

The Rise and Fall of Gatekeeper Mutations? The *BCR-ABL1* T315I Paradigm

Don L. Gibbons, MD, PhD¹; Sabrina Pricl, PhD²; Hagop Kantarjian, MD³; Jorge Cortes, MD³; and Alfonso Quintás-Cardama, MD³

The use of tyrosine kinase inhibitors (TKIs) has become an integral component of cancer therapy. Imatinib mesylate, a breakpoint cluster region-Abelson *BCR-ABL1* inhibitor, was the first TKI approved in cancer medicine and has served as a model for the development of similar agents for other cancers. An important drawback of TKI therapy is the development of resistance, frequently through the acquisition of mutations. Mutations at the gatekeeper residues of *BCR-ABL1* (eg, the threonine-to-isoleucine mutation at codon 315) and other oncogenic kinases have proven highly resistant to currently available TKIs. Advances in the structural biology of oncogenic kinases have facilitated the rational development of TKIs that are active against gatekeeper mutations. *Cancer* 2012;118:293-9. © 2011 American Cancer Society.

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The Paradigmatic T315I Gatekeeper Mutation

Phosphorylation of specific serine, threonine, or tyrosine residues is germane to cellular growth, and its control relies on the proper regulation of protein kinases. Protein kinases are plastic molecular switches that oscillate between “on” and “off” conformations according to diametrically opposite catalytic activity states.¹ All protein kinases catalyze the transfer of the γ -phosphate of adenosine triphosphate (ATP) to the hydroxyl group of serine, threonine, or tyrosine. To do so, they must adopt a catalytically active “on” conformation, which involves changes in the orientation of the C helix in the small N-terminal lobe and the activation area in the larger C-terminal lobe.² Kinase activation in the wrong temporospatial context can result in unbridled cell proliferation and malignant transformation. Such is the case in chronic myeloid leukemia (CML), a clonal malignancy characterized by unchecked myeloid proliferation driven by the constitutively active kinase activity of the breakpoint cluster region/Abelson murine leukemia 1 (*BCR-ABL1*) fusion oncoprotein.¹ The critical role of *BCR-ABL1* in the pathogenesis of CML is fueled the development of ATP mimetics capable of antagonizing its kinase activity. Tyrosine kinase inhibitors (TKIs) can be divided in type I, which compete directly with ATP for the ATP-binding site, and type II, which not only bind to the ATP-binding site but also occupy an adjacent hydrophobic space only accessible when the kinase is in its inactive conformation.³ The type II *BCR-ABL1* kinase inhibitor imatinib mesylate was the first TKI to be approved in cancer medicine and propelled the development of similar therapeutics for other cancer types. After 8 years of follow-up in the phase 3 International Randomized Study of Interferon versus STI571 (IRIS) trial, imatinib therapy produced cumulative rates of complete cytogenetic response (ie, no evidence of the Philadelphia chromosome in the bone marrow) and major molecular response (ie, a *BCR-ABL1/ABL1* ratio $\leq 0.1\%$) of 83% and 86%, respectively, among patients with CML in chronic phase (CP). These responses translated into event-free, progression-free, and overall survival rates of 81%, 92%, and 85% (93% considering only CML-related deaths), respectively.⁴ Despite these remarkable results, it is estimated that 20% to 30% of patients eventually will develop resistance to imatinib, which, in 40%, will be associated with the acquisition of *BCR-ABL1* kinase domain mutations.⁵ Although >100 *BCR-ABL1* point

Corresponding author: Alfonso Quintás-Cardama, MD, Department of Leukemia, Unit 428, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030; Fax: (713) 745-4009; aquintas@mdanderson.org

¹Department of Thoracic, Head/Neck Medical Oncology and Molecular and Cellular Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas; ²Molecular Simulation Engineering Laboratory, Department of Materials and Natural Resources, University of Trieste, Trieste, Italy; ³Department of Leukemia, The University of Texas MD Anderson Cancer Center, Houston, Texas

The first 2 authors contributed equally to this article.

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mutations have been reported,^{1,6-11} those at residues glycine 250 (Gly250), tyrosine 253 (Tyr253), glutamic acid 255 (Glu255), threonine 315 (Thr315), methionine 351 (Met351), and phenylalanine 359 (Phe359) account for >60% of all mutations.¹² Most troublesome among all mutations is the substitution of isoleucine (I) at the 315 position of the ABL1 kinase for threonine (T315I). Thr315 is known as the gatekeeper residue, because it maps to the periphery of the nucleotide-binding site of ABL1.¹ T315I is associated with an overall survival of 22 months in patients with CML-CP.¹³ Second-generation TKIs, such as nilotinib and dasatinib, when used in patients who have CML that is resistant to imatinib, produce complete cytogenetic response rates of 45% to 50%.^{14,15} Notwithstanding their remarkable activity against most *BCR-ABL1* mutants, neither nilotinib nor dasatinib is active in patients who carry the T315I mutation,^{14,15} supporting the notion that this mutation represents an important escape mechanism for CML cells withstanding high levels of selection pressure imposed by TKI therapy.

Why Is T315I (and, for That Matter, Any Gatekeeper Mutation) So Difficult to Target With ATP-Mimetic TKIs?

Structural studies have demonstrated that the location of the Thr315 residue plays a big role.¹⁶ Thr315 locates at the periphery of the nucleotide-binding site of ABL1 kinase within the hinge region of the enzymatic cleft, stabilizes imatinib binding through hydrogen-bond interactions, and regulates access to a deep hydrophobic pocket in the active site.¹⁷ It is noteworthy that, although imatinib and nilotinib bind *BCR-ABL1* kinase in the inactive conformation and dasatinib does so in the active conformation, all 3 TKIs make a critical hydrogen bond with the side chain hydroxyl group of Thr315.^{1,17,18} A mutation of the threonine gatekeeper residue to isoleucine prevents the formation of this critical hydrogen bond. Second, such mutation also causes steric hindrance between the large hydrophobic isoleucine residue and any of the 3 TKIs, thus blocking the access of the latter to the hydrophobic pocket in the proximity of Thr315.¹⁶ Third, Thr315 participates in a network of hydrophobic interactions when the kinase is in the active conformation. Its mutation to isoleucine promotes the assembly of an enzymatically active kinase conformation through the stabilization a series of hydrophobic interactions.^{2,19} Consequently, T315I results in complete insensitivity to imatinib, nilotinib, dasatinib, and bosutinib.^{16,17,20-24}

Is It Possible to Target *BCR-ABL1*^{T315I} With an ATP-Competitive Small Molecule?

In recent years, a series of dual inhibitors of *BCR-ABL1*^{T315I} and Aurora kinases have been tested in clinical trials, including MK-0457,²⁵ danusertib (PHA-739538),^{26,27} and XL-228.²⁸ Unfortunately, their administration involved inconvenient intravenous infusions, and responses were limited and typically short-lived, coinciding with the periods of drug administration. Furthermore, all of these agents have been associated with very high rates of grade 3/4 cytopenia and with significant nonhematologic toxicities from their activity against both malignant cells and normal cells, because Aurora kinases are involved in normal cell signaling. Interest in developing T315I inhibitors has been reignited with the irruption of ponatinib (AP24534) into the clinical arena (Table 1). The TKI ponatinib is obtained through a structure-guided drug-design strategy aimed at targeting the inactive conformation of the ABL1 kinase and at avoiding the interaction with the side chain of 315I.²⁹ Ponatinib potently inhibits both native (50% inhibitory concentration [IC₅₀], 0.37 nM) and T315I (IC₅₀, 2.0 nM) ABL1 kinases in addition to multiple *BCR-ABL1* mutations that confer high levels of resistance to other TKIs, SRC family of kinases, vascular endothelial growth factor receptor, fibroblast growth factor receptor 1, and platelet-derived growth factor receptor (PDGFR) tyrosine kinases, but not Aurora kinases.³⁰ A structural analysis of the cocrystal structure of the ponatinib analog AP24589 with *BCR-ABL1*^{T315I} indicated that the ability of ponatinib to inhibit the gatekeeper mutation resides in its slender, linear, triple-carbon ethynyl linker, which 1) prevents direct contact with the isoleucine residue and 2) promotes an extended conformation of the unbound inhibitor that favors binding to the inactive conformation that T315I adopts.²⁹

We have conducted *in silico* molecular simulations that have confirmed the ability of ponatinib to bind in a highly efficient manner to the T315I mutant isoform of *BCR-ABL1* by virtue of its ethynyl linker, which minimizes steric clash with the bulky isoleucine residue (Fig. 1a-c). Indeed, ponatinib derivatives with altered ethynyl linkers exhibit a markedly diminished activity against T315I in cellular assays, thus highlighting the tremendous importance of this region of the molecule in avoiding the steric hindrance posed by the mutant isoleucine residue.²⁹ In cell-based mutagenesis screens, ponatinib completely abrogated the emergence of resistant CML clones at concentrations ≥ 40 nM in cell-based mutagenesis screens and prolonged the survival of mice previously injected intravenously with Ba/F3 cells expressing *BCR-ABL1*^{T315I}.³⁰ In a

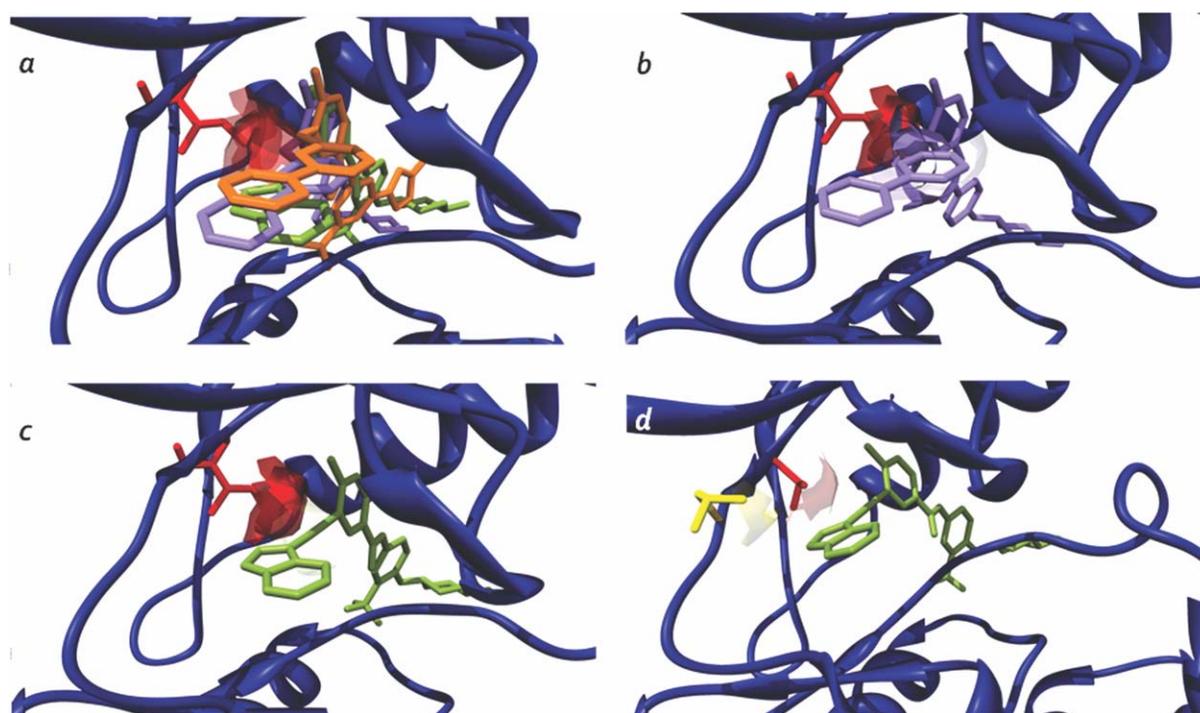


Figure 1. Ponatinib avoids the steric hindrance imposed by the threonine-to-isoleucine mutation at codon 315 (T315I). (a) This is a computer-simulated ribbon depiction of imatinib (purple), nilotinib (orange), and ponatinib (green) bound to a breakpoint cluster region/Abelson murine leukemia 1 (*BCR-ABL1*) T325I (*BCR-ABL1*^{T315I}) mutant. The mutant residue I315 is depicted as red sticks. (b,c) Highlights of the interaction of (b) imatinib and (c) ponatinib with the mutant residue I315 of *BCR-ABL* are shown. Note the difference in steric clash (portrayed as colored areas), accounting for the preserved affinity of ponatinib for the mutant protein with respect to the binding failure of imatinib. (d) A computer simulation is shown of ponatinib bound to the *BCR-ABL2* T315I/phenylalanine-to-leucine mutation at codon 317 (*BCR-ABL2*^{T315I/F317L}) double mutant. The mutant residues I315 and L317 are depicted as red and yellow sticks, respectively. Drug hydrogen atoms, water molecules, and counterions are omitted for clarity. Ponatinib inhibits *BCR-ABL1*^{T315I/F317L} with a 50% inhibitory concentration (IC_{50}) of 21 nM, which is 2.8-fold greater than the IC_{50} of *BCR-ABL1*^{T315I} (7.4 nM).

recently completed phase 1 study, ponatinib was administered orally at doses ranging from 2 to 60 mg daily to 74 patients, including 60 patients with CML (44 in CP, 7 in accelerated phase, and 9 in blastic phase), 4 patients with *BCR-ABL1*-positive acute lymphoblastic leukemia (ALL), and 10 patients with other myeloid malignancies. Of the 64 patients with Philadelphia chromosome-positive leukemia, 95% had failed at least 2 TKIs (65% had failed at least 3 TKIs), and 63% carried at least 1 *BCR-ABL1* mutation, including the T315I mutation in 28% of patients.³¹ The dose-limiting toxicity was pancreatic, which was reported in 4 of the 12 evaluable patients who received 60 mg daily. The most frequent grade 3/4 treatment-related, nonhematologic toxicities were thrombocytopenia (16%), neutropenia (7%), and elevation of lipase (7%), but only 4% of patients discontinued ponatinib therapy because of toxicity. Doses >30 mg daily consistently rendered plasma concentrations that were predicted to prevent the emergence of all *BCR-ABL1* mutations in

vitro (>40 nM).³¹ Among patients with CML-CP, the complete cytogenetic response rate was 53% (89% in patients with T315I), and the major molecular response rate was 42% (78% in patients with T315I), and most responses were maintained after 12 months of follow-up.³¹ These results, although preliminary, are remarkable and, if confirmed in an ongoing phase 2 study, will establish ponatinib as the drug of choice for patients who carry the T315I mutation. Furthermore, it is known that patients who fail sequential TKI therapy accumulate more than 1 *BCR-ABL1* mutation within the same malignant clone, and it has been demonstrated that this increases their oncogenicity.^{10,32} In addition to single point mutants, our in silico experiments also demonstrated that ponatinib binds with high affinity several double mutant isoforms of this protein, mainly by virtue of substantially reduced steric clashes and preserved favorable interaction with the mutant residues as well as other amino acids lining the drug binding site (Fig. 1d). A recently published

Table 1. Characteristics of Ponatinib Compared With Those of Nilotinib and Dasatinib Regarding Efficacy and Toxicity in Patients With Chronic Myeloid Leukemia

| Characteristic | Dasatinib | Nilotinib | Ponatinib |
|--|---------------------------------------|--|-----------------------|
| Potency relative to imatinib | 325-fold | 30-fold | 400- to 500-fold |
| Main target kinase(s) | SRC, ABL1 | ABL1 | SRC, ABL1, VEGF |
| Target ABL1 conformation | Active and inactive | Inactive | Inactive |
| Resistant mutations | T315I | T315I, E255V | None |
| Mutations with intermediate sensitivity | E255K/V, V299L, F317L | E255K/V, Y253F/H, Q252H, F359V | T315I, E255V |
| Standard dose (frontline, CP) | 100 mg/d | 300 mg twice daily | 45 mg/d |
| Main toxicities | Cytopenia, pleural effusion, bleeding | Cytopenia, bilirubin, and lipase elevation | Cytopenia, pancreatic |
| KIT inhibition (vs imatinib) | Increased | Similar | Increased |
| PDGFR inhibition (vs imatinib) | Increased | Similar | Increased |
| Clinical activity after failure to 2 TKIs ^a | + | + | +++ |
| Clinical activity against T315I | None | None | Highly active |
| Activity against CML stem cells | None | None | Unknown |

Abbreviations: ABL1, v-abl Abelson murine leukemia viral oncogene 1; Bcr-Abl, breakpoint cluster region/v-abl Abelson murine leukemia viral oncogene; CML, chronic myeloid leukemia; CP, chronic phase; E255K/V, glutamic acid-to-leucine or valine mutation at codon 255; E255V, glutamic acid-to-valine mutation at codon 255; F317L, phenylalanine-to-leucine mutation at codon 317; F359V, phenylalanine-to-valine mutation at codon 359; KIT, v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog; PDGFR platelet-derived growth factor receptor; Q252H, glutamine-to-histidine mutation at codon 252; SRC, sarcoma; T315I, threonine-to-isoleucine mutation at codon 315; TKIs, tyrosine kinase inhibitors; V299L, valine-to-leucine mutation at codon 299; VEGF, vascular endothelial growth factor; Y253F/H, tyrosine-to-phenylalanine or histidine mutation at codon 253.

^a + low; +++ high.

drug-resistance screen with AP24163, a TKI related to ponatinib, using randomly mutagenized *BCR-ABL1*^{T315I}, indicated that compound mutations involving T315I still can be recovered.³³ However, structural analyses of some of the most frequently encountered compound mutants identified in patients with imatinib-resistant CML who were treated at our institution in complex with ponatinib have indicated that ponatinib is able to bind to all double-mutant *BCR-ABL1* isoforms with IC₅₀ values that, although several-fold higher compared with the single point mutants, can be reached easily at 45 mg daily, the dose currently being tested in phase 2 studies.

Are There Alternative Ways to Inhibit Gatekeeper Mutations With TKIs?

Alternate ways to inhibit *BCR-ABL1* kinase activity while avoiding a direct interaction with the T315I gatekeeper mutation have been reported. One of the most attractive methods is that of targeting hydrophobic pockets remote from the catalytic domain, where classic TKIs clash with gatekeeper mutant residues. DCC-2036, which is in a novel TKI class known as *switch pocket inhibitors*, is undergoing clinical testing in a phase 1 study for patients who carry T315I or who have failed >2 TKIs. DCC-2036 is a multikinase inhibitor that binds to a pocket that governs the transition between the active and the inactive states of ABL1, thus locking the kinase into its inactive state through a non-ATP-competitive mechanism.³⁴ It

has been reported that gatekeeper mutants also can be inhibited by perturbing the flexibility of the P-loop.³⁵ Alternatively, T315I kinase potentially may be inhibited by binding to the autoregulatory allosteric myristate cleft at the N-terminus of ABL1, rendering a similar net effect of freezing the kinase in its inactive state in a selective, non-ATP-competitive manner.³⁶ Such is the mechanism of action of GNF-2, although this compound does not inhibit T315I.³⁶ However, the GNF-2 analog GNF-5 reportedly synergizes in vitro with nilotinib and imatinib to inhibit T315I.³⁷ Both DCC-2036 and GNF-5 represent attractive options that warrant clinical testing in patients with highly resistant *BCR-ABL1* mutations; but, ultimately, these novel agents for the treatment of CML resistant to conventional TKI therapies provide conceptual experimental templates that can be adapted to the development of targeted agents for highly resistant mutants driving the growth of other malignancies. In addition to TKIs, 2 other alternatives to the treatment of patients with CML who carry the T315I mutation are available: First, omacetaxine, a semisynthetic alkaloid with putative activity against CML stem cells,^{38,39} has demonstrated ability to induce cytogenetic and complete cytogenetic response rates of 41% and 18%, respectively, among patients with CML-CP who carry the T315I mutation.⁴⁰ Second, allogeneic stem cell transplantation can induce a complete molecular response, particularly in patients with CML-CP.⁴¹

What Are the Implications of These Advances in CML?

Gatekeeper mutations represent a common mechanism of escape for cancer cells to overcome the selection pressure imposed by effective TKIs. Prime examples of the latter are the PDGFR threonine-to-isoleucine or methionine mutation at codon 315 (PDGFR^{T674I/M}) in hypereosinophilic syndrome,⁴² the epidermal growth factor receptor (EGFR) threonine-to-methionine mutation at codon 790 (EGFR^{T790M}) in nonsmall cell lung cancer,⁴³⁻⁴⁵ and the v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT) threonine-to-isoleucine mutation at codon 670 (KIT^{T670I}) in gastrointestinal stromal tumors.⁴⁶ Experimental modeling of v-raf murine sarcoma viral oncogene homolog B1 (BRAF) kinase gatekeeper mutations suggest that this may also be a mechanism of TKI resistance in cancer cells driven by mutant BRAF kinase.⁴⁷ Similar to CML, gatekeeper mutations in these cancers typically are detected in patients who develop TKI resistance after an initial response to treatment. The development of ponatinib exemplifies how drug design driven by meticulous structural analyses can render compounds that override the hurdles imposed by gatekeeper mutations. An alternative approach is that of using functional pharmacologic screens of large libraries of small molecule inhibitors guided by molecular modeling predictions.⁴⁸ By means of such an approach, a covalent, irreversible, pyrimidine EGFR inhibitor with 30-fold to 100-fold greater potency against EGFR^{T790M} compared with native EGFR recently was identified.⁴⁸ It has been reported that a novel class of ATP-competitive TKIs led by HG-7-85-01 is capable of inhibiting *BCR-ABL1*^{T315I}, KIT^{T670I}, and PDGFR^{T674I/M} gatekeeper mutations because of its ability to accommodate both the native gatekeeper threonine as well as the above-mentioned hydrophobic mutant residues. Certainly, the prospect of a universal gatekeeper mutation inhibitor is attractive: however, is it realistically possible? The surface comparison of distinct tyrosine and serine-threonine kinases has revealed a set of 30 residues with highly conserved spatial positions associated with similar patterns of protein kinase activation.² This high level of conservation across different kinases involved in cancer development may unveil a vulnerability that could be therapeutically exploited. After all, the development of a gatekeeper mutation is a rather predictable (conserved?) process arising in cancer cells driven by oncokineses exposed to high levels of selection pressure by effective TKIs. The process customarily involves the substitution of an isoleucine or a

methionine residue by the native gatekeeper residue. This remarkably narrow repertoire of substitutions at the gatekeeper position has furnished structural biologists and chemists the opportunity to design and synthesize compounds, such as ponatinib or HG-7-85-01, versatile enough to avoid the structural constraints imposed by the gatekeeper mutants.

Lessons Learned and Unsolved Questions

Although nilotinib and dasatinib have proven more efficacious than imatinib as frontline therapy for CML-CP, a subset of patients will continue to fail therapy, which, in many patients, will be associated with the acquisition of *BCR-ABL1* mutations, including T315I. Ponatinib is an oral TKI that reportedly produced high rates of cytogenetic and molecular response with a favorable toxicity profile in a phase 1 study of patients who were resistant to multiple TKIs and/or who carried the T315I mutation. It appears that, almost 1 decade after the initial description of this gatekeeper mutation, an efficacious, safe, oral TKI finally will be clinically available. However, several questions still linger: First, what will be the role of ponatinib? Given its remarkable activity in patients with multi-TKI-resistant CML, it is reasonable to believe that its activity will be even greater as frontline therapy and perhaps superior to nilotinib or dasatinib. Second, will the use of ponatinib in the frontline setting prevent the clinical emergence of *BCR-ABL1* mutants? The *in vitro* characterization of this agent appears to suggest that possibility. However, it is uncertain whether alternative mechanisms of resistance will become more relevant, such as the development of complex compound *BCR-ABL1* mutations or mutations in tumor suppressors (eg, *p53*, *ARF* [an alternate reading frame product of the cyclin-dependent kinase inhibitor 2A locus], and *p16*). In that regard, it must be emphasized that, despite the remarkable preliminary activity demonstrated by ponatinib, a significant proportion of patients with the T315I mutation fail to respond appropriately to ponatinib, probably because of the coexistence of an alternative mechanism of resistance. Third, could ponatinib improve the outcomes of patients with *BCR-ABL1*-positive ALL? This may be possible given its high potency against native *BCR-ABL1* kinase, its mild toxicity profile, and its activity against T315I, a mutation that is particularly prevalent in patients with *BCR-ABL1*-positive ALL who fail TKI therapy. Finally, can ponatinib cure patients with newly diagnosed CML-CP? It is both tantalizing and naïve, in equal measure, to believe so.

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