A new mutation affecting the ATP pocket of kit receptor in patients with GIST showing acquired resistance to Imatinib: a coupled experimental and modeling investigation

S. Pricl[1], A. Coslanich[1], M. Fermeglia[1], M. Ferrone[1], M.S. Paneni[1], E. Tamborini[2], S. Pilotti[2], M.A. Pierotti[3]

[1]Computer-aided System Laboratory – MO.S:E., Department of Chemical, Environmental and Raw Materials Engineering, University of Trieste – Piazzale Europa 1, 34127 Trieste, Italy; [2]Experimental Molecular Pathology, Department of Pathology, Istituto Nazionale per lo Studio e la Cura dei Tumori, Via Venezian 1, 20133 Milano, Italy; [3]Department of Experimental Oncology, Istituto Nazionale per lo Studio e la Cura dei Tumori, Via Venezian 1, 20133 Milano, Italy

Introduction

A new era of targeted cancer therapy was inaugurated with the approval of Imatinib mesylate (or STI 571/Gleevec) for the treatment of chronic myeloid leukemia (CML). STI-571 is a phenyl-aminopyrimidine compound, initially identified from a high-throughput screen for inhibitors of protein kinase C and subsequently found to be a potent and selective inhibitor of the Abl, platelet-derived growth factor β receptor, and kit tyrosine kinases [1]. Imatinib binds in a pocket close to the ATP-binding site of the Abl catalytic domain, and effectively inhibits Abl kinase activity in vitro and in vivo at concentrations of 0.1-1.0 μM [2]. One year after its approval by FDA and EMEA for CML treatment, in 2001 Imatinib was also approved for advanced gastrointestinal stromal tumors (GISTs) chemotherapy. GISTs are the most common mesenchymal tumors of the gastrointestinal tract; they represent a spectrum of tumors, ranging from benign to highly malignant. In CML, Imatinib is highly effective both in early and late stages if the disease. Nonetheless, several relapses do occur after initial response, despite continued treatment [3]. In patients who developed resistance to Imatinib, reactivation of the Bcr-Abl signaling was observed, due to either a secondary mutation, resulting in a missense substitution of a residue belonging to the drug binding site and critical for binding, or to a progressive Bcr-Abl gene amplification [4]. 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 [5]. 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 in advanced GISTs, a point mutation in exon 14, observed only in Imatinib nonresponding metastases, was identified for the first time. This mutation, T2030C, results in the corresponding protein mutation T670I, belonging to the ATP pocket of the kit receptor. In this work, we present the results obtained from the application of combined detailed molecular modeling and experimental investigation techniques to the study of the interactions between T670I and Imatinib.

MODELING/EXPERIMENTAL DETAILS

The crystallized structure of c-kit in complex with Imatinib was chosen as starting point for all simulations [6]. All calculations were carried out using the Amber 6.0 suite of programs [7] using the all-atoms force filed by Cornell et al. [8]. All missing parameters for Imatinib were obtained performing *ab initio* calculations on a minimized structure. To calculate the energetics of binding and examining the effects of conformational change and dynamics induced by the T670I in kit, we applied the following ansatz: binding free energies ΔG_{bind} of Imatinib to wild

type and T670I mutant c-kit were obtained by applying the so-called molecular mechanics Poisson-Boltzmann surface area (MM/PBSA) method of Kollman et al. [9]. Solvation free energy was estimated by continuum solvent methods as the sum of electrostatic and non-polar contributions. The entropic contribution to ΔG_{bind} was estimated via normal mode analysis. The mutation T670I was introduced to the wild type crystal structure of the kit-Imatinib complex by swapping the mutant residue into the specific site. 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 T670I 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 $\Delta\Delta G_{bind}$ and standard deviations. Morphologic, immunophenologic, molecular and biochemical analyses were carried out on resected lesions, two responding and one progressing. A c-kit mutational analysis on genomic DNA obtained from the tissue block of the primary tumor excised elsewhere was performed concomitantly. Immuno-staining for CD117 was performed using the Dako antibody 1:250 diluted, following standard protocols. RNA and DNA were extracted, and the sequencing of the cDNA and genomic DNA was performed with an automated sequencing protocol, using kit sequence NCBI-#X06182 for comparison. Proteic extracts, immunoprecipitation experiments and Western Blotting analysis were performed according to standard procedures.

RESULTS AND DISCUSSION

The analysis of the entire c-kit cDNA sequence of the nonresponding metastases revealed a point mutation in exon 14, T2030C, resulting in the corresponding aminoacidic substitution T670I. This newly identified mutation, also confirmed at genomic level, was absent in the cDNA and genomic DNA from the corresponding metastases and from the primary tumor. Strong cytoplasmatic CD117 positivity marked the non-responding lesion, while the responding ones showed weak cytoplasmic immunoreactivity. Immunoprecipitation and Western Blotting experiments paralleled this observation. In fact, the non-responding lesion showed a highly expressed and phosphorylated kit receptor, whereas the responding ones showed a weak it expression and activation, almost below the detection level.

Interestingly, our alignment of kit primary sequence with the aberrant tyrosine kinase expressed by the Philadelphia chromosome, Bcr-Abl, revealed that the mutation residue T670I corresponds to T315I of Abl receptor [10], an absolutely analogous mutation which has been claimed to be the cause of acquired resistance to Imatinib in CML [11]. Mutating T to I at position 670 in the ATP binding domain of the kit receptor resulted in a calculated $\Delta\Delta G_{bind}$ of 1.95 kcal/mol with respect to the corresponding wild type structure. This value is in a very good agreement with the corresponding experimental finding of 1.77 kcal/mol verified for the same mutation in Bcr-Abl [12]. Several, stabilizing interaction between the receptor and the drug are not longer maintained in the presence of the T670I mutation. For instance, beside the absence of the fundamental H-bond between the aminopyridine nitrogen of Imatinib and the side chain $O\gamma 1$ atom of the gatekeeper residue T670, the topical salt link between D640 and K623 is substantially weakened, as the $O\varepsilon 1$ atom of D640 is also forming a stronger and shorter H-bond with the nitrogen of the Imatinib peptide bond linker. Superimposing the wild type structure on the mutated Imatinib complex structure clearly shows that two moieties of Imatinib (i.e., the phenyl and the piperazyl rings) are incompatible with the fully-assembled, autoinhibited structure of the c-kit kinase.

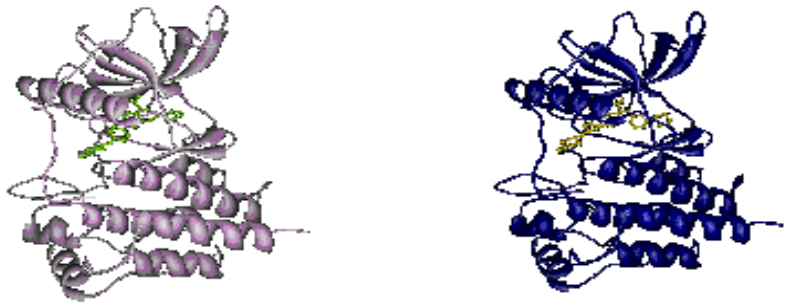

Figure 1. Snapshots taken from the equilibrium MD simulation of the wild type (left) and the T670I mutant (right) c-kit in complex with Imatinib.

CONCLUSIONS

A new mutation, T670I, affecting the ATP-binding pocket of the c-kit receptor was detected for the first time in patients with advanced GISTs undergoing Imatinib therapy. This mutation is superimposable to the substitution T315I already reported in CML, where it is related to acquired resistance to Imatinib. From the molecular point of view, the presence of this aminoacidic substitution results in a depletion of a number of fundamental interactions between the receptor and the drug. This, in turn, reflects in a distorted conformation of the ATP-binding pocket of the protein, which 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.

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