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Original Article

The Effect of Mutations on Drug Sensitivity and Kinase Activity of Fibroblast Growth Factor Receptors: A Combined Experimental and Theoretical Study

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ABSTRACT

Fibroblast growth factor receptors (FGFRs) are recognized therapeutic targets in cancer. We here describe insights underpinning the impact of mutations on FGFR1 and FGFR3 kinase activity and drug efficacy, using a combination of computational calculations and experimental approaches including cellular studies, X-ray crystallography and biophysical and biochemical measurements. Our findings reveal that some of the tested compounds, in particular TKI258, could provide therapeutic opportunity not only for patients with primary alterations in *FGFR* but also for acquired resistance due to the gatekeeper mutation. The accuracy of the computational methodologies applied here shows a potential for their wider application in studies of drug binding and in assessments of functional and mechanistic impacts of mutations, thus assisting efforts in precision medicine.

1. Introduction

Fibroblast growth factors and their receptors (FGFs and FGFRs) play a critical role in many physiological processes including embryogenesis, wound healing, inflammation and angiogenesis as well as adult tissue homeostasis (Beenken and Mohammadi, 2009). Compelling evidence also implicates activation of FGFRs (FGFR1–4) in pathogenesis of several developmental syndromes and a broad range of human malignancies. FGF/FGFR signalling contributes to tumour generation and progression through activating *FGFR* genomic alterations (driver point-mutations, fusions and amplifications) (Greulich and Pollock, 2011; Wesche et al., 2011; Sabnis and Bivona, 2013), as a positive regulator of tumour neoangiogenesis (Turner and Grose, 2010) and as a mediator of resistance to endocrine (Turner et al., 2010) and targeted therapies to related oncogenic pathways, in particular to signalling by other receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) (Wilson et al., 2012; Crystal et al., 2014).

The involvement of FGF/FGFRs in the pathology of many cancer types provides a strong rationale for development of effective agents for these targets and a large effort to develop FGFR inhibitors as anticancer treatments is underway (Brooks et al., 2012; Dieci et al., 2013). Some of the FGFR inhibitors such as TKI258 (dovitinib), levatinib, brivanib and AP24534 (ponatinib) also target a subset of other tyrosine kinases while AZD4547, PD173074, BGJ398 and JNJ-*493 appear to be selective for FGFR1–3. For the compounds already in clinical trials, important issues include optimising the management of emerging toxicity profiles and anticipated intrinsic target resistance as well as designing further trials to best match the target alterations with the proposed drug action. One of the bottlenecks in achieving such precision therapies is the lack of suitable approaches to functionally interpret vast quantities of genomic data. In particular, for FGFRs there are hundreds of mutations found in tumour samples (Greulich and Pollock, 2011; Wesche et al., 2011; Sabnis and Bivona, 2013) and their impact on FGFR activation cannot be predicted based on crystallographic insights alone; this is in part due to the considerable scope for allosteric effects inherent to protein kinases (Meharena et al., 2013).

Furthermore, the inhibitor binding can also be altered by various mutations. Prime examples are acquired intrinsic resistance mutations that have marred the success of tyrosine kinase inhibitors (TKIs) such as gefitinib, erlotinib and imanitib, prompting efforts for second- and third-line treatments (Daub et al., 2004; Azam and Daley, 2006; Gibbons et al., 2012). One resistance mechanism common to many

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kinase inhibitors is the mutation of the so-called "gatekeeper" residue that remains the most frequently detected drug-resistance mutation in the clinic. Examples include resistance to TKIs targeting breakpoint cluster region-abelson tyrosine kinase (BCR-ABL) fusion in chronic myelogenous leukaemia (Gorre et al., 2001; Shah et al., 2002), EGFR in nonsmall cell lung cancer (Kobayashi et al., 2005; Pao et al., 2005), platelet-derived growth factor receptor (PDGFR) in hypereosinophilic syndrome (Cools et al., 2003), KIT in gastrointestinal stromal tumours (Tamborini et al., 2006) and echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase (EML4-ALK) fusion in lung cancer (Choi et al., 2010b). Modelling in cell culture has also been successfully used to discover clinically relevant acquired resistance and the application of this approach to FGFR driven-cancer cells identified a gatekeeper substitution (Chell et al., 2013). Gatekeeper substitutions in FGFR have been also identified in clinical samples, however as primary cancer mutations rather than secondary mutations (Taylor et al., 2009; Shukla et al., 2012; Ang et al., 2015). Taking into account the widespread occurrence of acquired gatekeeper resistance in many kinases and initial laboratory and clinical observations for FGFR, occurrence of this phenomenon in FGFR is widely anticipated.

Further factors that can influence drug binding include, pre-existing mutations in the targeted kinase or subtle differences between closely related family members. This has also been documented for the FGFR family members (Brooks et al., 2012; Dieci et al., 2013; Byron et al., 2013) emphasizing the need for further characterisation that would inform treatment.

Here we apply a combination of approaches to address current limitations in assessing whether a specific mutation in a kinase domain affects the activity or alters drug sensitivity. We report striking differences between FGFR sequence variants with respect to the effect on kinase activity and the efficacy of tyrosine kinase inhibitors. Furthermore, we found that the gatekeeper variant, that enhances kinase activity, is not refractory to some of the inhibitors currently in clinical trials; in particular, TKI258 retained its efficacy towards FGFR1 and FGFR3 gatekeeper substitutions in vitro and in cells. These findings, supported by measurements of kinase activity, determination of binding constants and X-ray crystallography, are in good agreement with the values obtained independently by molecular dynamics simulations that also provide an in-depth insight into the allosteric communication between the mutated site and important functional motifs. The computational methods used here to calculate the binding and surface free energies could therefore have wider application in predicting the functional impact of disease mutations, drug binding and underpinning molecular mechanisms.

2. Methods

2.1. Protein Expression and Purification

All kinase domain constructs were cloned into pOPINS (OPPF, Oxford, UK) or pJ821 (DNA2.0, USA) and expressed in C41 (DE3) cells harbouring lambda phosphatase and human CDC37. Kinases were purified by a combination of Ni^{2+}-chelating, ion exchange and gel filtration chromatography. Protein purity was assessed by SDS-PAGE and preparations at >95% homogeneity were used for kinase assays and crystallography.

2.2. Kinase Assay

The ADP-Glo (Promega) methodology was used for all kinase assays following manufacturers instructions. Unless otherwise stated in the text, each kinase assay consisted of 20 μL, containing Kinase Buffer (40 mM Tris-HCl, 20 mM NaCl, 20 mM $MgCl_2$, 2 mM TCEP, 2 mM $MnCl_2$, Na_3VO_4, 0.1 mg/mL BSA, pH 8.0), 250 μM ATP, 0.4 mg/mL $polyGlu_4Tyr$ substrate, 0.5 μM kinase domain and inhibitors in selected experiments. In various experiments the concentrations of these components were varied and this is stated in the text or figure legends. In order to calculate valid inhibition constants (K_i), a number of kinetic parameters needed to be ascertained, in particular, the K_m of the kinases for ATP. A thorough Michaelis–Menten kinetic analysis was performed ensuring that all relevant parameters were in the linear range and that the appropriate concentrations of ATP, substrate and kinase were adhered to. All data were processed using Graphpad Prism and parameters presented in Supplemental Tables. Classical enzyme competitive inhibition approaches were found to be unsuitable to generate inhibition constants and therefore the Morrison approach (the quadratic velocity equation for tight-binding substrates) was utilized (Morrison, 1969).

2.3. Crystallisation and Crystallography

Crystals of FGFR1-2c Apo, native FGFR1-2c with TKI258 (FGFR1-2c TKI258) and FGFR1-$2c^{V561M}$ with TKI258 (FGFR1-$2c^{V561M}$ TKI258) were grown by both hanging and sitting drop methods in condition 20% PEG 5 K MME, 0.1 M Tris, pH 7.5, 0.2 M ammonium sulphate. X-ray diffraction data were recorded at Diamond Light Source in stations I02, I03 and I24 for FGFR1-2c Apo, FGFR1-2c TKI258 and FGFR1-$2c^{V561M}$ TKI258 respectively. The data were auto-processed using autoprocessing tools Xia2 (Winter et al., 2013) and FAST_DP (uses XDS (Kabsch, 2014)) at Diamond Light Source. The unmerged output from XDS was taken for each of the dataset and scaled using Aimless (Evans and Murshudov, 2013) software suite from CCP4 package (Winn et al., 2011). The FGFR1-2c Apo, FGFR1-2c TKI258 and FGFR1-$2c^{V561M}$ TKI258 were scaled to a high resolution of 2.3 Å, 1.96 Å and 1.96 Å units respectively. Please refer to Supplemental Table S5, X-ray data processing statistics. Phasing, refinement and structure validation are described in the Supplemental Material.

2.4. Computational Calculations

The binding and the surface free energy changes were calculated using multiple thermodynamic integration simulations based on methodology described in (Wan and Coveney, 2011). Further improvements and enhanced-sampling molecular dynamic simulations (parallel-tempering metadynamics) were according to (Bussi et al., 2006); the algorithms were previously used in calculating the conformational free energy surfaces of EGFR (Sutto and Gervasio, 2013), c-SRC and ABL (Lovera et al., 2012). Computational calculations are described further in Supplemental Material.

2.5. PDB ID Codes

The refined and validated structures of FGFR1-2c Apo, FGFR1-2c TKI258 and FGFR1-$2c^{V561M}$ TKI258 have been submitted to Protein Data Bank (PDB) and their PDB ID codes are 4UWY, 4UWZ and 4UX0, respectively.

3. Results

3.1. Kinase Activity and Inhibition of FGFR Variants by Non-selective and Selective Inhibitors

Mutations in the ATP binding pocket of protein kinases that prevent or reduce binding of inhibitory compounds most frequently occur at a particular residue in the hinge region between the N and C lobes, positioned to control access to a hydrophobic pocket that helps anchor kinase inhibitors to the active site. This, so called, gatekeeper residue corresponds to threonine in a number of kinases (including EGFR, PDGFR, KIT, ABL and SRC) but can also be other amino acid residues such as leucine, phenylalanine or valine (Fig. 1A). Among all FGFR members, the gatekeeper valine is conserved, however, the tyrosine residue in its proximity in FGFR1-3 corresponds to a cysteine in FGFR4 (Fig. 1A). FGFR4 is known to have reduced sensitivity to several inhibitors and the position of the tyrosine to cysteine replacement (close to the gatekeeper) is likely to contribute to such a difference. For the analyses of inhibitor sensitivity, we focused on a structurally well-defined