Brief report

Nilotinib exerts equipotent antiproliferative effects to imatinib and does not induce apoptosis in CD34+ CML cells

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Chronic myeloid leukemia (CML) stem and progenitor cells overexpress BcrAbl and are insensitive to imatinib mesylate (IM). We therefore investigated whether these cells were efficiently targeted by nilotinib. In K562, the inhibitory concentration (IC50) of nilotinib was 30 nM versus 600 nM for IM, consistent with its reported 20-fold-higher potency. However, in primary CD34+ CML cells, nilotinib and IM were equipotent for inhibition of BcrAbl activity, producing equivalent but incomplete reduction in CrkL phosphorylation at 5 μM. CML CD34+ cells were still able to expand over 72 hours with 5 μM of either drug, although there was a concentration-dependent restriction of amplification. As for IM, the most primitive cells (CFSEmax) persisted and accumulated over 72 hours with nilotinib and remained caspase-3 negative. Furthermore, nilotinib with IM led to further accumulation of this population, suggesting at least additive antiproliferative effects. These results confirmed that, like IM, the predominant effect of nilotinib is antiproliferative rather than proapoptotic. (Blood. 2007;109:4016-4019)

Introduction

Nilotinib (formerly AMN107; Novartis Pharma, Basel, Switzerland) is an Abl tyrosine kinase inhibitor (TKI) specifically developed to be more selective for BcrAbl and that also maintains activity against the most common mutations associated with clinical resistance to imatinib mesylate (IM).1-4 Similar to IM, nilotinib binds to the inactive conformation of Abl; however, an improved topologic fit to the protein binding surface likely contributes to its reported increased potency over IM both in vitro and in vivo.1,4

Our specific interest is not in IM resistance per se but in disease persistence in optimally responding patients. Our previous in vitro studies have highlighted a population of primary quiescent stem cells (QSCs) that persist and accumulate following IM exposure,3,8 suggesting that IM is more cytostatic than proapoptotic in this subpopulation. Furthermore, previous studies have demonstrated the existence of QSCs that can regenerate chronic myeloid leukemia (CML) populations in immunodeficient mice8 and that persist in patients even in durable complete cytogenetic response (CCR) on IM.9 Indeed it is clear that the majority of IM-treated patients remain positive for BcrAbl by quantitative reverse transcription–polymerase chain reaction (Q-RT-PCR).10,11

To prospectively identify the QSC pool, we recently studied CD34+CD38− cells that make up less than 5% of total CD34+ cells. In this subpopulation, we have observed a significant increase in BcrAbl mRNA expression, protein expression, and tyrosine kinase activity.12 To date, consecutive CML patient CD34+CD38− cells tested have not been shown to harbor any common BcrAbl point mutations associated with clinical resistance.12 Thus, if the QSCs are presumed to require significantly higher intracellular levels of tyrosine kinase inhibition to overcome the increased oncprotein expression and activity before initiating apoptosis, then a drug of increased potency would be required.

As nilotinib is reported to be 20-fold more potent than IM we compared the drugs in terms of inhibition of BcrAbl tyrosine kinase activity that in turn was correlated with antiproliferative activity and apoptosis induction in primary chronic phase CD34+ and more primitive CD34+CD38− stem and progenitor cells.

Materials and methods

Nilotinib and IM were supplied by Novartis and were stored at 10 mM in DMSO at −20°C or 100 mM in distilled water at 4°C, respectively. Primary CML cells were obtained with written informed consent, in accordance with the Declaration of Helsinki, from peripheral blood leukapheresis samples from newly diagnosed patients with chronic-phase CML (n = 8). Glasgow Royal Infirmary’s Local Research Ethics Committee approved the use of human tissue in this study protocol. CD34+ cells (> 95% CD34+) were enriched by positive selection and cultured in growth factor (5GF)-supplemented serum-free medium as previously described.5,7 Cell proliferation was assayed by tritiated-thymidine uptake as previously described.6,7 Intracellular phospho-CrkL (P-CrkL), intracellular active caspase-3, and CFSE (carboxyfluorescein succinimidyl ester) staining were performed and analyzed by flow cytometry as described elsewhere.5,7,13 Statistical analyses were performed using paired Student t test.

Results and discussion

Nilotinib seemed the ideal small molecule inhibitor of oncprotein kinase activity with reportedly increased potency (20-fold), greater...
Nilotinib reduces P-Crkl expression in K562 in a concentration-dependent manner. Nilotinib (0.05 μM versus 0.6 μM, respectively; Figure 1A). However, on trying to establish an IC50 for nilotinib in primary CML CD34+ cells supported with GF, it became evident that these cells could still proliferate despite the presence of this drug (Figure 1C) and clearly did not undergo apoptosis to any significant extent.

Next, we assayed the apoptotic response of primitive CML cells to nilotinib. It was evident that the most primitive cells (ie, those that remain relatively quiescent [CFSEmax]) persisted in the presence of nilotinib as we had observed previously with IM.5-7 These cells had not initiated apoptosis in response to the drug (ie, caspase-3neg) whereas the more mature cells in divisions 3, 4, and 5 (CFSEdim, CD34lo) were beginning to undergo apoptosis (caspase-3+; Figure 2Ai-xii).

On analysis of the percentage recovery of input, a way of numerically relating the contribution of the input cells to the surviving output cell number after drug treatment,5-7 nilotinib treatment resulted in an accumulation of primitive cells that had not divided (CFSEmax) or that had divided only once (Figure 2B), as compared with the no drug control. This effect was concentration dependent and was greater for IM than nilotinib at an equal concentration (5 μM). Importantly, the combination of nilotinib and IM (each at 5 μM) apparently exacerbated the accumulation observed with either agent alone, although this did not reach statistical significance.

The finding that nilotinib has potent antiproliferative activity resulting in the “accumulation” of viable quiescent CD34+ is supported by a report that nilotinib can bring about a G0/G1 cell cycle block of murine cells expressing wild-type BcrAbl just as seen with IM.9 Another study also found accumulation in G0/G1 of the IM-sensitive KBM5 human Ph+ cell line in response to nilotinib or IM,7 and similarly in a T315I-expressing counterpart cell line. Whereas many anticancer agents are considered potentially useful if they inhibit proliferation, inducing a G0/G1 block might protect CML stem and progenitor cells from damage by cytotoxic agents used in drug combinations. This could result in disease persistence and minimal residual disease that is therefore a negative finding in this population in terms of therapy.

We have recently developed a flow cytometry assay of Bcr-Abl tyrosine kinase activity through the phosphorylation status of the fastidious downstream adaptor molecule, CrkL.12,13 After 16 hours in culture with nilotinib, long enough for inhibition of CrkL phosphorylation but not for induction of apoptosis, the total CD34+ cell samples studied exhibited only partial and variable inhibition (range, 49% to 0% inhibition, n = 5; Figure 1D) of CrkL phosphorylation. This was despite demonstrable concentration-dependent inhibition of CrkL phosphorylation in K562 cells (up to 80% inhibition; Figure 1B). Furthermore, CrkL phosphorylation in the more primitive CD34+CD38− subpopulation is not inhibited by nilotinib (ie, <20% inhibition) in response to either 5 μM nilotinib or IM, confirming our previously published observation with IM.12

Figure 1. Proliferation, cell survival, and CrkL phosphorylation of Ph+ and Ph− cells after 72 hours of culture with nilotinib with or without IM. (A) The reportedly improved potency of the novel agent was confirmed in our study by [H]-thymidine uptake proliferation assays to determine IC50 for each cell type. In the Ph+ cell line K562, IC50 was calculated as 30 nM ± 10 nM for nilotinib as compared with 600 nM ± 60 nM for IM. The IC50 for the Ph− cell line HL60 was 1 μM for nilotinib and 10 μM for IM. (B) Nilotinib reduces P-Crkl expression in K562 in a concentration-dependent manner. Nilotinib (0.05 μM; dark blue line) and IM (1 μM; red line) are approximately equipotent in this regard, confirming the reported 20-fold increased potency of the new agent in cell lines. Baseline P-Crkl for Ph−HL60 cells is shown by the dark gray-filled peak; isotype control antibody staining is shown by the black-filled peak; and maximal P-Crkl expression is shown by the no drug control in the light gray-filled peak. (C) It was evident that in the majority of cases the total primary CML cell number surviving 72 hours of exposure to nilotinib, even at 5 μM, was greater than input, although there was a concentration-dependent restriction of the amplification of this output with respect to no drug 5GF-only control. Output cells were found to be Ph− by fluorescence in situ hybridization (FISH); however, the observed cellular proliferation in the presence of drug was not owing to normal (Ph+) cells. Representative data from 1 of 5 individual CML CD34+ cell samples is shown. (D) In total CD34+ cells from individual patients with CML, nilotinib either equally inhibited CrkL phosphorylation with imatinib at equal concentration (Figure 1D, left panel) or failed to inhibit CrkL phosphorylation (Figure 1D, right panel) at 16 hours. Black fill indicates isotype control; dark gray fill, baseline CrkL-P in Ph− cells; light gray fill, 5GF-only–treated CML cells; dark blue line, 5 μM-nilotinib–treated CML cells; red line, 5 μM-imatinib–treated CML cells.
These data suggest that we may need to find ways to increase the intracellular accumulation of drugs such as IM or nilotinib in order to overcome the greatly increased oncoprotein levels in this critical cell subset. Inadequate active uptake or overefficient drug extrusion via efflux transporters may also contribute to the presumed insufficiency in drug loading of stem cells, which may explain this drug insensitivity. Alternatively, the localization or conformation of the BcrAbl protein may preclude drug binding. Previously, we found no evidence of other classical mechanisms of resistance, such as point mutations that may emerge after drug treatment in this population. However, we cannot yet rule out the more novel concept that stem cells may not be oncogene addicted and can persist in the absence of a BcrAbl survival signal; we are currently undertaking knock-down experiments to investigate this possibility.

Nilotinib has shown substantial activity against advanced IM-resistant CML in recent phase 1/2 clinical trials. Clearly, the patients entered into these trials cannot be compared with IM-naive chronic-phase patients. It may be predicted from our in vitro study that, although nilotinib will undoubtedly be useful to induce cytogenetic responses in those patients with a proven IM-insensitive clone, simply increasing the spectrum of mutations against which a molecule is active will not elicit a cure unless that molecule can be targeted to the stem cell population sustaining the disease. In fact, as we have shown that the predominant effect of nilotinib is antiproliferative rather than proapoptotic, combining nilotinib or IM either with each other or with other drugs should be carefully considered from the point of view of merely inducing G0/G1 block without apoptosis. Furthermore, analysis should be included to delineate between cell death and cytostasis to enable adequate interpretation of drug effects in stem/progenitor cell populations.

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**Authorship**

Contribution: H.J., E.A., J.M., and T.H. participated in designing the research; H.J., E.A., and N.J. performed the research; H.J., E.A., N.J., and J.M. analyzed the data; and H.J., J.M., and T.H. wrote the paper. All authors checked the final version of the manuscript.

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