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Study of the role of "gatekeeper" mutations V654A and T670I of c-kit kinase in the interaction with inhibitors by means mixed molecular dynamics/docking approach

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Abstract:
The over-expression of *c-kit* proto-oncogene has been reported in hematopoietic cells, small cell lung cancer, and gastrointestinal stromal tumors. The clinical importance of c-kit expression in tumors focused the research towards inhibitors of this tyrosine kinase. Imatinib (Gleevec®) was the first compound used in therapy, but mutations on c-kit led to reduced effectiveness or ineffectiveness of this treatment. Other compounds are likely to be effective against mutants, such as Sunitinib (Sutent®), but the need for new and most effective inhibitors against mutants is still critical. We report mixed Molecular Dynamics/Docking study with the aim to unveil the molecular mechanism involved in the resistance of Imatinib, Sunitinib, and other known compounds against the "gatekeeper" mutants V654A e T670I. We tried to evidence strong and weak features of actual inhibitors in order to identify the guidelines to design new and most potent inhibitors against c-kit mutants.

Background:
The *c-kit* proto-oncogene encodes a transmembrane tyrosine kinase receptor which is activated by the stem cell factor (SCF), its natural ligand. C-kit protein plays a critical role in modulating histamine release from mast cells **[1-2],** following its binding with SCF which leads to dimerization and auto-phosphorylation at specific tyrosine residues. Moreover signaling by c-kit, plays an important role in cellular transformation and differentiation, including proliferation, survival, adhesion, and chemotaxis **[3].** The over-expression of *c-kit* proto-oncogene has been reported in hematopoietic cells, small cell lung cancer, and gastrointestinal stromal tumors **[4-6].** Furthermore, it has been demonstrated that mutations of c-kit protect human colon adenocarcinoma cells against apoptosis and enhance their invasive potential **[7].** The clinical importance of c-kit expression in tumors focused the research towards inhibitors of this tyrosine kinase. Imatinib (Gleevec®) was the first compound used in therapy, but mutations on TK1 domain, known also ATP-binding pocket, (V654A, T670I gatekeeper mutations of c-kit) led to reduced effectiveness or ineffectiveness of this treatment **[8].** Other compounds are likely to be effective against mutants, such as Sunitinib (Sutent®), but the need for new and most effective inhibitors is still critical. In order to understand which features of the inhibitors could be determinant in the interaction with wild type and/or mutant enzyme, in this paper is reported mixed Molecular Dynamics/Docking study with the aim to unveil the molecular mechanisms involved in the resistance of Imatinib, Sunitinib, and other known compounds against the "gatekeeper" mutants V654A e T670I. We tried to evidence strong and weak features of actual inhibitors **(Figure 1)** (Nilotinib, Sorafenib, Motesanib, PKC-412, a thienopyrimidine derivative TPD, an

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aminobenzoisoxazole derivative ABIOZ) in order to identify the guidelines to design new and most potent inhibitors against c-kit mutants.

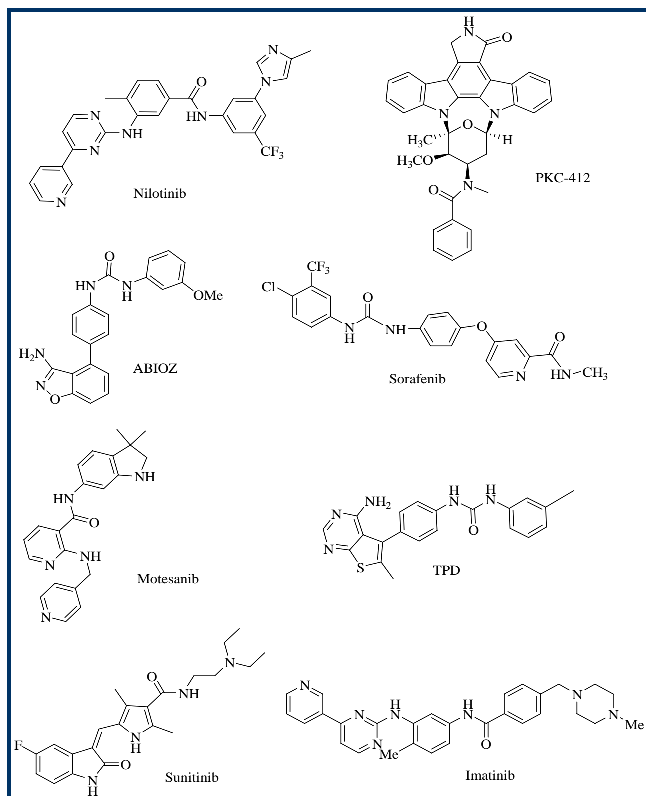

Figure 1: Known c-kit inhibitors

Methodology:
The three dimensional crystal structures of intracellular domain of tyrosine kinase c-kit complexed with Imatinib (Pdb: 1T46) and complexed with Sunitinib (Pdb: 3G0E) were used as receptor throughout the work. The two crystal structures, analyzed by means TM-align software **[9]**, revealed RMSD = 1.39 Å and TM-score = 0.95 (TM-score >0.5 means good structural alignment). Crystallized inhibitors were re-docked with the aim to evaluate the ability of Glide docking software **[10]** to reproduce the experimental conformation (Imatinib crystallized/docked RMSD = 0.43 Å; Sunitinib crystallized/docked RMSD = 0.51 Å). All the structures of compounds (Nilotinib, Sorafenib, Motesanib, PKC-412, TPD, ABIOZ) object of the study were prepared by means Ligprep **[11]**. The mixed Molecular Docking/Dynamics protocol, called Induced Fit Docking (IFD) **[10, 12]** was used. This approach combines, in an iterative fashion, the ligand docking techniques with those for modeling receptor conformational changes. The Glide docking software is used for ligand flexibility, while the refinement module in Prime program **[12]** is used to account for receptor flexibility: the side chain degrees of freedom are mainly sampled, while minor backbone movements are allowed through minimization. The strategy is to dock first ligands into a rigid receptor using a softened energy function such that steric clashes do not prevent at least one pose from assuming a conformation close to the correct one (ligand sampling step). Further, the receptor degrees of freedom are sampled, and a global ligand/receptor energy minimization is performed for many ligand poses which attempts to identify low free-energy conformations of the whole complex (protein sampling step). A second step of ligand docking is performed on the refined protein structures, using a hard potential function to sample ligand conformational space within the refined protein environment (ligand resampling step). Finally a composite score function is applied to rank the complexes, accounting for the receptor/ligand interaction energy as well as strain and solvation energies (scoring step). The composite score, used for final ranking of compounds, is reported in the equation: IFScore = GlideScore + 0.05 PrimeEnergy. c-kit crystallized structures were modified inserting "gatekeeper mutations" and optimized by means Prime to erase bumping and altered dihedral angles.

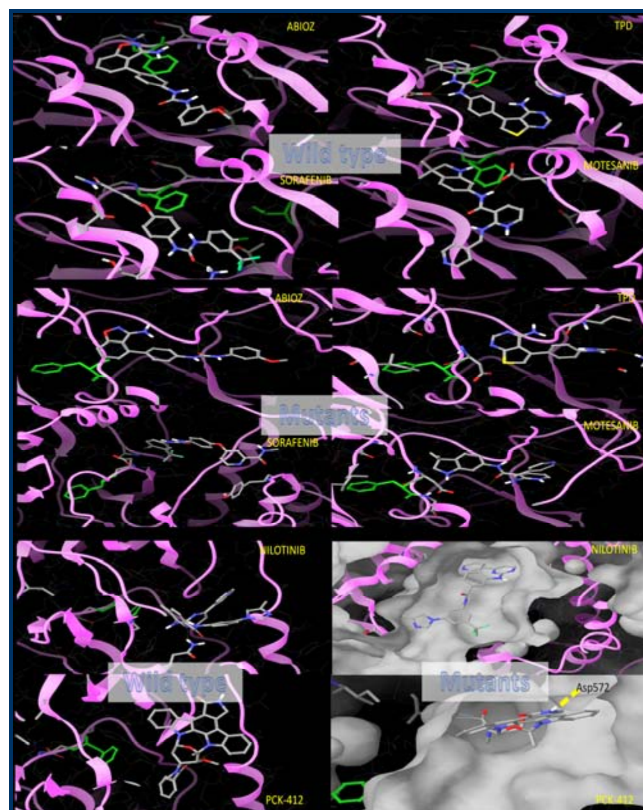

Figure 2: Docking poses of inhibitors within wild-type and mutant protein. In green Phe811 is represented.

Discussion:
Imatinib occupies hydrophobic pockets generated by Trp577 and Phe811 in the inactive conformation. Beyond these, it establishes three H-bonds with three different residues of ATP binding pocket: the first between NH of Asp810 and the oxygen atom of amide moiety; the second between the NH separating the aromatic rings and the oxygen of the gatekeeper residue Thr670; the third between the nitrogen atom of pyridine moiety and the NH of Cys673. Thus, not only hydrophobic and aromatic features are involved in the modulation of ligand-receptor interaction, but these are doubtless predominant. Also Sunitinib, bound to wild-type protein, forms one H-bond with Cys673 and π-stacking interaction between Phe811 and the fluoroindole moiety. Further, distances from gatekeeper residues Val654 and Thr670 are quite stable. The mutation T670I determines the loss of one of the H-bond between Imatinib and

c-kit, consequently a weaker binding could be the reason of minor effectiveness and therefore could lead to resistance. Introduction of mutations does not substantially influence binding mode of Sunitinib, as expected being this inhibitor effective against mutants. The principal difference regards Phe811 which is not oriented towards the binding pocket, but is rotated in the opposite side. Two H-bonds were formed: the first between the NH of indole moiety of Sunitinib and Glu640; the second between the peptide carbonyl of Ile789 and the amide NH of the inhibitor. Hydrophobic interaction with 654A remains unaltered due to the similar distance of mutant residue respect to wild-type residue (5.0 Å vs 4.78 Å), in the same manner the distance of 670I respect to the indole nitrogen is quite similar. The binding mode of four inhibitor (TPD, ABIOZ, Sorafenib, Motesanib) is really different. In fact for all compounds the same condition was verified: in wild type protein, the formation of one or more H-bond (with Asp723 and Gly676 for TPD; Lys593, Thr594, Asp723 for Sorafenib; Arg796, Gly676 for ABIOZ; Asp677 for Motesanib) together with the rotation and π-stacking interaction between each inhibitor and the phenyl ring of Phe811, turn out to be essential. In the mutant protein, docking scores are lower than wild-type ones: this could be justified by the loss of π-stacking interaction with Phe811. In fact, in all the cases the phenyl ring of Phe811 is oriented toward the opposite site of binding pocket, therefore opposite to inhibitors as verified for Sunitinib, but with the consistent difference that distances from mutant residues are longer than wild type, leading to the loss of interaction that could justify lower scores. Number and distribution of H-bonds keep constant, but some residues are different from those involved in wild-type and Ile789 is always involved in H-bonding with all these four inhibitors. For Nilotinib and PKC412, both in wild type and in mutant protein, the orientation of the phenyl ring of Phe811 turns out to be not influent even if rotated of 180° with respect to the inhibitors, because the hydrophobic pocket created by 654A and 670I is occupied by the pyridine ring of Nilotinib and by the phenyl ring of PKC412, which keeps this orientation also by means a H-bond with Asp572. Therefore, docking results stressed the fact that the presence of gatekeeper mutations lead to the change of key residue Phe811 orientation, which rotate almost 180° out of the binding pocket, so that selectivity or resistance to an inhibitor against mutant protein is related to the loss of Phe811 interaction in quantitative terms. This loss should be balanced with new hydrophobic interactions. In fact, Nilotinib and PKC412, able to establish new hydrophobic interactions with sub-pockets created by mutant residues seem to prove the key role of this type of interactions. Hydrogen bonds are important for binding, but not for selectivity or resistance. The presence of NH groups, both amidic or aromatic, favour the binding with c-kit, but residues involved in H-bonds are several and they do not turn out crucial to foresee the arise of resistance. By means information collected from docking analysis, it is possible to obtain useful and concrete guidelines to design new compounds able to keep the inhibitory activity also against the mutant protein. Design of new compounds should head towards inhibitors with strong hydrophobic ability, determined by aromatic moieties, also fused, and H-bond donor groups. Inhibitors which turn out most selective against mutant are about 17-18 Å long, while resistant compounds do not exceed 15 Å, therefore scaffold lengthening seems to lead to increase the interaction with gatekeeper residues which are located at 5 Å from the binding pocket.

Conclusion:

In this work we focused on the study of c-kit kinase inhibitors, overexpression of which was demonstrated the cue event of gastrointestinal stromal tumors (GISTs). We studied the role of gatekeeper mutations V654A and T670I, which are located into the activation loop (A-loop of catalytic domain), in relation to the binding of therapeutic drugs (Imatinib, Sunitinib and other important inhibitors). By means a mixed Molecular Dynamics/Docking approach we tried to understand the molecular mechanisms involved in the resistance or selectivity of Nilotinib, PKC412, Sorafenib, Motesanib, TPD, ABIOZ. The goal was to point out strong and weak features of known inhibitors suggesting valuable guidelines to the design of more potent inhibitors, and able to overcome resistance. This theoretical approach can reveal useful to develop an effective anti cancer drug.

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