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Treatment of Chronic Myeloid Leukaemia: Current Practice and Future Prospects

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1. Introduction

Chronic myeloid leukaemia (CML) is a cancer of the hematopoietic system that arises from the Philadelphia chromosome (Ph^1). This results from the reciprocal translocation of chromosomes 9 and 22 which generates a *Bcr-Abl* fusion gene encoding a 210kDa protein with constitutive tyrosine kinase activity (Ben-Neriah et al., 1986; Kuzrock et al., 1988). This constitutively active tyrosine kinase drives proliferation and survival through multiple downstream pathways (Reviewed in Cowan-Jacob et al., 2004; Ren et al., 2005). The disease is characterised by three stages; the chronic phase marked by an accumulation of mature granulocytes and myeloid precursors in the bone marrow and peripheral blood; the accelerated phase characterised by a rise in myeloid precursors and a blast crisis stage which is characterised by a marked accumulation of differentiation-arrested blast cells of either myeloid or lymphoid lineage (Calabretta & Perotti, 2004; Savage et al., 1997). The generation and clinical use of the Bcr-Abl tyrosine kinase inhibitor (TKI) imatinib mesylate (IM) has revolutionised the treatment of CML patients (Druker et al., 1996; Druker et al., 2006) and has become the standard line of therapy for CML patients. Following treatment with imatinib, over 90% of patients obtain a complete haematologic response and more than 80% achieve a complete cytogenetic response. However, there are limitations associated with IM therapy. The drug is highly effective in the chronic phase of the disease but the response of patients in blast crisis is limited (Hehlman & Saussele, 2008). Furthermore, in approximately 40% of patients, resistance develops i.e. resistance in 100 patient years (Gorre et al., 2001). Great progress has been made over the last ten years in elucidating the molecular mechanisms of IM-resistance *in vitro* but correlating any of these individual resistance mechanisms in a clinical sample does not always indicate that it alone drives clinical progression as additional modes of resistance may be at work. The mechanisms by which patients become resistant to IM therapy include Bcr-Abl dependent mechanisms such as an increase in the levels of Bcr-Abl mRNA expression and corresponding upregulation of protein levels and amplification of the Bcr-Abl gene (Mahon et al. 2000). Bcr-Abl independent mechanisms include activation of signalling pathways downstream of Bcr-Abl including the phosphatidylinositol 3-kinase (PI3K)/Akt cell survival pathway or activation of signalling pathways separate to that of the *Bcr-Abl* gene and an efflux of IM via multidrug resistant proteins such as p-glycoprotein (Capdeville et al., 2002). The most well-characterised cooperating pathway involves the Src Family Kinases (SFKs) which have

been demonstrated to play a role in altering responsiveness to TKIs as well as promoting disease progression (Danhauser-Riedl et al., 1996; Wilson et al., 2002).
However, the major factor influencing IM resistance is due to mutations at critical points in the kinase domain of the *Bcr-Abl* gene which interferes with the ability of IM to interact with the enzyme. To date over 60 amino acid substitutions in the kinase domain have been found. Of these T315I is one of the most common Bcr-Abl mutations identified in patients and importantly this is also associated with the highest degree of IM-resistance, preventing the formation of the critical hydrogen bond and changing the conformation of the Bcr-Abl protein in such a way to render the protein completely resistant to imatinib. The frequency of the T315I mutation in IM-resistant patients is reported to range between 2% and 20% with variability related to detection methods along with patient cohort characteristics and treatment (Nicolini et al., 2009). Recent data suggests that the survival rate of patients harbouring a T315I mutation is dependent on disease phase at the time of mutation detection, with chronic phase patients responding to some investigational compounds (Nicolini et al., 2009). For example, Legros et al, (2007) reported T315I transcript disappearance in an IM-resistant CML patient treated with homoharringtonine and Giles et al, (2007) reported that 3 patients harbouring the T315I mutation achieved clinical responses with the aurora kinase inhibitor MK-0457. A greater understanding of the molecular basis of IM-resistance has provided the molecular rationale for the development of second and now third generation therapies for patients with CML. Such therapies will play a key role in the control of CML over the next decade.

2. Second generation tyrosine kinase inhibitors

Shortly after the introduction of IM in the clinic, reports of primary and secondary resistance cases began to emerge which led to the search for agents that might overcome this problem. The first second-generation TKI that was clinically evaluated was dasatinib (BMS-354825) and it was approved by the FDA for treatment of all phases of IM-resistant CML in June 2006. Dasatinib is able to bind to both Bcr-Abl and Src family kinases and it was originally identified in a screen of compounds that demonstrated potent Src/Abl kinase inhibition with antiproliferative activity in CML cell lines and xenograft models systems (Lombardo et al., 2004). Nilotinib (AMN107) was subsequently developed by rational drug design based on the crystal structure of an Abl/imatinib complex, allowing researchers to optimise the potency and selectivity of the compound (Weisberg et al., 2005).
Dasatinib and nilotinib were initially evaluated in patients with IM-resistant or intolerant CML. In phase II clinical trials with a 24 month follow up, both dasatinib and nilotinib were shown to have efficacy in patients in the chronic phase of CML (see Table 1). In the nilotinib trial, intolerance to imatinib was defined as having intolerance with no major cytogenetic response whereas in the dasatinib trial, imatinib-intolerant patients included patients who had a major cytogenetic response. These results were very promising and led to further Phase 3 trials where these second-generation TKIs were compared directly with imatinib as front-line therapies. It would be of interest in the future to determine the optimal time point for switching to second line treatment.
Saglio et al., (2010), recently reported results from the Evaluating Nilotinib Efficacy and Safety in Clinical Trials-Newly Diagnosed Patients (ENESTnd) trial. In this phase 3, randomized, multicenter study, 846 patients with chronic phase CML received either nilotinib (at a dose of either 300 mg or 400 mg twice daily) or imatinib (at a dose of 400 mg

once daily). At 12 months, the rates of major molecular response for nilotinib (44% for the 300-mg dose and 43% for the 400-mg dose) were nearly twice that for imatinib (22%). The rates of complete cytogenetic response by 12 months were significantly higher for nilotinib (80% for the 300-mg dose and 78% for the 400-mg dose) than for imatinib (65%). Patients receiving nilotinib also had a significant improvement in the time to progression to the accelerated phase or blast crisis, as compared with those receiving imatinib. Based on these results the US FDA has granted accelerated approval of nilotinib for the treatment of patients with newly diagnosed CML in chronic phase.

Response	**Dasatinib**	**Nilotinib**
No. of patients	387	321
Percent imatinib-resistant	74	70
Percent imatinib-intolerant	26	30
Percent CHR	91	95
Percent McyR	62	59
Percent CCyR	53	44

Table 1. Summary of results of phase 2 studies of dasatinib and nilotinib in patients with chronic phase CML demonstrating either IM-resistance or intolerance. CHR. Complete hematologic response; McyR, major cytogenetic response; CCyR, complete cytogenetic response.

Similarly, Kantarjian et al., (2010a) has reported the results of a trial comparing dasatinib versus imatinib in treatment naïve CML patients (DASISION trial). 519 patients with newly diagnosed chronic phase CML were randomly assigned to receive dasatinib at a dose of 100 mg once daily (259 patients) or imatinib at a dose of 400 mg once daily (260 patients). After a follow-up of 12 months, the rate of complete cytogenetic response was higher with dasatinib than with imatinib (77% vs. 66%). The rate of major molecular response was higher with dasatinib than with imatinib (46% vs. 28%), and responses were achieved in a shorter time with dasatinib. Progression to the accelerated or blastic phase of CML occurred in 5 patients who were receiving dasatinib (1.9%) and in 9 patients who were receiving imatinib (3.5%) while the safety profiles of the two treatments were similar. These results also led to accelerated FDA approval in October 2010 for this second generation TKI for initial therapy of CML. Taken together, the results of these recent trials suggest that the best treatment for resistance may be preventing the emergence of resistance in the first place by using these alternative frontline therapies. In addition to second generation TKIs, modified imatinib-based regimes (e.g. increasing the dose of imatinib to 800mg/day) are also currently under evaluation. It would also be of interest to determine whether administration of two or more TKIs together or consecutively would improve disease control as compared to single-agent therapy but such a course of study would present many difficulties in terms of clinical trial design.

3. Combination approaches and investigational compounds

The currently available TKIs do not demonstrate efficacy against the T315I mutation suggesting the need for additional strategies such as combination approaches with alternative classes of agents. Prior to the introduction of imatinib, interferon (IFN) alpha-

based regimens were the gold standard for treatment of early chronic phase CML patients. The combination of IFN-alpha with imatinib was recently investigated in two large clinical trials, the French SPIRIT trial and the German CML Study IV. In the SPIRIT trial 636 patients were randomised 1:1:1:1 to receive either imatinib 400mg/day, 600mg/day, 400mg/day plus cytarabine or 400mg/day plus INF-alpha. After 18 months MMR rates were 41% versus 52% versus 53% versus 62% respectively (Preudhomme et al., 2010). In the German study CML IV, patients were given imaitinib 400mg/day versus 800mg/day versus 400mg/day plus IFN-alpha. Response rates were higher in the imatinib (800mg/day) cohort (CCyR 65%, MMR 54%) when compared to the imatinib (400mg/day) cohort with or without IFN-alpha (CCyR 52% and 51%; MMR 30% and 35%, respectively) suggesting in this case that high dose imatinib increases the rate of MMR at 12 months (Hehlmann et al., 2011). Furthermore, they demonstrated that achievement of MMR by month 12 is directly associated with improved survival (Hehlmann et al., 2011).

A number of investigational compounds, many of which are active against T315I mutants have also been identified and many are currently undergoing clinical trials and are summarised in Table 2. These can be subdivided into four classes; third generation TKIs, aurora kinase inhibitors, switch pocket inhibitors and apoptosis modulators.

3.1 Third generation TKIs

Despite the very promising results with dasatinib and nilotinib there is still room for improvement. Due to the fact that the currently available TKIs have no activity against T315I mutants, many investigational compounds are currently being clinically evaluated in this cohort of patients. Ponatinib (AP24534) is an orally bioavailable multi-targeted compound with activity against many kinases including native Bcr-Abl, the T315I mutant and other mutants (O'Hare et al., 2009). Ponatinib does not need to make a hydrogen bond with T315 therefore it can accommodate the side chain of the isoleucine residue in the T315I mutation (O'Hare et al., 2009). In kinase-based assays, ponatinib potently inhibited the activity of wild-type Bcr-Abl and the T315I mutant in the nanomolar range. Ponatinib also exhibited nanomolar activity against other kinases such as SRC, FGFR1, FLT3, KIT and VEGFR. A phase I trial of ponatinib has recently been completed and a phase II trial is currently underway. If ponatinib can be demonstrated to be a pan-Bcr-Abl inhibitor in the clinic and it is proven to be safe and effective it may be a future frontline therapeutic for CML.

Bosutinib, is a third generation TKI that is currently being developed by Pfizer. It inhibits Bcr-Abl with higher potency than imatinib but it also demonstrates activity against a number of other kinases including SFKs, c-Kit and PDGF receptors (Remsing-Rixet et al., 2009). Bosutinib is currently undergoing frontline testing against imatinib with promising results. This third generation TKI may shortly win FDA approval for initial therapy of CML (Bixby & Talpaz, 2011).

INNO-406 is an orally bioavailable dual Abl/Lyn kinase inhibitor which is up to 50 times more potent than imatinib against Bcr-Abl. Results of a Phase I trial with this TKI have recently been reported (Kantarjian et al., 2010b). INNO-406 was administered to 56 patients with imatinib resistance (n = 40) or intolerance (n = 16). Other previous treatments included nilotinib (n = 20 patients), dasatinib (n = 26 patients), and dasatinib/nilotinib (n = 9 patients). Of 31 patients with CML in chronic phase who received INNO-406, the major cytogenetic response rate was 19%. No responses were observed in patients who had CML in accelerated phase or in blastic phase. The maximium tolerated dose was identified at 240mg twice daily and further phase II studies are planned.

XL228 is an intravenously available multi-targeted TKI that has significant *in vitro* activity against the T315I mutant. It is currently been investigated in a small clinical trial of 27 patients where 20 of the patients harbour the highly resistant T315I (10 patients), F317L (7 patients) and V299L (3 patients) mutations. Preliminary clinical activity has been reported in a poster session at the 50th Annual American Society of Haematology in December 2008 (Abstract 3232) and looks promising. XL228 is being currently tested in two Phase I clinical trials, one for the treatment of CML or Ph+ acute lymphoblastic leukaemia (ALL), and the second for the treatment of advanced malignancies including lymphoma.

3.2 Aurora kinase inhibitors

Aurora kinases A and B are a group of serine/threonine kinases also known as mitotic kinases, that regulate the transition from G2 through cytokinesis. Aurora kinases are overexpressed in several types of solid tumours and some haematological malignancies including acute myeloid leukaemia (AML) (Ye et al., 2009). Inhibition of these kinases in leukaemia cells has been shown to result in aberrant mitosis which in turn can lead to mitotic catastrophe. Aurora kinase inhibitors are being developed as potential targeted therapies for cancer patients. There is much similarity between the ATP binding sites of aurora kinases and other kinases including Bcr-Abl.

Danusertib is an intravenously administered multi-targeted kinase inhibitor which demonstrates significant activity against various aurora kinases (Gontarewicz & Brummendorf, 2010). It has also demonstrated *in vitro* efficacy against native Bcr-Abl and the T315I mutant. A phase I clinical trial is currently underway with advanced phase CML patients resistant or intolerant to imatinib and/or a second generation TKI.

AT9283 is an Aurora kinase A and B inhibitor which is administered intravenously. It exhibits efficacy in the nanomolar range against Abl and the T315I mutant along with a range of other kinases including JAK 2 and 3 and FGF4 (Howard et al., 2009). Phase I trials are currently underway in the United States.

One potential problem associated with aurora kinase inhibitors is that they all require prolonged intravenous administration and response have frequently been associated with the periods at which the drug is administered.

3.3 Switch pocket inhibitors

Recently a series of non-ATP competitive multi-kinase inhibitors have been developed. Switch pocket inhibitors bind to amino acid residues that kinases use to undergo the conformational change from the inactive(closed) to the active(open) state and therefore they keep the kinase in the inactive conformation (Chan et al., 2011; Eide et al., 2011). An important structural feature of the Abl kinase is the presence of a series of hydrophobic residues that are stacked in a layer and help to stabilise the active conformation. Indeed the T315I mutant further stabilises the active conformation possibly leading to increased activity of the enzyme. DCC-2036 is one of the lead switch pocket inhibitors. It is an orally bioavailable compound that has demonstrated activity against both native Abl and the T315I mutant and a number of other kinases such as VEGFR2 (Chan et al., 2011; Eide et al., 2011). The compound is currently been evaluated in a phase 1 clinical trial for use in imatinib-resistant CML including patients with T315I mutation.

3.4 Apoptosis modulators

Certain compounds that function independently of kinase inhibition activity are also being developed. Omacetaxine is one such compound. Omacetaxine is a semisynthetic formulation of the alkaloid homoharringtone which can be administered subcutaneously. Homoharringtone has been shown to exhibit anti-tumoural effects by disrupting protein synthesis and downregulating the anti-apoptotic protein myeloid cell leukaemia-1 (MCL-1) (Tang et al., 2006). This leads to disruption of the mitochondrial membrane with release of cytochrome c, caspase activation resulting in apoptotic cell death (Tang et al., 2006). Omacetaxine is currently been evaluated in two multicentre Phase III clinical trials for patients with CML who have failed two or more TKIs or for patients with the T315I mutation. It may become the first drug to be approved for third-line therapy in CML.

Drug Class	Company	Targets	Activity against T315I mutant	References
TKIs				
Ponatinib	Ariad U.S.A.	ABL, FGFR1, FLT3, KIT, VEGFR	Yes	O'Hare et al., 2009
Bosutinib	Pfizer U.S.A.	ABL,CAMK2G, STE20, TEC	No	Bixby & Talpaz, 2011; Remsing Rix et al., 2009
INNO-406	CytRx U.S.A	ABL, KIT, LYN, PDGFR	No	Kantarjian et al., 2010b
XL228	Exelixis U.S.A	ABL, Aurora A, FGFR1-3, IGF1R, SRC	Yes	Not applicable
Aurora kinase inhibitors				
AT9283	Astex, U.K.	ABL, Aurora A & B, FLT3, JAK2, JAK3	Yes	Howard et al., 2009
Danusertib	Nerivano medical sciences, Italy	ABL, Aurora A & B,FGFR1, RET, TRK	Yes	Gontarewicz & Brummendorf , 2010
Switch pocket inhibitors				
DCC-2036	Deciphera, U.S.A.	ABL, FLT3, KDR, SFK, TIE2	Yes	Chan et al., 2011; Eide et al., 2011
Apoptosis modulators				
Omacetaxine	ChemGenex, Australia	Cytochrome c, MCL-1	Yes	Tang et al., 2006
Hsp90 inhibitor KOS-1022	Kosan, U.S.A.	Cytochrome c	Yes	Gorre et al., 2002

Table 2. Investigational compounds in chronic myeloid leukaemia

Heat shock protein 90 (Hsp90) is a chaperone protein that assists client proteins in folding and prevents protein misfolding and degradation by the proteasome. The Hsp90 antagonist, 17-allyamino-17-demethoxygeldanamycin (17-AAG), has been shown to cause release of

cytochrome c, caspase activation and apoptosis in native Bcr-Abl cells and those expressing the T315I mutant (Gorre et al., 2002). Clinical trials with the more soluble analogue of 17-AAG, 17-DMAG (KOS-1022) are currently ongoing.

Our group have recently reported the effects of representative members of the novel pro-apoptotic microtubule depolymerising pyrrolo-1,5-benzoxazepines or PBOX compounds on chemotherapy-refractory CML cells using a series of Bcr-Abl mutant cell lines, clinical *ex vivo* patient samples and an *in vivo* mouse model (Bright et al., 2010). The PBOX compounds potently reduced cell viability in cells expressing the E225K and H396P mutants as well as the highly resistant T315I mutant. The PBOX compounds also induced apoptosis in primary CML samples including those resistant to imatinib. In addition we have shown that the PBOXs enhance the apoptotic efficacy of imatinib in CML cell lines (Bright et al., 2009; Greene et al., 2007). Furthermore we have demonstrated the *in vivo* efficacy of a representative pro-apoptotic PBOX compound, PBOX-6, in a CML mouse model of the T315I Bcr-Abl mutant. Results from this study highlight the potential of these novel series of PBOX compounds as potential therapy against CML.

4. Stem cell transplantation

Prior to the advent of imatinib and other TKIs, allogeneic hematopoietic stem cell transplantation (HSCT) was the main therapeutic option for CML patients and indeed is the only known curative treatment for CML to date. After the initial results with imatinib were published, allogeneic transplantation began to decline as a frontline treatment for CML. However to date there is no prospective study that compares imatinib and HSCT as frontline treatments. A retrospective review of over 1000 patients who received an allogeneic transplant in the pre-imatinib era reported an overall survival rate of 47% after 8 years and a relapse rate of 33% after 5 years (Gratwohl et al., 1993). More recently, Saussele et al., reported the results of an analysis of a subgroup of the randomized German CML study IV. These patients received a transplant after imatinib failure and demonstrated a 91% survival after 3 years (Saussele et al., 2010). Imatinib does not appear to impair engraftment and the incidence of graft versus host disease and survival was the same as for patients in the same stage of the disease who were not treated with imatinib. Since TKIs have no harmful effect on the transplant outcome they can be used until a suitable donor is found and the transplant procedure is performed. On the recommendation of the European LeukemiaNet, allogeneic transplant is now considered for those patients who have failed treatment with a second-generation TKI, patients in the advanced or blastic phase of CML at the time of diagnosis (as these patients are not responsive to TKIs) or those with the T315I mutation (see Table 3). Transplantation may also be an option for those patients that develop mutations while undergoing second line therapy (Baccarani et al., 2009). Finally transplantation may possibly be an option for pediatric or young patients with a suitable donor as the long term effects of TKIs such as drug toxicity (Kerkala et al., 2006) and immune dysfunction (Dietz et al., 2004) have not been clearly identified to date. Finally the capacity to combine novel TKIs with allogeneic transplantation in high-risk patients will potentially improve survival but further studies are required. Unfortunately only a low percentage of patients receive a transplant for a variety of reasons such as age and lack of appropriate donors. In the German CML study IV, of the 1,242 CML patients involved, 84

patients underwent allogeneic HSCT, with a relatively young age of patients reported (median age of 36 years) (Saussele et al., 2010).

Chronic phase, frontline therapy	Imatinib (400mg daily)
Chronic phase, second-line therapy *IM-intolerance* *IM-failure*	Nilotinib (400mg twice daily) or Dasatinib (100mg daily) Nilotinib (400mg twice daily) or Dasatinib (100mg daily) or HSCT in low transplantation risk and high risk disease (e.g. T315I mutation)
Chronic Phase, third-line therapy *In case of dasatinib or nilotinib failure*	HSCT
Accelerated and blastic phase *Frontline* *Second-line*	Imatinib followed by HSCT wherever possible Nilotinib or Dasatinib followed by HSCT wherever possible

Table 3. Current recommendations for treatment of CML patients (modified from Baccarani et al., 2009)

5. Chronic myeloid leukaemia stem cells

There is also a mounting body of evidence suggesting that in many cancers, including CML, cancer stem cells (CSCs) evolve as a result of both genetic and epigenetic events that alter hematopoietic progenitor differentiation, survival and self-renewal. Hematopoietic stem cells (HSCs) are defined by their capacity for self-renewal and their ability to give rise to all mature haematopoietic cell lineages throughout an individual's lifetime. There is accumulating evidence to suggest that CML cells emerge due to expression of Bcr-Abl in normal HSCs. Transplantation of multipotent murine HSCs expressing Bcr-Abl into recipient mice induces a CML-like myeloproliferative disorder (Pear et al., 1998) whereas CML is not induced in committed murine haematopoietic progenitor cells expressing Bcr-Abl (Huntly et al., 2004). There is also accumulating evidence that the signalling pathways that control normal HSC fate also determine maintenance of stem cell function. Recently signalling pathways or molecules such as Wnt/β-catenin, hedgehog (Hh), promyelocytic leukaemia (PML) and forkhead box class O of transcription factors (FOXO) have been shown to control stem cell fate in both normal hematopoiesis and in CML.

5.1 Signalling pathways underlying maintenance of CML stem cells

The wnt/β-catenin signalling pathway is thought to play a role in maintenance of CML stem cells. There are numerous reports demonstrating that β-catenin regulates normal mouse HSC renewal (Reya et al., 2003; Zhao et al., 2007). Furthermore, Zhao et al., 2007 performed a series of mouse genetic studies demonstrating that conditional deletion of β-catenin reduced maintenance of CML stem cells in the chronic phase. Loss of β-catenin also suppressed infiltration of CML cells into the lung and liver of mice injected with CML stem cells (Zhao et al., 2007).

The hedgehog signalling pathway is also though to underlie stem cell fate in both normal hematopoiesis and CML. In the absence of Hh ligands, Patched (Ptch) a twelve-

transmembrane receptor inhibits smo, a seven-transmembrane receptor. The binding of Hh ligands such as Indian hedgehog, Desert hedgehog or Sonic hedgehog to the Patched receptor in turn activates Smo and this receptor activates downstream signalling events mediated through activation of Gli transcriptional effectors. Two recent studies have demonstrated that expansion of Bcr-Abl leukaemic stem cells is dependent on the hedgehog pathway. Conditional *Smo* deletion caused CML stem cell suppression and impaired CML progression (Dierks et al., 2008; Zhao et al., 2009). Furthermore expression of constitutively active Smo increased the frequency of CML stem cells and accelerated CML development (Zhao et al., 2009) demonstrating an essential requirement for the Hh signalling pathway in maintenance of CML stem cells.

The promyelocytic leukaemia (PML) protein is a tumour suppressor protein localising to PML nuclear bodies. It plays a role in a wide array of biological activities including apoptosis, senescence and the DNA damage response pathway. Ito et al., 2008 reported high expression of PML in normal HSCs and demonstrated that conditional deletion of *Pml* resulted in intensive cell cycling which in turn resulted in impaired self-renewal capacity. They also demonstrated the defective ability of $Pml^{-/-}$ CML stem cells to develop CML at the 3rd serial transplantation.

Forkhead box class O of transcription factors (FOXO) have been shown to control stem cell fate in both normal hematopoiesis and in CML. The FOXO family of transcription factors include FOXO1, FOXO3a, FOXO4 and FOXO6 and they are all downstream targets of the cell survival phosphatidylinositol-3-kinase-AKT signalling pathway. When a ligand such as a growth factor or insulin binds to its receptor and activates the PI3-K-AKT pathway, AKT phosporylates FOXOs preventing their translocation to the nucleus and causing their degradation. It is widely believed that Bcr-Abl activates AKT signalling and suppresses FOXOs which in turn enhances the proliferation or inhibits the apoptosis of CML cells. However, Naka et al., (2010) have recently shown that FOXO3a plays an essential role in the maintenance of CML stem cells through the use of a syngeneic transplantation system and a CML-like myeloproliferative disease mouse model. They demonstrated that cells with a nuclear localisation of FOXO3a and decreased AKT phosphorylation are enriched in CML stem cell population, despite expression of Bcr-Abl. They also found that the ability of CML stem cells to promote malignancy at the 3rd transplantation is significantly decreased by Foxo3a deficiency *in vivo*. In addition, they have shown that TGF-beta is a critical regulator of AKT activation in CML stem cells and control the localisation of FOXO3a. This suggests the potential of TGF-beta-FOXO signalling inhibitors in eradicating CML stem cells.

The transcription factor JunB has been shown to protect against myeloid malignancies including CML by limiting hematopoietic stem cell proliferation and differentiation. Inactivation of JunB deregulates the cell-cycle machinery and increases the proliferation of HSCs without impairing their self-renewal or regenerative potential *in vivo* (Santaguida et al., 2009). Such data increases our understanding of how defects in signalling pathways that control the proliferation of stem cells leads to an increase in their transformation ability.

5.2 Mechanisms of tyrosine kinase inhibitor resistance of CML stem cells

Many studies have shown that TKIs such as imatinib, dasatinib and nilotinib potently inhibit TKI in differentiated CML stem cells but are not as effective in quiescent CML stem cells. For example, the presence of detectable primitive leukaemic progenitor cells in CML patients with an established complete cytogenetic response after 5 years on imatinib

treatment has been demonstrated (Bhatia et al., 2003). Furthermore, patients with an apparent molecular remission of CML following cessation of imatinib treatment quickly relapse (Cortes et al., 2004). It has been suggested therefore that these quiescent stem cells may be a reservoir for relapse (Holyoake, 1999; Wang et al., 1998). Drugs that are capable of eradicating the CML stem cells would provide much improved treatment for CML patients. To date, a number of potential mechanisms mediating TKI-resistance of CML stem cells have been postulated.

Firstly FOXO has been suggested to contribute to resistance to TKI therapy. Komatsu et al., (2003), has previously reported that FOXO3a is a downstream effector of imatinib induced cell cycle arrest in Bcr-Abl expressing cells and that FOXO inactivation sensitises cells to imatinib treatment suggesting that FOXO contributes to resistance to TKI treatment. To study this further in CML stem cells, Naka et al., (2010) investigated the roles of Foxo3a in response to TKI therapy using a CML mouse model. They showed that Foxo3a deficiency sensitised CML stem cells to TKI treatment and suggested that Foxo3a plays diverse roles in CML stem cell and non-stem cells. In their model, FOXO activation protects CML stem cells against TKI treatment while in non-CML stem cells it induces apoptosis or cell cycle arrest. In this same paper, they provided both *in vitro* and *in vivo* data which demonstrates a role for the TGF-beta/FOXO signalling pathway in maintaining imatinib-resistant CML stem cells. Treatment of CML stem cells with a TGF-beta inhibitor, Ly364947, impaired their colony forming ability *in vitro* and a combination of TGF-beta inhibition, Foxo3a deficiency and imatinib treatment resulted in efficient depletion of CML *in vivo*. Thus inhibition of TGF-beta signalling may result in eradication of the reservoir of CML stem cells.

There have also been recent reports demonstrating that Bcr-Abl stimulates the proteasome mediated degradation of certain FOXO family members in an animal model and in samples taken from CML patients (Jagani et al., 2009). Treatment with the proteasome inhibitor, bortezomib, resulted in an inhibition of Bcr-Abl mediated downregulation of FOXO and a regression of leukaemia suggesting that bortezomib is a candidate therapeutic in the treatment of Bcr-Abl-induced leukaemia. Furthermore, recent data demonstrate that bortezomib has significant activity against CML stem cells and synergises with imatinib in a CML murine model (Heaney et al., 2010; Hu et al., 2009). Bortezomib has also been shown to inhibit proteosomal degradation of protein phosphatase 2A (PP2A). This in turn reactivates PP2A which is an important negative regulator of Bcr-Abl (Hu et al., 2009). However, due to the known toxicities of bortezomib, including myelosuppression, the likely initial clinical application of bortezomib in CML would be in resistant and advanced disease.

Other work has demonstrated that Hh signalling contributes to TKI resistance. Dierks et al., (2008) demonstrated that inhibition of the Hh signalling pathway with cyclopamine, which maintains Smo in its inactive form, impairs development of CML by CML stem cells. In addition, a combination of cyclopamine with nilotinib delayed the recurrence of the disease compared to treatment with nilotinib alone (Dierks et al., 2008).

Another key molecule that may control TKI resistance of CML stem cells is reported to be the arachidonate 5-lipoxygenase (*Alox5*) gene which encodes a lipoxygenase 5-LO (Chen et al., 2009). Gene expression profiling demonstrated that *Alox5* expression is up-regulated by Bcr-Abl. In the absence of Alox5, Bcr-Abl failed to induce CML in mice. This *Alox5* deficiency caused impairment of the function of CML stem cells but not normal hematopoietic stem cells by affecting their differentiation and cell division. This in turn caused a depletion of CML stem cells and a failure of CML development. Treatment of CML

mice with a 5-LO inhibitor, zileuton, also impaired the function of CML stem cells and prolonged survival of CML affected mice. These results demonstrate that a specific target gene can be found in CML stem cells and its inhibition can inhibit the function of these stem cells. It is of interest to note that upregulation of *Alox5* was not inhibited by treatment with TKIs which may go some way to explaining why imatinib does not affect CML stem cells.

PML as described above is also an important target of CML stem cell therapy. Ito et al., (2008) demonstrated the critical role of this tumour suppressor in CML stem cell maintenance, and presented a new therapeutic approach for targeting quiescent CML stem cells by pharmacological inhibition of PML. Treatment of mice with arsenic trioxide, which downregulates PML expression, completely eradicated CML stem cells when used in combination with the chemotherapeutic drug Ara-C. This suggests that targeting PML for degradation could be an attractive therapeutic approach for targeting CML stem cells.

Autophagy is a genetically controlled process whereby organelles and long lived proteins are sequestered and engulfed into vacuoles called autophagosomes. These autophagosomes then fuse with lysosomes to produce autolysosomes which are targeted for either destruction or recycling (Kroemer & Levine, 2008). In certain situations autophagy serves as an alternative to apoptosis and is thus called type II cell death whereas in other cellular contexts, such as starvation induced by growth factor withdrawal/metabolic stress, it serves as a cell survival mechanism allowing tumour cells to become metabolically dormant. It has recently been reported that imatinib not only induces apoptotic cell death in CML cells but also induces autophagy following the induction of ER stress (Bellodi et al., 2009). In addition, inhibition of autophagic cell death using pharmacological inhibitors of autophagosome-lysosome fusion (chloroquine and bafilomycin) enhanced imatinib-induced cell death in CML cell lines and primary CML cells including those expressing partially IM-resistant Bcr-Abl mutants. Furthermore and of even greater importance, CML stem cells were shown to be extremely sensitive to the combination treatment. Knockdown of the autophagy genes Atg5 and Atg7 in CML cells also enhanced TKI-induced cell death. These workers therefore postulated that TKI-induced autophagy may antagonise TKI-induced cell death through apoptosis and inhibition of autophagy may eliminate this survival mechanism by restoring sensitivity of CML stem cells to TKI therapy (Bellodi et al., 2009). This approach would avoid the necessity of targeting CML stem cells through Bcr-Abl-indpendent approaches. In addition normal stem cells would not be targeted as these autophagic inhibitors had little or no effect on normal progenitors. The results of the Bellodi study have recently led to a randomised phase II clinical trial of IM versus IM/hydrochloroquine in CML patients which is being initiated at a number of centres in the U.K. This is known as the CHOICES (chloroquine and imatinib combination to eliminate stem cells) trial.

Histone deacetylase inhibitors (HDACIs) are drugs that target histone deacetylase complexes which modulate chromatin acetylation resulting in changes in gene expression. These inhibitors have a wide variety of effects as they also inhibit deacetylation of chaperone proteins such as Hsp90, transcription factors and a variety of other signalling mediators. It has previously been shown that treatment of CML cells with HDACIs such as LBH589 resulted in a downregulation of Bcr-Abl and an induction of apoptosis (Fiskus et al., 2006). In addition synergistic effects were observed with HDACIs in combination with a variety of TKIs (dasatinib, nilotinib and imatinib). The HDACIs are thought to target Hsp90 which results in decreased chaperone activity of Hsp90 leading to increased proteosomal degradation of Bcr-Abl. A recent report has demonstrated that the HDACI LBH589 when

used in combination with imatinib induced apoptosis of quiescent CML stem cells with a subsequent lack of engraftment in immunodeficient mice (Zhang et al., 2010). Thus a further possibility for eradication of CML stem cells may lie in combining TKIs with HDACIs.
Table 4 below summarises the key signalling pathways/molecules that are thought to play a role in mediating resistance to TKI therapy and drugs that target these pathways/molecules that may have potential either alone or in combination for CML therapy.

Drug	**Drug target in CML stem cells**	**Combination therapy**	**Reference**
Ly364947	TGF-beta-FOXO signalling pathway	Ly364947 and imatinib improved survival of CML mice	Komatso et al., 2003
Cyclopamine	Smo in the hedgehog signalling pathway	Cyclopamine and nilotinib improved survival of CML mice	Dierks et al., 2008
Zileuton	5-lipoxygenase	Zileuton and imatinib prolonged survival of CML mice	Chen et al., 2009
Arsenic trioxide	PML	Arsenic trioxide and AraC prolonged survival of CML mice	Ito et al., 2008
Chloroquine	Autophagy	Chloroquine sensitised primary CML stem cells to imatinib-induced cell death	Bellodi et al., 2009
LBH589	HDACs	LBH589 and imatinib co-treatment induced apoptosis of CML stem cells and prevented subsequent engraftment in immunodeficient mice	Zhang et al., 2010

Table 4. Signalling pathway/key molecules underlying TKI-resistance in CML stem cells as potential drug targets

6. Immunotherapy for the treatment of CML

Clinical interest in immunotherapy still remains as allogeneic stem cell transplantation, which relys on a graft versus leukaemia effect, provides the only long-term eradication of CML. The differences in minor histocompatibility antigens between recipient and donor along with effector cells specifically targeted at leukaemic antigens contributes to the cure of the disease (Rezvani & Barret., 2008). Additional evidence that CML is a disease susceptible to immunotherapy is provided by reports demonstrating the benefit of allogeneic donor lymphocyte infusions following transplantation (Drobyski & Keever, 1993; Kolb et al., 1995).

As mentioned above, CML is a clonal disorder of pluripotent haematopoietic stem cells which is characterised by the Bcr-Abl fusion protein. This results from the reciprocal translocation of chromosomes 9 and 22 which generates a *Bcr-Abl* fusion gene (Ben-Neriah et al., 1986). The t(9;22) mRNA is translated to a chimeric Bcr-Abl protein of molecular weight 210kDa often referred to as the p210 protein. However different breakpoint areas in the bcr gene have been identified resulting in slight variations in fusion transcripts. The most commonly expressed transcripts are the b3a2 and b2a2 transcripts (Deininger & Goldman, 2000). This generates a neo-antigen which is tumour specific because it contains a new sequence of amino acids in the junctional region of p210 that are not present in normal hematopoietic stem cells. This in turn provides a unique target for immunotherapeutic intervention using a vaccine-based approach.

6.1 Antigen-specific targets in CML-Bcr-Abl junctional peptides

The junctional regions of p210 contain not only a unique sequence of amino acids but additionally a new amino acid is formed due to codon split during translocation. Thus a lysine in b3a2 and a glutamic acid in b2a2 is generated (Shtivelman et al., 2006). There have been many reports of immunogenicity of the fusion region derived peptides of p210 with respect to the major histocompatibility complex (MHC) class I and II. For example, the p210/b3a2-derived fusion protein amino acid sequences have been shown to bind to various class I HLA antigen molecules including A0201, A3, A11 and B8 (Berke et al., 2000) supporting the potential of these peptides as target for class I HLA-restricted T-cell cytotoxicity. However, presentation of other Bcr-Abl junctional peptides has not been established in other HLA types which somewhat limits the clinical potential of class I peptides to subpopulations with specific HLA alleles. Strategies have been implemented to improve the binding of HLA class I molecules by amino acid substitutions at key binding residues of Bcr-Abl peptides to try and overcome their somewhat poor immunogenicity (Pinilla-Ibarz et al., 2005). Interest has also developed in class II Bcr-Abl specific peptides although less is known regarding the interaction of Bcr-Abl peptides with HLA class II molecules (Mannering et al., 1997; Yasukawaet al., 1998). In addition several clinical trials have been initiated using peptide based vaccines to treat CML, often with concomitant treatment of interferon-alpha or imatinib (Bocchia et al., 2005; Cathcart et al., 2004; Pinilla-Ibarz et al., 2000; Rojas et al., 2007). Results of these trials are reviewed by Pinilla-Ibarz et al., (2009).

6.2 Selectively expressed and over-expressed antigens in CML

Another potential target for immunotherapy are antigens that are selectively expressed or over-expressed. Wilms' tumour antigen 1 (WT1) is a transcription factor that is over-expressed in many human leukaemias including CML and also in solid malignancies and several class I restricted epitopes have been identified to date (Ariyaratana & Loeb, 2007). The expression of WT1 in CML has been shown to correlate with disease progression. Many peptides have been designed and cytotoxic T-lymphocytes generated in the presence of some of these peptides were able to specifically target WT1-expressing leukemic cells while sparing normal progenitors (Oka et al., 2000).

The efficacy of WT1-based vaccines has been the study of a number of trials with patients with AML, breast cancer, lung cancer, myelodysplastic syndrome and mesothelioma with promising results (Chaise et al., 2008; Li Z et al., 2005; Oka et al., 2004).

Another promising target in immunotherapy is PR3, a serine protease which is stored in neutrophils and is over-expressed in 75% of CML patients. CD8+ T cells specific for PR3 have been identified in patients in remission following HSCT and correlated with cytogenetic remission (Moldrem et al., 2006).
Several other antigens have been reported as being over-expressed in CML including preferentially expressed antigen of melanoma (PRAME) (Rezvani et al., 2009) and human telomerase reverse transcriptase (hTERT) (Gannage et al., 2005) and these may also be useful for immunotherapy in leukaemia.
It is also important to note the differential and sequential expression of several tumour antigens in different phases of CML suggesting the importance of combining several antigens in the design of future vaccines. The safety and immunogenicity of a combined vaccine of two antigenic peptides, PR1 and WT1, has recently been described and supports further studies of immunisation strategies in CML patients (Rezvani et al., 2008).

6.3 Immunomodulatory effects of TKIs

It has been hypothesised that imatinib reduces the efficacy of graft versus leukemia effect or other T-cell-based immunotherapies. This is based on several studies reporting impaired T-cell specific proliferation and responses as well as the inhibition of antigen-specific memory T cells (Boissel et al., 2006; Mumprecht et al., 2006). Conversely, imatinib has also been demonstrated to initiate an increase in IFN-gamma-producing T cells following 3 months of treatment and it may restore the function of Th1 helper T cells (Aswald et al., 2002).
In vivo antitumour T-cell immunity has been observed in several clinical trials using both Bcr-Abl peptide vaccines and other cellular vaccines (Maslak et al., 2008 & Smith et al., 2006). The use of imatinib in conjunction with donor lymphocyte infusion for relapsed CML patients following HSCT has also been shown to be efficacious suggesting that the clinical effect of imatinib may actually be beneficial (Olavarria et al., 2007; Savani et al., 2005).
Second generation TKIs have also been shown to have immunomodulatory effects. For example, nilotinib has been shown to inhibit the expansion of CD8+ T lymphocytes specific for viral or leukemia antigens much more potently that the same inhibitory effect elicited by imatinib. These effects are thought to mediated through inhibition of phosphorylation of the Src family kinase Lck (Blake et al., 2008). Furthermore, dasatinib was found to inhibit T-cell receptor mediated signal transduction, cytokine production and *in vivo* T cell responses (Blake et al., 2008; Fei et al., 2008). Again the effect is thought to be mediated by the inhibition of Lck.

7. New application of old therapies

Interferon was the most efficacious drug in the treatment of patients in the chronic phase of the disease prior to the advent of TKIs. There is now evidence that interferon-alpha may interfere with stem cell retention in the microenvironment and that it activates dormant haematopoietic stem cells (Essers et al., 2009). In response to treatment of mice with interferon-alpha, HSCs efficiently exited the dormant G(o) and entered an active cell cycle. In addition, HSCs pretreated with interferon-alpha were eliminated by 5-fluorouracil treatment, which raises the possibility for new applications of type I interferons to target CML stem cells. Two large randomized studies show improved outcome when pegylated IFN-alpha is combined with imatinib (Hughes et al., 2010). It could be suggested that IFN-

alpha stimulates the quiescent stem cells to proliferate thereby increasing sensitivity to imatinib. Although imatinib and other TKIs are very efficient, they are rarely curative. IFN-alpha could be included in combination treatment protocols aimed at curing patients and thus could still be an important drug in CML treatment.

8. Concluding remarks

The understanding of the biology underlying CML has rapidly advanced in the last fifty years. From initially identifying a cytogenetic abnormality, we have gone on to translating this finding into treatment strategies for this disease. Imatinib has revolutionised the treatment of CML and for patients who fail this treatment, nilotinib and dasatinib may reduce the rate of progression of the disease. Indeed some of these second generation tyrosine kinase inhibitors may represent a better first-line treatment option for some patients with possible benefits including an improvement in side-effects and tolerability profiles, the ability to suppress a wider range of mutant clones and reaching a response milestone sooner thus avoiding or reducing the risk of relapse. Furthermore third generation drugs are in development that show activity against the T315I mutant, which has emerged as a common Bcr-Abl mutation based resistance mechanism. However, despite the enormous therapeutic benefits of TKIs these drugs do not eradicate leukaemia-initiating stem cells allowing the persistence of a reservoir of Bcr-Abl positive stem cells that are potentially responsible for disease progression. There is therefore a requirement to elucidate why CML stem cells are insensitive to TKIs and to define differences in quiescent versus proliferating CML stem cells. Thus current research should lead to development of novel therapeutic strategies that may eradicate the stem cell population and finally lead to a cure for CML. It is likely though that any new therapeutics for CML will be administered either following or in combination with a tyrosine kinase inhibitor.

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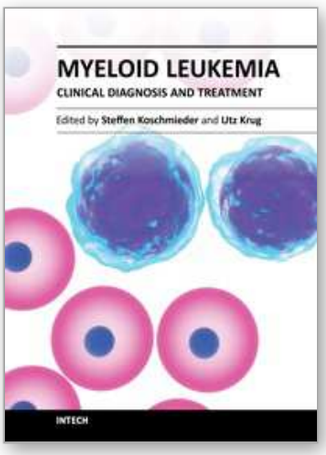

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This book comprises a series of chapters from experts in the field of diagnosis and treatment of myeloid leukemias from all over the world, including America, Europe, Africa and Asia. It contains both reviews on clinical aspects of acute (AML) and chronic myeloid leukemias (CML) and original publications covering specific clinical aspects of these important diseases. Covering the specifics of myeloid leukemia epidemiology, diagnosis, risk stratification and management by authors from different parts of the world, this book will be of interest to experienced hematologists as well as physicians in training and students from all around the globe.

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