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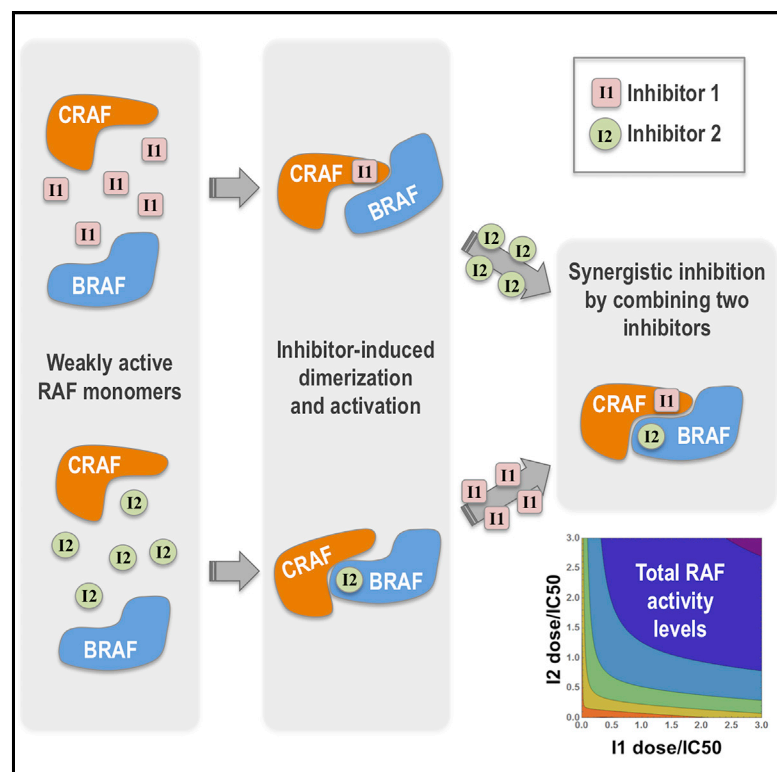
Cell Reports

Article

Drug Resistance Resulting from Kinase Dimerization Is Rationalized by Thermodynamic Factors Describing Allosteric Inhibitor Effects

Graphical Abstract



Highlights

- Allosteric kinase activation caused by dimerization conveys drug resistance
- Thermodynamic factors account for paradoxical kinase activation by a drug
- Accumulation of dimers harboring drug-bound and free protomers drives resistance
- Two inhibitors, ineffective on their own, when combined can abolish drug resistance

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In Brief

Kholodenko has developed a model that describes drug-facilitated dimerization and the emergence of differing drug affinities between free kinase monomers versus dimers. Importantly, the model suggests ways of overcoming drug resistance.

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Drug Resistance Resulting from Kinase Dimerization Is Rationalized by Thermodynamic Factors Describing Allosteric Inhibitor Effects

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SUMMARY

Treatment of cancer patients with ATP-competitive inhibitors of BRAF/CRAF kinases surprisingly increases total kinase activity, especially in wild-type BRAF cells, subverting the desired clinical outcome. Similar inhibition resistance is observed for numerous kinases involving homo/heterodimerization in their activation cycles. Here, I demonstrate that drug resistance resulting from kinase dimerization can be explained using thermodynamic principles. I show that allosteric regulation by inhibitors is described by thermodynamic factors that quantify inhibitor-induced changes in kinase dimerization and the difference in the drug affinity for a free monomer versus a dimer harboring one drug molecule. The analysis extends to kinase homo- and heterodimers, allows for their symmetric and asymmetric conformations, and predicts how thermodynamic factors influence dose-response dependencies. I show how two inhibitors, ineffective on their own, when combined can abolish drug resistance at lower doses than either inhibitor applied alone. Thus, the mechanistic models suggest ways to overcome resistance to kinase inhibitors.

INTRODUCTION

The human genome encodes over 500 protein kinases (Manning et al., 2002). Kinase oncogenic mutations are frequently found in human cancers, many driving the tumor progression and survival of cancer cells (Holderfield et al., 2014; Thomas et al., 2007). Pharmaceutical companies race to add new kinase inhibitors to the ever-increasing number of clinically approved drugs, and protein kinases are currently the second largest targeted protein group following G protein-coupled receptors (Cohen, 2002). Although protein kinase inhibitors often show impressive clinical responses, resistance inevitably occurs. Moreover, many kinase inhibitors have unexpected side effects, surprisingly activating signaling pathways by promoting kinase dimerization (Koppikar et al., 2012; Lito et al., 2013).

Homo- and heterodimerization are key events in the physiological and oncogenic activation of numerous kinases, including receptor tyrosine kinases and multiple cytoplasmic kinases (Bessman et al., 2014; Dey et al., 2005; Hu et al., 2013; Huang et al., 2014; Romano et al., 2014; Wang et al., 2012). In mitogen-activated protein kinase (MAPK) pathways, dimerization is essential for the activation of first-tier kinases (MAP3Ks), including MLK4 and MAP3K11 (Leung and Lassam, 1998). The MAP3Ks of the extracellular signal-regulated kinase (ERK) cascade, BRAF and CRAF (RAF-1, gene name), form homo- and heterodimers as intrinsic steps of their activation cycles (Freeman et al., 2013; Heidorn et al., 2010; Rushworth et al., 2006). The discovery of BRAF mutations, such as BRAF600E (Davies et al., 2002), which lead to oncogenic activation of BRAF and perpetual ERK activation, has made the MAPK pathway a primary target for new cancer drugs. Several BRAF and CRAF inhibitors are undergoing clinical trials or seeing use in the clinic (Rahman et al., 2014). However, nearly all existing RAF inhibitors suppress MAPK signaling only in tumors with mutated BRAF and wild-type RAS. In cells with wild-type BRAF, these inhibitors paradoxically increase the total CRAF/BRAF kinase activity due to inhibitor-induced homo- and heterodimerization of these kinases (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2010). RAF inhibitors are also ineffective in cells with constitutively active mutant RAS (Heidorn et al., 2010). Binding to active RAS drives RAF homo- and heterodimerization by inducing RAF conformational changes and bringing two RAF molecules into close vicinity at the plasma membrane (Kholodenko et al., 2000, 2010; Weber et al., 2001). Since RAF heterodimers have significantly higher kinase activity than monomers (Garnett et al., 2005; Lavoie and Therrien, 2015; Rushworth et al., 2006), RAF dimerization is thought to be a major mechanism causing resistance to RAF inhibitors in experiments and clinically (Heidorn et al., 2010; Poulikakos et al., 2011).

Although all the structural details of how existing ATP-competitive inhibitors induce RAF dimerization are not yet worked out, current models suggest that these inhibitors stabilize an active

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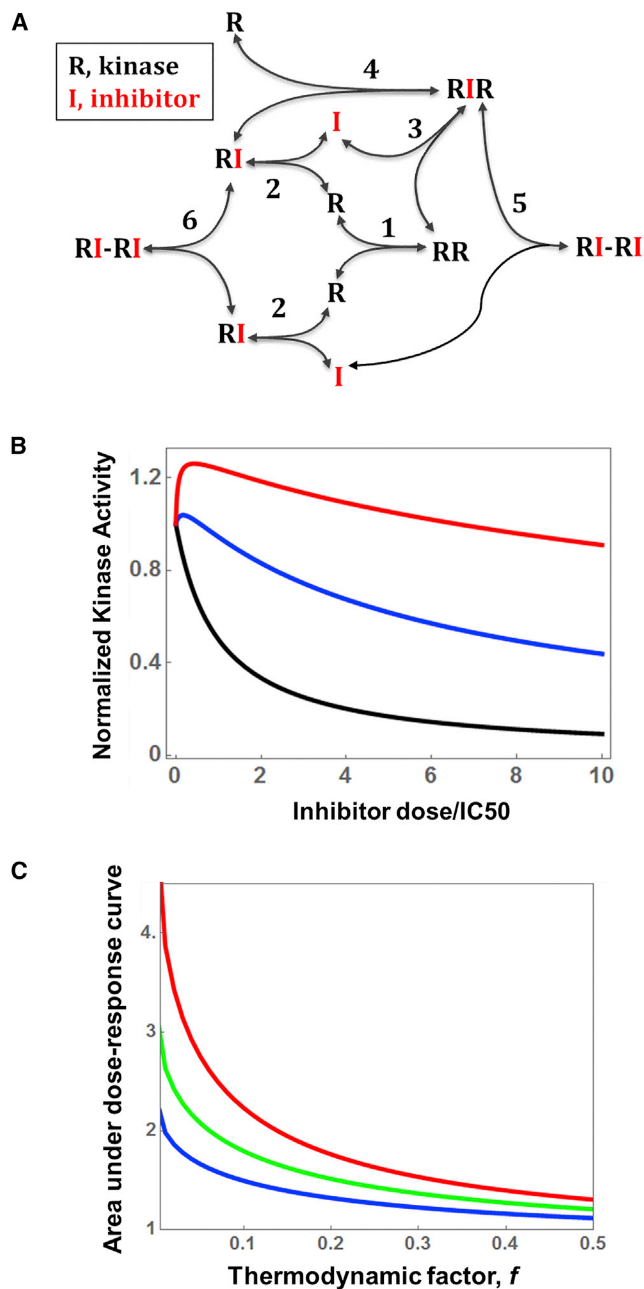

Figure 1. A Core Model of Kinase Dimerization and Inhibitor Binding

(A) Kinetic diagram is shown. R, kinase monomer; I, inhibitor (shown in red).

(B) Dose-response curves. Total kinase activity is normalized by the activity in the inhibitor's absence. Inhibitor concentration is normalized by IC_{50} calculated for kinase monomers, $IC_{50} = K_2$ (note that in cells under standard experimental conditions and also in vivo free inhibitor concentration can be considered as a parameter [Kholodenko et al., 1999]). (Black line) Inhibitor binding and kinase dimerization are thermodynamically independent events. (Blue and red lines) Inhibitors show paradoxical kinase activation due to allosteric effects. Thermodynamic factors, blue line, $g = 10$, $f = 0.05$; red, $g = 100$, $f = 0.01$.

(C) The dependence of the area under dose-response curves on the thermodynamic factor f. The area is calculated for $0 < [I]/IC_{50} \leq 10$ and normalized by the area calculated for $f = 1$, $g = 1$, which is the area under the black curve in (B), $g = 1$ (blue), 2 (green), 10 (red). In (B) and (C), activities of inhibitor-free dimers are assumed to be ten times higher than monomers and two times higher than activities of partially inhibited dimers (Rushworth et al., 2006). The ratio of the kinase abundance (R_{tot}) and K_1 is 0.1. See also Figure S1.

kinase conformation and unique side-to-side dimer configuration of the RAF kinase domains (Heidorn et al., 2010; Lavoie et al., 2013; Wu et al., 2012). Most recent data show that several sulfonamide RAF inhibitors can disrupt BRAF homodimers; however, the same inhibitors still markedly induce BRAF-CRAF heterodimers in cells, a feature that is not yet understood (Thevakumaran et al., 2015).

Here I show that inhibitors that increase kinase dimerization can paradoxically promote overall kinase activity by driving the formation of catalytically active dimers, carrying a single inhibitor molecule and a free protomer, with a low affinity for binding a second inhibitor molecule. The exact dimer affinity for the second inhibitor molecule is dictated by the inhibitor and the dimer structures. Fundamental thermodynamic principles suggest that this affinity is very different from the inhibitor affinity for a drug-free dimer. The analysis extends to both homo- and heterodimers, allowing for their asymmetric conformations (Bollag et al., 2010; Hu et al., 2013) and predicting the dose responses as functions of thermodynamic factors that characterize allosteric inhibitor effects. Modeling demonstrates that two structurally different inhibitors can synergize to suppress the activity of symmetric and asymmetric dimers much more efficiently than either inhibitor applied alone at considerably larger doses. The results explain the limited effectiveness of ATP-competitive inhibitors for numerous kinases that are allosterically regulated by dimerization, including RAF kinases, Janus kinase 2 (JAK2), eIF2a kinase PKR-like ER kinase, and the EGFR family members (Koppikar et al., 2012; Lavoie et al., 2013; Macdonald-Obermann et al., 2013).

RESULTS

A Core Model of a Symmetric Kinase Dimer and Allosteric Inhibitor Effects

The aim is to explore how inhibition efficacy depends on allosteric effects of inhibitors on kinase dimerization. I start with a core model of kinase interactions with inhibitor molecules, initially considering a symmetric dimer in which both protomers have similar conformations. In the inhibitor's absence, two kinase monomers (R) form a dimer (RR), which is shown as step 1 in the kinetic scheme (Figure 1A). The equilibrium dissociation constant (K_1) of this step is assumed to be known from experimental data (Lavoie et al., 2013; Rajakulendran et al., 2009). The inhibitor (I) can bind to R, yielding an inhibitor-bound monomer (RI), which is catalytically inactive (step 2). The dissociation constant (K_2) of inhibitor binding to kinase monomers also is assumed to be known (Bollag et al., 2010; Rahman et al., 2014). Likewise, I can bind to RR that harbors two I-binding sites, resulting in a dimer (RIR) that carries a single inhibitor molecule (step 3, K_3). Structural and biochemical data suggest that RIR dimers are catalytically active, since an inhibitor-bound protomer can transactivate the inhibitor-free protomer partner (Lavoie et al., 2014). Partially inhibited RIR dimers also are

formed when an inhibitor-bound monomer RI dimerizes with a free monomer R (step 4, K_4). The subsequent binding of I to RIR yields a completely inhibited dimer RI-RI (step5, K_5), which also can be formed by two inhibitor-bound, inactive monomers (step 6, K_6).

The kinetic scheme in Figure 1A shows that the dimers RIR and RI-RI each can be formed in more than one sequence of reactions, and, therefore, the scheme contains cyclic paths. Equilibrium constants of reactions along a cycle, in which the initial and final states are identical, satisfy so-called detailed balance relationships (see, for example, Ederer and Gilles, 2007; Hearon, 1953; and Kholodenko et al., 1999). These thermodynamic restrictions require the product of the equilibrium dissociation constants (K_ds) along a cycle to be equal to 1, as at equilibrium the net flux through any cycle vanishes, since the overall free energy change is zero. Detailed balance relationships, therefore, decrease the number of independent dissociation constants. When inhibitor binding and kinase dimerization are independent events, all K_ds are readily expressed solely in terms of dimerization (K_1) and inhibitor binding (K_2) constants. Taking into consideration the numbers of free and inhibitor-occupied sites on kinase dimers, one obtains the following: $K_3 = K_2/2$, $K_4 = K_1/2$, $K_5 = 2K_2$, and $K_6 = K_1$ (see the Supplemental Experimental Procedures).

Following reports that inhibitors increase dimerization of numerous kinases (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Koppikar et al., 2012; Macdonald-Obermann et al., 2013; Poulikakos et al., 2010; Wu et al., 2012), we now assume that the inhibitor binding to a monomer facilitates the dimerization with a free monomer, yielding the catalytically active RIR dimer (Lavoie et al., 2014; Rajakulendran et al., 2009). The inhibitor-induced change in the dimerization affinity is described by the facilitation factor (*f*), which relates the dimerization constants of free monomers (K_1) and inhibitor-bound and free monomers (K_4) as follows: $K_4 = f \cdot K_1/2$. Clearly, if the facilitation factor *f* is less than 1, the RIR dimers are stabilized by the inhibitor.

Thermodynamic restrictions then require that the first inhibitor molecule must bind to the kinase dimer (RR) with a dissociation constant (K_3) that is related by *the same* factor *f* to the dissociation constant (K_2) of inhibitor binding to a kinase monomer as follows: $K_3 = f \cdot K_2/2$ (see the Supplemental Experimental Procedures for derivations). This thermodynamic relation means that if the inhibitor increases dimerization $(f < 1)$, then it binds to an inhibitor-free dimer with a greater affinity than to a kinase monomer. Further, as shown in the Supplemental Experimental Procedures, thermodynamic restrictions imply that an additional thermodynamic factor (*g*) connects the dissociation constant (K_5) of the binding of the second inhibitor molecule to a partially inhibited dimer (RIR) and the dissociation constant (K_2) of the inhibitor binding to a monomer as follows: $K_5 = 2g \cdot K_2$. When $g > 1$, K_5 is greater than $2K_2$, meaning the second inhibitor molecule binds to partially inhibited dimers less effectively than the inhibitor binds to a kinase monomer. Finally, the dissociation constant (K_6) of the fully inhibited dimer (RI-RI) is related by the product of factors *f* and *g* to the dissociation constant (K_1) of the inhibitor-free dimer (RR) as follows: $K_6 = f \cdot g \cdot K_1$ (see the Supplemental Experimental Procedures).

Summarizing, allosteric regulation by an inhibitor can be described by two independent thermodynamic factors, *f* that quantifies the inhibitor-induced increase in dimerization affinity and *g* that defines the difference in the affinity of the second inhibitor molecule for a dimer and the inhibitor affinity for a monomer. The *g/f* ratio accounts for the affinity difference of the first and second inhibitor molecules binding to a dimer. When this ratio is less than 1, the binding of the second inhibitor molecule to a partially inhibited dimer is hindered by allosteric effects.

Thermodynamic Factors Determine the Dose-Response Curve

For each inhibitor dose, the total kinase activity is the sum of activities of inhibitor-free monomers, inhibitor-free dimers, and partially inhibited dimers. The factors *f* and *g* determine the ratios between the concentrations of inhibitor-free, partially inhibited, and fully inhibited dimers, thereby shaping the dependence of the total kinase activity on the inhibitor dose, termed the dose-response curve.

Comparing dose-response curves, it is convenient to express the inhibitor dose, [I], in terms of the ratio [I]/IC_{50}, where IC_{50} is the inhibitor dose that inhibits kinase monomer activity by 50%. With the [I] increase, the concentrations of inhibitor-free monomers and dimers decrease, whereas the concentrations of inhibitor-bound monomers and fully inhibited dimers increase (Figures S1A–S1C). The concentration of partially inhibited dimers (RIR) initially always rises, as more inhibitor-bound monomers become available, but with the further [I] increase it starts declining, since more dimers become fully inhibited (Hatzivassiliou et al., 2010). Exactly how the concentration dynamics of catalytically active and inactive kinase forms depend on the ratio [I]/IC_{50} is determined by the thermodynamic factors (*f* and *g*) and the ratio of the kinase abundance (R_{tot}) and the dimerization constant K_1 (see derivations in the Supplemental Experimental Procedures). When the ratio R_{tot}/K_1 is high, kinase dimerization is already saturated in the inhibitor absence, and the further dimerization affinity increase induced by the inhibitor does not have an appreciable effect on the dimer formation.

Thermodynamic Relationships Explain Paradoxical Kinase Activation by Inhibitors

In the absence of allosteric inhibitor effects, when thermodynamic factors equal 1, the ratio of the concentrations of all dimeric and monomeric forms does not change when [I] increases (Figure S1A). The total concentration of signaling-capable monomers and dimers ([R]+[RR]+[RIR]), and, therefore, total kinase activity will always decrease with inhibitor increase (Figures 1B and S1A; see the Supplemental Experimental Procedures for derivations). Thus, we conclude that inhibitors that do not allosterically regulate kinases always effectively inhibit kinase activity (provided that their IC_{50} amounts are in therapeutically reasonable ranges).

When an inhibitor facilitates kinase dimerization $(f < 1$ and $g \geq 1)$, the model implies that partially inhibited dimers, RIR, significantly build up while the formation of completely inhibited dimers, RI-RI, lags behind (until the inhibitor dose exceeds the IC_{50} by orders of magnitude, Figures S1B–S1D).

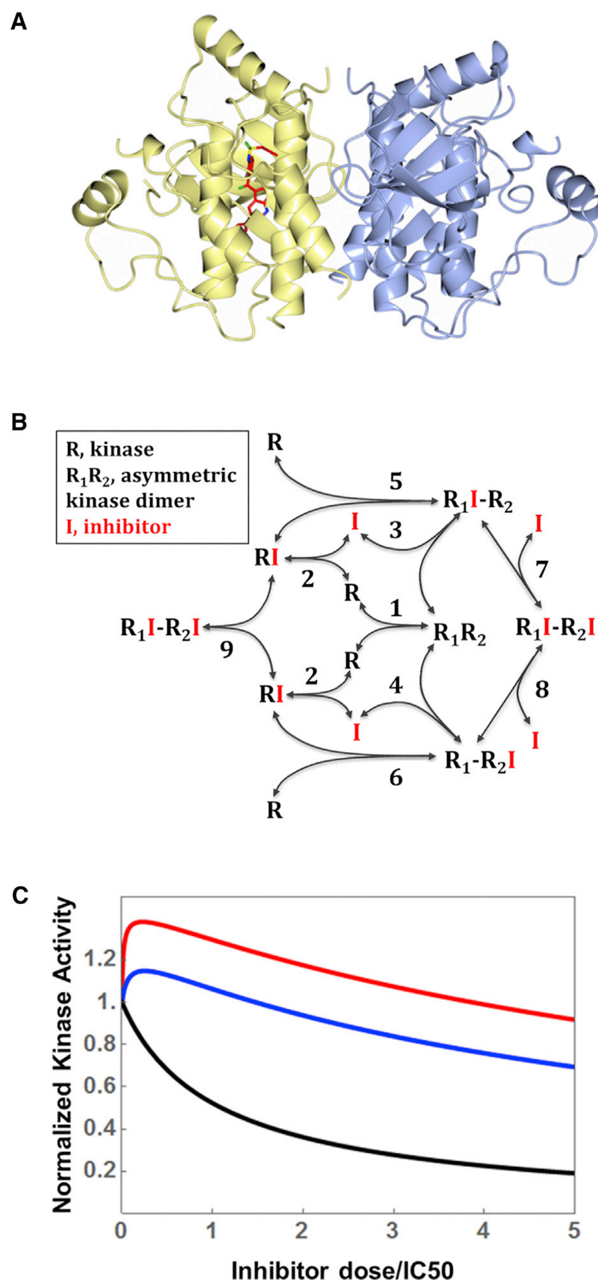

Figure 2. Asymmetric Kinase Dimerization and Allosteric Inhibitor Effects

(A) Structure of the asymmetric B-RAFV600E homodimer is derived from PDB: 3OG7 (Bollag et al., 2010). A protomer (shown in yellow) is bound to inhibitor PLX4032 (red).

(B) Kinetic scheme. R_1 and R_2 are two different protomer conformations in a dimer.

(C) Effect of the increase in the R_1-R_2I dimer accumulation on dose responses in the absence of inhibitor-induced facilitation of R_1I-R_2 dimer formation, g_1 = 0.01 (red line), g_1 = 0.05 (blue), g_1 = 1 (black). The remaining thermodynamic factors are as follows: f = 1 and g_2 = 10; normalization and other parameters are the same as in Figure 1. See also Figure S2.

In RIR dimers, the inhibitor-bound protomer is inactive, but the inhibitor-free protomer is allosterically activated by the conformational change, resulting in increased dimer activity (Rajakulendran et al., 2009). Since the RIR surge prevails over the decrease in inhibitor-free dimers (RR) and the RI-RI buildup, and dimer activity is often considerably higher than monomer activity (Rushworth et al., 2006), the inhibitor will paradoxically increase overall kinase activity within a wide concentration range (if f<1, see the Supplemental Experimental Procedures; Figure 1B). Only following the [I] increase over its IC_{50} by orders of magnitude do most dimers become fully inhibited and the total kinase activity eventually decreases (Figure 1B). One may conclude that allosteric kinase regulation by inhibitors, resulting in thermodynamic factors $f < 1$ and $g \geq 1$, can drive primary or acquired inhibitor resistance.

The roles of thermodynamic factors f and g in shaping the dose-response curve are different. Sufficiently low f values bring about paradoxical kinase activation, even when $g = 1$ (Figure S1E and derivations in the Supplemental Experimental Procedures). Notably, the more the inhibitor facilitates dimerization (the lower is f), the greater the maximum height of a dose-response curve is, if it exhibits a paradoxical surge in kinase activity (Figure S1D). On the other hand, even high g values are not sufficient to produce paradoxical activation when the inhibitor does not influence dimerization $(f = 1)$. Yet, kinase activity can still be resistant to inhibition when $g \gg 1$, if dimerization is efficient in the absence of inhibitor (Figure S1F). Therefore, such inhibitors are ineffective, even if they do not induce paradoxical kinase activation. For two inhibitors with the same IC_{50}, the decrease in the net kinase activity will be much steeper for the inhibitor that does not allosterically regulate the kinase $(f = 1,\ g = 1)$ than for an allosteric inhibitor $(f < 1,\ g \geq 1)$, regardless if there is a maximum on the dose-response curve (see Figures 1B, S1E, and S1F and the Supplemental Experimental Procedures). Thus, a more objective measure of kinase inhibition and resistance brought about by allosteric drug effects is to compare the areas under the dose-response curves. As Figure 1C demonstrates, this area and, therefore, resistance to inhibition, precipitously increases with decreasing f (for low f values) and increasing g. Summarizing, this core thermodynamic model is able to quantitatively explain paradoxical activity upswings, which are observed for numerous kinases as the effects of ATP-competitive inhibitors that allosterically promote kinase dimerization.

Asymmetric Kinase Homodimer

Recent data demonstrate that dimers of kinases, such as CRAF or BRAF, are structurally asymmetric (Hu et al., 2013; Bollag et al., 2010; Figure 2A). Therefore, one next may assume that monomer (R) dimerization yields an asymmetric dimer (R_1R_2) where protomers acquire two different conformations termed R_1 and R_2 (step 1 in the kinetic scheme in Figure 2B). This scheme resembles the kinetic diagram for a symmetric dimer (Figure 1A), but includes two distinct forms of partially inhibited dimers, R_1I-R_2 and R_1-R_2I, where an inhibitor is bound to a protomer in conformations R_1 and R_2, respectively. Transitions between different dimer conformations can be added to this scheme (Figure S2A), but, since at the thermodynamic

equilibrium the results remain the same, it is sufficient to analyze a diagram in Figure 2B.

As for a symmetric dimer, we describe the inhibitor-induced dimerization increase by the facilitation factor *f*, which relates the dimerization constants of free monomers (K_1, step 1) and inhibitor-bound and free monomers (K_5, step 5 yielding R_1I-R_2), as follows: $K_5 = f \cdot K_1$. Then, *the same* factor *f* links the inhibitor affinities for a free monomer (step 2, K_2) and a protomer (R_1) within the dimer R_1R_2 (step 3, K_3) as follows: $K_3 = f \cdot K_2$ (see the Supplemental Experimental Procedures). Therefore, if an inhibitor facilitates kinase dimerization ($f < 1$), this inhibitor is a more avid binder of a protomer (R_1) in the R_1R_2 dimer than a free monomer (R). In contrast, with a symmetric dimer, the affinities of the first inhibitor molecule for protomer conformations R_1 and R_2 in the asymmetric dimer are likely to be different. The difference in the corresponding dissociation constants, K_3 and K_4, is described by an additional thermodynamic factor (g_1) as follows: $K_4 = g_1 \cdot K_3$. This factor determines the ratio of the equilibrium concentrations [R_1I-R_2] and [R_2I-R_1], regardless of whether a transition between these two forms is explicitly incorporated in the kinetic scheme. The same factor then relates the dimerization constants of steps 5 and 6 (yielding R_1-R_2I) as follows: $K_6 = g_1 \cdot K_5$. Finally, as for a symmetric dimer, we introduce a thermodynamic factor (g_2) that relates two dissociation constants, K_2 and K_8, as follows: $K_8 = g_2 \cdot K_2$, where K_2 describes the affinity of the inhibitor binding to a monomer, while K_8 represents the affinity of the second inhibitor molecule binding to the protomer R_1 in a partially inhibited dimer, R_1-R_2I. The dissociation constant (K_7) of the second inhibitor molecule binding to the protomer R_2 in a partially inhibited dimer (R_1I-R_2) is as follows: $K_7 = g_1 \cdot g_2 \cdot K_2$. Then, the dimerization constants of inhibitor-bound monomers (K_9) and free monomers (K_1) are related by the product of all three thermodynamic factors as follows: $K_9 = f \cdot g_1 \cdot g_2 \cdot K_1$ (see the Supplemental Experimental Procedures for derivations). Summarizing, the dimerization constant K_1; the inhibitor affinity for kinase monomers K_2; and thermodynamic factors *f*, g_1, and g_2 determine all reaction K_ds for an asymmetric dimer.

A distinction between symmetric and asymmetric dimer descriptions is an extra parameter (g_1) that quantifies the difference in the inhibitor affinities for two protomer conformations in an asymmetric dimer, R_1R_2. When $g_1 = 1$, all results obtained for a symmetric dimer are applicable for an asymmetric dimer (such as shown in Figures 1 and S1 after substituting the factor *g* for its analog g_2). In particular, in the absence of allosteric inhibitor influence, the total concentration of asymmetric signaling dimers and inhibitor-free monomers ([R_1R_2] + [R_1I-R_2] + [R_1-R_2I] + [R]) always decreases with the increasing inhibitor dose, resulting in a net decrease in kinase activity (Figure S2B). Therefore, such drugs ($f = g_1 = g_2 = 1$) will effectively suppress kinases (Figure S2C). If the inhibitor promotes kinase dimerization ($f < 1$) and has similar affinities for both protomer conformations, then dose-response curves for an asymmetric dimer are the same as for a symmetric dimer, and inhibitor-induced paradoxical increases in kinase activity can be observed (Figure S2C).

When the inhibitor affinities for the protomers R_1 and R_2 are different, $g_1 < 1$ implies further inhibitor-induced facilitation of kinase dimerization that yields the asymmetric R_1-R_2I dimer, whereas $g_1 > 1$ means that the R_1-R_2I dimer is less preferably formed than the R_1I-R_2 dimer. When there is no inhibitor-induced facilitation of the R_1I-R_2 formation ($f = 1$), low $g_1 < 1$ values drive the R_1-R_2I accumulation, leading to paradoxical kinase activation (Figure 2C). If the inhibitor efficiently induces R_1I-R_2 dimerization ($f \ll 1$), the elevated R_1-R_2I accumulation further increases kinase resistance to inhibition only at high g_2 values (Figures S2D–S2F). Under this condition, increasing g_1 or g_2 similarly decreases inhibitor efficacy, which can be explained by the fact that the dissociation constants of reactions yielding fully inhibited dimers include the product of g_1 and g_2 (Figure S2G).

Summarizing, the formation of both symmetric and asymmetric kinase dimers causes resistance to allosteric inhibitors that facilitate dimerization, but there is a richer repertoire of possibilities allowed by thermodynamics for an asymmetric dimer. Subsequently given are surprising modeling predictions that two kinase inhibitors, each ineffective on its own, in combination can abolish drug resistance by preferably binding to different protomer conformations.

Overcoming Resistance by Combining Two Inhibitors that Are Ineffective when Applied Separately

While several reports in the literature confirm that kinase dimerization conveys resistance to inhibitors, the mechanism is unknown (Heidorn et al., 2010; Koppikar et al., 2012; Poulikakos et al., 2011). Here we found that when the dimerization is enhanced by an allosteric inhibitor, which poorly binds to kinase dimers already carrying one inhibitor molecule, resistance inevitably occurs. One may conjecture that, if the binding of an inhibitor to one protomer induces an allosteric change that alters the conformation of the other, drug-free protomer (Nussinov et al., 2013), this induced conformation could become a target for another inhibitor. In this scenario, two structurally different inhibitors against the same target could synergize to suppress the kinase activity of the dimer by binding to different conformations of its protomer constituents. Therefore, I next explore the inhibition of kinase monomers and dimers by a combination of two inhibitors, I_1 and I_2.

A diagram of kinase interactions with two inhibitors includes four kinetic graphs (Figure S3A). The first two of these graphs resemble the graph in Figure 2B where I is replaced by I_1 or I_2, whereas the remaining two graphs include dimers where protomers R_1 and R_2 have bound two different inhibitors, namely R_1I_1-R_2I_2 and R_1I_2-R_2I_1 (Figure 3A). I assume resistance to inhibition, considering the worst-case scenario when each inhibitor causes paradoxical kinase activation when applied separately. This means that the facilitation factors f_a and f_b are less than 1 (here and below subscripts "a" and "b" refer to inhibitors I_1 and I_2), and the factors g_{2a} and g_{2b}, which describe the binding of the second inhibitor molecule to partially inhibited dimers, are about or greater than 1.

Compared with single inhibitors, there are six reactions yielding dimers harboring two different inhibitors, I_1 and I_2 (Figure 3A). Expressing the K_ds of these reactions in terms of the K_ds of free monomer (R) dimerization and inhibitor binding to R requires two additional thermodynamic factors, g_{3a} and g_{3b} (see the Supplemental Experimental Procedures). The factor

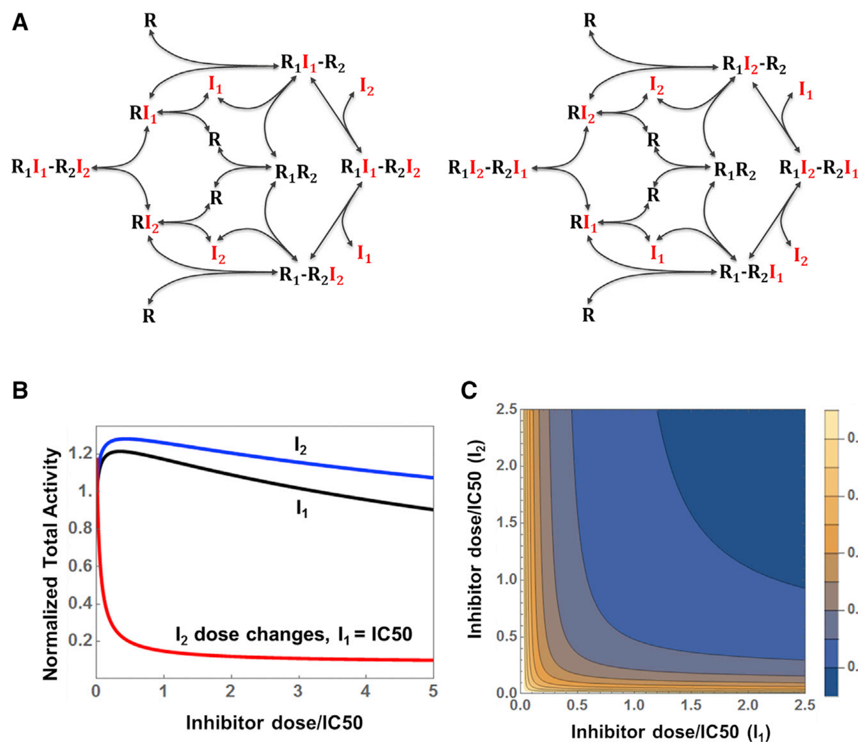

Figure 3. Effective Kinase Inhibition by a Combination of Two Inhibitors

(A) Kinetic schemes of kinase dimerization and interactions with two inhibitors, I_1 and I_2, are shown.

(B) Each inhibitor, I_1 (black line) or I_2 (blue), when applied separately causes paradoxical kinase activation. However, even low doses of I_2 abolish resistance when taken in combination with an IC_{50} dose of I_1, which is ineffective on its own (red line). $IC_{50} = K_{2a}$ for I_1; $IC_{50} = K_{2b}$ for I_2.

(C) Synergy between inhibitors is shown by lines of constant inhibition (termed Loewe isoboles). Thermodynamic factors for (B) and (C) are as follows: $f_a = 0.01$, $f_b = 0.005$, $g_{1a} = 2$, $g_{1b} = 5$, $g_{2a} = 10$, $g_{2b} = 20$, $g_{3a} = 0.01$, and $g_{3b} = 10$. The remaining parameters and normalization are the same as in Figure 1. See also Figure S3.

g_{3a} quantifies the difference in the K_ds of I_1 binding to a free monomer R versus to the protomer R_1 in the R_1-R_2I_2 dimer (yielding R_1I_1-R_2I_2). If I_1 preferably binds to the conformation R_1 in the R_1-R_2I_2 dimer, then g_{3a} is less than 1. Likewise, the factor g_{3b} describes the difference in the K_ds of I_2 binding to R versus to R_1 in the R_1-R_2I_1 dimer (yielding R_1I_2-R_2I_1). The dissociation constants of alternative reactions that yield fully inhibited dimers, R_1I_1-R_2I_2 or R_1I_2-R_2I_1, include the products $g_{1b} \cdot g_{3a}$ or $g_{1a} \cdot g_{3b}$ (see the Supplemental Experimental Procedures for derivations). Therefore, if one or both factors g_{3a} and g_{3b} are less than 1, the formation of fully inhibited dimers is prompted by combining two inhibitors.

Figure 3B shows that kinase activity is resistant to I_1 and I_2 applied separately (black and blue lines). However, when I_2 is applied in addition to the I_1 dose, which by itself only activates the kinase, the combination of I_1 and I_2 effectively inhibits kinase activity, thus overcoming resistance (red line). Similarly, there is no resistance if I_1 is applied on top of the I_2 dose that by itself only activates the kinase (Figure S3B). Interactions between two inhibitors or drugs also can be assessed by plotting inhibition effects across a two-dimensional range of drug doses, thereby creating a response surface (Keith et al., 2005; Yeh et al., 2009). Lines of constant inhibition on response surfaces are termed Loewe isoboles (Greco et al., 1995). For non-interacting drugs, Loewe isoboles are straight lines (Figure S3C; see the Supplemental Experimental Procedures where the constraints on thermodynamic factors defining two non-interacting inhibitors are derived). If two drugs synergize, Loewe isoboles are concave, since lesser drug doses result in the same inhibitory effect that these drugs would achieve if they would not interact. Convex isoboles indicate drug antagonism, because drug combinations require increased doses to achieve the same inhibition.

Figure 3C shows Loewe isoboles on the plane of the I_1 and I_2 doses normalized by IC_{50} for each inhibitor. We see that if binding of the second inhibitor molecule to a dimer is facilitated when two inhibitors are applied together compared to individual inhibitors, this leads to a remarkable synergy between inhibitors (Figure 3C). Importantly, two inhibitors that are individually ineffective, in combination can abolish inhibition resistance across a wide range of thermodynamic factors, provided that dimers bind two different inhibitors more effectively than two molecules of either inhibitor (Figures S3D and S3E). If the formation of dimers carrying two molecules of different inhibitors is impeded, these drugs show antagonism (Figure S3F).

Allosteric Regulation of BRAF-CRAF Heterodimers by ATP-Competitive Inhibitors

Next is an analysis of the paradoxical effect of enhancing the total RAF activity by RAF inhibitors, which remains a significant problem in clinic (Rahman et al., 2014). To that end, I develop a core model describing BRAF-CRAF heterodimerization and interactions with inhibitors. A kinetic scheme of the model is shown in Figure 4A (where BRAF and CRAF are denoted by B and C, respectively). Here, step 1 is heterodimerization of inhibitor-free BRAF and CRAF monomers with the dissociation constant K_1. The inhibitor I binds to BRAF and CRAF monomers (steps 2 and 3) with similar or different affinities, characterized by the dissociation constants, K_2 and K_3. The ratio of these constants, K_3/K_2, determines the inhibitor specificity. If the ratio is around 1, the inhibitor is termed a pan-RAF inhibitor, if it is much greater than 1, the inhibitor is BRAF specific.

ATP-competitive inhibitors stabilize an active RAF kinase domain conformation, prompting side-to-side dimerization of kinase domains (Lavoie and Therrien, 2015). As above, this is quantified by the facilitation factor *f* that relates two dimerization constants, K_1 and K_6, of inhibitor-free monomers (step 1) and inhibitor-bound BRAF (BI) and free CRAF (step 6), such that

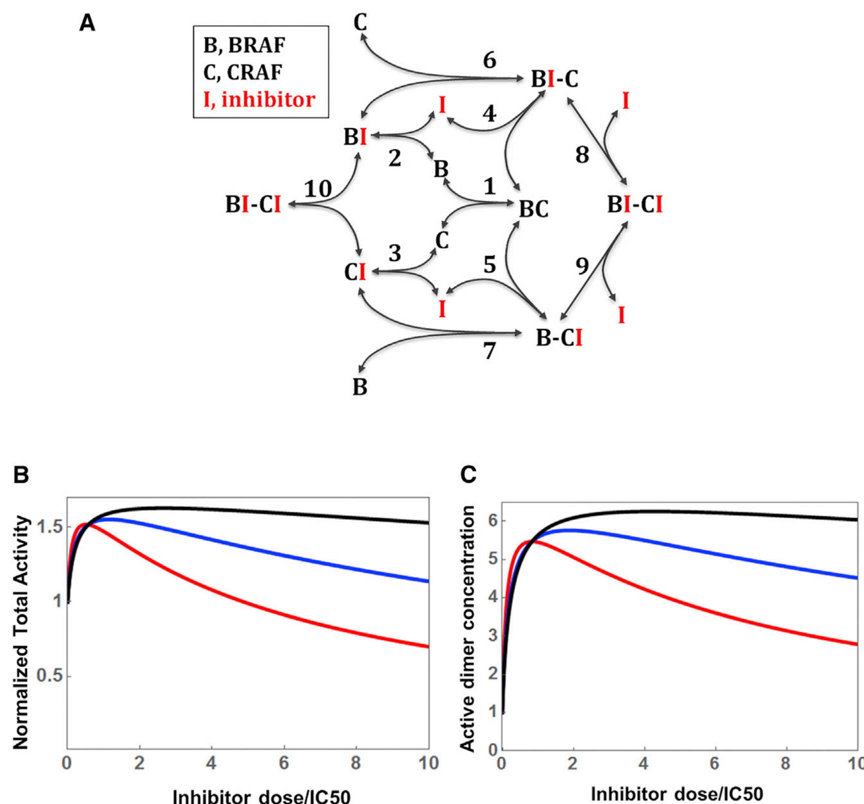

Figure 4. A Model of BRAF and CRAF Heterodimerization and Inhibitor Binding

(A) Kinetic diagram of BRAF and CRAF dimerization and inhibitor binding is shown.

(B) Sensitivity of dose-response curves to changes in inhibitor specificity for BRAF. Total kinase activity is normalized as in Figure 1; inhibitor dose is normalized by IC_{50} for BRAF monomers, $IC_{50} = K_2$.

(C) Dependence of the total concentration of active heterodimers ([BC] + [BI-C] + [B-CI]) on the inhibitor dose for different inhibitor specificities. Total active heterodimer concentration is normalized by its value with no inhibitor. Parameter values for (B) and (C) are as follows: g_1 = 1, g_2 = 10, f = 0.03, K_3/K_2 = 1 (red curves), 5 (blue), and 25 (black). Total concentrations of BRAF and CRAF are equal (RAF_{tot}), RAF_{tot}/K_1 = 0.1. Inhibitor-free heterodimers are twice as active as partially inhibited heterodimers and 15 times as active as BRAF monomers, and a BRAF monomer is twice as active as a CRAF monomer (Rushworth et al., 2006). See also Figure S4.

$K_6 = f \cdot K_1$. Thermodynamic restrictions then require that the dissociation constants, K_4 of inhibitor binding to a BRAF protomer in a BC heterodimer (step 4) and K_2 of inhibitor binding to a free BRAF monomer, must be related by the same facilitation factor f, such that $K_4 = f \cdot K_2$ (see the Supplemental Experimental Procedures).

Step 5 yields another form of partially active heterodimer, B-CI, where the inhibitor is bound to a CRAF protomer. Accordingly, an additional thermodynamic factor, g_1, is required (together with f) to relate the free energies of inhibitor binding to a CRAF protomer in a BC heterodimer (step 5) versus a CRAF monomer, such that $K_5 = f \cdot g_1 \cdot K_3$. The same two factors connect two dimerization constants, K_1 and K_7, of inhibitor-free monomers (step 1) and inhibitor-bound CRAF (CI) and free BRAF (step 7) as follows: $K_7 = f \cdot g_1 \cdot K_1$ (see the Supplemental Experimental Procedures). Similarly as to asymmetric homodimers, another thermodynamic factor (g_2) relates two dissociation constants, K_9 of inhibitor binding to a BRAF protomer in a partially inhibited dimer (B-CI) and K_2 of inhibitor binding to a monomeric BRAF, as follows: $K_9 = g_2 \cdot K_2$. Then, the dissociation constant (K_8) of inhibitor binding to a CRAF protomer in a partially inhibited dimer (BI-C) is as follows: $K_8 = g_1 \cdot g_2 \cdot K_3$. Thus, enhancement of BRAF-CRAF dimerization by a drug leads to its increased binding to one protomer in BC dimers, whereas the free protomer can be left with a much lesser affinity for this drug.

Finally, the dimerization constant for two inhibitor-bound monomers BI and CI (K_{10}) is related by the product of factors f, g_1, and g_2 to the dimerization constant of free BRAF and CRAF monomers as follows: $K_{10} = f \cdot g_1 \cdot g_2 \cdot K_1$ (see the Supplemental Experimental Procedures for derivation). Summarizing, the K_ds of all dimerization and inhibitor-binding reactions are expressed in terms of the dissociation constants of BRAF and CRAF dimerization; inhibitor binding to free BRAF and CRAF monomers; and thermodynamic factors f, g_1, and g_2.

Dose-Response Curves Show Resistance to Single Inhibitors that Promote Dimerization

If an inhibitor promotes dimerization, the model suggests that this inhibitor drives the formation of catalytically active BI-C and B-CI heterodimers, while the accumulation of completely inhibited BI-CI dimers lags behind (Figure S4A). The heterodimer activity is estimated to be 10–20 times higher than the activity of BRAF and CRAF monomers/homodimers (Freeman et al., 2013; Rushworth et al., 2006; see the Supplemental Experimental Procedures. As a result, dose-response curves show paradoxical increases in the total RAF kinase activity (Figure 4B), resembling the behavior of the total concentrations of catalytically active heterodimers (Figure 4C). Simulations suggest that specific BRAF inhibitors, which have higher affinity for BRAF versus CRAF, instigate paradoxical activation more significantly than pan-RAF inhibitors (provided that these inhibitors equally facilitate BRAF-CRAF dimerization; Figures 4B, 4C, and S4A). These results are consistent with a recent report that pan-RAF inhibitors are more effective than specific BRAF inhibitors in melanoma patients who have developed resistance (Girotti et al., 2015). One may conclude that inhibitor-induced increase in RAF dimerization and weak inhibitor affinities for partially inhibited heterodimers drive inhibition resistance, preventing kinase activity suppression at therapeutically relevant inhibitor doses (Figures S4B–S4D). Finally, since kinase activity of

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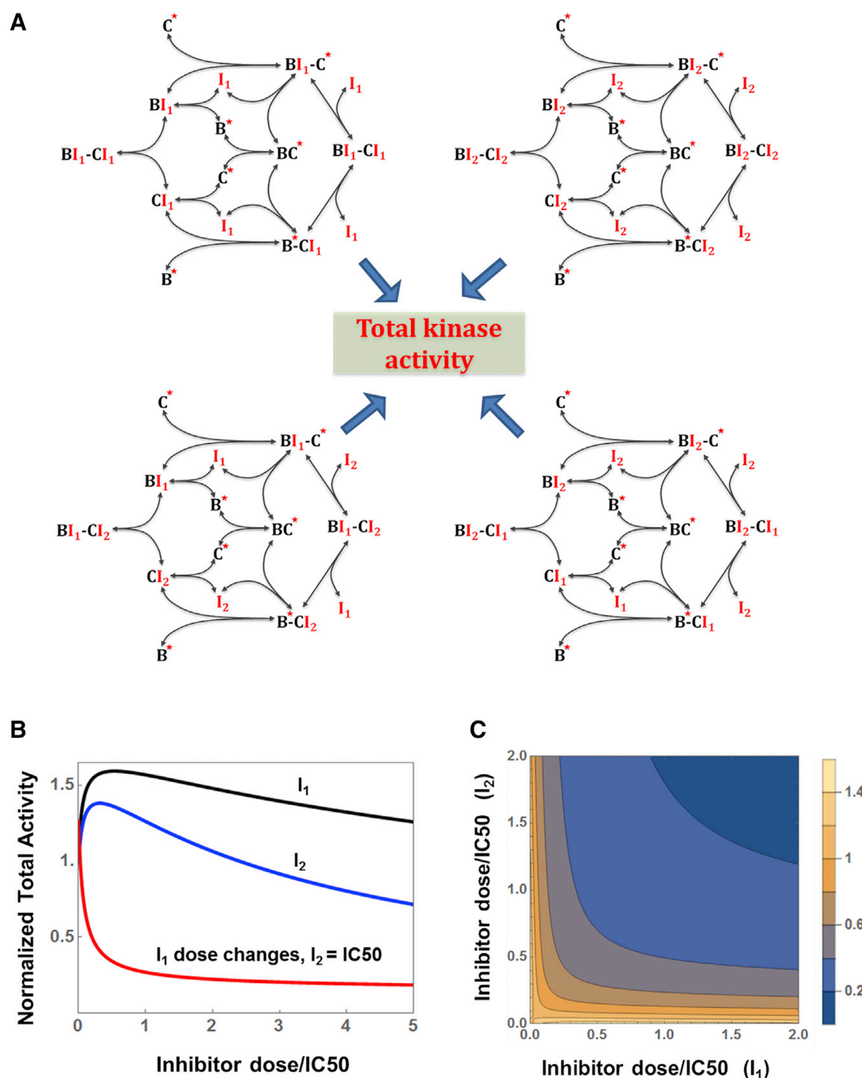

Figure 5. A Combination of Two Individually Ineffective Inhibitors Can Abolish BRAF/CRAF Inhibition Resistance

(A) Kinetic schemes of BRAF and CRAF heterodimerization and interactions with two inhibitors, I_1 and I_2. Active monomers and dimers are denoted by the red asterisks.

(B) Each inhibitor, I_1 (black line) and I_2 (blue), when applied separately causes paradoxical kinase activation. When taken in combination with an IC_{50} dose of I_2, which is ineffective on its own, even low doses of I_1 abolish resistance (red line). $IC_{50} = K_{2a}$ for I_1; $IC_{50} = K_{2b}$ for I_2.

(C) Loewe isoboles show synergy between these two inhibitors. Thermodynamic factors for (B) and (C) are as follows: $f_a = 0.01$, $f_b = 0.1$, $g_{1a} = 1$, $g_{1b} = 0.1$, $g_{2a} = 50$, $g_{2b} = 5$, $g_{3a} = 0.5$, and $g_{3b} = 0.05$. The remaining parameters and normalization are the same as in Figure 4. See also Figure S5.

heterodimers is significantly greater than homodimer activities (Garnett et al., 2005; Rushworth et al., 2006), the results obtained with this model are applicable to the cellular BRAF and RAF system, where both homodimers and heterodimers are formed under physiological conditions.

A Combination of Two Inhibitors Can Abolish BRAF-CRAF Inhibition Resistance

The difference in the BRAF-CRAF dimer affinities for the first and second inhibitor molecules depends on both the dimer and inhibitor structures and allosteric effects induced by the first inhibitor molecule. Allosteric interactions between inhibitors (Nussinov et al., 2013) may result in the tighter binding of two molecules of different inhibitors than two molecules of either inhibitor to a BRAF-CRAF dimer. Consequently, these two inhibitors can synergistically suppress BRAF and CRAF signaling. I analyzed this hypothesis using our core model of the BRAF and CRAF system with two different RAF inhibitors.

When two inhibitors (I_1 and I_2) are applied together, each inhibitor can bind separately and also in combination with another inhibitor, as shown by four kinetic graphs in Figures 5A and S5A. Two of these graphs present reactions that involve only I_1 or I_2, whereas the remaining two graphs include BRAF and CRAF heterodimers that have bound different inhibitors (BI_1-CI_2 or BI_2-CI_1). As in the case with asymmetric homodimers, two additional thermodynamic factors, g_{3a} and g_{3b}, are needed to express the K_ds of I_1 binding to B-CI_2 (g_{3a}) and I_2 binding to B-CI_1 (g_{3b}) in terms of the K_ds of I_1 and I_2 binding to free BRAF and CRAF monomers (see the Supplemental Experimental Procedures for derivations).

I showed that pan-RAF inhibitors suppress total kinase activity more efficiently than specific BRAF (or CRAF) inhibitors (Figures 4B, 4C, and S4A). Therefore, to analyze if a combination of two drugs can surmount resistance, consider an unfavorable situation when each pan-RAF inhibitor (I_1 and I_2) causes paradoxical kinase activation, when applied separately. One may conjecture that if the I_1 affinity for B-CI_2 heterodimer is higher than for B-CI_1 heterodimer, due to allosteric influence of I_2, there will be synergy between these two inhibitors. Testing this hypothesis, I found that, in this case, even low doses of I_1 already abolish resistance when taken in combination with an IC_{50} dose of I_2, which is ineffective on its own (Figure 5B). Likewise, the synergy will be observed if the I_2 affinity for B-CI_1 heterodimer is higher than for B-CI_2 heterodimer, due to allosteric influence of I_1 (Figure S5B). The analysis of Loewe isoboles demonstrates that synergy between two different inhibitors occurs in a wide range of kinetic constants and also extends to a combination of two specific BRAF inhibitors or a specific BRAF and a pan-RAF inhibitor (Figures 5C, S5D, and S5E). However, if binding of one inhibitor to a heterodimer

hinders binding of the other inhibitor, these two RAF inhibitors can show antagonism (Figure S5F). In this case, each inhibitor and their combination are ineffective. Instructively, at some values of thermodynamic factors, two drugs can show antagonism, synergy, or independence in different dose ranges (Figure S5G).

DISCUSSION

Discovered more than a decade ago, the paradoxical activation of the MAPK pathway by ATP-competitive RAF inhibitors caught the scientific community by surprise (Hall-Jackson et al., 1999). It took another 10 years to recognize that failure of oncogenic BRAF600E inhibitors to suppress proliferative signaling in patients with mutant RAS is related to RAS-dependent heterodimerization of BRAF and CRAF. In these dimers, kinase-dead or inhibited BRAF allosterically activates CRAF, driving MAPK signaling (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Wan et al., 2004). Paradoxical activation of mitogenic signaling by RAF inhibitors causes both drug resistance and clinical side effects, including frequent incidence of keratoacanthomas and squamous cell carcinomas (Rahman et al., 2014). This highlights the necessity of quantitative understanding of drug-induced signaling and genotyping tumors before using these drugs in patients. A similar cautioning is echoed by resistance of JAK-STAT signaling to JAK ATP-competitive inhibitors induced by homo- and heterodimerization of JAK kinases (Brooks et al., 2014; Koppikar et al., 2012).

Here I presented quantitative analysis of allosteric effects of ATP-competitive inhibitors on kinase dimerization and total activity. I showed that, in addition to the degree of promoting dimerization by an inhibitor, other thermodynamic factors, such as the drug affinity difference for protomers in asymmetric dimers or heterodimers and the inhibitor affinity for dimers already carrying single drug molecules, can drive inhibition resistance. My analysis suggests that upon dimerization the affinity of drug binding to one protomer increases, but then the second, unoccupied protomer experiences a steep reduction in affinity. This leads to an accumulation of kinase dimers that have one protomer bound to drug and the other not. As the drug-bound protomer allosterically activates the free protomer, this constellation brings about very high signaling activity upon inhibitor addition. In the Protein Data Bank (PDB), there are structures of BRAF and CRAF dimers co-crystallized with different RAF inhibitors, including PLX4032. Although during the co-crystallization the inhibitor concentrations were extremely high, several co-crystallized dimer structures show only one of the two protomers bound to inhibitor (Bollag et al., 2010). This suggests that the dimer affinity for the second inhibitor molecule precipitously drops compared to the affinity for the first inhibitor molecule, in line with my results.

Central to this analysis was a principle of microscopic reversibility and detailed balance relationships between the equilibrium dissociation constants. Resulting constraints on the concentrations of inhibitor-free and inhibitor-bound kinase monomers and homo- and heterodimers determine the total activity response to drug. In cells, kinase dimerization, as well as G protein-coupled receptor dimerization, usually is followed by kinetically irreversible steps with large free energy changes, such as phosphorylation or GTP hydrolysis. At first glance, these energy-generating steps suggest that signaling networks are not constrained by the detailed balance equations. However, this assertion misses the point that complex signaling networks with kinetically irreversible reactions contain subnetworks, in which inhibitor (or modulator) binding occurs without changes in the phosphorylation status of protein monomers and dimers. The corresponding free energy changes equal zero along cyclic reaction routes. Although being embedded in larger reaction networks where the net phosphorylation and dephosphorylation fluxes may not be zero, signaling subnetworks with no changes in the phosphorylation status practically always obey the detailed balance relationships (Colquhoun et al., 2004; Ederer and Gilles, 2007; Yang et al., 2006). For instance, reactions of inhibitor binding to phosphorylated protein monomers and dimers generate another reaction subnetwork in addition to the analyzed above subnetwork containing unphosphorylated kinase forms. This subnetwork is again restricted by the detailed balance equations. Of course, a cyclic reaction path that includes kinetically irreversible (de)phosphorylation reactions is accompanied by the free energy change, and the detailed balance relationships do not apply (Kholodenko et al., 1999; Markevich et al., 2004).

My mechanistic models show that pan-RAF inhibitors suppress BRAF/CRAF activity more effectively than specific RAF inhibitors. These models also suggest ways to surmount inhibition resistance by the combined action of two RAF inhibitors. For instance, if a specific inhibitor of oncogenic BRAF600E induces paradoxical ERK activation in wild-type cells, a combination with a pan-RAF inhibitor might be more effective. I demonstrate that even if each inhibitor is ineffective individually, but allosteric interaction of one inhibitor with a kinase dimer facilitates the binding of another inhibitor to this dimer, this inhibitor combination abolishes resistance (Figures 3 and 5). Thus, a choice of effective inhibitor combination can be aimed at selecting inhibitors that preferably associate with different protomer conformations in an asymmetric kinase dimer.

EXPERIMENTAL PROCEDURES

Reaction lists for all models and thermodynamic relationships between the equilibrium dissociation constants (K_ds) are given in the Supplemental Experimental Procedures (Reaction Lists S1–S5). Derivations of these relationships and the properties of dose-response curves are presented in the Supplemental Experimental Procedures. Equilibrium concentrations of different forms of kinase monomers and dimers are derived in the Supplemental Experimental Procedures. Numerically, all concentrations and total kinase activities are calculated with Mathematica software from equations given in the Supplemental Experimental Procedures.

Here a typical derivation of the relationships between the K_ds is given for a model of the allosteric regulation of BRAF–CRAF heterodimers by an inhibitor (Figure 4A). We first identify all cyclic paths in the kinetic scheme presented in Figure 4A. Equating the total free energy change along each cyclic path to zero, we obtain the following relationships between the free energy changes

(ΔG_i) of individual reactions (index i is the reaction number in the kinetic scheme; Figure 4A):

$$\Delta G_1 + \Delta G_4 + \Delta G_8 = \Delta G_1 + \Delta G_5 + \Delta G_9 = \Delta G_2 + \Delta G_6 + \Delta G_8 = \Delta G_3 + \Delta G_7 + \Delta G_9 = \Delta G_2 + \Delta G_3 + \Delta G_{10}. \quad \text{(Equation 1)}$$

The Gibbs free energy change is directly related to the equilibrium dissociation constant of a reaction,

$$\Delta G = RT \cdot \ln(K_d / c_0), \quad \text{(Equation 2)}$$

where R is the gas constant, T is temperature, and c_0 has a numerical value of 1 and units that are the reciprocal of the units of the concentration quotient to make the K_d/c_0 ratio dimensionless. Equation 2 shows that linear relationships (Equation 1) between the free energy changes are equivalent to constraints on the products of the K_ds of the corresponding reactions.

The K_ds of inhibitor binding to BRAF (K_2) and CRAF (K_3) monomers and dimerization (K_1) of inhibitor-free BRAF and CRAF monomers are assumed to be known (see the text). Since the matrix of the linear equation system (Equation 1) has rank 4, there are four independent linear relationships between three known $(\Delta G_1, \Delta G_2,$ and $\Delta G_3)$ and seven unknown $(\Delta G_4, \Delta G_5, \Delta G_6, \Delta G_7, \Delta G_8, \Delta G_9$, and $\Delta G_{10})$ free energy changes. Consequently, three independent thermodynamic factors are required to express seven unknown K_ds in terms of K_1, K_2, and K_3. I have introduced these factors as (1) the facilitation factor f that quantifies the extent of promoting dimerization by an inhibitor, $K_6 = f \cdot K_1$; (2) the factor g_1 that determines the difference in the affinities of the first inhibitor molecule for two protomers (BRAF and CRAF) in the BC heterodimer, $K_5/K_4 = g_1 \cdot K_3/K_2$; and (3) the factor g_2 that defines the difference in the affinity of the second inhibitor molecule for a BRAF protomer in a B-CI dimer and the inhibitor affinity for BRAF monomers, $K_9 = g_2 \cdot K_2$. Using these three relationships and solving linear Equation 1, we arrive at the expressions of all remaining K_ds in terms of K_1, K_2, and K_3 and thermodynamic factors f, g_1, and g_2 (see the text and Reaction List 4 in the Supplemental Experimental Procedures). According to Equation 2, the parameters f, g_1, and g_2 directly relate to the differences in the Gibbs free energy changes of the corresponding reactions, and this explains the logic of referring to these parameters as thermodynamic factors.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.08.014.

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