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A Hot Spot for Protein Kinase Inhibitor Sensitivity

ATP binding site-directed protein kinase inhibitors are potent weapons in the war on cancer. However, specific mutations at an inhibitor-sensitivity "hot spot" can render these molecules ineffective. In this issue of *Chemistry & Biology*, Daub and coworkers have used an array of known kinase inhibitors to systematically characterize the desensitizing effects of hot spot mutations [1].

The ubiquity of the protein kinases in anticancer therapeutic research is difficult to overstate [2]. Protein kinase-catalyzed protein phosphorylation controls aspects of essentially every known eukaryotic signaling pathway, and aberrant regulation of kinase activity has been widely implicated in oncogenesis. It is no longer simply a truism that small molecule protein kinase inhibitors may be important anticancer agents—it is well-established truth. STI571 has received wide attention in both the scientific and mainstream press as the first "target-based" cancer therapeutic (Figure 1) [3]. This molecule now represents a critical mode of treatment for chronic myelogenous leukemia (CML). More recent developments have shown that kinase inhibitor-based cancer treatment extends beyond CML. In the last year, two different inhibitors of epidermal growth factor receptor (EGFR) kinase have been approved for clinical use in the U.S.: ZD1839 (Gefitinib) for nonsmall cell lung cancer and Cetuximab for colorectal cancer. In addition, roughly a dozen other members of the protein kinase superfamily are the putative drug targets in ongoing anticancer clinical trials [2].

The sobering fact buried in this good news is that protein kinases can acquire insensitivity to small molecule inhibitors through amino acid mutations in the kinase active site. This phenomenon has been most thoroughly characterized for STI571's target, BCR-Abl, a constitutively active protein kinase fusion protein that is thought to be the causative agent of CML [4]. STI571-resistant CML patients harbor a variety of mutations in the kinase active site-encoding region of the *BCR-ABL* oncogene. Prevalent among these is a single base mutation (C to T) that converts amino acid 315 from threonine to isoleucine, a change in the BCR-Abl ATP binding site that precludes molecular recognition of STI571.

Interestingly, the existence of a kinase inhibitor-sensitivity hot spot was known prior to the discovery of STI571 resistance in CML. Position 315 (c-Abl numbering) had been previously termed a "molecular gate" that controls access to a portion of the kinase active site that is not used by ATP (see Figure 2) [5]. Kinase/inhibitor cocrystal structures have revealed that many potent kinase inhibitors make use of this "extra" pocket, allowing them to compete with ATP for active site binding affinity [6]. The effects of the 315 side chain on the inhibitor sensitivity of several kinase families were studied before the direct medical relevance of the hot spot was elucidated. Young and coworkers [7] and Goedert and coworkers [8] showed that the sensitivity of mitogen-activated protein kinases to pyridinylimidazole inhibitors is highly dependent on the nature of the amino acid at position 315. In a similar vein, Shokat and coworkers reported that both tyrosine and serine/threonine kinases could be sensitized to the nominally Src-family-selective inhibitor PP1 (Figure 1) through the introduction of small side chains at position 315 [9]. These studies led to the generation of an "orthogonal" inhibitor approach, which can be used to specifically target protein kinases that have been rationally mutated at position 315 [10]. More recent studies have shown that BCR-Abl's sensitivity to PD180970 (a pyrido[2,3-*d*]pyrimidine) [11] and the PD153035-sensitivity (a 4-anilinoquinazoline) of EGFR are also highly dependent on the side chain at position 315 [12]. If any constant theme emerges from these studies, it is that smaller amino acid side chains at 315 generally render a kinase more susceptible to inhibition by compounds that are capable of exploiting the open "gate." While minor exceptions exist, this generalization holds remarkably true over multiple kinase families.

Daub and coworkers use various inhibitor scaffolds to develop a more complete picture of the relationship between the nature of the 315 side chain and the sensitivity profile of protein kinases [1]. The authors clearly demonstrate that the importance of the 315 position as a determinant for kinase inhibitor sensitivity extends further than had been previously recognized. They use as their starting point the "natural" C to T mutation that renders BCR-Abl insensitive to inhibition by STI571. The equivalent base mutation (which, of course, leads to different amino acid changes in different kinases) also has profound effects on the sensitivity of EGFR (T766M) and βPDGFR (T681I). Critically, the threonine to methionine mutation in EGFR desensitizes the kinase to inhibition by ZD1839. This result highlights the possibility that ZD1839-resistant kinases could emerge in a fashion that is exactly analogous to the emergence of STI571-resistant BCR-Abl. The authors also show that mutation of the corresponding βPDGFR position to isoleucine desensitizes that receptor tyrosine kinase to AG1296, STI571, PP1, and PP58 (see Figures 1 and 2) and that similar hot spot methionine mutations in c-Src and FGFR also desensitize those kinases to PP58. Moreover, the authors show that the indolinone class of molecules (exemplified by SU6656) represents an exception to the general "big is bad" rule, i.e., indolinones can inhibit kinases that possess large side chains at position 315. The structural basis for the indolinone exception is clear: these molecules do not extend into the extra binding pocket that is exploited by the other inhibitor classes; they do not attempt to get past the "gate" [13].

What to Do with All of These Mutant-Specific Inhibitor Data? Daub and coworkers use their inhibitor-sensitivity data to devise a clever chemical genetic method for

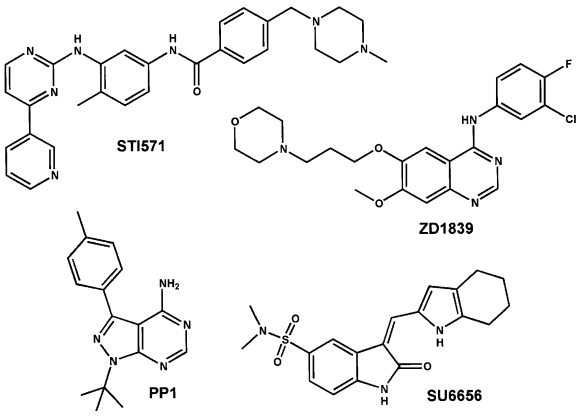

Figure 1. Chemical Structures of Some of the Kinase Inhibitors Discussed in This Article

The structure of PP58 is shown in Figure 2.

elucidating the functions of c-Src, a tyrosine kinase that is also a cancer drug target [14]. They show that the PP58 (a broad spectrum kinase inhibitor) can inhibit the c-Src-inhibitory kinase CSK. Thus, expression of PP58-desensitized c-Src in cells creates a system in which Src is specifically activated through inhibition of CSK. Since the PP58-desensitized c-Src is (presumably) the only cellular kinase that is activated by the addition of PP58, this system allows for c-Src-specific cell signaling events to be studied in isolation. This (rather counterintuitive) method of activating c-Src kinase through application of a general kinase *inhibitor* may prove to be a useful tool for the deconvolution of c-Src-mediated signaling cascades and for the identification of c-Src substrates.

What Else to Do with All of These Mutant-Specific Inhibitor Data? In theory, drugs that are specific for naturally occurring mutant oncogene products could be designed. However, the labor intensity of such an undertaking coupled with the small number of patients that express a given mutant kinase would most likely render this approach impractical. Nonetheless, emerging data suggest that mutant-specific kinase inhibition data may be very helpful in tailoring individual treatment programs for cancer patients. Paez et al. recently reported that a leucine to arginine mutation in the kinase active site confers increased ZD1839 sensitivity upon EGFR [15]. This mutation was discovered by sequencing the *EGFR* genes of lung cancer tumors. Importantly, presence of the mutation correlates with efficacy of treatment with ZD1839. Paez et al. suggest that similar genetic data may prove predictive for gauging a patient's prospects for effective lung cancer treatment with ZD1839. These results highlight the importance of gaining a molecular level understanding of the relationship between kinase active site mutations and inhibitor sensitivity. They also foretell a future when doctors and patients will know precisely which kinase inhibitors will successfully target which tumors, allowing futile treatments to be avoided—something to look forward to, indeed.

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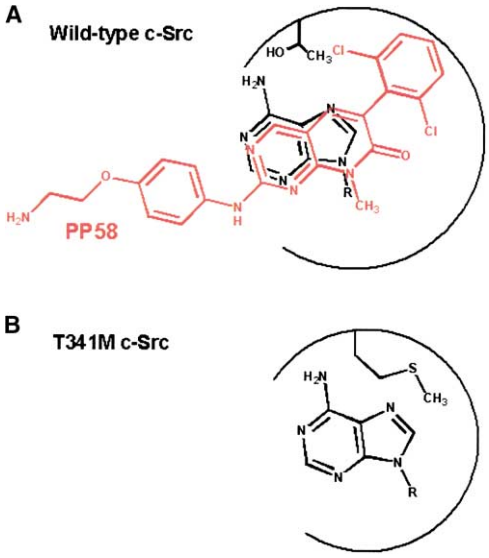

Figure 2. Schematic Picture of the "Gatekeeper" Side Chain in Protein Kinases

(A) Wild-type c-Src has a threonine side chain at the hot spot position (341). ATP (black) does not occupy the "extra" binding site that is filled by PP58 (red) and many other kinase inhibitors. "R" signifies the ribose triphosphate moiety of ATP.

(B) T341M c-Src can use ATP as a substrate, but PP58 binding is precluded by the smaller pocket.

Selected Reading

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Recombination of Fragmented Proteins

Directed evolution is a powerful method for generating novel molecules with desirable properties. In developing a new sensor to screen for protein-protein interactions, Tafelmeyer et al. [1] report a clever strategy to evolve heterodimeric "split proteins" from a monomer in this issue of *Chemistry & Biology*.

Two individually inactive protein fragments can sometimes recombine to form active heterodimers. Perhaps the most notable example of such a split-protein system is ribonuclease S (RNase S), a tightly associated heterodimer that is generated from two proteolytic fragments produced by the limited digestion of ribonuclease A by the protease subtilisin [2]. RNase S is a longstanding workhorse in both protein chemistry [3] and biotechnology [4]. In addition, a rationally designed split in ubiquitin was used to develop a sensor for screening protein-protein interactions [5] and has also inspired other sensors based on complementation of fragment pairs derived from a few other proteins. Muir et al. recently developed a split-protein application that harnesses a naturally heterodimeric intein to facilitate the in vivo ligation of an endogenously produced protein with an exogenously produced molecule [6]. Despite their diverse uses, the number of available split proteins appears quite limited.

The challenge of finding suitable fragment pairings may have thus far prevented more widespread application of split-protein systems. By performing activity selections on combinatorial libraries of fragment pairs, Tafelmeyer et al. [1] have surmounted this obstacle. In this way, they have identified a series of fragmentation sites within the $(\beta/\alpha)_8$-barrel enzyme N-(5′-phosphoribosyl) anthranilate isomerase, Trp1p, from *Saccharomyces cerevisiae* that can be used to make a sensor for protein-protein interactions (Figure 1). In essence, their strategy for library construction adapts an established method for generating random circular permutations of a given protein sequence. In this method, a circularized gene is randomly linearized to generate new termini [7]. Tafelmeyer et al. modified this procedure by afterwards inserting a DNA sequence containing a successive terminator and promoter between the sequence encoding the original N and C termini of Trp1p. By this scheme, a circular permutation generates a single break in the primary sequence and is followed by the unmasking of the original termini, effectively splitting the expressed protein in two. This process generates a library of plasmids that encode all possible fragment pairs (224 in all for Trp1p).

To isolate sensors of protein-protein interactions, the N- and C-terminal fragments of Trp1p were each produced as C-terminal and N-terminal fusions, respectively, to independently interacting polypeptides. The plasmid library was transformed into *trp1* yeast (which require an added source of Trp1p activity for survival in the absence of tryptophan) to select for functional split-Trp1p pairs. In this selection system, the only cells that grow are those in which the fragment pairs can reconstitute Trp1p activity.

The sequences identified by functional selection must be further screened to identify false positives (Figure 1). In this case, positives were subjected to a second round of selection after removing the fusion partner from the N-terminal fragment of Trp1p. Those split Trp1ps that either associate too tightly or possess one fragment large enough to possess activity by itself will complement the mutation independent of the interaction between the fusion partners and can thus be eliminated. Out of a library of approximately 1600 clones, Tafelmeyer et al. identified four Trp1p fragment pairs that survived the first selection but not the second. These four split Trp1ps thus function as sensors of protein-protein interactions [1].

Trp1p is a smart choice for this proof-of-principle experiment for generating tailored split proteins. In addition to the availability of a selection system for yeast cell growth, there is a wealth of knowledge about the structure and engineering of this $(\beta/\alpha)_8$-barrel scaffold. The existence of enzymatically active variants that have been either circularly permuted (in the homolog of Trp1p from *Escherichia coli*) [8] or rationally fragmented (in *S. cerevisiae* Trp1p) [9] augured well for the success of this approach.

For developing a split Trp1p that can act as a sensor for protein-protein interactions, the trick is to isolate fragments capable of only conditional heterodimerization. Heterodimers with a sufficiently high K_d in vivo require the interaction of fusion partners to raise the effective concentration of one fragment relative to the other. The rationally designed split Trp1p engineered by Eder and Kirschner [9] forms a complex between a large, well-structured N-terminal fragment and a small, poorly structured C-terminal fragment with a K_d of $\sim$0.2 μM and near-native catalytic efficiency. This observation may explain why most of the false positives identified in the search for sensors of protein-protein interactions were heterodimers that possessed relatively large N-terminal fragments [1]. The affinity and activity of Eder and