Structural basis of resistance of mutant RET protein-tyrosine kinase to its inhibitors nintedanib and vandetanib

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This article contains supporting text and Figs. S1 and S2.

The atomic coordinates and structure factors (codes 6NEC, 6NJA, and 6NE7) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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RET is a transmembrane growth factor receptor. Aberrantly activated RET is found in several types of human cancer and is a target for treating RET aberration-associated cancer. Multiple clinically relevant RET protein-tyrosine kinase inhibitors (TKIs) have been identified, but how TKIs bind to RET is unknown except for vandetanib. Nintedanib is a RET TKI that inhibits the vandetanib-resistant RET (G810A) mutant. Here, we determined the X-ray co-crystal structure of RET kinase domain–nintedanib complex to 1.87 Å resolution and a RET (G810A) kinase domain crystal structure to 1.99 Å resolution. We also identified a vandetanib-resistant RET (L881V) mutation previously found in familial medullary thyroid carcinoma. Drug-sensitivity profiling of RET (L881V) revealed that it remains sensitive to nintedanib. The RET–nintedanib co-crystal structure disclosed that Leu-730 in RET engages in hydrophobic interactions with the piperazine, anilino, and phenyl groups of nintedanib, providing a structural basis for explaining that the p.L730V mutation identified in nine independently isolated cell lines resistant to nintedanib. Comparisons of RET–nintedanib, RET (G810A), and RET–vandetanib crystal structures suggested that the solvent-front Ala-810 makes hydrophobic contacts with a methyl group and aniline in nintedanib and blocks water access to two oxygen atoms of vandetanib, resulting in an energetic penalty for burying polar groups. Of note, even though the p.L881V mutation did not affect sensitivity to nintedanib, RET (L881V) was resistant to nintedanib analogs lacking a phenyl group. These results provide structural insights into resistance of RET mutants against the TKIs nintedanib and vandetanib.

The RET protein-tyrosine kinase is a transmembrane receptor for glial cell line–derived neurotropic factor family of growth factors (1). Oncogenic mutations and fusions of RET lead to ligand-independent activation of the protein-tyrosine kinase, which are most often found in thyroid and non-small cell lung cancer (2, 3). A number of protein-tyrosine kinase inhibitors (TKIs)4 with anti-RET activity have been identified. Some of these have been tried in RET-associated cancers and have shown clinical efficacy, such as vandetanib (4, 5), cabozantinib (6), lenvatinib (7), BLU-667 (8), and LOXO-292 (9).

A mechanism of resistance to TKIs is acquisition of on-target mutations in the kinase domain that sterically hinder the TKI binding (10). Second and third generations of EGFR or ALK inhibitors have been successfully developed to inhibit some of these drug-resistant EGFR or ALK mutations, such as osimertinib (11), ceritinib (12), and brigatinib (13).

Mutations in the RET kinase domain that resulted in resistance to RET TKIs have also been found in the laboratory (8, 14, 15) and in the clinic (9, 16, 17). Predictably, more TKI-resistant RET mutations will be identified as more acquired RET TKI-resistant tumors from patients are analyzed. Because a number of clinically relevant RET TKIs have been identified (18), a RET kinase domain mutation resistant to one of the RET TKIs may be inhibited by another RET TKI with a different chemical
structure (15). Detailed knowledge of how each of different TKI binds to the RET kinase domain could facilitate the identification of a secondary drug to inhibit the acquired drug-resistant RET mutants. However, among the >10 known clinically applicable RET TKIs, only a co-crystal structure of the vandetanib-RET complex had been determined (19).

Nintedanib was identified by us and others as a multikinase TKI that inhibits RET (7, 15, 20). Nintedanib was effective in inhibiting the vandetanib-resistant RET(G810A) mutant (15) and a novel vandetanib-resistant RET(L881V) mutant identified in this study. RET(L881V) was reported previously in familial medullary thyroid carcinoma (1). To understand how nintedanib binds to RET, we determined the crystal structure of nintedanib-bound RET kinase domain protein. We also determined the crystal structure of an adenosine phosphate-bound RET(G810A) mutant kinase. Comparison of the vandetanib- and nintedanib-bound RET crystal structures showed that nintedanib has a phenyl group that is absent in vandetanib in a position near Leu-881. To assess whether the phenyl group in nintedanib may play a role in allowing nintedanib to inhibit the vandetanib-resistant RET(L881V), we synthesized nintedanib analogs lacking the phenyl group (compounds 1 and 2) and tested its activity in the vandetanib-resistant BaF3/KIF5B–RET(L881V) (BaF3/KR(L881V)) cells.

Results

We present three crystal structures of RET kinase and the results of cell assays using nintedanib analogs. The first crystal structure is of the TKI nintedanib bound to the WT RET kinase (PDB code 6NEC). We compare this structure to that of the WT protein bound by vandetanib (PDB code 2IVU) and the previously reported apo WT protein (PDB code 2IVT) and our new apo WT protein structure at a higher resolution (1.87 Å, PDB code 6NJA). We also compare our structure of apo mutant protein RET(G810A) (PDB code 6NE7). This mutation was identified as a vandetanib-resistant mutant that did not affect the sensitivity to nintedanib (15). Our analysis suggests that a phenyl ring in nintedanib would play a role in inhibiting the vandetanib-resistant mutant p.L881V. We tested this hypothesis by measuring the activity of nintedanib analogs missing the phenyl group in vandetanib-resistant BaF3/KR(L881V) cells.

Crystal structure of RET–nintedanib complex

To obtain crystals of the WT RET kinase–nintedanib complex, the protein was incubated with 350 µg/µl of nintedanib (10 molar excess) during the later stages of protein purification. The crystals of the complex were yellow in color and had leaf-like shapes (∼0.5 × 0.05 × 0.05 mm) that were different from the crystals of the WT protein. The diffraction data from the crystals of the complex showed that the crystals belonged to space group P21 with two proteins in the asymmetric unit (Table 1). The best crystal gave a diffraction limit of 1.87 Å. The structure (PDB code 6NEC) was solved by molecular replacement using MolRep and our structure of WT RET kinase stripped of all heteroatoms (PDB code 6NJA). Data in the resolution range of 35–2.15 Å were used. The correlation coefficient and F factor of the correct model was 0.7324 and 0.462, respectively. Likewise, correlation coefficient and F factor of the best scoring incorrect model was 0.5174 and 0.572, respectively. The initial Fo – Fc and 2Fo – Fc difference Fourier maps showed clear density for the bound nintedanib molecule. Eleven cycles of refinement and model building resulted in a final structure of the complex with Rwork and Rfactor of 19.7 and 23.4%, respectively. The first of the two molecules in the asymmetric unit (chain A, Fig. 1A) had electron density around residues from Glu–713 to Ala–828 and from Asp–842 to Arg–1012 and approximately one molecule of nintedanib (Fig. 1B).

The second protein molecule (chain C), when compared with molecule A, had significant electron density around residues Gly–700 to Leu–712; however, molecule C was missing electron density for residues Gly–823 to Ala–828, Asp–842 and from Tyr–900 to Arg–908. As with molecule A, molecule C also bound one molecule of nintedanib (PDB code 6NEC).

Superposition of the CA atoms of residues 715–819 and 847–1012 of molecule A with the equivalent CA atoms of molecule C showed a RMS deviation of 0.57 Å. The maximum difference was of 2.8 Å between CA atoms of residue Lys–1011 at the C termini of the molecules. The residues from positions 820 to 822 and 843 to 846, which were present in both molecules and which were located before and after the break in polypeptide chain, were not included in the superposition because they had different conformations.

The next largest shifts were in the glycine-rich loop (GRL) (residues Lys–728 to Val–739) and the loop connecting the β2 and β3 strands (residues Phe–744 to Tyr–752). The movement in the Phe–744–Tyr–752 loop could be explained by the difference of their contacts with symmetry-related molecules. The loop in molecule A interacts with residues 903–908 and 925–929 of a symmetry mate, whereas the loop in molecule C interacts with residues Arg–982–Gln–986 and Lys–994–Asp–1004 of another symmetry mate. The GRL does not make any interactions with symmetry-related molecules, so the causes of shifts between the two GRLs must be internal. Superposition of the coordinates of nintedanib from the two protein molecules of the asymmetric unit gave a RMS deviation of 0.073 Å when using the 26 most similar nonhydrogen atoms, which are also inside the ligand-binding pocket. The remaining 14 nonhydrogen atoms had large differences and were in the piperezine end of the nintedanib molecule (Fig. 1C). This end of the nintedanib molecule was exposed to the solvent at the mouth of the ATP-binding site, and the piperezines were flipped in opposite directions. The largest difference as 2.8 Å between the C40 carbon atoms. This difference in the inhibitors was reflected in weaker electron density and higher B-factors for these atoms (Fig. 1B).

In both copies of the complex, three polar atoms in the inhibitor makes four polar contacts with residues of polypeptide chain (Fig. 1D and Fig. S1). Atom N9 of the indole ring of the inhibitor formed a hydrogen bond with backbone carbonyl of Glu–805, which is in the hinge. The O10 oxygen atom of the inhibitor forms a hydrogen bond with main chain nitrogen of the hinge residue Ala–807. The O26 oxygen atom of the nintedanib molecule interacts with the Ne nitrogen atom of the side chain of Lys–758 and the main chain nitrogen of residue Asp–892.

The inhibitor molecule is also involved in several hydrophobic interactions: Lys–728 to Gly–731, Val–738 from β2 strand,
Co-crystal structure of RET and nintedanib complex

### Table 1
Data collection and refinement statistics for human RET, RET–nintedanib complex, and RET(G810A) mutant

| Parameters | RET | RET–nintedanib | RET(G810A) |
|------------|-----|---------------|------------|
| PDB code   | 6NJA | 6NEC | 6NE7 |
| **Data collection** | | | |
| Space group | C2 | P2₁ | C2 |
| Unit cell parameters | | | |
| a (Å) | 71.70 | 70.82 | 78.87 |
| b (Å) | 70.82 | 80.29 | 102.05 |
| c (Å) | 78.87 | 79.93 | 100.52 |
| α (°) | 102.05 | 100.52 | 102.26 |
| Protein molecules in asymmetric unit | 1 | 2 | 1 |
| Resolution range (Å) | 39.3–1.92 | 56.16–1.87 | 39.22–1.99 |
| High resolution bin (Å) | 1.97–1.92 | 1.92–1.87 | 2.02–1.99 |
| Completeness (%) | 97.3 (94.7) | 96.0 (80.4) | 96.6 (95) |
| Multiplicity | 3.4 (3.3) | 3.4 (2.9) | 3.3 (3.0) |
| Rmerge (%) | 4.1 (61.4) | 4.3 (39.7) | 7.9 (68.9) |
| Mean I/σ(I) | 25.3 (1.84) | 25.1 (2.5) | 13.66 (1.3) |
| B factor from Wilson plot (Å²) | 28.72 | 27.09 | 24.93 |
| Refinement | | | |
| Resolution range (Å) | 39.31–1.92 | 42.32–1.87 | 39.22–1.99 |
| High resolution bin (Å) | 1.97–1.92 | 1.92–1.87 | 2.04–1.99 |
| No. of reflections | 27,339 (1942) | 24,270 (1728) | 24,270 (1728) |
| No. of reflections in test set | 28,780 (1441) | 25,551 (1281) | 25,551 (1281) |
| Data cutoff (Å) | 1.97–1.92 | 1.92–1.87 | 2.04–1.99 |
| Data collection & refinement statistics | | | |
| Rmerge (%) | 18.7 (29.6) | 19.7 (29.1) | 20.04 (32.7) |
| Rwork (%) | 23.4 (38.3) | 23.4 (33.3) | 25.0 (36.9) |
| Protein atoms | 24,286 | 4675 | 2467 |
| Water molecules | 163 | 241 | 133 |
| Mean B (Å²) | 41.55 | 40.94 | 38.0 |
| Protein chains A, B ligand, anions, water | 41.15, 56.31, 46.23 | 38.00, 46.64, 41.25, 43.51 | 38.65, 39.5, 43.16 |
| All-atoms clashscore | 5 | 4 | 8 |
| Ramachandran plot: favored/outliers (%) | 99/0 | 97.86/0.0 | 98/0 |
| Rotamer outliers | 2 | 2 | 1 |
| Root mean square deviation from ideal geometry | 0.05 | 0.005 | 0.008 |
| Bond angles (°) | 1.31 | 1.28 | 1.50 |
| Maximum likelihood coordinate error (Å) | 0.111 | 0.108 | 0.141 |

* Rmerge (merging R factor) = \( \Sigma_i (I_i(hk)) - \langle I(hk) \rangle / \Sigma_i I_i(hk) \), where \( I_i(hk) \) is the intensity measured for the \( i \)th reflection and \( \langle I(hk) \rangle \) is the average intensity of all reflections with indices \( hk \).

* R factor = \( \Sigma_i [F_{obs}(hk)] - [F_{calc}(hk)] / \Sigma_i [F_{obs}(hk)] \). Rmerge is calculated in the same manner with 5% of the randomly selected reflections not included in the refinement.

### Ala-756 and Lys-758 from β3 strand, Glu-775 and Ile-779 from αC helix, Val-804 gatekeeper residue, Glu-805 to Gly-810 of the hinge, Leu-881 from β3 strand, and Ser-891 are the residues from RET participating in hydrophobic interactions (Fig. 1D and Fig. S1). The methyl carboxyl end of the inhibitor is bound to the back pocket I, which is just behind the gate area of RET molecule (21–23). The other end of the inhibitor molecule (the piperazine end) projects out of the front pocket into the solvent, but steric hindrance with the aromatic six-membered ring forces the piperazine ring out of the plane formed by the conjugated ring system of the remainder of the inhibitor molecule (Fig. 1C). The free inhibitor would adopt something close to this conformation one-third of the time. One face of the piperazine ring covers the backbone atoms of residues Lys-728, Thr-729, and Leu-730 from solvent (Fig. 1C). Single polar interaction of this part of inhibitor molecule is with a water molecule and involves the O33 atom of the inhibitor.

The conformation of the RET kinase domain in the nintedanib complex suggests that it is in the active state for the following reasons. First, the conformation of the activation structure (Asp-892–Glu-921) corresponds to the active conformation. Second, the side chain of Asp-892, the second Asp of the KEDD signature quartet, was oriented inward and formed a hydrogen bond with the backbone N of Lys-758. Third, the αC helix is in the “in” conformation and its Glu-775 forms a hydrogen bond with the side chain of Lys-808 of the β3 strand. Unexpectedly, the GRL has a closed conformation characteristic of the inactive form of the enzyme despite the presence of inhibitor molecule.

### Crystal structures of RET(G810A) and RET kinase

The best crystal of RET(G810A) gave 1.99 Å diffraction (Table 1). The unit cell of the crystal of RET(G810A) was isomorphous with those of the WT RET kinase (PDB code 6NJA), so the structure was determined by molecular substitution with the coordinates of the RET kinase (Table 1). Although the protein was co-crystallized with ATP, only the base atoms were visible in the electron density map.

The unit cell of the crystal of our RET kinase was isomorphous with that of the previous structure determined at lower resolution (PDB code 2IVT). This structure was used as the starting model in structure determination by molecular substitution. Our higher resolution structure was then used as a starting model in the structure determination of our two structures as mentioned above.

Superposition of coordinates of molecule A of the RET–nintedanib complex (PDB code 6NEC) with RET(G810A) (PDB code 6NE7) gave an RMS deviation of 0.211 Å between 157 matching pairs of CA atoms in the C-terminal lobe. Based on comparison of the two structures, it is predicted that the Ala-
810 of RET(G810A) makes a hydrophobic contact of 3.5 Å with the methyl group on the nintedanib and would makes unfavorable close contacts with the phenyl ring of aniline in nintedanib (Fig. 2A). These close contacts could be relieved by shifts of few tenths of an angstrom by the nintedanib. The surface of the mouth of the binding pocket is very similar in shape and position between the two structures. The introduction of a bulkier serine residue at site 810 would cause steric clashes with both the methyl group and the phenyl ring of nintedanib. Consistently, RET(G810A) has a buried cavity. Similarly identified as a vandetanib-resistant mutation (15).

Superposition of RET(A810) with RET–vandetanib shows that the CB carbon atom of Ala-810 (blue) blocks the access of water to the OAT oxygen atom of vandetanib (white carbons) (Fig. 2B). The CB carbon atom reduces access of the OAS oxygen atom of vandetanib to water but to a lesser extent. The reduced access to water reduces the probability of H-bond formation. Buried polar groups with unsatisfied H-bonds leads to an energetic penalty (24). Consistently, RET(G810A) was originally identified as a vandetanib-resistant mutation (14).

The molecular surface of the back of the binding pocket is less similar (Fig. 2C). The RET(G810A) has a buried cavity. Movement of the side chain of residue Leu-779 causes the cavity behind the back of the binding pocket to open and merge with the rest of the binding pocket in the structure of RET–nintedanib.

Comparison of RET kinase complexes with nintedanib and vandetanib

Superposition of coordinates of RET–vandetanib (PDB code 2IVU) with coordinates of molecule A of the RET–nintedanib complex (PDB code 6NEC) gave an RMS deviation of 0.568 Å between 273 matching pairs of CA atoms. The largest movements were located in the GRL, the hinge region, the activation loop, the nintedanib bound (PDB code 6NEC). There were two copies of the protein in the asymmetric unit. The copy labeled chain A is shown. The N-terminal lobe is at the top, and the C-terminal lobe is at the bottom. The nintedanib is shown as a stick model that is colored by atom type with cyan carbons. The αC helix is colored blue. The activation loop is colored green, and the glycine-rich loop is colored orange. Site 810 is colored magenta. The remainder of the protein is colored gray. The molecular surface is colored as in in A, D, LigPlot diagram of the interactions between the nintedanib and the surrounding atoms in the chain A protein.
Co-crystal structure of RET and nintedanib complex

Figure 2. Comparison of ligand-binding pocket of the RET kinase–nintedanib complex (PDB code 6NEC) with those of other crystal structures of RET kinase. A, view of the mouth of the binding site showing close contacts between Ala-810 and the nintedanib from the superposed structure. The mutant protein RET(G810A) kinase (6NE7, salmon-colored carbon atoms) was superposed onto the RET kinase–nintedanib complex (PDB code 6NEC, chain A, cyan-colored carbon atoms) using the CA atoms of the C-terminal domain (residues 847–1012). The molecular surface of the ligand-binding pocket was generated in PyMOL. Only the base atoms of the nucleotide were seen in the electron density map of RET(G810A). B, superposition of RET kinase–vandetanib complex (PDB code 2IVU, gray-colored carbon atoms) and the RET kinase–nintedanib complex (PDB code 6NEC, chain A, cyan-colored carbon atoms) on the crystal structure of RET(G810A) kinase (PDB code 6NE7, salmon-colored carbon atoms). The transparent molecular surface of RET(G810A) is shown. The distances from Ala-810 of RET(G810A) to either carbon atoms in nintedanib of PDB code 6NEC or oxygen atoms in vandetanib of PDB code 2IVU are shown. C, comparison of the back end of the ligand-binding pocket for the RET(G810A) (6NE7, salmon-colored carbon atoms) and the RET kinase–nintedanib complex (6NEC, chain A, cyan-colored carbon atoms). The molecular surfaces of the cavities and pockets for both structures were computed in PyMOL. The RET(G810A) has an isolated cavity beyond the end of its binding pocket (right side of figure). The side chain on Leu-779 shifts in the structure of the RET kinase–nintedanib complex to cause the buried cavity to merge with the binding pocket. The terminal methyl group of Leu-881 creates a notch for the six-membered ring of nintedanib to slip into by shape complementarity (left side of the image).

(nintedanib) and the nintedanib from the superposed structure. The mutant protein RET(G810A) kinase (6NE7, salmon-colored carbon atoms) was superposed onto the RET kinase–nintedanib complex (PDB code 6NEC, chain A, cyan-colored carbon atoms) using the CA atoms of the C-terminal domain (residues 847–1012). The molecular surface of the ligand-binding pocket was generated in PyMOL. Only the base atoms of the nucleotide were seen in the electron density map of RET(G810A). B, superposition of RET kinase–vandetanib complex (PDB code 2IVU, gray-colored carbon atoms) and the RET kinase–nintedanib complex (PDB code 6NEC, chain A, cyan-colored carbon atoms) on the crystal structure of RET(G810A) kinase (PDB code 6NE7, salmon-colored carbon atoms). The transparent molecular surface of RET(G810A) is shown. The distances from Ala-810 of RET(G810A) to either carbon atoms in nintedanib of PDB code 6NEC or oxygen atoms in vandetanib of PDB code 2IVU are shown. C, comparison of the back end of the ligand-binding pocket for the RET(G810A) (6NE7, salmon-colored carbon atoms) and the RET kinase–nintedanib complex (6NEC, chain A, cyan-colored carbon atoms). The molecular surfaces of the cavities and pockets for both structures were computed in PyMOL. The RET(G810A) has an isolated cavity beyond the end of its binding pocket (right side of figure). The side chain on Leu-779 shifts in the structure of the RET kinase–nintedanib complex to cause the buried cavity to merge with the binding pocket. The terminal methyl group of Leu-881 creates a notch for the six-membered ring of nintedanib to slip into by shape complementarity (left side of the image).

Ala-807) between the enzyme and the inhibitor. In the vandetanib complex, the backbone O atom of Glu-805 has the same conformation as in nintedanib complex, but its counterpart is a carbon atom. To avoid a clash between these atoms in the vandetanib complex, the hinge loop is pushed away from inhibitor molecule; the largest shift is 1.23 Å between CA atoms of residues Glu-805.

The p.L881V mutant is sensitive to nintedanib but resistant to vandetanib. This site is on the C-terminal lobe surface of the binding pocket. Inspection of the superposition of 6NEC and 2IVU shows that the CD1 carbon atom of Leu-881 makes favorable hydrophobic interactions with the face of the ring in nintedanib (Fig. 3B). The replacement of the leucine with a valine would place the methyl group of the side chain of Val-811 approximately an angstrom further away from the phenyl ring. However, there is room in the binding pocket for this ring to settle upon this methyl group and restore a favorable interaction. The phenyl ringsettles in a notch in the molecular surface between the side chain methyl group and the backbone of site 810. This notch would be shallower in the structure of the Leu → Val-881 mutant but sufficient for the binding of the phenyl ring of nintedanib. Similar shape complementarity is much weaker in the vandetanib complex with RET kinase. It is possible in the Leu → Val-881 mutation would further weaken the shape complementarity with vandetanib and thereby dramatically reduce its binding affinity.

Identification and characterization of the vandetanib-resistant RET(L881V) mutation

In an effort to identify TKI-resistant RET mutations, we subjected BaF3/KR cells (14) to the chemical mutagen N-ethyl-N-nitrosoure (ENU) and selected for vandetanib-resistant cells. A vandetanib-resistant cell line was found to contain p.L881V mutation in the KIF5B–RET kinase domain (Fig. 4A). A BaF3/KR(L881V) cell line containing the p.L881V mutation was recreated (see “Experimental procedures”) and further analyzed.

Vandetanib had a 7.1-fold higher IC50 in BaF3/KR(L881V) cells than in BaF3/KR cells. In the presence of IL-3, a 14.2-fold higher IC50 of vandetanib was observed in BaF3/KR cells, demonstrating specificity. Profiling BaF3/KR(L881V) cells with cabozantinib, lenvatinib, and nintedanib showed that cabozantinib had a 4.7-fold higher IC50 and lenvatinib had a 12.7-fold higher IC50 than in BaF3/KR cells, whereas nintedanib had similar IC50 values in BaF3/KR(L881V) and BaF3/KR cells (Fig. 4C). Consistently, immunoblotting analyses of pRET(Y905) and cleaved PARP showed that vandetanib, cabozantinib, and lenvatinib were less potent in reducing pRET(Y905) and inducing apoptosis in BaF3/KR(L881V) cells, whereas nintedanib had a similar potency in inhibiting pRET(Y905) and inducing cleaved PARP between BaF3/KR and BaF3/KR(L881V) cells (Fig. 4D). Thus, the p.L881V mutation in the RET kinase domain of KIF5B–RET caused drug resistance to vandetanib, cabozantinib, and lenvatinib, but not to nintedanib.

BaF3/KR(L881V) is resistant to a nintedanib analog lacking the phenyl group

The nintedanib-bound RET co-crystal structure showed that nintedanib had a phenyl group (b in Fig. 5A) that together with the six-carbon ring of the indole group provided hydrophobic interaction with Leu-881. In the vandetanib-bound RET structure (PDB code 2IVU), the corresponding positions were a methoxy group (a in Fig. 5A) connected to the quinazolin rings, both of them were at a slightly longer distance from Leu-881 than the phenyl and the indole groups of nintedanib (Fig. 3B). To determine whether the phenyl group is critical for binding of nintedanib to Val-881 in the mutant protein, we synthesized two nintedanib analogs lacking the phenyl group, compound 1, and compound 2 (Fig. 5A) and compared their activities in BaF3/KR and BaF3/KR(L881V) cells.

Removal of the phenyl group from nintedanib changed the IC50 from 0.077 μM (nintedanib) to 1.13 μM (compound 1) in
BaF3/KR cells (Figs. 4C and 5B), suggesting that the phenyl group of nintedanib contributes to binding of nintedanib to the RET kinase. The substitution of the methylester group in compound 1 with a longer ethylester group in compound 2 (IC\textsubscript{50}/H11005 \(1.4\) \(\mu\)M) had a minimal impact on the inhibitory activity in BaF3/KR cells (Fig. 5B). Importantly, compound 1 had a 2.0-fold and compound 2 had a 2.3-fold higher IC\textsubscript{50} in BaF3/KR(L881V) cells than in BaF3/KR cells (Fig. 5B).

In another experiment, we replaced the O-methyl group of vandetanib with a benzyl group to yield compound 3 and tested compound 3 in BaF3/KR and BaF3/KR(L881V) cells. The result showed that compound 3 had IC\textsubscript{50} >30 \(\mu\)M in both cell lines (Fig. 5C), indicating that the benzyl group was too large to fit well into the drug-binding site. Nevertheless, in contrast to vandetanib that displayed different sensitivities in BaF3/KR and BaF3/KR(L881V) cells, the response curves to compound 3 were similar in these two cell lines.

**Discussion**

Although over 10 TKIs with potent RET kinase inhibitor activity have been identified, the structural details of how these TKIs bind to the RET kinase remain largely uncharacterized. We report here three crystal structures of RET kinase domain: a RET–nintedanib complex, a RET(G810A) mutant, and a WT RET of higher resolution than the one determined previously.

Previously, we isolated nine nintedanib-resistant BaF3/KR cell lines by culturing BaF3/KR cells with increasing concentrations of nintedanib and identified the p.L730V mutation in the kinase domain of the KIF5B–RET oncogene in these cells (15). Some of these progressed to double p.L730V/p.V804M mutations upon future culturing the cells with higher concentrations of the drug (15). The RET–nintedanib co-crystal structure reveals that the methylpiperazine group of nintedanib was extended outside of the solvent front of the RET ATP-binding pocket and formed hydrophobic interaction with several residues, including Leu-730, in the N-terminal portion of the GRL. In addition, the aniline and phenyl components also interacted with Leu-730. These multiple interactions between Leu-730 and nintedanib provide a structural basis for explaining why nine independently isolated nintedanib-resistant RET mutations in the previous study had the p.L730V mutation.

RET(G810A/S) were identified as vandetanib-resistant mutations in previous studies (14, 15). Gly-810 is located in the hinge strand connecting the N-lobe (residues 713–805) and the C-lobe (residue 812–1013) of RET kinase at the solvent front of ATP-binding pocket. The RET(G810A/S) mutations are at a paralogous position to the osimertinib-resistant EGFR(G796S/R) mutations, crizotinib-resistant ALK(G1202R) and ROS1(G2032R) mutations, and entrectinib-resistant NTRK1(G595R)/NTRK3(G623R) mutations (25–28). The RET(G810A) mutant remains sensitive to nintedanib, whereas the RET(G810S) mutant is resistant to nintedanib (15). Comparison of crystal structures of the RET–nintedanib complex, RET(G810A), and the RET–vandetanib complex suggested that the added methyl group in the p.G810A mutant could form new hydrophobic interactions with a methyl group in nintedanib, which is absent in vandetanib, and with a phenyl ring in nintedanib. However, the RET–nintedanib co-crystal structure also suggested that substitution of Gly-810 with the larger, polar Ser at this position could not be tolerated. Consistently, the RET(G810S) mutant is resistant to nintedanib (15).

RET(L881V) was reported as a germ-line mutation in familial medullary thyroid carcinoma (1). Here, we identify p.L881V as a vandetanib-resistant mutation. Among the four RET TKIs tested in this study, only the sensitivity of nintedanib was not affected by the p.L881V mutation. Based on the co-crystal structures of RET–nintedanib and RET–vandetanib, we predicted that a phenyl ring in nintedanib, which is absent in vandetanib, would play an important role in retaining the inhibiting activity against RET(L881V). Experimental data using nintedanib analogs lacking this phenyl ring support this notion.
Co-crystal structure of RET and nintedanib complex

Figure 4. The vandetanib-resistant KIF5B–RET(L881V) mutation is nintedanib-sensitive. 

A, DNA sequencing of KIF5B–RET isolated from a vandetanib-resistant BaF3/KR-derived cell line identified a C → G mutation indicating the p.L881V mutation. B, comparison of KIF5B–RET and KIF5B–RET(L881V) protein expression and activation (pRETY905) in BaF3/KR and recreated BaF3/KR(L881V) cells by immunoblotting. C, sensitivities of BaF3/KR (red) and BaF3/KR(L881V) (black) cells to vandetanib, cabozantinib, lenvatinib, and nintedanib in the cell viability assay. Control experiments for TKI specificity were performed in BaF3 cells cultured in the presence of 1 ng/ml IL-3 (blue). D, concentration-dependent inhibition of KIF5B–RET and KIF5B–RET(L881V) kinase activity and induction of apoptosis by vandetanib (VDT), cabozantinib (CBT), lenvatinib (LVT), and nintedanib (NTD) in BaF3/KR and BaF3/KR(L881V) cells determined by immunoblotting of pRETY905 and cleaved PARP. After transferring the proteins from gels to nitrocellulose filters, the filters were cut at or near the dashed lines. The upper parts and the lower parts of the filters were used separately for immunoblotting with indicated antibodies. Immunoblots of pRET, RET, and FLAG were performed using samples loaded onto different gels.

Figure 5. Assessment of the phenyl group in nintedanib for inhibition of p. L881V mutation. 

A, chemical structures of vandetanib, nintedanib, nintedanib analogs compound 1 and compound 2, and vandetanib analog compound 3. Vandetanib has a methoxy group (dotted red circle a), whereas nintedanib has a phenyl group (dotted red circle b) at a comparable position of interacting with RETL881 in the crystal structures (PDB codes 2IVU and 6NEC). B, sensitivities of BaF3/KR and BaF3/KR(L881V) cells to compounds 1 and 2. C, sensitivities of BaF3/KR and BaF3/KR(L881V) cells to compound 3.
These results demonstrate that the knowledge of RET-TKI co-crystal structures could provide novel insights to predict and explain interactions between TKIs and RET kinase mutations.

**Experimental procedures**

**Materials and methods**

BacPAK baculovirus expression system was obtained from Clontech. Nintedanib, cabozantinib, lenvatinib, and vandetanib were from LC Laboratories (Woburn, MA).

**Recombinant protein production and purification**

The coding sequence for a GST–RET kinase domain fusion protein was constructed by PCR using pGEX6P1 and KIF5B–RET cDNA (14) as the templates and linker oligonucleotides. After PreScission protease digestion, the RET kinase domain protein fragment had a GPLSL sequence at the N-terminal followed by RET amino acid residues 705–1013. The GST-RET coding sequence was subcloned into pBacPAK9. Recombinant baculovirus for expression of GST-RET was generated by cotransfection of SF21 cells with Bsu36I-digested BacPAK6 and pBacPAK9-GST-RET DNA according to the supplier’s instructions. Recombinant baculovirus for expression of GST-RET(G810A) was generated similarly. Plaque-purified viruses were amplified, titrated, and used to infect SF21 cells at a multiplicity of infection of 5 in multiple 1-liter cultures incubated at 27 °C and 80 rpm. The cells were harvested 72 h after infection for protein purification.

Frozen cell pellets (1 liter culture/each) were thawed and resuspended in 20 ml of buffer A (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.02% thyglycerol) plus 1 mg/ml lysozyme and sonicated. The cell lysates were centrifuged (20000 rpm, 45 min). The supernatant was mixed with GSH-Sepharose 4 Fast Flow (GE Healthcare) at 4 °C overnight. After washing twice with buffer A and buffer B (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10% glycerol, 1 mM EDTA, and 1 mM DTT), the GSH-Sepharose with bound proteins were incubated with 2.5 mM ATP and 5 mM MgCl₂ in buffer B (20 ml) for 4 h at 4 °C. For RET–nintedanib complex preparation, 10 μl of nintedanib ethanesulfonate (10 mg/ml) was mixed at 4 °C overnight with protein bound to the GSH-Sepharose beads. The beads were washed three times with 5 volumes of buffer B and then digested with PreScission protease at 4 °C overnight. The soluble fraction was collected and concentrated using Amicon Ultra-15. Purities of protein were >90% as determined by SDS-PAGE and Coomassie Blue stain.

**Crystallization, data collection, and structure determination**

The hanging-drop method was used for crystallization by vapor diffusion at room temperature. The crystallization drops contained 2 μl of protein and 2 μl of reservoir solution, against which drops were equilibrated. The reservoir solutions were sodium formate (1.1 to 2.3 M) and 100 mM sodium citrate at pH 5.5. Crystals appeared in 2 weeks. The crystals of apo protein were transparent with dimensions of 0.1 × 0.1 × 0.05 mm. The crystals of the RET–nintedanib complex were yellow, had the shape of a flower petal, and were much more bulky. The crystals were dipped into N-paratone and flash-cooled in liquid nitro-gen. Diffraction data for all structures were collected at the Stanford Synchrotron Radiation Lightsource Beamlines 7-1 and 14-1 at −173 °C. The diffraction images were processed with the program HKL2000 (29). The structures were determined by molecular replacement using the program Molrep (30) and difference Fourier maps. The models were checked, corrected, and initially validated with the computer graphics program Coot (31). The coordinates were refined with Refmac (32). The coordinates of nintedanib were made with the program PRODRG (33). The structure was validated with MolProbity run inside of Coot (34). The final validation was carried out by the validation server of the OneDep deposition system of the wwPDB (35). Comparison of the structures were done with Coot and the superpose program from the CCP4 suite (36). The figures were made with UCSF Chimera (37) or PyMOL (38).

**Co-crystal structure of RET and nintedanib complex**

The BaF3 cell line stably expresses a KIF5B–RET fusion protein (BaF3/KR) has been reported (14, 15). Mutagenesis of BaF3/KR cells using ENU was performed similar to that described (39). ENU-treated cells were cultured in the presence of 2.4 μM vandetanib for 3 weeks to select for the drug–resistant cells, after which drug-resistant cell clones were isolated from semisolid methylcellulose cultures. Genomic DNA was purified, and the KIF5B–RET kinase domain-coding region was sequenced in both strands to check for the presence of mutation(s). All five clones (nonindependent) analyzed had the p.L881V mutation that was due to a C → G change at the L881V codon (CTG → GTG). No other mutation in the KIF5B–RET kinase domain was found.

To avoid possible unknown mutation caused by ENU in BaF3/KR(L881V) cells, BaF3/KR(L881V) cells were recreated by infecting BaF3 cells with lentiviruses encoding the KIF5B–RET(L881V) mutant similar to that described (15). The KIF5B–RET(L881V) cDNA was generated by site-directed mutagenesis using PCR. The DNA sequence was verified by DNA sequencing.

The cell viability assay was performed using the CellTiter-Glo reagent (Promega, Madison, WI) (15). The data were from three biological replicates each performed in three technical replicates. Curve fitting was performed using the GraphPad Prism 6 software as described (40). Cell lysate preparation and immunoblotting analysis were performed similar to methods described in Refs. 15 and 41.

**Synthesis of nintedanib and vandetanib analogs**

Compound 1 (methyl (3Z)-3-[[4-[methyl-[2-(4-methyl-piperazin-1-yl)acetyl]amino]anilino]methylidene]-2-oxo-1H-indole-6-carboxylate; PubChem CID: 11605072) and compound 2 were synthesized as described in the supporting procedures. The purities of compounds 1 and 2 were 95.2 and 97.8%, respectively (Fig. S1).

To substitute the O-methyl group in vandetanib with a bulkier component, vandetanib was demethylated to yield hydroxyl-vandetanib. Compound 3 was made by coupling benzyl bromide with hydroxyl-vandetanib as described in the supporting procedures.
Co-crystal structure of RET and nintedanib complex

**Author contributions**—S. T. T., T. S., X. L., Q. H., P. T., M. Z., J. C., B. H. M. M., and J. W. data curation; S. T. T., T. S., X. L., B. H. M. M., and J. W. formal analysis; S. T. T., T. S., X. L., Q. H., P. T., M. Z., J. C., B. H. M. M., and J. W. visualization; S. T. T., T. S., X. L., P. T., M. Z., J. C., B. H. M. M., and J. W. methodology; S. T. T., T. S., X. L., M. Z., B. H. M. M., and J. W. writing-original draft; S. T. T., T. S., X. L., P. T., F. H., J. C., B. H. M. M., and J. W. writing-review and editing; F. H., J. C., B. H. M. M., and J. W. resources; B. H. M. M. and J. W. conceptualization; B. H. M. M. and J. W. methodology; S. S. T., T. S., X. L., Q. H., P. T., M. Z., J. C., B. H. M. M., and J. W. visualization; B. H. M. M. and J. W. funding acquisition; B. H. M. M. and J. W. validation; B. H. M. M. and J. W. project administration.

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