Prediction of Resistance to Small Molecule FLT3 Inhibitors: Implications for Molecularly Targeted Therapy of Acute Leukemia

Jan Cools,1,4 Nicole Mentens,4 Pascal Furet,5 Doriano Fabbro,5 Jennifer J. Clark,1,2,3 James D. Griffin,2 Peter Marynen,4 and D. Gary Gilliland1,2,3

1Division of Hematology, Department of Medicine, Brigham and Women’s Hospital; 2Dana-Farber Cancer Institute; and 3Howard Hughes Medical Institute, Harvard Medical School, Boston, Massachusetts; 4Department of Human Genetics, Flanders Interuniversity Institute for Biotechnology, University of Leuven, Leuven, Belgium; 5Novartis Pharma, Basel, Switzerland.

Abstract

Mutations in the receptor tyrosine kinase FLT3 occur frequently in patients with acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL). Small molecules that selectively inhibit FLT3 kinase activity induce apoptosis in blasts from AML patients with FLT3 mutations and prolong survival in animal models of FLT3-induced myeloproliferative disease. A spectrum of structurally different small molecules with activity against FLT3 have been described, and their efficacy for treatment of AML and ALL is now being investigated in clinical trials.

Here, we describe the results of an in vitro screen designed to identify mutations in the ATP-binding pocket of FLT3 that confer resistance to tyrosine kinase inhibitors. Mutations at four different positions (Ala-627, Asn-676, Phe-691, and Gly-697) were identified that confer varying degrees of resistance to PKC412, SU5614, or K-252a. FLT3 proteins mutated at Ala-627, Asn-676, or Phe-691 remained sensitive to higher concentrations of the inhibitors, but the G697R mutation conferred high-level resistance to each of these inhibitors as well as to six additional experimental inhibitors. These data provide insights into potential mechanisms of acquired resistance of FLT3 to small molecule inhibitors and indicate that the G697R mutation may be a clinically problematic resistance mutation that warrants proactive screening for additional inhibitors.

Introduction

The receptor tyrosine kinase FLT3 is mutated in ~30% of acute myeloid leukemia (AML) and in a subset of acute lymphoblastic leukemia (ALL; refs. 1 and 2). Activating mutations in FLT3 are most frequently internal tandem duplications within the juxtamembrane region and less frequently point mutations, insertions, or deletions in the kinase domain. These mutant forms of FLT3 are constitutively activated tyrosine kinases that transform hematopoietic cells through activation of several intracellular signaling pathways. Mutant FLT3 cooperates with oncogenic transcription factors to induce acute leukemia (reviewed in refs. 1 and 2). Inhibition of FLT3 kinase activity using small-molecule inhibitors induces apoptosis in cell lines with FLT3-activating mutations and prolongs survival of mice expressing mutant FLT3 in their bone marrow cells (3–6). Phase I/II clinical trials with FLT3 inhibitors (PKC412, MLN518, SU11248, and CEP-701) are ongoing (2), with some promising initial results (7–9).

Together, these data support an important role for FLT3 inhibitors as molecularly targeted therapy for acute leukemias harboring FLT3 mutations. However, despite potent inhibition of protein tyrosine kinases with small molecule inhibitors, the development of resistance due to acquired point mutations in the target kinase is now emerging as a new problem for the treatment of leukemia patients, especially those with acute leukemia (10, 11). One solution for this problem would be to combine small molecule inhibitors with a different chemical structure but target the same kinase. Inhibition of FLT3 provides a unique model to test this strategy, because there are currently four structurally different inhibitors in clinical trials. We report here the development of an in vitro screening system to identify point mutations within the ATP-binding region of FLT3 that confer resistance to PKC412. We also explored cross-resistance with other inhibitors as a way to predict which combinations of inhibitors, if any, would be successful in preventing the development of resistance.

Materials and Methods

Vector and Library Construction. The open reading frame of FLT3 containing an internal tandem duplication (W51 mutation; ref. 4) was subcloned into the blunted EcoRI site of the retroviral MSCV-neo vector (BD Clontech, Palo Alto, CA). This vector was further modified by mutating the ‘ACTAGG’ sequence to ‘CCTAGG’, generating a unique AvrII site. These alterations did not change the amino acid sequence. The 420-bp region containing the ATP-binding pocket of the kinase domain of FLT3 was amplified by PCR, using the primers 5'-gaaatttagagtttgggaagg and 5'-catatattaactcatc-ACAGT (in the presence of 50 μmol/L MnCl2, which is known to reduce the fidelity of Taq (12). Under these conditions, 80% of the PCR products contained mutations, with 50% of the products containing a 1-bp change. The PCR products were cut with AvrII and EcoRI and ligated into the modified MSCV-FLT3-ITD vector. The library contained approximately 28,000 independent clones.

Viral Production and Cell Growth. Production of retroviral vectors and transduction of Ba/F3 cells was described previously (4). Ba/F3 cells expressing the different FLT3 constructs became interleukin-3 (IL-3)-independent and were grown in RPMI without IL-3. For dose-response curves, Ba/F3 cells were grown in 24-well plates for 24 hours in the presence of different concentrations of inhibitor. Viable cell numbers were determined using the AqueousOne Solution (Promega, Madison, WI).

Inhibitors. The kinase inhibitors SU5614 (6), K-252a (similar to CEP-701; ref.3),(5-hydroxy-1H-2-indolyl)(1H-2-indolyl) methanone (D-64406), 5-butanoyl-1H-2-(2-thienyl)-imidazolo[5,4-g] quinoxaline (AGL2043), 3-(3-thienyl)-6-(4-methoxyphenyl) pyrazole [1,5a]-pyrimidine (TMPPP), and 1-phenyl-3-H-oxa-2,3-diaza-cyclopenta[aj]inden (GTP-14564) were purchased from Calbiochem (Merck, Nottingham, United Kingdom). PKC412 (5) was provided by Novartis. All inhibitors were stored as 10 mM/L stock solutions in dimethyl sulfoxide at ~20°C.

Deoxyribonucleic Acid Isolation and Polymerase Chain Reaction. Genomic DNA was isolated from cell lines using the QIAnaamp DNA mini kit (Qiagen, Germantown, MD). The mutated region was amplified from genomic DNA with the primers 5'-gaaatttagagtttgggaagg and 5'-catatattaactcatccAgCT (in the presence of 50 μmol/L MnCl2, which is known to reduce the fidelity of Taq (12). Under these conditions, 80% of the PCR products contained mutations, with 50% of the products containing a 1-bp change. The PCR products were cut with AvrII and EcoRI and ligated into the modified MSCV-FLT3-ITD vector. The library contained approximately 28,000 independent clones.

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Requests for reprints: Jan Cools, Department of Human Genetics—VIB4, Campus Gasthuisberg O&N box 602, Herestraat 49, B-3000 Leuven, Belgium. Phone: 32-16-345948; Fax: 32-16-347166; E-mail: jan.cools@med.kuleuven.ac.be.

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Results

Identification of Mutations Conferring Resistance to PKC412. Constitutively activated FLT3 kinase transforms the Ba/F3 cell line to IL-3-independent growth. Treatment of the transformed cells with FLT3 inhibitors results in dephosphorylation of mutated FLT3 and induces apoptosis (3-6). The Ba/F3 cell line is thus an ideal system for the identification and study of mutations that confer resistance to small molecule FLT3 inhibitors.

We randomly mutated the ATP-binding region of a construct containing the FLT3 open reading frame with an internal tandem duplication (FLT3-ITD). Point mutations were introduced by PCR amplification in the presence of MnCl₂, and the mutated region was subcloned in the FLT3-ITD cDNA (Fig. 1). A library was generated containing ~28,000 clones (~22,400 mutants). Ba/F3 cells were transduced with this library, selected for IL-3-independent growth, and then selected for growth in the presence of PKC412 (150 nmol/L). After selection, DNA was extracted from surviving cells, amplified by PCR, and cloned. Sequence analysis of cloned PCR products revealed the presence of a limited number of point mutations. Each of the mutations that was identified was regenerated using site-directed mutagenesis and tested again in Ba/F3 cells for resistance to PKC412. Only mutants that increased the 50% inhibitory concentration (IC₅₀) for PKC412 at least 2-fold were selected for additional study. Seven mutations were retained, located at four different positions (Fig. 1).

The mutated residues were also positioned on a model of the FLT3 kinase domain in complex with PKC412 (Fig. 2). The mutated residues were also positioned on a model of the FLT3 kinase domain in complex with PKC412.
other small molecule inhibitors (GTP-14564, AGL2043, D-64406, D-65476, TMPPP, and DQPPC).

All of these inhibitors potently inhibited the growth of Ba/F3 cells expressing FLT3-ITD, and growth inhibition correlated with inhibition of FLT3 phosphorylation (Fig. 4; data not shown). In contrast, none of these inhibitors potently inhibited the growth of Ba/F3 cells expressing the G697R mutant. DQPPC was toxic to Ba/F3 cells greater than 1 μmol/L, but even at that concentration, it had no effect on tyrosine kinase activity of the G697R resistance mutant as assessed by Western blotting. Although a 50% growth inhibition was observed for the related inhibitors D-64406 and D-65476 at 10 μmol/L (Fig. 4B), these inhibitors also had no effect on the tyrosine phosphorylation of the G697R mutant, even at higher concentrations up to 50 μmol/L. (Fig. 4A; data not shown). The G697R mutation thus conferred a high-level resistance to all inhibitors tested.

**Discussion**

Inhibition of tyrosine kinases by selective small molecule inhibitors is emerging as a new strategy for treatment of hematologic malignancies and solid tumors, including leukemias, gastrointestinal stromal cell tumors, and non-small cell lung cancer (11, 18–20). Several types of cancer have been successfully treated with imatinib, but mutation of the target kinases has been observed as one mechanism for the development of resistance (10, 11, 21). Studies with imatinib in more advanced disease (chronic myelogenous leukemia in blast crisis) have demonstrated the clinical significance of this problem and indicate that the majority of cases of clinical resistance can be ascribed to acquired or preexisting mutations in BCR-ABL (10). Most of these mutations can be inhibited by other ABL inhibitors such as BMS-354825 and AP23464, but a few of the BCR-ABL mutations, including the T315I mutation, make the ABL kinase highly resistant to imatinib, BMS-354825, and AP23464 (16, 17, 21).

Work on the use of BMS-354825 and AP23464 for the inhibition of imatinib-resistant BCR-ABL mutants and on PKC412 for the treatment of an imatinib-resistant FIP1L1-PDGFRα mutant suggests that the use of combinations of kinase inhibitors could be a way to prevent or treat resistant disease (15–17). Targeted therapy for FLT3 mutation-positive AML is currently being investigated in clinical trials, with the unique situation that not one, but four structurally different small molecules are tested for the inhibition of FLT3. Here, we describe the first insights into resistance mutations in FLT3 and especially into the question of whether combinations of different inhibitors could be used to prevent the development of resistance.

We have identified and characterized several mutations in FLT3 that confer resistance to PKC412, SU5614, and K-252a (similar to CEP-701). Although it is difficult to predict clinical resistance based on in vitro studies, our results indicate that mutations at three of the four identified positions remain sensitive to the inhibitors tested in this study, and it remains to be determined whether any of these three mutations could result in clinical resistance in AML patients treated with these inhibitors. The G697R mutation, however, was identified as a mutation conferring high-level resistance to all tested inhibitors.

These results are in agreement with data from a model of the FLT3 kinase domain in complex with PKC412. This model confirms that Gly-697 is in contact with a phenyl ring of PKC412 and predicts that mutation of Gly-697 to a larger amino acid will create a steric clash with the inhibitor, weakening its binding affinity. Phe-691 is in contact with another phenyl ring of PKC412, and its mutation to a smaller residue would remove favorable hydrophobic contacts. Mod-
eling suggests that leucine makes more contacts with PKC412 than isoleucine, which may explain why F691L confers a lower level of resistance to PKC412 than F691I. The two other mutated positions are not in direct contact with PKC412. However, modeling suggests that mutation of Ala-627 to a larger residue would destabilize the conformation of the P-loop (residues of this loop make contact with PKC412) and that mutation of Asn-676 may destabilize the conformation of the hinge segment, which makes H-bonds with the lactam ring of PKC412.

Our study identifies several potential resistance mutations in the ATP-binding region of FLT3 that confer resistance to small molecule kinase inhibitors. Of these mutations, the G697R mutation confers high-level resistance to all tested inhibitors, including three classes of inhibitors that are currently tested in clinical trials. These observations indicate that the G697R mutation has potential to confer clinical resistance to any of the small molecule inhibitors of FLT3 currently under evaluation in clinical trails. We predict that AML patients who develop resistance to a FLT3 inhibitor due to the G697R mutation would not respond to any of the other FLT3 inhibitors tested in our study. Treatment of AML patients with FLT3 inhibitors will provide...
additional insights into the clinical importance of the G697R mutation, but these data indicate that it may be of value to proactively screen for small molecule FLT3 inhibitors with activity against the G697R mutant.

References
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