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

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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).

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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 blunt 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’-gaaattagattgtaggaggaag and 5’-catatcatctactctctcag, in the presence of 50 μm/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-64006), 5-butanone-1H-2(1H-2-indolyl)-1H-2-indolyl)methanone (D-65476), 4-(6-dimethylamino-4-oxa-2-indolyl)-(1H-2-indolyl)-N-(4-phenoxypyphenyl)-1-piperazinecarboxamidine (DQPPC), 1,2-dimethyl-6-(2-thienyl)-imidazol[5,4-g]quinazoline (AGL2043), 3-(3-thienyl)-6-(4-methoxyphenyl)pyrazolo[1,5-a]pyrimidine (TMPPP), and 1-phenyl-3-H-oxa-2,3-diaza-cyclopenta[ajinden (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 QIamp DNA mini kit (Qiagen, Germantown, MD). The mutated region was amplified from genomic DNA with primers specific to the mutated allele and normal allele. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced with an ABI Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The sequencing results were analyzed using the Sequencing Analysis software (version 5.0, Applied Biosystems).
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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).

Characterization of the Identified Mutants. To determine the level of resistance that each of the identified mutations conferred to PKC412, we generated dose-response curves of the growth of Ba/F3 cells transformed by the different mutants. Increasing concentrations of PKC412 were added to the Ba/F3 cells in a range from 0 to 400 nmol/L. Concentrations greater than 400 nmol/L of PKC412 could not be used in Ba/F3 cells, because these are toxic, even to Ba/F3 cells grown in the presence of IL-3 (15). The growth of Ba/F3 cells expressing the nonmutated FLT3-ITD construct was potently inhibited by PKC412 (IC₅₀ = 35 nmol/L), whereas Ba/F3 cells expressing the mutant forms were less sensitive to PKC412 with IC₅₀ ranging from 70 to >400 nmol/L. The G697R mutation conferred the highest degree of resistance to PKC412, and an IC₅₀ could not be reached in this assay (IC₅₀ > 400 nmol/L; Fig. 3).

These data were confirmed by analysis of the phosphorylation status of FLT3 in Ba/F3 cells by Western blotting. No effect on FLT3 phosphorylation was observed for the G697R mutant, even at 400 nmol/L PKC412 (Fig. 4; data not shown), confirming that this mutation conferred high-level resistance to PKC412. To test higher concentrations of PKC412, we used an in vitro kinase assay of a recombinant FLT3-D835Y protein harboring the G697R mutation. In this assay, no decrease on kinase activity was observed for the G697R mutant in the presence of concentrations of PKC412 up to 10 μmol/L (data not shown). Based on these observations, the G697R mutant was selected for additional analysis.

DNA using the HF-2 kit (BD Clontech), cloned in pGEM-T-easy (Promega), and sequenced.

Western Blotting. Ba/F3 cells were incubated with the inhibitors for 2 hours. Total cell lysates were analyzed by standard Western blotting procedures using the monoclonal anti-phospho-FLT3, polyclonal anti-FLT3 (Cell Signaling, Beverly, MA), and antimouse/antirabbit (AP Biotech, Uppsala, Sweden) peroxidase-labeled antibodies.

Modeling. A homology model of the kinase domain of FLT3 in complex with PKC412 was constructed based on the atomic coordinates of the crystal structure of the Lck kinase in complex with staurosporine (Protein Data Bank code 1QPJ; ref. 13). This crystal structure of a member of the tyrosine kinase family in complex with the parent compound of PKC412 is a suitable template for PKC412 in the Lck structure was given to PKC412 in the same binding orientation as staurosporine. The positions of the four mutated amino acids are shown with all their atoms represented, whereas Ala-627 and Asn-676 are in direct contact with PKC412. Gly-697 and Phe-691 are shown with all their atoms represented, whereas Ala-627 and Asn-676 are represented by their C atoms.
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-

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). 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 trials. 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

Fig. 4. Characterization of cross resistance of the FLT3-ITD-G697R mutant to different FLT3 inhibitors. A, analysis of phosphorylation of FLT3-ITD and FLT3-ITD-G697R mutant after treatment with different concentrations of inhibitor. Although phosphorylation of FLT3-ITD was potently inhibited by all inhibitors, no effect was observed on the phosphorylation of the G697R mutant. B, dose-response curves of Ba/F3 cells expressing FLT3-ITD or FLT3-ITD-G697R treated with six additional experimental FLT3 inhibitors. Treatment with D-64406 and D-65476 resulted in a reduction of cell growth at concentrations >10 μmol/L, which is not observed in cells treated in the presence of IL-3. However, as shown in A, phosphorylation of the G697R mutant was not affected, suggesting that the effect on cell growth was not due to direct inhibition of FLT3-ITD-G697R. DQPPC inhibited cell growth of Ba/F3 cells at concentrations >1 μmol/L, even in the presence of IL-3, indicating that this is due to an aspecific toxic effect.
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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