Resistance to Avapritinib in PDGFRA-Driven GIST Is Caused by Secondary Mutations in the PDGFRA Kinase Domain 🔐

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ABSTRACT

Gastrointestinal stromal tumors (GIST) harboring activating mutations of PDGFRA respond to imatinib, with the notable exception of the most common mutation, D842V. Avapritinib is a novel, potent KIT/PDGFRA inhibitor with substantial clinical activity in patients with the D842V genotype. To date, only a minority of PDGFRA-mutant patients treated with avapritinib have developed secondary resistance. Tumor and plasma biopsies in 6 of 7 patients with PDGFRA primary mutations who progressed on avapritinib or imatinib had secondary resistance mutations within PDGFRA exons 13, 14, and 15 that interfere with avapritinib binding. Secondary PDGFRA mutations causing V658A, N659K, Y676C, and G680R substitutions were found in 2 or more patients each, representing recurrent mechanisms of PDGFRA GIST drug resistance. Notably, most PDGFRA-mutant GISTs refractory to avapritinib remain dependent on the PDGFRA oncogenic signal. Inhibitors that target PDGFRA protein stability or inhibition of PDGFRA-dependent signaling pathways may overcome avapritinib resistance.

SIGNIFICANCE: Here, we provide the first description of avapritinib resistance mechanisms in PDGFRA-mutant GIST.

INTRODUCTION

Gastrointestinal stromal tumors (GIST) are the most common sarcoma subtype and are thought to arise from oncogenic transformation of interstitial cells of Cajal (ICC) precursors found in the smooth muscle wall of the stomach and intestines (1, 2). Mutations of KIT or PDGFRA, both of which are intrinsically expressed at high levels during ICC differentiation, lead to constitutive activation of these kinases (3). Activating mutations in either KIT or PDGFRA are found in most GISTs and appear to function as the initiating oncogenic events (3-5).

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PDGFRA and KIT map to adjacent genomic loci on Ch4q12, and their protein structures are highly homologous. Both belong to the type III receptor tyrosine kinase family, which is characterized by an extracellular ligand binding domain, a transmembrane domain, and an intracellular catalytic domain (6). Effective biochemical inhibition of mutant forms of KIT and PDGFRA strongly correlates with clinical response in patients with GIST with metastatic disease (7). Primary, activating KIT mutations are most commonly found in the intracellular juxtamembrane domain (JM; exon 11) or in the extracellular dimerization domain (exon 9), with only rare cases involving the ATP-binding domain (exon 13) or activation loop (exon 17; ref. 3). In contrast, in PDGFRAmutant GIST, the majority of primary oncogenic mutations are found in the activation loop (AL; exon 18) and commonly involve codon 842, and more rarely affect the JM (exon 12) or ATP-binding domain (exon 14). GISTs that do not exhibit mutations in either KIT or PDGFRA often display alternative mechanisms (e.g., mutations of NF1, PI3K, BRAF, KRAS, FGFR, and NTRK3) to constitutively activate the same downstream pathways that are typically activated by oncogenic KIT or PDGFRA (8, 9).

The spectrum of primary KIT or PDGFRA mutations found in GIST is broad, involving point mutations, deletions, and insertions-deletions, and the various oncogenic mutants exhibit different enzymatic, cellular, and protein dynamic properties (7, 10). Not surprisingly, these mutations are also predictive for the response to KIT and PDGFRA inhibitors (7, 11, 12). As seen in other kinase-driven cancers treated with kinase inhibitors, KIT-mutant GISTs that progress after response to KIT inhibitors very frequently contain secondary mutations within the ATP-binding domain (exons 13-14) or the AL (exons 17-18) of KIT, as these types of mutations disrupt drug binding (11, 12). In contrast, little is known about mechanisms of resistance in PDGFRA-mutant GIST-except for primary or secondary PDGFRA^{D842V} mutations. This particular mutation is resistant to the initially approved kinase inhibitors for GIST, and therefore patients with advanced $PDGFRA^{D842V}$ -mutant

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GIST have historically had a poor prognosis (13, 14). Recently, avapritinib (BLU-285), a novel, fourth-generation type I kinase inhibitor specifically designed to inhibit PDGFRA^{D842V}, has been shown to be highly potent against this mutation, with biochemical IC₅₀ values in the low nanomolar range (15). Clinical data suggest efficacy of avapritinib for D842Vmutant GIST that parallels or even exceeds the activity of imatinib in KIT exon 11-mutant GIST (15, 16). Notably, in January 2020, avapritinib was approved by the FDA for first-line treatment of GIST with PDGFRA mutations in exon 18. Despite the profound clinical responses of patients with PDGFRA-mutant GIST, some patients with PDGFRA mutations treated within the recent NAVIGATOR trial [NCT02508532; A Phase I Study of BLU-285 in Patients with Gastrointestinal Stromal Tumors (GIST) and Other Relapsed and Refractory Solid Tumors] have progressed following an initial response to avapritinib. Here we describe, for the first time, the mechanisms of resistance to avapritinib in PDGFRA-mutant GIST based on 7 patient cases and investigate salvage therapies to overcome resistance in vitro.

RESULTS

Clinical Evidence and Significance of Resistance

The NAVIGATOR trial is a two-part, open-label, doseescalation/expansion study that investigated avapritinib in patients with PDGFRA^{D842V}-mutant GIST or GIST with other mutations that had progressed on imatinib or post-imatinib tyrosine kinase inhibitors (16). In total, 43 patients with PDGFRA exon 18-mutant GIST have been treated within the trial at the 300-mg and 400-mg dose levels, of whom 84% had an objective radiologic response, as assessed by mRECIST1.1 criteria. As of November 2019, 11 patients, representing 26% of this cohort, have experienced true on-treatment progression. Given the lack of therapeutic alternatives, most patients continued treatment beyond progression, but some underwent routine diagnostic biopsies or therapeutic resections of progressing lesions. Median overall survival from time after documented progression was only 5.2 months and a mere 0.5 months from the last dose of avapritinib (Supplementary Fig. S1A). We sought to identify mechanisms of resistance in these patients.

Circulating tumor DNA (ctDNA) is potentially useful in investigating resistance to kinase inhibitors, as plasma sequencing potentially offers a broader picture of clonal heterogeneity than needle biopsy of a single lesion. Recently, several clinical studies investigated ctDNA levels in patients with *KIT*-mutant GIST, with most patients having a low abundance of ctDNA compared with many common cancers (17). As an exploratory study within the NAVIGATOR trial, we investigated the usefulness of plasma sequencing for detection of mutational information from PDGFRA-mutant GIST (18). ctDNA was isolated from 60 patients at baseline and analyzed by either BEAMing (beads, emulsion, amplification, magnetics-digital PCR) or panel next-generation sequencing (NGS). The genotype of the primary PDGFRA mutation was known for all patients based on local tumor biopsy sequencing results. In total, mutant ctDNA was detected in 63% (38/60) of patients (Fig. 1A). Using BEAMing technology, the detection rate of the primary mutation was 64% (14/22) compared with 53% (20/38) for NGS. In patients with detectable ctDNA, the mean allelic frequency (MAF) of the primary mutations was 3.49% (Fig. 1A). In plasma samples that were analyzed by panel NGS, only the primary PDGFRA mutation was detected. We then calculated the sum of all target lesions of each patient and correlated tumor load with levels of mutant plasma DNA. In patients with detectable ctDNA, the median sum of target lesions (as per mRECIST version 1.1) was 18.2 cm, compared with 7.5 cm for those without detectable ctDNA. This suggests that ctDNA detection might be influenced, and potentially limited, by individual patient tumor burden.

Among the 11 patients harboring PDGFRA exon 18 mutations progressing on avapritinib, pre- and post-progression plasma and/or tumor samples were available for four patients. Patients 1, 2, and 3 harbored typical D842V point mutations, whereas patient 4 had an in-frame deletion in exon 18 (I843_ D846del, also referred to as delDIMH842-845 in the literature). These patients had received avapritinib at initial doses of 60 to 300 mg that resulted in tumor shrinkage by mRECIST version 1.1 or reduction of density of metastatic lesions, with maximum responses observed after 6 to 8 months of treatment and concordant disappearance of mutant ctDNA from plasma (Fig. 1B). Evidence of progression was found by radiographic imaging after 8.5 to 17 months of therapy, albeit the first progression in patient 4 was detected during a treatment break to manage side effects (Fig. 1B). Mutant ctDNA became detectable again in the plasma at the time of clinical progression in all four patients. All four patients underwent diagnostic biopsies and/or surgical removal of tumors postprogression.

Mutational analysis was performed on post-progression plasma and/or tumor samples to identify mechanisms of resistance. Although pretreatment samples of all patients included in this analysis exclusively showed primary *PDGFRA* mutations, post-progression samples revealed various mechanisms of resistance. Patients 1, 3, and 4 had several novel *PDGFRA* mutations detected using both plasma and tumor sequencing. These novel mutations were found in

Figure 1. Correlation of tumor size and treatment with the detection of mutant DNA in plasma. **A**, Plasma sequencing of 60 patients treated within the NAVIGATOR trial at baseline using either BEAMing or NGS sequencing. Bar graphs show allelic frequency of *PDGFRA* mutations in ctDNA. Detection rate of mutant DNA depending on the detection method; X = method used. For non-D842V row 1 = 1843 D846del; 2 = N659K; 3 = D1842 843V. **B**, CT images of avapritinib-treated patients with *PDGFRA*-mutant GIST at baseline, at the time of best response, and at time of progression. Total tumor diameter over treatment time and MAF of *PDGFRA* primary mutations as measured by BEAMing (patients 1 and 2) or PGDx PlasmaSelect64 assay (patients 3 and 4) is plotted below (%surgery, lesion classification using mRECIST1.1 criteria). Note re patient 3: The deep pelvic lesion depicted at baseline was located closer to the pelvic inlet in the follow-up scan (BR) and was later surgically resected. BR, best response; P, progressive disease.

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exons 13, 14, and 15, involving codons 598, 652, 658, 674, 676, and 680 (Table 1). Interestingly, in the case of patient 1, one lesion included compound (intra-allelic) secondary mutations G652E and V658A, whereas other lesions in this patient showed only V658A. Exome sequencing of the resistant tumor in patient 1 revealed this mutation to be homozygous (Supplementary Table S1), confirming that most, if not all, mutations must be in cis. In addition, mutations of codons 658 and 680 were found independently in other patients whose GIST had different primary mutations, suggesting that codon 658 and 680 mutations represent a recurrent cause of resistance. To exclude clonal hematopoiesis as a source for mutant PDGFRA in plasma, we analyzed all plasma samples from the NAVIGATOR, VOYAGER, and the ARROW studies for the presence of PDGFRA mutations. Notably, no mutations of PDGFRA were found in non-PDGFRA-mutant patients from the VOYAGER (GIST; n =417) or ARROW (non-small cell lung cancer; n = 398) studies (Supplementary Table S2).

Whole-exome sequencing of pre- and post-progression tumor samples was performed in patient 1 to gain a more complete view of mutations that could be contributing to avapritinib resistance, but only *PDGFRA* mutations were identified as relevant genomic findings (Supplementary Table S1).

Only one patient (patient 2) lacked secondary PDGFRA mutations post-progression. Plasma sequencing identified mutations in genes that could constitutively activate the PI3K pathway and/ or the MAPK pathway (TSC1 and NF1, respectively). In the context of KIT-mutant GIST, these mutations have been shown to supplant KIT as drivers (19) and confer resistance to KIT inhibitors in vitro (18). For avapritinib-naïve PDGFRA-mutant GIST, mutations involving these pathways have not been reported (14). These events are likely underreported in routine pathology reports but may become more relevant with the development of more potent KIT inhibitors. In the present study, both genomic events were found in post-progression tumor or plasma samples in only a single patient; further studies are needed to determine the clinical relevance of these mutations in the context of PDGFRA-mutant GIST. As baseline tumor material was not available, it is unclear if these mutations were preexisting or emerged during avapritinib treatment (19).

Little is known about mechanisms of imatinib resistance in patients with primary, imatinib-sensitive (non-D842V) PDGFRA mutations. We hypothesized that mutations that confer resistance to imatinib might also confer cross-resistance to avapritinib. This information could provide clinically relevant information to guide the management of patients with PDGFRA-mutant GIST. In addition to patients treated within the NAVIGATOR trial, we further searched institutional databases for patients having secondary PDGFRA mutations that could represent potential mechanisms for imatinib resistance. We identified one patient (patient 5) with a primary PDGFRA exon 14 mutation, N659K, who had previously progressed on imatinib, sunitinib, and regorafenib (Table 1). Post-progression samples from this patient's tumors, taken following progression on imatinib and sunitinib, harbored a second mutation resulting in a T674R substitution affecting the gatekeeper residue (20). Through participation in a compassionate-use program, patient 5 was treated with avapritinib at a dose of 200 mg

daily but exhibited rapid progression (Supplementary Fig. S1B). This suggests that mutations at the gatekeeper residue may also confer resistance to avapritinib. Two other patients with imatinib-sensitive, *PDGFRA* exon 18 primary mutations were both found to have the G680R secondary mutation in the plasma with an allelic frequency of 8.8% (patient 6) and 0.8% (patient 7). Patient 7 received avapritinib afterward within the VOYAGER trial and attained a partial response as best response but had a relatively short progression-free survival of 11.1 months compared with the median progression-free survival of patients treated in the NAVIGATOR study.

Validation of Secondary Resistance Mutations *In Vitro*

To validate the putative resistance mutations found in clinical samples, we created cell line models to assess the effects of imatinib and avapritinib on PDGFRA tyrosine phosphorylation. First, we created Ba/F3 cell lines driven by the primary mutations observed in patients from the NAVIGATOR trial (PDGFRA^{D842V} or PDGFRA^{I843-D846del}). As expected, the primary mutation seen in patients 1 to 3, PDGFRA^{D842V}, was highly sensitive to avapritinib ($IC_{50} = 4 \text{ nmol/L}$), but resistant to imatinib (Fig. 2A-I; Supplementary Figs. S2A-S2M and S3A-S3I). We then investigated the effect of the secondary mutations seen in patient 1 on sensitivity to avapritinib and observed different levels of avapritinib resistance with each mutation. The mutant kinase with an exon 13 secondary mutation, PDGFRA^{D842V/V598F}, conferred significant resistance to avapritinib (IC₅₀ = 464 nmol/L). The double mutant PDGFRA^{D842V/V658A} exhibited a greater than 5-fold higher IC₅₀ value for inhibition of PDGFRA phosphorylation (26 nmol/L) compared with PDGFRA^{D842V} (Fig. 2; Supplementary Fig. S2). The addition of a third mutation, resulting in PDGFRA^{D842V/V658A/G652E}, further increased resistance with a more than 40-fold higher IC50 value (170 nmol/L) compared with the D842V-only mutant. The addition of G680R to D842V (PDGFRA^{D842V/G680R}) was associated with the highest level of resistance to avapritinib with an IC_{50} value for PDGFRA phosphorylation above 1,000 nmol/L. Despite being identified at a low allelic fraction in the plasma of patient 1, we confirmed that $\text{PDGFRA}^{\text{D842V/QGPins}}$ conferred biochemical resistance to avapritinib ($IC_{50} = 102 \text{ nmol/L}$). In addition, the Y676C secondary mutation identified in patient 3 increased the IC₅₀ value of avapritinib by more than 100fold over PDGFRA^{D842V} alone (IC₅₀ = 484 nmol/L).

We then used a Ba/F3 model driven by PDGFRA^{I843-D846del} to model the mutations seen in patient 4. This primary mutation was sensitive to avapritinib (IC₅₀ = 2 nmol/L), as well as imatinib, as expected (Fig. 2; Supplementary Figs. S2 and S3). Of the three secondary mutations found in patient 4, two (V658A and G680R) were also seen in patient 1 in the context of the PDGFRA^{D842V} primary mutation. Similar to what was seen in patient 1 (PDGFRA^{D842V}), these mutations conferred resistance to avapritinib in the context of PDGFRA^{I843-D846del}, but to different extents: PDGFRA^{I843-D846del/V658A} had an IC₅₀ value 24-fold higher than the primary PDGFRA^{I843-D846del} alone (48 nmol/L), whereas PDGFRA^{I843-D846del/G680R} was highly resistant (>1,000 nmol/L). The exon 14 mutation N659K was seen as a secondary mutation in conjunction with PDGFRA^{I843-D846del}

Table 1. Oncogenic mutations found in patients with PDGFRA-mutant GIST progressing on kinase-inhibitory treatment

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	Patient	Treatment stage	PDGFRA mutation	Exon	Sample origin	Allelic fraction	Method
NAVIGATOR trial	1	Pre-ava	D842V	18	Tumor	89%	А
					Plasma	4.4%	В
		Post-ava	D842V	18	Tumor	91%	А
			G652E ^a	14		89.8%	
			V658Aª	14		90.3%	
			D842V	18	Plasma	12.1%	С
			V598F	13		1.1%	
			G652E	14		7.1%	
			V658A	14		1.2%	
			G680R ^b	15		3.3%	
			G680Rc	15		0.06%	
			V658A	14		1.5%	D
			G680R	15		9.1%	
			QGPins	14		0.2%	
	2	Pre-ava	D842V	18	Tumor	n.k.	Е
					Plasma	0.48%	В
		Post-ava	D842V	18	Tumor ^d	88.2%	А
			D842V	18	Plasma ^e	4.1%	D
	3	Pre-ava	D842V	18	Tumor	40.1%	F
					Plasma	0.74%	С
		Post-ava	D842V	18	Tumor ^f	39.3%	G
			D842V	18	Tumor	40.9%	G
			D842V	18	Plasma	1.2%	D
			Y676C	15		0.9%	
	4	Pre-ava	1843_D846del	18	Tumor	50% ^f	Е
					Plasma	1.05%	С
		Post-ava	1843_D846del	18	Tumor #1	96%	Н
			G680R	15		37%	
			1843_D846del	18	Tumor #2	76%	Н
			V658A	14		14%	
			G680R	15	Plasma	0.8%	Н
			N659K	14		0.9%	
Other	5	Pre-im	N659K	14	Tumor	n.k.	В
		Post-im	N659K	14	Tumor	34%	G
			T674R	15		33%	
			N659K	14	Plasma	2.2%	D
			T674R	15		1.4%	
	6	Pre-im/su	unknown				-
		Post-im/su	D842_D846delinsE	18	Plasma	16.3%	G
			G680R	15		8.8%	
	7	Pre-im/su/sora	unknown				-
		Post-im/su/sora	D842_D846delinsV	18	Plasma	11.1%	G
			G680R	15		0.8%	
			Y676C	15		0.2%	

NOTE: Pathogenic mutations found in pre- and post-progression tumor and circulating plasma DNA in patients treated in the NAVIGATOR trial (patients 1–4; ava, avapritinib) as well as genomic findings in three patients with non-D842V GIST pretreated with imatinib (im) and sunitinib (su; patients 5 and 6) and with imatinib, sunitinib, and sorafenib (sora; patient 7), in a routine clinical setting. Primary mutations are boldface. In patients 6 and 7, baseline sequencing was not available but D842V_D846delins are previously described primary mutations. Methods used for tumor sequencing:

A: Whole-exome sequencing (Genewiz); B: BEAMing; C: NGS Panel Sequencing (PGDx Plasma Select64); D: NGS Panel Sequencing (Guardant 360); E: Sanger sequencing; F: NGS Panel Sequencing (PGDx Cancer Select 125); G: NGS Panel Sequencing (Qiagen customized cancer panel CCP V3.0, University Hospital Essen, Germany); H: OHSU NGS panel.

^aMutations found on the same allele.

^b2038G>A.

°2038G>C.

^dAdditional downstream mutation found: TSC1 deletion (AF 10.9%) and

eNF1 F2156V (AF 0.1%).

^fProgression during treatment break.

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Figure 2. PDGFRA-mutant models display varying levels of avapritinib resistance. **A-I**, Immunoblot quantification of the effect of avapritinib on phosphorylation of PDGFRA mutants observed in patient 1 (**A-D**) or patient 4 (**F-H**) modeled in Ba/F3 cell lines, and PDGFRA mutants observed in imatinibresistant patients 3 (**E**) and 5 (**I**). Mutations involving N659K were not able to support IL3-independent Ba/F3 growth; these mutants were modeled using a transfection system in CHO cells. Data points represent average of at least 3 independent experiments with SEM shown. Nonlinear regression curve shown for each data set was used to calculate IC₅₀ (GraphPad Prism).

--- I843-D846del/N659K

1843-D846del

in patient 4. When tested in our model, PDGFRA^{I843-D846del/N659K} increased the IC₅₀ value for avapritinib 15-fold (30 nmol/L). Of the secondary mutations seen in patient 4, all also conferred resistance to imatinib compared with PDGFRA^{I843-D846del} alone (Supplementary Fig. S3). Of note, in the context of the imatinib-sensitive PDGFRA^{I843-D846del} primary mutation, cells with additional G680R remained moderately sensitive to imatinib (IC₅₀ = 46 nmol/L), whereas PDGFRA^{I843-D846del/N659K} and PDGFRA^{I843-D846del/N659K} conferred resistance to imatinib (IC₅₀ > 200 nmol/L; Supplementary Fig. S3).

--- I843-D846del/G680R

1843-D846del

In addition, we modeled both the primary mutation (PDGFRA^{N659K}) and secondary mutation (PDGFRA^{N659K/T674R}) seen in patient 5, who had previously progressed on imatinib and sunitinib before tissue analysis and rapidly progressed on avapritinib afterward. We found that PDGFRA^{N659K} was resistant to imatinib (IC₅₀ = 253 nmol/L) and also to avapritinib (IC₅₀ = 91 nmol/L) compared with PDGFRA^{D842V} (Fig. 2; Supplementary Figs. S2 and S3). PDGFRA^{N659K/T674R} conferred strong resistance to both avapritinib (IC₅₀ > 1,000 nmol/L), suggesting the

gatekeeper residue also plays an important role in avapritinib binding.

--- N659K

N659K/T674R

PDGFRA-Driven GIST Models Recapitulate Clinical Resistance

Prior to this study, no *PDGFRA*-mutant human GIST cell lines were available. We were able to derive a GIST cell line from a post-progression tumor sample from patient 1, which we used to investigate avapritinib resistance in this patient (Fig. 3A). This primary *PDGFRA*-mutated cell line (Trsh1) harbors the same triple mutant (PDGFRA^{D842V/V658A/G652E}) that was found in the tumor biopsy. Both the cell line and the tumor biopsy had identical short tandem repeat profiles (Supplementary Fig. S4). This cell line was confirmed to express activated PDGFRA, which demonstrated biochemical resistance to avapritinib and other currently approved GIST therapies, as predicted by Ba/F3 modeling and clinical data (Fig. 3B). Further, Trsh1 cells showed activated PDGFRAdependent signaling, which was inhibited by tanespimycin (Fig. 3C). These cells remained dependent on PDGFRA

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Figure 3. Patient-derived PDGFRA-mutant cell line demonstrates avapritinib resistance *in vitro*. Validation of Trsh1 cell line derived from a tumor from patient 1 with PDGFRA^{D842V/V658A/G652E} mutation. **A**, Microscopic bright field image of Trsh1 growing at passage 19. **B**, Sulforhodamine B (SRB) assays to determine sensitivity of Trsh1 to approved KIT/PDGFRA inhibitors (some data points from this panel also appear in Fig. 5A). **C**, Immunoblot of Trsh1 cells untreated or treated (24 hours) with 500 nmol/L tanespimycin.

signaling, as knockdown of PDGFRA reduced cell viability (Supplementary Fig. S5A and S5B).

In Silico Modeling of Mutations in PDGFRA

Because the resistance mutations identified in these *PDGFRA*-mutant patients treated with avapritinib are novel, we evaluated potential structural explanations for the observed resistance. The kinase domain of type III receptor tyrosine kinases is subject to an equilibrium between inactive and active conformations (6). In the inactive state, the active site is blocked by the autoinhibitory conformation of the JM. To this end, D842 stabilizes the autoinhibited conformation within the AL (Fig. 4A). In the active and catalytically competent state, the JM is stabilized distant from the binding site by tyrosine side-chain phosphorylation, the helix α C takes an inward rotated position, and the AL adopts an extended

conformation with a characteristic DFG-*in* motif. This conformation is favored by the PDGFRA^{D842V} mutation, which destabilizes the helical segments within the AL. Notably, in this conformation, type I inhibitors, such as avapritinib or crenolanib, can access the binding site for efficient kinase inhibition (6).

To better understand the conformational consequences of these mutations on protein structure, we performed *in silico* modeling for the most common PDGFRA mutants. Because the D842V mutation facilitates avapritinib binding, we assessed its binding mode using docking studies with a D842V-mutated molecular dynamics (MD) simulated homology model obtained for PDGFRA using KIT as the template structure. The binding mode is characterized by a hydrogen bond of the core scaffold with C677 within the hinge region. The fluorobenzyl moiety is wrapped



Figure 4. Conformational consequences of avapritinib-resistance *PDGFRA* mutations. *In silico* modeling of human PDGFRA. **A**, D842 within the AL (yellow) stabilizes the AL in the inactive DFG-out conformation by forming a network of polar contacts within the helical turn motif. **B**, Binding mode of avapritinib (MD simulation of a homology model, docked with avapritinib). **C**, Mutation G680R within the binding site sterically interferes with avapritinib binding. The mutations T6741 (gray; PDB ID: 6A32) and V658A alter the hydrophobic properties of the binding cleft and result in reduced binding affinity. **D**, Alignment of MD simulation snapshots with ADP-bound PDGFRA harboring D842V/V658A or D842V/V658A/G652E mutations. **E**, Alignment of an MD simulation snapshot with PDGFRA-P653 H654insQGP mutation (gray and red) with similarly mutated EGFR-D770 N771insNPG (blue and gray; PDB ID: 6A32; G680R was applied using the Mutagenesis Wizard of PyMoI) with ABL^{T3151} (PDB ID: 3IK3) and RET^{G810A} (PDB ID: 6NE7).

around the catalytic lysine (K627) and the DFG's aspartate (D836), which are engaged in polar contacts (Fig. 4B). This binding mode is incompatible with the mutation G680R within the kinase front pocket, which sterically interferes with avapritinib binding (Fig. 4C). The threonine-toisoleucine mutation, T674I, was used to assess the gatekeeper moiety by an alignment with a crystal structure of PDGFRA^{T6741} (PDB ID: 6A32, shown in gray; Fig. 4C), which disrupts a polar water-mediated network between A625, L627, and avapritinib. The valine at position 658 is in close interaction proximity to the avapritinib core binding site, and its substitution to alanine (V658A) might account for a loss in binding affinity resulting in drug resistance (Fig. 4C). Tyrosine at codon 676 serves as a "roof" for avapritinib binding and the interaction is lost with the cysteine substitution (Y676C; Fig. 4C).

Modeling of mutations located next to the regulatory helix α C, that is, PDGFRA^{G652E} and PDGFRA^{QGPins}, however, did not result in an altered binding site (Fig. 4D and E). These mutations might act through altered dynamics of the adjacent helix or the binding motif recognized by interacting proteins. PDGFRA^{QGPins}, PDGFRA^{T674I}, and PDGFRA^{G680R} correspond to analogous resistance mutations in other oncogenic kinases (Fig. 4E and F), further supporting the hypothesis that these mutations confer drug resistance.

Overcoming Resistance to Avapritinib

We evaluated whether mutations that emerged following treatment with avapritinib described herein are crossresistant to other drugs or drug classes that are currently in clinical development for treatment of GIST. Crenolanib is another type I kinase inhibitor that has shown efficacy



Figure 5. Focused drug screen in avapritinib-resistant GIST cell lines. **A**, SRB assays to compare the efficacy of different inhibitors in parental GIST-T1, GIST-T1^{D842V}, and Trsh1 (some data points from this panel also appear in Fig. 3A). **B**, Caspase Glo assays to determine induction of caspase cleavage for parental GIST-T1, GIST-T1^{D842V}, and Trsh1 to approved KIT/PDGFRA inhibitors or inhibitors in clinical development. **C**, Validation of substrate inhibition by western blot in parental GIST-T1, GIST-T1^{D842V}, Trsh1, and CRISPR-generated cell line T1- $\alpha^{D842V/G652E/V658A}$. (continued on next page)

GIST-T1D842V

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against PDGFRA^{D842V}, whereas ripretinib (DCC-2618) is a type II switch pocket inhibitor that recently was approved as a fourth-line treatment for advanced GIST (6, 21–23). Using our Ba/F3 models, we determined the effect of these additional kinase inhibitors on the various avapritinib resistance mutations to identify salvage therapies. In general, no other inhibitor outcompeted avapritinib against the secondary mutations examined (Supplementary Fig. S6A–S6M). One exception was the PDGFRA^{D842V/V598F} mutation, which was resistant to all approved drugs but sensitive to crenolanib (IC₅₀ = 7 nmol/L; Supplementary Fig. S6B). Another exception was PDGFRA^{I843-D846del/G680R}, which was more sensitive to imatinib (IC₅₀ = 46 nmol/L) and ripretinib (IC₅₀ = 200 nmol/L), compared with an IC₅₀ value more than 3,000 nmol/L for avapritinib (Supplementary Fig. S6I).

To confirm these results in a GIST cellular context, we evaluated the effect of these drugs on the viability of our *PDGFRA*-mutant GIST cell line, Trsh1, and the reference cell line GIST-T1^{D842V}, expressing exogenous PDGFRA^{D842V}. GIST-T1^{D842V} was resistant to imatinib and ripretinib, but strongly inhibited by avapritinib and crenolanib (Fig. 5A). Unfortunately, Trsh1 (PDGFRA^{D842V/G652E/V658A}) exhibited resistance to all direct KIT/PDGFRA inhibitors, indicating these drugs cannot be used as salvage therapy for this specific mutation (Fig. 5A–C).

Trsh1^{D842V/G652E/V658A}

To assess further secondary mutations in a GIST context, we used CRISPR/Cas9-mediated gene editing to generate additional isogenic GIST-T1 sublines (designated T1- α), harboring mutations identified in patients within the endogenous *PDGFRA* gene (Fig. 5D and E). Parallel Cas9-mediated

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T1-α^{D842V/G652E/V658A}

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Figure 5. (Continued) D, Overview of PDGFRA protein domains and exons involved in avapritinib resistance. Exons 13–15 (and their respective amino acid ranges), comprising the ATP-binding domain, are shown in more detail. Mutated amino acid residues discovered in patients and their approximate position are displayed. **E**, Secondary mutations discovered in patients were modeled in an isogenic cell line panel of GIST-T1. All cell lines carry the activating D842V mutation in exon 18. Additional resistance mutations were introduced into the ATP-binding domain. The pseudo heat map displays IC₅₀ values (nmol/L) for different PDGFRA and HSP90 inhibitors in each subline: green = sensitive; yellow = intermediate; red = resistant. IM, imatinib; Ava, avapritinib; Creno, crenolanib; Rip, ripretinib; Tram, trametinib; Tanes, tanespimycin; Lumi, luminespib; Ganet, ganetespib; n.r. = not reached at 10,000 nmol/L.

knockout of mutant KIT led to a kinase switch and complete dependence upon mutant PDGFRA activity. Interrogating this panel for inhibitor sensitivity, we confirmed that although D842V-only mutants were sensitive to avapritinib and crenolanib, all clinically observed secondary mutations conferred resistance to avapritinib.

Based on these data, there is currently no direct inhibitor of PDGFRA available that potently inhibits these PDGFRA double and triple mutants. Possible exceptions could be mutations involving codons V598 and T674, where crenolanib may be active. Of note, the substitution at T674 was found in the context of an N659K primary mutation and not a D842V. Given the homology to KIT and ABL, we have additionally modeled both isoleucine and arginine substitutions as those are likely to be affected in the common D842V primary mutation (Fig. 5E; ref. 24).

In preclinical models of KIT-mutant GIST, inhibitors of HSP90 as well as inhibitors of KIT-activated signaling pathways can overcome resistance regardless of the secondary resistance mutation, but this has not been tested in PDGFRA-mutant GIST (20, 21). Notably, the HSP90 inhibitor tanespimycin (17-AAG), a derivative of the antibiotic geldanamycin, exerted potent activity in the multiresistant Trsh1 cell line (IC_{50} value: 70 nmol/L; Fig. 5A). Several other HSP90 inhibitors currently in clinical development show equal or more potent inhibition of triple-mutant PDGFRA, with luminespib (8 nmol/L) and ganetespib (60 nmol/L) exhibiting the lowest total growth inhibition (TGI; Supplementary Fig. S7). Furthermore, HSP90 inhibition was the only treatment associated with induction of apoptosis as measured by caspase-3/7 activation (Fig. 5B). Immunoblotting studies revealed potent inhibition of PDGFRA expression and signaling in the GIST-T1 parental and isogenic sublines, as well as Trsh1, upon tanespimycin treatment (Fig. 5C).

Notably, all cell lines in this panel also proved to be highly sensitive to HSP90 inhibition by tanespimycin (IC₅₀ = 9–75 nmol/L), luminespib (IC₅₀ = 5–35 nmol/L), and ganetespib (IC₅₀ = 8–35 nmol/L; Fig. 5E). We further tested inhibitors of downstream pathways of PDGFRA as potential salvage treatments in avapritinib-resistant cells. In addition, trametinib, an inhibitor of MEK, inhibited growth of Trsh1 cells when near-complete target inhibition (pERK) was reached (100 nmol/L; Fig. 5A and C).

DISCUSSION

Activating mutations of the PDGFRA receptor tyrosine kinase were described more than 15 years ago, but until recently, no inhibitors with significant clinical activity against the notoriously resistant D842V mutation had been identified (4). Avapritinib has been shown to be a highly potent inhibitor of PDGFRA^{D842V} with IC_{50} values in the subnanomolar range (15). A phase I trial of avapritinib revealed an 84% objective response rate and tumor shrinkage in more than 95% in this D842V-mutant cohort. Tumor shrinkage was evident even in the earliest dose cohorts of the escalation phase (16). In January 2020, avapritinib was approved by the FDA for the treatment of GIST with mutations in PDGFRA exon 18. Thus, avapritinib represents a therapeutic breakthrough with activity similar to that of imatinib against untreated, advanced KIT exon 11-mutant GIST. To date, only a minority of patients with PDGFRA-mutant tumors treated with avapritinib have developed resistance-underscoring the unique potency of avapritinib against the PDGFRA^{D842V} activating mutation. However, patients whose tumors develop avapritinib resistance have extremely poor outcomes, with a median overall survival of 5.2 months after clinical progression and a median survival of <1 month after stopping avapritinib (Supplementary Fig. S1A). Understanding mechanisms of avapritinib resistance and identification of salvage therapies are therefore of utmost importance in this subgroup of patients.

Currently, plasma sequencing of ctDNA has not been clinically validated for routine use in the management of patients with GIST but represents a potential tool for the early detection of resistant tumor clones. We report that the primary PDGFRA mutation (e.g., D842V) is detectable in the majority of patients using BEAMing or NGS technology, with the latter showing a slightly lower detection rate (Fig. 1A). To date, only a minority of patients have progressed on avapritinib, and because the protocol did not include mandatory postprogression plasma sample collection, only limited numbers of such samples were available. Nonetheless, we were able to identify resistance mutations via plasma sequencing in all seven patients for whom plasma was available. No secondary PDGFRA mutations were found in the pretreatment plasma sequencing samples of patients in the NAVIGATOR study. This supports the hypothesis that those mutations arise or are selected for during avapritinib treatment. It should be noted that the panel sequencing of baseline samples only included exons 12, 14, and 18 but not exon 13 or exon 15 of PDGFRA, as mutations in the latter exon had at the time not been reported. Post-progression tumor samples revealed only single resistance mutations whereas several mutations were found in the plasma samples (patients 1 and 2)-underscoring the polyclonal fashion of avapritinib resistance, which is well known for KIT-mutant GIST (25).

We are aware that clonal hematopoiesis may account for a wealth of mutations (up to 50%) that are found in the plasma. This is particularly important for ctDNA mutations found at low allelic frequencies. We have therefore reviewed analyses from plasma from 219 patients with GIST from the NAVIGATOR trial (Supplementary Table S2) with longitudinal sampling performed on a subset of patients. Twenty-seven patients were found to have PDGFRA mutations. Of those, 21 represented the known primary mutation and 6 patients had variants of unknown significance, none of which represented mutations that we have described above. At post-baseline assessments, the only mutations that were found were those reported herein. The plasma assay was also used to evaluate 817 plasma samples from 398 patients from the ARROW trial. No patient had a PDGFRA mutation, although the detection threshold was as low as 0.05% allelic frequency. In contrast, mutations that are characteristic of clonal hematopoiesis of indeterminate potential (e.g., DNMT3A mutations, JAK2^{V617F}) were commonly observed with MAF as low as 0.08%.

In addition, baseline plasma samples from the phase III VOYAGER study for patients with imatinib- and sunitinibresistant GIST (n = 430) were analyzed. The vast majority of patients in this trial had been treated with potent inhibitors of wild-type PDGFRA (imatinib and sunitinib at a minimum). Again, only two patients were found to have mutations in *PDGFRA* exon 18 in the plasma, both of whom had known *PDGFRA*-mutant GIST (i.e., patients 6 and 7; Table 1). We therefore conclude that the resistance mutations we found in the plasma are most likely due to avapritinib-resistant tumor clones.

We are the first to describe GIST with secondary mutations within *PDGFRA* (apart from D842V as a secondary mutation

to an exon 12 PDGFRA mutation; ref. 23) and test their sensitivity to avapritinib and other kinase inhibitors using novel and unique PDGFRA-mutant models. In one avapritinibresistant patient, we also report mutations that constitutively activate pathways typically activated by PDGFRA (PI3K and MAPK pathways), which have recently been described as genomic events that drive resistance in some cases of KITmutant GIST as well (18, 19). Our investigation focused on resistance conferred by secondary PDGFRA mutations using our novel PDGFRA-mutant models. Although secondary mutations within the ATP-binding pocket and the AL are well understood in KIT-mutant GIST (11, 26-28), resistance mutations have not been well described in PDGFRA-mutant GIST, even in patients with imatinib-sensitive PDGFRA mutations (29). Here, we report that PDGFRA-mutant GIST tumors with clinical avapritinib resistance utilize similar escape mechanisms as those found in imatinib-resistant KITmutant GIST.

The secondary mutations that confer avapritinib resistance described in this study were found exclusively in exons 13, 14, and 15, which encode the ATP-binding pocket. These PDGFRA resistance mutations are novel both in the context of PDGFRAinhibitory treatment but also in a pan-cancer context. A query of The Cancer Genome Atlas database (10,967 samples from 10,953 patients across 32 tumor histologies) revealed no mutations at PDGFRA codons 598, 652, 658, 674, or 676. A single mutation at codon 680 (G680E) was seen in a patient with uterine serous carcinoma. A second query in a nonredundant data set of 47,005 samples from 178 trials yielded a single PDGFRA mutation Y676H in a patient with uterine endometrioid cancer. All other reported PDGFRA mutations were mutations in codon 842 (V/Y/I) that were found in GIST (n = 9), glioblastomas (n = 2), high-grade gliomas (n = 1), and neuroblastomas (n = 1).

In silico studies modeling secondary mutations found in patient-derived samples demonstrated steric hindrance with avapritinib. Notably, some PDGFRA secondary mutations occur at homologous residues to those of typical resistance mutations in other oncogenic kinases. The PDGFRAV658A mutant corresponds to KIT^{V654A} and PDGFRA^{T674I} to the KIT gatekeeper mutant T670I, which again corresponds with BCR-ABL T315I found in chronic myelogenous leukemia (CML; refs. 12, 30). The G680R secondary mutation is predicted to increase resistance to avapritinib due to a steric clash resulting in a solvent-front mutation (Fig. 4C). Notably, PDGFRAG680R resembles the substitution mutation found in drug-resistant RET-driven cancers (G810A; Fig. 4F; ref. 31). Solvent-front mutations involving a glycine have also been described for EGFR (G796S), ALK (G1202R), TRKA (G595R), TRKC (G623R), and ROS (G2032R; refs. 32-36). Interestingly, this mutation conferred the strongest resistance to imatinib in our biochemical assays, but its resistance to imatinib was dependent upon the context of the primary mutation, such that imatinib outperformed avapritinib in the context of PDGFRA^{I843-D846del/G680R} (Supplementary Fig. S6I). In contrast with the resistance mutations described above, V598F does not directly interfere with the inhibitor, and the side chain is far from the drug-binding site. However, V598 is located adjacent to the glycine-rich loop, which is a highly flexible element and crucial for substrate and ligand

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binding (37, 38). We therefore assume an influence of V598F on the dynamics of the G-rich loop results in drug resistance.

Patient 7, harboring a non-D842V primary mutation, responded to avapritinib despite a pretreatment G680R secondary mutation found at low allelic frequency in plasma only. As we did not find evidence for these mutations to reflect clonal hematopoiesis, it is likely that this clone represented a small fraction of the patient's tumor load and would not prevent the overall disease assessment from achieving a partial remission, before continued expansion of the clone resulted in clinical progression. The median allelic frequency of the primary mutation was >10 times higher than the resistance mutation. This case underscores that although plasma sequencing is a powerful technology for discovery of resistance mutations, it is not yet a validated predictive marker.

Although most of the secondary resistance mutations were point mutations, PDGFRA^{D842V/QGPins} also conferred moderate resistance to avapritinib (Figs. 2 and 5). Importantly, it closely aligns with an insertion mutation (D770_N771insNPG) found in a subset of patients with EGFR-mutant lung cancer (Fig. 4E). This insertion has been described to be at the pivot point of the helix αC , and the inserted residues sterically inhibit the reorientation of the helix αC characteristic of the inactive state (39) and interfere with an adjacent electrostatic switch interaction that is proposed to stabilize an inactive EGFR dimer (40). Our data also show that resistance mutations emerging from imatinib treatment in patients with imatinib-sensitive PDGFRA mutations may cause cross-resistance to avapritinib (patient 5). Studying imatinib resistance in patients with non-D842V-mutant tumors will therefore be important to better understand cross-resistance against PDGFRA inhibitors. Patient 1 was found to have a compound mutation in exon 14. This is noteworthy, as compound mutations have been described for BCR-ABL in CML (41), rarely in the context of KIT (11) and not yet in PDGFRA-driven GIST. Exome sequencing of the resistant tumor revealed this mutation to be homozygous (Supplementary Table S1), confirming that most, if not all, mutations must be in cis. This was also true for the Trsh1 cell line. Notably, looking at individual NGS reads reveals that V652E and V658A are observed both in cis and in trans in the aligned reads that span both codons ("spanning reads"). This is consistent with multiple mutational events (i.e., independent development of V652E and V658A as well as sequential mutations in the same allele). Fitting to the observation of multiple events, one postprogression plasma sample of this same patient showed two different base exchanges, leading to the same G680R substitution mutation albeit at highly different allelic frequencies (Table 1). Our functional studies show that the compound mutation is more resistant than the V658A mutation alone, which would support the hypothesis that the mutations occurred sequentially.

Apart from avapritinib, two other drugs show evidence of activity against *PDGFRA*-mutant GIST. Crenolanib, a benzimidazole compound, has been reported to be a potent inhibitor of PDGFRA and PDGFRB and was the first drug to show promising activity against D842V mutants *in vitro*, and preliminary clinical data also reported activity in patients (23, 42). A randomized phase III trial is currently ongoing (NCT02847429). Ripretinib (DCC-2618), a "switch-control" inhibitor of KIT and PDGFRA, has been shown to potently inhibit PDGFRA fusions and intragenic point mutants in biochemical assays (21). We found cross-resistance for ripretinib and crenolanib in most avapritinib-resistant models-in fact, ripretinib showed low potency against all D842V mutations, even the primary mutation, when tested in a GIST-specific context (Fig. 5E; $IC_{50} \ge 1,000 \text{ nmol/L}$). To our knowledge, clinical outcomes for ripretinib treatment of D842V-mutant patients have not yet been reported. Of note, T1- α -D842V/T674I had biochemical resistance to avapritinib and crenolanib. For the sublines with secondary mutations in codon 674, crenolanib showed an IC₅₀ of 250 nmol/L for the isoleucine (T674I) and 40 nmol/L for the arginine substitution (T674R). This suggests that crenolanib might have clinical activity against lesions harboring this particular resistance mutation. The IC_{50} for avapritinib was at 750 nmol/L, which is considered above clinically relevant levels. In agreement with these observations, patient 5, who had a secondary T674R mutation, did not respond to avapritinib at a dose of 200 mg daily.

Of note, we did not find evidence for escape mechanisms apart from resistance mutations in PDGFRA and its downstream signaling pathways. In patient 1, whole-exome sequencing studies comparing baseline and post-progression tumor samples revealed no other acquired mutations than those found in *PDGFRA*. After filtering for nonsynonymous variants and likely nonpathogenic variants, only *PDGFRA* secondary resistance mutations remained as differentially mutated genes.

Our results strongly support the hypothesis that for avapritinib-resistant GIST, salvage treatments should focus on improving PDGFRA inhibitors or combining avapritinib with inhibitors of downstream effectors. Notably, patientderived cells harboring PDGFRA triple mutations remain strongly dependent on the oncogenic signal of PDGFRA (Supplementary Fig. S5). PDGFRA, just like KIT, is a client of the HSP90 chaperone (43). Consequently, HSP90 inhibition completely abrogated PDGFRA phosphorylation-regardless of the secondary or tertiary resistance mutations (Fig. 5C). Both KIT-mutant and PDGFRA-mutant GIST seem uniquely dependent on their respective kinases, and, with both being substrates for HSP90, an indirect inhibition could represent a rational strategy. Although first- and second-generation HSP90 inhibitors have failed to provide lasting clinical benefit in GIST, metabolic studies have provided proof-of-concept that efficient KIT inhibition can be achieved at least in the short term. Very recently, a novel HSP90 inhibitor has been found active in KIT-mutant GIST (44). Therefore, next-generation HSP90 inhibitors remain a valid therapeutic concept warranting further preclinical and clinical testing for PDGFRA-mutant GIST as well.

Notably, in GIST cells harboring *KIT* mutations, *PDGFRA* regulates proliferation by stabilizing ETV1 (45), and this stabilization of ETV1 expression is dependent on ERK pathway signaling (46, 47). We found that inhibition of MEK/ERK using trametinib resulted in effective growth inhibition *in vitro* even in highly avapritinib-resistant sublines. Although clinical trials using MEK inhibitors have yet shown modest activity in *KIT*-mutant GIST (48), these results further underscore that avapritinib-resistant tumors remain dependent on oncogenic PDGFRA signaling.

In conclusion, our data show for the first time that resistance mechanisms in avapritinib-resistant PDGFRA-mutant GIST mirror those seen in imatinib-resistant KIT-mutant GIST. These consist of secondary PDGFRA mutations, particularly in the kinase domain, and less commonly mutations that constitutively activate PDGFRA downstream signaling intermediates. Future diagnostic sequencing panels should include PDGFRA exons 13, 14, and 15 for the detection of resistance mutations. Based on our in vitro studies, most but not all of these mutations appear to confer cross-resistance to other PDGFRA inhibitors currently in clinical development. Our data also provide first evidence that the oncogenic PDGFRA signal remains the dominant oncogenic driver at the time of progression and that salvage treatments should focus on improving PDGFRA-inhibitory strategies and also targeting the PDGFRA-dependent RAS-RAF-MAPK pathway.

METHODS

Patients

The NAVIGATOR trial is a phase I dose-escalation trial investigating avapritinib in patients with metastatic GIST refractory to IM and at least one other kinase inhibitor or a D842V mutation in PDGFRA. Eligibility criteria included provision of written informed consent, age ≥18 years, Eastern Cooperative Oncology Group performance status ≤2 and adequate end-organ function. Patient 1, a 67-year-old man, patient 2, a 76-year-old man, and patient 3, a 64-year-old man, all had a primary PDGFRA^{D842V} mutation and did not receive any previous systemic treatment. Patient 4 is a 70-year-old man with a primary DIMH-PDGFRA mutation in exon 18 who was included in the trial after having failed imatinib, sunitinib, and regorafenib treatment. Patient 5 is a 52-year-old man with a primary PDGFRA^{N659K} mutation in PDGFRA exon 14, who gained a secondary T674R mutation following imatinib and sunitinib treatment. Radiologic response to avapritinib was measured according to mRECIST criteria version 1.1 (49), and RECIST terms were used to describe tumor lesion measurements. Patient 6, who had a primary, imatinib-sensitive D842_D846delinsE mutation, had discontinued imatinib for unknown reasons and progressed on sunitinib when the plasma sample was taken. Patient 7 had a D842_D846delinsV PDGFRA primary mutation and had already progressed on imatinib, sunitinib, and sorafenib before plasma sequencing. Two secondary mutations reported within the deleted segment (M844I and M844K) were interpreted as sequencing artifacts.

Sequencing Analyses

During the escalation phase of the NAVIGATOR trial, mutant plasma DNA from patients with PDGFRA mutations was analyzed by BEAMing analyses and during the expansion phase using the PlasmaSelect gene panel assay (Personal Genome Diagnostics PGDx). BEAMing is a digital PCR approach that measures 5 specific PDG-FRA mutations (V561D, T674I, D842V, D842-H845del, and I843_ D846del). Sequenced DNA was aligned to the RAS sequence within the reference human genome to report mutations with a sensitivity of 0.1% mutant DNA, which is the limit of detection for the assay (50). The PlasmaSelect is an NGS-based panel sequencing assay that covers full coding sequences of both PDGFRA and KIT as well as 58 additional genes that may confer resistance to tyrosine kinase inhibitors. Plasma sequencing was performed on cycle 1 day 1 as well as at the end of treatment, when possible.

For 3 patients, post-progression samples were also analyzed using the Guardant360 system, which covers 73 genes, including KIT, PDGFRA, and common genes associated with tyrosine kinase inhibitor resistance as described previously (51).

For the VOYAGER trial (NCT03465722), plasma sequencing was performed at screening using Guardant360. For the ARROW trial (NCT03037385), plasma was analyzed using the PGDx PlasmaSelect64 assay, which evaluates a targeted panel of 64 cancer genes including PDGFRA.

Novel sequence variants were deposited at the Sequence Read Archive (SRA accession numbers SAMN16095462, SAMN16095463, SAMN16095464, SAMN16095465, SAMN16226685, SAMN16226686, and PRJNA664602).

Whole-Exome Sequencing

Whole-exome sequencing was done in a Clinical Laboratory Improvement Amendments (CLIA)-compliant laboratory (Genewiz). Results were filtered for somatic, nonsynonymous mutations and for variants that were found in the OMIM or OMIA databases. Variants were removed if the MAF was greater than 5% in the population or was reported in the 1000 genomes project (52).

Cell Lines, Reagents, and Antibodies

GIST-T1 and Trsh1 were established from human metastatic GIST. GIST-T1 contains a 57-bp deletion in KIT exon 11 (47). Trsh1 was established from tumor tissue that was obtained by a diagnostic biopsy of a growing lesion following avapritinib treatment (patient 1). The cell line harbors a primary PDGFRA exon 18 D842V mutation and two intra-allelic secondary mutations in exon 14 (V658A and G652E). GIST-T1^{D842V} is a subline of GIST-T1 that was created by transducing cells with D842V-mutant PDGFRA. Endogenous KIT was knocked out using CRISPR/Cas9. Knockout was verified at protein and DNA levels. All cell lines were grown in Iscove's Modified Dulbecco's Medium with 10% FBS at 37°C and 5% CO2. All cell lines were regularly authenticated by sequencing of endogenous mutations in KIT, confirmation of KIT expression, and response to KIT inhibitor treatment. In the course of this study, all cell lines were regularly tested for Mycoplasma contamination by PCR and the MycoAlert Mycoplasma Detection Kit (Lonza).

Generation of Ba/F3 Cell Lines

Ba/F3 cells were grown in RPMI-1640 medium with 10% FBS at 37°C and 5% CO2. Lentiviruses were produced by transient transfection of HEK-293T cells with ViraPower Lentiviral Packaging Mix (Invitrogen, #K497500) plus the pLENTI-C-Myc-DDK-IRES-Neo vector (Origene) containing full-length PDGFRA cDNA encoding each of the described mutations. Ba/F3 cells were then transduced with filtered lentivirus and polybrene via spinoculation and supplemented with murine IL3 (Cell Signaling Technology, #8923C). Transduced, IL3-dependent Ba/ F3 cells were then selected for 1 week in G418 (Sigma, #A1720). In order to generate IL3-independent clones, G418-resistant Ba/F3 cells were plated in limiting dilutions in the absence of IL3 and grown for 1 to 3 weeks (48). Full sequencing of the PDGFRA cDNA was performed for each clonal IL3-independent Ba/F3 cell line. The PDGFRA mutation P653_H654insQGP is abbreviated as QGPins in the text and figures.

Gene Editing by CRISPR/Cas9

Further cell lines with endogenous PDGFRA mutations (T1 α ^{D842V}, $T1\alpha^{D842V/QGPins}, \ T1\alpha^{D842V/G652E}, \ T1\alpha^{D842V/V658A}, \ T1\alpha^{D842V/G652E/V658A},$ $T1\alpha^{D842V/T670I}, T1\alpha^{D842V/G680R})$ were generated by CRISPR/Cas9-mediated gene editing, as described previously (18), with the following deviations: We used recombinant Cas9 from IDT Labs (60 µmol/L) in a 1:2 ratio with specific single guide RNA (sgRNA). First, $PDGFRA^{D842V}$ was introduced into parental GIST-T1, and cells were selected with IM 200 nmol/L until outgrowth of a resistant population, which carried heterozygous PDGFRAD842V. In a second round of editing,



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one sgRNA specific for *PDGFRA*-resistance mutations (exons 14 and 15) and an ssODN template to introduce the desired mutation (P653_H654insQGP, G652E, V658A, G652E/V658A, T674I, T674R, and G680R, respectively) was combined with a guide targeting exon 1 (for G652E, G652E/V658A, T674I, and G680R) or mutant exon 11 (for D842V-only, QGPins, and V658A) of KIT. Cells were then treated with avapritinib 100 nmol/L, and after outgrowth of a resistant population, single-cell clones were derived. Heterozygous D842V and in *cis* secondary mutations as well as frameshift mutations in mutant exon 1 and 11 of *KIT* were confirmed by Sanger sequencing and/or next-generation panel sequencing. Strong reduction of KIT expression and phosphorylation were confirmed by Western blotting. For a complete list of sequences used in CRISPR/Cas9 experiments, see Supporting Information.

CHO Transfection

Chinese Hamster Ovary (CHO) cells were cultured in FK12 medium + 10% FBS at 37° C and 5% CO₂. Cells were plated and transfected with various *PDGFRA*-mutant expressing plasmids (pcDNA 3.1+). After 24 hours, cells were treated with drug at indicated doses for 90 minutes and then harvested for immunoblotting.

SRB Cell Viability Assay

The sulforhodamine B (SRB) assay was used according to the method of Skehan and colleagues (53). Cells were plated at 5,000-10,000 cells/well in a 96-well flat-bottom plate and cultured for 24 hours. An untreated control plate was measured at the time of treatment for normalization, that is, to define the concentration of TGI and half TGI (TGI₅₀). Cells were then incubated with media containing inhibitors or solvent control (DMSO). After 72 hours, cells were fixed and stained with SRB (0.4%, Sigma-Aldrich), and absorption was measured with a Genion luminometer (Tecan). All measurements were carried out in triplicate/quadruplicate wells for at least two times, and a representative example is shown.

CellTiter-Glo Cell Viability Assay

Cells were plated, transfected, and incubated in opaque 96-well plates (Corning). Cell viability was measured using the CellTiter-Glo reagent (Promega) according to the manufacturer's instructions.

Assessment of Apoptosis

For assessment of caspase-3/7 activation by Caspase Glo luminescent assay (Promega), cells were prepared as above and treated for 16 hours. Then Caspase Glo reagent was added according to the manufacturer's protocol, and resulting luminescence was measured.

Western Blotting

Ba/F3 and CHO drug experiments were performed for 90 minutes before harvesting whole-cell protein lysates for immunoblotting. For GIST cells, whole-cell protein lysates were prepared as previously described (54). Protein concentrations were determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories). Proteins were separated by SDS/PAGE as described by Laemmli and colleagues (55) and transferred to Hybond-P membranes (Amersham Pharmacia Biotech). Changes in protein expression and phosphorylation as visualized by chemiluminescence (ECL chemi-luminescent reagent, Amersham Pharmacia Biotech) were captured and quantified using a FUJI LAS3000 system with Science Lab 2001 ImageGauge 4.0 software (Fujifilm Medial Systems).

PDGFRA Knockdown Studies

For an inducible knockdown of the *PDGFRA* gene, the Trsh1 cells were transduced with lentiviral particles: one control (nontargeting) and three *PDGFRA* ORF-targeting shRNAs in doxycycline-inducible

SMARTvectors (GE Healthcare/Dharmacon, product #V3SH11255-01EG5156). The virus was packaged using the 293T cell line transfected with second-generation lentiviral packaging plasmids psPAX2 and pMD2. Selection for cells harboring expressable proviral integrants was started 3 days after transduction with puromycin at 2 μ g/mL.

In Silico Modeling Studies for PDGFRA

The KIT crystal structure with the PDB ID 1PKG was used to generate homology models of PDGFRA mutants in the activated state (D842V, D842V/V658A, D842V/V658A/G652E, and D842V/P653_ H654insQGP). The homology modeling was performed with the "Homology Modelling" tool as implemented in MOE2015 (Molecular Operating Environment) Version 2015.10; Chemical Computing Group ULC). We generated 10 backbone models and 25 side-chain models per protein and retained ADP and the Mg²⁺ ion of the template structure as environment for induced fit.

For the validation of the homology models, the generated PDGFRA structures were subjected to 400 ns MD simulations using the software package AMBER2018 (56). Details regarding the MD simulations can be found elsewhere (52). The ion parameters by Li and colleagues were used (57). The parameters for ADP (58) were obtained from the AMBER parameter database (http://research.bmh.manchester.ac.uk/bryce/amber/). The resulting MD snapshots were clustered according to the RMSD of the ATP binding site-constituting residues using the hierarchical agglomerative approach (average linkage, RMSD threshold = 1.5 Å) as implemented in CPPTRAJ (59), and representative snapshots were extracted.

The representative snapshots of the PDGFRA^{D842V} structure were used for a molecular ensemble docking of avapritinib with GOLD (60). We used all residues within 10 Å of ADP as the binding site definition and performed 100 GA runs, and the "early termination" option was disabled. Apart from that, the default settings were applied. The resulting binding modes were visually inspected and the binding mode exerting the most favorable interactions with the binding site and showing the lowest number of clashes was chosen for a subsequent MD simulation.

The MD simulation of the hypothetical avapritinib binding mode in PDGFRA^{D842V} was performed as described above using the AMBER2018 package. Avapritinib was parameterized using the GAFF force field (61) and AM1-BCC charges with antechamber (58). The simulation was performed until a stable binding mode of avapritinib was observed (1000 ns). PyMOL was used for generating the figures (The PyMOL Molecular Graphics System, Version 2.2.0; Schrödinger, LLC).

Reagents and Antibodies

A list of reagents/drugs and antibodies is provided in Supplementary Table S3.

Authors' Disclosures

S. Bauer reports grants and personal fees from Novartis and Blueprint Medicines, personal fees from Deciphera, Bayer, Exelixis, Daichii-Sankyo, Plexxikon, and grants from Incyte during the conduct of the study; personal fees from Pharmamar, ADC Therapeutics, and Lilly outside the submitted work. J. Falkenhorst reports personal fees from Pharmamar and nonfinancial support from Eli Lilly (travel support) outside the submitted work. E. Wardelmann reports personal fees from Nanobiotix, Roche, Lilly, Bristol Myers Squibb, Bayer, and Novartis and grants from Diaceutics during the conduct of the study. H.-U. Schildhaus reports grants from Novartis Oncology, personal fees from Roche, MSD, BMS, Pfizer, and Takeda, and other from Blueprint Medicine outside the submitted work. J.A. Fletcher reports grants from David Foundation during the conduct of the study; personal fees from Deciphera

Avapritinib Resistance in PDGFRA-Mutant GIST

Pharmaceuticals and Novartis outside the submitted work; in addition, Dr Fletcher has a patent for activating mutations of PDGFRA issued and a patent for Treatment of Gastrointestinal Stromal Tumors licensed to Novartis. S. Miller reports personal fees from Blueprint Medicines (employee of Blueprint Medicines) and other from Blueprint Medicines (own stock in Blueprint Medicines) outside the submitted work. O. Schmidt-Kittler reports personal fees from Blueprint Medicines (employee and shareholder of Blueprint Medicines) during the conduct of the study; personal fees from Blueprint Medicines (employee and shareholder of Blueprint Medicines) outside the submitted work. D. Rauh reports grants from DFG (research grant by the German government) during the conduct of the study; other from PearlRiver Bio GmbH (cofounder and shareholder), CDL Therapeutics GmbH (co-founder and shareholder), and Pfizer, AZ, Sanofi, BI, and Bayer (consulting and lecture fees) outside the submitted work. M.C. Heinrich reports grants from US Veterans Administration (I01 BX000338), Jonathan David Foundation, and GIST Cancer Research Fund, personal fees from Molecular MD and Blueprint Medicines during the conduct of the study; personal fees from Deciphera Pharmaceuticals and personal fees from Novartis outside the submitted work; in addition, Dr Heinrich has a patent for Treatment of Gastrointestinal Stromal Tumors issued, licensed, and with royalties paid from Novartis and a patent for activating mutations of PDGFRA issued. J. Lategahn reports personal fees from PearlRiver Bio GmbH (Dr Lategahn is shareholder and full-time employee of PearlRiver Bio GmbH) outside the submitted work. No disclosures were reported by the other authors.

Authors' Contributions

S. Grunewald: Resources, data curation, investigation, methodology, writing-review and editing. L.R. Klug: Conceptualization, data curation, investigation, visualization, methodology, writing-review and editing. T. Muhlenberg: Data curation, validation, investigation, writing-review and editing. J. Lategahn: Validation, investigation, writing-review and editing. J. Falkenhorst: Data curation, visualization, writing-review and editing. A. Town: Data curation, investigation. C. Ehrt: Data curation, visualization, writingreview and editing. E. Wardelmann: Investigation, writing-review and editing. W. Hartmann: Investigation, writing-review and editing. H.-U. Schildhaus: Investigation, writing-review and editing. J. Treckmann: Resources, writing-review and editing. J.A. Fletcher: Data curation, validation, investigation, writing-review and editing. S. Jung: Investigation, writing-review and editing. P. Czodrowski: Investigation, visualization, writing-review and editing. S. Miller: Data curation, investigation, visualization, methodology, writingreview and editing. O. Schmidt-Kittler: Validation, visualization, methodology, writing-review and editing. D. Rauh: Conceptualization, resources, validation, visualization, writing-review and editing. M.C. Heinrich: Conceptualization, resources, data curation, formal analysis, funding acquisition, investigation, visualization, methodology, writing-original draft, writing-review and editing. S. Bauer: Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing-original draft, project administration, writingreview and editing.

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