Targeted therapy in GIST: *in silico* modeling for prediction of resistance

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Abstract | Elucidation of the genetic processes leading to neoplastic transformation has identified cancer-promoting molecular alterations that can be selectively targeted by rationally designed therapeutic agents. Protein kinases are druggable targets and have been studied intensively. New methodologies—including crystallography and three-dimensional modeling—have allowed the rational design of potent and selective kinase inhibitors that have already reached the clinical stage. However, despite the clinical success of kinase-targeted therapies, most patients that respond eventually relapse as a result of acquired resistance. Darwinian-type selection of secondary mutations seems to have a major role in this resistance. The emergence and/or expansion of tumor clones containing new mutations in the target kinase and that are drug-insensitive have been observed after chronic treatment. The resistance mechanisms to tyrosine kinase inhibitors, in particular secondary resistant mutations as a consequence of treatment, will be discussed in detail. In particular, this Review will focus on *KIT* and *PDGFRA* mutations, which are involved in the pathogenesis of gastrointestinal stromal tumors. Harnessing the selection of mutated variants developed to overcome these resistance mechanisms is an ongoing goal of current research and new strategies to overcome drug resistance is being envisaged.

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Introduction

Protein kinases are commonly implicated in cancer.[1] The human genome encodes more than 500 protein kinases, many of which have been identified as interesting targets for drug discovery. Nonetheless, few kinase inhibitors have been licensed and, despite early clinical success, drug resistance has been observed in patients who initially responded to such therapies. The resumption of tumor growth as a consequence of acquired drug resistance may be promoted by different mechanisms, the most common being the presence of secondary mutations in the target tyrosine kinase. These genetic alterations and resulting protein sequence changes often alter the structure of the kinase catalytic domain, causing it to have a lower affinity for the inhibitor. Such changes have been suggested by preclinical studies as well as by gene sequencing in patients who developed secondary resistance in the clinical setting.[2] The intrinsic properties of these mutations can affect the structural state of protein kinases, their affinity for ATP and catalytic activity. Moreover, these mutations can exert global dynamic effects on the structure of the kinase (active and inactive form), alter its binding-site characteristics and change the movements of amino acid residues. Understanding these aspects can shed new light on drug resistance mechanisms.

In this context, a relatively fast and noninvasive global approach based on an *in silico* analysis (that is, using computer-based molecular simulation) represents a useful tool that, coupled with traditional biochemical molecular evidence, may help overcome this problem. *In silico* analysis, which uses the three-dimensional (3D) structure of the receptor protein kinase, has multiple uses. The method can predict structural changes introduced by mutations, measure the strength of the interactions between the protein model and the drug, determine whether the drug can be effective or not by calculating the binding affinity (or binding free energy), and evaluate to what extent decreasing efficacy could still be counteracted by increasing the drug dose or by using a different compound (Supplementary Box 1 online).

In this Review, we discuss KIT and PDGFRA receptor tyrosine kinases (RTKs) in the context of gastrointestinal stromal tumors (GISTs, Box 1). We focus on the major challenge in kinase drug discovery—that is, the emergence of resistance—and discuss techniques to predict and help prevent this adverse clinical event.

RTK structure and function

RTKs consist of an extracellular domain, a transmembrane domain and an intracellular region. Physiologically, RTK activation resulting in phosphorylation in intracellular tyrosine residues that is achieved by binding cognate ligands, triggers a cascade of biological reactions leading to the on/off switch of the genes involved in cell growth, differentiation and survival. Deregulation of RTK function may be caused by gene mutation, amplification, protein

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Key points

- The pathogenetic role of constitutively activated receptor tyrosine kinases (RTKs) and drugs that specifically target this alteration in cancer has provided a new therapeutic opportunity
- Despite encouraging early therapeutic results, the development of resistance can occur after a variable period of chronic treatment
- The emergence of secondary mutations that affect the tyrosine kinase domain of RTKs reduce the drug binding affinity to the enzymatic pocket of the receptor; this resistance can be overcome by the development of drugs that bind efficiently the new mutated RTK forms
- A new *in silico* approach is consistent with both biochemical and molecular data and patient clinical outcome and could support clinical decisions to increase the drug dose or administer a different drug
- *In silico* molecular modeling can be used to predict the occurrence of all activating but drug-resistant secondary mutations and to develop a multi-drug targeted prevention strategy

Box 1 | History of the GIST and spectrum of its clinical presentation

The gastrointestinal stromal tumor (GIST) 'revolution' started in 1998 with the demonstration that a substantial subset of lesions harbor mutations in the gene encoding the receptor KIT leading to constitutive ligand-independent phosphorylation.[55] The second breakthrough was 5 years later, when a subset of GISTs lacking KIT mutations harbored activating mutations in the related PDGFRA.[56,57] Approximately 5–10% of GISTs lack mutations in either kinase; in these cases occasional *BRAF* mutations are reported[20] and, anecdotally, *KRAS* mutations. Sporadic GISTs are commonly found in the stomach (60%), small intestine (25%), rectum (5%) and esophagus (83%).[58]

GISTs may develop in syndromes such as neurofibromatosis type 1 and Carney triad or Carney/Stratakis dyad, which have wild-type *KIT* and *PDGFRA* status. Remarkably, dyad paraganglioma and GIST occur in patients carrying germline mutations of genes encoding succinate dehydrogenase subunits supporting the notion that these genes, rather than constitutively active tyrosine kinases, may be responsible for GIST formation in these patients.[59] Consistently with this view, it has been recently reported that a number of sporadic pediatric wild-type *KIT/PDGFRA* GISTs carry a loss of function mutation of succinate dehydrogenase subunits.[60]

translocation, an autocrine–paracrine loop not mediated by gene alteration, and signaling pathway deregulation.[3]

Core domain of RTKs

Protein kinases transmit and amplify intracellular signals through selective phosphorylation of residues on other proteins, often other kinases. Similar to all protein kinases, RTKs contain a catalytic domain (core domain) where ATP binds. The binding of a specific ligand to a docking site in the extracellular domain of the receptor, followed by receptor dimerization via tyrosine residues within the cytoplasmic domain, marks the first step of receptor activation. RTK autophosphorylation[4] activates the kinase and increases its intrinsic tyrosine kinase activity. This event creates phosphorylated tyrosine residues that become binding sites for intracellular adapter molecules, bringing signal transduction components close together.

The protein kinase cytoplasmic portion is structurally conserved among all serine/threonine and tyrosine kinases, and consists of a smaller N-terminal lobe (N-lobe) and a larger C-terminal lobe (C-lobe) connected by a strand known as a hinge region kinase insert.[5] The N-lobe consists of a β-sheet and a conserved α-helix. The C-lobe is mainly α-helical and contains a segment, the activation-loop (or A-loop), which includes residues that are phosphorylated in many kinases. The core domain that contains the ATP binding site is sandwiched between the two lobes. This domain sits beneath the P-loop (that is, the phosphate-binding loop or glycine-rich loop) that has a role in determining the shape of the ATP binding site.

In the active kinase, a characteristic DFG-motif or catalytic triad, which is located immediately before the A-loop, adopts a conformation whereby the aspartic acid and phenylalanine are oriented toward the binding site (DFG-in or open or active conformation of the kinase, Figure 1a). Different inactive states have been identified. One of these states is called DFG-out (closed or inactive conformation of the kinase). Under normal physiological conditions, the intracellular portion of the kinase exists in a regulated state with very low activity. However, in the presence of an activating mutation, the equilibrium between the DFG-in and DFG-out forms is shifted toward the active open (DFG-in) conformation of the receptor. This change allows enhanced binding of ATP and, hence, increased level of phosphorylation of the tyrosine kinase domain.[6] Notably, the DFG-out conformation, where the phenylalanine occupies part of the ATP binding site, alters the accessibility of the binding site within the pocket (Figure 1b). The DFG-motif represents a key point because the majority of the detected mutations destabilize the A-loop and, consequently, alter the structure of the catalytic triad. Moreover, several drugs target specifically either the DFG-in (for example, dasatinib) or the DGF-out (imatinib, nilotinib and sunitinib) form.

A new class of tyrosine kinase inhibitors is currently in development. These new molecules target the so-called 'switch pocket', a distinct region adjacent to the ATP binding site of the protein that is unique for each tyrosine kinase (or its subfamily). This region is involved in the regulation of the tyrosine kinase catalytic activity: the phosphorylation of specific amino acids alters the domain conformation inducing a switch of the structure of the domain itself.[7,8]

Mutations in KIT and PDGFRA

KIT and *PDGFRA* are both located on chromosome 4q12 and encode receptors belonging to the type III tyrosine kinase family and share a similar structure. The cognate ligands are stem-cell factor and platelet-derived growth factor (PDGFA and B), respectively. *KIT* and *PDGFRA* mutations are causally involved in the development of GISTs and the response to current therapies. They are also believed to be mutually exclusive and are subdivided into two categories: primary, linked to pathogenesis, and secondary, related to treatment or disease progression. About 80% of GISTs harbor primary *KIT* mutations that generally occur in the juxtamembrane domain (exon 11, termed 'mutational hot-spot'), whereas the majority of *PDGFRA* mutations (about 65%) affect the tyrosine kinase 2 domain (exon 18, Figure 2). All of the mutations that cause RTK activation affect the downstream

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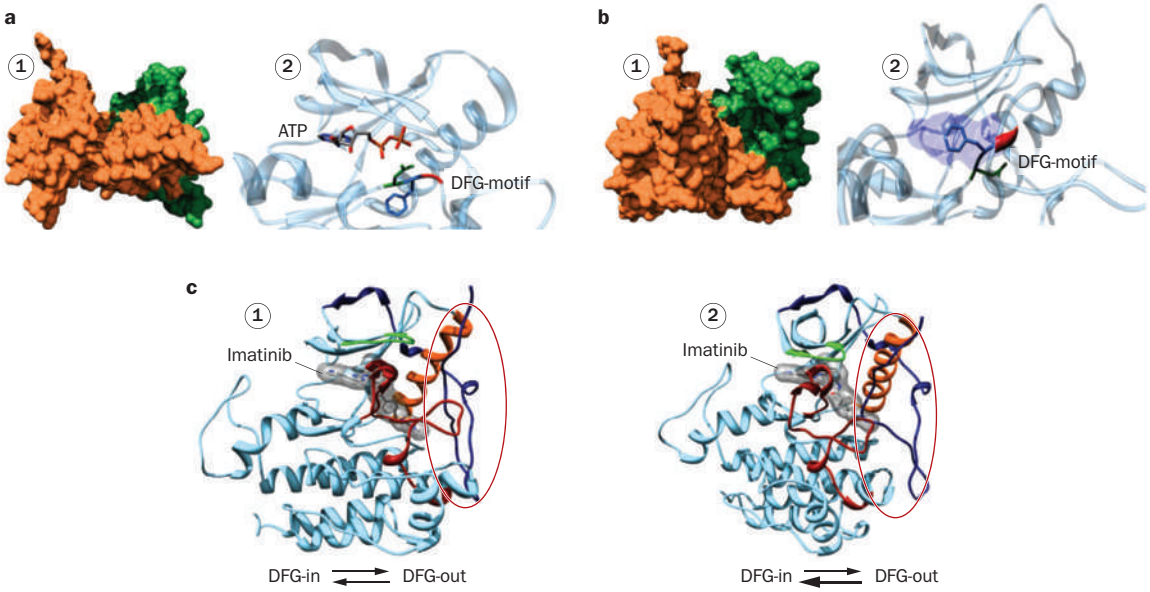

Figure 1 | KIT core conformations. **a** | Active kinase. (1) 3D model of the open form of KIT governed by the DFG motif. (2) Zoom on the DFG-in motif (open, active form). D810 is colored green, F811 blue, and G812 red. ATP is depicted in atom-colored sticks (C, gray; O, red; N, blue, P, orange). Hydrogen atoms are omitted. **b** | Inactive kinase. (1) 3D model of the closed form of KIT. (2) Zoom on the DFG-out motif (closed, inactive form). Note that F811 (dark blue) points inwards into the kinase ATP-binding pocket, thereby preventing ATP binding and kinase phosphorylation. The ATP is represented by its van der Waals surface (light purple). **c** | *In silico* 3D structures of (1) wild-type and (2) mutatated-Δ559 KIT in complex with imatinib. The juxtamembrane hairpin motif (dark blue) is highlighted in the red circles. Imatinib is in atom-colored sticks, (C, gray; O, red; N, blue), and its molecular surface is in orange. The α-helix C (orange), P-loop (green), and A-loop (dark red) are also highlighted. Hydrogen atoms, water molecules, ions and counterions are omitted for clarity. Arrows depict the equilibrium of the kinase between the open and closed form.

PI3K/AKT and RAS/MAPK pathways and mammalian target of rapamycin (mTOR) and its effectors (S6 kinase and 4EBP1).

Primary activating mutation types

A wide spectrum of GIST primary mutations has been reported. These mutations can be divided into two categories on the basis of their location: mutations of the receptor extracellular and cytoplasmic juxtamembrane domains, and mutations of the two enzymatic domains (tyrosine kinase 1 and tyrosine kinase 2, Figure 2). Primary *KIT* mutations have been reported to cluster in the extracellular (exon 9), juxtamembrane (exon 11), tyrosine kinase 1 (exon 13), and tyrosine kinase 2 (exon 17) domains. Mutations in aggregate primary exon 13 and 17, account for 1–2% of the total mutations.[9,10] Mutations in *KIT* exon 9, followed by those in exon 17 and 13 are overrepresented among intestinal GISTs and correlate with spindle-cell morphology. Primary *PDGFRA* mutations are mainly identified in exon 18, followed by exon 12 and 14. *PDGFRA* mutations occur almost exclusively in GISTs of the stomach and omentum and correlate with epithelioid morphology. All of these mutations lead to conformational changes that ultimately perturb the 3D structure of the receptor.

Imatinib (Gleevec®, Novartis AG Corporation, Basel, Switzerland) is the first effective small-molecule tyrosine kinase inhibitor originally approved for the treatment of chronic myeloid leukemia. Besides inhibiting *BCR-ABL*, imatinib blocks the activity of several other tyrosine kinases, including KIT and PDGFRA. The recognition of the inactive conformation of KIT and PDGFRA by imatinib enables it to bind in a pocket largely coincident with the ATP-binding site, thus preventing kinase activation by restricting the conformational transition of the A-loop.[6]

Imatinib response depends on *KIT* and *PDGFRA* mutational status. GISTs carrying *KIT* exon 11 mutations (juxtamembrane domain) respond much better to targeted treatment than tumors with exon 9 mutations (extracellular domain) or wild-type *KIT* and *PDGFRA*. By contrast, primary mutations affecting the tyrosine kinase domains do not generally respond to imatinib.[11] From this evidence, it seems that *KIT* and *PDGFRA* mutational status has a strong predictive value. Moreover, among the primary responsive mutations of the regulatory domains (extracellular and juxtamembrane), the drug-response modulation leads to primary imatinib resistance.

In-frame deletions including the deletion of valine 559 (Δ559) are the most frequently detected primary mutations affecting *KIT* exon 11.[9] This mutation induces a substantial modification in the conformation of the juxtamembrane domain that, in turn, results in a shift of the dynamic equilibrium of the kinase toward the open, active form (DFG-in, Figure 1c). When the receptor is in its closed form, the mutated juxtamembrane is able to better accommodate imatinib, thereby enhancing binding affinity. This finding is consistent with *in silico*

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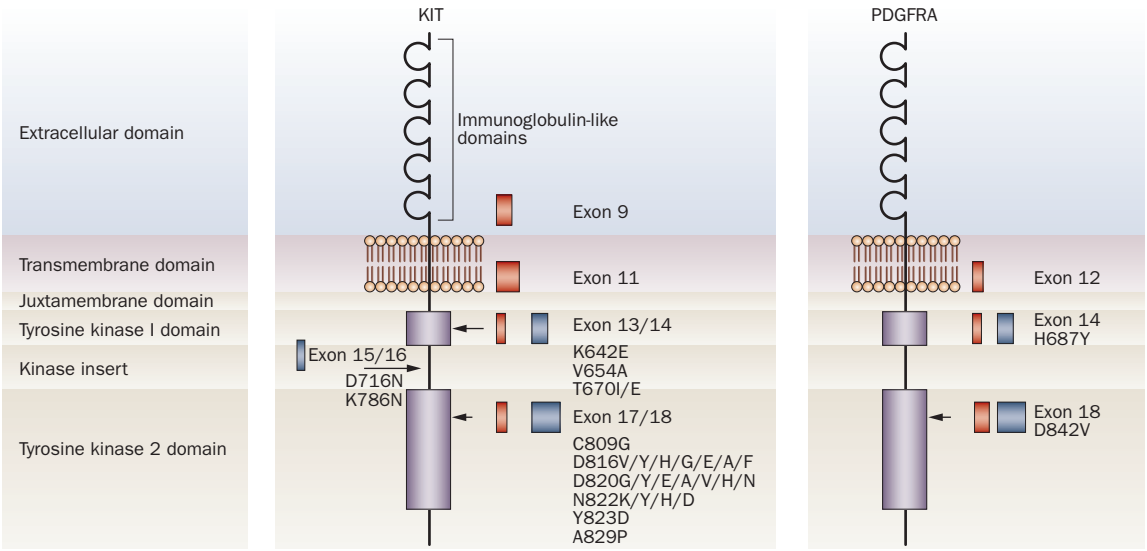

Figure 2 | *KIT* and *PDGFRA* mutations and correllation to protein structure. *KIT* and *PDGFRA* mutations are subdivided into primary mutations (red), present at the disease onset, and secondary mutations (blue), which develop during treatment. The reported frequency of primary and secondary mutations is represented by the thickness of the rectangles. All types of primary mutations mostly affect exon 11. Occasionally, point mutations may occur in exons 9, 13, and 17. Insertion mutations are very rare. The only deletion reported to occur outside the KIT juxtamembrane domain in gastrointestinal stromal tumors involves exon 14 (tyrosine kinase 1). Secondary mutations mainly affect KIT. Over 10 imatinib-resistant secondary mutations have been reported, most of which are missense mutations. KIT secondary mutations mainly occur in the activation loop and are likely to increase the propensity of the enzyme to adopt its active form. The observed secondary *PDGFRA* mutations are D842V (equivalent to the KIT D816E/H) and H687Y, both missense mutations.

prediction. Molecular modeling shows how the sterical hindrance exerted by the hairpin portion of the juxtamembrane domain at the ATP pocket entrance, which partly interferes with imatinib binding to the wild-type receptor, is removed in mutated exon 11 *KIT*. This mutation results in a higher affinity of the mutated isoform for imatinib with respect to its wild-type receptor (Figure 1c). An exception is represented by the L576P mutation of *KIT* exon 11 affecting the juxtamembrane domain, which shows a minimal response rate to imatinib (Figure 3a–c), an observation fully supported by molecular modeling prediction.[12] Accordingly, computer simulations of the L576P mutant *KIT* in complex with imatinib reveal that the L576P mutation imposes significant energetic constraints on several amino acids of the imatinib binding pocket. As a consequence, the ATP pocket shape is altered, and the strong binding of imatinib is no longer preserved (Supplementary Figures 1 and 2 online). This evidence is substantiated by the corresponding *in silico* predicted free energy of binding value (Supplementary Box 2 online).

Regarding the prognostic value of *KIT* and *PDGFRA* mutational status, data based on pre-imatinib clinical and molecular findings indicated that untreated patients carrying *KIT* exon 11 deletions had a worse outcome and a high risk of metastasis.[9,13] Nonetheless, point mutations or duplications that occur in the same exon correlate with less-aggressive tumor behavior, similar to most *PDGFRA* mutations.[14,15] In any case, complete radiological responses are rare (<5%) and after a median of about 2 years an acquired resistance to imatinib is generally observed.

Mechanisms of secondary resistance

A number of mechanisms have a role in GIST imatinib-resistance. Such mechanisms include the pharmacokinetic metabolic variability, alterations in the transporter enzymes, and the hitherto unexamined contribution of *KIT* and/or *PDGFRA* polymorphisms or copy number alterations,[16] which have already been demonstrated in other histotypes.[17] However, several crucial resistance mechanisms have been envisaged and reported.

First, pathological activation of downstream signaling pathways such as PI3K/AKT[18] and RAS/RAF/MEK/MAPK can occur, both converging on mTOR.[19] A BRAF mutation-based activation pathway may occasionally occur (7% of wild-type GISTs) in both imatinib-naive and imatinib-resistant GISTs without *KIT* and *PDGFRA* mutations. In GISTs where the activation of BRAF triggers MAPK pathway, MEK inhibitors could be included in the treatment plan.[20] Similarly, in neurofibromatosis type 1 GISTs the activation of the MAPK cascade is achieved through the loss of neurofibromin, which is a negative regulator of RAS signaling.[21]

Second, activation of an alternative tyrosine kinase receptor (for example, the oncogenic RTK, AXL) and loss of KIT expression may occur. Overexpression of AXL induces imatinib resistance through a kinase switch from KIT to AXL. Strong AXL expression and lack of KIT expression have been demonstrated in two imatinib-resistant patients with GISTs.[22] Molecular modeling and an *in vitro* assay using the novel KIT–AXL kinase inhibitor MP470 confirmed the results showing no imatinib binding to mutated KIT but efficient binding to MP470.[22]

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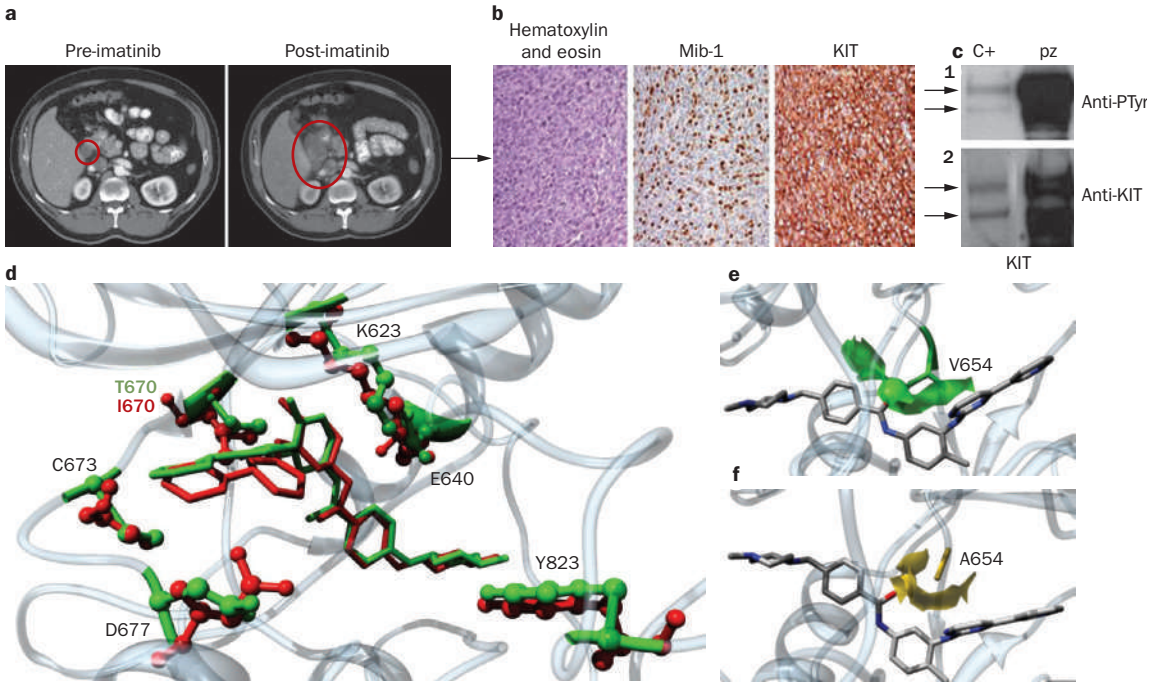

Figure 3 | Imaging, biochemical and molecular evidence and modeling of KIT. **a–c** | *L576P KIT* primary mutation: receptor activation and imatinib resistance. **a** | CT scan of a peritoneal nodule near to the duodenum (red circle) in progression after imatinib 400 mg/day. **b** | Surgical specimen lacking any evidence of imatinib response and showing evidence of high cellularity, high rate of the proliferative marker Ki-67 (Mib 1), and dot-like KIT decoration. **c** | (1) Immunoprecipitation and (2) Western blotting analysis performed on a matched pair of frozen tissue (pz) confirms KIT activation and expression. **d–f** | Comparison of **d** | *T670I* and **e,f** | *V654A* secondary *KIT* mutations. **d** | The larger molecular volume of the mutated isoleucine (I670) side chain with respect to the wild-type threonine (T670) induces imatinib (green and red sticks) to assume a shifted position within the pocket. Residues affected by the mutation are depicted in green and red sticks and balls. **e,f** | The decrease in imatinib binding affinity is mainly due to the smaller molecular volume of alanine (A654) compared with valine (V654) that favors the loss of packing interaction in the mutated alanine variant compared with the wild-type counterpart. Surface complementarity between imatinib (atom-colored sticks) and either **e** | wild-type valine (green sticks) or **f** | mutated alanine (gold sticks).

Third, amplification or loss of *KIT* and/or *PDGFRA*[23] may occur in rare instances.[24,25] This type of alteration may also be observed at disease onset (primary resistance).

Fourth, resistance caused by dedifferentiation (that is, the histological progression to, or change to a higher grade sarcoma) in the absence of secondary mutations may be observed. Indeed, disease progression and resistance have been described in advanced imatinib-treated GISTs harboring primary *KIT* and *PDGFRA* mutations in the absence of secondary resistant mutations but in the presence of evidence of dedifferentiation.[26]

Fifth, under drug pressure, the acquisition of secondary *KIT* or *PDGFRA* mutations represents the most important and frequent mechanism of secondary resistance. Remarkably, this resistance, which correlates with clinical progression, is due to the outgrowth of multiple resistant clones that often contain different secondary mutations.[25]

Structural perturbations

Secondary mutations (Box 2 and Figure 2) in the kinase domain of *KIT* and sometimes in *PDGFRA*, are accompanied by concomitant reactivation of the corresponding tyrosine kinase even in the presence of imatinib. The structural alterations induced by these mutations may confer drug resistance by two distinct pathways. One such pathway introduces a perturbation in the general architecture of the ATP pocket, as is the case of the two missense mutations T670I and V654A affecting the tyrosine kinase 1 domain[27] (Figure 3d–f). The other pathway induces a transition from the autoinhibited (closed) form toward the activated (open) form, as exemplified by D820N (Supplementary Figure 3 online) and D816V mutations in the tyrosine kinase 2 domain of KIT.[28]

The molecular mechanisms underlying these two pathways can be analyzed to a finer level using molecular modeling. For instance, our computer studies clearly show that, for the first pathway, the T670I mutation induces substantial modifications in the ATP-binding pocket of the imatinib-sensitive KIT (Δ559), resulting in a calculated free energy of binding difference ($\Delta\Delta G_{bind}$, Supplementary Box 1 onlin) for imatinib for the wild type KIT of –3.84 kcal/mol.[27] By contrast, the V654A mutation shows only limited modifications, leading to a $\Delta\Delta G_{bind}$ for imatinib equal to –1.52 kcal/mol. Consistently, Δ559 + T670I KIT was completely insensitive to all tested imatinib doses, whereas Δ559 + V654A KIT was sensitive to the highest imatinib dose tested.

In the second pathway, the most common primary mutation affecting *PDGFRA* exon 18, the D842V point mutation (homologous to D816V in *KIT*), is insensitive

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Box 2 | Secondary mutations in GISTs

All secondary mutations affect KIT with only two reported exceptions where acquired resistance was due to PDGFRA alterations in gastrointestinal stromal tumors (GISTs) characterized by *KIT* mutations.[24,61] These mutations occur after a long period of imatinib treatment (median 27 months). They are typically observed in patients with KIT-mutated exon 11 GISTs,[62] are rare in KIT exon 9 and do not generally occur in GIST patients with a wild-type genotype.[25,34] Other mutations in different lesions or simultaneous evolution of multiple clones in one lesion have been reported.[63] Combining the highly sensitive, allele-specific PCR with denaturing high-performance liquid chromatography (D-HPLC) technique can improve the sensitivity of mutation detection from about 15% to about 5%. KIT-tyrosine kinase inhibitor-resistant mutations were detected in 90% of tumor samples after treatment using these techniques (against the previously reported 45–70%).[25,62,64] Furthermore, in three cases, two (34%) secondary *KIT* mutations were observed in the same metastasis. Notably, patients treated with sunitinib showed a wider spectrum of mutations (1–5) compared with those treated with imatinib (1–2), and displayed a prevalence of *KIT* exon 17 mutations (60%), in keeping with *in vitro* studies showing that KIT activation loop mutations are sunitinib resistant.

to imatinib because it affects the A-loop of the catalytic domain and leads to constitutively activated kinase.[6] In *in silico* experiments with this mutation, the mutant receptor has $\Delta\Delta G_{bind}$ of –4.43 kcal/mol and is less likely to bind to imatinib than the wild-type protein. Notably, the imatinib insensitivity is dramatically reversed by a deletion of the same residue in ΔDIMH842–845, once again underscoring the importance of structural state resulting from the changes in the residues involved in this specific case (Supplementary Figure 4 online).[29] The in-frame deletion of the four residues D842–H845 in the core domain of PDGFRA does not negatively interfere with the conformation of the imatinib binding site; on the contrary, it is beneficial to the binding in that it favors a better accommodation of the inhibitor within the kinase binding pocket. Accordingly, the calculated affinity of this PDGFRA mutant isoform for the inhibitor is even slightly higher than that of the wild type receptor ($\Delta\Delta G_{bind}$ = +0.89 kcal/mol).

Molecular modeling in clinical settings

Molecular dynamic simulations of imatinib at the atomic level in complex with KIT receptors with T670I and V654A mutations indicate that these missense substitutions alter the conformation of the receptor drug-binding pocket, although to a different extent. In fact, the replacement of a threonine with an isoleucine at position 670 of KIT introduces several substantial modifications in the conformation of other residues, which induces a collapse of the ATP-pocket. Conversely, the presence of an alanine replacing a valine at position 654 of the same RTK results in only moderate structural alterations.[27] Thus, although both T670I and V654A missense mutations cause imatinib-acquired resistance, the former is far more resistant to imatinib than the latter. On the basis of these data, it was predicted that the effect of the V654A mutation might be subverted by a dose escalation of the inhibitor that was expected to recapture a clinical response[27] (Figure 3d–f). This example clearly illustrates how the tight coupling of biochemical analysis of mutated receptors (testing the actual imatinib resistance) and molecular modeling can yield vital information to medical oncologists, and even indicate the most suitable dose for escaping secondary resistance.

Molecular modeling provides more than just the criteria to determine, at a molecular level, the success or failure of a given tyrosine kinase targeted therapy in GIST. Molecular modeling may have a more important role in overcoming mutation resistance, as highlighted in the T670X model.[30] Negri *et al.*[30] assessed why only isoleucine is found in place of threonine at position 670 of KIT in unresponsive patients with GIST, and investigated all the mutations permitted by genetic code at this RTK position. The six different alternatives to the wild-type residue threonine produced six functionally different KIT receptors; these included two inactivated KITs (T670R and T670P, loss-of-function substitutions), and four constitutively activated KITs (T670A, T670S, T670K and T670I, activating mutations). Notably, all mutants in this second set were found to be imatinib-sensitive except for the one with the isoleucine mutant residue, which was totally imatinib-resistant.[30] According to this model, we can assume that a Darwinian-type selection is occurring because, among all possible variant forms allowed by the genetic code, only those clones providing a selective advantage emerge in the presence of the drug. All of these findings were confirmed by *in silico* experiments and the thermodynamic information gathered from this molecular model completely match the *in vitro* data.[30] Taken together, the evidence favors the utility of such an *in silico* approach in a clinical setting for predicting all the possible mutations, including those not yet detected in patients with GIST.

RTK inhibitors

The essential role of the ATP pocket in maintaining kinase function is beginning to be eludicaded, making it a major focus for kinase inhibitor research. Most of the current inhibitors inactivate KIT by binding to the tyrosine kinase 1 domain (exons 13 and 14) and locking the complex in the closed conformation (DFG-out). However, because the similarities in binding sites of different kinases at the common substrate ATP may limit their selectivity, other sites of the receptor have been exploited. A study on structural mapping of kinase genetic variants and their mutants coupled with crystal structure data showed that kinase cancer mutations preferentially cluster in the P-loop and A-loop (mutational hot-spots), and that the most relevant mutations destabilize the inactive tyrosine kinase form favoring a stabilization of its active form.[31] In fact, mutations in the A-loop are an ongoing pharmacological challenge. The A-loop acts like a gate swinging back and forth, promoting the protein conformational change from the inactive to the active conformation. Thus, the conformational flexibility of these proteins promotes the emergence of resistance.

The shift of equilibrium away from inactive state is most certainly believed to be the key determinant of the loss in binding capacity of the inhibitor to the receptor. In fact, mutations in the tyrosine kinase 2 domain generally contribute to maintaining the gate in its open state (DFG-in). Licensed kinase inhibitors that bind to the

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open or active form are classified as type I inhibitors, for example, dasatinib, whereas those that bind to the closed or inactive form are termed type II inhibitors, for example, imatinib, nilotinib and sunitinib.

Sunitinib

Sunitinib has been proved to be effective in imatinib-resistant GISTs that have secondary mutations in the ATP binding domain (V654A, exon 13 and T670I, exon 14), whereas mutations within the A-loop seemed to be resistant (D816V and D816H on exon 17) to the drug.[32] Experimental measurements of kinase auto-activation rates together with molecular modeling seem to support the following hypothesis: the shifting of the A-loop towards an open, active conformation, coupled to an accelerated autophosphorylation of the mutant RTK, is the mechanism underlying the resistance to sunitinib exhibited by D816H and D816V mutants.[33] On the other hand, the binding mode of sunitinib to KIT is slightly different from that of imatinib, the former reaching less deep into the tyrosine kinase binding pocket than the latter. Accordingly, in the presence of sunitinib, the longer side chain of the isoleucine residue in the KIT T670 mutant can fit in the cavity without inducing a dramatic conformational change of the binding site and, therefore, without impairing substantially the affinity of the protein for the inhibitor.[33] Notably, the clinical response for primary *KIT* exon 9 mutant and wild-type GISTs was higher in patients treated with sunitinib than in those treated with imatinib.[34] Molecular modeling studies are ongoing to shed light on these findings.

Nilotinib

Among the second generation KIT and PDGFRA kinase inhibitors, nilotinib (which is structurally related to imatinib), seems to be 30 times more potent than imatinib against wild-type *KIT*. Nilotinib has also shown encouraging results in patients who failed imatinib and sunitinib treatment.[35] Our preliminary (unpublished) calculations seem to be in line with these clinical findings. Despite the significant chemical and structural similarities between imatinib and nilotinib, our *in silico* evidence indicates that nilotinib assumes a slightly different conformation within the binding pocket, allowing for a tighter fit and, hence, a better binding. However, preliminary clinical data suggest that substantial benefits are restricted to first-line treated patients,[36] whereas in advanced GISTs no statistically significant differences were observed in progression-free survival or overall survival between the nilotinib and control arm (the control arm more frequently consisted of cases treated with imatinib or sunitinib, but also cases for which the treatment was stopped).[37]

Dasatinib

Dasatinib is a potent inhibitor of imatinib-resistant, wild-type, and mutated KIT. This potency is possibly a consequence of its ability to inhibit the DFG-in (active) form of the kinase, and many of the mutations are thought to destabilize the inactive form in favor of the active one. In fact, dasatinib targets the active conformation, which is highly conserved and thus shared by other kinases.[38] Notably, dasatinib is a potent inhibitor of D816V and D816F[39] as well as PDGFRA D842V, as demonstrated by *ex vivo* and *in vitro* experiments.[40] Crystallization of dasatinib in different tyrosine kinase complexes clearly reveals that this drug binds to the active state of the kinase. Therefore, all effects caused by the presence of mutations that disrupt the inactive state of the kinase (by reverting the conformation of the A-loop toward an open conformation) may affect the affinity of dasatinib for the corresponding mutant isoforms.

Table 1 | Inhibitors against alternative targets for the treatment of GIST

Drug name	Company	Phase of development
Drugs that inhibit HSP90 resulting in the proteasomal degradation of oncogenic client proteins		
IPI-504 (retaspimycin)	Infinity Pharmaceuticals in conjunction with MedImmune (Astra Zeneca)	Phase III trial was suspended due to safety concerns
STA-9090	Synta Pharmaceuticals Corp.	Phase II
BIIB021	Biogen Idec	Phase II
BIIB028	Biogen Idec	Phase I
SNX-5422	Serenex	Phase I
XL888	Exelixis	Phase I
AUY922	Novartis	Phase I
AT13387	Astex Therapeutics	Phase I
Drug that inhibits the targeted proteolysis via the 26S proteasome		
Bortezomib (Velcade)	Millenium Pharmaceuticals	Currently in combination trials
Drugs that inhibit mTOR		
Everolimus (RAD001)	Novartis	Phase II
Ridaforolimus (Deforolimus, AP23573)	Ariad Pharmaceuticals	Phase III
Drug that inhibits PI3K and mTOR		
PF-04691502	Pfizer Oncology	Phase I
Drugs that deacetylate by histone deacetylase inhibitors, leading to an accumulation of both hyperacetylated histones and transcription factors		
Vorinostat (Zolinza, SAHA)	Patheon, Inc. (Merck)	Phase I
CUDC101	Curis, Inc.	Phase I
Panobinostat	Novartis	Phase I

Abbreviation: GIST, gastrointestinal stromal tumor; HSP90, heat shock protein 90; mTOR, mammalian target of rapamycin.

Other kinase inhibitors

The sensitivity of *KIT T670I*, *KIT V654A* and *PDGFRA D842V* mutations to PKC412 was tested in cell cultures: these mutated receptors proved to be sensitive to the treatment.[24] Moreover, PKC412 was a potent inhibitor of imatinib-resistant D816V KIT.[41,42] Our *in silico* models of KIT and PDGFRA reveal that the side chain of D816 in KIT is involved in a hydrogen bond with N819, which has an essential role in maintaining the correct DGF-out conformation of the inactive kinase form. Clearly, this bond is no longer present when valine is substituted at this position. Owing to the repositioning of the side chain, other important hydrogen-bonds (that is, with A597 and K818) are no longer dominant interactions. The

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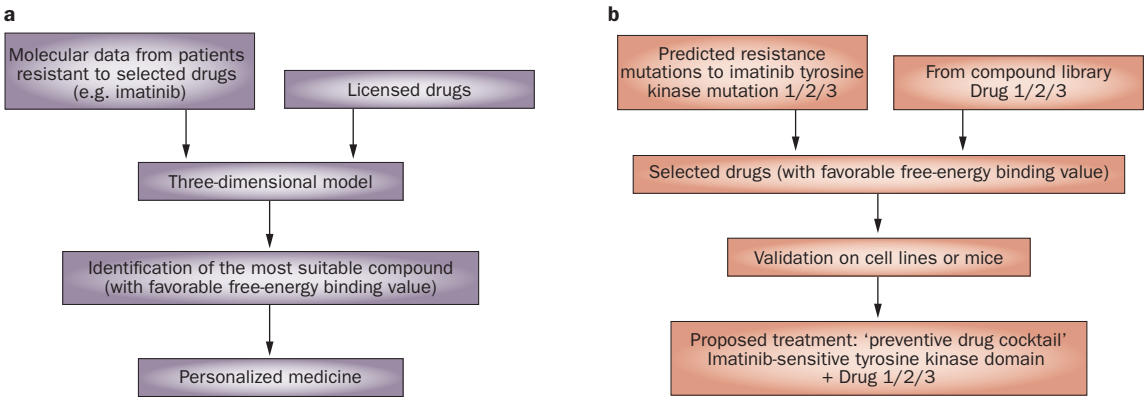

Figure 4 | Clinical prediction of treatment outcomes with molecular modeling. **a** | Current approaches. The type of secondary mutation in KIT and/or PDGFRA is assessed, followed by three-dimensional modeling of the candidate receptor carrying the mutation of interest. Licensed drugs are screened in order to choose the compound with the most favorable free energy of binding value. **b** | The new strategy includes a computer-based approach in order to predict all new possible activating but drug-resistant receptor tyrosine kinase mutations. The three-dimensional models of the receptors carrying these mutations will be used to screen compound libraries, select and develop drugs to be combined in a preventive cocktail capable of inhibiting all the resistant mutations.

calculated free energy of binding for Δ559 + D816KIT with imatinib is –10.02 kcal/mol, whereas the corresponding value for Δ559 + V816KIT with imatinib is –6.89 kcal/mol. These results clearly indicate that mutation of the key aspartic acid residue, D816KIT, is instrumental in destabilizing the inactive state of this protein, resulting in a lower affinity for imatinib.

PKC412 is a competitive inhibitor that targets the open kinase form. Its mechanism of action is, therefore, clearly different from that of imatinib, which targets the kinase inactive form. In the open conformation, the 816 residue does not seem to be involved in any particular intramolecular interaction, and the calculated free energies of binding for the wild type and mutant forms are very similar. Accordingly, the D842V mutation does not seem to interfere with PCK412 binding to PDGFRA, in keeping with the clinical response to this drug.[43]

Regarding the switch pocket kinase inhibitors, if they are able to target the switch pocket kinase domain, in principle they should also prevent the binding of the phosphorylated switch by competitively binding to this region and thus blocking the conformational activation of the kinase. The compounds currently in development seem to be highly effective, even at a very low concentration, not only against wild-type KIT and PDGFRA but also against the most common KIT and PDGFRA drug-resistant mutations (V654A, T670I, D816H, D816V and D842V) detected in recurrent patients treated with the clinically available tyrosine kinase inhibitors.[44]

Alternative targets

Inhibitors in development for GIST treatment are listed in Table 1. In particular, *in vitro* data suggested that IPI-504, a heat shock protein 90 inhibitor, might be a therapeutic option for GIST harboring D842V.[40,45] Among the new treatment options, the agents in development are histone deacetylase inhibitors,[46] and bortezomib, which inhibits KIT through transcriptional downregulation with strong apoptotic effects.[47] Another promising compound is RAD001, which inhibits mTOR and is now in phase II testing.[48,18] However, even though this compound displays an acceptable toxicity profile, its efficacy in the clinical setting seems to be limited in patients with advanced GISTs[49] and those treated with imatinib and sunitinib.[50] Unfortunately, *in silico* data does not help to elucidate why the efficacy is limited.

Conclusions

With the exception of two recently described KIT mutations (S709F and K818R),[51,52] the most commonly reported KIT/PDGFRA secondary mutations affect 13 different positions along the protein primary sequence. These mutations cluster into three domain regions (tyrosine kinase 1, kinase insert, tyrosine kinase 2), the last of which accounts for six different amino acidic substitutions in KIT: C809, D816, D820, N822, Y823, A829.[9,53] These substitutions result in 21 variants. However, from our experimental and *in silico* observations, we know that, from a functional standpoint, the overwhelming majority of these mutations exert similar effects in that their presence induces a change of the kinase toward its active (open) conformation, thus narrowing the number of ATP-competitive inhibitors. The challenge is, therefore, to overcome the development of secondary mutations that, in a tumor as heterogeneous as GIST, can occur in different combinations of up to five different types in the same patient (Box 2).

The promising switch pocket inhibitors currently in development aim to target kinases directly. However, other proposed new strategies are aimed at inducing KIT oncoprotein degradation to obtain a strong apoptotic effect[47] or inhibiting KIT-dependent PI3K downstream signaling using mTOR inhibitors.[18] Nonetheless, we think that these proteins constitute extremely promising targets. Realizing this promise is dependant on the synergistic action of alternative and more powerful techniques, including computer-based approaches such as those outlined here, to promptly predict new, possible RTK mutations.

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The Darwinian selection of mutated variants of target proteins could be harnessed with the intelligent design of drugs directed against the single new variants. This innovative multi-drug targeted prevention, which can potentially change the natural history of a disease, has been proposed in the setting of chronic myeloid leukemia. The positive results obtained with the newly introduced drugs nilotinib and dasatinib suggested that a "combination of two or three kinase inhibitors, when carefully selected to cover all known resistant mutations, could shut off all mechanisms of escape"[54] (Figure 4). A word of caution is mandatory because additional adverse effects caused a synergism in toxic effects may require drug concentrations to be tailored *ad hoc*. However, by predicting the mutations, and classifying them according to their degree of probability, it could be possible to administer a drug as soon as patients display such mutations, so that disease recurrence can be prevented, rather than waiting for the mutations to emerge to select the right compound.

Whether secondary resistance might be safely avoided or delayed by the combined administration of TKIs upfront or by a sequential administration strategy remains an open question. Nonetheless, the possibility to predict secondary resistance mutations, and hence to select the best active inhibitors and the optimal doses to treat the affected patients represents an effective tool to face this problem with a measure of optimism.

Review criteria

Data for this Review were compiled by searching the PubMed database. The search terms used were "receptor tyrosine kinases", "GIST" and "molecular modeling". Only articles published in English before July 28 2010 were considered. The ASCO 2010 website was also searched.

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Author contributions

M. A. Pierotti, E. Tamborini, T. Negri, S. Pricl and S. Pilotti contributed equally to the literature review, discussions of the content, writing the article and to review and/or editing of the manuscript before submission.

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