Leukemia (2015) **29,** 2390–2392

www.nature.com/leu

ORIGINAL ARTICLE

FLT3 D835 mutations confer differential resistance to type II FLT3 inhibitors

CC Smith[1,2,6], K Lin[1,6], A Stecula[3], A Sali[3,4,5] and NP Shah[1,2]

Activating mutations in FLT3 occur in ~30% of adult acute myeloid leukemia, primarily consisting of internal tandem duplication (ITD) mutations (~25%) and point mutations in the tyrosine kinase domain (~5%), commonly at the activation loop residue D835. Secondary kinase domain mutations in FLT3-ITD, particularly at the D835 residue are frequently associated with acquired clinical resistance to effective FLT3 tyrosine kinase inhibitors (TKIs). Molecular docking studies have suggested that D835 mutations primarily confer resistance by stabilizing an active Asp-Phe-Gly in ('DFG-in') kinase conformation unfavorable to the binding of type II FLT3 TKIs, which target a 'DFG-out' inactive conformation. We profiled the activity of active type II FLT3 TKIs against D835 kinase domain mutants that have been clinically detected to date. We found that type II inhibitors (quizartinib, sorafenib, ponatinib and PLX3397) retain activity against specific D835 substitutions. Modeling studies suggest that bulky hydrophobic substitutions (D835Y/V/I/F) at this residue are particularly resistant, whereas mutations that preserve interactions between D835 and S838 are relatively sensitive (D835E/N). All mutants retain sensitivity to the type I inhibitor crenolanib. These results suggest that patients with relatively sensitive D835 mutations should be included in clinical trials of type II FLT3 TKIs.

Leukemia (2015) **29,** 2390–2392; doi:10.1038/leu.2015.165

INTRODUCTION

Activating mutations in FLT3 occur commonly in acute myeloid leukemia, including internal tandem duplication (ITD) and point mutations in the tyrosine kinase domain, typically at the activation loop (AL) residue D835. Recent studies of potent FLT3 inhibitors in FLT3 mutant patients has demonstrated clinical activity for sorafenib,[1] quizartinib (AC220),[2] ponatinib[3] and crenolanib.[4] However, duration of response to FLT3 inhibitors is limited by resistance conferring secondary kinase domain mutations. Highly resistant FLT3-ITD AL D835V/Y/F and gatekeeper F691L mutations confer acquired clinical resistance to quizartinib[5] and acquired D835Y/H and F691L mutations have been detected at the time of resistance to sorafenib.[1,6]

The most common residue implicated in clinical resistance to FLT3 tyrosine kinase inhibitor (TKI) therapy is D835.[1,5–7] Molecular docking analysis suggests that D835 mutants induce an active 'Asp-Phe-Gly (DFG)-in' kinase conformation unfavorable for binding by type II inhibitors such as sorafenib, quizartinib, ponatinib and PLX3397.[5,7] Type I inhibitors (for example, crenolanib) bind a 'DFG-in' conformation and retain activity against D835 mutants.[8] Despite the fact that D835 mutations have been commonly associated with *in vitro* and clinical resistance to type II FLT3 inhibitors, differences in the spectrum of D835 mutations identified at the time of clinical resistance to FLT3 TKIs (for example, D835H mutations observed with sorafenib but not quizartinib resistance) suggest that relative resistance of D835 substitutions to type II FLT3 TKIs is not uniform, although the number of cases analyzed to date is small. *In vitro* mutagenesis screens have identified different resistant D835 substitutions for individual FLT3 TKIs.[5] Nevertheless, clinical trials of type II FLT3 inhibitors commonly exclude patients with any FLT3 D835 mutation because of a prevailing assumption that all FLT3 D835 substitutions uniformly confer resistance to type II inhibitors. We sought to experimentally determine the degree of resistance conferred by individual D835 mutations and to further characterize molecular mechanisms underlying this resistance with the goal of informing clinical trial design and molecular testing.

MATERIALS AND METHODS

Ba/F3 cells were obtained from the laboratory of Charles Sawyers and have not been authenticated. They were tested and confirmed to be mycoplasma-free. Cell lines were created and proliferation assays performed as previously described.[5] Technical triplicates were performed for each experiment and experiments were independently replicated at least three times. Quizartinib, sorafenib, ponatinib and crenolanib were purchased from Selleckchem (Houston, TX, USA) and PLX3397 was obtained under a materials transfer agreement from Plexxikon Inc (Berkeley, CA, USA). Comparative protein structure models of FLT3 mutants were created with MODELLER 9.14 (San Francisco, CA, USA),[9] using the crystal structures of the autoinhibited FLT3 (PDB ID 1RJB)[10] and the co-crystal structure of FLT3 with quizartinib (PDB ID 4RT7)[7] as templates. For each D835 mutant, we generated 100 models using the automodel class with default settings, separately for each template. The models had acceptable protein orientation-dependent statistically optimized atomic potential (SOAP-Protein) scores.[11] They were clustered visually into up to five classes based on the conformation of the mutated side chain.

[1]Division of Hematology/Oncology, University of California, San Francisco, CA, USA; [2]Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, CA, USA; [3]Department of Bioengineering and Therapeutic Sciences, University of California, San Francisco, CA, USA; [4]California Institute for Quantitative Biosciences, University of California, San Francisco, CA, USA and [5]Department of Pharmaceutical Chemistry, University of California, San Francisco, CA, USA. Correspondence: Dr NP Shah, Division of Hematology/Oncology, University of California, San Francisco School of Medicine, 505 Parnassus Avenue, Box 1270, San Francisco 94143, CA, USA.
E-mail: nshah@medicine.ucsf.edu
[6]These authors contributed equally to this work.
Received 14 April 2015; revised 1 June 2015; accepted 2 June 2015; accepted article preview online 25 June 2015; advance online publication, 17 July 2015

RESULTS AND DISCUSSION

We profiled all D835 substitutions previously reported to cause FLT3 TKI resistance in patients,[1,5,6] as well as D835 mutations occurring in patients as cataloged in the Sanger COSMIC database or the Cancer Genome Atlas. Inhibitory concentration 50 (IC_{50}) for proliferation of Ba/F3 cells expressing FLT3-ITD D835 mutants profiled for the clinically active FLT3 inhibitors quizartinib,[2] sorafenib,[1] ponatinib,[3] PLX3397[7] and crenolanib[4] is shown in Supplementary Table S1 and are in general, in keeping with previously reported values.[5,6,8,12,13] Relative resistance compared with FLT3-ITD is shown in Figure 1. Surprisingly, individual D835 substitutions conferred a wide range of resistance to all tested type II inhibitors. As previously reported,[5,12] FLT3-ITD D835V/Y/F mutations cause a high degree of resistance to all type II inhibitors. Deletion of the D835 residue or substitution with the bulky residue isoleucine also resulted in a high degree of resistance. The basic substitution D835H caused intermediate resistance, which may explain why this residue has been observed in clinical resistance to sorafenib[1] but not to the more potent inhibitor quizartinib.[5] Overall, D835A/E/G/N mutations conferred the least degree of resistance to the type II inhibitors. Consistent with our experimental observations, we identified only highly resistant D835 mutations (D835V/Y/F) in patients who relapsed after responding to quizartinib.[5] As expected, D835 mutations retained sensitivity to the type I inhibitor crenolanib and consistent with previous reports, it is expected that other type I inhibitors such as sunitinib, would also retain activity against these mutations.[6]

Type II inhibitors bind to the conformation coupled to the DFG-out position of the kinase AL (residues 829–856 in FLT3).[14] As previously noted, D835 is predicted to have a critical role in the stabilization of the DFG-out conformation by serving as an amino-terminal capping residue for the short, one-turn α-helix.[5,10,15] Alpha helices have a macrodipole, with a positive pole near the N-terminus and a negative pole near the C-terminus.[16,17] Short helices in particular may be stabilized with single residues forming favorable interactions with the helix dipole near the ends of the helix. The presumed negative charge of the D835 side chain at the N-terminus of the short α-helix spanning residues 835–839 is an example of such an interaction. D835 may also stabilize the helix by forming a hydrogen bond with either the main chain amide or the side chain hydroxyl of S838, as seen in the crystal structures of the autoinhibited and quizartinib bound FLT3 structures, respectively. Similar interactions are observed for the equivalent aspartic acid residue in the KIT structure (PDB ID 1T45).[15]

It is not straightforward to rationalize the effect of mutations near or in the short α-helix on the distant drug-binding active site. Nevertheless, it has been suggested that the short α-helix, which is part of the AL, is coupled to the drug-binding site over a distance.[5] Therefore, we focus here on describing the impact of D835 mutations on the short α-helix. We modeled each of the mutants using the crystal structures of FLT3 in the autoinhibited state and bound to quizartinib. It is possible to correlate three categories of resistance to type II inhibitors with predicted structural features of each mutant (Figure 2).

The most sensitive mutants (D835E/N) are characterized by the predicted preservation of the hydrogen bond between D835 and S838 based on models utilizing both the apo and holo structures as templates. Side chains of the D835E/N mutations are predicted to form hydrogen bonds with the side chain hydroxyl group of S838, the main chain amide group of S838, or the main chain amide group of M837, depending on the modeled conformations of the neighboring side chains. As a result, the short α-helix and thus the coupled DFG-out conformation are conserved, retaining type II inhibitor binding. In contrast, the short or lacking side chains of the residues in the more resistant mutants (D835G/A) cannot form any hydrogen bonds. Thus, the short α-helix may not form, shifting the equilibrium from the DFG-out to the DFG-in conformation, rationalizing the observed resistance.

The most highly resistant mutants (D835Y/V/I/F) are large and bulky hydrophobic amino acid residues. In addition to an inability to hydrogen bond with S838, these large side chains are predicted to be sterically incompatible with the short α-helix in models based on both the apo and holo structures, thus further shifting the equilibrium toward the active DFG-in conformation.

Unlike the mutations discussed above, the models of the moderately resistant D835H mutation based each of the two template structures differ from each other. We assume the model based on the quizartinib template is more accurate because holo states are generally more accurately modeled based on holo than apo templates. In addition, to accommodate the binding mode of quizartinib, the DFG motif is predicted to be displaced out of the DFG pocket (Figure 2), thus creating an essential edge-to-face aromatic interaction between F830 and the middle phenyl ring of quizartinib.[7] As a result, the predicted side chain conformations of the D835H mutant are unable to form any hydrogen bonds. The

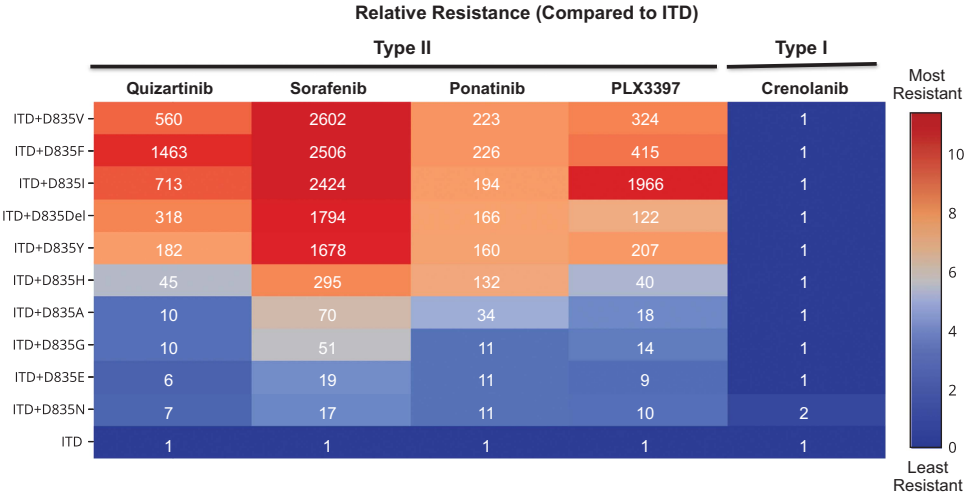

Figure 1. Relative resistance of FLT3 inhibitors to FLT3-ITD kinase domain mutations compared with ITD alone. Blue indicates most sensitive; red indicates most resistant. Number indicates fold-resistance compared with ITD alone for each inhibitor.

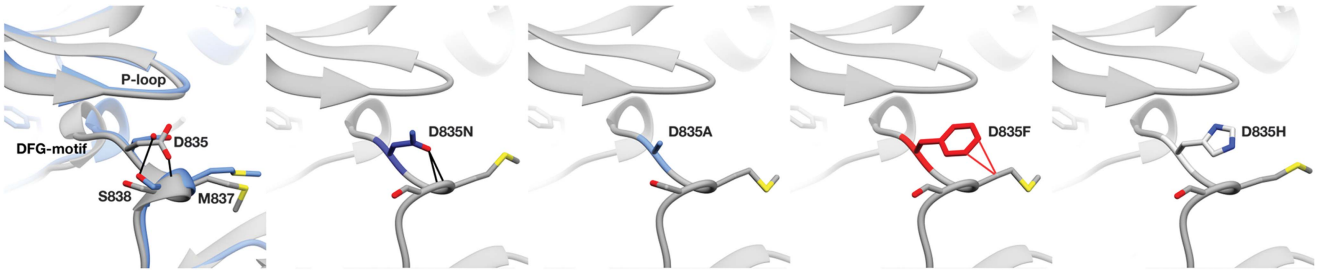

Figure 2. Comparative models of FLT3 mutants. Cartoon representation of the activation loop, where residue 835 is highlighted in the center of each panel. Predicted hydrogen bonds and steric clashes are indicated by solid black and red lines, respectively. The crystal structures of FLT3 in the autoinhibited state (PDB ID 1RJB)[10] (gray) and bound to quizartinib (PDB ID 4RT7)[7] (blue) are shown in the leftmost panel. Predicted orientations of mutant side chains from the three categories of resistance to type II inhibitors and histidine are shown in the other panels. All models shown are based on the FLT3 structure bound to quizartinib.

intermediary type II inhibitor resistance of the D835H mutation is therefore predicted to be a consequence of its inability to both accommodate the binding mode of the type II inhibitors and preserve the hydrogen bonds that stabilize the short α-helix in the autoinhibited conformation. Our conclusions based on the holo state model are in agreement with those based on an apo state model.[6]

Our data suggest that some clinically relevant D835 mutants retain sensitivity to type II inhibitors at clinically achievable drug concentrations and propose a molecular mechanism for differences in sensitivity for individual D835 mutants to type II FLT3 TKIs. Although it has been long recognized that the D835 residue has an important role in stabilizing the inactive conformation of FLT3,[5] our molecular modeling studies implicate critical hydrogen bonding interactions with the side chain hydroxyl group of S838, the main chain amide group of S838, and the main chain amide group of M837 that mediate formation of a short α-helix essential to the DFG-out conformation necessary for type II inhibitor binding. Mutations that preserve this short α-helix do not appreciably bias the kinase active conformation and therefore cause nominal resistance. Notably, current clinical assays report only the presence or absence of D835 mutations. From a practical perspective, our findings argue that the common practice of uniformly excluding patients with any D835 mutation from participation in type II FLT3 TKI clinical trials is misguided, as less resistant D835 substitutions such as D835N/E/G/A may retain sensitivity to TKI therapy. The development of clinical assays that report the exact nature of amino acid substitution(s) at D835 is therefore indicated. Further studies to elucidate molecular mechanisms of resistance mediated by FLT3 kinase domain mutants to type II TKIs will require co-crystal structural analyses, particularly with type I inhibitors bound to the active conformation of FLT3.

CONFLICT OF INTEREST

NPS has received research funding for the conduct of clinical trials from ARIAD Pharmaceuticals and Ambit Biosciences. NPS has received research funding from Daiichi-Sankyo and Plexxikon Inc. CCS has received research funding for the conduct of clinical trials from Plexxikon Inc. and Astellas Pharma. CCS, KL and A. Stecula designed experiments, performed research, analyzed data and wrote the manuscript. NPS and AS designed experiments, analyzed data and wrote the manuscript.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Cancer Institute (1R01 CA166616-01) (NPS) and the Leukemia and Lymphoma Society (CCS and NPS). CCS is an ASH Faculty Scholar and recipient of a Hellman Family Foundation Early Career Faculty Award. NPS acknowledges the generous support of Arthur Kern, Mark Maymar and the Edward S Ageno family. The authors thank Evan Massi for technical assistance.

REFERENCES

1 Man CH, Fung TK, Ho C, Han HH, Chow HC, Ma AC *et al.* Sorafenib treatment of FLT3-ITD(+) acute myeloid leukemia: favorable initial outcome and mechanisms of subsequent nonresponsiveness associated with the emergence of a D835 mutation. *Blood* 2012; **119**: 5133–5143.
2 Tallman MS, Schiller G, Trone D, Gammon G, Goldberg S, Perl AE *et al.* Results of a phase 2 randomized, open-label, study of lower doses of quizartinib (AC220; ASP2689) in subjects with FLT3-ITD positive relapsed or refractory acute myeloid leukemia (AML). *Blood* 2013; **122**: 494.
3 Shah NP, Talpaz M, Deininger MW, Mauro MJ, Flinn IW, Bixby D *et al.* Ponatinib in patients with refractory acute myeloid leukaemia: findings from a phase 1 study. *Br J Haematol* 2013; **162**: 548–552.
4 Randhawa JK, Kantarjian HM, Borthakur G, Thompson PA, Konopleva M, Daver N *et al.* Results of a phase II study of crenolanib in relapsed/refractory acute myeloid leukemia patients (Pts) with activating FLT3 mutations. *ASH Annual Meeting Abstracts*; presented at Annual Meeting of the American Society of Hematology in San Francisco, CA, USA, 2014, vol. 124, pp. 389.
5 Smith CC, Wang Q, Chin CS, Salerno S, Damon LE, Levis MJ *et al.* Validation of ITD mutations in FLT3 as a therapeutic target in human acute myeloid leukaemia. *Nature* 2012; **485**: 260–263.
6 Baker SD, Zimmerman EI, Wang YD, Orwick S, Zatechka DS, Buaboonnam J *et al.* Emergence of polyclonal FLT3 tyrosine kinase domain mutations during sequential therapy with sorafenib and sunitinib in FLT3-ITD-positive acute myeloid leukemia. *Clin Cancer Res* 2013; **19**: 5758–5768.
7 Smith CC, Zhang C, Lin K, Lasater EA, Zhang Y, Massi E *et al.* Characterizing and overriding the structural mechanism of the quizartinib-resistant FLT3 'gatekeeper' F691L mutation with PLX3397. *Cancer Discov* 2015; **5**: 668–679.
8 Smith CC, Lasater EA, Lin KC, Wang Q, McCreery MQ, Stewart WK *et al.* Crenolanib is a selective type I pan-FLT3 inhibitor. *Proc Natl Acad Sci USA* 2014; **111**: 5319–5324.
9 Sali A, Blundell TL. Comparative protein modelling by satisfaction of spatial restraints. *J Mol Biol* 1993; **234**: 779–815.
10 Griffith J, Black J, Faerman C, Swenson L, Wynn M, Lu F *et al.* The structural basis for autoinhibition of FLT3 by the juxtamembrane domain. *Mol Cell* 2004; **13**: 169–178.
11 Dong GQ, Fan H, Schneidman-Duhovny D, Webb B, Sali A. Optimized atomic statistical potentials: assessment of protein interfaces and loops. *Bioinformatics* 2013; **29**: 3158–3166.
12 Smith CC, Lasater EA, Zhu X, Lin KC, Stewart WK, Damon LE *et al.* Activity of ponatinib against clinically-relevant AC220-resistant kinase domain mutants of FLT3-ITD. *Blood* 2013; **121**: 3165–3171.
13 Galanis A, Ma H, Rajkhowa T, Ramachandran A, Small D, Cortes J *et al.* Crenolanib is a potent inhibitor of FLT3 with activity against resistance-conferring point mutants. *Blood* 2014; **123**: 94–100.
14 Wodicka LM, Ciceri P, Davis MI, Hunt JP, Floyd M, Salerno S *et al.* Activation state-dependent binding of small molecule kinase inhibitors: structural insights from biochemistry. *Chem Biol* 2010; **17**: 1241–1249.
15 Mol CD, Dougan DR, Schneider TR, Skene RJ, Kraus ML, Scheibe DN *et al.* Structural basis for the autoinhibition and STI-571 inhibition of c-Kit tyrosine kinase. *J Biol Chem* 2004; **279**: 31655–31663.
16 Fairman R, Shoemaker KR, York EJ, Stewart JM, Baldwin RL. Further studies of the helix dipole model: effects of a free alpha-NH3+ or alpha-COO- group on helix stability. *Proteins* 1989; **5**: 1–7.
17 Hol WG, van Duijnen PT, Berendsen HJ. The alpha-helix dipole and the properties of proteins. *Nature* 1978; **273**: 443–446.

Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)