Crystal Structure of the T315I Abl Mutant in Complex with the Aurora Kinases Inhibitor PHA-739358

Michele Modugno, Elena Casale, Chiara Soncini, Pamela Rosettani, Riccardo Colombo, Rosita Lupi, Luisa Rusconi, Daniele Fancelli, Patrizia Carpinelli, Alexander D. Cameron, Antonella Isacchi, and Jürgen Moll

Nerviano Medical Sciences S.r.l.-Oncology, Milan, Italy

Abstract

Mutations in the kinase domain of Bcr-Abl are the most common cause of resistance to therapy with imatinib in patients with chronic myelogenous leukemia (CML). Second-generation Bcr-Abl inhibitors are able to overcome most imatinib-resistant mutants, with the exception of the frequent T315I substitution, which is emerging as a major cause of resistance to these drugs in CML patients. Structural studies could be used to support the drug design process for the development of inhibitors able to target the T315I substitution, but until now no crystal structure of the T315I Abl mutant has been solved. We show here the first crystal structure of the kinase domain of Abl T315I in complex with PHA-739358, an Aurora kinase inhibitor currently in clinical development for solid and hematologic malignancies. This compound inhibits in vitro the kinase activity of wild-type Abl and of several mutants, including T315I. The cocrystal structure of T315I Abl kinase domain provides the structural basis for this activity: the inhibitor associates with an active conformation of the kinase domain in the ATP-binding pocket and lacks the steric hindrance imposed by the substitution of threonine by isoleucine. [Cancer Res 2007;67(17):7987–90]

Introduction

Imatinib, an ATP-competitive inhibitor that targets the tyrosine kinase activity of Bcr-Abl, is the first-line treatment for patients with chronic myelogenous leukemia (CML; ref. 1). Newly diagnosed patients in chronic phase treated with imatinib generally achieve durable responses, whereas patients in advanced phases of CML frequently develop resistance to imatinib therapy, which is often due to the emergence of mutant forms of Bcr-Abl bearing point mutations in the kinase domain (1). Crystallographic studies provided a rationale for the mechanisms by which mutant forms of Bcr-Abl were resistant to therapy. Imatinib binds and stabilizes an inactive conformation of the Abl kinase, in which the inhibitor interacts with the aromatic side chain of the conserved Phe of the Asp-Phe-Gly (DFG) motif, displacing the activation loop from its normal position and forcing it to adopt a substrate-like conformation, termed the "DFG-out" conformation (2). Mutations in the kinase domain of Abl that cause imatinib resistance either directly interfere with the inhibitor interaction or prevent the adoption of this inactive conformation required for imatinib binding.

Second-generation ATP-competitive Bcr-Abl inhibitors that are capable of targeting the majority of imatinib-resistant mutants are currently in clinical development, but none is able to target the T315I mutant (3–5). The T315I substitution is one of the most common mutations found in imatinib-resistant patients and the clinical importance of this mutation is expected to grow because it is predicted to represent the major mechanism of resistance to second-generation inhibitors, such as dasatinib and nilotinib (6–8). Therefore, inhibitors that target the T315I mutation are needed to address this emerging unmet medical need in CML. Two ATP-competitive inhibitors that were designed to target the Aurora kinases and are in clinical development, MK-0457 (VX-680) and PHA-739358, have been reported to be active on T315I Abl mutant (9). Indeed, MK-0457 is active ex vivo against cells from patients bearing the Abl T315I mutation (10) and clinical activity on patients with T315I mutated Abl has been reported (11). A crystal structure of this inhibitor with another mutant of Abl (H396P), but not with T315I, has been solved (10).

In this report, we describe the first crystal structure of the catalytic domain of the Abl T315I mutant in complex with PHA-739358. Our results provide a possible structural explanation for the activity of PHA-739358 on the T315I mutation.

Materials and Methods

Protein expression, purification, and characterization. The kinase domain of human c-Abl (residues 229–512, Abl-1a isoform) was expressed in High5 insect cells using a baculovirus expression vector based on pVL1393 (Invitrogen) to express an NH2-terminally HisGST-tagged protein. The imatinib-resistant mutants were generated by site-directed mutagenesis using the QuikChange Mutagenesis kit (Stratagene).

For protein purification, cells were lysed by sonication in lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.02% CHAPS, 20 mmol/L DTT, 10% glycerol, protease inhibitors) and cleared lysates were loaded on a glutathione-Sepharose 4B (Amersham Biosciences) column. For preparation of dephosphorylated protein, resin-bound protein was incubated for 2 h at 30°C with YopH phosphatase (Sigma-Aldrich). Recombinant proteins were cleaved with PreScission protease (Amersham Biosciences) and eluted.

Molecular weight and phosphorylation levels of all proteins were analyzed by liquid chromatography/electrospray ionization-mass spectrometry. Nondephosphorylated wild-type (WT) and mutant Abl kinase domain proteins yielded a distribution of forms with different phosphorylation levels, ranging from a prevalent 1P form to more highly phosphorylated forms. Phosphorylation of Tyr932 in the activation loop was confirmed by immunoblotting using anti-phospho-Abl-Tyr932 (Cell Signaling). YopH-treated WT kinase domain was entirely unphosphorylated.

Crystallography. The complex of Abl T315I with PHA-739358 was prepared essentially as described (2). The protein was crystallized using the hanging drop technique. Crystals were grown at 4°C mixing equal volumes of the protein
solution with the well solution consisting of 20% polyethylene glycol 4000, 1 mol/L HEPES (pH 7.0), and 0.1 mol/L MgCl₂. 

Data were collected at European Synchrotron Radiation Facility beamline ID14-EH1 and processed using the HKL package (12). The structure was solved by molecular replacement with AmoRe (13) using the structure of Abl in complex with PD173955 (PDB ID 1M52) as the search model. Model building of the protein and inhibitor was carried out with O (14). Initial refinement was done with CNX (15), whereas the final round of refinement was done with Refmac (13). A summary of the diffraction data and the refinement statistics is shown in Supplementary Table S1. Protein coordinates have been deposited in the protein databank with code 2v7a.

**Abl kinase assay.** Reaction mixtures (total volume, 20 µL) containing 15 nmol/L of enzyme, 10 µmol/L myelin basic protein as substrate (Sigma-Aldrich), 10 mmol/L HEPES (pH 7.5), 5 mmol/L MgCl₂, 1 mmol/L MnCl₂, 1 mmol/L DTT, 15 mmol/L Na₃VO₄, 4% DMSO, 3 µmol/L ATP, 0.1 µCi [γ⁻³²P]ATP, and the indicated amount of inhibitors were incubated for 30 min at 30°C. Reactions were terminated by addition of gel loading buffer and loaded onto a 10% SDS-polyacrylamide gel. Radioactive signals on resolved, dried gels were revealed using a Molecular Dynamics PhosphorImager.

**ATP site-dependent displacement assay.** Serial dilutions of test compounds were prepared in assay buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L MgCl₂, 1 mmol/L MnCl₂, 1 mmol/L DTT, 3 µmol/L Na₃VO₄]. The enzymes were present at a final concentration of 10 nmol/L. The probe was used at concentrations equal to 10-fold the K₀ for each of the protein tested (2.3, 1.5, 0.2, and 0.8 µmol/L, respectively, for Abl WT 0P, WT phosphorylated, E255V, T315I, and M351T). The mixture of enzyme and probe was added to the already diluted compounds to have a final reaction mixture of 70 µL directly in a black 384-well plate (Corning). Plates were read on a fusion α-FP HT (Packard) with excitation 425/20 nm and emission 525/20 nm with fluorescence intensity. Data analysis was done using DynaFit.

**Results and Discussion**

**In vitro inhibition of WT and imatinib-resistant Abl mutants by PHA-739358.** PHA-739358 has been identified and was developed as an ATP-competitive inhibitor of the Aurora kinases (16). The inhibitor shows high antitumor activity in vivo (16), with favorable chemico-physical and pharmacokinetic properties, and is currently being evaluated in clinical trials in solid and hematologic malignancies, including CML. PHA-739358 showed a significant cross-reactivity with Abl in a biochemical assay, with an IC₅₀ of 25 nmol/L (16). We tested whether PHA-739358 was capable of inhibiting the tyrosine kinase activity of the imatinib-resistant Abl mutants in a biochemical kinase assay using WT Abl kinase domain in parallel with three of the most frequent imatinib-resistant mutants: T315I, E255V, and M351T (1). As shown in Fig. 1, as expected, imatinib was active only on WT Abl, being more active on the dephosphorylated protein (2). The E255V and T315I mutants were insensitive, whereas the M351T mutant showed inhibition only at high imatinib concentration, in agreement with previous data reporting that this mutant is not completely insensitive to imatinib (17). On the contrary, PHA-739358 efficiently inhibits substrate phosphorylation by WT and mutant proteins, being even more active against the T315I mutant (Fig. 1).

To confirm and quantify the *in vitro* kinase assay data, an ATP site-dependent displacement assay was set up for the quantitative affinity evaluation for recombinant WT and Abl kinase domain mutants. The assay is based on the significant increase in fluorescence intensity due to indolinone binding to the Abl kinase domains. Because indolinones show extremely low levels of fluorescence per se, the change in intensity provides a direct readout of the extent of probe binding to Abl kinase domains. The probe used is capable of binding all forms of the Abl kinase domain with high affinities (0.22, 0.33, 0.15, 0.02, and 0.08 µmol/L, respectively, for Abl WT 0P, WT phosphorylated, E255V, T315I, and M351T) with a 1:1 stoichiometry. The Z′ evaluated at 10 nmol/L proteins and saturating probe concentrations were above 0.8 (data not shown). ATP-Y§S competes with the probe for binding to Abl kinase domains, resulting in a decrease in fluorescence intensity in comparison with the probe alone. The full displacement by ATP-Y§S indicates that the indolinone binds in the ATP pocket. In agreement, binding constants (Kᵦ) were determined by fitting with a pure competitive mechanism.

Inhibitor affinities for different Abl forms measured using this assay are shown in Table 1. Imatinib showed a higher affinity for the dephosphorylated compared with the phosphorylated form of Abl (0.021 and 0.23 µmol/L, respectively), whereas no displacement was observed on the T315I mutant, in agreement with published data (18). Lower affinity was observed for the E255V and M351T mutants. On the contrary, MK-0457, an Aurora inhibitor that exhibits cross-reactivity with Abl (10), binds all forms of Abl with a similar potency. Similarly, PHA-739358 binds all of the Abl forms, although with a higher affinity than MK-0457. Furthermore, PHA-739358 shows a higher affinity for the T315I mutant (0.005 µmol/L) in agreement with the kinase activity assay described above.

**Table 1. Inhibitor affinities for Abl forms**

<table>
<thead>
<tr>
<th></th>
<th>WT 0P</th>
<th>WT E255V</th>
<th>T315I</th>
<th>M351T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imatinib</td>
<td>0.021</td>
<td>0.230</td>
<td>0.610</td>
<td>0.0% displacement</td>
</tr>
<tr>
<td>PHA-739358</td>
<td>0.014</td>
<td>0.021</td>
<td>0.014</td>
<td>0.005</td>
</tr>
<tr>
<td>MK-0457</td>
<td>0.067</td>
<td>0.083</td>
<td>0.205</td>
<td>0.085</td>
</tr>
</tbody>
</table>

**Figure 1.** Comparison of inhibitory activity of PHA-739358 versus imatinib. Imatinib and PHA-739358 activity were tested in parallel on recombinant WT Abl kinase domain and on a panel of imatinib-resistant mutants representative of the most frequent mutations observed in imatinib-resistant patients.
Crystal structure of T315I Abl complexed with PHA-739358.

To understand the structural basis of the capability of PHA-739358 to bind and inhibit the T315I mutant, we determined the crystal structure of the inhibitor-protein complex (Fig. 2A). The protein is in the typical conformation of active kinases, with the activation loop in the extended DFG "in" conformation. Indeed, Asp381 points into the active site and interacts with Mg2+ ion that occupies a position similar to the one usually seen in the structures of kinases in complex with ATP. The glycine loop adopts an extended conformation, in contrast to the other publicly available Abl structures where the loop is more distorted, which could be due to the specific binding mode of our inhibitor. The purified T315I Abl kinase domain used for crystallization experiments is predominantly phosphorylated on the activation loop at Tyr393, whereas Tyr253, Tyr257, and Tyr264 are phosphorylated at lower levels. However, in the crystal structure, only phosphorylation in position Tyr393 can clearly be seen. The phosphoryl moiety of Tyr393 is situated in a basic pocket formed by the side chains of Arg256, Arg257, His396, and Lys285 and forms a salt bridge with Arg386. These interactions probably stabilize the active conformation of the activation loop, which is, however, very similar to the structures reported for dasatinib in complex with the WT Abl kinase domain (19) and of MK-0457 in complex with the Abl mutant H396P (10).

The inhibitor and the mutated gatekeeper residue Ile315 are well defined in the electron density maps. The mutation of the threonine to the more bulky isoleucine does not seem to cause any widespread conformational changes but creates a steric hindrance that would interfere with the binding of inhibitors, such as imatinib, nilotinib, and dasatinib, which make use of the hydrophobic pocket. The binding mode of PHA-739358 is very similar to that reported for the complex of the same compound with Aurora A (Fig. 2B and D; ref. 16), although the conformation of the proteins around the ATP-binding site shows some differences because in the Aurora A structure the DFG motif is more similar to the "out" conformation. However, all of the essential contacts between PHA-739358 and Abl T315I involve highly conserved elements. The molecule makes three hydrogen bonds with the protein backbone of the hinge region: the two nitrogen atoms of the pyrrolopyrazole core interact with the carbonyl oxygen of Glu316 and with the amide nitrogen of Met286, whereas the nitrogen of the amide group hydrogen bonds to the carbonyl oxygen of Met318. In addition, the side chain nitrogen of the conserved Lys271 is within hydrogen bonding distance of the oxygen of the carbonyl group and the oxygen of the methoxy group. As in the Aurora structure, the benzyl group packs against Leu263 (Leu210 in Aurora), whereas the N-methyl-piperazine points toward the solvent accessible area of the kinase pocket. The gatekeeper residue in the Aurora kinases is Leu210, a large and hydrophobic residue very similar to isoleucine, and we have observed that PHA-739358 binds in the ATP-binding pocket of Aurora A without any steric hindrance with the gatekeeper residue. Indeed, the cocrystal structure reported here reveals that the compound is bound to the Abl T315I kinase domain in a mode that accommodates the substitution of isoleucine and we have observed that PHA-739358 binds in the ATP-binding pocket of Aurora A without any steric hindrance with the gatekeeper residue. Indeed, the cocrystal structure reported here reveals that the compound is bound to the Abl T315I kinase domain in a mode that accommodates the substitution of isoleucine and we have observed that PHA-739358 binds in the ATP-binding pocket of Aurora A without any steri...
the side chain oxygen of threonine is lost (Fig. 3A and B). On the contrary, both PHA-739358 and MK-0457 bind in such a way to avoid the gatekeeper residue (Fig. 3C) and this provides an explanation for the ability of both compounds to accommodate the isoleucine substitution. Furthermore, the pyrrolopyrazole scaffold of PHA-739358 is situated within van der Waals distance of the side chain of Ile315 mimicking the interaction between the inhibitor and Leu310 in Aurora A. It is possible that this favorable hydrophobic packing interaction may explain why PHA-739358 is more active against the mutant than the WT protein. Our results suggest that PHA-739358 could represent a valuable novel agent to target the T315I Bcr-Abl mutation, and preclinical and clinical data are in progress to support this concept.

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**References**


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