Three novel patient-derived BCR/ABL mutants show different sensitivity to second and third generation tyrosine kinase inhibitors

Sara Redaelli,[1]* Luca Mologni,[1] Roberta Rostagno,[1] Rocco Piazza,[1] Vera Magistroni,[1] Monica Ceccon,[1] Michela Viltadi,[1] Daniel Flynn,[2] and Carlo Gambacorti-Passerini[1]

BCR/ABL (Breakpoint Cluster Region protein/Abelson tyrosine-protein kinase 1) kinase domain (KD) mutations represent the most frequently described mechanism of resistance to the treatment with tyrosine kinase inhibitors (TKI) in patients with chronic myeloid leukemia (CML). Mutations may impair TKI activity by directly or indirectly impairing the drug binding to the protein. We report the discovery of three new BCR/ABL mutations, L248R, T315V, and F317R identified in two patients with CML (L248R and T315V) and in one patient with Ph+ acute lymphoblastic leukemia (ALL) (F317R). Mutations were screened against second-generation (bosutinib, nilotinib, and dasatinib), as well as third-generation TKIs (ponatinib/AP-24534 and DCC-2036). Furthermore, the activity profile of ponatinib and DCC-2036 against a panel of 24 clinically relevant BCR/ABL mutants is presented and compared to the other TKIs. The IC50 values for each TKI against the mutants and the IC50 increase over wild type BCR/ABL (relative resistance, RR) were calculated to define four resistance levels: sensitive (RR $\leq$ 2), moderately resistant (2 $<$ RR $\leq$ 4), resistant (4 $<$ RR $\leq$ 10), or highly resistant (RR $>$ 10). L248R and T315V showed high resistance to imatinib, bosutinib, dasatinib, and nilotinib, intermediate resistance to ponatinib, but were sensitive to DCC-2036. Interestingly, F317R showed a moderate resistance to imatinib and nilotinib, but is resistant/highly resistant to dasatinib, bosutinib, ponatinib, and DCC-2036. The availability of drugs activity profiles may become a useful tool for clinicians dealing with the treatment of drug-resistant CML patients.

Since its development, imatinib established a new paradigm for the targeted therapy of CML. Following imatinib, several other TKIs were developed, aimed to improve both the activity and the selectivity against BCR/ABL, the deregulated tyrosine kinase responsible for the disease. Nilotinib, dasatinib, and bosutinib are generally referred to as second generation TKIs and have been widely characterized in the past years; nilotinib and dasatinib were approved in 2010 as first-line treatment for CML [1,2]. In addition, two novel compounds, bosutinib [3,4] and ponatinib [5,6] are being developed.

Resistance to imatinib therapy may develop in a subset of patients, based on the phase and type of disease [7]. In a high proportion of cases, the decreased efficacy of imatinib and other TKIs is due to point mutations in the protein sequence [7,8]. To date, more than 70 individual mutations, conferring variable resistance, have been described in patients with CML [8]. The mutation of the threonine 315 to an isoleucine (T315I) was reported to be highly resistant not only to imatinib but also to all second generation TKIs. Recently, new compounds were developed to specifically overcome resistance generated by this mutation. Among them ponatinib (AP-24534) is a pan BCR/ABL inhibitor [5,9] active against wild-type and T315I BCR/ABL as well as other clinically relevant mutated forms of the kinase and is presently in a Phase II clinical trial. DCC-2036 [10] was designed to bind into the switch pocket of ABL and to block kinase conformational activation. Like imatinib, nilotinib, and ponatinib, DCC-2036 binds ABL in an inactive conformation. DCC-2036 was reported as potently active against the gatekeeper mutant, and is presently in Phase I clinical trials.

Here, we report the discovery and characterization of three novel mutated forms of BCR/ABL derived from patients under TKI therapy.

L248R was identified in a patient with lymphoid Blast Crisis (BC) CML (Patient no. 1), *in cis* with a pre-existing mutation. The patient initially presented with an imatinib-resistant F359I mutation. The patient started bosutinib, which, according to our previous study [11], shows the lowest RR index for this mutation. Cytogenetic and molecular responses were obtained with bosutinib; however, hematological relapse developed after 1 year; a mutation analysis at the time of relapse revealed the presence of a L248R+F359I clone.

Mutation at position T315V was identified in a BC-CML patient (Patient no. 2), who was diagnosed in august 2008 with high white blood cells (WBC) count and a single extramedullary chloroma lesion at D4-5 level. The patient was initially treated with high dose (HD) Ara-C, followed by imatinib 800 mg. Due to the increase of the chloroma size, imatinib was stopped and the patient was subsequently treated with dasatinib 70 mg twice daily with disappearance of the lesion and development of complete cytogenetic response (CCyR). Hematological relapse occurred in June 2009 and analysis of the mutation status revealed the presence of a T315A clone, known to drive a complete resistance against dasatinib. In January 2010, the patient was treated with a combination of Idarubicin + HD Ara-C and, since February 2010, with nilotinib 400 mg BID, achieving a partial cytogenetic response. In June 2010, the patient relapsed and a new mutation screening showed the presence of a T315V mutation. For this reason an unrelated bone marrow transplant (UBMT) was performed in December 2010.

The third patient (Patient no. 3) was diagnosed with Ph+ ALL in February 2009. The patient received dasatinib (70 mg/die) achieving CCyR and major molecular response (MMR) in April 2009. However, in May 2010, the patient relapsed and the mutational screening revealed the F317R mutation. In June 2010, the patient received nilotinib (800 mg/die) leading to a new CCyR and MMR.

All the mutations were identified by direct sequencing; the analysis of chromatograms clearly demonstrated that mutated nucleotides are present as unique variants (Fig. 1). Therefore, double mutations cannot be ascribed to co-existence of two clones.

The Ba/F3 murine cell line was stably transfected with pcDNA3 vector containing either the wild type or the newly described mutated forms of BCR/ABL. The activity of bosutinib, imatinib, dasatinib, and nilotinib against T315V, L248R, F317R, F359I, and L248R+F359I was assessed (Table I).

The relative IC50 increase over wild type BCR/ABL (RR) was calculated. We classified RR values in four categories: sensitive (RR $\leq$ 2), moderately resistant (RR between 2 and 4), resistant (RR between 4 and 10), or highly resistant (RR$>$10).

In our experiments, T315V, L248R, and the double mutant L248R+F359I showed high resistance level to four TKIs (imatinib, bosutinib, dasatinib, and nilotinib). The single mutation F359I is highly resistant to nilotinib (RR 16.3), resistant to imatinib (RR 6.0) while it confers only moderate resistance to bosutinib and dasatinib (RR 2.9 and 3.0, respectively). F317R is moderately resistant to imatinib and nilotinib (RR 2.3) and highly resistant to bosutinib (RR 33.5) and dasatinib (RR 114.8).

Recently new compounds were developed to overcome resistance generated by the T315I mutant. Among them ponatinib, a pan BCR/ABL inhibitor [5,9], and the switch pocket inhibitor DCC-2036 [10] were reported as potently active against the T315I mutation.

When tested in our proliferation assay, DCC-2036 showed activity against both T315V (RR 0.6) and L248R (RR 0.4), while ponatinib encountered moderate resistance against T315V (RR 2.1) and resistance by L248R (RR 6.2). The double mutant L248R+F359I was resistant to ponatinib (RR = 17.7) but sensitive to DCC-2036 (RR = 1.0). Neither of the new compounds was able to inhibit the F317R mutant, which scored as resistant to ponatinib (RR 4.9) and highly resistant to DCC-2036 (RR 21).

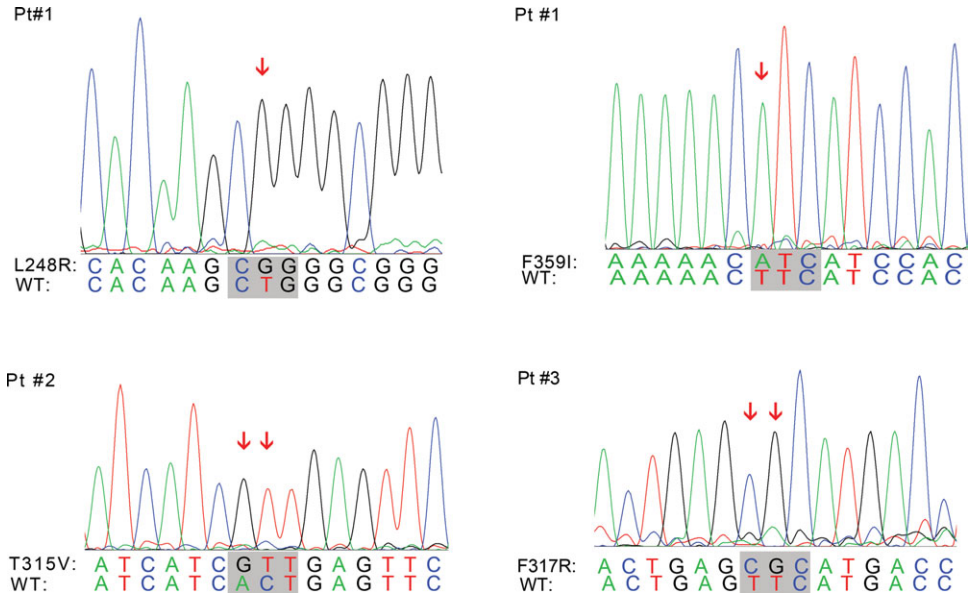

Figure 1. Sequencing chromatograms for the three patients analyzed. For all the mutations the WT sequence is reported as reference and the codon affected by the nucleotide change is shaded. The red arrows correspond to the mutation site. Chromatograms for Patient no. 1 refer to the same sequencing reaction and have been divided in two parts to allow visualization of both mutations.

TABLE I. Activity of Imatinib, Bosutinib, Dasatinib, Nilotinib, Ponatinib, and DCC-2036 Against Mutated Form of BCR/ABL

		IC50-fold increase (WT = 1)					
		Imatinib	Bosutinib	Dasatinib	Nilotinib	Ponatinib	DCC-2036
	Parental	10.8	38.3	568.3	38.4	570.0	13.1
	WT	1	1	1	1	1	1
P-loop	M244V	0.9	0.9	2.0	1.2	3.2	0.8
	L248R	14.6	22.9	12.5	30.2	6.2	0.4
	L248V	3.5	3.5	5.1	2.8	3.4	1.3
	G250E	6.9	4.3	4.4	4.6	6.0	3.0
	Q252H	1.4	0.8	3.1	2.6	6.1	2.1
	Y253F	3.6	1.0	1.6	3.2	3.7	2.3
	Y253H	8.7	0.6	2.6	36.8	2.6	2.7
	E255K	6.0	9.5	5.6	6.7	8.4	3.5
	E255V	17.0	5.5	3.4	10.3	12.9	2.1
C-helix	D276G	2.2	0.6	1.4	2.0	2.1	4.5
	E279K	3.6	1.0	1.6	2.0	3.0	6.5
	E292L	0.7	1.1	1.3	1.8	2.0	1.0
ATP binding region	V299L	1.5	26.1	8.7	1.3	0.6	0.3
	T315A	1.7	6.0	58.9	2.7	0.4	0.4
	T315I	17.5	45.4	75.0	39.4	3.0	0.7
	T315V	12.2	29.3	738.8	57.0	2.1	0.6
	F317L	2.6	2.4	4.5	2.2	0.7	1.1
	F317R	2.3	33.5	114.8	2.3	4.9	21.0
	F317V	0.4	11.5	21.3	0.5	2.3	6.6
SH2-contact	M343T	1.2	1.1	0.9	0.8	0.9	1.0
	M351T	1.8	0.7	0.9	0.4	1.2	2.2
Substrate binding region	F359I	6.0	2.9	3.0	16.3	2.9	0.7
	F359V	2.9	0.9	1.5	5.2	4.4	0.9
A-loop	L384M	1.3	0.5	2.2	2.3	2.2	0.9
	H396P	2.4	0.4	1.1	2.4	1.4	1.5
	H396R	3.9	0.8	1.6	3.1	5.9	0.7
C-terminal lobe	F486S	8.1	2.3	3.0	1.9	2.1	0.5
	L248R + F359I	11.7	39.3	13.7	96.2	17.7	1.0
Sensitive	≤2						
Moderately resistant	2.1–4						
Resistant	4.1–10						
Highly resistant	>10						

For each mutant the relative IC50 increase over wild type BCR/ABL was calculated. Results represent the average of at least three independent experiments.

The activity profile of ponatinib and DCC-2036 against L248R, L248R+F359I, T315V, F317R, and a panel of 24 additional mutated forms of BCR/ABL covering the most common patient mutations is presented in Table I. Activity of bosutinib, imatinib, dasatinib, nilotinib is reported for a comparison [11].

According to our data, only mutation E255V is classified as "highly resistant" to ponatinib, in addition to L248R+F359I, and 6/27 mutations are considered "resistant" to ponatinib (L248R, G250E, G252H, E255K, T315V, F317R, F359V, and H396R). DCC-2036 has only one mutation, F317R, identified as highly resistant, while 3/27 mutants are considered resistant (D276G, E279K, and F317V). The use of these information will certainly result useful to clinicians treating Ph+ leukemia; a clinical correlation between in vitro data and clinical results has already been established [12].

TABLE II. IC50 Values for Bosutinib, Imatinib, Dasatinib, and Nilotinib, Ponatinib and DCC-2036 Against the Mutated Forms of BCR/ABL Expressed in Ba/F3 Transfected Cells

	IC50 values (nM)					
	Imatinib	Bosutinib	Dasatinib	Nilotinib	Ponatinib	DCC-2036
Parental	5,683.0	1,594.0	1,040.0	679.9	1,197.0	299.3
WT	527.0	41.6	1.8	17.7	2.1	22.9
M244V	453.6	39.3	3.6	21.1	6.7	18.3
L248R	7,680.0	954.3	22.9	533.4	13.0	8.7
L248V	1,866.0	147.4	9.4	49.5	7.1	29.4
G250E	3,613.0	179.2	8.1	80.7	12.5	68.6
Q252H	733.6	33.7	5.6	46.8	12.9	48.6
Y253F	1,888.0	40.0	2.9	57.2	7.8	51.6
Y253H	4,589.0	24.0	4.7	651.3	5.4	61.3
E255K	3,174.0	394.0	10.3	1,18.4	17.6	81.1
E255V	8,953.0	230.1	6.3	1,82.3	27.2	48.0
D276G	1,147.0	25.0	2.6	35.3	4.4	101.9
E279K	1,872.0	39.7	3.0	36.3	6.2	149.2
E292L	360.3	47.0	2.5	32.2	4.2	22.8
V299L	8,13.7	1,086.0	15.8	23.7	1.2	6.5
T315A	890.5	249.9	107.8	47.6	0.9	10.2
T315I	9,221.0	1,890.0	137.3	697.1	6.3	15.3
T315V	6,408.0	1,220.0	1,352.0	1,008.0	4.5	12.6
F317L	1,370.0	100.7	8.2	39.2	1.5	25.1
F317R	1,213.0	1,395.0	210.0	40.0	10.4	481.3
F317V	190.3	479.6	39.1	8.1	4.9	150.8
M343T	630.6	46.1	1.6	14.1	2.0	22.1
M351T	926.6	29.1	1.6	7.8	2.6	49.9
F359I	3,147.0	122.2	5.5	288.4	6.2	16.9
F359V	1,509.0	38.6	2.7	91.3	9.2	21.1
L384M	674.4	19.5	4.0	41.2	4.6	20.1
H396P	1,280.0	18.1	2.0	42.7	2.8	33.4
H396R	2,058.0	33.7	3.0	54.8	12.5	15.6
F486S	4,267.0	96.1	5.6	32.8	4.3	10.6
L248R + F359I	6,165.0	1,635.0	25.1	1,701.0	37.2	23.0

As a reference, IC50 values for each drug against parental BaF/3 cell as well as BCR/ABL wild type are provided. Results are the average of at least three independent experiments.

However the heterogeneity of a tumor needs to be considered, knowing that sequencing will identify only dominant clones, present in at least 25–30% of the total population, and that clones present in small numbers, which could be selected and cause subsequent relapses, will be missed.

We present here three mutated forms of BCR/ABL identified for the first time in patient's CML samples. These mutants are not affected by 1st and 2nd generation TKIs, with a level of resistance similar to the highly resistant T315I mutation. L248R was previously reported only in vitro during a mutagenesis screen involving imatinib [13] and in a study with the T315I inhibitor SGX393 [14]. L248R was never isolated from a clinical sample. The gatekeeper T315V mutation and F317R involve residues with previously unidentified amino acid substitutions.

To our knowledge this is the first time that mutations other than T315I are described conferring pan-inhibitor resistance.

Ponatinib was optimized using a structure-based drug design in order to have multiple contact points with the kinase. This feature allows the activity to be unaffected by the disruption of one contact point due to a single point mutation. The simultaneous presence of two sequence modifications was predicted to be able to substantially impair the drug activity [5]. Indeed, our results show that the double mutant L248R+F359I is able to confer a high resistance index to ponatinib, higher than the single mutant L248R.

It is important to note (Table II) that the ratio in IC50 values for ponatinib between Ba/F3 parental cells (1197 nM) and Ba/F3-BCR/ABL wild type (2.1 nM) is pretty high (570) indicating a large theoretical therapeutic window. This compares favorably to the values obtained for imatinib (10.8), DCC-2036 (13.1), bosutinib (38.3), and nilotinib (38.4), while dasatinib (568.3) shows a similar value. This "therapeutic window" could render some of the mutants with high RR values to ponatinib and dasatinib still sensitive to non-toxic doses of these inhibitors. For example, the IC50 against the double L248R+F359I mutant is 37.2 nM, a value that is 15-fold higher compared to wild-type BCR/ABL (and therefore classified as highly resistant), but largely below the toxicity level defined by the activity against parental cells. It remains to be seen whether drug concentrations achievable in patients will be enough to fill this 15-fold sensitivity gap between wild-type and double mutant kinase. In this regard, a Phase I study showed that doses higher than 30 mg result in a plasma concentration of 40 nM [6]. Although plasma levels do not always correlate with intracellular concentrations of a drug [15,16], such a concentration should be sufficient to target the double mutant L248R+F359I.

Rational drug design behind DCC-2036 was based upon the development of an inhibitor which binds into the pocket that controls conformational activation of ABL. Through binding into this "switch pocket," DCC-2036 stabilizes ABL in the inactive conformation despite the presence of oncogenic mutations which predispose ABL to adopt active conformations. This binding mode is consistent with the retention of potency versus both the L248R p-loop mutation and the L248+F359I double mutation of BCR/ABL.

Mutation of phenylalanine 317 to arginine, according to our in vitro data, is able to strongly impair the activity of DCC-2036. Molecular modeling analysis indicated that activity against this mutant correlates with a drug's occupancy of the ATP pocket. Indeed, RR of the six compounds and their pocket filling followed the same order (Das>Bos>DCC>Pon>Im>Nil).

In conclusion, we describe three novel mutations that are highly resistant in vitro to the commonly used BCR/ABL TKIs but which might be inhibited in vitro by ponatinib or DCC-2036. While this fact is encouraging for patients affected by Ph+ leukemias, one should not overlook the extreme heterogeneity that can be present in advanced cancers, especially acute leukemias, in which billions of clones with different mutations exist and can be selected. As an example, it is likely that the T315V mutation (GTT codon) evolved from the previously identified T315A clone (GCT codon) as a consequence of a second mutational event occurred in the same codon. The targeting of more than a single pathogenic event in a highly heterogeneous cancer could produce better results [17].

Materials and Methods

Chemicals. Bosutinib (SKI-606, Wyeth Pharmaceuticals, Pearl River, NY) was kindly provided by Dr. Frank Boschelli (Wyeth Pharmaceuticals). Nilotinib (AMN-107, Novartis Pharmaceuticals), imatinib mesylate, and dasatinib (BMS 354825, Bristol-Meyer Squibb) were synthesized by Dr. Enrico Rosso (University of Venice, Italy); DCC-2036 (Deciphera Pharmaceuticals, Lawrence, KS 66044, USA) was kindly provided by Dr. Daniel Flynn; ponatinib (ARIAD Pharmaceuticals, Cambridge, MA) was supplied by the company. Bosutinib, dasatinib, nilotinib, ponatinib, and DCC-2036 were dissolved in dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO) at 10 mmol/L

as stock concentration. Imatinib was dissolved in water to obtain a 10 mmol/L stock solution. Tritiated thymidine was purchased from Perkin Elmer (Perkin Elmer, Waltham, MA).

Patients. Patients included in this study had been treated at the Clinical Research Unit, San Gerardo Hospital, Monza, Italy. All the patients gave a written informed consent to the study.

Sequence analysis of the BCR-ABL kinase domain. Ficoll paque plus gradient (GE Healthcare, USA) was applied to peripheral blood samples and total RNA was extracted from the recovered mononuclear cells using standard Trizol reagent protocol (Life Technologies Ltd, UK). RNA (1 μg) was retrotranscribed and amplified using Titan One Tube RT-PCR System (Roche Applied Science, Indianapolis, IN) and the following primers: p210: Bcr_b2c_for (5′-CAGATGCTGACCAACTCGTGT-3′) and Abl7_rev (5′-CCGGAAGGAGCTGCTGCGTT-3′); p190: Bcr_p190ext_for5′-GCACCTCTGAGCAGGAGAAGCG −3′. A nested PCR was performed using the first PCR product diluted 1:100 and the primers Bcr_b2c_int_for (5′-GTGAAACTCCAGACTGTCCA-3′) Abl4rev (5′-CTTCTCTAGCAGCTCATACAC-3′) Bcr_p190int_for 5′-AATGAGAACCTCACCTCCAG-3′ that allow the amplification of the whole KD of Abl. PCR product was purified and sequenced using Abl4rev primer. The presence of mutations was verified by the analysis of sequences chromatograms.

Cell lines and culture. Murine pro-B cell line Ba/F3 was maintained in RPMI 1640 (BioWhittaker Europe, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 units/mL penicillin G, 80 μg/mL gentamicin, and 20 mmol/L 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), in a humidified atmosphere at 37°C and 5% CO_2 plus Chinese hamster ovary–conditioned supernatant as a source of interleukin-3 (IL-3). Cell line was purchased from the American Type Culture Collection, where cells are routinely verified using polyphasic (genotypic and phenotypic) testing to confirm identity.

Site-directed mutagenesis and Ba/F3 stable transfection. Ba/F3 p210 cells were obtained by transfecting the IL-3-dependent murine hematopoietic Ba/F3 cell line with a pcDNA3 vector containing p210 Bcr-Abl cDNA [18]. The same plasmid was used as a template for the in vitro site-directed mutagenesis. Plasmids carrying mutations at positions M244V, L248R, L248V, G250E, Q252H, Y253F, Y253H, E255K, E255V, D276G, E279K, E292L, V299L, T315A, T315I, T315V, F317L, F317R, F317V, M343T, M351T, F359I, F359V, L384M, H396P, H396R, F486S, L248R + F359I were generated with "QuikChange II XL Site-Directed Mutagenesis kit"(Stratagene, La Jolla, CA) according to the manufacturer instructions.

For the transfection, 10^7 Ba/F3 cells were resuspended in 450 μL RPMI 1640, and 30 μg linearized plasmid in phosphate buffered saline (PBS) were added. Cells were incubated on ice for 2 min and then electroporated (270 V, 975 μF) using a GenePulser XCell (Bio-Rad, Hercules, CA). Cells were then placed back on ice for 2 min and finally transferred into 10 mL RPMI 1640 with FBS (10%) plus IL-3 at standard concentrations [19]. After 24 hr, 1 mg/mL G418 (Gibco-Invitrogen, Carlsbad, CA) was added and the cells were cultured until day 14 when IL-3 was withdrawn. Cells were grown under selection with G418 for 4 weeks.

Proliferation assay. Cells were seeded at a concentration of 10^4cells/well in 96-well round bottom cell culture plates with complete medium and in presence of increasing concentration of inhibitors. Cell proliferation was measured at 72 hr using the tritiated thymidine incorporation assay as described previously [20]. Each test was performed in triplicate and repeated at least twice.

Statistical analysis. All statistical analyses were performed using GraphPad Prism 4 (GraphPad Software, Inc.).

[1]*Department of Clinical Medicine and Prevention, University of Milano-Bicocca, S. Gerardo Hospital, Monza, Milan, Italy;*
[2]*Deciphera Pharmaceuticals LLC, Lawrence, Kansas;*
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**Correspondence to: Sara Redaelli, PhD, University of Milano-Bicocca, S.Gerardo Hospital, Dept. Clinical Medicine and Prevention, Via Cadore, 48 - 20900 Monza (MB), Italy. E-mail: sara.redaelli@unimib.it*
Conflict of interest: Carlo Gambacorti-Passerini has research grant from Pfizer, the owner of bosutinib. Daniel Flynn is employee at Deciphera pharmaceuticals, the owner of DCC-2036.
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