NDEL1-PDGFRB FUSION GENE IN A MYELOID MALIGNANCY WITH EOSINOPHILIA ASSOCIATED WITH RESISTANCE TO TYROSINE KINASE INHIBITORS

Short title: TKI-resistant myeloid malignancy with NDEL1-PDGFRB

Konstantin Byrgazov[1], Maria Gorna[2], Gregor Hoermann[3], Margit Koenig[1], Raphael Ulreich[4], Martin Benesch[4], Volker Strenger[4], Herwig Lackner[4], Wolfgang Schwinger[4], Petra Sovinz[4], Oskar A.Haas[1], Marry van den Heuvel-Eibrink[5], Charlotte M.Niemeyer[6], Oliver Hantschel[7], Peter Valent[8], Giulio Superti-Furga[2], Christian Urban[4], Michael N.Dworzak[1,9], Thomas Lion[1,10*]

[1] - Children's Cancer Research Institute; Vienna, 1090; Austria.

[2] - Center for Molecular Medicine (CeMM) of the Austrian Academy of Sciences; Vienna, 1090; Austria.

[3] - Department of Laboratory Medicine; Medical University of Vienna; Vienna, 1090; Austria.

[4] - Department of Pediatrics and Adolescent Medicine; Medical University of Graz; Graz, 8036; Austria.

[5] - Erasmus MC-Sophia Children's Hospital; Pediatric Oncology/Hematology; Rotterdam, 3015, and Princess Máxima Center for Pediatric Oncology; Utrecht, 3584; The Netherlands.

[6] - Department of Pediatrics and Adolescent Medicine; University of Freiburg; Freiburg, 79106; Germany.

[7] - Swiss Institute for Experimental Cancer Research (ISREC); School of Life Sciences; École Polytechnique Fédérale de Lausanne (EPFL); Lausanne, CH-1015; Switzerland.

[8] - Department of Internal Medicine I; Division of Hematology and Hemostaseology and Ludwig Boltzmann Cluster Oncology; Medical University of Vienna; Vienna, 1090; Austria.

[9] - St. Anna Children's Hospital; Vienna, 1090; Austria.

[10] –Department of Pediatrics, Medical University of Vienna; Vienna, 1090; Austria.

*Correspondence: thomas.lion@ccri.at

We have identified a novel fusion gene *NDEL1-PDGFRB* in an 18-month old child with a myeloid neoplasm and eosinophilia[1, 2]. In contrast to earlier data on fusion genes involving *PDGRFB* in myeloid malignancies, which were generally responsive to treatment with imatinib[3], the patient presented became refractory to both imatinib and nilotinib. Sequencing of a pertinent gene panel including *NRAS*, *KRAS*, *NF1*, *PTPN11*, *CBL*, *FLT3*, *c-KIT*, *PDGFRA*, *CSF1R*, *CSF3R*, *SF3B1*, *SRSF2*, *ZRSR2*, *SH2B3*, *RUNX1*, *EZH2*, *ASXL1*, *SETBP1*, *DNMT3A*, *TET2*, *PTEN* at the time of diagnosis and both relapses revealed no mutations. However, sequence analysis of the entire tyrosine kinase domain (TKD) of *PDGFRB* revealed the D850E mutation in the activation loop (A-loop). This mutation was identified in peripheral blood (PB) and bone marrow (BM) specimens from both relapses in virtually all cells belonging to the leukemic clone, but was undetectable in the diagnostic PB or BM. In order to elucidate the structural effects mediated by the D850E mutation in the PDGFRβ TKD, we have generated structure models of the kinase domain both in active (DFG-*in*) and inactive (DFG-*out*) conformations, which interact preferentially with type-I and type-II TKIs, respectively (Fig.1)[4]. Since the structure of PDGFRβ TKD at the level of atomic resolution is not yet available, we have modelled the kinase domain on the basis of crystallographic structures of closely related homologous proteins including c-KIT, CSF-1R and VEGFR2. All structural models indicated that the observed type-II TKI resistance of cells expressing the D850E mutation in NDEL1-PDGFRβ was conceivably related to stabilization of the A-loop in the active conformation. In this conformation, the DFG triad serving as a hypomochlion for the A-loop, adopts the so-called DFG-*in* position[5]. The modelled structure of the inactive DFG-*out* conformation (Fig.1A;orange) revealed the typical auto-inhibitory interaction between D850 and the amino acid at the +3 position, R853, which is commonly observed in inactive TKDs of other receptor tyrosine kinases (RTKs) from the PDGFR family, and is believed to

stabilize the A-loop in the inactive conformation (Fig.1B)[6-8]. However, modelling of the mutant PDGFRβ TKD in the inactive conformation could not explain the resistance to type-II TKIs and the enhanced kinase activity addressed below, because the negatively charged E850 is also able to form a salt bridge with the positively charged side chain of R853. By contrast, the DFG-*in* model suggested the occurrence of two intriguing amino acid interactions upon transition of the A-loop from inactive to the active state (Fig.1A;green). One interaction implicated the negatively charged D850 and the positively charged, conserved H657 in the αC-helix (Fig.1C), which is expected to stabilize the A-loop in the active conformation[9]. This interaction can be further enhanced by the D850E mutation, because the longer side chain of glutamate in comparison to aspartate brings the negatively charged carboxylic group 1.1 Å closer to the positively charged histidine. This increases the stability of the A-loop in the active conformation (Fig.1D), because the forces of electrostatic interaction between opposite charges increase with the second power of decreasing distance, and become largely ineffective at distances exceeding 4.5 Å[10]. The structural model also suggested that the mutation H657K would have an effect similar to the mutation D850E in terms of stabilizing the active conformation (Fig.1E). The other interaction involved R853 and E946 in the C-lobe of the TKD. The +3 position to D850 is one of the least conserved positions in the A-loop of RTKs from the PDGFR family (Fig.1H), and the arginine at this position in PDGFRβ (R853) has the longest side chain among all members. The DFG-*in* model suggested that the positively charged side chain of R853 can reach a distance of approximately 2.7 Å to the negatively charged carboxyl group of E946, which may facilitate electrostatic bonds and provide additional stabilization of the DFG-*in* conformation of the PDGFRβ TKD. The structural model therefore suggested resistance of *NDEL1-PDGFRB* with the D850E mutation to type-II TKIs, which can only bind to the inactive conformation

of the PDGFRβ TKD, but indicated sensitivity to type-I TKIs binding to the active conformation. To address the predictions provided by the protein model, we have introduced several mutations affecting the aforementioned interactions, and tested the sensitivity of generated constructs against a panel of TKIs.

To assess the oncogenic potential of the newly identified fusion gene, the murine cell line Ba/F3 was stably transduced with wildtype or mutant *NDEL1-PDGFRB* constructs by employing a transposon-based system[11]. In addition to the D850E mutation observed in the patient, a construct carrying the H657K mutation was generated. This mutation was expected to strengthen the electrostatic interaction between D850 and the αC-helix, thus stabilizing the DFG-*in* conformation of the PDGFRβ TKD (Fig.1E). In order to determine the influence of R853 in the PDGRFβ TKD on the kinase activity and TKI-sensitivity, constructs carrying R853H were generated (Fig.1G). Ba/F3 cells expressing the H657K and D850E mutant versions of *NDEL1-PDGFRB* displayed an elevated *in vitro* kinase activity, whereas constructs with the R853H mutation showed a kinase activity identical to cells carrying wildtype *NDEL1-PDGFRB*. The phosphorylation level of PDGFRβ and one of its downstream targets, Erk, which is activated via the Ras-pathway[12], was higher in Ba/F3-NDEL1-PDGFRB cells carrying the H657K or D850E mutations in comparison to wildtype or R853H-carrying fusion gene constructs (Fig.2B).

The *in vitro* responsiveness of Ba/F3-NDEL1-PDGFRB cells to different TKIs of type-I (dasatinib, midostaurin, pacritinib) and type-II (imatinib, nilotinib, sorafenib) was determined by MTT assays. Cells expressing wildtype *NDEL1-PDGFRB* were sensitive to all TKIs tested (Fig.2A). By contrast, proliferation of Ba/F3 cells carrying the D850E mutation in the *NDEL1-PDGFRB* gene could only be inhibited by the indicated type-I TKIs at sub-micromolar concentrations (Fig.2A). The observation of

TKI resistance apparently induced by the D850E mutation in the kinase domain of PDGFRβ was in contrast to the same amino acid exchange at the corresponding site in PDGFRα (D842E)[13]. This finding raised questions regarding important structural differences between the two highly homologous RTKs. While PDGFRβ displays an arginine in the +3 position to the mutation site (R853), PDGFRα has the much shorter and less basic histidine in the corresponding position (H845) (Fig.1H). It appeared conceivable therefore that interaction between the side chains of R853 and E946 in the mutant PDGFRβ TKD could stabilize the active conformation in the presence of H657K or D850E mutations, thus mediating resistance to type-II TKIs. To address this notion, the mutation R853H was introduced into *NDEL1-PDGFRB* constructs, thus mimicking the sequence of the A-loop in the PDGFRα TKD (Fig.1H). In line with the properties of the D842E mutation in the FIP1L1-PDGFRα fusion, this change restored the TKI type-II sensitivity of cells carrying one of the activating mutations, H657K or D850E, in NDEL1-PDGFRβ (Fig.2).

The position of the newly identified mutation D850E in PDGFRβ corresponds to well-known mutation sites within the activation loop of other members of the PDGFR-RTK family including c-KIT (D816)[14], PDGFRα (D842)[13, 15], and FLT3 (D835)[5] (Fig.1H). Mutations converting the aspartate residue at the indicated positions into a bulky hydrophobic residue were shown to mediate resistance to type-II TKIs[5, 15, 16]. Structural studies revealed that the interaction of the residues D816 in c-KIT and D835 in FLT3 with the residue at the +3 position (N819 in c-KIT and S838 in FLT3) may maintain the auto-inhibitory inactive conformation of the A-loop[6]. Notably, clinically relevant activating mutations D816V in c-KIT and D835V/Y in FLT3 converting the aspartate into a residue with bulky hydrophobic or aromatic side chain would disrupt the hydrophilic interactions with the corresponding residue at +3

position. However, a conversion of aspartate to glutamate, representing an exchange between two hydrophilic and negatively charged amino acids at this position, has never been associated with resistance to type-II TKIs[5, 13, 16]. In marked contrast to the clinical and *in vitro* data presented, the identical mutation at the corresponding site in the tyrosine kinase domain of PDGFRα (D842E) in the fusion gene *FIP1L1-PDGFRA* associated with chronic eosinophilic leukemia did not cause any significant increase in IC_{50} values of the type-II TKIs imatinib, nilotinib, and sorafenib[13] (Fig.2A). Similarly, the same mutation at the corresponding site of FLT3 associated with acute myeloid leukemia, D835E, did not significantly increase the IC_{50} to the type-II TKIs sunitinib and sorafenib[5, 16] but enhanced the kinase activity of FLT3, and mediated growth factor-independent proliferation of the murine hematopoietic cell line Ba/F3[17]. However, although these analogies are in line with the enhanced kinase activity of the D850E PDGFRβ mutant, they do not explain the observed resistance to type-II TKIs. In this regard, it is important to examine the protein model in the context of intramolecular interactions specific for PDGFRβ TKD. Protein modelling of the PDGFRβ TKD suggested that the residue in +3 position, R853, which differs from corresponding sites in all other RTKs of the PDGFR family, mediates a critical amino acid interaction stabilizing the A-loop in the active conformation, thereby preventing the interaction of type-II TKIs with their target sites in the kinase domain. This notion was confirmed by mutating R853 to histidine, the amino acid present at the corresponding site in PDGFRα, which decreased the kinase activity and restored sensitivity of NDEL1-PDGFRβ with D850E or H657K to type-II TKIs. The findings not only confirm the important role of R853 in establishing the resistant phenotype of the mutant *NDEL1-PDGFRB*, but also underline the potential of protein modelling for prediction of sensitivity and resistance to TKI treatment. The data presented provide

new insights into specific amino acid interactions in mutant RTKs which are of clinical relevance for improved selection of appropriate TKI treatment.

Acknowledgement

This work was supported by the Austrian Science Fund (FWF), SFB Grants F4705-B20 (T.L.), F4704-B20 (P.V.), and F4711-B20 (G. S.-F.).

Conflict of Interest

The authors declare no conflict of interests.

References

1. Valent P, Klion AD, Horny HP, Roufosse F, Gotlib J, Weller PF*, et al.* Contemporary consensus proposal on criteria and classification of eosinophilic disorders and related syndromes. *The Journal of allergy and clinical immunology* 2012 Sep; **130**(3)**:** 607-612 e609.

2. Arber DA, Orazi A, Hasserjian R, Thiele J, Borowitz MJ, Le Beau MM*, et al.* The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood* 2016 May 19; **127**(20)**:** 2391-2405.

3. Cheah CY, Burbury K, Apperley JF, Huguet F, Pitini V, Gardembas M*, et al.* Patients with myeloid malignancies bearing PDGFRB fusion genes achieve durable long-term remissions with imatinib. *Blood* 2014 Jun 5; **123**(23)**:** 3574-3577.

4. Garuti L, Roberti M, Bottegoni G. Non-ATP competitive protein kinase inhibitors. *Current medicinal chemistry* 2010; **17**(25)**:** 2804-2821.

5. Smith CC, Lin K, Stecula A, Sali A, Shah NP. FLT3 D835 mutations confer differential resistance to type II FLT3 inhibitors. *Leukemia* 2015 Jun 25.

6. 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. *The Journal of biological chemistry* 2004 Jul 23; **279**(30)**:** 31655-31663.

7. McTigue M, Murray BW, Chen JH, Deng YL, Solowiej J, Kania RS. Molecular conformations, interactions, and properties associated with drug efficiency and clinical performance among VEGFR TK inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* 2012 Nov 6; **109**(45)**:** 18281-18289.

8. Illig CR, Manthey CL, Wall MJ, Meegalla SK, Chen J, Wilson KJ*, et al.* Optimization of a potent class of arylamide colony-stimulating factor-1 receptor inhibitors leading to anti-inflammatory clinical candidate 4-cyano-N-[2-(1-cyclohexen-1-yl)-4-[1-[(dimethylamino)acetyl]-4-piperidinyl]pheny l]-1H-imidazole-2-carboxamide (JNJ-28312141). *Journal of medicinal chemistry* 2011 Nov 24; **54**(22)**:** 7860-7883.

9. Elling C, Erben P, Walz C, Frickenhaus M, Schemionek M, Stehling M*, et al.* Novel imatinib-sensitive PDGFRA-activating point mutations in hypereosinophilic syndrome induce growth factor independence and leukemia-like disease. *Blood* 2011 Mar 10; **117**(10)**:** 2935-2943.

10. Donald JE, Kulp DW, DeGrado WF. Salt bridges: geometrically specific, designable interactions. *Proteins* 2011 Mar; **79**(3)**:** 898-915.

11. Wachter K, Kowarz E, Marschalek R. Functional characterisation of different MLL fusion proteins by using inducible Sleeping Beauty vectors. *Cancer letters* 2014 Oct 1; **352**(2)**:** 196-202.

12. Demoulin JB, Essaghir A. PDGF receptor signaling networks in normal and cancer cells. *Cytokine & growth factor reviews* 2014 Jun; **25**(3)**:** 273-283.

13. von Bubnoff N, Gorantla SP, Engh RA, Oliveira TM, Thone S, Aberg E*, et al.* The low frequency of clinical resistance to PDGFR inhibitors in myeloid neoplasms with abnormalities of PDGFRA might be related to the limited repertoire of possible PDGFRA kinase domain mutations in vitro. *Oncogene* 2011 Feb 24; **30**(8)**:** 933-943.

14. Sotlar K, Fridrich C, Mall A, Jaussi R, Bultmann B, Valent P*, et al.* Detection of c-kit point mutation Asp-816 --> Val in microdissected pooled single mast cells and leukemic cells in a patient with systemic mastocytosis and concomitant chronic myelomonocytic leukemia. *Leukemia research* 2002 Nov; **26**(11)**:** 979-984.

15. Weisberg E, Wright RD, Jiang J, Ray A, Moreno D, Manley PW*, et al.* Effects of PKC412, nilotinib, and imatinib against GIST-associated PDGFRA mutants with differential imatinib sensitivity. *Gastroenterology* 2006 Dec; **131**(6)**:** 1734-1742.

16. von Bubnoff N, Engh RA, Aberg E, Sanger J, Peschel C, Duyster J. FMS-like tyrosine kinase 3-internal tandem duplication tyrosine kinase inhibitors display a nonoverlapping profile of resistance mutations in vitro. *Cancer research* 2009 Apr 1; **69**(7)**:** 3032-3041.

17. Clark JJ, Cools J, Curley DP, Yu JC, Lokker NA, Giese NA*, et al.* Variable sensitivity of FLT3 activation loop mutations to the small molecule tyrosine kinase inhibitor MLN518. *Blood* 2004 Nov 1; **104**(9)**:** 2867-2872.

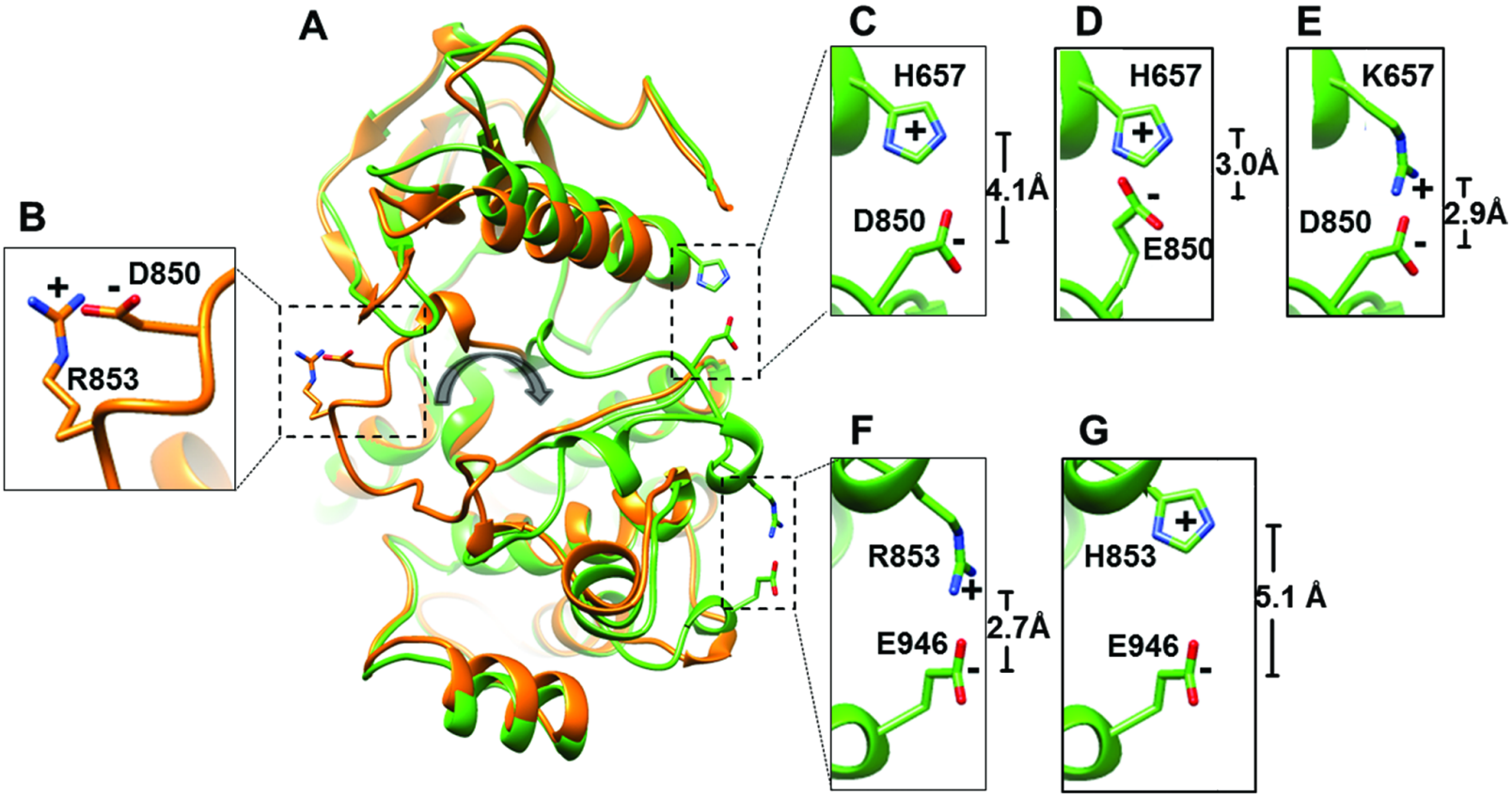
Figure Legends

Figure 1. Protein models of the PDGFRβ TKD structure

(A) The modelled DFG-*out* (orange) and DFG-*in* (green) conformations of PDGFRβ TKD are displayed. The zoom-in windows show the relevant stabilizing electrostatic interactions with the corresponding distances between charges: (B) D850-R853 in the DFG-*out* model; (C) H657-D850, (D) H657-E850, (E) K657-D850, (F) R853-E946, and (G) H853-E946 in the DFG-*in* model. Oxygen atoms carrying negative charge are marked in red, nitrogen atoms carrying positive charge in blue. The grey arrow in the center indicates rotation of the A-loop upon transition from inactive to active state. (H) Sequence alignment of RTKs from the PDGFR family depicting the region covering αC-helices and A-loops of PDGFRα, PDGFRβ, FLT3, CSF-1R, and c-Kit.

Figure 2. TKI-responsiveness of wildtype and mutant *NDEL1-PDGFRB* genes

(A) Displayed are IC_{50} values of different TKIs against Ba/F3 cells expressing wildtype (wt) or mutant NDEL1-PDGFRβ fusion proteins. The corresponding IC_{50} values for Ba/F3 expressing FIP1L1-PDGFRα WT and D842E are given for comparison. (B) Western blot analysis of Ba/F3 cells transduced with wildtype or mutant (R = R853H, H = H657K, HR = H657K/R853H, D = D850E, and DR = D850E/R853H) *NDEL1-PDGFRB* genes. The phosphorylation levels of NDEL1-PDGFRβ at Y751 and Y857, and Erk are displayed. Shown are also the total expression levels of NDEL1-PDGFRβ, Erk, and the control gene Gapdh upon mock treatment for 4 h with DMSO (indicated by "-") or with 100 nM nilotinib (indicated by "+").



H

	H657	D850	+3
PDGFRb	ALMSELKIMSHLG	KICDFGLARDIMRDSNY	
PDGFRa	ALMSELKIMTHLG	KICDFGLARDIMHDSNY	
FLT3	ALMSELKMMTQLG	KICDFGLARDIMSDSNY	
CSF1R	ALMSELKIMSHLG	KIGDFGLARDIMNDSNY	
c-Kit	ALMSELKVLSYLG	KICDFGLARDIKNDSNY	
Consensus	ALMSELKIMSHLG	KICDFGLARDIMNDSNY	

Conservation (100% / 0%)

αC-helix

A-loop

A

	TKI	NDEL1-PDGFRβ WT	R853H	D850E	DR	H657K	HR	FIP1L1-PDGFRα WT	D842E
Type II	IMA	80	50	>5000	15	>5000	15	2	4
	NIL	90	40	2200	12	2100	10	8	12
	SOR	40	30	2100	15	1900	10	1	2
Type I	DAS	20	10	25	12	25	10	7	N/A
	MID	25	20	15	15	10	12	30	N/A
	PAC	30	25	10	12	15	13	N/A	N/A

B