

Original article

A novel FIP1L1-PDGFR α mutant destabilizing the inactive conformation of the kinase domain in chronic eosinophilic leukemia/hypereosinophilic syndrome

Background: The Fip1-like-1–platelet-derived growth factor receptor α (FIP1L1-PDGFR α) gene fusion is a common cause of chronic eosinophilic leukemia (CEL)/hypereosinophilic syndrome (HES), and patients suffering from this particular subgroup of CEL/HES respond to low-dose imatinib therapy. However, some patients may develop imatinib resistance because of an acquired T674I mutation, which is believed to prevent drug binding through steric hindrance.

Methods: In an imatinib resistant FIP1L1-PDGFR α positive patient, we analyzed the molecular structure of the fusion gene and analyzed the effect of several kinase inhibitors on FIP1L1-PDGFR α -mediated proliferative responses *in vitro*.

Results: Sequencing of the FIP1L1-PDGFR α fusion gene revealed the occurrence of a S601P mutation, which is located within the nucleotide binding loop. In agreement with the clinical observations, imatinib did not inhibit the proliferation of S601P mutant FIP1L1-PDGFR α -transduced Ba/F3 cells. Moreover, sorafenib, which has been described to inhibit T674I mutant FIP1L1-PDGFR α , failed to block S601P mutant FIP1L1-PDGFR α . Structural modeling revealed that the newly identified S601P mutated form of PDGFR α destabilizes the inactive conformation of the kinase domain that is necessary to bind imatinib as well as sorafenib.

Conclusions: We identified a novel mutation in FIP1L1-PDGFR α resulting in both imatinib and sorafenib resistance. The identification of novel drug-resistant FIP1L1-PDGFR α variants may help to develop the next generation of target-directed compounds for CEL/HES and other leukemias.

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Chronic eosinophilic leukemias (CEL) and the myeloproliferative forms of hypereosinophilic syndrome (HES) are rare chronic disorders associated with hypereosinophilia and caused by mutations in hematopoietic precursors (1). One subgroup of these disorders is the result of a fusion between the Fip1-like-1 (FIP1L1) and platelet-derived growth factor receptor α (PDGFR α) genes (2). Many of the FIP1L1-PDGFR α positive CEL/HES patients were previously considered as patients suffering from idiopathic HES. The fact that some idiopathic HES patients responded to the kinase inhibitor imatinib (3, 4), resulted in the identification of the FIP1L1-PDGFR α -positive CEL/HES subgroup (5). The imatinib response rate in these patients is nearly 100% and the drug can induce molecular remission (2, 4).

Five FIP1L1-PDGFR α -positive CEL/HES patients have been described who developed resistance to imatinib. In four patients, the resistance was due to a T674I mutation within the kinase domain of FIP1L1-PDGFR α (2, 6–8). The T674I mutation causes resistance presum-

ably through steric hindrance mechanisms (9). Recently, we described a fifth patient, who presented concurrent S601P and L629P mutations in FIP1L1-PDGFR α (10). Here, we investigated molecular mechanisms in order to understand how the two new mutations are linked to clinical imatinib resistance. The S601P and L629P mutations were individually analyzed by the introduction of mutant FIP1L1-PDGFR α gene fusion products into Ba/F3 cells as well as by structural modeling.

Materials and methods

Immunoblotting

Blood eosinophils were purified as previously described (11, 12). EOL-1 cells (6) were obtained from DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). 10^6 cells/ml were washed with phosphate-buffered saline supplemented with protease inhibitor cocktail (Sigma-Aldrich, Buchs, Switzerland) and lysed with RIPA buffer (50 mM Tris-HCl,

pH 7.4, 150 mM NaCl, 0.25% sodium deoxycholate, 1% Nonidet P-40, 1 mM EGTA supplemented with protease inhibitor cocktail). Gel electrophoresis and immunoblotting were performed as described (12, 13). Anti-PDGFR α and anti-phosphotyrosine (clone 4G10) antibodies were from Upstate (Lake Placid, NY, USA).

Inhibitors

Imatinib, sorafenib, and gefitinib were purchased from ChemieTek (Indianapolis, IN, USA). Cmp-584 is an imatinib analog, which binds with higher affinity to BCR-ABL compared with imatinib, and synthesized as described (14). The inhibitors were reconstituted in dimethyl sulfoxide and further diluted in RPMI 1640.

FIP1L1-PDGFR α mutagenesis and retroviral gene transfer

The complete FIP1L1-PDGFR α fusion gene construct was obtained from Dr Jan Cools (Department of Molecular and Developmental Genetics, VIB, Leuven, Belgium) (2, 15). The FIP1L1-PDGFR α cDNA was used in a retroviral expression construct variant of the vector murine stem-cell virus, which contains an expression cassette for the enhanced green fluorescent protein (EGFP). Mutagenesis of wild-type FIP1L1-PDGFR α was performed using the QuikChange site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands). The FIP1L1-PDGFR α cDNAs with S601P and/or L629P mutations (numbering according to the PDGFR α sequence, accession no. M22734) were generated and verified by sequencing. Ba/F3 cells were infected as previously described (15) and transduced cells were sorted from EGFP-negative cells using FACS Vantage cell sorter (BD Biosciences, Basel, Switzerland). Sorted cells were re-analyzed by flow cytometry (FACS Calibur; BD Biosciences) before proliferation assays were performed.

Proliferation assay

Ba/F3 cells (DSMZ) transduced by FIP1L1-PDGFR α variants were grown in the presence and absence of the indicated concentrations of several different tyrosine kinase inhibitors and cell proliferation was assessed by the incorporation of methyl- ^3H -thymidine (GE Healthcare Europe GmbH, Otelfingen, Switzerland) after 24 h using a liquid scintillation counter (Wallac ADL, Hünenberg, Switzerland) (13). In these experiments, Ba/F3 cells (3×10^5 cells/ml) were grown in RPMI 1640 with 10% heat-inactivated fetal bovine serum (complete culture medium; Life Technologies, Basel, Switzerland) in the absence of interleukin (IL)-3. Experiments were performed in triplicate. Dose-response curves were fitted using the GRAPHPAD PRISM (Prism 5.01) software (GraphPad Software, Inc., La Jolla, CA, USA).

Structural modeling and representation

Two states of the wild-type PDGFR α kinase domain, namely inactive and active, were generated by homology modeling based on the crystal structures of c-kit in its inactive [Protein Databank (PDB) code 1T45] (16) and active state (code 1PKG) (17). The mutated forms of these proteins were also produced and investigated. The three-dimensional structural models were generated by means of MODELLER 9 version 1 (18) applying the fast protocol for modeling building. The subsequent refinement of theoretical models was performed with molecular dynamics simulation (MD). The GROMACS 3.3.1 software package (19) was used to calculate the 5-ns trajectories and to analyze them. Multiple sequence alignments were

calculated with the MALIGN module within BODIL suite of programs (20) and rendered by TEXSHADE (21). Figures in which atomic coordinates are shown were generated with PYMOL (22).

Results and discussion

We recently described a FIP1L1-PDGFR α -positive CEL/HES patient with primary imatinib resistance in association with S601P and L629P mutations (10). As assessed by immunoblotting, isolated blood eosinophils from this patient demonstrated expression of FIP1L1-PDGFR α , which was autophosphorylated, indicating that the kinase was activated (Fig. 1). In purified blood eosinophils from three normal donors, no FIP1L1-PDGFR α was detectable. As a positive control, we used the FIP1L1-PDGFR α -positive cell line EOL-1 in these experiments. The FIP1L1-PDGFR α was detected at 95 kDa.

To investigate the pharmacological consequences of S601P and L629P mutations in FIP1L1-PDGFR α (10), we used the system of mutant FIP1L1-PDGFR α -transduced Ba/F3 cells to study the effect of imatinib and other tyrosine kinase inhibitors on cell growth. We generated Ba/F3 cells expressing wild-type FIP1L1-PDGFR α , S601P mutant FIP1L1-PDGFR α , L629P mutant FIP1L1-PDGFR α , and S601P/L629P double mutant FIP1L1-PDGFR α . After transduction and sorting, all cells were EGFP positive, indicating that they expressed the fusion protein (Fig. 2A).

Retroviral FIP1L1-PDGFR α gene transfer in Ba/F3 cells resulted in IL-3-independent growth as previously reported (2, 15). Proliferation of the genetically modified cells was analyzed in the presence and absence of imatinib and other tyrosine kinase inhibitors. Imatinib showed

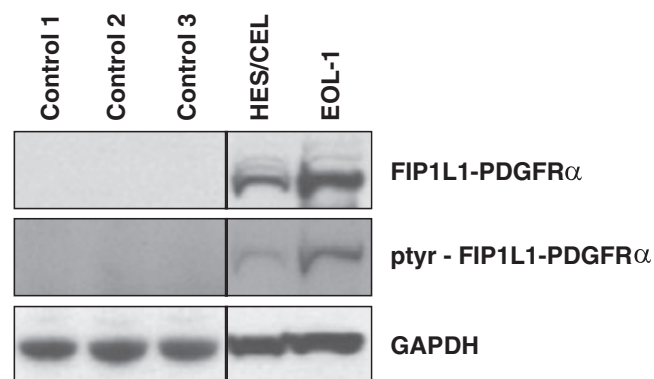


Figure 1. Expression and tyrosine phosphorylation status of FIP1L1-PDGFR α in purified blood eosinophils from a HES/CEL patient and in three control eosinophil populations as assessed by immunoblotting. As a positive control, FIP1L1-PDGFR α -positive EOL-1 cells were used. FIP1L1-PDGFR α was detected at 95 kDa. Filters were also probed with anti-GAPDH antibody to ensure equal loading of the gels.

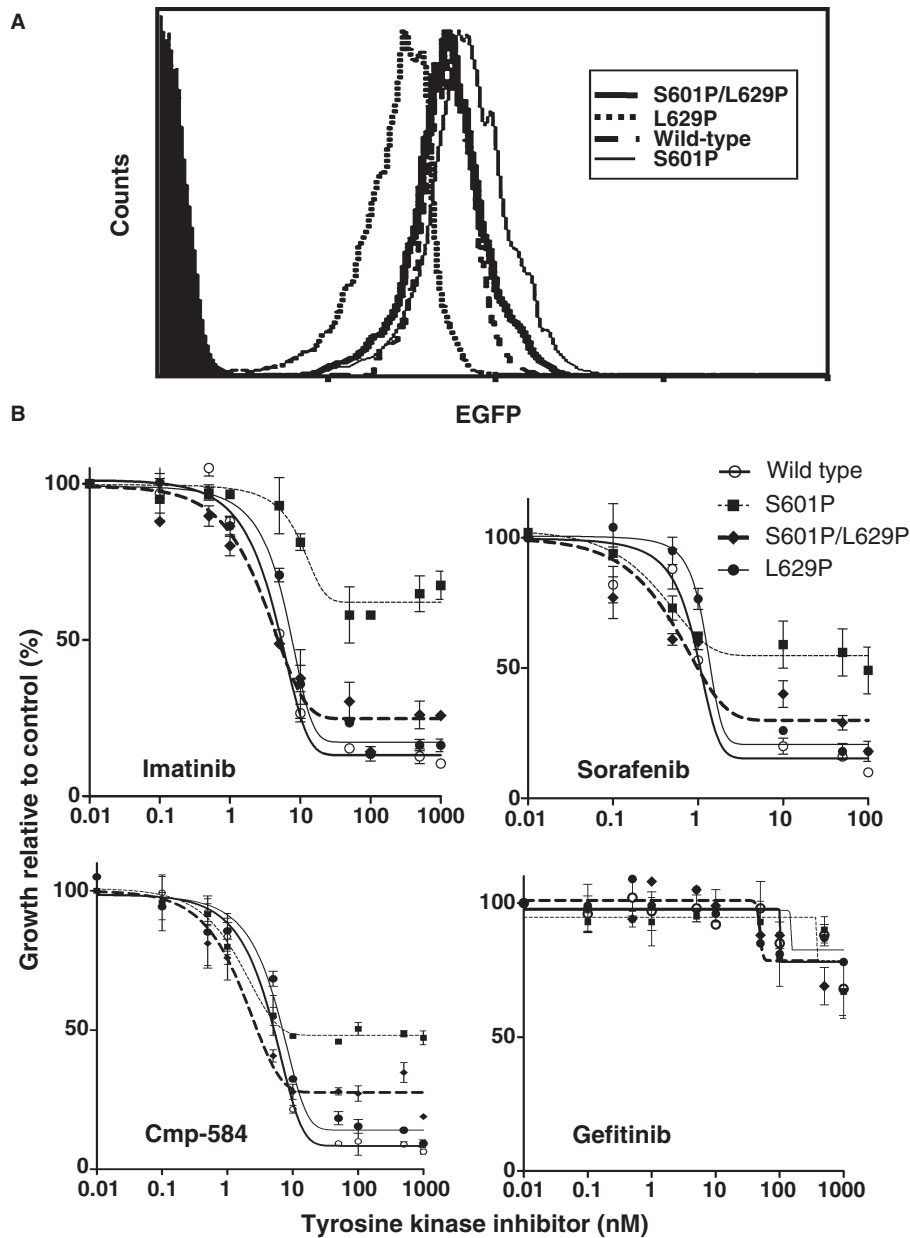


Figure 2. Imatinib, sorafenib, and cmp-584 inhibit wild-type but not S601P mutant FIP1L1-PDGFR α . (A) Ba/F3 cells were transduced with wild-type and the indicated mutants of FIP1L1-PDGFR α . All cells expressed the fusion proteins at high level as assessed by flow cytometry. (B) Concentration-effect curves show efficacy of the indicated tyrosine kinase inhibitors. Mean $\pm$ SD of triplicate measurements are shown.

potent inhibition of wild-type FIP1L1-PDGFR α under these conditions as previously described (2, 15). In contrast, imatinib showed no activity against S601P mutant FIP1L1-PDGFR α until a concentration of 100 nM. L629P mutant FIP1L1-PDGFR α , however, was imatinib sensitive. Interestingly, S601P/L629P double mutant FIP1L1-PDGFR α was partially sensitive to imatinib at 100 nM (Fig. 2B), a finding, which was not clinically relevant (10).

Sorafenib has been shown to inhibit FIP1L1-PDGFR α including its T674I mutated form (15). Therefore,

we screened sorafenib and two other inhibitors whether they might be able to block S601P mutant FIP1L1-PDGFR α . Sorafenib showed activity against wild-type FIP1L1-PDGFR α as expected. However, in contrast to T674I mutant FIP1L1-PDGFR α , which is sorafenib sensitive (15), this drug had no effect on S601P mutant FIP1L1-PDGFR α . We also used cmp-584, which has been described to bind BCR-ABL with higher affinity compared with imatinib (14). Cmp-584, although effectively blocking wild-type FIP1L1-PDGFR α , did not reduce the growth of Ba/F3 cells transduced with S601P

mutant FIP1L1-PDGFR. Therefore, resistance against imatinib cannot be broken with higher drug affinity. Gefitinib, which targets the ATP cleft within the tyrosine kinase epidermal growth factor receptor (23), did not inhibit wild-type or any mutated form of FIP1L1-PDGFR and served as a negative control in these assays (Fig. 2B).

Imatinib and cmp-584 are known to target the inactive conformation of BCR-ABL (14, 24). Sorafenib has been shown to target the inactive conformation of B-raf and the binding is characterized by the absence of a crucial interaction with the gatekeeper amino acid, explaining its activity against T674I (25). As sorafenib blocked T674I but not S601P FIP1L1-PDGFR, we hypothesized that the molecular mechanisms causing imatinib resistance differ between these two mutations. Because the structure of PDGFRA has not been resolved with experimental techniques, we used a theoretical approach based on homology modeling to gain structural insights into the molecular mechanism of imatinib/sorafenib resistance. The members of the PDGFR family, namely PDGFRA, PDGFRB, c-Kit, M-CSF receptor (FMS), and FMS-like tyrosine kinase 3 (Flt-3) share good sequence similarity (51–67%) within the kinase domain (Fig. 3A). Moreover, comparisons between the crystallographic structures of the kinase domains of c-Kit, FMS, and Flt-3, deposited at the PDB, revealed the same folding (16, 17, 26, 27), suggesting comparable plasticity of these proteins. The available structures of c-Kit were taken as representative for the active (PDB code: 1PKG) and inactive states (PDB code: 1T45) of the kinase domains of the whole PDGFR family (16, 17) and used as templates for the homology modeling.

The comparison of the two crystallographic structures of c-Kit (1T45 and 1PKG) as well as the inactive and active conformation models of PDGFRA revealed conformational changes while undergoing enzyme activation (Fig. 3B). The juxtamembrane domain is displaced by the movement of the activation loop (A loop) leaving the binding site free to bind ATP and the protein substrate. The main kinase domain movements involve the A loop, including the catalytically essential DFG motif, and the N-terminal lobe, especially helix C. As the imatinib, cmp-584, and sorafenib binding site is located at the interface between the two lobes of the kinase domain (16), both S601P and L629P mutations are not involved in direct interaction with the ligand.

The mutation S601P in PDGFRA (labeled P1) occurs in the loop between the first two strands (Fig. 3). This element is either called the 'nucleotide-binding loop' for its function, as part of the ATP-binding site, or the 'Gly-rich loop' for the three glycine residues highly conserved in the protein tyrosine kinase family. The glycines donate high plasticity at this part of the protein necessary to adapt to the ATP molecule. Furthermore, the Gly-rich loop forms the roof of the binding site of the pyridinyl-

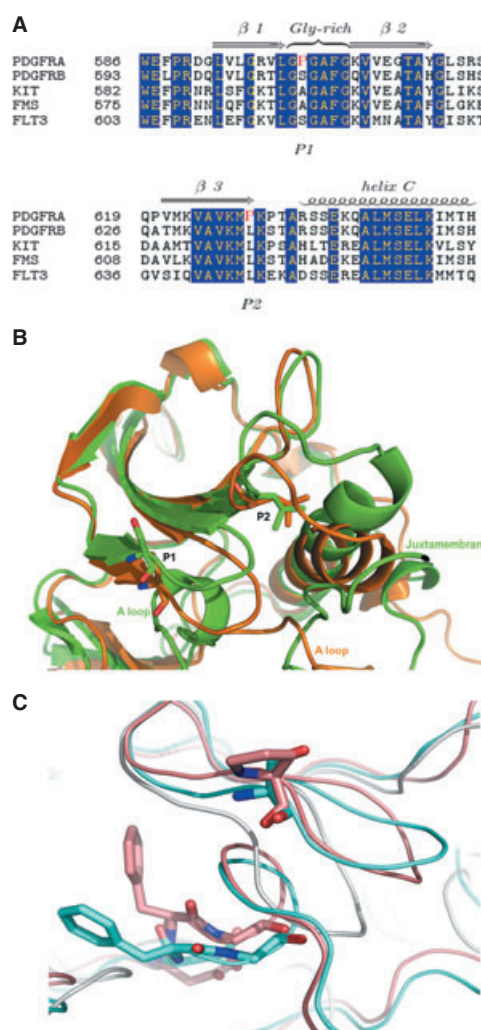


Figure 3. Structural rearrangements in wild-type and mutant PDGFRA models. (A) Multiple sequence alignment of the N-terminal lobe until the end of helix C of mutated PDGFRA with PDGFRB, c-Kit, FMS, and Flt3. The mutations resulted in changes of the amino acid sequence within the PDGFRA kinase domain (marked in red and labeled with P1 and P2, respectively): S601P and L629P. The conserved residues are shadowed in blue and some of the secondary structures are labeled. Alignment was rendered with Texshade. (B) Conformational changes of the N-terminal lobe upon activation: Superposition of the active (orange) and inactive (green) states of the PDGFRA kinase domain that is comparable with the superposition of the c-Kit in the active (1PKG) and inactive (1T45) conformations used as templates. Positions of the two mutations on the PDGFRA kinase domain are labeled P1 and P2. (C) 4–5 ns MD averaged structure of S601P mutant and wild-type PDGFRA. The backbone of PDGFRA models is represented as cartoons and color coded for the mutated (P601, cyan) and the wild-type (S601, green) inactive forms. The active conformation of the wild-type and S601P mutant PDGFRA has not been displayed because they are equivalent. The residues at P1 position and of the DFG motif (Asp836, Phe837, Gly838) are represented as sticks. Atoms are color coded for oxygen (red), nitrogen (blue), and carbon (cyan for the mutant and green for the wild-type).

pyrimidino moiety of imatinib and cmp-584, as well as of the substituted pyridinyl moiety of sorafenib.

The analysis of the different structures reveals that in the inactive state the Gly-rich loop packs hydrophobically right above the N-terminus of the A loop (Fig. 3B). The analysis of the ϕ angle of the amino acids at position 601 in different conformations suggests that the mutation to proline at this position would bend the backbone of the protein, because proline has more restricted values of ϕ angle compared to all other residues. This, most likely, causes the disruption of the interaction between the Gly-rich loop and the A loop disfavoring the inactive state.

A confirmation of this hypothesis was obtained by the structural refinement of the wild-type and mutated models of the PDGFR α catalytic domain in the inactive state performed using MD simulation. The atomic movements of the protein models were traced by means of root mean square deviation (RMSD) analysis of the obtained MD trajectories. We found that helix C is the structural element with the most structural deviations. A comparison between the different mutants and wild-type PDGFR α shows that the S601P mutant has the highest RMSD values for helix C (averaged $\text{RMSD}_{2\text{ns}-5\text{ns}} = 1.7 \text{ \AA} \pm 0.2$), followed by the S601P/L629P double mutant (averaged $\text{RMSD}_{2\text{ns}-5\text{ns}} = 1.4 \text{ \AA} \pm 0.3$), and then by both L629P mutant (averaged $\text{RMSD}_{2\text{ns}-5\text{ns}} = 1 \text{ \AA} \pm 0.3$) and wild-type protein (averaged $\text{RMSD}_{2\text{ns}-5\text{ns}} = 1 \text{ \AA} \pm 0.2$) (data not shown).

The helix C movement influenced directly the whole N-terminal lobe conformation and is essential for tyrosine kinase activation (Fig. 3B). Therefore, the results of the RMSD analysis suggest that the S601P mutation has the strongest effect on destabilizing the inactive conformation, while L629P behaves like wild-type PDGFR α . This correlates with the drug sensitivity experimental data showing imatinib resistance in case of S601P and sensitivity in case of L629P. Interestingly, the double mutant situates in between the single mutants regarding both helix C movement and drug sensitivity.

The comparison between the average structure over the last nanosecond of MD simulation of wild-type and S601P mutant (Fig. 3C) shows that helix C of the mutant has a more pronounced downward movement in the direction of the catalytic loop. This conformational change is the same as seen in association with kinase activation (Fig. 3B). On the other hand, the structural

variation of the A loop associated with activation was not seen in our simulations. This is due to the longer timescale needed for the event to occur compared with the simulation time. However, similarly to the helix C, the conformational change of the DFG motif toward the active form seems to be facilitated by the S601P mutation. In agreement with the results of the RMSD analysis, the structural comparison of the average structures of the other mutants shows that L629P behaves like wild-type, while S601P/L629P experiences a less pronounced movement compared with S601P (data not shown). Taken together, our structural modeling studies suggest that the S601P mutation leads to destabilization of the inactive conformation inducing a dominance of the active form of PDGFR α kinase domain that is unable to bind imatinib (16).

The fact that most FIP1L1-PDGFR α positive CEL/HES patients respond to imatinib demonstrates that PDGFR α is not destabilized by the fusion to FIP1L1 and is kinetically accessible to the drug. In contrast to the T674I mutation in FIP1L1-PDGFR α that occurs at the same position as the T315I mutation in BCR-ABL and sterically prevents imatinib binding by introducing a large isoleucine side chain into the gate-keeper position that abolishes an essential interaction with the inhibitor, the S601P mutation in FIP1L1-PDGFR α confers drug resistance through an allosteric mechanism. Similar imatinib drug resistant variants that shift the equilibrium toward the active kinase formation have been described in BCR-ABL (28, 29). In light of the data reported here, an appealing strategy for suppressing resistance is to treat imatinib-resistant CEL/HES patients with a kinase inhibitor with less stringent conformational requirements for binding to PDGFR α than the inhibitors analyzed in this study (24, 30). The identification of imatinib-resistant FIP1L1-PDGFR α fusion proteins may serve to inform development of next generation target-directed compounds.

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