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Enhanced selectivity for inhibition of analog-sensitive protein kinases through scaffold optimization

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Abstract—The ability to inhibit any protein kinase of interest with a small molecule is enabled by a combination of genetics and chemistry. Genetics is used to modify the active site of a single kinase to render it distinct from all naturally occurring kinases. Next, organic synthesis is used to develop a small molecule, which does not bind to wild-type kinases but is a potent inhibitor of the engineered kinase. This approach, termed chemical genetics, has been used to generate highly potent mutant kinase-specific inhibitors based on a pyrazolopyrimidine scaffold. Here, we asked if the selectivity of the resulting pyrazolopyrimidines could be improved, as they inhibit several wild-type kinases with low-micromolar IC_{50} values. Our approach to improve the selectivity of allele-specific inhibitors was to explore a second kinase inhibitor scaffold. A series of 6,9-disubstituted purines was designed, synthesized, and evaluated for inhibitory activity against several kinases in vitro and in vivo. Several purines proved to be potent inhibitors against the analog-sensitive kinases and exhibited greater selectivity than the existing pyrazolopyrimidines.

1. Introduction

Potent and selective inhibitors of protein kinases are valuable tools for probing the cellular functions of kinases.[1,2] However, due to the large number of protein kinases in a cell and their highly homologous active sites, it has proven difficult to find specific inhibitors for individual kinases.[2,3] Our laboratory has developed a chemical approach, which employs genetics to circumvent the specificity problem associated with conventional small-molecule inhibitors of protein kinases.[4,5] The approach exploits a conserved, large hydrophobic residue in the kinase active site (termed the gatekeeper),[6] which makes direct contact with the N^6 amino group of ATP. When this residue is mutated from the naturally occurring bulky residue (methionine, leucine, phenylalanine, threonine, etc.) to glycine or alanine, a novel pocket not found in any *w*ild-*t*ype (*WT*) kinase is created within the kinase of interest. Such engineered kinases, termed *a*nalog-*s*ensitive (*as*) alleles, can thus be potently targeted by inhibitor analogs that contain substituents which occupy this enlarged ATP binding pocket and are occluded from binding to *WT* kinases because they lack the additional pocket.

The utility of an inhibitor, which specifically targets a non-naturally occurring protein kinase is only realized when the *WT* kinase can be replaced with the engineered form in cells or animals. Advances in genetics allow for precise introduction of a single mutation in many single-cell eukaryotes such as the budding yeast with ease. Consequently, the chemical genetic approach has been extensively applied to the study of various yeast kinases enabling selective pharmacological blockage of these kinases for the first time.[7] Analogous genetic manipulation in higher eukaryotes is technically much more challenging and thus represents a significant barrier to the use of chemical genetics to study mammalian protein kinases. Despite the technical barrier, genetically engineered mouse models have been created, which carry an *as* allele in place of the *WT* form for a number of protein kinases allowing for the first in vivo assessment of the effects of a mono-specific protein kinase inhibitor.[8,9] While requiring the extra effort of genetic manipulation compared to traditional pharmacology, chemical genetics allows for a critical control experiment, which addresses the target selectivity of a pharmacological agent that is frequently a major question facing protein kinase inhibitors. An isogeneic *WT* control animal or cell can be treated with the *as* specific kinase inhibitor in parallel to the cell or animal which carries the mutant *as* kinase, thus providing an assessment of any 'off-target' effects of the allele-specific inhibitor.

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We initially designed and synthesized a small panel of analogs based on the pyrazolopyrimidine scaffold [exemplified by PP1 (Fig. 1A),[10] a nonselective kinase inhibitor], and showed that some PP1 analogs potently inhibit the engineered kinases.[4,5] However, PP1 analogs still affect certain *WT* kinases such as v-Src and Fyn. For example, 1NA-PP1, one of the most effective allele-specific inhibitors discovered so far, inhibits *WT* v-Src with an IC_{50} of 1 μM (Table 1), suggesting that it could inhibit kinases like v-Src in the cell when used at mid-micromolar or higher concentrations. This has restricted the use of PP1-derived inhibitors to relatively low concentrations. To overcome this limitation, we sought to develop allele-specific kinase inhibitors with improved selectivity over the existing PP1 analogs.

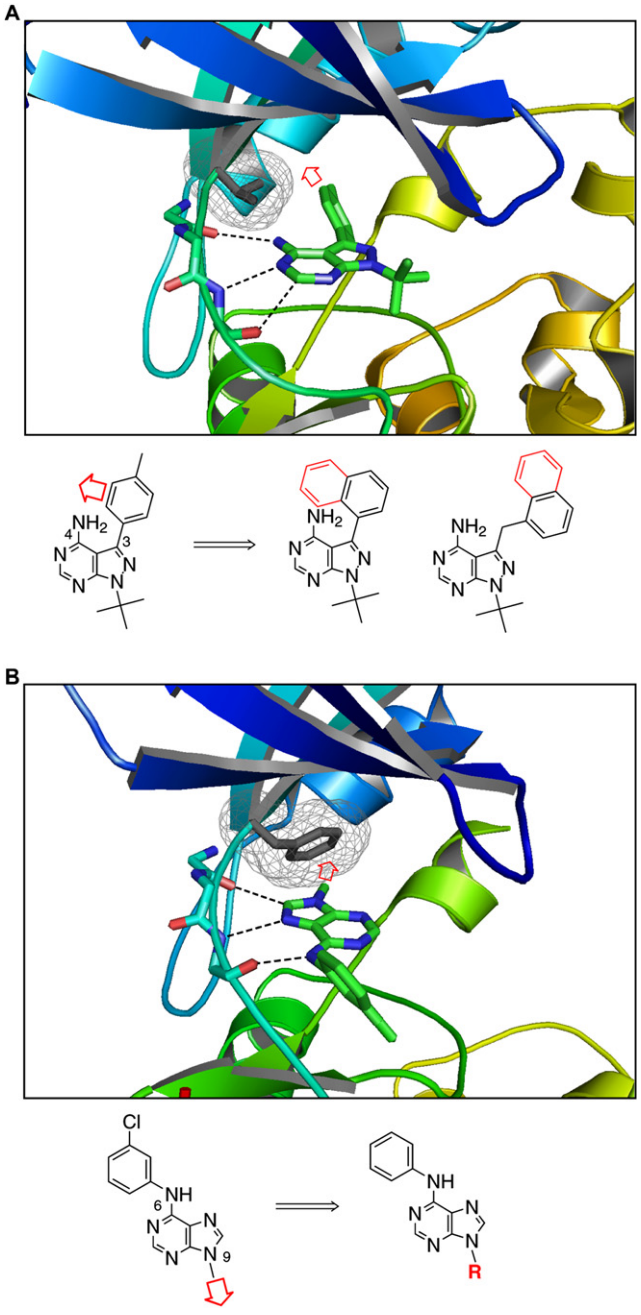

Figure 1. (A) Crystal structure of Hck–PP1 complex at the top and the structures of PP1, 1NM-PP1, and 1NA-PP1 at the bottom. The gatekeeper residue (Thr338) in Hck is highlighted gray with meshed surface and the H-bonds between PP1 and the kinase hinge region are shown in dashed line. The red arrow indicates the point and direction of derivatization on PP1 to generate allele-specific kinase inhibitors. (B) Crystal structure of CDK2–PVA (a purvalanol analog) complex at the top and the structures of PVA and its N9-modified derivatives at the bottom. The gatekeeper residue (Phe80) in CDK2 is highlighted gray with meshed surface and the hydrogen bonds between PVB and the kinase hinge region are shown in dashed line. The red arrow indicates the point and direction of derivatization on PVA to generate allele-specific inhibitors for protein kinases.

In principle, the challenge of improving allele specificity can be met by either increasing the inhibitor potency toward *as* kinases or decreasing the potency toward *WT* kinases. The crystal structure of Hck–PP1 complex shows that the pyrazolopyrimidine ring of PP1 occupies the base-binding pocket within the kinase active site in a nearly identical manner to the adenine ring of ATP while the *p*-tolyl group at the 3-position projects from the pyrazolopyrimidine core into a deep hydrophobic pocket in the kinase active site (Fig. 1A).[11] The gatekeeper residue in Hck, T338, forms one side of this hydrophobic pocket and directly stacks on one face of the tolyl ring. The crystal structure reveals that the N^4 amino and the 3-tolyl groups of PP1 are in direct contact of T338. As one of its two N–H bonds points directly at T338, the N^4 position was first chosen for derivatization to generate allele-specific inhibitors. However, when N^4-substituted pyrazolopyrimidines were synthesized, it was found that due to a steric clash with the 3-tolyl group the N^4 substituent is forced into the cis conformation at the C4–N bond, which disrupted a key H-bond between PP1 and the kinase hinge region (Fig. 1A).[12] Subsequently, 3-position expanded analogs of PP1 were created to introduce a steric clash with the gatekeeper residue.[4] This effort succeeded in providing two pyrazolopyrimidine-based allele-specific kinase inhibitors, 1NA-PP1 and 1NM-PP1, which have been featured in the studies of various kinases. However, the extra moiety in these PP1 derivatives such as 1NA-PP1 is not oriented directly toward the gatekeeper and thus could only form a partial steric clash with T338 (Fig. 1A). It is conceivable that *WT* Hck could accommodate 1NA-PP1 through mild conformational reorganization, which may explain why 1NA-PP1 retains the ability to inhibit unmodified Src family kinases at micromolar concentrations (Table 1). The pyrazolopyrimidine scaffold dictates the relative orientation of the 3-substituent to the gatekeeper residue and makes it difficult to design PP1 derivatives with a more direct clash with the gatekeeper residue than 1NA-PP1.

We reasoned that inhibitors based on a different scaffold could possess greater selectivity with respect to *as* kinases if the scaffold could provide a platform for positioning substituents directly toward the gatekeeper residue while still maintaining unperturbed interactions with the hinge region. After examining the various protein kinase inhibitors reported in the literature, we selected the purine scaffold (exemplified by purvalanol) because it presents a distinct binding mode from PP1 and has potential to be derivatized to induce a direct steric clash with the gatekeeper residue.[13] Structural studies reveal that despite their isosteric structures purine and pyrazolopyrimidine are situated within the kinase active site with completely different orientations (Fig. 1). As a result, the frequently observed hydrogen bonds between