Supplementary Material

Supplementary Computational Analysis of EGFR Mutations (See Supplementary Figure 1A)

G735S: This mutation has previously been associated with increased proliferation anchorage-independence, invasion, phosphorylation, activation of secondary pathways AKT, STAT3 and MAPK (1). This mutation replaces a flexible glycine in the tight β2-β3 loop of the N-terminal kinase lobe with a serine, which has less backbone flexibility. This replacement may influence the structure of this loop region. The β2-β3 loop is however away from the active site or the N-terminal site for allosteric asymmetric kinase activation. Therefore, the G735S action is most likely linked to a subtle distortion of the N-terminal lobe that affects the interaction of the N-terminal lobe with ATP/lapatinib, or other molecules.

L792F: No phenotype has been described for this mutation. L792 is positioned at the back of the ATP and lapatinib binding pocket of EGFR. L792 is in direct contact with lapatinib in the inactive kinase form, and close to a short helix (residues 995-1002) of a C-terminal kinase domain extension that binds to the back of the ATP/lapatinib binding pocket in the inactive kinase form, but not the active form. Substitution of L792 with a bulkier phenylalanine will alter the binding pocket for lapatinib and may affect the interaction with residues 995-1002.

E804D: This mutation has not been described previously, however the mutation E804G was reported to have the same phenotype as G735S (described above) (1). E804D is a homologous substitution, away from the sites for binding ATP, lapatinib or substrates. E804D is not involved in kinase fold stability, and E804D is therefore not anticipated to have a significant direct effect on kinase activity. Potential effects might be linked to E804D affecting substrate binding or the interaction with residues 995-1002, or long range charge interactions.

N842I: In the EGFR:lapatinib complex, N842 is involved in a hydrogen-bond network that links N842 to D855, D837, and the catalytic K745. In the wild-type, the same residues are participating in the site and function for phosphotransfer and substrate binding. The substitution of N842 with a hydrophobic isoleucine will lead to remodeling of this site, and thus is likely to affect ATP/TKI binding and substrate recognition.

V843I: V843I is adjacent to N842, however points away from the active side, into the hydrophobic core of the C-terminal kinase lobe. By substituting a valine with a bulkier isoleucine, V843I is predicted to affect the local surface and dynamics of the ATP/drug binding pocket of the C-terminal kinase lobe, which may explain why V843I has been associated with familial predisposition to lung cancer and resistance to tyrosine kinase inhibitors in patients (2).

G857E: G857 is positioned at the N-terminal end of the activation loop, in contact with the αC helix. This region undergoes significant rearrangement upon going from the active (ATP-bound) to the inactive (lapatinib-bound) kinase conformation. In particular, for this rearrangement, G857 has to slide across the αC helix. Incorporation of the much larger and charged glutamic acid is predicted to significantly increase the energy required for this structural transition. In the inactive kinase conformation, G857E is predicted to affect the shape of the lapatinib binding pocket, by requiring a local rearrangement of the N-terminal region of the activation loop and/or the αC helix. In the active conformation G857E, where a glutamic acid would be much less constrained than in the inactive kinase conformation, E857 would be able to stabilize the activation loop in its active position through ionic interactions with K857. Similar to the mutation L858R (described by Shan et al. (3)), G857E may stabilize EGFR and promote receptor dimerization and lateral signal amplification.

Supplementary Computational Analysis of HER2 Mutations

L726F: *In silico* structural analysis showed that L726F affects the shape of the ATP/TKI binding pocket (Figure 1B and Supplementary figure 1B). The substitution of L726 by the much bulkier phenylalanine leads to partial occlusion of the ATP/TKI binding pocket (Fig. 1B, central panel). This occlusion does not appear to prevent binding of ATP in the active (open) state of the kinase (Fig. 1B, right panel), suggesting that L726F does not block enzymatic activity. The steric obstruction caused by L726F appears to become more cumbersome in the inactive (closed) conformation (Fig. 1B, central panel). Especially, L726F would lead to significant clashes with lapatinib, which binds to HER2 in the inactive state (Fig. 1B, central panel). L726F is also at the interface between the ATP/TKI pocket and a short α-helix (residues 1004-1009) of the C-terminal extension that wraps around the back of the kinase domain (Figure 1A). This α-helix is present in one of the two molecules of the HER2 crystal structures (in its active-like form), and may influence the stability of the active state. Our structural analysis therefore strongly suggests that L726F hampers binding of lapatinb, but not ATP. Additionally, L726F may also enhance the stability of the active versus inactive kinase state, although this prediction is less certain.

V794M: This mutation is implied in contacts between the N-terminal β-sheet and the αC helix, and also lies close to the surface that is used by the receiver kinase to asymmetrically bind to the activator kinase (Fig. 1C, left panel). This position suggests the following complementary modes of action. Firstly, V794M could reinforce the interaction between the αC helix and the β-sheet, and thus stabilize the αC helix in its active conformation. Structural and functional data suggest that the particularly glycine-rich αC-β4 loop of HER2 destabilizes the active conformation of the αC helix (4, 5), which may explain the low intrinsic kinase activity of HER2. V794M may reduce this intrinsic flexibility of αC. Secondly, V794M may affect the affinity of the interaction between receiver and activator kinase. The longer methionine side chain may increase hydrophobicity and compactness of this interface stabilizing dimers with HER2-V794M

as the receiver, and hence increase activation of HER2 (Supplementary Figure 1B). Also in the activator:receiver complex showing the receiver kinase in an inactive conformation, V794M would fill a cavity, and hence may increase propensity to form dimers (Supplementary Figure 1B; van der Waals sphere representation). Thirdly, since αC is part of the receiver:activator interface, stabilization of αC by V794M may also influence the strength of this dimer interaction. Finally αC also delineates a part of the kinase pocket that is exploited by TKIs, including lapatinb. Hence V794M may affect TKI binding. Together, our analysis predicts that V794M promotes activation of HER2 by enhancing dimerization and stabilizing the αC helix, and may affect binding of TKIs such as lapatinib.

D808N: Located on the C-terminal lobe is in proximity of bound ATP (4-5 Å from the ribose, > 7 Å from the γ-phosphosphate) and the substrate binding site. By altering long-range charge interactions D808N may increase affinity and turnover of ATP. By affecting the side chain orientation of R849, D808N may also influence the solvent structure and characteristics of the substrate binding site. While not providing a clear prediction for the effects of D808N on kinase activity, our structural analysis nonetheless suggests that D808N acts through a mechanism distinct from those used by either L729F or V794M. D808 is coordinating the methylsulfonylethylamino group of lapatinib, suggesting that its substitution with asparagine may influence lapatinib response (Fig. 1C, right panel).

Supplementary Computational Analysis of HER4 Mutations (See Supplementary Figure 1C)

G785S: G785 is located in the β-strand that follows the αC helix. The G785 mutation will create local deformations because of the bulkier serine side chain. These local deformations may influence the shape of the lapatinib binding cavity and possibly the interaction with an asymmetrically activating kinase molecule.

R838Q: R838 is located at the C-terminal end of a long helix of the C-terminal lobe. Since R838 solvent exposed, and away from the active site of the site for asymmetric activation, its substitution to glutamine is unlikely to affect the active site directly. If this mutation proves to have a significant effect on kinase activity, then it might be linked to unknown regulatory interactions of this residue. For example, R838Q is close to the αC-β4 loop that was suggested to serve as a docking site for HSP90 in HER2 (6). R838Q is also in a capping position of the second α-helix of the C-terminal lobe, and hence R838Q might influence stability of this helix.

M887I: Positioned at the C-terminal basis of the activation loop, M887 might influence conformation and dynamics of this loop. Although hydrophobic too, a bulkier isoleucine at this position may influence activation loop conformation and dynamics. In so doing, it may affect the position and dynamics of the N-terminal lobe, and hence alter dimerization characteristics in the receiver kinase.

Computational analysis of neratinib (See Supplementary figure S4C)

Neratib, a 4-(arylamino)quinoline-3-carbonitrile compound, is a potent irreversible inhibitor of both EGFR and HER. Both, lapatinib and neratinib have characteristic aniline substitutions, leading to an outward rotation of the C-helix upon binding (7, 8). Despite the covalent bond formed between the neratinib crotonamide Michael-acceptor group and a cysteine on the side of the active side (C804 in HER2), both neratinib and lapatinib engage very similar non-covalent bonds with the active site of the kinase. Thus both inhibitors bind the kinase in its closed conformation and use a very similar binding mode (8). The response of the HER2 mutants to neratinib is therefore expected to be similar to their response to lapatinib if these mutants block the initial (non-covalent) binding of the inhibitor. Consequently, L726F is predicted to inhibit binding of neratinib by the same steric mechanism as for lapatinib. However, once neratinib bound covalently, it is no longer in a competitive, reversible equilibrium.

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