Selective strong synergism of Ruxolitinib and second generation tyrosine kinase inhibitors to overcome bone marrow stroma related drug resistance in chronic myelogenous leukemia

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A B S T R A C T

The IC50 of TKIs is significantly increased when BCR-ABL+ K562 cell line is cultured in stroma conditioned media produced by BM mesenchymal cells. In particular, while the Imatinib IC50 in the stromal cocultures was well above the in vivo through levels of the drug, the IC50s of second generation TKIs were still below their through levels. Moreover, we provide a formal comparison of the synergy between first and second generation TKIs with the JAK inhibitor Ruxolitinib to overcome BM stroma related TKI resistance. Taken together, our data provide a rationale for the therapeutic combination of TKIs and Ruxolitinib with the aim to eradicate primary BCR-ABL+ cells homed in BM niches.

1. Introduction

Most patients with CML in chronic phase (CML-CP) treated with Imatinib achieve long term complete cytogenetic response (CCR), while only a minority achieve complete molecular response (CMR) [1]. Moreover, although second-generation TKIs yield higher rates of CMR than Imatinib [2,3], there is still no evidence that CMR results in CML stem cell eradication in the majority of patients. Several experimental evidences and clinical observations indicate that most patients achieving CCR have BCR-ABL transcripts detectable by RT-PCR, and that BCR-ABL-expressing leukemia stem cells persist in the BM of patients with CML even in sustained undetectable molecular residual disease [4]. In addition, Imatinib discontinuation studies demonstrated that only a minority of CML patients, i.e. those with more than a 4-log reduction of minimal residual disease, is eligible for discontinuation trials [5], suggesting that new additional therapeutic strategies are required to eradicate the disease in a larger number of patients. Noteworthy, recent evidences suggest that upon TKI treatment pressure, CML stem cell survival is due to BCR-ABL kinase independent mechanisms [6–9]. Thus, the curative treatment strategies for CML must also include the inhibition of survival pathways in addition to those activated by the hallmark BCR-ABL oncoprotein. Ph+ leukemic stem cell (LSC) viability is supported by an aberrant activation of pro-survival and self-renewal pathways regulated by both cell-intrinsic factors, including β-catenin and Sonic Hedgehog (Shh) [10,11], and cell-extrinsic stroma related stimulations [12]. Enhanced survival of LSC in protective microenvironments, provided by the BM niche [11], as well as the physiological dormancy of cells in these niches, also contributes to their persistence despite TKI treatment. In this regard, it is important to note that the BM microenvironment is a source of both extracellular matrices and high local concentrations of cytokines and growth factors, all involved in the signal transduction mostly mediated by the activation of JAK–STAT pathways [13]. In particular, previous studies have shown that stroma-mediated drug resistance is mediated by the increase in the phosphorylation level of Stat3 (Tyr705) [14], and that JAK inhibitor potentiates Nilotinib-mediated cell death in the context of the BM microenvironment.

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although the observed synergistic effect was nullified by toxic effect toward non-leukemic cells. In this paper, we show that the TKI IC50 modulation related to BM stroma microenvironment may account for the differential level of response observed in patients treated with first or second generation of TKIs. Moreover, we prove that either Nilotinib or Dasatinib (used at in vivo applicable concentration) are very strongly synergetic with JAK inhibitor Ruxolitinib to overcome the BM stroma related TKI resistance in either CML cell lines or CD34+ progenitor cells from CML patients, with minimal toxic effect on progenitor cells from healthy donors at the applied concentrations.

2. Materials/subjects and methods

2.1. Cell lines and tumor cells

K562 cells (DSMZ, Braunschweig, Germany) were maintained in culture in complete medium (defined as Regular Media [RM]; as previously described [17]. Cell line viability was assessed by trypan blue exclusion. In selected experiments, cell lines were incubated for 72 h in the presence of the required concentrations of Imatinib, Nilotinib (kindly provided by Novartis, Basel, Switzerland), Dasatinib (kindly provided by Bristol-Myers Squibb) or Ruxolitinib (INCB018424, Selleckchem, Houston, TX; Apo) was measured by Annexin-V Kit and cell cycle was assessed by BD Flow Cytometry (BD Pharmingen). Following the manufacturer’s instructions. The human stroma cell line HS-5 was cultured in α-MEM with 10% FBS and sub-cultured following the manufacturer’s instructions.

2.2. Samples from CML patients and healthy donors

BM samples were collected from six newly diagnosed patients with CML-CP, three CML patients in molecular response (MR*) [18] and three healthy donors (HD) after approval by the Institutional Review Board (IRB) of the Federico II University of Naples. Mononuclear cells were isolated from BM or PB aspirates, as previously described [19]. CD34+ hematopoietic progenitor cells were selected by the Direct CD34 Progenitor Cell Isolation Kit (Miltenyi Biotech Inc., Auburn, CA), and cultured in AIM-V serum-free media (Invitrogen) at a starting density of 1 x 10^6 cells/ml. When specified, the following cytokines were included: high growth factor (GF) cocktail [20]: 100 ng/ml SCF, 100 ng/ml IL-7, 20 ng/ml G-CSF, 20 ng/ml IL-3, and 20 ng/ml IL-6 (all from R&D Systems). Depending on the specific experiment, TKIs were added as follows: 1000 nM Imatinib, 400 nM Nilotinib and 2.5 nM Dasatinib [21]. MICA-1+ cells were selected from 8 BM samples of patients with CML-CP and two BM samples of HDs, using a MicroBead kit (Miltenyi) following the manufacturer’s instructions.

2.3. Generation of mesenchymal SCM

HS-5 cell line was plated to achieve 80% confluence. Thereafter, the complete media was removed, and the adherent cells were washed with PBS. Then, HS-5 were cultured in serum-free media for 24 h. HS-5 serum-free supernatant (named HS-5/SCM) was removed from the cells and filtered through a 0.22 μm filter. SCM from HD and patients with CML was obtained with the same procedure after at least two cell passages of two mesenchymal stromal cell lines obtained from HDs (named HD/SCM) and eight stroma cell lines obtained each from a BM sample of a CML patient (named CML/SCM). After SCM collection, mesenchymal stromal cell lines were analyzed by human MSC Phenotyping Kit (Miltenyi). HS-5/SCM and CML/SCMs were analyzed for the concentration of soluble factors by Bio-Plex assay (Bio-Rad), according to the manufacturer’s instructions.

2.4. Colony Forming Cell (CFC) assay

1 x 10^6 cells CD34+ cells isolated from BM of six CML donors and three HDs were treated with TKIs alone or in combination with Ruxolitinib, plated in triplicate in methylcellulose medium supplemented with recombinant cytokines (MethoCult; StemCell Technologies), and incubated at 37° C. Colony-forming units (CFU) were scored using a high-quality inverted microscope after 2 weeks of culture [22].

2.5. Statistical analysis

All data are presented as mean ± 1 SD. The Student’s t test was used to evaluate the statistical significance of differences using the non parametric Mann–Whitney test, with a p value <0.05 indicating a significant difference. IC50 was calculated based on the level of residual viable cells (Annexin-Vneg/PIneg/ve) after treatment with TKIs at increasing doses. Data were analyzed by a specific software (MasterPlex ReaderFt). The combination index (CI) was defined as previously specified [23], and calculated by CompuSyn software based on Chou’s median-effect equation [24,25]. In particular, based on the CI value, we defined a very strong synergism (CI <0.1), a strong synergism (0.1 <CI <0.3) and a synergism (0.3 <CI <0.7), according to the manufacturer’s instructions.

3. Results

3.1. The IC50 of either first or second generation TKIs significantly increases when Ph+ cell line are treated in the presence of HS-5/SCM

K562 cells were exposed to increasing doses of Imatinib, Nilotinib or Dasatinib (range 10^–1–10^8 nM) in either the presence or absence of HS-5/SCM, to quantify the effect of exposure to HS-5/SCM on the modulation of TKI activity. Thereafter, cell viability was monitored by Annexin-V/PI staining after 72 h. As shown in Fig. 1A, we observed that the IC50 of Imatinib, Nilotinib and Dasatinib were significantly increased when K562 cells were cultured in the presence of HS-5/SCM (IC(0.5)HS:SCM: 7957.16 nM, 889.06 nM, 2.85 nM, respectively) respect to the control cultures in RM (IC(0.5): 545.46 nM, 13.93 nM, and 1.12 nM, respectively).

We also investigated whether apoptosis was only delayed in the presence of HS-5/SCM. To this aim, we cultured K562 cell line with TKIs at the calculated IC(0.5)HS:SCM for an additional 3 days in the presence of HS-5/SCM or RM. On day 6, cells were re-plated in RM for a total of 16 days without addition of TKIs. Interestingly, only the residual K562 cells cultured in HS-5/SCM (Fig. 1C) kept a significant ability to proliferate after TKI withdrawal, whereas those exposed to TKI without stromal protection (Fig. 1B) failed to re-expand once TKI exposure was suspended. All these effects were directly mediated by soluble factors released by BM stroma, since HS-5/SCM withdrawal before TKI exposure resulted in the complete absence of protection against the TKI effect (data not shown). Moreover, serial dilutions of HS-5/SCM, ranging from 50% to 0.05%, resulted in a dose dependent effect on resistance to TKI exposure (Supplemental Fig. 1).

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3.2. CML derived BM mesenchymal cell lines produce several STAT3 activating cytokines

Since it was already reported that BM stroma soluble factors induce STAT3 activation [14], we also quantified the amounts of several cytokines by Bioplex ELISA assay, in HS-5/SCM and CML/SCM derived from eight BM mesenchymal stromal cell lines generated from untreated CP-CML patients (Supplemental Fig. 2). IL-1α, IL-1β, IL-7, IL-15 and MIP-1α were expressed exclusively by HS-5 cell line, while SDF-1α, TRAIL and HGF cytokines were found also in CML derived stroma. Moreover, most of the other cytokines tested, whereas present in significantly higher levels in HS-5/SCM, were also produced in CML/SCM (M-CSF, SCF, PDGF-bb, IL-6, IL-8, IL-10, IL-12, G-CSF, GM-CSF, TNF-α and VEGF).

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3.3. Second generation TKIs differentially synergize with Ruxolitinib to overcome stroma related drug resistance in CML cell line

Recent convincing experimental evidences have shown that BM stroma conditioned media activates Jak2–Tyk2–Stat3 pathway in CML cells [14,16] and that turning off the JAK pathway using the JAK inhibitor sensitizes CML cells to Nilotinib treatment [15]. In the era of TKI combinatorial therapy, we next sought to determine a potential differential level of synergism with Ruxolitinib between first and second generation of TKIs.

Whereas Ruxolitinib, as single agent, showed only a modest effect on overall cell viability after 72 h of treatment, the
exposure of JAK inhibitor combined with TKIs in the context of BM stroma SCM showed significantly greater toxicity in K562 cells than a single TKI treatment. In particular, in the presence of HS-5/SCM, K562 cell line viability was significantly inhibited by the combination of 1000 nM Imatinib and 300 nM Ruxolitinib (Fig. 2B), 0.5 nM Dasatinib and 100 nM Ruxolitinib (Fig. 3B), 50 nM Nilotinib and 100 nM Ruxolitinib (Fig. 4B), respect to the use of each single drug. Moreover, CI values indicate that either Imatinib, Nilotinib or Dasatinib were very synergistic with the JAK inhibitor in the presence of HS-5/SCM. In particular, 2 nM Dasatinib (Fig. 3D) or 50 nM

Fig. 1. Analysis of Imatinib, Nilotinib and Dasatinib IC50 modulation by mesenchymal stroma cells exposition. (A) TKI IC50 of K562 cell viability in the presence of HS-5/SCM or RM. (B) Viability of K562 cells treated with TKIs in RM. (C) K562 cells treated with TKIs in the presence of HS-5/SCM are able to further proliferate.

Fig. 2. Strong synergism of Imatinib and JAK2 inhibitor Ruxolitinib on overcoming stroma derived TKI resistance. K562 cells exposed to Imatinib and/or Ruxolitinib. Cell viability was assessed after treatment in RM (A) or in HS-5/SCM (B). Dose matrix of cytotoxic effects in K562 relative to Imatinib and/or Ruxolitinib treatment in RM (C) or in HS-5/SCM (D). Gray scale color indicates CI values.
Fig. 3. Very strong synergism of Dasatinib and JAK2 inhibitor Ruxolitinib on overcoming stroma derived TKI resistance. K562 cells exposed to Dasatinib and/or Ruxolitinib. Cell viability was assessed after treatment in RM (A) or in HS-5/SCM (B). Dose matrix of cytotoxic effects in K562 relative to Dasatinib and/or Ruxolitinib treatment in RM (C) or in HS-5/SCM (D). Gray scale color indicates CI values.

Fig. 4. Very strong synergism of Nilotinib and JAK2 inhibitor Ruxolitinib on overcoming stroma derived TKI resistance. K562 cells exposed to Nilotinib and/or Ruxolitinib. Cell viability was assessed after treatment in RM (A) or in HS-5/SCM (B). Dose matrix of cytotoxic effects in K562 relative to Nilotinib and/or Ruxolitinib treatment in RM (C) or in HS-5/SCM (D). Gray scale color indicates CI values.
Nilotinib (Fig. 4D) combined with 300 nM of Ruxolitinib achieve a greater synergism, corresponding to the definition of very strong synergism (CI: 0.09 and 0.06, respectively) than Imatinib 1000 nM that show to be strongly synergic with the in vitro treatment of 300 nM of Ruxolitinib (CI: 0.24).

3.4. Selective synergistic effect of JAK inhibitor Ruxolitinib and TKIs to overcome stroma related drug resistance in CML progenitor sparing normal CD34+ cells

Whereas it has been shown that turning off the JAK pathway using the JAK inhibitor sensitizes CML cells to Nilotinib treatment [15], a significant suppression of normal hematopoietic cells were also observed, suggesting that the toxic effect of this drug combination could limit its clinical relevance [16]. Thus, we sought to evaluate the effect of TKIs and Ruxolitinib combination in primary CD34+ cells from CML patients and BM healthy donors. Co-treatment of BM CD34+ cells from six CML patients with Imatinib, Nilotinib or Dasatinib and Ruxolitinib (Fig. 5A) for 72 h resulted in a significant reduction of cell viability, independently of the presence of either HS-5/SCM or high growth factor cocktail. In addition, although Ruxolitinib as single agent slightly affect cell viability of both CML CD34+ cells (Fig. 5A) and HD (Fig. 5C) in culture containing HS-5/SCM, this effect was significantly synergic with TKIs only in reducing CML CD34+ cell viability, but not in CD34+ primary cells from CML patients in MR4 (Fig. 6) or from HD (Fig. 5C). Therefore, the synergistic effect of TKIs and Ruxolitinib seems to be selective for CD34+ cells derived from patients with CML, and it is clearly discernible also in a functional assay (Fig. 5B). Indeed, when we tested the ability of Ruxolitinib to functionally impair the formation of CFUs, alone or in combination with TKIs, we observed that Imatinib, Nilotinib, Dasatinib and Ruxolitinib used as single drugs demonstrated only a moderate ability to impair colony outgrowth in the presence of HS-5/SCM (79%, 66%, 52% and 45% colonies, respectively, relative to untreated controls) in CML CD34+ cells (Fig. 5B). However, Ruxolitinib in combination with Imatinib, Nilotinib or Dasatinib showed an enhanced activity in suppressing colony outgrowth over each single agent (p < 0.01) in the presence of HS-5/SCM in CML CD34+ cells (Fig. 5B), but not in HD CD34+ cells (Fig. 5D).

4. Discussion

Second generation TKI agents have improved the ‘suppression rates’ in terms of both deepness and duration of the response, leading to a further reduction of blastic transformation [2,26,27]. However, even in case of second generation TKIs, there is a great variability in the leukemic residual burden among CML patients under treatment. Thus, the possibility of CML eradication is still under investigation and not frequently seen in patients. The most plausible explanation for the latter issue may be the low sensitivity observed in immature or staminal BCR-ABL cells during TKI
treatment [28]. Despite several mechanisms have been proposed, including increased BCR-ABL levels and activity [29], so far the molecular basis of this clinical observation is still poorly understood. However, both clinical and in vitro preclinical data, in which residual BCR-ABL immature cells were isolated despite successful treatment with Imatinib mesylate [4], have shown that CML stem cell survival is not dependent on BCR-ABL kinase activity [6,8], and that, consequently, BCR-ABL independent mechanisms may be responsible for primary and secondary TKI resistance in CML patients.

In this regard, since BM niches represent a rich milieu regulating hematopoietic stem cell self-renewal, proliferation and differentiation, we investigated its role in the modulation of TKI effects on CML cells. The results of our study confirm that mesenchymal stromal microenvironment holds also a relevant role in regulating BCR-ABL leukemia cell fate under TKI treatment. Indeed, applying an in vitro model based on the co-culture of CML cells and BM stromal derived HS-5 cell line, we demonstrated that IC50s significantly increase for the three clinically available TKIs. In particular, in case of Imatinib and Nilotinib, IC50 increased by approximately one log. However, while the Imatinib IC50 in the stromal co-cultures was well above the through levels of the drug when administered at the standard dose of 400 mg OD (7957 nM vs 1600 nM), the IC50s of second generation TKIs in the same conditions were still below their corresponding through levels at the steady-state (889 nM vs 1700 nM for Nilotinib 400 mg BID, 2.85 nM vs 5.5 nM for Dasatinib 100 mg OD). These observations may account for the higher activity of these drugs against the BCR-ABL immature cell compartment, and hence provide a plausible explanation for the deeper molecular responses observed in CML patients treated with second generation TKIs compared to those observed in CML patients treated with Imatinib.

Importantly, our data also show that stromal soluble factors preserve viability of CML cells under treatment with TKIs, since they maintain a good proliferating capability in subsequent long term sub-cultures. Indeed, despite TKI treatment at concentrations corresponding to their stromal co-culture IC50s, BCR-ABL cells show an initial proliferation arrest and then, only those treated in the presence of stromal soluble factors retain their ability to re-start proliferation in long term sub-cultures after TKI withdrawal. Thus, stromal cells provide long lasting protection to CML cells against apoptosis, resembling the clinical situation in which Ph+ stem cells homed in BM niches under TKI treatment survive in small numbers and in a sort of quiescent status, but are ready to resume proliferation upon TKI discontinuation. Moreover, since we demonstrated that CML cells are protected from TKI activity by soluble factors either derived from immortalized HS-5 cell line or stroma CML cell lines (data not shown), we performed a quantitative evaluation of cytokines present in this two cellular settings. The majority of the investigated cytokines, such as M-CSF, SCF, PDGF-bb, IL-6, IL-8, IL-10, IL-12, G-CSF, GM-CSF, TNF-a and VEGF but not the chemotactic factor SDF-1, whereas present in significantly higher levels in HS-5/SCM, were also found in CML/SCM.

In particular, previous studies showed that stroma-mediated drug resistance is due to the increase in the phosphorylation level of Stat3 (Tyr705) [14], thus indicating that the stromal protection passes through the activation of this clinically relevant pathway, which is known to sustain cell viability in vivo, and to trigger the activation of Jak2–Tyk2 signaling. Moreover, it was observed that turning off the JAK pathway using a JAK inhibitor sensitizes CML cells to Nilotinib treatment in the context of the BM microenvironment [15,16]. Thus, we investigated whether Ruxolitinib differentially synergizes with Imatinib, Nilotinib and Dasatinib to overcome stroma related TKI resistance in CML cell line or CD34+ progenitor cells from CML patients.

Our data indicate that Ruxolitinib effectively strongly synergizes with Imatinib, and achieves a very strong synergism with second generation TKIs, either Dasatinib or Nilotinib, being able to induce apoptosis in both K562 cell line and CD34+ progenitor CML cells resistant to the elimination by the single TKI agent, in the presence of BM stroma signaling. Indeed, co-treatment of Ph+ CD34+ cells with Ruxolitinib and matinib, Nilotinib or Dasatinib significantly decreased CFU outgrowth, providing evidence that it is able to overcome stromal protection. In contrast with Traer et al. [16], we prove that the JAK inhibitor Ruxolitinib, instead the TG101209, used by Traer et al., allows to achieve a therapeutic window of drug combination that spare progenitor cells from healthy donors or CML patients in MR4.

From a clinical point of view, although second-generation TKIs achieve a deeper and prolonged cytogenetic and molecular response rate than Imatinib in patients with CML-CP [3,30,31], the use of TKIs as a single agent seems unable to eradicate the disease, despite the increase in MR4.0 rate.

Our data strongly support the hypothesis that a drug combination of Ruxolitinib and second generation TKIs (either Nilotinib or Dasatinib) may be more effective in eradicating leukemia rather than the single agent TKI, with minimal toxic effect on normal hematopoietic cells. However, the in vivo side effects related to the proposed drug combination, need to be determined by a specific clinical trial.

The combined deep down-regulation of BCR/ABL oncogene plus the down-regulation of the signaling induced by the soluble factors present in the stroma BM microenvironment through the JAK/STAT pathway may be likely relevant in vivo for the treatment of CML patients and may significantly increase the rate of MR4.0, which is the only condition that may prelude the decision of treatment interruption.

Conflict of interest statement

All authors declare no conflicts of interest.

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Contributions. C.Q., B.D.A., S.E., S.C., M.R., I.C. and N.E. performed experiments. C.Q., B.D.A., S.E., S.C. and F.P. designed the research and analyzed the data. N.P., A.M.R., S.P., M.P., I.L. and F.P. provided patient’s samples. C.Q., B.D.A., G.M., G.S. and F.P. provided expertise in Molecular Biology and analyzed the data. C.Q., B.D.A. and F.P. wrote the manuscript. All authors reviewed the manuscript.

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