’-AAGACCAACGCAGGATCCAT-3’ and reverse 5’-GAGTTTCACCCATTCCACGTACA-3’ primers.
3.2.1.3 Nucleofection of KCL22 cell line using AMAXA standard nucleofector kits

The KCL22 cells were passaged 1 day prior to nucleofection aiming to achieve a concentration of $1 \times 10^6$ cells/ml. Only cells with a viability of at least 85% were used for transfection. Cell viability was determined using the countess chamber. 10µl of KCL22 cells growing in supplemented RPMI (Sigma Aldrich, Gillingham, UK) were mixed gently with 10µl of 0.4% trypan blue. The 20µl solution was then placed in the counting chamber (Invitrogen, UK).

Nucleofector solution was prepared by the adding 0.5 ml supplement (from Amaxa standard Nucleofector kits) to 2.25 mls of Nucleofector solution (AMAXA Biosystems, Cologne, Germany). The solution was mixed gently and stored at 4°C.
3.2.1.4 The nucleofection process

1x10^6 KCL22 cells suspended in RPMI were centrifuged at 90xg for 10 minutes. The supernatant was then discarded leaving the cell pellet only. The cell pellet was resuspended in 100µl nucleofector solution at 24°C. The 100µl of resuspended cells were then added to either

1. Reaction 1: 1µg pmaxGFP
2. Reaction 2: 2.5µg pmaxGFP
3. Reaction 3: 1µg pcDNA3.1-SLCO1A2
4. Reaction 4: 2.5µg pcDNA3.1-SLCO1A2
5. Reaction 5: 1µg pcDNA3.1-Empty vector
6. Reaction 6: 200µl RPMI (control)

Reactions 1 and 2 were used to determine the optimal amount of pmaxGFP required for optimal transfection efficacy. All nucleofector kits have a plasmid encoding maxGFP. This is a green fluorescent protein which can be visualised under confocal microscopy. Reactions 3 and 4 were used to determine which concentration of pcDNA3.1-SLCO1A2 resulted in the best transfection of pcDNA3.1-SLCO1A2 into the KCL22 cell line. Reaction 5 enabled growth and culture of the mock transfected cell line. Reaction 6 was the control.

The samples were placed in an Amava certified cuvette. Program T19 was selected as this had previously been shown by Dr Athina Giannoudis to have the most efficient transfection capacity for KCL22 cells. After completion of the T19 program, 500µl of prewarmed RPMI (containing 10% foetal calf serum supplements) was added to the cuvette. The samples were then transferred to a 12 well plate containing 1ml of culture medium (RPMI, 10% foetal calf serum). This procedure
was performed on all the reactions 1-6. The cells were then incubated in a humidified 37°C/5% CO₂ incubator.
3.2.1.5 Transfection efficiency

The transfection efficiency was determined by the percentage of KCL22 cells successfully transfected with the pmaxGFP. The cells were analysed by flow cytometry, with the physical characteristics of the transfected cells showing increased forward scatter due to their larger size compared to the parental KCL22 cells. Figure 3.1 shows at 6 and 24 hours post-transfection the average transfection efficiency was 24.87% and 54.7% respectively. At 48 hours the transfection efficiency was 79.22%.

Figure 3.1 Flow cytometry demonstrating transfection efficiency using cell characteristics on flow cytometry
3.2.1.6 Confocal microscopy

In order to carry out confocal microscopy, an aliquot of cells growing in each of the 12 well plates was taken. To this was added 10µl of 4% paraformaldehyde (PFA) (Sigma-Aldrich, Gillingham, UK). The 4% paraformaldehyde was prepared by adding 4g of paraformaldehyde to 90 mls of water and heating to 60°C. 10µl of NaOH was added along with 10ml of phosphate buffered saline (PBS). The cells and PFA are then centrifuged and the supernatant discarded. The cells were then washed in PBS twice and placed on a poly-L-Lysine slide. After 15 minutes, excess liquid was removed and the slide was placed on the confocal microscope.

Confocal microscopy demonstrated that the transfection had been effective. The cells which are green are successfully transfected KCL22 cells with the pmaxGFP. The cells staining red are KCL22 cells with unsuccessful transfection. Figure 3.2 shows the confocal appearances when KCL22 cells were transfected with 2.5µg of pmaxGFP (reaction 2), while Figure 3.3 shows the confocal appearances when KCL22 cells were transfected using 1µg of pmaxGFP (reaction 1). The higher concentration of pmaxGFP produced superior transfection efficiency. Figure 3.4 shows the confocal appearances when KCL22 cells were transfected using only RPMI (reaction 6). All the cells are red as there is no fluorescent green tag. In view of these finding cells from reaction 4 (2.5 µg of pcDNA3.1-SLC01A2), which had higher transfection efficiency were passaged with 100 µl of RPMI while all the other reactions were discarded.
Figure 3.2: Confocal microscopy images of reaction 2 (ie plasmid pcDNA3.1-SLCO1A2 2.5µg)

Figure 3.3: Confocal images of reaction 1 (ie plasmid pcDNA3.1-SLCO1A2 1µg)
3.2.1.7 Selection and cloning

After 48 hours the transfected cells (reaction 4 – using the higher plasmid pcDNA3.1-SLCO1A2 concentration) were placed into a 50ml tube and centrifuged at 400xg for 10 minutes. The 48 hour time point was associated with superior transfection efficiency (see Figure 3.1). The supernatant was discarded and the cells were resuspended in 10 mls of supplemented RPMI (1% L-glutamine, penicillin/streptomycin, 10% foetal calf serum and 1mg/ml G418). One ml of cell suspension and 1ml of G418 (with a concentration of 1mg/ml) was then added to 8mls of ClonacellCell-TCS. The ClonaCell-TCS medium is provided in the Amaxa Nucleofection kits and was allowed to warm to room temperature. The tubes were mixed by gentle inversion and allowed to stand for 10 minutes. 9.5 mls of the clonaCell-TCS, cell suspension and G418 solution were placed in 100mm petri dishes and incubated at 37°C in 5% CO₂ and 95% humidity. The plates were left for 7-10 days. After this period distinct colonies were identified and specific isolated
colonies were aseptically removed and placed in a 96-well plate containing 200µl of RPMI and G418 with a final concentration of 1mg/ml (Figure 3.5). The cells were then incubated at 37°C in 5% CO₂ for 2 days without feeding. A further 150µl of supplemented RPMI was added to the 96 well plates. As the cell density increased, the cells were transferred to 24 well plates after 7 days and then finally 6 well plates after a subsequent 7-10 days.

Figure 3.5 Macroscopic colonies of KCL22 cells transfected with pcDNA3.1-SLCO1A2. The colonies are then aseptically transferred and grown in RPMI supplemented G418. The parental cell lines will not form colonies as the clonaCell-TCS has G418 present.

The transfected KCL22 pcDNA3.1-SLCO1A2 clones were then assessed for cDNA expression of SLCO1A2 as described in the next section. The transfected pcDNA3.1-mock transfected cells (Reaction 5) were also passaged and used as a control for subsequent time course assays.
3.2.2 RNA isolation and cDNA synthesis from transfected cell lines

In total 20 stable cell lines of KCL22 cells transfected with SLCO1A2 were successfully grown and able to be analysed for SLCO1A2 expression, by RNA extraction and cDNA synthesis.

RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Valencia, California, USA). The transfected KCL22 clones were cultured in G418 containing RPMI medium. A cell concentration of $1 \times 10^6$/ml was required prior to RNA extraction. The cells were counted using the Invitrogen cell counting chamber (Invitrogen). The manufacturer's protocol was followed and is briefly described in this section. $1 \times 10^6$ cells were centrifuged at 300xg for 5 minutes. The supernatant was discarded and 350µl of RLT buffer was added to the cells and mixed thoroughly. The lysate was then transferred into a microcentrifuge and pipetted to remove cell clumps. The lysate was then transferred to a QIAshedder spin column and centrifuged for 2 minutes at 300xg. 350µl of 70% ethanol was then added to the lysate and mixed vigorously. The 700µl solution was then added to an RNeasy spin column with a collection tube and centrifuged for 15 seconds at 8000xg. The flow through was discarded. 700µl of Buffer RW1 was added to the RNeasy spin column, and centrifuged for 15 seconds at 8000xg and the flowthrough was discarded. 500µl of Buffer RPE was added to the RNeasy spin column and again centrifuged at 8000xg for 15 seconds. The washing with Buffer RPE was performed twice. The RNeasy spin column was then placed in a clean collection tube. 50µl RNase-free water was added to the spin column and centrifuged for 60 seconds at 8000xg to elute the RNA. The RNA concentration and purity was determined by adding 1µl to the Nanodrop 2000 (Thermo scientific, Paisley, UK). The RNA concentration varied between 120ng/ml to 280ng/ml.
3.2.3 cDNA synthesis

1µl of random hexamers (250ng/µl), 1µl dNTP mix (Promega, UK), RNA (1µG total) and DNase/RNase free water were gently mixed in a 0.5ml Eppendorf tube. The total volume was made up to 12µl. The reaction was incubated for 65°C for 5 minutes. The reaction was then placed on ice for 5 minutes and then spun down at 300xg. 4µl of 5x buffer reaction, 2µl 0.1M DTT and 1µl DNase/RNase free water were then added to the contents of the Eppendorf and incubated for 2 minutes at 25°C. Superscript II Reverse Transcriptase 2000U/ml (Invitrogen, UK) was then added to the solution and pipetted vigorously. The reaction was then incubated for 10 minutes at 25°C and then 50 minutes at 42°C, and finally 15 minutes at 70°C. The cDNA was then stored at -20°C. Following this procedure cDNA for all 20 KCL22 pcDNA3.1-SLCO1A2 clones were obtained.
3.2.4 TaqMan low density array and single gene expression assay (RT-PCR)

TaqMan Gene expression assays for SLCO1A2 were purchased from Applied Biosystems (Paisley, UK). This allows for the quantification of PCR product in real time. The Taqman assay consists of a custom made forward and reverse primer. The forward and reverse primer concentrations supplied by Applied Biosciences are 900nM. The TaqMan MGB probe was designed to cover an exon-exon junction (250nM). The ID number purchased for SLCO1A2 was Hs00366488_m1. The ID number purchased for GAPDH was Hs99999905_m1 (endogenous control).
The cDNA from the 20 pcDNA3.1-SLCO1A2 clones were quantified on the Nanodrop 2000. 100ng of cDNA was added to 1µl of 20X TaqMan Gene Expression Assay, 10µl 2X TaqMan Gene Expression Master Mix and RNase free water to make a total volume of 20µl. The reactions were run in triplicate. The expression of GAPDH was used as a control gene expression parameter. The reactions were loaded onto a 384-well plate. The plate was sealed and centrifuged for 10 seconds. The real time PCR amplifications were run in an ABI Prism 7900HT System (Foster City, California, USA) using the following conditions: 50°C for 2 minutes, 10 minutes at 95°C. This was then followed by 40 cycles of denaturation and annealing/extension at 95°C for 15 seconds and 60°C for 1 minute respectively.

The comparative C<sub>T</sub> method was used to measure the relative expression of a particular clone compared to the expression of GAPDH. The comparative method uses the 2<sup>ΔΔCT</sup> to give a relative quantification of SLCO1A2 expression compared to an endogenous control (GAPDH). The levels of these amplicons in the transfected KLC22-SLCO1A2 clones are compared to the parental KCL22. Table 3.1 gives the fold difference in the transfected SLCO1A2 clones compared to the KCL22 parental
cell line. The ΔΔC\textsubscript{T} values are also included. Figure 3.6 shows all 20 SLCO1A2 clones with their relative expressions of SLCO1A2 compared to the parental KCL22 cell line.
Table 3.1: The fold difference in the transfected cell lines of SLCO1A2 relative to parental KCL22 cell line.

<table>
<thead>
<tr>
<th>Sample</th>
<th>SLCO1A2 Average C_T</th>
<th>GAPDH Average C_T</th>
<th>ΔCT SLCO1A2-GAPDH</th>
<th>ΔΔCT Transfected clones – ΔC_T KCL22</th>
<th>Fold difference in SLCO1A2 relative to parental KCL22 cell line 2^ΔΔCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCL22 parental cell line</td>
<td>43.99</td>
<td>24.67</td>
<td>19.32</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Clone 1</td>
<td>33.22</td>
<td>21.98</td>
<td>11.24</td>
<td>-8.08</td>
<td>270.59</td>
</tr>
<tr>
<td>Clone 2</td>
<td>34.34</td>
<td>21.82</td>
<td>12.53</td>
<td>-6.8</td>
<td>111.43</td>
</tr>
<tr>
<td>Clone 3</td>
<td>31.92</td>
<td>20.49</td>
<td>11.43</td>
<td>-7.89</td>
<td>238.21</td>
</tr>
<tr>
<td>Clone 5</td>
<td>32.36</td>
<td>22.40</td>
<td>9.96</td>
<td>-9.36</td>
<td>657.18</td>
</tr>
<tr>
<td>Clone 6</td>
<td>33.93</td>
<td>21.70</td>
<td>12.23</td>
<td>-7.01</td>
<td>136.23</td>
</tr>
<tr>
<td>Clone 7</td>
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<td>26.77</td>
<td>15.87</td>
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<td>10.91</td>
</tr>
<tr>
<td>Clone 8</td>
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<td>20.53</td>
<td>13.69</td>
<td>-5.63</td>
<td>49.52</td>
</tr>
<tr>
<td>Clone 9</td>
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<td>14.42</td>
<td>-4.9</td>
<td>29.84</td>
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<tr>
<td>Clone 10</td>
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<td>13.89</td>
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</tr>
<tr>
<td>Clone 11</td>
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<td>11.7</td>
<td>-7.62</td>
<td>196.72</td>
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<tr>
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<td>17.33</td>
<td>-1.99</td>
<td>3.97</td>
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<tr>
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<td>21.84</td>
<td>17.24</td>
<td>-2.08</td>
<td>4.22</td>
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<td>11.4</td>
<td>-7.92</td>
<td>242.19</td>
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<td>Clone 15</td>
<td>33.85</td>
<td>23.02</td>
<td>10.83</td>
<td>-8.49</td>
<td>359.53</td>
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<tr>
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<td>30.93</td>
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<td>11.31</td>
<td>-8.01</td>
<td>317.36</td>
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<tr>
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<td>12.92</td>
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<td>84.44</td>
</tr>
<tr>
<td>Clone 18</td>
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<td>11.28</td>
<td>-8.04</td>
<td>263.19</td>
</tr>
<tr>
<td>Clone 19</td>
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<td>20.7</td>
<td>10.9</td>
<td>-8.42</td>
<td>342.51</td>
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<td>Clone 20</td>
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<td>10.07</td>
<td>-9.24</td>
<td>608.87</td>
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</tbody>
</table>
The highest expressing cell lines were Clone 4 and Clone 5. Unfortunately these clones had a reduced viability ranging between 60-70%. They were slow growing and eventually became infected. Clone 15 was successfully grown and cultured in RPMI with G418 at a concentration of 1mg/ml. Clones with expression levels greater than 400x that of KCL22 cell lines were not growing and had poor viability. Clone 15 was therefore used in the following radioactive uptake experiments. Clone 20 was initially used but was very slow growing and became infected.
Radioactive uptake experiments

[^3H] Estrone-3-sulphate (E-3-S) was purchased from PerkinElmer (Waltham, Massachusetts, USA). E-3-S is a known substrate of OATP1A2. The specific activity of E-3-S was 2.009 TBq/mmol. The final concentration of E-3-S used in uptake experiments was 2μM. [14C]-imatinib and [14C]-nilotinib were provided by Novartis, Basel with specific activities of 3.386 MBq/mg and 3.48 MBq/mg respectively. The final concentrations of imatinib and nilotinib were both 5μM. [14C]-dasatinib was provided by Bristol Myers Squibb Pharmaceuticals (New York, USA). Dasatinib had a specific activity of 1.18 MBq/mg; the final concentration of dasatinib used in experiments was 150nM. The final concentrations of imatinib, nilotinib and dasatinib were selected on the basis of known concentrations achievable in patient blood samples. All radioactive drugs were dissolved in transport buffer (1% HEPES in Hanks Buffered salt solution HBSS).

Naringin, a known inhibitor of SLCO1A2, was purchased from Sigma-Aldrich (Gillingham, UK). The naringin concentration used in inhibitor assays was 100μM. This concentration has been used in other publications.

Cells were supplemented with fresh RPMI prior to TKI radioactive uptake experiments. 1x10^6 cells were centrifuged at 3500 rpm for 2 minutes. The supernatant was discarded and the pellet resuspended in 500μl of transport buffer and kept in the incubator at 37.6°C, 5% CO2. Radioactive TKI with the appropriate amount of non-radioactive TKI was prepared in 500μl of transport buffer, with or without naringin. The cells were then centrifuged and the supernatant again discarded. The cell pellet was then added to 500μl of prepared TKI or E-3-S. The
cells were incubated for 0, 30, 60 and 120 minutes in duplicate. After the specific period of incubation, the cells were centrifuged at 3500 rpm and 200µl of the supernatant was added to 4ml of scintillation fluid (Meridian, Epsom, Surrey). The remaining supernatant was discarded without dislodging the cell pellet. The cell pellet was washed with 500µl of cold HBSS and then centrifuged at 6000 rpm for 3 minutes. This was repeated twice. After the second wash, 100µl of distilled water was added and vigorously pipetted. The cells were solubilised by the addition of 200µl of lysis buffer (10% sodium dodecanyl sulphate (SDS) and 1M sodium hydroxide). The cells were incubated for 1 hour and then the contents were transferred to 4 mls of scintillation fluid. The radioactivity was measured using a scintillation counter 1500 Tri Carb LS Counter Packard (Perkin Elmer, Waltham, Massachusetts, USA).

3.2.6 Cell culture

KCL22-SLCO1A2, KCL22-pcDNA3.1 (mock transfected) and KCL22 (parental cell line) cells were cultured in RPMI 1640 (Sigma Aldrich) and supplemented with 1% streptomycin/penicillin, 1% glutamine and 10% foetal calf serum. Cell viability was measured using the Countess cell counting chambers (Invitrogen). 10µl cell suspension was added to 10µl tryptan blue and placed in the Countess chamber. Radioactive uptake experiments were performed on samples with cell viabilities of greater than 85%.
3.3 Results

3.3.1 Radioactive uptake experiments with estrone-3 sulphate

Figure 3.7 shows the uptake of E-3-S into parental KCL22 cells, mock transfected cells, Clone 15 and Clone 20 at 37°C. The SLCO1A2 transfected clones had high expression of SLCO1A2 compared to the parental cell line. They were used to assess the functional activity of the clones. The results show clone 20 and clone 15 have functionally active OATP1A2 protein, which actively influxes E-3-S into the intracellular compartment. Clone 20 (highest expression of SLCO1A2) and clone 15 had a significantly higher E-3-S uptake compared to the unmanipulated and mock transfected cell line at 60 minutes (ANOVA, p<0.05). Clone 20 achieved maximal uptake of E-3-S at between 30-60 minutes. Clone 15 achieved maximal uptake of E-3-S at 60 minutes. Clone 20 had a higher E-3-S uptake at 30 minutes when compared to Clone 15; however this was not statistically significant (t-test, p=0.19).

The increased uptake of imatinib by the SLCO1A2 transfected clones is explained by higher functional OATP1A2 actively transporting imatinib into the cell. When the time course assay was performed at 4°C (Figure 3.8), there was no difference in imatinib transport between the parental KCL22, mock transfected and the SLCO1A2 clones (ANOVA, p=0.67). The mock transfected cell line and KCL22 were accumulating E-3-S by simple diffusion. Active transport achieves rapid accumulation of drug into the cell.
These results show that the stable SLCO1A2 clones 15 and 20 have been transfected correctly and are expressing functional OATP1A2 protein.

Figure 3.7: Time course assay for E-3-S uptake by clone 20, clone 15, mock transfected KCL22 cells and the KCL22 parental cell line at 37°C. Experiments were performed in duplicate and repeated on 3 occasions (mean with error bars corresponding to standard error).
Figure 3.8: Time course assay for E-3-S uptake by clone 20, clone 15, mock transfected KCL22 cells and the KCL22 parental cell line at 4°C. Experiments were performed in duplicate and repeated on 3 occasions.

3.3.2 The effect of naringin on E-3-S uptake by unmanipulated, mock transfected and clone 15 cell lines

Figure 3.9 shows the effect of the SLCO1A2 inhibitor naringin on the uptake of E-3-S into SLCO1A2 transfected cells. The reaction was performed at 1 hour incubation (maximal uptake of E-3-S) with and without naringin. The addition of naringin significantly reduces the uptake of E-3-S into the transfected clone 15 (t-test, p=0.03). This demonstrates that E-3-S influx into the transfected cell lines is via
active drug transport. There was no difference in E-3-S uptake when naringin was added to unmanipulated or mock transfected cell lines.

![E-3-S uptake with/without naringin](image)

Figure 3.9: E-3-S uptake at 60 minutes with or without the OATP1A2 inhibitor naringin. Experiments were performed in duplicate and repeated on 3 occasions.

### 3.3.3 Effect of pH and temperature on E-3-S uptake

The pH can influence drug transporter mediated uptake. The pH in the small bowel where absorption takes place is alkaline, while the pH of the stomach is acidic. Figure 3.10 shows acidic pH increases E-3-S uptake significantly compared to that at pH 8.0 (ANOVA, p=0.04). This shows OATP1A2 transport is pH dependent and could have clinical implications for imatinib transport and drug distribution.
3.3.4 Imatinib transport

A time course assay for imatinib uptake was performed using clone 15, mock transfected cells and unmanipulated parental KCL22 cells. Figure 3.11 shows clone 15 has greater imatinib uptake at the 60 minute time point when compared to mock transfected and unmanipulated cells at 37˚C (ANOVA, p=0.02). When the experiment is performed at 4˚C (Figure 3.12) there is no difference in imatinib uptake between the clone 15, mock transfected and unmanipulated cells (ANOVA, p=0.58). This shows imatinib is actively transported by OATP1A2.
Figure 3.11: Time course assay for imatinib uptake using SLCO1A2 transfected, mock transfected and unmanipulated cells at 37˚C. All experiments were performed in duplicate and repeated on 3 occasions.
3.3.4.1 Inhibitor studies on imatinib uptake

Figure 3.12: Time course assay for imatinib uptake using SLCO1A2 transfected, mock transfected and unmanipulated cells at 4˚C. All experiments were performed in duplicate and repeated on 3 occasions.

Figure 3.13 shows the effect of naringin incubation on imatinib uptake at 60 minutes. Naringin significantly reduces the uptake of imatinib (t-test, p=0.03). At 60 minutes the average imatinib uptake into the cells is 176 ng per million cells, but in the presence of naringin this is reduced to 136ng per million cells. This confirms that OATP1A2 may contribute to imatinib uptake.
3.3.4.2 Imatinib uptake into CML cell lines with high and low SLCO1A2 mRNA expression

The results of the imatinib time course assays confirm that imatinib is a substrate for OATP1A2. However, the effect of imatinib uptake by OATP1A2 in patient samples needed to be evaluated to assess whether OATP1A2 has an important clinical role in the pharmacokinetics of imatinib. Imatinib uptake was compared between primary CML cells with high (n=3) and low (n=3) SLCO1A2 expression. Low SLCO1A2 expression was defined as mRNA expression <0.02 (calibrated to GAPDH and normalised to healthy individuals), while high SLCO1A2 expression was defined as mRNA expression >0.08 (calibrated to GAPDH and normalised to healthy individuals). Unfortunately the cell viability of these cells was between 70-80%. This was markedly less than the viability of cell lines which were always
>85%. The imatinib uptake in patient samples with low SLCO1A2 was 53.7 ng per million cells (Figure 3.14) compared to 54.5 ng per million cells in samples with high SLCO1A2 expression (Figure 3.15, p=0.57). There was no difference in uptake between patients with high and low OATP1A2 expression (Figures 3.14 and 3.15).

![Imatinib uptake using primary CML cells with low SLCO1A2 mRNA expression](image)

**Figure 3.14**: Imatinib uptake in primary CML cells with low SLCO1A2 expression (n=3).

The addition of naringin had no effect in primary samples. Amantadine and verapamil are inhibitors of OCT1 and were used at 100µM concentrations. The addition of amantadine and verapamil also had minimal effect (all the primary cells studied with low and high SLCO1A2 levels had low OCT1 levels).
The imatinib uptake at 60 minutes in patient cells is less than the uptake of imatinib observed in the cell lines, most probably due to a combination of reduced cell viability of the patient cell samples and the primary cells having a varying number of mature neutrophils to promyelocytes.

### 3.3.5 Nilotinib transport

Nilotinib transport was assessed using clone 15 (high expressing SLCO1A2), the mock transfected cell line and the unmanipulated parental cell line. Figure 3.16 shows there is no difference in nilotinib uptake between the 3 cell lines (one way ANOVA, p=0.45). Nilotinib achieves a high concentration rapidly due to its lipophilic properties and does not require OATP1A2 to influx into the cell. Nilotinib uptake
intracellularly varied between 950ng-1050ng per million cells. This maximum uptake was achieved within 30 minutes.

Figure 3.16. Time course assay for nilotinib uptake using clone 15, the mock transfected cell line and the unmanipulated parental cell line. Experiments were performed in duplicate on 3 occasions.
3.3.5.1 The effect of naringin on nilotinib uptake

Figure 3.17 shows the effect of naringin on nilotinib uptake. It has no effect on nilotinib uptake, again confirming nilotinib is not a substrate for OATP1A2.

![Nilotinib uptake with/without naringin](image)

Figure 3.17: The effect of naringin on nilotinib uptake in KCL22, KCL22 mock transfected and SLCO1A2 transfected cell lines. Experiments were performed in duplicate on 3 occasions.

3.3.6 Dasatinib transport

Dasatinib uptake was also assessed using clone 15, with the mock transfected cell line and unmanipulated cells as controls (Figure 3.18). There was no significant difference in dasatinib uptake between all 3 cell lines (one way ANOVA, p=0.34). This suggests dasatinib is not a substrate for OATP1A2. Dasatinib therefore influxes into the cell via OATP1A2 independent mechanisms. Maximal uptake of dasatinib is achieved at 60
minutes. Again, dasatinib is a lipophilic drug and can diffuse rapidly into the intracellular compartment.

Figure 3.18: Time course assay for dasatinib uptake using clone 15, KCL22 mock transfected cell line and KCL22 parental cell line. Experiments performed in duplicate on 3 occasions.
3.3.6.1 The effect of naringin on dasatinib uptake

Figure 3.19 shows that dasatinib uptake is not affected by the addition of naringin. This demonstrates dasatinib uptake is independent of OATP1A2 mediated transport. There was no significant reduction in dasatinib uptake when naringin was added to all 3 cell lines (one way ANOVA, \( p=0.58 \)).

![Dasatinib uptake with/without naringin](image)

**Figure 3.19**: The effect of naringin on dasatinib uptake using transfected SLCO1A2 clones.
3.3.7 The prognostic significance of messenger RNA expression levels of SLCO1A2 in imatinib treated patients

The Taqman low density array (TDLA) was used to quantify mRNA expression levels of SLCO1A2 at baseline for CML patients. The assay was specific for SLCO1A2 (Applied Biosystems) and the ID number was SLCO1A2: Hs00366488_m1.

Sixty patients had mRNA expression levels of SLCO1A2 analysed at diagnosis. These patients were categorised into

a) Patients achieving a CCR at 12 months (n=26). These patients were further divided into optimal response n=13 (MMR at 12 months) and warning category n=13 (CCR but not in MMR at 12 months) as per ELN criteria 2013

b) Patients failing to achieve a CCR at 12 months (failure as per ELN criteria 2013) (n=27)

c) Patients who later progressed to blast crisis (n=7).

The mRNA expression levels were calibrated to normal samples and normalised to GAPDH, using the comparative Ct method. All 60 patients were treated with imatinib as first line TKI.

The mRNA expression levels of SLCO1A2 at diagnosis were also assessed to determine if they could predict patient outcomes as per the ELN 2013 criteria. Patients who failed to achieve a CCR at 12 months or were in the warning category, or had an optimal response or who later progressed to blast crisis had SLCO1A2 mRNA expression levels of 0.156, 0.004, 0.002 and 0.017 respectively (Figure 3.20). There was no difference in the mRNA levels at diagnosis in all 4 groups (one way ANOVA, p=0.463). The corresponding F statistic was 0.866.
3.3.8 The prognostic significance of messenger RNA expression levels of SLCO1A2 in nilotinib and dasatinib treated patients

The mRNA expression levels of SLCO1A2 were also analysed for patients treated with nilotinib and dasatinib as first line treatment (Figure 3.21). Six patients with first line nilotinib and four patients with first line dasatinib had their mRNA expression levels of SLCO1A2 at diagnosis analysed. All ten patients achieved a CCR at 12 months.
3.3.9 The correlation between mRNA expression levels of hOCT1 and SLCO1A2

The Hu et al (2008) paper suggested that hOCT1 (SLC22A1) may be a surrogate marker for other drug transporters which could potentially have a greater role in imatinib influx. The messenger RNA expression levels of hOCT1 were performed by Dr Athina Giannoudis (using primers to avoid the M420del SNP). Spearman's rank correlation was used to assess the relationship between these 2 different drug transporter mRNA levels at baseline. The graph below (Figure 3.22) shows that the messenger RNA expression levels of SLCO1A2 and hOCT1 are moderately positively correlated (r=0.468). The mean mRNA expression for OCT1 and SLCO1A2 were 0.182 and 0.023 respectively (t-test, p<0.05).
The correlation between SLCO1A2 mRNA and hOCT mRNA levels

Figure 3.22: The correlation between hOCT1 (SLC22A1) and SLCO1A2 mRNA expression levels at baseline in 60 CML patients ($r=0.468$).

3.3.10 Drug drug interactions in CML patients at the OATP1A2 level

Patient characteristics and outcomes were collected for 160 CML patients treated with imatinib. The concomitant medications were recorded from case note records and repeat prescriptions from the pharmacy department. Studies have shown amitriptyline, carbamazepine, chlorpromazine, saquinavir and verapamil are inhibitors of OATP1A2 (Lu et al, 2015).

Twelve patients treated with first line imatinib were noted to be on OATP1A2 inhibitors. Four of the twelve patients were noted to be on amitriptyline, the indication for amitriptyline was neuropathic pain. Four patients were on carbamazepine, also for the treatment of neuropathic pain. Three patients were on verapamil for blood pressure control. One patient was on saquinavir for treatment of
HIV. All 12 patients were on these medications for at least 6 months. Verapamil is a broad inhibitor of numerous drug transporters including hOCT1, but has been demonstrated to also inhibit OATP1A2. The table 3.2 below shows the characteristics of the 12 patients on concomitant medications which are OATP1A2 inhibitors.

Six patients were low risk Sokal score, five patients were intermediate Sokal score and one patient had a high risk Sokal score. There were 7 males and 5 females. The mean age of the group was 55.7 years (range 24.6-80.5). Patients 5, 7, 8, 10 and 12 did not achieve CCR at time of analysis. Patient 5 was switched to nilotinib, but was censored at 60 months. Patients 2, 5, 7, 8, 9, 10, 11 and 12 failed to achieve MMR at the time of analysis. Only patients who received concomitant medications inhibiting OATP1A2 continually for greater than 6 months were included in this analysis. Two further imatinib treated patients were on tricyclic antidepressants, which are also OATP1A2 inhibitors, but they were excluded from this analysis due to limited exposure of less than 2 months.
Table 3.2 The characteristics of patients with comediations which are OATP1A2 inhibitors. An asterisk denotes failure to achieve CCR or MMR.

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Sex</th>
<th>Age /yr</th>
<th>Sokal score</th>
<th>Comedications</th>
<th>Medical history</th>
<th>Time to CCR/months</th>
<th>Time to MMR/ months</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>61.2</td>
<td>Low</td>
<td>Amitriptyline</td>
<td>Neuropathic pain</td>
<td>6.4</td>
<td>17.7</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>47.5</td>
<td>Low</td>
<td>Amitriptyline</td>
<td>Neuropathic pain</td>
<td>4.3</td>
<td>7.9*</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>71.8</td>
<td>Intermediate</td>
<td>Verapamil</td>
<td>Hypertension</td>
<td>3.4</td>
<td>24.9</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>69.5</td>
<td>Low</td>
<td>Carbamazepine</td>
<td>Neuropathic pain</td>
<td>8.8</td>
<td>26.4</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>65.2</td>
<td>Intermediate</td>
<td>Carbamazepine</td>
<td>Epilepsy</td>
<td>58.6*</td>
<td>58.6*</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>37.1</td>
<td>Low</td>
<td>Saquinavir</td>
<td>HIV</td>
<td>18.4</td>
<td>34.5</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>24.6</td>
<td>High</td>
<td>Amitriptyline</td>
<td>Neuropathic pain</td>
<td>21.3*</td>
<td>21.3*</td>
</tr>
<tr>
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<td>Neuropathic pain</td>
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<td>19.6*</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>59.7</td>
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<td>Carbamazepine</td>
<td>Neuropathic pain</td>
<td>28.6</td>
<td>47.4*</td>
</tr>
<tr>
<td>10</td>
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<td>Intermediate</td>
<td>Amitriptyline</td>
<td>Neuropathic pain</td>
<td>11.7*</td>
<td>18.7*</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>48.7</td>
<td>Low</td>
<td>Verapamil</td>
<td>Learning difficulties</td>
<td>6.0</td>
<td>37.7*</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>80.5</td>
<td>Intermediate</td>
<td>Verapamil</td>
<td>Hypertension</td>
<td>15.2*</td>
<td>15.2*</td>
</tr>
</tbody>
</table>
3.3.11 The effect of concomitant inhibiting OATP1A2 medications on achieving CCR and MMR on imatinib treated patients

As described above there were 12 patients treated with imatinib and a concomitant OATP1A2 inhibitor. Seven out of the 12 (58%) patients receiving imatinib and concomitant OATP1A2 inhibitor achieved a CCR at time of analysis. The mean time to CCR in the OATP1A2 inhibitor group was 10.7 months, while the mean time to CCR in the group not on OATP1A2 inhibitors was 12.4 months. Figure 3.23 shows that there was no difference in time to achieve CCR between the two groups using the Kaplan-Meier method (p=0.73). Although the groups are unbalanced with respect to sample size, these results suggest OATP1A2 inhibitors do not affect achievement of CCR.
Figure 3.23: The effect of OATP1A2 inhibitor concomitant medications and imatinib on the cumulative achievement of CCR
Four out of the 12 patients on OATP1A2 inhibitors eventually achieved a MMR at the time of analysis at 60 months. Patient 5 was switched to second generation TKI after 10 months due to intolerance. When the two groups were analysed there was no difference in cumulative MMR rates (Figure 3.24, p=0.57). This suggests that OATP1A2 inhibitors in CML patients treated with imatinib have no significant effect on the achievement of MMR. Caution needs to be applied when interpreting these results due to the imbalance in the sample size of the 2 groups.

Figure 3.24: The effect of OATP1A2 inhibiting concomitant comedications on achieving MMR with imatinib.
3.3.12 The effect of OATP1A2 inhibiting concomitant medication on overall and progression free survival in imatinib treated patients

Two out of the 12 patients in the OATP1A2 concomitant medication group died from CML related causes. Both these patients progressed to blast crisis from chronic phase CML. Figure 3.25 shows that concomitant OATP1A2 inhibitors have no effect on overall survival (p=0.49) when compared to patients not receiving an OATP1A2 inhibitor. Figure 3.26 similarly shows that progression free survival is not influenced by OATP1A2 inhibitors (p=0.73).

![Figure 3.25: The effect of concomitant OATP1A2 inhibitor comedications and imatinib on overall survival](chart.png)
Figure 3.26: The effect of OATP1A2 inhibitor comedications and imatinib on progression free survival
3.4 Discussion

The results above confirm that imatinib is a substrate for OATP1A2. This is evidenced by significantly increased imatinib uptake into the OATP1A2 transfected cell line compared to the mock transfected and KCL22 parental cell line. Imatinib uptake is reduced with the co-incubation of naringin.

The mRNA expression levels of SLCO1A2 at diagnosis do not predict patients with a poor outcome after treatment imatinib. All the patients treated with nilotinib (n=6) and dasatinib (n=4) achieved a CCR at 12 months. It is therefore not possible to interpret the mRNA of SLCO1A2 in second generation TKI treated patients. The mRNA expression levels of SLC22A1 and SLCO1A2 are weakly positively correlated with each other.

In a population study, inhibitors of OATP1A2 have no significant effect on CCR or MMR rate. Inhibitors of OATP1A2 also have no significant effect on progression or overall survival in CML patients treated with imatinib. However, caution needs to be applied when interpreting these results due to difference in the sample size between the two groups. The factor which may have influenced the results is that the CML patients were treated by different haematology teams and this could also have introduced variation.
4. The role of OCTN1 and OCTN2 in the transport of TKIs in CML

4.1 Introduction

The OCTN family of transmembrane proteins are part of the SLC family of drug transporters. They have 12 transmembrane domains in common with other SLC drug transporters. There is a high degree of amino acid sequence concordance in the first transmembrane loop between the OCTN family and other SLC family drug transporters, while the C-terminus of the OCTN protein has the least amino acid sequence concordance with other members of the SLC family. The nucleotide binding motif for the OCTN family is predicted to be between the intracellular transmembrane 4 and 5 segments (Indiveri et al, 2013).

The physiological role of OCTN1 (SLC22A4) is not clear, though acetylcholine is a known substrate (Pochini et al, 2012). OCTN1 is expressed predominately in the spinal cord, bone marrow, liver, intestine and renal epithelium. The regulation of acetylcholine is important as this neurotransmitter can regulate the cytoskeleton by affecting the organisation of the proteins involved in the composition of the cytoskeleton (Wessler et al, 2008). OCTN1 mainly transports organic cations, with tetraethylammonium (TEA) a well-known substrate. OCTN1 may have a possible clinical role in the removal of cationic xenobiotics via the renal tubules (Tamai et al, 2004). Recent studies have shown it transports carnitine and ergothioneine (Yabuuchi et al, 1999, Grundemann et al, 2005).

OCTN2 (SLC22A5) is a protein that transports carnitine into the cell. Carnitine has a vital role in the oxidation of fatty acids in the mitochondria. OCTN2 uses a Na
dependent antiport or symport to transport carnitine in a 1:1 ratio (Indiveri et al, 2010). OCTN2 expression is predominately in the intestine, kidney, placenta, liver and testis (Wagner et al, 2000).

OCTN2 has been shown to transport imatinib in HEK293 transfected cell lines. It was therefore hypothesised that the OCTN family of drug transporters may be important in imatinib uptake. The role of OCTN1 in the transport of imatinib is not clear. The role of the OCTN family of drug transporters in active transport of imatinib is disputed. Polymorphisms of the OCTN1 gene (TT rs1050152) have been associated with inferior MMR and CMR rates (Angelini et al, 2013).

4.1.1 Aims

The aims of this chapter are to:

1. Determine whether OCTN1 and OCTN2 are able to transport imatinib, nilotinib or dasatinib

2. Determine mRNA expressions of OCTN1 and OCTN2 at diagnosis to determine if low levels of OCTN predict poor outcome

3. Assess whether OCT1 mRNA levels correlate with OCTN1/OCTN2 mRNA expression levels
4.2 Methods

4.2.1 Radioactive drugs used in uptake experiments

$^{14}$C-tetraethylammonium bromide (TEA) was purchased from PerkinElmer (Boston, Massachusetts, USA). The specific activity was 118.4 MBq/mmol. The final concentration of TEA used in uptake experiments was 5µM. TEA is a known substrate for both OCTN1 and OCTN2. $[^{14}\text{C}]-$imatinib and $[^{14}\text{C}]-$nilotinib were provided by Novartis, Basel with specific activities of 3.386 MBq/mg and 3.48 MBq/mg respectively. The final concentrations of imatinib and nilotinib were both 5µM. This was achieved by adding non radiolabelled imatinib (3.4µM) and nilotinib (4µM) respectively to the final concentration. $[^{14}\text{C}]-$dasatinib was provided by Bristol Myers Squibb Pharmaceuticals (New York, USA). Dasatinib had a specific activity of 1.18 MBq/mg; the final concentration of dasatinib used in experiments was 150nM. The final concentrations of imatinib, nilotinib and dasatinib were selected on the basis of known concentrations achievable in patient blood samples. All radioactive drugs were dissolved in transport buffer (1% HEPES in Hanks Buffered salt solution HBSS).

Verapamil was purchased from Sigma-Aldrich, UK. Verapamil was used at a concentration of 100µM (as used in previous experiments by Thomas et al, 2004).
4.2.2 Radioactive uptake experiments

Cells were supplemented with fresh RPMI prior to TKI radioactive uptake experiments. 1x10^6 cells were centrifuged at 3500 rpm for 2 minutes. The supernatant was discarded and the pellet resuspended in 500µl of transport buffer and kept in the incubator at 37.6°C, 5% CO₂. Radioactive TKI with the appropriate amount of non-radioactive TKI was prepared in 500µl of transport buffer, with or without naringin. The cells were then centrifuged and the supernatant again discarded. The cell pellet was then added to 500µl of prepared TKI or E-3-S. The cells were incubated for 0, 30, 60 and 120 minutes in duplicate. After the specific period of incubation, the cells were centrifuged at 3500 rpm and 200µl of the supernatant was added to 4ml of scintillation fluid (Meridian, Epsom, Surrey). The remaining supernatant was discarded without dislodging the cell pellet. The cell pellet was washed with 500µl of cold HBSS and then centrifuged at 6000 rpm for 3 minutes. This was repeated twice. After the second wash, 100µl of distilled water was added and vigorously pipetted. The cells were solubilised by the addition of 200µl of lysis buffer (10% sodium dodecanyl sulphate (SDS) and 1M sodium hydroxide). The cells were incubated for 1 hour and then the contents were transferred to 4 mls of scintillation fluid. The radioactivity was measured using a scintillation counter 1500 Tri Carb LS Counter Packard (Perkin Elmer, Waltham, Massachusetts, USA).
4.2.3 Cell culture

KCL22 cells were transfected using AMAXA nucleofection technology (Lonza) with either pcDNA3.1-OCTN1 or pcDNA3.1-OCTN2 (kind gifts from Prof A. Tsuji and Y. Kato, Kanazawa, Japan). The cells were selected and grown in 1mg/ml neomycin. After successful selection they were grown in RPMI 1640 (Biosera, UK) and supplemented with 1% streptomycin/penicillin, 1% glutamine and 10% foetal calf serum. Cell viability was measured using the Countess cell counting chambers (Invitrogen, UK). The transfection process was performed by Dr T. Siriboonpiputtana, and the mRNA expression levels of the transfected clones were quantified using Taqman PCR. The clones selected for the present work were shown to have increased expression compared to the parental KCL22 cell line.

4.2.4 TaqMan low density array and OCTN1 and OCTN2 gene expression assays

The Taqman low density array (TDLA) was used to quantify mRNA expression levels of OCTN1 and OCTN2 at baseline for CML patients. The assay was specific for OCTN1 (Applied Biosystems) and the ID number was OCTN1 SLC22A4: Hs00268200_m1. The ID number used for the OCTN2 assay (Applied Biosciences) was OCTN2 SLC22A5: Hs00161895_m1. The ID number purchased for GAPDH was Hs99999905_m1 (endogenous control). The mRNA from patients with CML at baseline was calibrated to normal samples and normalised to the endogenous control GAPDH.
The cDNA from the CML patient samples at baseline were quantified on the Nanodrop 2000. 100ng of cDNA was added to 1µl of 20X TaqMan Gene Expression Assay, 10µl 2X TaqMan Gene Expression Master Mix and RNase free water to make a total volume of 20µl. The reactions were run in triplicate. The expression of GAPDH was used as a control gene expression parameter. The reactions were loaded onto a 384-well plate. The plate was sealed and centrifuged for 10 seconds. The real time PCR amplifications were run in an ABI Prism 7900HT System using the following conditions: 50°C for 2 minutes, 10 minutes at 95°C. This was then followed by 40 cycles of denaturation and annealing/extension at 95°C for 15 seconds and 60°C for 1 minute respectively.

4.3 Results

4.3.1 Radioactive uptake experiments with Tetraethylammonium bromide (TEA)

Figure 4.1 shows the uptake of TEA into the unmanipulated parental cells, mock transfected cell line and the OCTN1 transfected cell line. TEA is a known substrate for OCTN1 (the transfected OCTN1 cells had low hOCT1 expression). The OCTN1 transfected cell line had a significantly increased TEA uptake at 30 minutes compared to both unmanipulated parental cells and the mock transfected cell line (one way ANOVA, p=0.04). The average uptake of TEA at 30 minutes for the OCTN1 transfected cell line was 42.11 pmoles, compared with 5.79 and 5.18 pmoles for the parental cell line and the mock transfected cell line respectively. After 30 minutes, TEA uptake had reached a plateau with no further intracellular accumulation.
Figure 4.2 shows the uptake of TEA into OCTN2 transfected KCL22 cells, together with unmanipulated parental and mock transfected cells as controls. There was a significantly increased uptake of TEA at 30 minutes for the OCTN2 transfected clone which was quantified at 56.9 pmoles of TEA per million cells compared to 4.7 and 5.9 pmoles for the parental KCL22 and mock transfected cell lines (one way ANOVA, p=0.04).

The results confirm that OCTN1 and OCTN2 transport TEA, and the clones are therefore expressing functional protein. This allows the cells to be used to assess whether they transport the TKIs.

Figure 4.1. Tetraethylammonium uptake by OCTN1 transfected clone, unmanipulated parental cells and mock transfected cell lines. Experiments were performed in duplicate and repeated on 3 occasions.
4.3.2 The effect of Verapamil on TEA uptake by OCTN1 and OCTN2 parental and mock transfected cells

Figure 4.3 shows the effect of verapamil on TEA uptake by OCTN1 and OCTN2 transfected clones. Verapamil is a known inhibitor of both OCTN1 and OCTN2; it also known to inhibit hOCT1 and MDR1. At 30 minutes, there was 40.2 pmoles of TEA uptake into the OCTN1 transfected cells, but when the experiment was repeated with verapamil the TEA uptake was significantly lower at only 14.9 pmoles (t-test, p<0.05). This is consistent with verapamil inhibiting the active uptake of TEA by OCTN1.

In OCTN2 transfected cells TEA uptake at 30 minutes was 58.7 pmoles without inhibitor, but only 16.6 pmoles of TEA with verapamil (t-test, p<0.05). This demonstrates that OCTN2 is also contributing to TEA uptake, but verapamil blocks this effect.
Figure 4.3. TEA uptake with or without inhibitor using OCTN1 and OCTN2 transfected cells, unmanipulated and mock transfected cells. Experiments were performed in duplicate and repeated on 3 occasions.

4.3.3 Imatinib uptake by OCTN1 and OCTN2 transfected KCL22 cells

Figure 4.4 below shows the time course assay for imatinib uptake into the OCTN1 transfected cell line, with unmanipulated parental and mock transfected cells as control. At 30 minutes, imatinib uptake was 79.3 ng for the OCTN1 transfected cell line, 77.6 ng for the mock transfected cells and 80.3 ng for the parental cells. There was no difference in imatinib uptake between the cell lines (one way ANOVA, p=0.64).
Figure 4.4. Imatinib uptake by OCTN1 transfected cells. Experiments were performed in duplicate and repeated on 3 occasions.

Figure 4.5 shows the uptake of imatinib by the OCTN2 transfected cells. At 30 minutes, the imatinib uptake was 84.6 ng for OCTN2, 80.1 ng for the mock transfected cell line and 80.8 ng for the parental KCL22 cell line. There was no difference in imatinib uptake (one way ANOVA, p=0.74).
Figure 4.5. Imatinib uptake by OCTN2 transfected cells. Experiments were performed in duplicate and repeated on 3 occasions.

Imatinib uptake into OCTN1 and OCTN2 cell lines was performed with or without verapamil, shown in Figure 4.6. There was no difference in imatinib uptake with the addition of verapamil between the different cell line lines (OCTN1 t-test, p=0.53; OCTN2 t-test, p=0.75).
Figure 4.6 Imatinib uptake into OCTN1 and OCTN2 cells with or without verapamil. Experiments were performed in duplicate and repeated on 3 occasions.

4.3.4 Nilotinib transport by OCTN1 and OCTN2

A time course assay was used to assess nilotinib uptake by OCTN1 and OCTN2 overexpressing cell lines. Figure 4.7 shows that at 30 minutes, there was 982ng nilotinib uptake into the OCTN1 cell line, 1003 ng nilotinib uptake into the parental KCL22 cell line and 963 ng nilotinib uptake into the mock transfected cell line. There was no difference in nilotinib uptake between all 3 cell lines (one way ANOVA, p=0.65).

Figure 4.8 shows nilotinib uptake into OCTN2 transfected cell lines. At 30 minutes, nilotinib uptake into the OCTN2 transfected, parental and mock transfected cell lines was 1013 ng, 1003 ng and 1033 ng respectively. There was no difference in nilotinib uptake between the 3 cell lines (one way ANOVA, p=0.82).
Figure 4.7. Time course assay for nilotinib uptake using OCTN1 transfected cells. Experiments were performed in duplicate and repeated on 3 occasions.
Figure 4.8. Time course assay for nilotinib uptake using OCTN2 transfected cells. Experiments were performed in duplicate and repeated on 3 occasions.
Nilotinib uptake was assessed with or without verapamil. Figure 4.9 below shows verapamil has no effect on nilotinib uptake by OCTN1 and OCTN2 (OCTN1 t-test p=0.67; OCTN2 t-test p=0.51).

![Graph showing nilotinib uptake into OCTN1 and OCTN2 transfected cell lines with or without verapamil.](image)

**Figure 4.9** Nilotinib uptake into OCTN1 and OCTN2 cell lines with or without verapamil. Experiments were performed in duplicate on 3 occasions.

### 4.3.5 Dasatinib transport by OCTN1 and OCTN2

Dasatinib uptake into OCTN1 and OCTN2 transfected cells was also assessed using time course assays. Figure 4.10 shows dasatinib uptake by the OCTN1 transfected cells, parental and mock transfected cells. At 30 minutes, there was no difference in dasatinib uptake between all 3 cell lines (one way ANOVA, p=0.76), with 237ng, 263 ng and 256 ng of dasatinib accumulation for the OCTN1 transfected cells, parental cells and mock transfected cells respectively.
Figure 4.11 shows dasatinib uptake by the OCTN2 transfected cell line, parental cell line and mock transfected cell line. There is no difference in dasatinib uptake between all 3 cell lines. At 30 minutes, the dasatinib uptake into OCTN2, parental KCL22 cell line and mock transfected cell line was 246ng, 263ng and 256ng respectively (one way ANOVA, p=0.65).

Figure 4.10. Time course assay for dasatinib uptake using OCTN1 transfected cells, mock transfected and parental cells. Experiments were performed in duplicate and repeated on 3 occasions.
Figure 4.11 Time course assay for dasatinib uptake using OCTN2 transfected cells, unmanipulated parental and mock transfected cells. Experiments were performed in duplicate on 3 occasions.

Figure 4.12 below shows the uptake of dasatinib into OCTN1 and OCTN2 with or without verapamil. At 30 minutes, there was no difference in dasatinib uptake with or without verapamil (OCTN1 transfected t-test, p=0.59; OCTN2 transfected t-test, p=0.78).
Figure 4.12. Dasatinib uptake by OCTN1 and OCTN2 transfected cells at 30 minutes. Experiments were performed in duplicate and repeated on 3 occasions.

4.3.6 The prognostic significance of mRNA expression levels of OCTN1 and OCTN2 at diagnosis for imatinib treated patients

Sixty patients who were treated with imatinib were studied at diagnosis of chronic phase of CML. They had their diagnostic mRNA expression levels of OCTN1 and OCTN2 measured at diagnosis in chronic phase. Twenty six patients achieved a CCR at 12 months; this group included 13 patients who had an optimal response (MMR at 12 months) and 13 patients who were in the warning category as per the ELN 2013 criteria at 12 months. 27 patients failed to achieve a CCR at 12 months and had failed TKI as per ELN criteria. Seven patients were categorised as blast crisis (these patients initially presented in chronic phase of CML but progressed to blast crisis during the course of their disease).
The mRNA expression levels of OCTN1 at diagnosis for patients treated with imatinib was 0.908 for the patients deemed to have failed imatinib (as per ELN 2013), 0.25 for patients in the warning category, 0.392 for patients in the optimal response and 0.255 for the future blast crisis group (Figure 4.13). There was no difference in mRNA level of OCTN1 between all 4 groups (one way ANOVA p=0.391, F=1.01).

![Figure 4.13. mRNA expression levels of OCTN1 in patients treated with imatinib at diagnosis and categorised as per ELN 2013 criteria. Error bars represent standard error.](image)

The mRNA expression levels of OCTN2 at diagnosis for patients failing imatinib was 0.283, 0.754 for patients in the warning category, 0.147 for patients in the optimal response category and 0.23 for the future blast crisis group (Figure 4.14) as per the ELN criteria. There was no difference in mRNA expression of OCTN1 between all 4 groups (one way ANOVA p=0.391, F=1.12).
Figure 4.14. mRNA expression levels of OCTN2 in patients treated with imatinib at diagnosis and categorised as per ELN 2013 criteria. Error bars represent standard error.

This finding suggests OCTN1 and OCTN2 expression levels at diagnosis are not of prognostic significance.
4.3.7 The prognostic significance of mRNA expression levels of OCTN1 and OCTN2 in patients treated with nilotinib

Six patients treated with nilotinib as first line treatment had their mRNA expression levels of OCTN1 and OCTN2 measured at diagnosis. All 6 patients achieved a CCR at 12 months. The average mRNA expression of OCTN1 and OCTN2 at diagnosis was 0.15 and 0.13 respectively. Figure 4.15 below shows the RNA expression levels of OCTN1 and OCTN2 at diagnosis in nilotinib treated patients.

Figure 4.15. mRNA expression levels of OCTN1 and OCTN2 at diagnosis in nilotinib treated patients in chronic phase CML.
4.3.8 The prognostic significance of mRNA expression levels of OCTN1 and OCTN2 in patients treated with dasatinib

There were 4 patients treated with dasatinib who had their OCTN1 and OCTN2 mRNA expression levels analysed. All 4 patients achieved a CCR. Figure 4.16 shows the mRNA expression levels of OCTN1 and OCTN2 in dasatinib treated patients.

![Figure 4.16](image-url) mRNA expression of OCTN1 and OCTN2 at diagnosis (Dasatinib treated patients n=4)

**Figure 4.16.** mRNA expression levels of OCTN1 and OCTN2 at diagnosis in dasatinib treated patients in chronic phase CML.
4.3.9 The correlation between mRNA expression levels of hOCT1, OCTN1 and OCTN2

As previously suggested by Hu et al, hOCT1 maybe a surrogate marker for other drug transporters involved in imatinib transport. The mRNA expression levels of hOCT1 were compared to the expression levels of OCTN1 and OCTN2 at diagnosis. In total 60 patient samples were analysed.

Figure 4.17 shows that there is no association between mRNA expressions levels of hOCT1 and OCTN1. The Spearman correlation was $r=0.16$.

![The correlation between OCTN1 mRNA and hOCT1 mRNA levels](image)

Figure 4.17. The correlation between mRNA levels of hOCT1 and OCTN1 at diagnosis in chronic phase CML patients. Spearman rank $r=0.16$. 
hOCT1 is also not correlated with OCTN2 (Spearman rank correlation of $r = 0.04$).

**Figure 4.18.** The correlation between mRNA levels of hOCT1 and OCTN2 at diagnosis in chronic phase CML patients. Spearman rank $r=0.04$. 

![The correlation between OCTN2 mRNA and hOCT1 mRNA levels](image)
4.4 Discussion

The above experiments show imatinib, nilotinib and dasatinib are not substrates for OCTN1 and OCTN2. There was no difference in accumulation of these TKIs when the transfected cells were compared to the mock transfected and parental cells. The addition of inhibitor had no effect on TKI uptake.

The mRNA expressions levels of OCTN1 and OCTN2 at diagnosis, in imatinib treated patients, do not predict patients destined to fail initial TKIs as per the ELN criteria. The mRNA levels were not significantly different in patients achieving optimal response, the warning category or failure. This is in contrast to published data suggesting OCT1 levels/activity can predict outcome in CML. The OCTN1 and OCTN2 levels in nilotinib and dasatinib treated patients are limited to only 6 and 4 patients respectively. All nilotinib and dasatinib treated patients had an optimal response. Larger sample sizes are required to confirm if OCTN1 and OCTN2 levels have a prognostic value role in second generation TKI treated patients.

There is no correlation between mRNA expression levels of hOCT1 when compared to those of OCTN1 and OCTN2. This would suggest hOCT1 is not a surrogate marker for OCTN1 or OCTN2. This again shows hOCT1 is the most important determinant of imatinib response.
5. The effect of metformin and TKI clinical efficacy

5.1 Introduction

Pharmacovigilance has a central role in safeguarding the use of drugs in clinical practice. It is important to understand the potential harm associated with certain medications and to assess the risk of adverse drug reactions (Moore et al, 1985). Numerous studies have shown adverse drug reactions (ADRs) can lead to increase in morbidity and admission to hospital (Bordet et al, 2001; Lee et al, 2000). The elderly population are at most risk of clinically important drug-drug interactions due to polypharmacy. These drug-drug interactions can cause serious ADRs and potentially reduce the intracellular concentration of certain drugs leading to poor outcomes (Johnell et al, 2007).

Drug drug interaction can occur due to pharmacokinetic factors such as drug absorption and distribution, metabolism and excretion. Changes in pH and gastric motility can adversely affect drug absorption. A change in the concentration of albumin and alpha1 acid glycoprotein can also influence the concentration of the drug in the unbound physiologically active state. The Cytochrome P450 (CYP) family can reduce the concentration of active drug by oxidation, reduction or hydrolysis. Clinically relevant drug drug interactions usually involve inhibition of the CYP family of enzymes. These combined effects can result in reduced bioavailability. Drug elimination via the renal system, bile and faeces can also influence drug drug interaction. However, another major cause of drug drug interactions are pharmacokinetic factors such as inhibition of drug transporters at the cellular level leading to reduced intracellular uptake of the desired active drug (Caterina et al, 2013).
Imatinib as previously discussed is known to be a substrate of hOCT1. CML is generally diagnosed in elderly patients with other comorbidities. A large number of frequently used medications in the elderly are substrates for hOCT1. To date, studies have focused mainly on positively identifying hOCT1 substrates and have largely neglected the role of hOCT1 inhibition leading to clinically relevant drug drug interactions (Thomas et al, 2004; Davies et al, 2009).

Researchers have studied medication frequently used in clinical practice to determine their degree of OCT1 inhibition. They used OCT1 transfected HEK293 cells to assess (4-(4-(dimethylamino)styryl)-N-methylpyridinium (ASP+) transport. ASP+ is a recognised substrate for OCT1. The degree of OCT1 inhibition by a particular drug was determined by comparing the amount of ASP+ uptake into HEK-OCT1 transfected cells with and without incubation with the drug under evaluation. A list of inhibitors has been identified (Ahlin et al, 2008). Studies have shown protease inhibitors, used in the treatment of HIV, such as nelfinavir, ritonavir and saquinavir, are inhibitors of OCT1. These protease inhibitors reduced the uptake of MPP+ (another known OCT1 substrate), at clinically relevant concentrations. Interestingly, pentamidine and trimethoprim were also noted to inhibit OCT1 (Jung et al, 2008). Other studies have investigated the drug drug interaction between metformin and repaglinide which are dependent on OCT1. Metformin uptake was markedly reduced by concomitant incubation with repaglinide (Bachmakov et al, 2008). The beta blocker group of drugs including atenolol, metoprolol and propranolol have been demonstrated to be inhibitors of OCT1 (Umehara et al, 2008). The NSAID family of drugs have been shown to inhibit OCT1, though they are not substrates of OCT1 (Khamdang et al, 2002). This is of relevance as many patients with imatinib have musculoskeletal pain and require NSAIDs. Indeed, imatinib functional uptake is reduced in the presence of ibuprofen. It has been
suggested ibuprofen and imatinib should not be prescribed in combination (Wang et al, 2012).

Type II diabetes is increasing in frequency in the western world. Patients with diabetes have a higher incidence of colorectal, breast and endometrial cancer (Chowdhury et al, 2010). Diabetes and cancer are increasingly being recognised in the same individuals. It is therefore not uncommon to encounter concurrent use of imatinib and metformin.

Metformin is a biguanide and is commonly used in type II diabetes mellitus. It lowers the blood glucose and can be administered as monotherapy or in combination treatment with insulin. It increases cellular glucose uptake while limiting the amount of glucose uptake via the gastrointestinal tract (Li et al, 2012). It also limits gluconeogenesis in the liver. Metformin has a half-life of 5 hours and is not subjected to first pass metabolism. Metformin is distributed in many tissues via the OCT1 transporter and is excreted via the kidney. The therapeutic concentration of metformin is generally accepted as 1-40 µM (Christensen et al, 2011).

The main drug transporters involved in metformin uptake at the brush border are plasma membrane monoamine transporter (PMAT, SLC29A4) and OCT3 (SLC22A3) (Zhou et al, 2007). At the basolateral border, hOCT1 transports metformin into the portal system (Muller et al, 2005). Metformin is transported in the portal system to the liver. The liver has OCT1 expression at the basolateral membrane and influxes metformin into the hepatocyte. OCT3 is also thought to have a role in metformin uptake (Nies et al, 2009). Mice with deficient OCT1 have reduced hepatocyte uptake, supporting the crucial role OCT1 has in hepatic uptake (Shu et al, 2007). Human multidrug and toxin extrusion 1 (MATE1) may have a role in metformin excretion from the liver and kidney, as this transporter is highly
expressed in these organs and is known to transport metformin (Otsuka et al, 2005). Once metformin is in the systemic circulation it is influxed into the renal epithelial cells by OCT2, which is located at the basolateral membrane (Takane et al, 2008). Intracellular metformin is then transported into the lumen by MATE1 (Kusuhara et al, 2011) and excreted in the urine.

Genetic polymorphisms in the OCT1 drug transporter have been studied extensively. Polymorphisms of OCT1 including R61C, M420del and G465R were found to change the pharmacokinetics of metformin in healthy individuals. These individuals were found to have high concentrations of metformin once the drug was administered and also higher peak concentrations (Shu et al, 2008). Another study of healthy volunteers with these genetic polymorphisms again showed altered metformin pharmacokinetics with reduced liver uptake but ultimately there was no difference in the area under the curve (Tzvetkov et al, 2009).

As previously mentioned metformin is not metabolised by the liver and thus high metformin concentrations may interact with imatinib at a drug transporter level and lead to failure of imatinib treatment. This would be considered a serious ADR and lead to poor clinical outcomes for CML patients.
5.1.1 Aims

The aim of the work in this chapter was to:

1. Determine whether increasing concentrations of metformin inhibit OCT1 facilitated imatinib uptake
2. Assess whether there is a potential for drug drug interactions between metformin and imatinib at clinically relevant concentrations
3. Determine whether patients receiving concomitant metformin and imatinib have failure of response to imatinib.

5.2 Materials and methods

5.2.1 Cell lines

The CML cell line KCL22 was used for drug transporter experiments, since this cell line has low levels of hOCT1 mRNA expression. RPMI 1640 (Sigma-Aldrich) with additives including 1% L-glutamine, penicillin/streptomycin, and 10% foetal calf serum was used to culture cells. The KCL22 cells were transfected using nucleofection techniques (AMAXA Biosystems, Cologne, Germany). In this way, two stable cell lines were produced (high expressing KCL22-hOCT1 transfected cell line and a KCL22-pcDNA3.1 mock transfected cell line). The KCL22-hOCT1 transfected cell lines were made by Dr Athina Giannoudis.
5.2.2 Radioactive uptake experiments

Radioactive tyrosine kinase inhibitors were used to assess drug transport. $^{14}\text{C-Imatinib}$ (a kind gift from Novartis, Basel, Switzerland) had a specific activity of 3.386MBq/mg. $^{14}\text{C-Imatinib}$ was added to non-radioactive imatinib to achieve a final concentration of 5µM. $^{14}\text{C-Nilotinib}$ (also a kind gift from Novartis, Basel, Switzerland) had a specific activity of 118.4MBq/mmol. Nilotinib was used at a final concentration of 5µM. $^{14}\text{C-Dasatinib}$ (Custom made by Bristol Myers Squibb Pharmaceuticals, New York, NY) had a specific activity of 1.18MBq/mg. Dasatinib was used at a final concentration of 150nM. All these concentrations are clinically relevant concentrations at standard administered doses of TKI.

$1\times10^6$ cells were incubated with TKI containing transport medium, with or without metformin. Metformin (Sigma Aldrich, UK) was used at a final concentration 40µM to perform a time course assay. The time course assay was continued for up to 2 hours. This concentration was used as this is a recognised clinically relevant in vivo concentration of metformin. To determine the IC50, varying concentrations of metformin were used ranging from 0- 10000 µM.

Cells and media were incubated for 2 hours while performing a time course assay. To perform the IC50, the cells were incubated for 30 minutes. At the appropriate time point, the cells were centrifuged at 3000 rpm and 200µl of supernatant was added to 4ml of scintillation fluid (Meridian, Epson, Surrey). The remaining supernatant was discarded without dislodging the cell pellet. The cell pellet was washed with 500µl of cold HBSS and then centrifuged at 6000 rpm for 3 minutes at 4°C. This was repeated twice. after the second wash, 100µl of distilled water was added to the pellet and they were vigorously vortexed and then solubilised using
200µl of lysis buffer. This was allowed to stand for 1 hour. The solution was mixed with 3.5 ml of scintillation fluid and radioactivity counted using a beta counter.

All experiments were performed in duplicate on 4 separate occasions. An ANOVA was used to compare multiple results for significance.

5.2.3 Cell counting

A cell count determination was made by adding 10µl of cells growing in supplemented RPMI to 10µl of 0.4% trypan blue. The cells and trypan blue were placed in an Invitrogen counting chamber.

5.2.4 MTT assay

Fifty µl of 2x10^6 cells/ml were placed in individual 96 well plates. Stock solutions of 10µM imatinib (or nilotinib 8 µM or dasatinib 300nM) and varying concentrations of metformin (ranging from 0-20mM) were prepared. 50µl of the drug solution (TKI and metformin) in transport buffer was used to incubate the 50µl of 2x10^6 cells in the well. The cells were incubated for 60 minutes at 37°C and 5% CO₂. Twenty microlitres of MTT solution was added (5mg MTT and 1ml Hanks solution) to the well for 2 hours at 37°C. One hundred microlitres of lysis buffer (50% dimethylformamide and 20% SDS) was added and the solution left overnight for 12 hours at 37°C and wrapped in foil. A plate reader was used to measure optical density. A control of cells and transport buffer was also included.
5.2.5 Patient analysis

From inspection of pharmacy and trial entry records 8 patients were found to have received repeat prescriptions for both TKIs and metformin. The CCR and MMR rates for these patients were analysed to assess the effect of metformin on imatinib efficacy. Three further patients had diabetes and were prescribed TKIs in combination with either insulin or gliclazide.

5.3 Results

5.3.1 Imatinib uptake with or without metformin (40µM) co incubation

A time course assay was performed to assess imatinib uptake into hOCT1 transfected cell lines with or without metformin. The OCT1 transfected cells used in these experiments were provided by Dr Athina Giannoudis. Four time course assays were done with imatinib only, or imatinib and metformin 40µM using both hOCT1 transfected and mock transfected cells (Figure 5.1).
Figure 5.1. A time course assay for imatinib uptake into hOCT1 transfected and mock transfected cells with or without imatinib. Experiments were performed in duplicate on 3 occasions. All values represent mean +/- error bars.

Figure 5.1 shows imatinib uptake with or without metformin (at 40µM) into hOCT1 transfected and mock transfected cells over 3 hours. The graph shows increased imatinib uptake when hOCT1 transfected cell lines were used, when compared to the mock transfected cell line (t-test, p=0.02). This confirms imatinib uptake is mediated by hOCT1. There was no difference in imatinib uptake when the hOCT1 transfected cell lines were incubated with or without metformin. The error bars overlap suggesting a metformin concentration of 40µM does not inhibit imatinib uptake. There was no difference in imatinib uptake when using the mock transfected cell lines with or without metformin. This is as expected as the mock transfected cell line does not increase imatinib uptake, again confirming hOCT1 is important for imatinib uptake.
5.3.2 The inhibitory effects of metformin on TKI uptake

The uptake of the three main TKIs used in clinical practice imatinib, nilotinib and dasatinib were assessed with varying concentrations of metformin. The concentrations of metformin used were 0, 10, 100, 500, 1000 and 10000 µM concentrations. The cells were incubated in the transport medium for 60 minutes.

![Inhibitory effects of varying log concentrations of metformin on imatinib uptake](image)

**Figure 5.2.** The uptake of imatinib at 60 minutes incubation with varying concentrations of metformin using both mock transfected and hOCT1 transfected cells.

The concentration of metformin was increased from 0-10000µM in order to determine the IC50 of metformin. Therefore 10000µl corresponded to a log concentration of metformin 4. The grey line signifies a 30 minute preincubation with metformin to determine if the hOCT1 transporter when saturated with metformin would reduce imatinib uptake.
Figure 5.2 shows metformin concentrations up to 500µM have minimal inhibitory effect on imatinib uptake. Metformin concentrations greater than 500µM showed a reduction in imatinib uptake. The preincubation with metformin for an extra 30 minutes with the cell lines had no significant effect on imatinib uptake. The control for this experiment is the mock transfected cells and metformin.

5.3.3 Calculation of IC50 for metformin

5.3.3.1 Imatinib

To calculate the IC50 (concentration of metformin required to inhibit 50% of imatinib uptake) the uptake of imatinib by the hOCT1 transfected cell lines is subtracted from the uptake of imatinib by the mock transfected cells, at the corresponding metformin concentrations Figure 5.3). This eliminates the effect of passive diffusion and therefore assumes the remaining imatinib uptake is solely due to OCT1 expression facilitating imatinib uptake.

Figure 5.3. The IC50 for metformin. Imatinib uptake with varying concentrations of metformin. Experiments were performed in duplicate on 3 occasions.
The IC50 for metformin is 764µM. This concentration of metformin reduces imatinib uptake by 50%. However, this concentration is 20 times greater than the upper limit of the Cmax in humans at steady state (based on metformin 1 g twice daily).

5.3.3.2 Nilotinib

Figure 5.4. Nilotinib uptake with varying concentrations of metformin. Experiments were performed in duplicate on 3 occasions.

Figure 5.4 shows the uptake of nilotinib with varying concentrations of metformin. There is no difference in nilotinib uptake between the mock transfected and hOCT1 transfected cells with increasing metformin concentrations. This suggests high concentrations of metformin have no influence on nilotinib uptake.
5.3.3.3 Dasatinib

Figure 5.5. Dasatinib uptake with varying concentrations of metformin. Experiments were performed in duplicate on 3 occasions.

Figure 5.5 shows the uptake of dasatinib with increasing concentrations of metformin. There is no inhibitory effect by metformin on dasatinib uptake. Interestingly, the transfected hOCT1 cell line has an increased dasatinib uptake compared to the mock transfected cell line (t-test, p<0.05).

5.3.4 MTT assay results

5.3.4.1 Imatinib

There is no difference in cell viability at 0 minutes, between cells incubated with imatinib only or cells incubated with imatinib and varying concentrations of metformin (one way ANOVA; F=1.27, p<0.05). However, at 30 minutes there is a
difference in cell viability between the cells incubated in imatinib and varying concentrations of metformin (one way ANOVA; F=16.9, p<0.05). This difference in cell viability is more pronounced when cells are incubated with metformin for longer than 30 minutes. When cells are incubated in imatinib only, they have a cell viability of 94% at 60 minutes. However, when the cells are incubated in 10000µM concentration of metformin the cell viability is 80% at 60 minutes (Figure 5.6).

Figure 5.6. Percentage cell viability for hOCT1 transfected cells incubated in imatinib 5µM and varying concentrations of metformin.
5.3.4.2 Nilotinib

At 0 minutes incubation, there is no difference in cell viability between cells incubated in nilotinib only compared to cells incubated in nilotinib and varying concentrations of metformin (one way ANOVA; F=1.03, p=0.45). However, after incubation with nilotinib and varying concentrations of metformin for 30 minutes there is a significant difference in cell viability (one way ANOVA; F=8.98, p<0.05). Increasing concentrations of metformin are associated with reduced cell viability especially beyond 30 minutes (Figure 5.7).

![Cell viability % with varying concentrations of metformin](image)

Figure 5.7 Cell viability using hOCT1 transfected cells with nilotinib 4µM and varying concentrations of metformin.
5.3.4.3 Dasatinib

There is a significant difference in cell viability in cells treated with dasatinib alone and with cells treated with dasatinib and varying metformin concentration (Figure 5.8). At 2 hours there is 72% viability when cells were treated with 10000µM metformin, as compared to a cell viability of 95% at 2 hours with dasatinib alone (t-test, p<0.05).

Figure 5.8 Cell viability of hOCT1 transfected cells in dasatinib only and varying concentrations of metformin.
5.3.5 Patient population with concurrent diabetes and CML treated with TKIs

There were 11 out of 192 patients diagnosed with concurrent diabetes and CML in the Merseyside, North Wales and Chester region between the periods January 2003-December 2009. Six patients were on imatinib and metformin and 2 patients were on nilotinib and metformin. There were 4 males and 4 females and the average age of the CML patients with diabetes was 58.8 years (35.9–84.9). There were 4 patients with a low Sokal score, 3 patients with an intermediate Sokal score and 1 patient with a high Sokal score. All the eight patients had the Philadelphia chromosome present with no other cytogenetic abnormalities at presentation. There were also 3 further patients with CML who were prescribed other diabetic medication. Two patients were on imatinib and gliclazide and 1 patient was on imatinib on insulin.

Four of the six patients (66.7%) on imatinib and metformin achieved a CCR. Figure 5.9 shows there is no difference in cumulative CCR rates between imatinib and metformin treated patients compared to imatinib only patients (p=0.87). Three of the 6 patients treated with imatinib and metformin achieved a MMR during the course of their treatment. Figure 5.10 shows patients treated with imatinib and metformin have no difference in MMR rates compared to patients treated with imatinib only (p=0.64). Patient 7 was switched from imatinib to nilotinib due to intolerance; however, he had achieved CCR prior to switching to nilotinib. Patient 7 achieved a MMR while on nilotinib, and was therefore censored at 60 months. The 2 patients on nilotinib and metformin both achieved a CCR but only 1 achieved a MMR. All 8 patients were alive at time of analysis with no progression of disease. The table 5.1 below gives the characteristics of the group. The 3 patients with CML and either
gliclazide or insulin treatment all achieved a CCR but only 1 achieved a MMR at 29.8 months.

Table 5.1. The characteristics of patients with TKIs and diabetic medication

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Sex</th>
<th>Age (Years)</th>
<th>Sokal score</th>
<th>Comedications</th>
<th>Time to CCR (months)</th>
<th>Time to MMR (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>59.6</td>
<td>Intermediate</td>
<td>Imatinib/metformin</td>
<td>6.7</td>
<td>16.7</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>45.9</td>
<td>Low</td>
<td>Imatinib/metformin</td>
<td>4.6</td>
<td>24.6</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>64.7</td>
<td>Intermediate</td>
<td>Imatinib/metformin</td>
<td>21.1</td>
<td>39.5*</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>84.9</td>
<td>Intermediate</td>
<td>Nilotinib/metformin</td>
<td>6.8</td>
<td>31.6</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>53.9</td>
<td>Low</td>
<td>Nilotinib/metformin</td>
<td>7.9</td>
<td>NA</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>35.9</td>
<td>Low</td>
<td>Imatinib/metformin</td>
<td>13.6</td>
<td>32.8*</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>80.3</td>
<td>High</td>
<td>Imatinib/metformin</td>
<td>8.8</td>
<td>28.7</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>44.9</td>
<td>Low</td>
<td>Imatinib/metformin</td>
<td>23.5nil</td>
<td>47.6</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>76.8</td>
<td>Low</td>
<td>Imatinib/gliclazide</td>
<td>29.5</td>
<td>41.5*</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>67.5</td>
<td>Intermediate</td>
<td>Imatinib/Gliclazide</td>
<td>18.7</td>
<td>29.8</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>81.7</td>
<td>High</td>
<td>Imatinib/Insulin</td>
<td>19.7</td>
<td>38.6*</td>
</tr>
</tbody>
</table>
Figure 5.9. The cumulative CCR rates of imatinib/metformin treated patients compared to imatinib only treated patients
Figure 5.10. The cumulative MMR rates of imatinib/metformin treated patients compared to imatinib only patients.
5.4 Discussion

This study has shown metformin is a weak inhibitor of OCT1 and reduces imatinib uptake at concentrations of metformin greater than 500 µM. This concentration is much higher than clinically relevant metformin concentrations which range between 1-40 micromolar concentrations. At 40 µM metformin, there is no reduction in imatinib uptake. This would suggest metformin does not affect the outcome of imatinib treatment in CML. This is supported by 75% of patients on both imatinib and metformin achieving CCR at 12 months. Also metformin does not interact with dasatinib or nilotinib, and has no effect on uptake of these second generation TKIs.

In order to predict drug drug interactions, CML cell lines were pre incubated with metformin for 30 minutes prior to being assessed for imatinib uptake. In vivo the active drug may be metabolised and the active metabolite may also be a substrate/inhibitor of OCT1. The interaction of N-desmethyliimatinib was not assessed with metformin.

Metformin was preincubated with CML cells to exclude the possibility that metformin may bind to the OCT1 domain and therefore impede imatinib uptake. However, this was not the case and imatinib uptake was not reduced at normal therapeutic concentrations of metformin.

The MTT assay shows CML cells incubated in high concentrations of metformin greater than or equal to 1000µM have increased cell death. The reduction in imatinib uptake with very high concentrations of metformin may potentially be due to reduced viability of cells.
It is interesting to note that the patients on concomitant imatinib and metformin had good response in terms of the CML. This would support the finding that metformin is a weak inhibitor of OCT1. However, 4 patients eventually required switching to insulin. This was necessitated by high blood sugars. Potentially, imatinib at 5 micromolar concentrations (therapeutic concentration) may inhibit metformin uptake and lead to high systemic concentrations of metformin. High systemic concentrations of metformin can be associated with lactic acidosis. This is a serious adverse side effect of metformin and can result in renal failure and death. Renal failure could increase the imatinib concentrations and cause other adverse reactions. This could be investigated further by incubating the cells at 40µM concentration of \(^{14}\)C-metformin and varying the concentration of imatinib. Increases in imatinib concentration could lead to high concentration of metformin in the supernatant and reduced uptake by the CML lines. This may be a potential explanation as to why the 3 patients had to switch to insulin. However, it is well known patients on metformin require insulin to control their blood sugars due to factors such as weight and increasing creatinine. In vivo experiments using HPLC tracing to monitor metformin concentration could also be performed in patients taking imatinib. This would allow the area under the curve for metformin to the determined in the presence of imatinib. The peak concentration of metformin could be determined and pharmacokinetic studies could then show the potential effect of OCT1 inhibition by imatinib on metformin.

This study shows metformin is a weak inhibitor of OCT1. It does not reduce imatinib uptake at therapeutic concentrations. However, further studies are necessary to determine if imatinib could potentially inhibit metformin uptake into the cells and thus cause potentially high toxic metformin concentrations in the systemic circulation.
6. Conclusion

6.1.1 Comparison of results from population studies versus phase 3 clinical trials in CML

The population study of CML patients in Merseyside and North Wales showed important differences from the results of large phase 3 clinical trials such as the IRIS, ENESTnd and DASISION study. The IRIS study compared imatinib 400mg daily versus low dose cytarabine and interferon, while the ENESTnd study compared nilotinib 300mg bd or 400mg bd versus imatinib 400mg daily and the DASISION study compared dasatinib 100mg daily versus imatinib 400mg daily. In our population study only 59% of the patients achieved a CCR at 12 months with a cumulative CCR rate at 5 years of 65%. The MMR rate at 18 months was 47% with a cumulative MMR rate of 51% at 5 years. This is in marked contrast to the IRIS study which showed that imatinib treated patients had a 69% CCR rate at 12 months and a cumulative CCR rate at 5 years of 87%. The ENESTnd study showed the control group (imatinib) had a CCR rate of 65% at 12 months and a cumulative incidence of major molecular response of 44% at 2 years. The DASISION study, a phase 3 trial comparing imatinib and dasatinib, showed imatinib produced a 73% CCR rate at 12 months.

There is a difference between the CCR rates published in clinical trials compared to real life experiences. My study shows that 41% patients would have been classified as imatinib failures at 12 months and therefore required switching to a second generation TKI, as per the ELN criteria. IRIS and DASISION have much higher CCR rates. The MMR rate was 49% at 2 years in my population study which is similar to the MMR rates of 44% (ENESTnd) and 46% (DASISION).
6.1.2 Possible explanations for differences in primary outcomes between general unselected populations and clinical trials

The differences between my population study and clinical trial data can be explained by strict inclusion and exclusion criteria in the latter. The IRIS study excluded patients above the age of 70 years old, those with creatinine and liver function tests greater than 1.5 times the upper limit of normal, performance status higher than ECOG 3 and within 4 weeks of a major surgical procedure. Patients with other serious co morbidities were also excluded, thus skewing the trial patients to be younger and have less co-morbidity. The IRIS study also censored patients if they had poor response to treatment (11%), withdrawal of consent (5%) and severe side effects (4%). The IRIS study may have censored patients in a non-random fashion, with patients with poor risk characteristics more likely to have been censored. Thus, only a population study of unselected patients with chronic phase CML can reliably determine the efficacy of imatinib.

A population study performed by Lucas et al showed that only 41% patients achieved a CCR at 12 months, and at 24 months 49% had either failed imatinib or were intolerant of imatinib. Another single centre experience of imatinib outcomes performed by de Lavallade et al (2008) showed imatinib was associated with a CCR rate of 57.4% at 12 months. This is similar to the results obtained by my population study. A CML study in the West of Scotland and Lothian region again showed CCR rates roughly in the region of 55% at 12 months. This study also showed only 52.7% of patients at 5 years had a satisfactory response to imatinib as the ELN criteria.
6.1.3 First line use of 2nd generation TKIs in CML

Imatinib is an effective drug in a proportion of chronic phase CML patients but there are 41% of patients who would fail imatinib at 12 months, if the ELN criteria are applied to the population study. This raises the question of whether a second generation TKI should be used as first line treatment in all chronic phase CML.

Between 2007 and 2013, patients in the UK were able to participate in SPIRIT2 and between 2007 and 2008 they were able to enter ENESTnd. SPIRIT2 was a phase 3 randomised study comparing imatinib 400mg daily versus dasatinib 100mg daily.

In the Merseyside and North Wales region during the period 2003-2009, 20 patients were treated with second generation TKI as first line treatment. The population study showed second generation TKI produced a CCR rate of 85% at 12 months and an MMR rate of 60% at 18 months. These responses are superior to the results with imatinib. Second generation TKIs clearly achieve more rapid responses and more patients achieve both CCR and MMR. The ENESTnd study showed nilotinib 300mg bd produced a CCR rate of 80% while nilotinib 400mg produced a CCR rate of 78% at 12 months. The MMR rates published in the ENESTnd at 12 and 24 months for nilotinib 300mg bd were 55% and 71% respectively. The DASISION study at 24 months showed dasatinib produced a CCR and MMR rate of 86% and 64% respectively at 24 months. The population study results for frontline second generation therapy are broadly in keeping with those published in the large phase 3 clinical trials. However, this is a small group of patients and caution needs to be applied in interpreting outcomes from such a group. A large real life experience of second generation TKIs will be useful to determine primary outcomes such as CCR and MMR.
6.1.4 Clinical efficacy of 2\textsuperscript{nd} generation TKI in imatinib failure/intolerance

The population study recorded the outcomes of 33 patients who were switched from imatinib to a second generation TKI (nilotinib or dasatinib). Eighteen patients were deemed to have failed imatinib as per the ELN criteria and 15 patients were intolerant of imatinib. At 12 months 70% of patients had achieved a MMR. This would suggest second generation TKIs are effective therapeutic agents in imatinib resistant or intolerant patients. This rate of response appears to be higher than in published clinical trials, though the number of patients in my study was small.

A 321 patient study of imatinib resistant (70%) and intolerant (30%) patients treated with nilotinib as second line treatment showed 44% of patients achieved a CCR at 24 months. Imatinib intolerant patients had a higher CCR rate of 51% compared to 41% of imatinib resistant patients. At 24 months, 28% of patients overall achieved a MMR, with patients starting nilotinib with a complete haematological response having a higher rate of MMR. The progression free and overall survival at 24 months was 64% and 87% respectively (Kantarjian et al, 2011).

A large study of 1067 patients either intolerant or resistant to imatinib was treated with dasatinib as second line treatment. The overall CCR rate at 12 months was 51%, with imatinib resistant patients having a lower CCR rate of 45% compared to intolerant patients with a CCR rate of 75%. The overall MMR rate at 24 months was 40%, with imatinib resistant patients having a MMR rate of 34% and imatinib intolerant patients having a MMR rate of 63% at 24 months. This study showed patients failing to achieve a CCR at 12 months post dasatinib had inferior progression free and overall survival. MMR at 12 months predicted for improved
progression free survival, however there was no significant predictive effect on overall survival (Hocchaus et al, 2009).

The response to second generation TKIs in imatinib failure/intolerance in my population is much higher than in clinical trials. This is surprising considering the general population has patients with higher comorbidities than clinical trials. Previous studies have shown factors such as high and intermediate Sokal score, additional cytogenetic abnormalities, >95% Philadelphia chromosome positivity prior to starting second generation TKI, >6 months from imatinib failure to second generation TKI and loss of complete haematological response prior to starting second generation TKI are significant in predicting CCR. Scoring systems incorporating best cytogenetic response while on imatinib, Sokal score and recurrent neutropenia have been able to predict patients likely to respond to second generation TKI (Milojkovic et al, 2010). In clinical trials the patients included may have had skewed characteristics with a higher proportion of these risk factors. Patients in real life practice would potentially switch to a second generation TKI within 3 months of failure. A Scottish experience of second generation TKIs found that in 39 imatinib failures who were switched to a second generation TKI, 25(64%) had a satisfactory response, with 20 improving the depth of their response (Gallipoli et al, 2011). Large real life experiences of outcomes in imatinib failure/intolerant patients are required to determine the efficacy of second generation TKIs. The SPIRIT 3 study will enable clinicians to determine how effective ponatinib can be in imatinib or nilotinib resistance.

The results of the population study show second generation TKIs improve progression free survival in imatinib failure/intolerant patients. There were significantly more progressions prior to January 2005 compared to patients diagnosed post 2005, yet the overall survival was not significantly different between pre and post 2005 population. This is compatible with the view second generation
TKIs can be used effectively to salvage imatinib resistant patients. However, other factors such as increased physician experience with imatinib and introduction of the ELN criteria could also have influenced the outcome.

There have been other studies demonstrating second generation TKIs can improve survival outcomes. A large study compared the outcome of 104 imatinib failure patients salvaged with second generation TKI versus a historical cohort of 246 patients who had failed interferon. The patients treated with second generation TKIs had a superior overall survival compared to the historical cohort, though this was only applicable to patients who achieved a CCR (Ibrahim et al, 2011). However, my population study used a control group of patients treated with imatinib prior to 2005 as opposed to patients treated with interferon. A historical control group of interferon recipients is not an optimal comparator. Also, failure was defined differently in the imatinib failure group and interferon group, thus making the groups difficult to compare as the interferon treated group may have been less fit. My population study analysed only 33 patients requiring salvage with a second generation TKI, so it is difficult to determine which second generation TKI is superior in imatinib failure. A 597 patient study retrospectively analysed 301 imatinib failure/intolerant patients treated with nilotinib compared to 296 imatinib failure/intolerant patients treated with dasatinib. Nilotinib was associated with superior progression free survival; however the median follow up was only 11 months (Griffen et al, 2013).
6.1.5 Imatinib front line therapy is an effective strategy

The results of the population study show imatinib achieves a 59% CCR rate at 12 months. This shows imatinib is an effective treatment in CML. The CML patients developing imatinib resistance/failure have a 70% rate of salvage with second generation TKI. Imatinib is therefore still an effective treatment in CML patients, although frontline second generation TKI are associated with faster and deeper molecular response. A health economic analysis will help determine whether using frontline imatinib is cost effective, knowing that roughly 40% of patients will fail the drug.

Large studies such as TIDEL II (Yeung et al, 2015) showed patients initially treated with imatinib failing to achieve BCR-ABL<10% at 3 months were salvaged with either a dose increase in imatinib or a switch to nilotinib. This trial had MMR rates of 64% and 73% at 12 and 24 months respectively. The overall survival was >95% at 3 years. This suggests imatinib front line therapy is safe and effective in CML patients, and if resistance/failure occurs nilotinib is an efficacious agent.

6.2 Drug transporters

6.2.1 The role of SLCO1A2 in imatinib transport

As described previously 40% of patients fail imatinib due to either intolerance or resistance. Pharmacological causes of drug resistance such as low hOCT1 expression and low functional activity have been associated with inferior outcomes in CML patients treated with imatinib (Thomas et al, 2004 and White et al, 2006).
However, other studies have found contradictory results with some showing hOCT1 does not transport imatinib (Nies et al, 2013) and others showing only a slight increase in imatinib uptake with high hOCT1 expression (Hu et al, 2008). This could potentially imply that hOCT1 is a surrogate marker for other drug transporters which may have an important function in imatinib uptake.

SLCO1A2 is a gene encoding the OATP1A2 drug transporter and has been speculated to transport imatinib. Xenopus laevis oocytes injected with SLCO1A2 cDNA has been shown to increase imatinib uptake compared to water injected controls (Hu et al, 2008). I used a stably transfected CML cell line KCL22 into which I successfully transfected the SLCO1A2 gene. Expression was confirmed by using RQ-PCR and high expressing clones were selected. The high SLCO1A2 expressing clones showed higher imatinib uptake compared to the mock transfected and parental cell line. Imatinib transport into the SLCO1A2 transfected cell line was reduced if the pH was >8, or on addition of naringin or if the temperature was reduced to 4˚C. This shows that imatinib transport is an active process and drug transporter dependent. The increased uptake of imatinib in acidic conditions would suggest maximal uptake of imatinib is from the first part duodenum, where there are acidic conditions. There is high expression of SLCO1A2 in the gastrointestinal system. Therefore patients with small bowel malabsorption syndromes or previous gut surgery may have reduced absorption of imatinib and reduced bioavailability.

6.2.2 Gene polymorphisms of the SLCO1A2 gene and effect on CML outcome

Yamakawa et al have also recently shown that imatinib is actively transported by OATP1A2 in both Caco-2 intestinal cells and K562 CML cell lines (Yamakawa et al, 2011). They also determined the role of polymorphisms in the SLCO1A2 gene with
respect to imatinib clearance. Patients with certain polymorphisms in the promoter sequence of SLCO1A2 such as the SLCO1A2 –361GG genotype had significantly reduced imatinib clearance compared to patients with the SLCO1A2 –361GA and SLCO1A2 –361AA genotypes. The patients with SLCO1A2 –1105GA/–1032GA or SLCO1A2 –1105AA/–1032GA/–189_– 188insA genotype had a non-significant trend to lower imatinib clearance compared to patients with the SLCO1A2 –1105GG/–1032GG genotype. This study also analysed the effect of SLCO1A2 polymorphisms on CML outcomes with imatinib. In their cohort of 34 patients, 15 patients achieved a MMR and 15 patients achieved a CCR. This would suggest at a clinical level OATP1A2 polymorphisms do not influence clinical outcome.

6.2.3 Prognostic significance of SLCO1A2

I also analysed the expression of SLCO1A2 mRNA in CML patient samples. Generally CML patient samples had low expression of SLCO1A2, compared to normal controls. This suggests mature neutrophils and myelocytes (polymorphonuclear cells) in CML patients have low expression of SLCO1A2. It would be useful to determine if the CML blast cells have high expression of SLCO1A2 which could potentially make them susceptible to cell death. SLCO1A2 has been shown to be mainly expressed in the intestinal cells, liver and renal cells.

My study also compared whether there was a difference in imatinib uptake between patients with high versus low SLCO1A2 mRNA expression. There was no difference in imatinib uptake between patients with high or low SLCO1A2 expression. This would suggest OATP1A2 expression does not have an important influence on imatinib uptake. There was also no difference in mRNA expression of SLCO1A2 between patients progressing to blast crisis, patients achieving a CCR or patients
failing to achieve a CCR. This suggests SLCO1A2 is not a useful biomarker at predicting outcome in CML patients. This is in contrast to OCT1 levels which have been shown to predict response to imatinib. I did not perform a functional SLCO1A2 activity assay using naringin and imatinib, which has been done on OCT1 patient samples using prazosin as an inhibitor. It was noted that patient CML cells had reduced imatinib uptake compared to the transfected cell line, primarily due to reduced cell viability in patient samples. My results also show that OCT1 and SLCO1A2 mRNA expression are moderately positively correlated with an $r=0.468$.

### 6.2.4 Drug drug interactions at the SLCO1A2 level

SLCO1A2 transports a diverse range of xenobiotics. A postulated mechanism of imatinib resistance was drug drug interactions at the SLCO1A2 level. Reduced imatinib influx into a cell could be due to another co-administered drug impeding OATP1A2 mediated imatinib transport. The main inhibitors of SLCO1A2 such as antiepileptics and protease inhibitors used in HIV treatment were not commonly encountered in CML patients. In total 12 patients were on inhibitors of SLCO1A2. The concentrations of these medications were not measured in the patient plasma and these medications will have differing levels of inhibition at the SLCO1A2 level. Accepting these limitations in study analysis, these inhibitors did not appear to affect CML outcomes. Caution needs to be taken when interpreting these results in view of the imbalance between the groups. These results would again suggest SLCO1A2 does not have a major role in imatinib resistance, and OCT1 is the major drug transporter associated with imatinib resistance.
6.2.5 The role of OCTN1 and OCTN2 in imatinib transport

The roles of OCTN1 and OCTN2 were also assessed to determine if they were involved in imatinib transport. My results show imatinib is not a substrate of OCTN1 and OCTN2. The levels of OCTN1 and OCTN2 are very weakly correlated to OCT1. There was no difference in mRNA expressions levels of OCTN1 and OCTN2 in patients achieving optimal response, failing imatinib or falling in the caution category as per ELN criteria. OCTN1 and OCTN2 mRNA have no predictive role in determining imatinib failure. Other groups have also reported imatinib is not a substrate for OCTN1, but they showed OCTN2 was a possible transporter of imatinib. However, this has not been reproduced and they did not use a CML cell line for imatinib uptake instead using the HEK cell line. Interestingly, the OCTN1 rs1050152-C allele was associated with higher cumulative incidences of MMR and CMR. Patients with the CC or CT genotype had superior MMR rates compared to patients with TT genotype which is the minor carrier. However, the significance of this is not clear as OCTN1 does not transport imatinib, thus it maybe that this result was due to chance (Angelini et al, 2013).

Nilotinib and dasatinib are not transported by SLCO1A2, OCTN1 or OCTN2. These drugs are presumably lipophilic and can diffuse through the cell membrane.
6.3 Drug drug interactions

6.3.1 The interaction between metformin and imatinib

Chapter 5 assessed the interaction between imatinib and metformin in CML patients for potential drug drug interactions between imatinib and metformin. There is an increasing prevalence of type II Diabetes in the developed world (Chowdhury et al, 2010), and there is a suggestion diabetic patients are more likely to develop certain cancers such a pancreatic and colorectal cancer (Huxley et al, 2005; Larsson et al, 2005). Recently, studies have shown metformin has a protective role against cancer compared to other diabetic medication (Currie et al, 2009). The mechanism whereby metformin inhibits cancer is not clear but may be due to AMP kinase activation. AMP kinase activation reduces cell proliferation via cell cycle regulation inhibition of protein synthesis, thereby conserving energy. It is likely metformin in combination with cancer medication will become more widespread. Insulin and insulin growth factors can potentially cause cancer proliferation (Zakikhani et al, 2006).

Both imatinib and metformin are known substrates of hOCT1 and therefore drug inhibition is possible at a drug transporter level. The adverse outcomes associated with these drug interactions could be detrimental to health. If metformin is a potent inhibitor of imatinib then the treatment of CML could be compromised.

Only 6 patients were on a combination of imatinib and metformin. Both imatinib and metformin have been shown to be transported by hOCT1. The results indicate that increasing concentrations of metformin inhibit imatinib uptake. At 768µM metformin
there is a 50% reduction in imatinib uptake. However, this concentration of metformin is not achievable in clinical practice. The normal therapeutic concentration of metformin varies between 1-40µM. At therapeutic concentrations of metformin, imatinib uptake is not affected. This suggests metformin does not adversely affect CML outcome in imatinib treated patients. However, it is not clear whether the diabetic control of patients on imatinib and metformin is affected. Imatinib may have a higher affinity for the OCT1 drug transporter and therefore inhibit metformin uptake. This would lead to elevated blood sugars and poor diabetic control. To prove this hypothesis, the concentration of metformin would need to be kept constant and increasing concentrations of imatinib used. If concentrations of imatinib at 5µM, led to reduced metformin uptake pharmacokinetic studies would be helpful to determine the concentration of metformin in patient plasma. This is of relevance as high concentrations of metformin can cause lactic acidosis and renal impairment.

6.4 Summary

My CML population study has demonstrated that the outcomes in imatinib treated CML patients are inferior to the results published in phase 3 clinical trials. Only 59% of imatinib treated CML patient achieved a CCR at 12 months. The maximal MMR rate while on imatinib was 50% at 36 months. The study also showed that imatinib failure/intolerant patients, when switched to a second generation TKI had a high MMR rate of 73%. The introduction of second generation TKIs also improved the PFS but not overall survival. This would therefore suggest first line imatinib is an effective treatment for newly diagnosed CML patients, and patients failing imatinib can still achieve good durable responses. A health economic analysis would answer
how cost effective frontline imatinib is compared to using second generation TKI as frontline treatment.

I have demonstrated that imatinib is a substrate for OATP1A2. However, OATP1A2 does not transport nilotinib or dasatinib. The mRNA expression levels of SLCO1A2 at diagnosis in imatinib treated do not correlate to outcome as per the ELN criteria. Patients with concomitant OATP1A2 inhibitors and imatinib had similar outcomes to imatinib only treated patients. This suggests OATP1A2 transports imatinib but is not a significant prognostic marker to determine imatinib failure.

I have also shown that imatinib, nilotinib and dasatinib are not transported by OCTN1 or OCTN2. The mRNA expression levels of OCTN1 and OCTN2 do not correlate to patient outcome.

Metformin and imatinib are known substrates for hOCT1. In my thesis I have shown metformin at therapeutic concentrations has no effect on imatinib intracellular uptake. However, metformin concentrations greater than 768µM reduce imatinib uptake by 50%. Patients on metformin and imatinib had similar outcomes to imatinib only treated patients. Further studies assessing the interaction between imatinib and other commonly used prescription drugs which are known inhibitors of hOCT1 would be useful to guide the clinician in avoiding any significant imatinib drug interactions.


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