Phospho-CRKL monitoring for the assessment of BCR-ABL activity in imatinib-resistant chronic myeloid leukemia or Ph+ acute lymphoblastic leukemia patients treated with nilotinib

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ABSTRACT

Actual BCR-ABL kinase inhibition *in vivo* as determined by phospho-CRKL (pCRKL) monitoring has been recognized as a prognostic parameter in patients with chronic myelogenous leukemia treated with imatinib. We report a biomarker sub-study of the international phase I clinical trial of nilotinib (AMN107) using the established pCRKL assay in imatinib-resistant chronic myeloid leukemia or Ph+ acute lymphoblastic leukemia. A minimum dose (200 mg) required for effective BCR-ABL inhibition in imatinib resistant/intolerant leukemia was determined. The pre-clinical activity profile of nilotinib against mutant BCR-ABL was largely confirmed. Substantial differences between peripheral blood baseline pCRKL/CRKL ratios were observed when comparing chronic myeloid leukemia with Ph+ acute lymphoblastic leukemia. Finally, rapid BCR-ABL-reactivation shortly after starting nilotinib treatment was seen in acute lymphoblastic leukemia patients with progressive disease carrying the P-loop mutations Y253H, E255K, or mutation T315I. Monitoring the actual BCR-ABL inhibition in nilotinib treated patients using pCRKL as a surrogate is a means to establish effective dosing and to characterize resistance mechanisms against nilotinib.

Key words: CRKL, BCR-ABL, nilotinib, resistance

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Introduction

Imatinib has revolutionized CML and Ph+ ALL therapy and is now standard medical treatment.[1,2] While imatinib monotherapy produces high response rates in early stages of CML (chronic phase; CP), more advanced CML (accelerated phase and blast crisis; AP, BC) and Ph+ ALL are prone to development of resistance.[3] *In vitro* and *in vivo* studies have revealed that the reactivation of BCR-ABL signaling in the presence of continued imatinib-treatment is the major cause of resistance. In the majority of patients, reactivation can be traced to the emergence of BCR-ABL kinase domain mutations which impair the binding of imatinib with differential levels of resistance induction (low, intermediate, high, complete).[4,5] Measurement of BCR-ABL kinase inhibition *in vivo* can predict response to first-line treatment with imatinib.[6] Phosphorylated CRKL (pCRKL), a BCR-ABL adaptor protein, serves as a surrogate of BCR-ABL activity *in vivo*.[7] On electrophoresis pCRKL migrates more slowly than the unphosphorylated CRKL.[8] Nilotinib (formerly AMN107, Novartis Pharmaceuticals, Basel, Switzerland) is ≥20-fold more potent than imatinib in killing wild-type BCR-ABL-expressing cell lines.[9,10] Nilotinib maintains activity against 32/33 imatinib-resistant BCR-ABL mutants, but has no significant activity against the T315I mutant *in vitro*. Here we report the pCRKL monitoring of patients with imatinib resistant or intolerant CML or Ph+

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ALL treated with nilotinib in an open label dose escalation phase I protocol.[11]

Design and Methods

Clinical study design

The clinical study design has already been published.[11] The study was conducted in accordance with the Declaration of Helsinki and was approved by the institutional review board. Patients were successively assigned to one of nine dose cohorts, ranging from 50 to 1,200 mg administered once daily (qd) and from 800 to 1,200 mg administered as split dosage twice daily (400 bid, 600 bid). Patients reported were recruited from June 2004 at Frankfurt University and represent a sub-population of the international study cohort.

Sample collection and preparation

Approximately 10 mL of EDTA-anticoagulated peripheral blood was collected from patients at the study site (Frankfurt) and shipped at room temperature to the laboratory (Mannheim) within 24 hours. Samples were collected at baseline, and at least once per treatment cycle (1 cycle = 28 days); in the low dose cohorts (50 and 100 mg qd) usually once a week.

Detection, quantification and mutation analysis of BCR-ABL

RNA-extraction, cDNA synthesis, qualitative and quantitative BCR-ABL PCR were performed as previously described[12,13] Mutation analysis was performed by denaturing high-performance liquid chromatography (D-HPLC) essentially as described by Soverini *et al*.[14] with slight modifications of the primers and temperatures.[15] The estimated proportion of mutant clone was derived from the nucleotide associated peaks as provided by sequence analysis.

Protein extraction and Western blot analysis

Western blot analysis of total leukocyte lysates to determine the level of pCRKL was performed according to published protocols.[4,6] Immunoblots were generated using the anti-CRKL antibody (C-20, Santa Cruz, Heidelberg, Germany), which detects the phosphorylated and unphosphorylated protein fractions separated by band shift. Autoradiographs were scanned using the ChemiDoc XRS system (BioRad) to quantitate the signal intensity and to calculate the ratio pCRKL/total CRKL. Validation experiments using 10 randomly selected patient samples with three independent experiments revealed a coefficient of variability (CV) of 14% (3-22%).

Table 1. Patient and sample characteristics.

	Total n	BCR-ABL unmutated	mutant	Baseline sample for protein analysis (n)	Median follow-up (days, range)
CML CP	1	0	1	1	130
CML AP	13	8	5	12	299 (55-429)
CML my BC	7	6	1	7	85 (41-386)
ALL Ph+ / CML ly BC	12	2	10	9	56 (6-304)
Total	*33*	*16*	*17*	*29*	*107 (6-429)*

Data analysis

Significant differences between treatment conditions were analyzed using the *t* test. Data analysis was performed using the GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego CA, and MS Excel (Seattle, WA, USA).

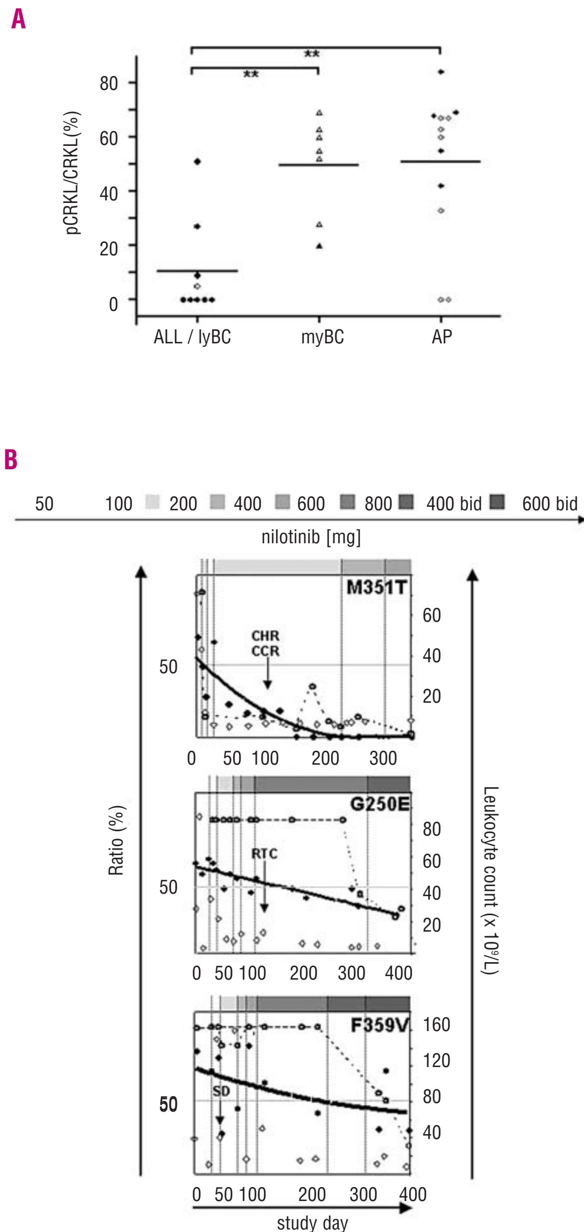
Results and Discussion

Patients' baseline characteristics

Patients' baseline characteristics are summarized in Table 1. Kinase domain mutations were detected in 17/33 patients. Detection of CRKL prior to treatment with nilotinib revealed > 50% pCRKL in the PB of CML patients (AP, myBC) (Figure 1A). By contrast, Ph+ ALL and lyBC patients show a significantly lower pCRKL/CRKL ratio with pCRKL being <10% in the PB of 7/9 patients ($p<0.01$). In line with low pCRKL in ALL/lyBC is a trend towards lower leukocyte counts and a statistically significant lower BCR-ABL/ABL ratio in PB of ALL/lyBC patients ($p<0.01$, *data not shown*). The discrepancy of the pCRKL detectability in myeloid compared with lymphoblastic Ph+ leukemia is reminiscent of the fact that in ALL PCR-based detection of BCR-ABL transcripts is preferably performed in the bone marrow (BM), whereas quantification of BCR-ABL by RT-PCR reveals comparable results in BM and PB of CML patients.[12,16] We therefore hypothesize that low pCRKL levels in the PB of lyBC/Ph+ ALL patients might rather reflect differential compartmentalization of the leukemic clone than indicating BCR-ABL dependency of the disease in general. However, serial and parallel BM/PB analysis would be needed to prove this. From this preliminary data we conclude that CRKL-monitoring at early time-points after start of treatment in the PB appears feasible in AP/BC CML. By contrast, low or undetectable pCRKL in Ph+ ALL prior to treatment onset does not allow serial *in vivo* assessment of actual kinase inhibition in PB leukocytes by Western blot analysis.

pCRKL at progressive disease in Ph+ ALL

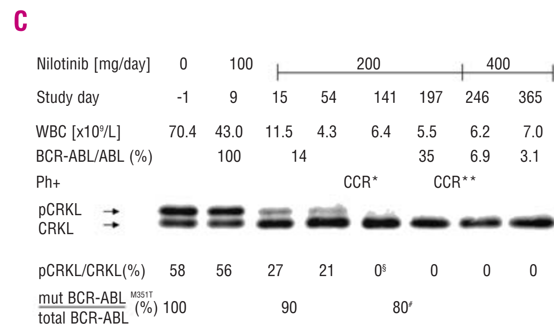
According to sample availability, we were able to analyze 7 ALL/lyBC patients with available baseline and PD samples. Mutations, pCRKL/CRKL, BCR-ABL/ABL ratios, and estimated mutant clone fraction according to sequencing profile are shown in Table 2. All patients except one (M244V) were recruited in cohorts with starting daily doses ≥200 mg of nilotinib. Doses at progressive disease in all cases were >200 mg. A significant increase in pCRKL/CRKL at PD was seen in all but the M244V patient. Patients carrying com-



plete or high resistance mutations (T315I, E255K, Y253H) showed very short time to progression (TTP) with concomitant CRKL-phosphorylation within a 28 day time period indicating BCR-ABL dependent progressive leukemic involvement of the PB. It is widely accepted that rapid leukemic progression on imatinib treatment reflects selection of highly resistant clones, and discontinuation of inhibitor treatment has the potential to slow down disease progression.[3,17] Whether continued treatment with imatinib increases the oncogenic fitness of highly resistant BCR-ABL-mutants besides providing the mutant clone with a selection advantage is currently under investigation.[18,19]

pCRKL-response to intrapatient dose-escalation of nilotinib

Three patients with AP-CML entering the protocol in the 50 mg (2x) or 100 mg (1x) starting dose cohorts were available for dose-response analysis. They carried resistance mutations, with one patient expressing low resistant mutant M351T, and the remaining patients express-



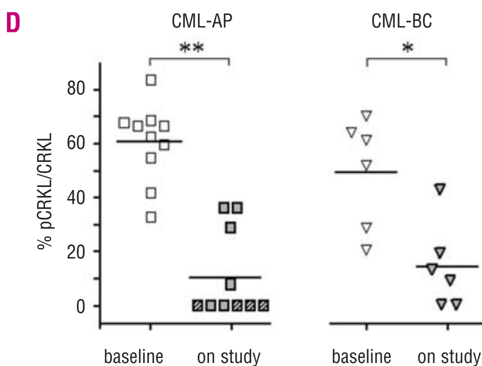


Figure 1. Baseline pCRKL/CRKL ratio prior to study entry (A). Nilotinib naive patients were analyzed according to stage of disease (x-axis) based on their pCRKL/CRKL ratio (y-axis). Patients tested mutation positive by D-HPLC and direct sequencing are indicated with black symbols, mutation negative patients appear as transparent symbols. The mean pCRKL/CRKL ratio is shown as a black line. $^{**}p<0.01$. Intrapatient dose escalation of nilotinib leads to mutation dependent pCRKL response (B, C). Monitoring results of 3 patients carrying BCR-ABL kinase mutations with differential sensitivity to nilotinib (M351T [left] low resistant; G250 and F359V intermediate resistant). Dose escalation is shown as increasingly darker grey shades. Serial pCRKL/CRKL (●) and BCR-ABL/ABL (○) measurements are shown in percent (y-axis left) over time (study day; x-axis). The bold line indicates a best-fit analysis of pCRKL/CRKL-values. The right y-axis relates to the 10^9/L with the corresponding data points shown as ◇. First appearance of best response is indicated by arrows (CHR: complete hematologic response; CCR: complete cytogenetic response; RTC: reversal to chronic phase; SD: stable disease). A representative immunoblot demonstrating the CRKL-expression of the patient expressing BCR-ABL^{M351T} is shown. (C). Response evaluation results for leukocytes (WBC), quantitative BCR-ABL/ABL mRNA measurement, cytogenetic evaluation (CCR; complete cytogenetic response), calculated pCRKL/CRKL ratio in percent, and the estimated fraction of mutant BCR-ABL as assessed by estimating the relative size of the nucleotide peaks by sequencing. *study day 113; **day 225; #day 169; §: 0 is an operational arbitrary value due to the undetectability of phosphorylated CRKL. Stage dependent CRKL-response in AP and myBC CML (D). Best pCRKL-inhibition on study is depicted as pCRKL/CRKL ratio in percent (y-axis) and compared with baseline pCRKL/CRKL ratios (prior to start of study treatment). Data points represent individual patients with the mean shown as a black line. Only patients with available baseline and on study data were selected for analysis. Hatched symbols represent patients who achieved complete hematologic response. $^{**}p<0.0001$; $^{*}p<0.05$.

Table 2. Clinical and molecular parameters of Ph+ lymphoblastic leukemias (Ph+ ALL and lyBC) at baseline and time of progressive disease. Patient selection is based on sample availability at baseline and progressive disease.

Mutation		pCRKL/totalCRKL (%)	BCR-ABL/ABL (%)	Mutant /total BCR-ABL (%)	WBC /μL	TTP [days]	Nilotinib dose [mg]
M244V*	B	27	35	100	5.8		
	PD	27	50	n/a	16.7	37	200 qd
G250E**	B	0	29	90	2.7		
	PD	25	19	n/a	14.5	93	400 qd
Y253H#	B	0	100 [BM]	100 [BM]	8.2		
	PD	16	100	100	10.7	14	400 bid
E255K#	B	9	100	50	15.5		
	PD	74	100		63.4	29	800 qd
L384M/H396P**	B	0	11	50/33	3.4		
	PD	32	N/a	n/a	3.9	57	600 qd
T315I§	B	0	29	100	9.5		
	PD	62	100		27.8	6	600 qd
T315I§	B	0	11	50	3.9		
	PD	55	100		19.3	28	600 qd

*B: baseline; PD: progressive disease. n/a: not assessed. [BM]: analysis was made from bone marrow due to sample availability. TTP: time to progressive disease. *low resistant; **intermediate resistant; #high resistant; §completely resistant according to Weisberg et al.[10]*

ing the intermediate resistant mutants G250E and F359V. All 3 of the patients presented with leukocytosis between 30×10^9/L and 80×10^9/L. BCR-ABL/ABL ratio was 100% by quantitative PCR in all 3 patients (Figure 1B). The M351T-positive patient rapidly responded with pCRKL-suppression below 50% after dose increase to 200 mg qd (Figure 1B,C). Complete hematologic remission (CHR) and complete cytogenetic remission (CCR) were achieved at day 113 with low level pCRKL/CRKL still detectable. pCRKL became undetectable at day 169 after start of treatment. pCRKL kinetics of the patients with mutations G250E and F359V are consistent with the known less sensitive *in vitro* response to nilotinib.[10] Interestingly, BCR-ABL/ABL reduction in all 3 patients is associated with preceding pCRKL-inhibition below 50%. In a recently published clinical study that prospectively analyzed the pCRKL/CRKL ratio in CML patients treated with imatinib, the 50% threshold of kinase inhibition was identified as prognostically relevant.[6] Therefore, decreased clinical activity associated with less potent pCRKL-reduction corroborates the importance of effective actual BCR-ABL-inhibition in patients treated with nilotinib.

CRKL inhibition may translate into disease control in some patients with advanced Ph+ leukemia

We also investigated whether inhibition of pCRKL by nilotinib is associated with clinically meaningful response. A separate analysis for accelerated phase and BC CML was performed for patients with detectable CRKL-phosphorylation at baseline. Baseline pCRKL/CRKL and overall best pCRKL/CRKL-inhibition on nilotinib are shown in Figure 1D. Both populations show significant pCRKL-inhibition after starting study treatment. In AP, 6/10 (60%) patients achieved complete CRKL-inhibition with 4 of them developing CHR over time. BC patients showed complete pCRKL-inhibition in 2/6 patients (33%) with none of the patients achieving CHR over time. Effective CRKL-inhibition despite resistant/progressive disease was also observed in patients treated with imatinib.[4] This observation may reflect the more complex biology of advanced disease with recruitment of additional oncogenic signals in addition to BCR-ABL, and is therefore indicative of BCR-ABL-independent disease.[20] Patients with BCR-ABL-independent disease are not predicted to benefit from alternative BCR-ABL-selective treatment.

To summarize, our preliminiary data support serial PB pCRKL- monitoring of patients treated with nilotinib for Ph+ myeloid leukemia as a potential means to establish effective kinase inhibition or to detect BCR-ABL reactivation in nilotinib-resistant patients. The limitations of this assay have been shown in advanced disease, where effective CRKL-inhibition was not always associated with disease control (CML-BC), or where possible compartmentalization to the bone marrow prevents pCRKL-analysis in the PB (Ph+ ALL). It is hoped that with the introduction of flow cytometry based assays pCRKL monitoring becomes more feasible for clinical routine.[21] Given the increasing evidence for stem cell resistance of BCR-ABL inhibitors, detection of BCR-ABL inhibition *in vivo* by flow cytometry may allow us to specifically gate for the residual leukemic population within the stem cell compartment.

Authorship and Disclosures

PL designed research and wrote paper; SHE, JD, TE and PE performed research, HK, NH, AB, and MCM analyzed data; LW and OGO treated patients; YS and MD designed the clinical study; RH wrote the paper; AH designed study, wrote the paper. YS and MD are employees of Novartis, the manufacturer of nilotinib. RH, AH and OGO received research funding by Novartis. The other authors reported no potential conflicts of interest.

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Front-line treatment of Philadelphia positive chronic myeloid leukemia with imatinib and interferon-α: 5-year outcome

Francesca Palandri,[1] Ilaria Iacobucci,[1] Fausto Castagnetti,[1] Nicoletta Testoni,[1] Angela Poerio,[1] Marilina Amabile,[1] Massimo Breccia,[2] Tamara Intermesoli,[3] Francesco Iuliano,[4] Giovanna Rege-Cambrin,[5] Mario Tiribelli,[6] Maurizio Miglino,[7] Fabrizio Pane,[8] Giuseppe Saglio,[5] Giovanni Martinelli,[1] Gianantonio Rosti,[1] and Michele Baccarani[2] on behalf of the GIMEMA Working Party on CML

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ABSTRACT

In 2004, we reported the short-term results of a multicentric, phase 2 study of imatinib 400 mg daily and pegylated interferon-α in the treatment of 76 early chronic phase Philadelphia-positive chronic myeloid leukemia patients. In this report, we update the results with an observation time of five years. After two years of treatment, all but 10 patients (13%) had discontinued pegylated interferon-α. The complete cytogenetic response rate at five years was 87%, and 94% of complete cytogenetic responders maintained the complete cytogenetic response after five years. All but one complete cytogenetic response also achieved a major molecular response. These data confirm the excellent response to imatinib front-line and the stability of the complete cytogenetic response. Any possible additional benefit of the combination with interferon-α remains uncertain, due to low patient compliance.

Key words: chronic myeloid leukemia, imatinib, long-term results, interferon-alpha

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Introduction

Imatinib mesylate (Glivec, Novartis Pharma) is a small molecule inhibiting, among others, the Bcr-Abl encoded protein kinase.[1,2] Imatinib was first tested and registered in a rapid sequence between 1998 and 2000, for the treatment of blast crisis,[3,4] accelerated phase[5,6] and late chronic phase patients resistant or intolerant to interferon-α.[7,8] The potency of imatinib was such that a prospective randomized study of imatinib *vs.* interferon-α in early chronic phase, treatment-naïve, patients was initiated in 2000.[9,10] This study, called IRIS (International Randomized Study of imatinib *vs.* interferon-α and low dose arabinosyl cytosine) led to an impressive change in the front-line management of CML, with imatinib almost completely replacing both interferon-α and allogeneic stem cell transplantation (alloSCT).[11] Since the mechanisms of action of imatinib and interferon-α are different, in 2001 the Italian Cooperative Study Group on CML (now the GIMEMA CML Working Party) carried out an exploratory phase 2 study of the combination of imatinib and interferon-α, to evaluate the safety of the combination, appropriate dosage and patients' compliance. Seventy-six consecutive, previously untreated, CML patients were treated with imatinib 400 mg daily and a pegylated preparation of human recombinant interferon-α2b (PegIntron; PegIFNα; Schering Plough, NJ, USA) at a variable dose (50, 100 and 150 μg/week).

The results of this study were published in 2004,[12] reporting that 45 out of 76 (59%) patients discontinued PegIFNα during the first year of treatment, that the frequency and the

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