CML: Case Closed?

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Chronic myeloid leukaemia (CML) is a malignant haemopoietic stem cell disorder characterised by the t(9;22)(q34;q11) reciprocal chromosomal translocation, the functional consequence of which is the Bcr-Abl oncoprotein. In 1998, imatinib, a tyrosine kinase inhibitor belonging to the 2-phenylaminopyrimidine group of pharmacological compounds, was introduced into the armamentarium of drugs for the treatment of CML, and has since revolutionised its management. Imatinib has a high affinity for the ATP-binding site of Abl, in addition to other kinases such as PDGFR, Kit and Arg, and clinical trials have validated the promise of this molecular targeted therapy. In the more advanced phases of CML, imatinib was able to induce a major (complete or partial) cytogenetic response in 16-60% of patients [1,2,3]. In a phase III trial comparing imatinib with interferon-α plus cytosine arabinoside in newly diagnosed chronic phase CML, 85% of patients treated with imatinib attained a major cytogenetic response (MCyR) after a median follow-up of 19 months, compared to 22% in patients treated with the latter combination [4]. A recent update has shown a further increase in MCyR of up to 92% of patients in the imatinib arm after a median follow-up of 54 months [5]. In view of its high efficacy and low toxicity, imatinib has now replaced interferon-α as frontline treatment for CML patients who are not eligible for allogeneic stem cell transplantation[6].

Clinical resistance to imatinib

The efficacy of imatinib in CML is remarkable, but the development of resistance and the persistence of minimal residual disease have dampened the initial enthusiasm for this much heralded 'magic bullet'. Resistance can be defined on the basis of its time of onset. Primary resistance is a failure to achieve a significant haematological or cytogenetic response, whereas secondary or acquired resistance is the progressive reappearance of the leukaemic clone after an initial response to the drug. Resistance is also defined on the basis of clinical and laboratory criteria used for detection of leukaemia, which includes haematological, cytogenetic and molecular resistance. Haematological resistance is a lack of normalisation of peripheral blood counts and spleen size; cytogenetic resistance is a failure to achieve a MCyR, i.e. less than 35% Philadelphia (Ph) chromosome positivity; and molecular resistance represents the failure to achieve or the loss of complete or major molecular response (MMR). MMR can be defined as a 3 or more log-reduction of *BCR-ABL*/control gene ratio from a laboratory standardised baseline or an international scale converted *BCR-ABL*/control gene ratio of < 0.1% [7]. The attainment of a MCyR or MMR has an impact on progression-free survival. Early chronic phase patients who achieved a MCyR after 12 months of imatinib had a 96% rate of survival without progression to accelerated phase or blast crisis at 54 months, compared to 81% who did not achieve a MCyR [5]. Achieving a complete cytogenetic response (CCyR) and a MMR in this cohort of patients translated to a 100% rate of survival without progression to the more advanced phases, compared to 95% in those who achieved a CCyR but not a MMR, and 89% in those who do not achieve a CCyR [5]. Recently, the European Leukaemia Net refined response criteria allowing

resistance to be categorised into 2 groups, 'sub-optimal response' and 'failure to respond' (Table I). Continuing imatinib treatment is unlikely to be beneficial in those with failure to respond, while the suboptimal responders may still have a benefit in continuing, although with a less favourable long term outcome [8].

Molecular basis of resistance

Ever since the first reports of resistance were described in 2000, the mechanisms of resistance to imatinib have been extensively studied and three major mechanisms have been identified. The two most common affect the *BCR-ABL* gene itself, namely mutations in its tyrosine kinase domain and overexpression of the Bcr-Abl protein due to amplification of the *BCR-ABL* gene [9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24,25,26]. The third mechanism is less well characterised and understood, and is represented by phenomena which lead to resistance independent of Bcr-Abl. These include upregulation of the drug efflux pumps [27,9,28,29,30], downregulation of drug influx transporters [31,32,33], binding of the α1-acid glycoprotein (AGP) [34], overexpression of Lyn, a Src kinase [35], and other Bcr-Abl- independent mechanisms [36].

Mutations in the Abl kinase domain

The emergence and selection of clones exhibiting point mutations in the Abl kinase domain is the most frequently identified mechanism of resistance in patients treated with imatinib and is more common in acquired than in primary resistance [37]. These mutations are not *induced* by imatinib, but rather, just like antibiotic resistance in bacteria, arise through a process whereby the drug itself *selects* for rare *pre-existing* mutant clones, which gradually outgrow drug-sensitive cells [38].

Mutations can be categorised into 4 groups: *(i)* those which directly impair imatinib binding; *(ii)* those within the ATP binding site; *(iii)* those within the activation loop, preventing the kinase from achieving the conformation required for imatinib binding; and *(iv)* those within the catalytic domain.

The substitution of the amino acid threonine with isoleucine at position 315 of the Abl protein was the first mutation to be detected in resistant patients [10]. Based on the crystal structure of the catalytic domain of Abl complexed to a variant of imatinib [39], this substitution was predicted to reduce the affinity for the drug in two ways. Firstly, the oxygen atom provided by the side chain of threonine 315 is not present, and this prevents the formation of a hydrogen bond with the secondary amino group of imatinib. Secondly, isoleucine contains an extra hydrocarbon group on its side chain and this sterically inhibits the binding of the inhibitor [10]. Another amino acid that makes contact with imatinib is phenylalanine 317, and its mutation to leucine also leads to resistance.

Mutations can also cluster within the ATP-binding loop (phosphate or P-loop). This domain is a highly conserved glycine-rich sequence that spans amino acids 248-256 and interacts with imatinib through hydrogen and van der Waals bonds [37]. These mutations modify the flexibility of the P-loop and destabilise the conformation required for imatinib binding [14]. Apart from imatinib insensitivity, a feature of clinical relevance is that imatinib-treated patients who harbour P-loop mutations have been suggested to have a worse prognosis than those with non-P-loop mutations [19]. However, this has not yet been confirmed in larger series.

The activation loop of the Abl kinase begins at amino acid 381 with a highly conserved motif of 3 amino acid residues (Aspartate-Phenylalanine-Glycine). This region of the kinase can adopt a closed (inactive) or an open (active) conformation. Imatinib forces Abl into the inactive conformation and is incapable of binding to the active configuration [40]. Mutations in the activation loop may disturb the energetic balance required to stabilise the closed conformation of the loop and thus favour the open, active conformation [14].

Finally, some amino acid substitutions cluster in the catalytic domain, a region that has a close topologic relation to the base of the activation loop. Mutations in this region can also influence the binding of imatinib [14].

At least 73 different point mutations leading to substitution of 50 amino acids in the Abl kinase domain have been isolated from CML patients resistant to imatinib so far, and this number is likely to increase with more sensitive methods of detection.

The detection of a Ph-positive clone harbouring an Abl kinase domain mutation is associated with resistance to imatinib and may be associated with progression to a more advanced phase disease [19,26]. Using highly sensitive assays, mutations have also been detected in patients in complete cytogenetic response, and in imatinib-naïve advanced phase

but not chronic phase patients [24,25]. However, detection of mutations in these patients did not always result in progressive disease while on imatinib. It is likely that mutant clones in the presence of low leukaemic burden or low levels of mutant clones do not have a similar clinical impact as clones which are detected when disease burden is rising or high [25,41]. Furthermore, the probability of detecting a clone is low when *BCR-ABL* transcript levels are stable or declining [21].

Bcr-Abl overexpression

Overexpression of the Bcr-Abl protein due to amplification of the *BCR-ABL* gene was first observed *in vitro* when resistant CML cell lines were generated by exposure to gradually increasing doses of imatinib [27,9,42,43]. This phenomenon has been reported in a relatively small proportion of patients, with an overall percentage of 18% [17,10,37], but this may be an underestimate if its detection is only based on the cytogenetic findings of Ph chromosome duplication. In one study, 3 out of 11 CML patients in blast crisis who relapsed after initially responding to imatinib were shown to have multiple copies of the *BCR-ABL* gene by fluorescence *in-situ* hybridisation (FISH) [10]. In another study, 7 out of 55 patients showed a more than 10-fold increase in *BCR-ABL* transcript levels and 2 out of the 32 patients evaluated were found to have genomic amplification of *BCR-ABL* by FISH [37]. In the latter 2 patients, resistance was primary and not acquired. Overexpression of Bcr-Abl leads to resistance by increasing the amount of target protein needed to be inhibited by the therapeutic dose of the drug. It is also possible that a transient overexpression of Bcr-Abl may be an early phenomenon in the establishment of imatinib resistance, preceding the emergence of a dominant clone with a mutant kinase domain, as suggested by kinetic studies in cell lines [44]

Drug efflux and influx transporters

Multidrug resistance (MDR) due to cross-resistance of mammalian cells to a number of anticancer agents following exposure to one such drug is a well described mechanism of resistance in cancer therapy. This is mediated by an increased expression at the cell surface of the *MDR1* gene product, Pgp, an energy dependent efflux pump, which reduces intracellular drug concentrations and leads to ineffective levels of the drug reaching its target [45,46]. Imatinib and other tyrosine kinase inhibitors are substrates of Pgp, and the intracellular levels of imatinib were shown to be significantly lower in Pgp-expressing cells [47,48,31,49,50]. An imatinib-resistant CML cell line generated by gradual exposure to increasing doses of the drug was shown to exhibit Pgp overexpression, and *MDR1* overexpression in CML cell lines also confers resistance to imatinib [9,28]. Pgp overexpression has not been reported in patients who are resistant to imatinib. However, the addition of a Pgp pump inhibitor, PSC833, to cultures of imatinib-treated cells from drug-resistant CML patients produced a significant decrease in colony formation, thus suggesting that *MDR1* overexpression may play a role in clinical imatinib resistance [28].

Recently, two other drug transporters, breast cancer resistance protein (BCRP)/ABCG2 and human organic cation transporter 1 (hOCT1), have been implicated as possible mechanisms for promoting imatinib resistance. Imatinib has been variably reported to be a substrate and/or an inhibitor for the BCRP/ABCG2 drug efflux pump which is overexpressed in many human tumours and also found to be functionally expressed in CML stem cells [51,52,53,54,29,55,30]. The drug transporter, hOCT1 mediates the active transport of imatinib into cells, and inhibition of hOCT1 decreases the intracellular concentration of imatinib, which may predict for a less favourable molecular response [31,33]. The *hOCT1* gene was also found to be expressed in significantly higher levels in patients who achieved a complete cytogenetic response to imatinib than in those who were more than 65% Ph chromosome positive after 10 months of treatment [32]. This would suggest that patients with low baseline expression of *hOCT1* may not achieve a complete cytogenetic response because of insufficient intracelluar levels of imatinib.

Bcr-Abl independent mechanisms

The Src family kinases, Lyn and Hck, are activated in *BCR-ABL*-expressing cell lines. Lyn is overexpressed and activated in an imatinib-resistant CML cell line generated by incubation of the parental line in increasing concentrations of imatinib and in samples from CML patients who were resistant to imatinib [35]. Lyn suppression by a Src kinase inhibitor resulted in reduced proliferation and survival of the imatinib-resistant but not the sensitive cell line [35].

Microarray analysis have shown that transcripts from genes with anti-apoptotic or malignant transformation properties and with involvement in

signal transduction/ transcriptional regulation are overexpressed in CML cells innately resistant to imatinib. This would suggest that pathways downstream of Bcr-Abl and independent of its kinase activity may be important factors which confer resistance to imatinib [36]. The phosphatidylinositol-3 (PI-3) kinase/Akt pathway is an important downstream pathway activated by Bcr-Abl and is essential for the proliferation of *BCR-ABL*-positive cells [56]. The mammalian target of rapamycin (mTOR) is a serine-threonine kinase activated by the PI-3 kinase. Treatment with imatinib was shown to activate the PI-3 kinase/Akt/mTOR pathway and this activation was important in mediating cell survival during the early development of imatinib resistance before overt resistance developed [57].

Overcoming imatinib resistance

Since the discovery of imatinib resistance it became clear that there is an urgency to discover and develop novel compounds or combinations capable of circumventing it. A number of potent Abl kinase inhibitors with *in vitro* and *in vivo* activity in wild-type and Abl kinase mutant Bcr-Abl cell lines have been identified. Two of these compounds are now in clinical trials. Pre-clinical studies have also provided evidence that combination therapy may have an important role in preventing or combating imatinib-resistance.

Dual Src/Abl kinase inhibitors

The disruption of the proto-oncogene Src is associated with the pathogenesis of human cancers [58]. Synthetic small molecule inhibitors of Src-family kinases have been developed and these compounds, eg, PD180970, AP23464, SKI606, CGP76030, BMS-354825, also inhibit Bcr-Abl, Kit and PDGFβ receptors, and have *in vitro* anti-proliferative activity in imatinib-sensitive and -resistant CML cells[59,60,61,62,63,64]. Dasatinib (BMS-354825, Bristol Myers Squibbs) was found to be more potent than imatinib, and was capable of inhibiting the proliferation and kinase activity of wild type Bcr-Abl cell lines at picomolar concentrations [65]. Similar to previously developed Src/Abl kinase inhibitors, dasatinib is also active against the imatinib-resistant active conformation of the kinase domain. *In vitro* assays revealed that dasatinib inhibited the kinase activity and proliferation of 14 out of 15 clinically relevant Bcr-Abl mutant cell lines. However, the T315I mutant remained resistant, even at micromolar concentrations of the drug. *In vivo* studies in murine models have confirmed the activity of dasatinib in inhibiting the leukaemic cell growth and prolonged the survival of mice harbouring the wild type Bcr-Abl and the M351T, but not the T315I mutant [63].

Clinical trials of dasatinib in imatinib-resistant and -intolerant CML and Ph chromosome-positive acute lymphoid leukaemia (Ph+ALL) are currently in progress, and the haematological and cytogenetic responses are summarised in Table II [66,67,68,69,70]. Dasatinib is well tolerated but grade 3-4 myelosuppression is common, especially in the advance phases. Non-haematological side effects include diarrhoea, nausea, headache, peripheral oedema and pleural effusion.

Second generation Abl kinase inhibitors

The *N*-methylpiperazine moiety was originally incorporated into imatinib to improve its solubility and oral bioavailability. Substitution of this amide moiety with alternative binding groups, while maintaining H-bond interactions to Glu286 and Asp381, led to the discovery of a more potent compoud, nilotinib (AMN107, Novartis). Nilotinib also inhibits the activity of Arg, Kit, and PDGFα and β receptors, but not Src kinase. At submicromolar concentrations it is 10 to 50 times more potent than imatinib in inhibiting the proliferation and autophosphorylation of wild-type Bcr-Abl cell lines and of most of the clinically relevant Bcr-Abl mutants, except the T315I mutant. Nilotinib was also superior to imatinib in reducing leukaemic burden and prolonging the survival of mice transplanted with marrow transduced with wild-type Bcr-Abl, the M351T and E255V mutants [71]. Results from phase I clinical trials with nilotinib are summarised in Table III [72] .

Substrate-competitive inhibitors

Adaphostin is a tyrphostin which alters the binding of peptide substrates rather than the ATP-binding site. Imatinib-resistant cell lines were shown to remain sensitive to the inhibitory effects of adaphostin [73]. Adaphostin does not target Bcr-Abl specifically but it selectively inhibited colony formation from primary CML cells [73]. It has also been shown to induce cytotoxicity by generation of reactive oxygen species [74].

The resistance of the T315I mutant to the Src/Abl kinase inhibitors and nilotinib poses a therapeutic challenge, and it is likely that this mutant will remain insensitive to other ATP-competitive inhibitors. A substrate-competitive inhibitor of

Bcr-Abl, ON012380, was recently reported to have potent *in vitro* inhibitory activity in cell lines expressing wild-type Bcr-Abl and all the Bcr-Abl mutants, including the T315I mutant. The activity against the T315I mutant was confirmed *in vivo* in mice expressing this form of Bcr-Abl protein where treatment with ON012380 caused a decrease in leukaemic cells [75].

Allosteric inhibitors

A recent class of Bcr-Abl inhibitor compounds was uncovered by differential cytoxicity screen in a 384-well format of approximately 50,000 combinatorially derived kinase-directed heterocycles [76]. This is a class of compounds which exert their activity through a newly described allosteric, non-ATP competitive mechanism, potentially involving binding to the myristate pocket in the C-loop of the Bcr-Abl kinase domain.

Other compounds with activity against imatinib-resistance

Many compounds have now been described to exhibit *in vitro* activity against imatinib-resistant cells. These include the inhibitors of the Bcr-Abl chaperone heat shock protein 90, geldanamycin and 17-allylaminogeldanamycin (17-AAG)[77]; the MEK kinase inhibitor, PD184352[78]; the cyclin-dependent kinase inhibitor, flavopiridol[79]; the histone deacetylase inhibitors, LBH589[80], suberoylanilide hydroxamic acid (SAHA) and sodium butyrate[81]; the proteasome inhibitor, bortezomib[82]; and the dual Abl/Lyn kinase inhibitor, NS-187[83].

Other small molecule compounds have also been identified to have *in vitro* anti-proliferative activity against the T315I mutant and these include a phosphoinositide-dependent kinase-1 inhibitor, OSU-03012 [84]; an Aurora kinase inhibitor, VX-680 [85]; a p38 inhibitor, BIRB-796[85]; and an Abl kinase inhibitor, SGX-70430[86].

Combination therapy with inhibitors of effectors in Bcr-Abl downstream pathways

Targeting Bcr-Abl downstream pathways is another attractive strategy for overcoming and possibly preventing resistance. Farnesyl transferase inhibitors (FTI) inhibit protein farnesylation and antagonise the oncogenecity of Ras, a protein that plays a central role in leukaemogenic transformation by Bcr-Abl. Lonafarnib (SCH66336, Schering-Plough) is an FTI which inhibits the proliferation of Bcr-Abl wild type, overexpressing and T315I mutant cell lines. While the drug itself did not induce apoptosis, it enhanced imatinib-induced apoptosis in the first two cell lines but not the T315I mutant [87]. Lonafarnib also enhances imatinib-induced cytotoxicity in primitive quiescent CML cells, a population of cells known to persist in vitro in imatinib-treated primary CML cells [88]. Nitrogen-containing bisphosphonates (e.g., zoledronic acid), via their inhibition of Ras prenylation, have also been effective in inducing apoptosis and inhibiting proliferation of imatinib-sensitive and -resistant CML cells [89,90].

The PI-3 kinase/Akt/mTOR pathway effectors are also candidates for targeted molecular therapy. The combination of the mTOR inhibitor, rapamycin or its derivative, RAD001, with imatinib was effective in overcoming imatinib-resistance in cell lines which overexpressed Bcr-Abl or harboured mutants which retained a moderate sensitivity to imatinib. However this combination was not effective in mutants which were highly resistant to imatinib, for example, T315I and E255K [91,92]. A novel phosphoinositide-dependent kinase-1 inhibitor, OSU-03012, acting via an Akt-dependent mechanism, was shown to synergise with imatinib in inducing apoptosis and inhibiting proliferation in both the T315I and E255K mutants [84].

The Jak-STAT pathway is the third major pathway downstream of Bcr-Abl. Mycophenolic acid (MPA), an inosine monophosphate dehydrogenase inhibitor that depletes intracellular guanine nucleotides, reduced phosphorylation of STAT5 and S6 ribosomal protein, a substrate downstream of mTOR. When combined with imatinib, MPA produced synergistic antiproliferative and pro-apoptotic effects in imatinib-sensitive CML cells. However the effect on imatinib-resistant cells was not determined [93].

Conclusion

The introduction of imatinib has represented a major achievement for the treatment of CML but the development of resistance to this drug has become a potential therapeutic dilemma. It is clear, therefore, that monotherapy with imatinib may not be the best option in CML, or at least not for all patients. Therapeutic approaches to circumvent the problem of imatinib-resistance are now possible with novel compounds and combinations being investigated pre-clinically and clinically. Dasatinib and nilotinib represent the first of the newer generation tyrosine kinase inhibitors to have a good safety profile and efficacy in imatinib-resistant

and -intolerant CML patients. It is not possible, currently, to determine which is more effective or less toxic. However, subclones with hitherto unseen Bcr-Abl mutants will probably develop in response to these new small-molecule inhibitors, leading again to resistance to these compounds. One strategy to delay or suppress the emergence of these mutants is, therefore, to inhibit Bcr-Abl downstream signalling pathways and, possibly, to achieve a synergistic combination with the newer Abl kinase inhibitors.

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