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A Tumor (GIST), a Mutated Gene (c-kit), and a Molecular Inhibitor (Imatinib): Insights from Computer Simulations

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Prepared for presentation at 2003 AIChE Annual Meeting, November 16-21, 2003, Food, Pharmaceutical, Bioengineering and CoFe

Abstract
Deregulation of c-kit has been implicated in the etiology of a number of cancers, including gastrointestinal stromal tumors (GISTs). Most of GISTs carry mutations in c-kit, resulting in an enhanced and constitutive ligand-independent tyrosine kinase activity that appears to play a key role in the pathogenesis of these tumors.
Imatinib (STI571, Glivec®, Gleevec™), a 2-phenylaminopyrimidine derivative, has shown remarkable efficacy in the treatment of GISTs, which are notoriously unresponsive to cancer chemotherapy.
In this work we report the results obtained from a combined effort of bringing together clinical evidences on a newly discovered c-kit mutation in GISTs, showing acquired resistance to Imatinib, and a possible explanation, based on molecular simulations, of the mechanism by which this mutation may results in GISTs resistant to Imatinib.

Introduction
Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the gastrointestinal tract. GIST may develop anywhere along the gastrointestinal tract, but most often it arises in the stomach and, less commonly, in the intestine. GIST represents a spectrum of tumors that range from benign to highly malignant. Predicting the clinical behavior of a particular GIST is imprecise. In general, the malignant potential of GIST depends primarily on tumor size, mitotic activity, and anatomical origin. Tumors from the small intestine often behave more aggressively than tumors from the stomach; however, most GISTs fall into a 'gray zone' and, therefore, the ultimate determination of malignancy depends on the development of tumor recurrence or metastasis.
The development of Imatinib mesylate (STI571, Glivec®, Gleevec™) by Novartis Pharmaceuticals (Basel, Switzerland), is a landmark achievement in cancer therapy. Imatinib is an inhibitor of a number of tyrosine kinases, including the intracellular kinase Abl, the growth factor receptors kit, the platelet derived growth factor (pdgf), and their oncogenic activated forms (Mauro et al., 2002). This action is due to competition with ATP for the ATP-binding site of the kinases. By preventing ATP binding, the drug is able to avoid the complex downstream signaling in the cell. The application of Imatinib to GISTs was a direct result of 1) its selective inhibition of the kit receptor tyrosine kinase, which is constitutively active in most GISTs; 2) its efficacy and minimal toxicity in patients with chronic myeloid leukemia (CML), where it inhibits the Bcr-Abl fusion protein that possesses constitutive tyrosine kinase activity from a balanced translocation between chromosomes 9 and 22; 3) the parallels between the pathogenesis of GIST and CML; and 4) the lack of effective alternative for metastatic GISTs.
In CML, Imatinib is highly effective both in early and late stages of the disease. Nonetheless, several relapses do occur after initial response, despite continued treatment (La Roseé et al., 2002). In patients who developed resistance to Imatinib, reactivation of the Bcr-Abl signaling was observed, due to either a secondary mutation, resulting in the substitution of one or more residues belonging to drug binding site and critical for binding, or to a progressive Bcr-Abl gene amplification (Bonn, 2001; Nimmanapalli and Bhalla, 2002).
In GISTs, primary resistance seems to involve at least 15% of patients with advanced disease, and its occurrence could be correlated with different c-kit mutations. *c-kit* is an oncogene located on chromosome 4 that is the cellular homolog of *v-kit* from the Hardy-Zuckerman 4 feline sarcoma virus (Besmer et al., 1986). The gene encodes a transmembrane receptor tyrosine kinase called Kit, which is expressed by the interstitial cells of Cajal (from which GIST is thought to originate), hematopoietic cells, melanocytes, and mast cells. The natural ligand for Kit is known variably as Kit ligand, stem cell factor, Steel factor, or mast cell growth factor. Binding of kit ligand induces dimerization and autophosphorilation of Kit. This, in turn, increases the interaction with and phosphorilation of signal transduction proteins. The result is activation of a cascade of intracellular

proteins that promote cell survival and proliferation.
In 1998, Hirota et al. firstly reported that a mutation of *c-kit* was found in five patients with GIST (Hirota et al., 1998). The mutations were located in exon 11 of the gene, and resulted in activation of the kit-receptor. Since then, mutations in other regions of the c-kit gene, including exons 9, 13, and 17, have also been reported (see, for instance, Lux et al, 2001). The reported prevalence of *c-kit* mutations in GIST has varied considerably, the discrepancy in prevalence being possibly related to different methods of detection and the type of tissue analyzed. The presence of *c-kit* mutation has been reported to adversely affect survival in patients with GIST (Ernst et al., 1998); whether the presence of *c-kit* mutation alone actually influences clinical outcome remains, however, uncertain.
Imatinib has been approved by FDA and EMEA from treatment of advanced GIST in 2002. At the Istituto Nazionale per lo Studio e la Cura dei Tumori di Milano, among a series of 105 patients enrolled in a Phase III, prospective controlled trial on Imatinib mesylate at two dose levels in advanced GISTs (Casali et al., 2002), a point mutation in exon 14, observed only in Imatinib non-responding metastases, was identified for the first time (Tamborini et al, 2003). This mutation, T2030C, results in the corresponding protein point mutation Thr670Ile, belonging to the ATP pocket of the kit receptor. Interestingly, the alignment of kit primary sequence with other tyrosine kinase receptors revealed that this residue corresponds to Thr315 of Abl receptor and, in this respect, the Thr760Ile mutation strictly parallel another mutation, which has been claimed to be the caused of acquired resistance to Imatinib in CML: Thr315Ile in Bcr-Abl protein (Gorre et al., 2001).
Previously reported work, based on the crystallographic structure of Bcr-Abl in complex with Imatinib, postulated that the mutation Thr315Ile in the tyrosine kinase domain of Bcr-Abl both alters the three-dimensional structure of the ATP pocket, thus decreasing the sensitivity to Bcr-Abl for Imatinib (Schindler et al, 2000), and does not features a fundamental hydrogen bond, critical for binding with Imatinib (Corbin et al., 2002). These speculations, however, were not supported by further investigations.
Considering the high homology between the ATP-binding pocket of c-kit and Bcr-Abl, in this work, we therefore present, for the first time, the results obtained from the application of detailed molecular modeling techniques to the study of the interactions between Thr760Ile (i.e., Thr315Ile in Bcr-Abl) and Imatinib.

Computational details
The starting model was the X-ray crystal structure of the complex of c-kit and Imatinib. All calculations were carried out using the Amber 6.0 suite of programs (Case et al., 1999) using the all-atoms force filed by Cornell et al. (1995). All missing parameters for Imatinib were obtained performing *ab initio* calculations on a minimized structure. The corresponding partial charges were obtained using the standard multiple molecule RESP fit (Cornell et al., 1993). To calculate the energetics of binding and examining the effects of conformational change and dynamics induced by the Thr315Ile mutation, we applied the following ansatz: binding free energies ΔG of Imatinib to wild type and Thr315Ile mutant Bcr-Abl were obtained by applying the so-called molecular mechanics Poisson-Boltzmann surface area (MM/PBSA) method of Kollman et al (Srinivasan et al., 1998). Computational mutagenesis studies with MM/PBSA, allowed us to calculated the relative binding of wild type and mutant protein-inhibitor association. To generate the mutant structure and estimate the relevant ΔG, we applied a method proposed by Reyes and Kollman (2000), consisting in performing a separate molecular dynamics (MD) simulation on the mutated structure. Although more computationally expensive, this method gives more detailed insights on the structure and dynamics of the mutant. All energy analyses were performed separately for each complex and monomers of both mutant and wild type structures. The molecular mechanical energies were obtained with the *anal* module of the Amber 6.0 suite of programs, with the Cornell et al. force field. All intra-solute pairwise interactions were accumulated without a distance-based cut-off. Solvation free energy was estimated by continuum solvent methods as the sum of electrostatic and non-polar contributions. The

firsts were estimated by finite-difference solution of the Poisson-Boltzmann equation, as implemented in the Delphi package (Sharp and Honig, 1990). The non-polar contribution was estimated as a linear function of the solvent-accessible surface area, which implicitly includes the solute-solvent van der Waals interactions.

The mutation Thr315Ile was introduced to the wild type crystal structure of the c-kit-Imatinib complex using InsighII (from Accelrys, San Diego CA, USA) by swapping the mutant residue into the specific site (Lovell et al., 2000). Starting mutant side chain orientation was selected using the side chain rotamer library method. Both the wild type and its mutant structure were relaxed using the *sander* module of Amber. We run 300 ps MD simulation of the Thr315Ile mutant complex at 300K with explicit water and counterions. A total of 25 snapshots from the mutant MD trajectory were then analyzed by MM/PBSA. The difference between the average mutant trajectory binding and wild type trajectory binding was calculated to yield the $\Delta\Delta G$ and standard deviations.

Results and Discussion

Mutating Thr to Ile at position 315 in the ATP-binding domain of the Bcr-Abl results in a calculated $\Delta\Delta G$ of binding of 1.74 kcal/mol with respect to the corresponding wild type structure. This value is in a very good agreement with the corresponding experimental finding of > 1.07 kcal/mol.

Co-crystallization of the Abl kinase domain with Imatinib identified a number of amino acid residues within the ATP binding pocket predicted to contact the inhibitor (Nagar et al., 2002). These included hydrogen bonds with Thr315, Met290, Glu286, Lys271, His361, Ile 360, and the peptide backbone at Asp381 and Met318, as well as hydrophobic interactions with Ile313, Phe382, Val256, Tyr253 and Leu370. In particular, Corbin et al. (2002) showed that not only the Thr315Ile mutation exhibited no significant inhibition at Imatinib concentrations 200-fold higher that the IC_{50} value of the wild type Abl kinase, but also demonstrated a 2-fold increase in its ATP affinity relative to the wild type: Accordingly, these authors suggested that this mutation not only eliminates a critical hydrogen bond with Imatinib but also increases the affinity of ATP binding relative to Imatinib, contributing to resistance.

Figure 1 shows two snapshots taken from the MD simulation of the mutant at the beginning (10 ps) and at the end (300 ps) of the simulation period, respectively.

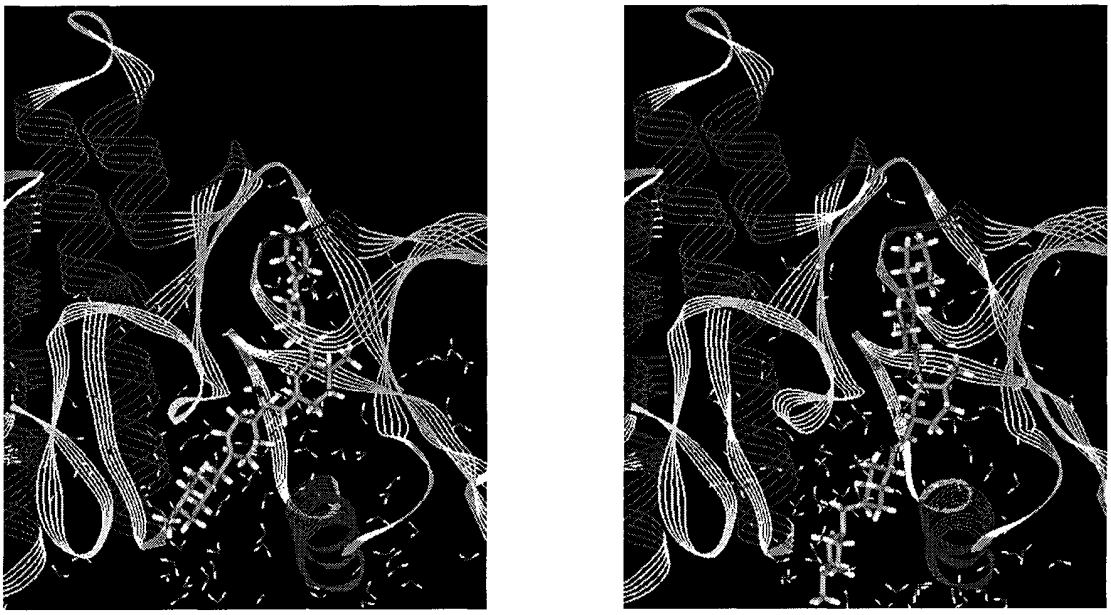

Figure 1. Shapshots taken from the MD simulation of the Thr315Ile Bcr-Abl mutant at the beginning (10 ps, left) and at the end (300 ps, right) of the simulation period.

The analysis of the relevant trajectory clearly reveals that, with respect to the wild type system, the missing of the supposed-to-be critical H-bond is not the only cause for the failing of receptor inhibition by Imatinib. Besides the obvious (and somewhat aleatory) dynamic nature of the water-mediated stabilizing interactions, we can see that the interaction of the drug with the activation loop is drastically changed, the drug progressively drifting away from it during the considered time interval. In details, not only several of the wild type/Imatinib stabilizing H-bonds no longer exist in the mutant, but also a plethora of van der Waals and hydrophobic interactions are drastically, unfavorably changed in the mutant trajectory. As an example for all, the role played by the conformation of Phe382 of the well-conserved DFG motif, pointing towards the APT binding site (Figure 2, left), and thought crucial for the proper binding of Imatinib (Nagar et al., 2002), is no longer maintained in the mutant trajectory, resulting in the net loss of a favorable stabilizing interaction.

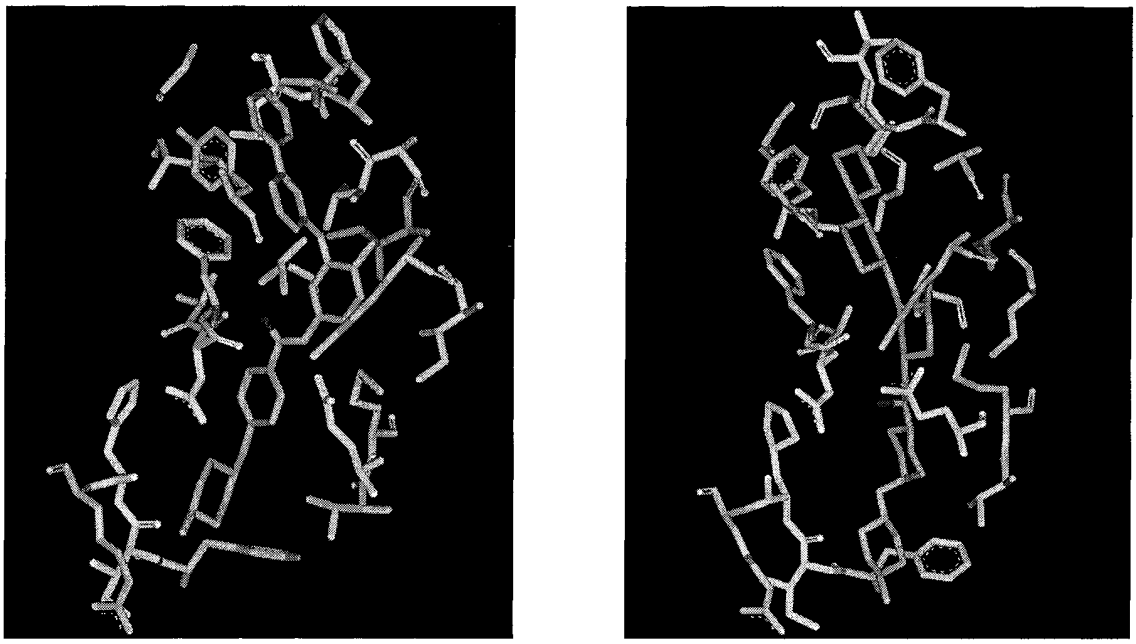

Figure 2. ATP-binding pocket of Thr315Ile Bcr-Abl after10 ps (left) and 300 ps (right) of MD simulation. Amino acids interacting with Imatinib via van der Waals/hydrophobic interactions are colored in pink; residues able to H-bond the drug in the crystal structure are colored in yellow; mutation 315 is colored in green.

Some structural rearrangements in the conformation of the ATP-binding pocket of Bcr-Abl are clearly visible in Figure 2. Also, a modification of the activation loop can be envisaged, on the basis of the analysis of both wild type and mutant protein (see Figure 1), although longer simulations, being currently carried out by our group (Pricl et al., 2003), are undoubtedly required to analyze these effects in details. These evidences would clearly, favorable support the hypothesis of Nagar et al. (2002) that Imatinib requires a specific conformation of the kinase receptor before it can bind.

Conclusions

Imatinib is mainly evaluated as an adjuvant therapy after complete resection of primary GIST because 1) the risk of recurrence after surgical resection alone is high, 2) conventional chemotherapy is ineffective in preventing or treating recurrent disease, and 3) it has demonstrated considerable activity in metastatic GISTs. Nonetheless, there are a number of unanswered questions regarding the use of Imatinib in patients with GISTs. Firstly, the type of *c-kit* mutation will predict the clinical response to Imatinib therapy? What will be the duration of response in patients with Imatinib-

sensitive tumors? What mechanisms are at the bases of Imatinib resistance? (gene amplification and additional mutations have already been identified in the development of resistance to Imatinib in CML).

Exploiting the high homology of the ATP-binding pocket of c-kit in GISTs and Bcr-Abl in CMLs, we performed a preliminary molecular dynamics investigation of the binding of Imatinib to both wild type and its Thr315Ile mutant. Besides the good agreement between calculated and experimental $\Delta\Delta G$ of binding, these experiments have highlighted the fact that the presence of this mutation results in a distorted conformation of the ATP-binding pocket of the protein. This, in turn, confers to Imatinib a lesser ability to jam between the protein activation loop and helix αC, and hence to prevent the protein from assuming an active conformation.

References

Besmer P, Murphy JE, George PC et al. (1986). *Nature*, **320**, 415-421.

Bonn D. (2001). *The Lancet Oncology*, **2**, 651.

Casali PG, Verweij J, Zalcberg J et al. (2002). *Am. Soc. Clin. Oncol. Proc.*, **21**, 413a.

Case DA, Pearlman DA, Caldwell JW et al. (1999). *AMBER 6.0*, University of California, San Francisco CA, USA.

Corbin AS, Buchdunger E, Pascal F and Drucker BJ. (2002). *J. Biol. Chem.*, **277**, 32214-33219.

Cornell WD, Cieplak P, Bayly CI and Kollman PA. (1993). *J. Am. Chem. Soc.*, **115**, 9620-9631.

Cornell WD, Cieplak P, Bayly CI et al. (1995). *J. Am. Chem. Soc.*, **117**, 5179-5197.

Ernst SI, Hubbs AE, Przygodzki RM, Emory TS, Sobin LH and O'Leary TJ. (1998). *Lab. Invest.*, **78**, 1633-1636.

Gorre ME, Mohammed M, Ellwood K et al. (2002). *Science*, **293**, 876-880.

Hirota S, Isozaki K, Moriyama Y et al. (1998). *Science*, **279**, 577-580.

La Roseé P., O'Dwyer ME and Drucker BJ. (2002). *Leukemia*, **16**, 1213-1219.

Lovell SC, Word JM, Richardson JS, Richardson DC. (2000). *Proteins: Struct. Funct. and Genet.*, **40**, 389-408.

Lux ML, Rubin BP, Biase TL et al. (2000). *Am. J. Pathol.*, **156**, 791-795.

Mauro MJ, O'Dwyer ME, Heinrich, MC and Drucker BJ. (2002). *J. Clin. Pathol.*, **20**, 325-334.

Nagar B, Bornmann WG, Pellicena P et al. (2002). *Cancer Res.*, **62**, 4236-4243.

Nimmanapalli R and Bhalla K. (2002). *Curr. Opin. Oncol.*, **14**, 616-620.

Pricl S, Coslanich A, Ferrone M et al. (2003). Manuscript in preparation.

Reyes CM and Kollman PA. (2000). *J. Mol. Biol.*, **295**, 1-6.

Schindler T, Bornmann WG, Pellicena P, Miller WT, Clarkson B and Kuryan J. (2000). *Science*, **289**, 1938-1942.

Sharp KA and Honig B. (1990). *Annu Rev. Biophys. Biophys. Chem.*, **19**, 301-332.

Srinivasan J, Miller J, Kollman PA and Case DA. (1998). *J. Biol. Struct. Dyn.*, **16**, 671-682.

Tamborini E, Albertini V, Bonadiman L et al. (2003). *New Engl. J. Med.*, submitted.

Acknowledgments

Authors wish to thank the Italian Ministry for Instruction, University and Research (MIUR, Rome, Italy) for financial support.