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A Back-to-Front Fragment-Based Drug Design Search Strategy Targeting the DFG-Out Pocket of Protein Tyrosine Kinases

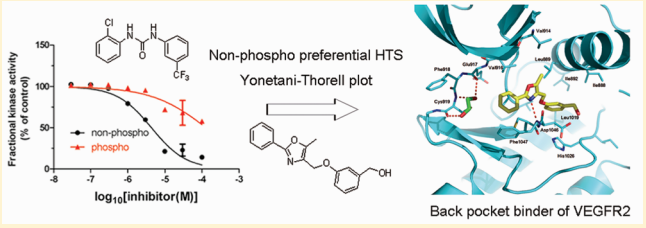
Hidehisa Iwata,[†] Hideyuki Oki,[†] Kengo Okada,[†] Terufumi Takagi,[†] Michiko Tawada,[†] Yasushi Miyazaki,[†] Shinichi Imamura,[‡] Akira Hori,[‡] J. David Lawson,[§] Mark S. Hixon,[§] Hiroyuki Kimura,[†] and Hiroshi Miki*[,†]

[†]Discovery Research Laboratories, [‡]Oncology Drug Discovery Unit, Pharmaceutical Research Division, Takeda Pharmaceutical Company Ltd., 26-1, Muraoka-Higashi 2-chome, Fujisawa, Kanagawa 251-8555, Japan

[§]Takeda California Inc., 10410 Science Center Drive, San Diego, California 92121, United States

Supporting Information

ABSTRACT: We present a straightforward process for the discovery of novel back pocket-binding fragment molecules against protein tyrosine kinases. The approach begins by screening against the nonphosphorylated target kinase with subsequent counterscreening of hits against the phosphorylated enzyme. Back pocket-binding fragments are inactive against the phosphorylated kinase. Fragment molecules are of insufficient size to span both regions of the ATP binding pocket; thus, the outcome is binary (back pocket-binding or hinge-binding). Next, fragments with the appropriate binding profile are assayed in combination with a known hinge-binding fragment and subsequently with a known back pocket-binding fragment. Confirmation of back pocket-binding by Yonetani–Theorell plot analysis progresses candidate fragments to crystallization trials. The method is exemplified by a fragment screening campaign against vascular endothelial growth factor receptor 2, and a novel back pocket-binding fragment is presented.



KEYWORDS: *back-to-front, FBDD, DFG-out, VEGFR2, phosphorylation, Yonetani–Theorell Plot*

There are more than 90 known protein tyrosine kinases (PTKs) in the human kinome.[1] Mechanistically, PTKs transfer the γ-phosphate of ATP to a substrate protein's tyrosine residue that, in turn, modulates the acceptor protein's function. These phosphorylation events form the backbone of many cellular signaling cascades including those in which disregulation is implicated in diseases ranging from diabetes to cancer.[2]

The ATP binding pocket, located adjacent to the peptide binding cleft and within the interlobal hinge, has proven to be one of the best binding sites for small molecule inhibitors known.[3] Although ATP is a substrate common to all protein kinases, most ATP binding pockets contain exploitable unique features affording potential selectivity against other kinases.

Phosphorylation of the PTK activation loop—a loop containing one or more phosphorylatable residues (tyrosine, serine, or threonine) and anchored by a conserved Asp-Phe-Gly (DFG) motif—leads to a large shift in the conformational equilibrium toward the activated state of the enzyme.[4] In the nonphosphorylated state, the DFG motif is arranged with the Phe side chain flipped out of the hydrophobic core of the protein (DFG-out), thus interfering with ATP access to the active site.[5,6] When phosphorylated, the equilibrium shifts toward the active conformation in which the Phe side chain is buried within the core of the protein (DFG-in), allowing ATP to access the site and positioning the catalytic residues for activity. The active conformation is part of the DFG-in family of conformations.

DFG-out inhibitors are necessarily selective for the inactive enzyme form since, in addition to the hinge region (part of the main ATP binding pocket), they interact with an additional region known as the back pocket, which does not exist in a DFG-in conformation. These inhibitors often demonstrate notable advantages over DFG-in inhibitors including a longer residence time[7,8] and, for some kinases, greater selectivity since the back pocket contains more characteristics unique to a targeted kinase than does the front pocket.[9] In addition, the structural distinctions of DFG-out inhibitors can help avoid intellectual property (IP) issues.[10]

Fragment-based drug design (FBDD) is an increasingly popular approach to lead discovery.[11] The "rule of three" (molecular mass $\leq$300 amu, cLog $P \leq 3$ H-bond donors and H-bond acceptors each $\leq$3) classically describes fragmentlike molecules.[12] Such constraints result in hits against a target having weak IC_{50} values, typically >100 μM. Harnessing the full potential of FBDD requires coupling to a structure-based drug design campaign (SBDD). This allows facile expansion of the fragment into a leadlike molecule through an understanding of

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the fragment's binding mode and opportunities for generating additional, favorable interactions. A FBDD approach enables the exploration of more chemical diversity, higher hit rates, and higher ligand efficiency (LE) of hits as compared to traditional high-throughput screening (HTS).[13] Evidence suggests that best-in-class drugs have the greatest LE and highest binding enthalpy.[14] These parameters are optimized with a FBDD approach.[15]

Applying a FBDD approach toward the discovery of novel kinase back pocket binders is particularly challenging and has essentially eluded the pharmaceutical industry to date. The strategy is known as "back-to-front" and involves starting from a putative back pocket-binding fragment hit and subsequently elaborating toward the hinge. Back pocket-binding fragments are key to this approach because they provide the starting points for optimization.[16,17]

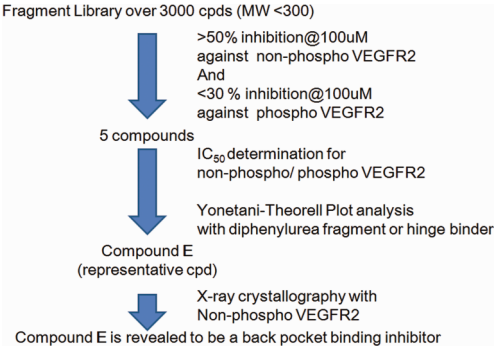
Developing an effective and efficient assay is the main challenge in the application of FBDD to the back pocket. Typically, the activated kinase is used for enzymatic screening of compound libraries. While technically easier to follow the inhibition kinetics relative to using an unactivated kinase, the activated form is thermodynamically unfavorable toward back pocket-binding compounds. This is particularly problematic when examining fragment molecules, which, by their very nature, bind weakly.

ATP-competitive ligand displacement assays are an alternative approach. Although this approach is amenable to screening against the unphosphorylated/unactivated enzyme, a ligand must be displaced from the active site, which again adds a thermodynamic burden for a weak binder to overcome. Most recently, Simard et al. have approached the problem by chemically modifying the kinase activation loop with a fluorophore and observing distinct intrinsic fluorescence signatures when fragments bind within the back pocket.[18,19] Among the challenges of this approach to new PTK targets are finding suitable amino acids on the activation loop amenable to labeling and providing distinct fluorescent signatures for back pocket binding as well as ensuring that the labeling event itself does not create an artificial pocket confirmation.

This letter establishes a straightforward process for the discovery of novel back pocket-binding fragment molecules. The essence of this approach is to screen against the nonphosphorylated form of the target kinase followed by counterscreening the hits against the phosphorylated enzyme form. Fragment molecules are of insufficient size to span both regions of the ATP-binding pocket; thus, the outcome is binary (back pocket-binding or hinge-binding). Fragments that bind in the back pocket should suffer a substantial loss of potency against the phosphorylated form of the kinase. Fragments demonstrating the appropriate binding profile are then assayed in combination with a known hinge-binding fragment and subsequently with a known back pocket-binding fragment. Confirmation of back pocket-binding by Yonetani–Theorell plot analysis progresses candidate fragments to crystallization trials (Scheme 1).

It is established that (1) compounds exploiting only the hinge-binding region lack selectivity between phosphorylation states of the kinase and (2) inhibitors that access the back pocket are selective for the unphosphorylated state of the kinase. Would fragment molecules capable of only interactions within the back pocket also display such selective behavior? To that end, we examined known hinge-binding fragments as well as back pocket-binding fragments for differences in inhibitory

Scheme 1. Compound Progression Strategy for the Discovery of Fragments Binding in the Back Pocket



activity between nonphosphorylated and phosphorylated receptor tyrosine kinase VEGFR2.

Generating a fragment that engages the hinge-binding region of a kinase is straightforward; for example, one can generate a truncated analogue of Staurosporine. Such hinge-binding fragments are pan-kinase inhibitors. The design of a back pocket-binding fragment is a little more challenging and tends to be more kinase family-specific. A retro-analysis of type II inhibitors for the VEGFR family of RTKs reveals that diarylureas are a common motif engaging the back pocket. We designed back pocket fragment **B** based on previously reported **20d**, a time-dependent and selective inhibitor for nonphosphorylated VEGFR2.[20] Fragment **B** displayed more than 20-fold selectivity for nonphosphorylated over phosphorylated VEGFR2 with IC_{50} values of 4.4 and over 100 μM, respectively

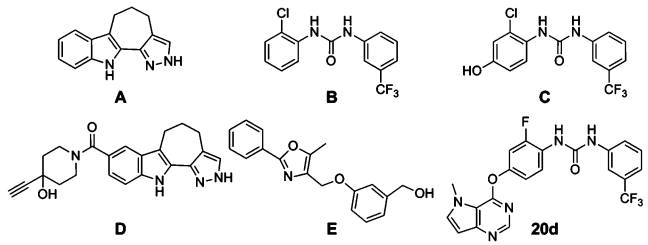


Figure 1. Chemical structures of the compound discussed within this study.

(Figure 2b and Supporting Information, Table 1). Fragment **C**, structurally similar to **B**, was also selective for the nonphosphorylated enzyme (Supporting Information, Figure 1 and Table 1). Thus, even fragment molecules whose interactions are limited to back pocket binding preferentially inhibit nonphosphorylated VEGFR2. In contrast, **A**, which binds to the hinge region (Figure 3d), displayed no significant difference in inhibition between the nonphosphorylated and the phosphorylated catalytic domains of VEGFR2, with IC_{50} values of 620 and 860 nM, respectively (Figure 2a and Supporting Information, Table 1). In addition to activity-based comparisons, we performed thermal shift assays of phosphorylated and nonphosphorylated VEGFR2, obtaining results consistent with the assays above (Supporting Information, Figure 2 and Table 2).

Because fragments **A** and **B** bind to distinct regions of the ATP-binding pocket, they open the possibility that both fragments could bind simultaneously, that is, nonmutually

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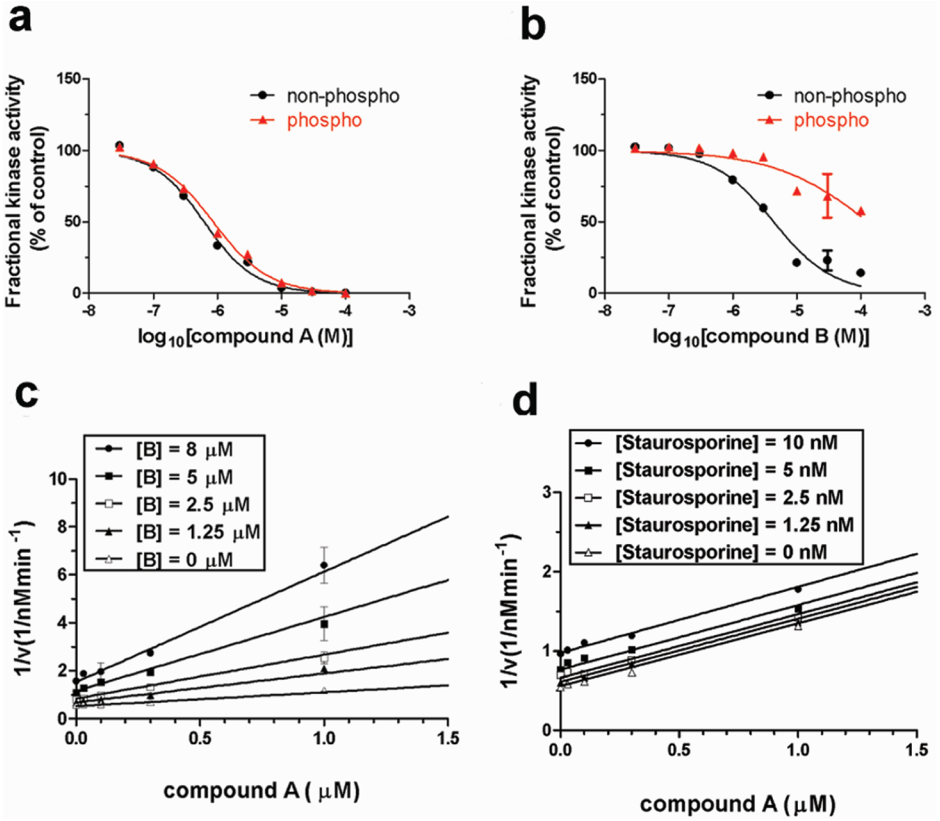

Figure 2. Inhibitory activity and Yonetani–Theorell plots analysis of known fragments. (a) Inhibitory activity of compound **A** for nonphosphorylated VEGFR2 (black) and phosphorylated VEGFR2 (red). (b) Inhibitory activity of compound **B** for nonphosphorylated VEGFR2 (black) and phosphorylated VEGFR2 (red). In both figures, data represent the mean ± SD (n = 4). (c) Yonetani–Theorell plots for the inhibition of VEGFR2 by various combinations of **B** (0, △; 1.25, ▲; 2.5, □; 5, ■; and 8 μM, ●) and **A**. (d) Yonetani–Theorell plots for the inhibition of VEGFR2 activity by various combinations of staurosporine (0, △; 1.25, ▲; 2.5, □; 5, ■; and 10 nM, ●) and **A**. In both figures, error bars are from the average of two replicate experiments.

exclusive. Investigating this possibility, we performed a Yonetani–Theorell analysis.[21,22]

Two inhibitors with overlapping binding sites will be mutually exclusive in their binding and a plot of $1/v$ versus the concentration of one inhibitor at fixed concentrations of the second inhibitor will produce a family of parallel lines as a diagnostic pattern. In contrast, if the two inhibitors are not mutually exclusive, the same plot will produce a family of intersecting lines.

$$\frac{1}{v} = \frac{1}{V_0} \times \left(1 + \frac{[A]}{K_{iA}} + \frac{[B]}{K_{iB}} + \frac{[A][B]}{\alpha K_{iA} K_{iB}}\right) \quad (1)$$

$$\% \text{ inhibition} = 100\% \left(1 - \frac{1}{2 + \frac{[\text{fragment}]}{\beta}}\right) \quad (2)$$

$$\beta = \frac{[\text{fragment}]}{\frac{V_0}{v} - 1}$$

Equation 1 is a general solution to a two inhibitor combination and contains the inhibitor interaction parameter α. The magnitude of α describes the degree of interaction taking values of less than 1, equal to 1, and greater than 1, reflecting synergistic binding, independent binding, and antagonistic binding, respectively. The results from Yonetani–Theorell analysis indicated that **A** and **B** bind to nonphosphorylated VEGFR2 simultaneously and synergistically since the lines on the plot converge approximately at the x-axis (Figure 2c), producing an α-value for this combination of 0.21 (each compound binds 5-fold more potently in the presence of the other). In contrast, the combination of **A** with Staurosporine produces mutually exclusive inhibition, revealed in the plot as a series of parallel lines (Figure 2d) affording an α > 10 (mutually exclusive binding). A fluorescent probe displacement assay using fluorescent labeled **D**, an analogue of **A**, supported the result of the Yonetani–Theorell analysis above since **A** could displace the fluorescent probe but **B** could not (Supporting Information, Figure 3).

Pursuant to the above strategy, a proprietary fragment library of 3000 compounds was assembled. Approximately 66% of the compounds were rule-of-3 compliant, and the remaining compounds violated no more than one rule. The library was screened against unactivated VEGFR2 with follow-up counter-screening of hits against the activated kinase. Five fragments were observed to meet the selectivity cutoff values (Scheme 1), one of which, **E**, a nonurea-based compound (Figure 1), is described further in this report.

Fragment **E** demonstrated an IC_{50} of 50 μM (Cheng Prussoff calculated K_i of 25 μM) against unactivated VEGFR2 and an IC_{50} well over 100 μM (8% inhibition at 100 μM) against phosphorylated VEGFR2 (Supporting Information, Figure 1). Follow up examination of **E** by Yonetani–Theorell plot analysis in combination with back pocket binder **B** displayed mutually

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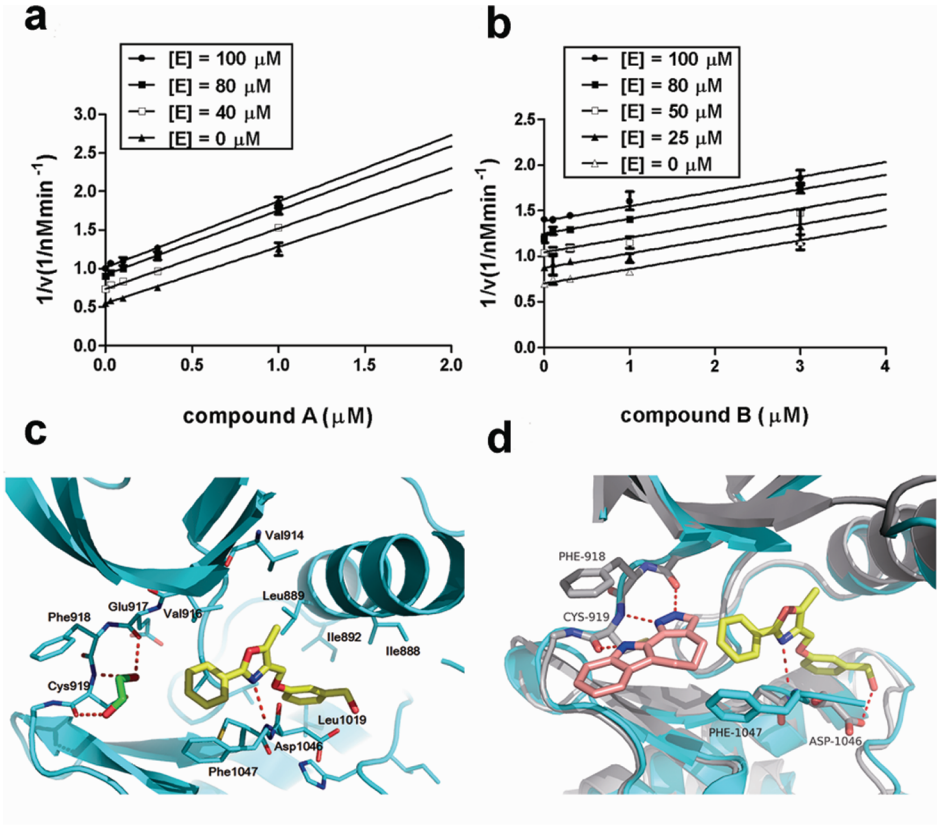

Figure 3. Yonetani–Theorell plots of **E** and crystal structure analysis of **E** bound to the VEGFR2. (a) Yonetani–Theorell plots for the inhibition of VEGFR2 by various combinations of **E** (0, ▲; 40, □; 80, ■; and 100 μM, ●) and **A**. (b) Yonetani–Theorell plots for the inhibition of VEGFR2 activity by various combinations of **E** (0, △; 25, ▲; 50, □; 80, ■; and 100 μM, ●) and **B**. In both figures, error bars are from the average of two replicate experiments. (c) X-ray cocrystal structure of **E** in complex with nonphosphorylated VEGFR2. (d) Overlay comparison of **E** (yellow ligand and blue protein) and **A** (pink ligand and gray protein) in complex with nonphosphorylated VEGFR2. In both figures, H-bonds are depicted as red dashes, and the P loop has been hidden for clarity.

exclusive binding (Figure 3b and Supporting Information, Table 3). Conversely, **E** in combination with **A** produced a nonmutually exclusive interaction although the α was 4.9, indicating some antagonism between the binding of **A** and **E** (Figure 3a). With these encouraging results, we progressed **E** into crystallization trials.

The crystal structure of VEGFR2 cocomplexed with **E** was solved by molecular replacement to 2.5 Å resolution. The final structure was refined to an *R* factor of 21.0%, with a free *R* of 26.2% (Supporting Information, Table 5). This structure has been deposited in the PDB (accession code 3VHK).

Compound **E** binds mainly within the back pocket although the phenyl ring proximal to the hinge does slightly overlap with the nucleotide binding site (Figure 3c). While the ligand does not interact directly with the hinge, an ethylene glycol molecule bridges between the terminal phenyl ring and the hinge, contacting both and making hydrogen bonds to hinge backbone atoms in Glu917 and Cys919. In addition, this phenyl ring is flanked by Phe1047 in the DFG loop, forming an edge to face aromatic interaction. In addition, **E** makes a hydrogen bond between the backbone NH of Asp1046 and the nitrogen of the oxazole ring at a distance of 3.1 Å, similar to what was observed in the cocomplex structure with compound **20d** (PDB ID code 3VHE) (Supporting Information, Figure 4).[23] Finally, the 3-hydroxylmethyl-phenyl ring of **E** is deeply positioned in the hydrophobic portion of the back pocket, surrounded by Ile888, Lu889, Ile892, Val898, Leu1019, His1026, and Ile1044.

Tetracyclic compound **A** interacts with the hinge in a typical fashion satisfying the donor–acceptor–donor pharmacophore with its pyrazole and indole moieties. H-bonding to the hinge occurs at the backbone carbonyl and amide NH of Cys919 and the backbone carbonyl of Glu917 (PDB ID code 3VID). VEGFR2 cocomplex crystal structure of **A** overlaid with the cocomplex structure of **E** shows that the proximity of two fragments suggests linking them together to form a more potent inhibitor (Figure 3d). The two compounds sit within 1.4 Å of each other. The partial antagonism observed is consistent with such close proximity. In addition, because **E** is a nonurea-based compound, it was thought to be an appropriate fragment for a back-to-front design strategy. As hypothesized, **E** became an essential fragment that, prosecuted via SBDD, led to low nanomolar inhibitors with long residence times. These resulting inhibitors will be the subject of a future publication.

In this study, novel back pocket binding fragment **E** was discovered through our screening strategy (Scheme 1) by use of known hinge and back pocket-binding fragments. For campaigns against PTK outside of the VEGFR family, one should adopt a retro-analysis approach of known type II inhibitors against that kinase family with the goal of deconstruction to a back pocket fragment.

The back-to-front approach described in this communication has even broader utility. In the absence of a back pocket-binding fragment, execution of step one in our scheme yields those fragments/compounds selective for the unphosphorylated enzyme. Broadly, these compounds fall into one of two

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bins, the back pocket binders and the allosteric inhibitors, both of which are novel hits for a lead generation effort. A follow up assay of the hits in combination with **A** serves a rapid confirmation that these hits engage the kinase remote from the hinge binding region.

We chose to conduct full Yonetani−Theorell analysis as a follow up for the hits in step one, a necessity since this study was our proof of concept. When a greater number of hits are produced, one can gain throughput at the cost of some accuracy by screening the inhibitor pairs (**A** and fragment hit) at their respective IC_{50} values. The null hypothesis is that the compounds are mutually exclusive and the combination should produce 67% inhibition (eq 1, α = infinity). Combinations with **A** that produce greater than 67% inhibition are binned as interesting. If the hits are solubility limited such that an IC_{50} cannot be determined, eq 2[24] affords a practical approximation of the null hypothesis.

A FBDD methodology for the discovery of back pocket inhibitors has been elusive as a result of both the fluidity of the back pocket dynamics and the difficulty in executing an appropriate screen against the unactivated kinase. Paradoxically, weak binding of a fragment within this pocket will, when optimally included in a larger molecule engaging the hinge region, create a much more potent and selective inhibitor than could be achieved in its absence. This study reports the first robust, broadly applicable FBDD screening strategy capable of discovering kinase back pocket-binding fragments.

■ ASSOCIATED CONTENT

Supporting Information

Materials, characterization of all compounds, full experimental procedures, supporting figures, and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +81-466-32-2747. Fax: +81-466-29-4484. E-mail: Miki_Hiroshi@takeda.co.jp.

Notes

The authors declare no competing financial interest.

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(24) Assumes that the hinge binder is present at its IC_{50} and that the fragment was assayed alone under the same assay conditions (especially [ATP]) as in the combination assay. β is the single point estimated IC_{50} where V_0 is the control kinase activity and v is the kinase activity in the presence of the fragment.