BCR-ABL1 compound mutants display differential and dose-dependent responses to ponatinib

The third-generation tyrosine kinase inhibitor (TKI) ponatinib exerts a strong anti-neoplastic effect in all stages of chronic myeloid leukemia (CML) as well as in Philadelphia(Ph)-positive acute lymphoblastic leukemia (ALL). It is capable of suppressing the kinase activity of BCR-ABL1 carrying any single mutation in the tyrosine kinase domain (TKD), including the gate-keeper mutation T315I.[1] Nevertheless, resistance to ponatinib can evolve in sub-clones carrying *BCR-ABL1* variants with two or more mutations on the same allele, if the IC_{50} values for this TKI exceed the maximum achievable effective plasma levels.[2,3] These so-called compound mutations (CMs) represent a powerful mechanism of resistance to all currently available TKIs.[2] Our data indicate that individual CMs confer high resistance to ponatinib, thus precluding clinical use of this particular TKI. However, several CMs only display low to intermediate resistance to ponatinib, suggesting that successful suppression of the mutant subclones is feasible, provided that the appropriate dosing regimen is applied.

The occurrence of CMs in CML has been principally linked to sequential treatment with different TKIs,[3] and is far more frequently observed in Ph-positive ALL.[4] Although CMs have been suggested not to confer primary/secondary resistance to ponatinib in the chronic phase of CML,[5] they still pose a major problem in advanced stages of CML and Ph-positive ALL.[2] The identification of their responsiveness to ponatinib is therefore of paramount importance for subsequent clinical management. Therapy with ponatinib may, however, be associated with serious side effects,[6] and reports on apparently dose-dependent severe adverse events provided the basis for reduction of the recommended daily dose from 45 mg to 30 or even 15 mg.[7] These modifications in the dosing regimen imply that the achievable levels of effective average plasma concentrations (efCave) of ponatinib decrease from 28 nM to 23 and 10 nM, respectively.[8] In this regard, stringent analysis of the *in vitro* responses of CMs to ponatinib could permit assessment of the required effective dose in the clinical setting. This notion is supported by exemplary courses of three patients displaying the CM F317L/E459K who were treated within the Ponatinib Ph-positive ALL and CML Evaluation (PACE) trial, a phase 2 clinical study with ponatinib in heavily pretreated patients with resistant or intolerant Ph-positive leukemia. Two patients who had received 42 and 45 mg daily (efCave ~26 and 28 nM) achieved durable major molecular response (MMR) in 168 and 87 days, respectively, whereas the other patient with this CM, who had received an average daily dose of 32 mg (corresponding to an efCave of ~24 nM), needed 583 days to reach MMR, and was withdrawn from the study 82 days after having achieved this.[5] The latter observation is in line with an inadequately low dose of ponatinib, but it is necessary to consider the possibility that the slow response may have been attributable to a mutation-independent mechanism.

To assess the anticipated responses to ponatinib based on *in vitro* testing, we introduced a panel of 28 BCR-ABL1 CMs as well as various single mutations as controls into Ba/F3 cells (Table 1). This cellular background was selected because the majority of published heat maps indicating the TKI responses of individual *BCR-ABL1* mutations had been established in this model.[1,2,9] Our recently published observations indicate that the *in vitro* data on TKI resistance of some mutations presented in earlier reports may be overestimated.[10] This is likely attributable to the testing of cells inadvertently carrying multiple insertions of mutant *BCR-ABL1* cassettes in the genome.[10] We have therefore established a transposon-based approach to rapid and efficient transfection of mutant *BCR-ABL1* constructs into cells, and implemented a flow cytometry-assisted selection method facilitating targeted enrichment of cells carrying single gene construct insertions in the genome. This protocol was demonstrated to provide *BCR-ABL1* expression levels similar to those observed in patient specimens and to permit unbiased testing of TKI sensitivity *in vitro*.[10]

Table 1. *In vitro* **responses of native and mutant BCR-ABL1 to ponatinib.**

BCR-ABL1		IC_{50}, nM	$\pm\Delta IC_{50}$, nM
Native		2.1	0.3
G250E		5.7	1.2
E255V		12.5	1.6
V299L		4.3	1.1
T315I		10.0	1.3
F317L		2.7	0.8
F359V		3.5	0.7
L384M		5.7	1.2
L387M		5.6	1.2
E459K		4.9	0.9
M244V	E459K	5.2	1.1
M244V	M351T	5.2	1.2
G250E	M351T	9.8	1.9
Y253H	F359V	23.7	1.7
E255V	M351T	10.2	2.3
V299L	G250E	8.5	2.1
V299L	E255V	16.2*	1.2
V299L	L298V	4.5	0.9
V299L	F359V	10.7*	1.1
V299L	M351T	4.4*	1.2
V299L	L384M	8.7	2.1
V299L	L387M	8.5	1.9
V299L	E459K	9.1	2.3
T315I	G250E	120.0*	13.0
T315I	E255V	250.0*	18.0
T315I	F311L	150.0	14.0
T315I	F359V	92.0*	10.0
T315I	L384M	53.0	5.0
T315I	L387M	55.0	6.0
T315I	H396R	72.5	9.5
T315I	E459K	62.0	8.0
F317I	E255V	35.0	2.2
F317L	E255V	30.0	1.5
F317L	V299L	5.5*	1.2
F317L	M351T	2.7	0.8
F317L	F359V	41.0*	5.0
F317L	L384M	33.0	2.0
F317L	L387M	5.4	1.1
F317L	E459K	28.5	2.0

The indicated *BCR-ABL1* constructs were expressed at equivalent levels in Ba/F3 cells, and responses to ponatinib were determined using survival assays.[10] The indicated nanomolar IC_{50} values for ponatinib and the corresponding variation ($\pm\Delta IC_{50}$) are based on three biological and a minimum of four technical replicates. Mutations highly sensitive to ponatinib are highlighted in green, whereas increasing resistance to this TKI is indicated in yellow>orange>red. *Compound mutations previously characterized *in vitro*.[2]

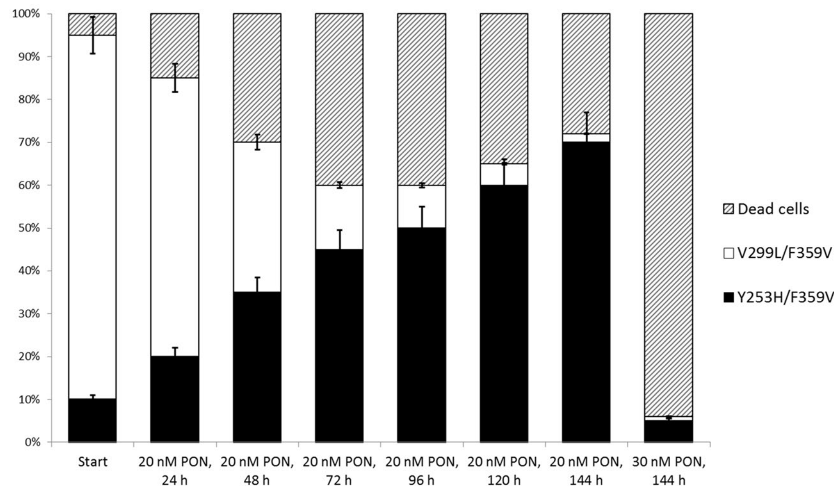

Figure 1. Competitive co-culture of BaF3 cells expressing BCR-ABL1$^{Y253H/F359V}$ and BCR-ABL1$^{V299L/F359V}$. BaF3 cells expressing BCR-ABL1$^{Y253H/F359V}$ (black columns) and BCR-ABL1$^{V299L/F359V}$ (white columns) were mixed at a 1:9 ratio and incubated in the presence of 20 or 30 nM ponatinib. Clonal evolution was monitored by fluorescence of the co-expressed fluorescent proteins ZsGreen for BCR-ABL1$^{Y253H/F359V}$ and TdTomato for BCR-ABL1$^{V299L/F359V}$. The results were confirmed by Sanger sequencing of the BCR-ABL1 TKD using gDNA and cDNA isolated and prepared at the indicated time points. The proportion of dead cells was calculated using Annexin/PI staining (striped columns). The indicated error bars are based on two biological and three technical replicates.

The IC_{50} values of ponatinib were determined by employing a widely used *in vitro* proliferation assay.[10] Most CMs involving sites with no previous evidence for implication in resistance to ponatinib displayed IC_{50} values below 10 nM. This efCave is readily achievable with the 15 mg daily dose of ponatinib,[8] thus suggesting high sensitivity to treatment with this TKI. This finding supports the notion that CMs do not necessarily confer resistance to ponatinib, unless specific sites including both T315 and F317 in particular are affected.[5] CMs revealing elevated resistance to ponatinib *in vitro* almost invariably included mutations at these two sites. In fact, the only CM constellation within the tested panel bearing neither T315I nor F317L and displaying reduced sensitivity to ponatinib was Y253H/F359V, with an IC_{50} value of 23.7 ± 1.7 nM (Table 1). This *in vitro* assessment is supported by a clinical observation made within the PACE trial: a patient with CML had presented with a V299L/F359V prior to therapy with ponatinib, a constellation displaying an IC_{50} for this TKI in the range of 10 nM (Table 1). However, the patient showed signs of progressive disease under an average daily ponatinib dose of 26 mg, corresponding to an efCave of ~20 nM.[2] Mutational analysis at this time revealed the Y253H/F359V which displays an IC_{50} value for ponatinib beyond the efCave apparently achieved in this patient. It is conceivable, therefore, that the actual dose of ponatinib may not have been sufficient to suppress the kinase activity of BCR-ABL1$^{Y253H/F359V}$, thus resulting in disease progression. Competitive *in vitro* co-culture experiments have demonstrated that Ba/F3 cells expressing BCR-ABL1$^{Y253H/F359V}$ have a proliferative advantage over Ba/F3 carrying BCR-ABL1$^{V299L/F359V}$ in the presence of 20 nM ponatinib, whereas a significant decrease in survival of both cell lines is observed at a 30 nM concentration of ponatinib (Figure 1). This example highlights the potential relevance of selecting the appropriate dose of ponatinib by considering the IC_{50} of mutations identified prior to the initiation of treatment or during therapy. It is necessary to consider, however, that the prevalence of mutant subclones is elevated in advanced disease stages, and higher doses of ponatinib may be generally required in this instance in order to prevent clonal selection and expansion, even in the absence of mutations amenable to detection by currently available approaches.

In agreement with earlier reports, CMs involving the T315I mutation displayed the highest IC_{50} values for ponatinib, clearly exceeding the clinically achievable plasma concentration of the drug.[2,11] These mutational constellations included combinations of T315I with the P-loop mutations G250E and E255V as well as T315I/F359V, which arise during treatment with ponatinib, as has been described earlier.[2,3] Our analysis of these CMs also indicated high levels of resistance to ponatinib, but the IC_{50} values determined were lower than reported previously,[2] conceivably due to the technical reasons as outlined above. In addition to constellations already shown to confer resistance to ponatinib, we identified CMs involving T315I and residues at sites displaying very distinct functional properties, which also showed IC_{50} values for ponatinib clearly above the efCave of 28 nM. The additional sites affected included the south lobe mutation E459K and the A-loop mutations L384M, L387M and H396R. Similar to A-loop mutations, it has been suggested that the E459K mutation destabilizes the inactive conformation of the ABL1 TKD.[12] This functional property may play a critical role, as ponatinib is a type II TKI which binds primarily to the inactive conformation of the BCR-ABL1 TKD[13] and as such requires considerably higher concentrations of the drug in order to inhibit the kinase activity. The CM T315I/L384M was suggested and confirmed *in vitro* to confer resistance to ponatinib at the clinically achievable plasma levels, but this constellation has not been reported to date in patients with Ph-positive leukemia. By contrast, the compound mutations T315I/L387M, T315I/H396R and T315I/E459K have been observed in the clinical setting,[2] but susceptibility to ponatinib has only been characterized by *in vitro* analysis for T315I/H396R.[2] Intriguingly, the CM T315I/H396R has shown sensitivity to axitinib,[14] a Food and Drug Administration (FDA)-approved inhibitor of the vascular endothelial growth factor receptor (VEGFR), which also displays activity against the BCR-ABL1T315I mutant.[14,15] Likewise, we demonstrated that other CMs combining T315I with A-loop mutations, including T315I/L384M, T315I/L387M, and T315I/E459K, respond to axitinib with IC_{50} values <100 nM (Table 2), suggesting clinical sensitivity to this drug.[14] However, axitinib at clinically achievable concentrations was not effective against ponatinib-resistant CMs affecting the F317 position (Table 2).

Therapy with ponatinib has been associated with con-

siderable cardiovascular toxicity and other potentially serious side effects which appear to be dose-related.[6,7] However, as highlighted by the data presented, current strategies that aim at decreasing the dose of ponatinib should carefully consider the presence and type of mutations in the *BCR-ABL1* TKD in order to enable effective treatment. It would therefore be highly desirable to implement testing of the effective plasma drug concentrations and monitor the kinetics of mutant subclones covering, in addition, compound mutations[16] in routine diagnostic surveillance so as to provide a basis for optimized clinical management of patients treated with ponatinib. Nevertheless, despite all the evidence regarding the important role of mutations in limiting the efficacy of TKI treatment, other mechanisms of resistance which remain undetermined by current experimental and diagnostic evidence may operate in mutant BCR-ABL1 cells, and can affect clinical responses to therapy.

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Funding: this work was supported by the Austrian Science Fund (FWF), SFB Grants F4705-B20 (TL) and F4704-B20 (PV). The authors declare no conflict of interests.

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doi:10.3324/haematol.2017.176347

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

Table 2. *In vitro* responses of selected BCR-ABL1 CMs to ponatinib and axitinib.

BCR-ABL1		Ponatinib IC_{50}, nM	Axitinib IC_{50}, nM
Native		2.1	550.0
T315I		10.0	122.5
F317L		2.7	1020.0
T315I	G250E	120.0	713.0
T315I	E255V	250.0	718.0
T315I	L384M	53.0	75.0
T315I	L387M	55.0	76.5
T315I	H396R	72.5	95.2
T315I	E459K	62.0	80.5
F317I	E255V	35.0	1120.0
F317L	E255V	30.0	980.0
F317L	F359V	41.0	856.0
F317L	L384M	33.0	715.5
F317L	E459K	28.5	754.0

The indicated *BCR-ABL1* constructs were expressed at equivalent levels in Ba/F3 cells, and responses to axitinib and ponatinib were determined using survival assays.[10] The indicated nanomolar IC_{50} values are based on three biological and a minimum of four technical replicates. Mutations sensitive to axitinib (IC_{50} < 150 nM) are highlighted in green, whereas resistance to this TKI is indicated in red.

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