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Acquisition of Inhibitor-Sensitive Protein Kinases through Protein Design

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ABSTRACT. Protein phosphorylation is the major post-translational modification used by eukaryotic cells to control cellular signaling. Protein kinases have emerged as attractive drug targets because heightened protein kinase activity has been associated with several proliferative diseases, most notably cancer and restenosis. Until now, it has been very difficult to confirm the utility of protein kinases as inhibitor targets because very few small molecules that selectively inhibit one particular kinase are known. Discovery of highly specific kinase inhibitors has been slow because the protein family contains approximately 2000 members, all of which share a conserved active site fold. Recent work in several laboratories has sought to circumvent the problem of kinase structural degeneracy by engineering drug sensitivity into Src family tyrosine kinases and mitogen-activated protein kinases through site-directed mutagenesis. By introducing a unique non-naturally occurring amino acid into a conserved region of the enzyme's binding site, a target protein kinase can be rapidly sensitized to a small molecule. Introduction of the engineered kinase into a cell line or animal model should greatly expedite the investigation of protein kinase inhibition as a viable drug treatment. The purpose of this review is to summarize these recent advances in protein kinase drug sensitization. PHARMACOL. THER. 82(2–3):337–346, 1999. © 1999 Elsevier Science Inc. All rights reserved.

KEY WORDS. Protein kinase inhibitors, Src family tyrosine kinases, mitogen-activated protein kinases (MAPKs), protein engineering, PPI, pyridinylimidazoles.

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ABBREVIATIONS. EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; PKC, protein kinase C; SAPK, stress-activated protein kinase.

1. INTRODUCTION

Protein phosphorylation is used by all cells as an essential control element of many different cell signaling events (Shokat, 1995). The body of cellular processes regulated by protein phosphorylation is impossible to list. In fact, there may be no cellular signal transduction pathway that is not phospho-regulated in one way or another. Even higher order processes, such as sensory perception and memory, can be analyzed at the level of individual protein phosphorylation events (Philpot *et al.*, 1997).

The first discovery of the role of protein phosphorylation was made in the context of phosphorylase regulation by cy-

elic AMP in glucose metabolism. Since these seminal studies, hundreds of protein kinases have been discovered using a variety of strategies. The sheer number of protein kinases (roughly 2% of the proteins in the human genome) has made their study a challenge to contemporary biochemical and genetic methods (Hunter, 1987). The interconnectivity of many cell signaling pathways complicates the problem further.

Our laboratory seeks to develop new chemical approaches aimed at deciphering the precise components of the interconnected web of kinase-mediated signal transduction. In recent years, the term “chemical genetics” has been coined to encapsulate the idea that small molecule ligands that are specific for a given gene product would be invaluable tools in dissecting complex biochemical networks

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(Mitchison, 1994). Traditionally, medicinal chemists have pursued these same small molecule probes (as drugs) by generating chemical diversity (synthesizing many compounds), which are focused on a particular protein target, one target at a time. The stunning size and conserved active site fold of the protein kinase super-family presents two serious problems for this approach. First, the simple generation of a small molecule inhibitor that is unique for a single family member out of a group of highly homologous proteins that may number as many as 2000 is a daunting chemical problem. When this problem is multiplied by the presence of 2000 target protein kinases, the goal of the chemical genetics vision appears unreachable. In addition, even when it appears that a uniquely specific inhibitor of a given protein kinase has been discovered, there is currently no assay that can confirm the selectivity across the breadth of the kinase super-family. Taken to its extreme, this problem points out the need for up to 2000 *in vitro* assays to unambiguously confirm the selectivity of each putative specific kinase inhibitor. Despite the size of the problem, traditional inhibitor screens have produced a number of highly selective kinase inhibitors. The bulk of the success has come in the identification of inhibitors for the receptor tyrosine kinases, particularly the epidermal growth factor receptor (EGFR) (Fry *et al.*, 1994; Traxler *et al.*, 1997b) and the platelet-derived growth factor receptor (Buchdunger *et al.*, 1995). This work has been reviewed previously (Showalter and Kraker, 1997).

Recently, our laboratory has described a novel combination of synthetic chemistry and genetic manipulation that approaches the chemical genetics problem of protein kinase inhibitor design from an angle opposite that of traditional medicinal chemistry (Bishop *et al.*, 1998). The crux of this strategy is to overcome the problem of genetic redundancy in Src family tyrosine kinase active sites by introducing structural diversity in the target kinase through site-directed mutagenesis. The design of small molecules that specifically bind and inhibit the engineered target is thus greatly facilitated. In addition, recent work in multiple laboratories has shown that point mutations in the ATP-binding sites of multiple mitogen-activated protein kinase (MAPK) family members are sufficient to confer sensitivity to pyridinylimidazole inhibitors, molecules that previously were known to inhibit p38 MAPK selectively (Wilson *et al.*, 1997; Evers *et al.*, 1998; Gum *et al.*, 1998). The purpose of this review is to summarize these recent advances in the use of combined chemical and genetic approaches for protein kinase inhibitor design, as well as the utility of these advances in studying the cellular roles of individual protein kinases.

2. CHEMICAL GENETIC DESIGN OF Src FAMILY TYROSINE KINASE INHIBITORS

2.1. Inhibitors of Wild-Type Src Family Tyrosine Kinases

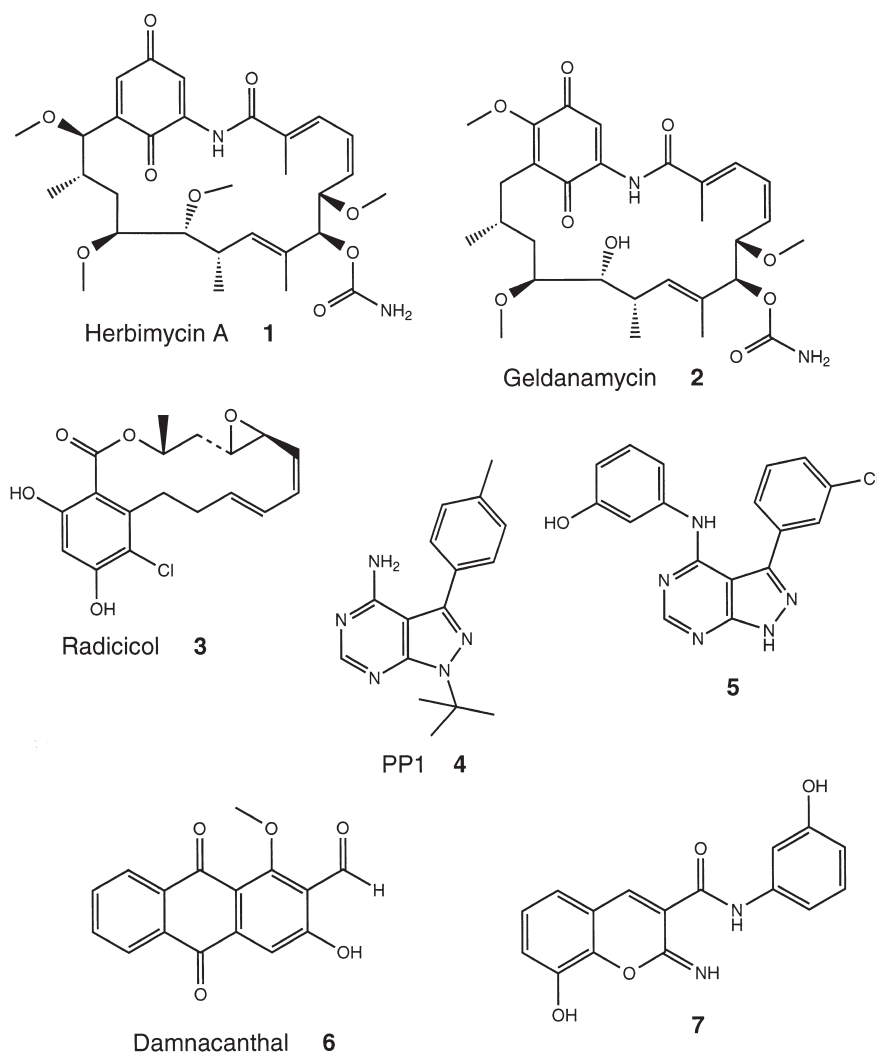
Members of the Src family of protein tyrosine kinases are vital cytosolic components of the signal transduction ma-

chinery in cellular processes as diverse as lymphocyte activation, cell adhesion, and oncogenesis (Bolen *et al.*, 1992). The prototypical Src family members, *v*-Src and *c*-Src, have been widely studied as inhibitor targets (Levitzki, 1996). Because of its putative role in human cancer, *c*-Src may prove to be a potential drug target. Elevated *c*-Src activity is found in tissue samples taken from human breast, lung, and colon tumors (Levitzki, 1996). In addition, the inhibition of Lck has been investigated extensively because of its importance in the early steps of T-cell receptor-mediated immune response (Li *et al.*, 1991; Faltynek *et al.*, 1995; Hanke *et al.*, 1996). Very few data are available concerning *in vitro* or cellular inhibition of the remaining known Src family kinases (Fyn, Lyn, Hck, Yes, Fgr, Blk, Yrk).

While the field of Src family kinase inhibitors has been reviewed previously (Levitzki, 1996; Showalter and Kraker, 1997), it will be instructive for this review of new inhibitor design approaches to mention a few of the most potent and selective Src family blockers identified to date (see Fig. 1 for structures). Historically, the benzoquinone ansamycins, herbimycin A (1) and geldanamycin (2), as well as another macrocycle, radicicol (3), have received much attention due to their ability to reverse the transformed morphology of fibroblasts that express activated forms of the Src tyrosine kinase (Uehara *et al.*, 1986; Murakami *et al.*, 1988; Kwon *et al.*, 1992). While these natural products do inhibit Src *in vitro*, little has been reported concerning their selectivity between different tyrosine kinases. In addition, the reversion of transformed phenotype due to ansamycin or radicicol treatment recently has been shown to proceed through a mechanism independent of kinase inhibition. From the emerging data, it appears that these molecules bind to heat-shock protein-90 in transformed cell lines and subsequently target the heat-shock protein-90-associated oncogenic tyrosine kinases for proteasomic degradation (Whitesell *et al.*, 1992, 1994; Sepp-Lorenzino *et al.*, 1995; Stebbins *et al.*, 1997; Schulte *et al.*, 1998; Sharma *et al.*, 1998).

Several molecules have appeared in the literature that potentially inhibit Src family kinases, but are not able to discriminate between the Src members (or no Src family selectivity is reported). Most notably, Hanke *et al.* (1996) reported that the pyrazolo[3,4-*d*]pyrimidine PP1 (4) inhibits both Fyn and Lck at an IC_{50} of ~ 5 nM. While this molecule is selective with respect to the non-Src family kinases (EGFR, ZAP-70, JAK-2, protein kinase A [PKA]), it is only 4-fold selective with respect to Hck and 30-fold with respect to *c*-Src. In addition, work in our laboratory has shown that PP1 also effectively inhibits the non-Src family member tyrosine kinase, *c*-Abl ($IC_{50} \approx 30$ nM) (Y. Liu, A. C. Bishop and K. M. Shokat, unpublished results). In an attempt to discover more potent and selective EGFR blockers, Traxler *et al.* (1997a) have discovered the most potent *c*-Src inhibitor published to date (5, IC_{50} 2 nM). This molecule shows moderate to strong selectivity with respect to EGFR, *v*-Abl, protein kinase C (PKC) α , and cyclin-dependent kinase-1, but no data for Src family members other than *c*-Src are reported.

FIGURE 1. Inhibitors of wild-type Src family tyrosine kinases (see text for references).



Through traditional inhibitor screens, no molecules that demonstrate high selectivity between different Src family kinases have been identified. The most effective to date are the natural product damnacanthal (**6**) (Faltynek *et al.*, 1995) and a 3-(*N*-phenyl)carbamoyl-2-iminochromene (**7**) synthesized by Huang *et al.* (1995). Damnacanthal (IC_{50} for Lck, 17 nM) demonstrated 7- and 20-fold selectivity for Lck over Src and Fyn, respectively. The most selective iminochromene strongly inhibited *c*-Src (IC_{50} 120 nM), with approximately 18-fold selectivity with respect to Lck, <285-fold selectivity over Lyn, and 570-fold selectivity over Fyn. Unfortunately, the authors of this report did not describe any *in vitro* inhibition of non-Src family kinases or demonstrate any cellular kinase inhibition.

2.2. Engineering a Unique Pocket in Src Family Tyrosine Kinases

It has been shown in several systems that protein engineering can confer unique small molecule specificity on a rationally designed protein (Clackson, 1998). A recent series of papers from our laboratory has shown that Src family kinases can be re-engineered in a systematic fashion to con-

tain a unique pocket in the ATP-binding site (Shah *et al.*, 1997; Bishop *et al.*, 1998; Liu *et al.*, 1998a,b).

By examining protein kinase crystal structures, Shah *et al.* (1997) recognized that there is a structurally conserved amino acid residue at the base of the ATP binding pocket that is in close contact with the exocyclic amine (N^6) of ATP's adenine ring (Ile338 in *v*-Src, from this point on referred to as position 338) (DeBondt *et al.*, 1993; Zheng *et al.*, 1993). Analysis of protein kinase sequence alignments confirmed that residue 338 contains a bulky side chain (usually Thr, Ile, Leu, Met, or Phe) in all known eukaryotic protein kinases (Hanks and Quinn, 1991). Thus, it was inferred that mutation of residue 338 to a smaller amino acid (e.g., glycine) would create a novel binding pocket that could uniquely bind small molecules that would be excluded by every wild-type kinase in the genome. Initial work showed that residue 338 could be mutated to glycine without altering the phosphoacceptor specificity (Shah *et al.*, 1997) or biological function of the *v*-Src kinase (K. Shah and K. M. Shokat, unpublished results). The space-creating mutation caused only a modest drop in k_{cat} and a modest increase in the K_m for ATP. It was also shown that the mutation strategy could be readily extended to the Src

family kinase Fyn, implying that the mutation of position 338 to glycine could be used as a general tool for systematically expanding the binding pocket of a given target protein kinase (Liu *et al.*, 1998a).

Subsequent modeling studies by Liu *et al.* (1998b) predicted that the 338 position of protein kinases acts as a "molecular gate" to a natural binding pocket surrounding N⁷ of ATP, a pocket that is not utilized in ATP binding. Thus, removal of the gate (mutation of 338 to a small amino acid) would be expected to give access to a significantly larger unique binding pocket than would be expected from most single-point mutations. All of these data suggested that genetic introduction of a single-point mutation should confer unique drug sensitivity to the engineered Src family kinase, if a suitable inhibitor could be designed and synthesized.

2.3. Design and Synthesis of Inhibitors Targeted to the Engineered Kinase

To identify a cell-permeable small molecule that uniquely inhibits Ile338Gly v-Src, Bishop and colleagues tethered bulky chemical groups to the previously described Src family inhibitor PP1 (Hanke *et al.*, 1996; Bishop *et al.*, 1998). Through modeling of PP1 in the active site of the Src family kinase Hck, it was predicted that PP1 would bind in an orientation analogous to that of ATP, therefore presenting its exocyclic amine (N⁴) to the space surrounding residue 338 (Sicheri *et al.*, 1997). This amine was used as a chemical hook to which they could attach hydrophobic moieties that would prevent binding of the derivatized molecules to wild-type protein kinases. The same hook would concurrently generate novel van der Waals interactions with the engineered v-Src. From a panel of 10 PP1 analogues, the prediction of binding orientation was confirmed by the finding that all of the molecules were preferentially bound by the engineered v-Src with respect to the wild type (see Fig. 2). The analogue that was over 20-fold more potent for Ile338Gly [4-(*p*-*tert*-butyl)benzamido-1-*tert*-butyl-3-phenylpyrazolo[3,4-*d*]pyrimidine, IC₅₀ 430 nM, 17] than any other in the panel contains a surprising large 11-carbon substituent, providing further support for the validity of the 338 "molecular gate" hypothesis. The selectivity of 17 was confirmed against wild-type v-Src, Fyn, Abl, PKA, and PKCδ, all of which were inhibited at least 700-fold less effectively than the target kinase. Bishop *et al.* (1998) also showed that the equivalent mutation in Fyn also made it sensitive to inhibition by 17 (IC₅₀ 830 nM), showing that a chemical genetic approach to protein/small molecule recognition can rapidly circumvent the selectivity problem inherent in the structural degeneracy of homologous protein families.

To identify more potent inhibitors of the engineered Src family kinases, we have subsequently generated an expanded panel of PP1 analogues, consisting of over 25 molecules with increased chemical diversity at the N⁴ and N(1) positions of the pyrazolo[3,4-*d*] pyrimidine ring. Screening of these putative inhibitors against Ile338Gly v-Src yielded

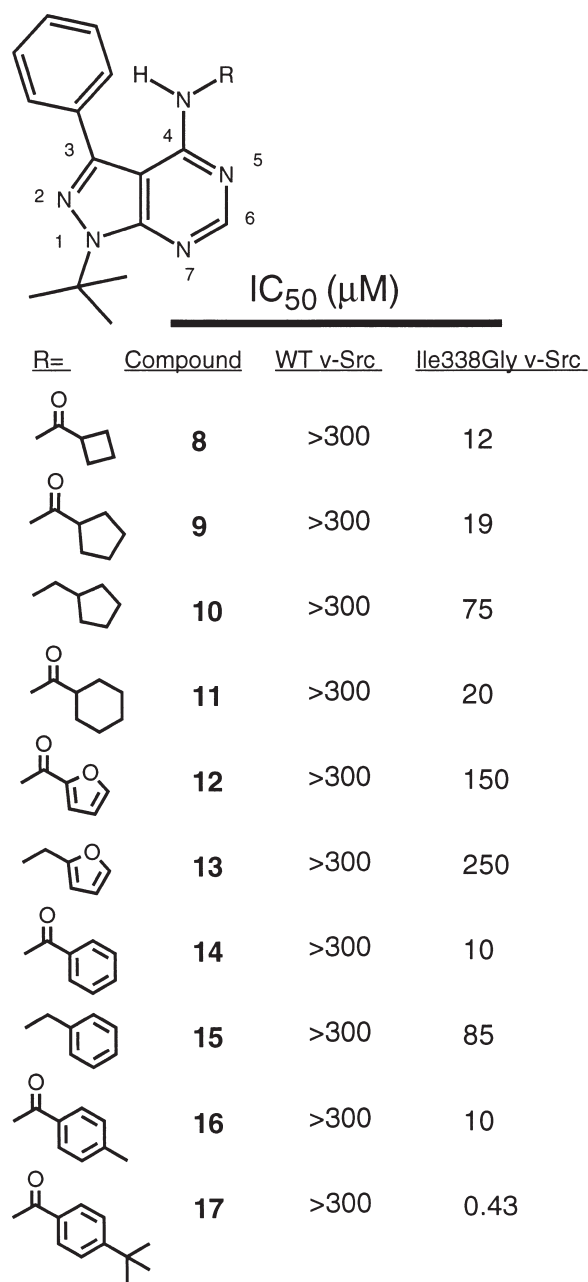


FIGURE 2. Selective inhibition of engineered v-Src by N⁴ derivatized PP1 analogues. Modified from Bishop *et al.* (1998).

no potent inhibitors. All of the molecules in Fig. 3 were found to have an IC₅₀ for the target kinase of greater than 10 μM (A. C. Bishop and K. M. Shokat, unpublished results). Examination of the cumulative data from both inhibitor panels strongly suggests that any alkyl substitution at N⁴ greatly reduces binding of the PP1 analogues for both wild-type and engineered Src kinases. However, acyl substitutions at N⁴ are very well tolerated. The basis for this dichotomy is not well understood and currently is under investigation.

All compounds: IC₅₀ (Ile338Gly v-Src) >10 μM

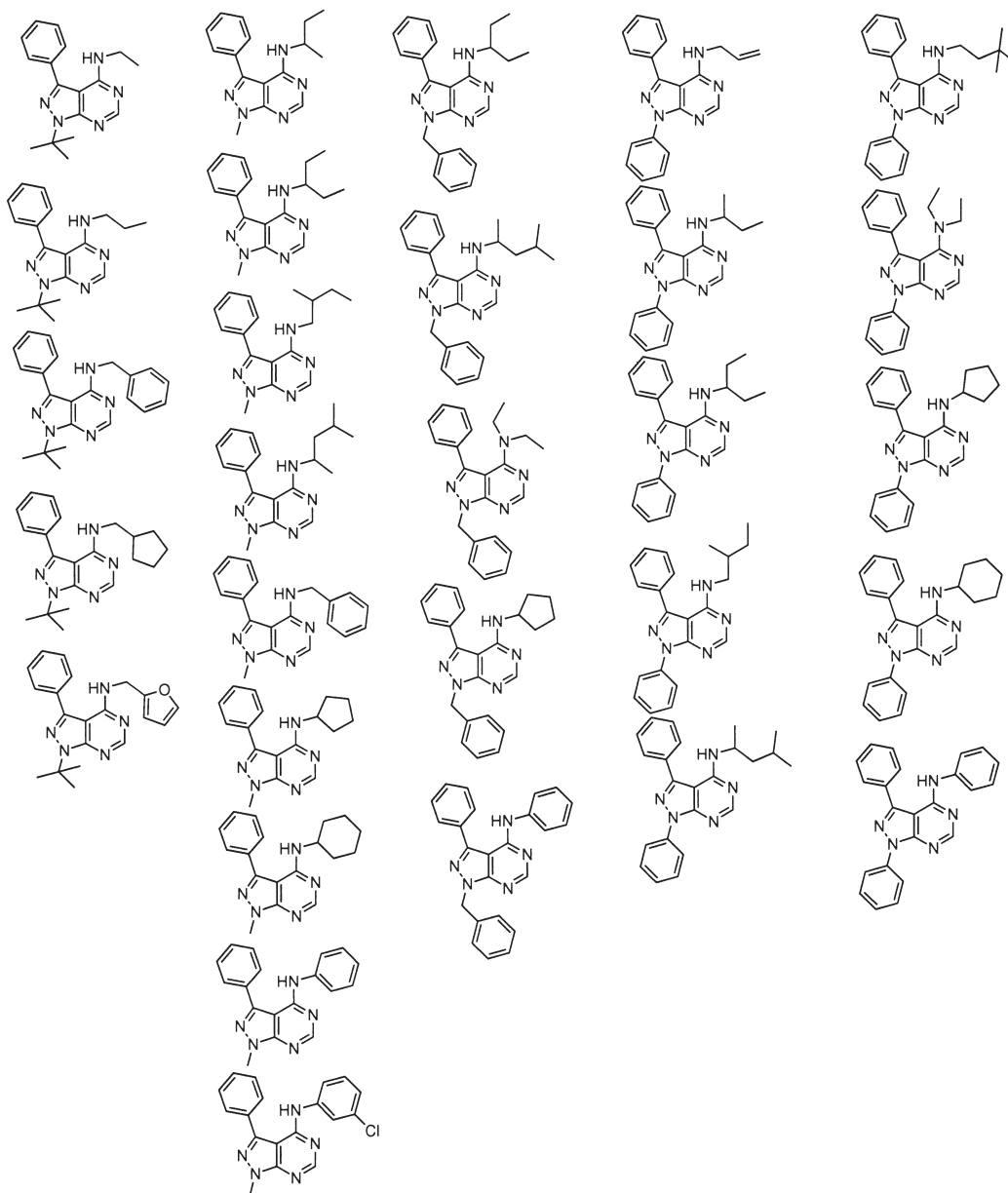


FIGURE 3. Second-generation panel of N(1)- and N⁴-diversified PP1 analogues.

2.4. Unique Cellular Target Inhibition through Chemical Genetic Design

Identification of a selective enzyme inhibitor through chemical genetic design is only useful if the inhibitor can be used in a relevant cellular context to probe the target's function inside the cell. To demonstrate cellular inhibition of the target kinase by **17**, Bishop *et al.* (1998) treated NIH 3T3 fibroblasts, which expressed either wild-type or Ile338Gly v-Src, with the mutant-specific inhibitor. The authors found that at 100 μM, **17** had no effect on the phosphotyrosine level of cells expressing wild-type v-Src, whereas the

phosphotyrosine level of cells that expressed the target kinase was moderately reduced. More strikingly, under prolonged drug treatment, it was found that the Ile338Gly expressing cells selectively reverted to the flattened morphology of non-transformed fibroblasts. This phenotype was confirmed by staining the actin stress fibers that are characteristic of normal fibroblasts (see Fig. 4). No change in the morphology or actin organization was observed for the wild-type v-Src-expressing cells. Thus, it was demonstrated that a small molecule inhibitor, which is uniquely selective for a tyrosine kinase oncogene product, can revert

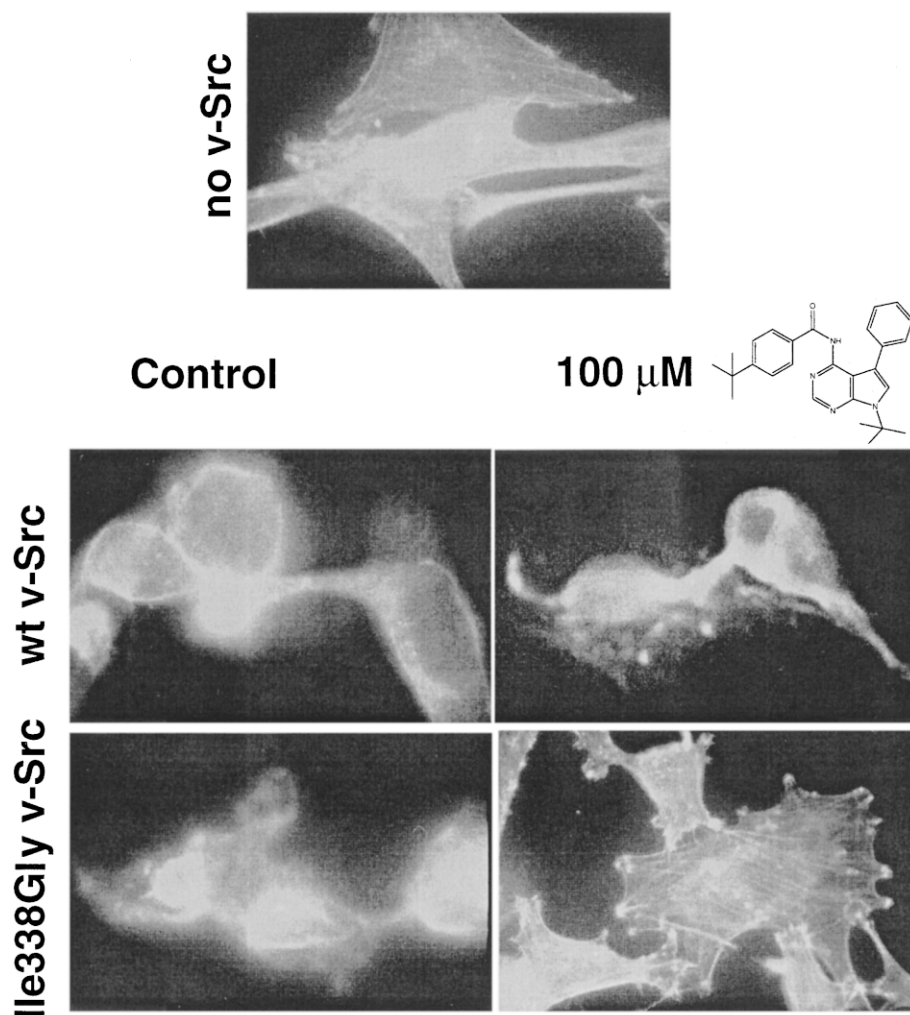


FIGURE 4. Selective reversion of transformed morphology in fibroblasts expressing Ile338Gly v-Src. Cells were stained with phalloidin-FITC and visualized by fluorescence microscopy. Modified from Bishop *et al.* (1998).

the morphological changes associated with cellular transformation. Only through chemical genetic design coupled with the built-in control of the wild-type target homologue could such a determination of cellular specificity be made.

3. GENETIC MANIPULATION OF MITOGEN-ACTIVATED PROTEIN KINASE HOMOLOGUES TO GENERATE NOVEL INHIBITOR SENSITIVITY

3.1. Pyridinylimidazoles

Lee *et al.* (1994) reported a remarkable finding. In one of the more striking demonstrations of the power of chemical genetics, the authors used a radiolabeled 2,4-diaryl-5-pyridin-4-yl-imidazole (SB 206718, **18**; see Fig. 5) to directly photoaffinity label this small molecule's direct cellular targets in the human cell line THP.1. It was known that this class of pyridinylimidazoles potently inhibits the production of cytokines (interleukin-1 and tumor necrosis factor), which are associated with inflammatory response. Surprisingly, the authors were able to isolate a single radiolabeled protein target from the cellular milieu and subsequently clone two cDNAs that were identical to one another outside of a 75-nucleotide region. The cloned genes encode

MAPK homologues cytokine-suppressive anti-inflammatory drug-binding protein-1 and -2 (also named Stress-Activated Protein Kinase [SAPK] 2a or p38).

The stunning selectivity of the pyridinylimidazole family for p38 led to further investigation of the structure-activity relationships of these molecules (Boehm *et al.*, 1996; Gallagher *et al.*, 1997). It was found by Gallagher *et al.* that both the 4-phenyl and 5-pyrid-4-yl groups are required for maximum inhibition of p38. Halogens at the *meta* or *para* position of the 4-phenyl ring yield the most potent inhibitors of p38, with the most widely used pyridinylimidazole analogue containing a *para*-fluoro moiety (SB 203580, **19**). As can be seen from the structures in Fig. 5, potent pyridinylimidazole p38 inhibitors can contain substitutions at either C(2) or N(1). The IC_{50} (p38) for SB 203580 is 42 nM, but it does not inhibit the MAPK extracellular signal-regulated kinase (ERK)2 ($IC_{50} > 10 \mu\text{M}$) and does not inhibit PKA ($IC_{50} > 100 \mu\text{M}$) or PKC α ($IC_{50} > 100 \mu\text{M}$).

3.2. Structural Studies on p38/Pyridinylimidazole Complexes

Predictably, the high specificity of the pyridinylimidazole compounds led to a series of structural studies aimed at de-

3.3. Pyridinylimidazole Sensitivity through Site-Directed Mutagenesis

From their structural studies, along with analysis of the sequence alignments of the MAPK family, both Tong *et al.* (1997) and Wilson *et al.* (1997) proposed that Thr106 (equivalent to 338 of Src) may be an important residue for the determination of the observed specificity of pyridinylimidazole inhibitors for p38. The side chain of this residue helps make up the *para*-fluoro phenyl ring binding pocket. The sequence alignment of MAPKs shown in Fig. 6 shows that only p38 and p38 β 2 have threonine at position 106. These enzymes are uniquely sensitive to pyridinylimidazoles. All of the other MAPK homologues possess a larger side chain (Met in SAPK3, SAPK4, c-Jun N-terminal kinase [JNK]1, and JNK2 β or Gln in ERK1 and ERK2) at this position. To test the hypothesis that this inhibitor sensitivity was mediated by the side chain of residue 106, Wilson *et al.* (1997) mutated Thr106 of p38 to both methionine and alanine. As predicted, Thr106Met showed resistance to pyridinylimidazoles that was equivalent to SAPK3 ($IC_{50} \gg 10 \mu\text{M}$ for both VK-19911 and SB 202190), while Thr106Ala p38 retained its sensitivity to inhibition.

Two recent papers have expanded the scope of the inquiry into MAPK acquisition of SB 203580 sensitivity through active site mutations (Eyers *et al.*, 1998; Gum *et al.*, 1998). Eyers *et al.* carried out a systematic investigation of Thr106 mutations in five MAPKs. The authors found that mutation of the wild-type methionines of SAPK3 and SAPK4 to threonine conferred inhibition sensitivity that was directly comparable with that of wild-type SAPK2b ($IC_{50} \approx 300 \text{ nM}$), but still 10-fold lower than SAPK2a. Even smaller side chains (Ala, Gly) were then introduced in all four of these enzymes. For each of these SAPKs, the SB 203580 sensitivity for 106 mutations lies in the pattern Ala > Gly > Thr \gg Met, suggesting that the methyl side chain of alanine is the ideal size to pack with the *para*-fluoro-phenyl ring efficiently. Not surprisingly, mutation of the corresponding methionine of the less closely related JNK1 to threonine and alanine also increased its SB 203580 sensitivity, but to a substantially lower level than was demonstrated with SAPK3 and SAPK4. Through investigation of the primary sequences of these kinases, the authors realized that all of the SAPKs and JNKs contained a Leu residue at the position 2 residues N-terminal to Thr106 of p38, with the exception of JNK1 (Ile). Thus, it was demonstrated that while the single mutation of this isoleucine to leucine changed the inhibitor sensitivity of JNK1 only very slightly, the double mutant (Met106Ala, Ile104Leu) possessed SB 203580 sensitivity ($IC_{50} 30 \text{ nM}$) that was comparable with that of the SAPK3 alanine mutant.

A report from Gum *et al.* (1998) took a somewhat different approach to the problem. These authors mutated 21 residues in the ATP-binding site of p38, and assayed both the kinase activity and radiolabeled SB206718 crosslinking sensitivity of these mutants. It was discovered that kinase

activity can be decoupled from pyridinylimidazole binding capability, as two mutants were discovered that were dead enzymes, but were still efficiently photoaffinity labeled by SB 206718 (K152A, A157L). In a more directed mutagenesis strategy that strongly parallels the work by Eyers *et al.* (1998), the authors investigated the effect of exchanging not only the 106 position in the various SAPKs, but also triple mutations of residues 106–108. In these positions, p38 contains the amino acids T-H-L, SAPKs 3 and 4 contain M-P-F, and JNK1 contains M-E-L. When the 106–108 region of SAPK3 and SAPK4 were replaced with T-H-L, the resulting mutants each demonstrated SB 203580 sensitivity that was equivalent to each other and to wild-type p38 ($IC_{50} \sim 0.5\text{--}1 \mu\text{M}$). This represented an ~ 10 -fold gain over the single Met106Thr mutations of SAPK3 and SAPK4. (Note: due to different assays, the inhibition data from Eyers *et al.* and Gum *et al.* should not be compared directly.) The more distant relative JNK1 fit the paradigm. Mutations of the M-E motif of JNK1 to T-H of p38 led to SB 203580 sensitivity that was comparable with that of wild-type p38 and roughly an order of magnitude greater than the single mutant.

4. CONCLUSIONS AND PERSPECTIVES

In a span of a little over a year, two functionally important and structurally distinct classes of kinases, the Src family tyrosine kinases and the MAP serine/threonine kinases, have been shown to be amenable to a systematic and generalizable drug-sensitization strategy. Amazingly, analogous mutations of an equivalently positioned residue are sufficient in both families to confer potent small molecule sensitivity on the target kinase. This consistency in the mutagenesis data implies that the limiting factor in using engineered kinase/inhibitor systems in biological research will lie in the identification of the uniquely specific small molecules. This becomes particularly apparent when it is realized that in the MAPK sensitization studies, all of the experiments started with an incredibly specific small molecule, and through protein design, it was made less specific. By contrast, the Src family studies started with a weakly selective inhibitor and combined chemical and protein design to generate a unique kinase/inhibitor pair that was functional in a cellular context.

These first steps in the identification and characterization of “chemical sensitive mutants” of protein kinases have demonstrated that the field holds great promise. Since transfected cell lines, and even “knock-in” mice, can now be generated rapidly, this ability to genetically “program” the kinase of interest for unique inhibition by a small molecule should greatly expedite the process of testing the effects of selective inhibition of a given kinase in a whole cell or in an animal model. Furthermore, the unique pairing of a designed target/small molecule pair will allow for the unambiguous assignment of the activity of a specific kinase to an induced phenotype.

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References

- Bishop, A. C., Shah, K., Liu, Y., Witucki, L., Kung, C. and Shokat, K. M. (1998) Design of allele-specific inhibitors to probe protein kinase signaling. *Curr. Biol.* 8: 257–266.
- Boehm, J. C., Smietana, J. M., Sorenson, M. E., Garigipati, R. S., Gallagher, T. F., Sheldrake, P. L., Bradbeer, J., Badger, A. M., Laydon, J. T., Lee, J. C., Hilleagass, L. M., Griswold, D. E., Breton, J. J., Chabot-Fletcher, M. C. and Adams, J. L. (1996) 1-Substituted 4-aryl-5-pyridinylimidazoles: a new class of cytokine suppressive drugs with low 5-lipoxygenase and cyclooxygenase inhibitory potency. *J. Med. Chem.* 39: 3929–3937.
- Bolen, J. B., Rowley, R. B., Spana, C. and Tsygankov, A. Y. (1992) The Src family of tyrosine protein kinases in hemopoietic signal transduction. *FASEB J.* 6: 3403–3409.
- Buchdunger, E., Zimmermann, J., Mett, H., Meyer, T., Muller, M., Regenass, U. and Lydon, N. B. (1995) Selective inhibition of the platelet-derived growth factor signal transduction pathway by a protein-tyrosine kinase inhibitor of the 2-phenylaminopyrimidine class. *Proc. Natl. Acad. Sci. USA* 92: 2558–2562.
- Clackson, T. (1998) Redesigning small molecule-protein interfaces. *Curr. Opin. Struct. Biol.* 8: 451–458.
- DeBodt, H. L., Rosenblatt, J., Jancarik, J., Jones, H. D., Morgan, D. O. and Kim, S.-H. (1993) Crystal structure of cyclin-dependent kinase 2. *Nature* 363: 595–601.
- Eyers, P. A., Craxton, M., Morrice, N., Cohen, P. and Goedert, M. (1998) Conversion of SB 203580-insensitive MAP kinase family members to drug-sensitive forms by a single amino acid substitution. *Chem. Biol.* 5: 321–328.
- Faltynek, C. R., Schroeder, J., Mauvais, P., Miller, D., Wang, S., Murphy, D., Lehr, R., Kelley, M., Maycock, A., Michne, W., Miski, M. and Thunberg, A. L. (1995) Damnacanthal is a highly potent, selective inhibitor of p56lck tyrosine kinase activity. *Biochemistry* 34: 12404–12410.
- Fry, D. W., Kraker, A. J., McMichael, A., Ambroso, L. A., Nelson, J. M., Leopold, W. R., Connors, R. W. and Bridges, A. J. (1994) A specific inhibitor of the epidermal growth factor receptor tyrosine kinase. *Science* 265: 1093–1095.
- Gallagher, T. F., Seibel, G. L., Kassis, S., Laydon, J. T., Blumenthal, M. J., Lee, J. C., Lee, D., Boehm, J. C., Fier-Thompson, S. M., Abt, J. W., Sorenson, M. E., Smietana, J. M., Hall, R. F., Garigipati, R. S., Bender, P. E., Erhard, K. F., Krog, A. J., Hofmann, G. A., Sheldrake, P. L., McDonnell, P. C., Kumar, S., Young, P. R. and Adams, J. L. (1997) Regulation of stress-induced cytokine production by pyridinylimidazoles; inhibition of CSBP kinase. *Bioorg. Med. Chem.* 5: 49–64.
- Gum, R. J., McLaughlin, M. M., Kumar, S., Wang, Z., Bower, M. J., Lee, J. C., Adams, J. L., Livi, G. P., Goldsmith, E. J. and Young, P. R. (1998) Acquisition of sensitivity of stress-activated protein kinases to the p38 inhibitor, SB 203580, by alteration of one or more amino acids within the ATP binding pocket. *J. Biol. Chem.* 273: 15605–15610.
- Hanke, J. H., Gardner, J. P., Dow, R. L., Changelian, P. S., Brissette, W. H., Weringer, E. J., Pollok, B. A. and Connelly, P. A. (1996) Discovery of a novel, potent, and src family-selective tyrosine kinase inhibitor. *J. Biol. Chem.* 271: 695–701.
- Hanks, S. K. and Quinn, A. M. (1991) Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members. *Methods Enzymol.* 200: 38–62.
- Huang, C. K., Wu, F. Y. and Ai, Y. X. (1995) Polyhydroxylated 3-(N-phenyl) carbamoyl-2-iminochrome derivatives as potent inhibitors of tyrosine kinase p60^{c-src}. *Bioorg. Med. Chem. Lett.* 5: 2423–2428.
- Hunter, T. (1987) A thousand and one protein kinases. *Cell* 50: 823–829.
- Kwon, H. J., Yoshida, M., Fukui, Y., Horinouchi, S. and Beppu, T. (1992) Potent and specific inhibition of p60 v-src protein kinase both *in vivo* and *in vitro* by radicicol. *Cancer Res.* 52: 6926–6930.
- Lee, J. C., Laydon, J. T., McDonnell, P. C., Gallagher, T. F., Kumar, S., Green, D., McNulty, D., Blumenthal, M. J., Heys, J. R., Landvatter, S. W., Strickler, J. E., McLaughlin, M. M., Siemens, I. R., Fisher, S. M., Livi, G. P., White, J. R., Adams, J. L. and Young, P. R. (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* 372: 739–745.
- Levitzi, A. (1996) SRC as a target for anti-cancer drugs. *Anti-cancer Drug Des.* 11: 175–182.
- Li, Z. H., Burke, T. R. and Bolen, J. B. (1991) Analysis of styryl-based inhibitors of the lymphocyte tyrosine protein kinase p56^{lck}. *Biochem. Biophys. Res. Commun.* 180: 1048–1056.
- Liu, Y., Shah, K., Yang, F., Witucki, L. and Shokat, K. (1998a) Engineering Src family protein kinases with unnatural nucleotide specificity. *Chem. Biol.* 5: 91–101.
- Liu, Y., Shah, K., Yang, F., Witucki, L. and Shokat, K. (1998b) A molecular gate which controls unnatural ATP analogue recognition by the tyrosine kinase v-Src. *Bioorg. Med. Chem.* 6: 1219–1226.
- Mitchison, T. J. (1994) Towards a pharmacological genetics. *Chem. Biol.* 1: 3–6.
- Murakami, Y., Mizuno, S., Hori, M. and Uehara, Y. (1988) Reversal of transformed phenotypes by herbimycin A in src oncogene expressed rat fibroblasts. *Cancer Res.* 48: 1587–1590.
- Philpot, B. D., Lim, J. H., Halpain, S. and Brunjes, P. C. (1997) Experience-dependent modifications in MAP2 phosphorylation in rat olfactory bulb. *J. Neurosci.* 17: 9596–9604.
- Schulte, T. W., Akinaga, S., Soga, S., Sullivan, W., Stensgard, B., Toft, D. and Neckers, L. M. (1998) Antibiotic radicicol binds to the N-terminal domain of Hsp90 and shares important biologic activities with geldanamycin. *Cell Stress Chaperones* 3: 100–108.
- Sepp-Lorenzino, L., Ma, Z., Leibold, D. E., Vinitzky, A. and Rosen, N. (1995) Herbimycin A induces the 20 S proteasome- and ubiquitin-dependent degradation of receptor tyrosine kinases. *J. Biol. Chem.* 270: 16580–16587.
- Shah, K., Liu, Y., Deirmengian, C. and Shokat, K. M. (1997) Engineering unnatural nucleotide specificity for Rous sarcoma virus tyrosine kinase to uniquely label its direct substrates. *Proc. Natl. Acad. Sci. USA* 94: 3565–3570.
- Sharma, S. V., Agatsuma, T. and Nakano, H. (1998) Targeting of the protein chaperone, HSP90, by the transformation suppressing agent, radicicol. *Oncogene* 16: 2639–2645.
- Shokat, K. M. (1995) Tyrosine kinases: modular signaling enzymes with tunable specificities. *Chem. Biol.* 2: 509–514.
- Showalter, H. D. and Kraker, A. J. (1997) Small molecule inhibitors of the platelet-derived growth factor receptor, the fibroblast growth factor receptor, and Src family tyrosine kinases. *Pharmacol. Ther.* 76: 55–71.
- Sicheri, F., Moarefi, I. and Kuriyan, J. (1997) Crystal structure of the Src family tyrosine kinase Hck. *Nature* 385: 602–609.

- Stebbins, C. E., Russo, A. A., Schneider, C., Rosen, N., Hartl, F. U. and Pavletich, N. P. (1997) Crystal structure of an hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent. *Cell* 89: 239–250.
- Tong, L., Pav, S., White, D. M., Rogers, S., Crane, K. M., Cywin, C. L., Brown, M. L. and Pargellis, C. A. (1997) A highly specific inhibitor of human p38 MAP kinase binds in the ATP pocket. *Nature Struct. Biol.* 4: 311–316.
- Traxler, P., Bold, G., Frei, J., Lang, M., Lydon, N., Mett, H., Buchdunger, E., Meyer, T., Mueller, M. and Furet, P. (1997a) Use of a pharmacophore model for the design of EGF-R tyrosine kinase inhibitors: 4-(phenylamino)pyrazolo[3,4-*d*]pyrimidines. *J. Med. Chem.* 40: 3601–3616.
- Traxler, P., Furet, P., Mett, H., Buchdunger, E., Meyer, T. and Lydon, N. (1997b) Design and synthesis of novel tyrosine kinase inhibitors using a pharmacophore model of the ATP-binding site of the EGF-R. *J. Pharm. Belg.* 52: 88–96.
- Uehara, Y., Hori, M., Takeuchi, T. and Umezawa, H. (1986) Phenotypic change from transformed to normal induced by benzoquinonoid ansamycins accompanies inactivation of p60src in rat kidney cells infected with Rous sarcoma virus. *Mol. Cell. Biol.* 6: 2198–2206.
- Whitesell, L., Shifrin, S. D., Schwab, G. and Neckers, L. M. (1992) Benzoquinonoid ansamycins possess selective tumoricidal activity unrelated to src kinase inhibition. *Cancer Res.* 52: 1721–1728.
- Whitesell, L., Mimnaugh, E. G., De Costa, B., Myers, C. E. and Neckers, L. M. (1994) Inhibition of heat shock protein hsp90-pp60^{v-src} heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. *Proc. Natl. Acad. Sci. USA* 91: 8324–8328.
- Wilson, K. P., McCaffrey, P. G., Hsiao, K., Pazhanisamy, S., Galullo, V., Bemis, G. W., Fitzgibbon, M. J., Caron, P. R., Murcko, M. A. and Su, M. S. (1997) The structural basis for the specificity of pyridinylimidazole inhibitors of p38 MAP kinase. *Chem. Biol.* 4: 423–431.
- Young, P. R., McLaughlin, M. M., Kumar, S., Kassis, S., Doyle, M. L., McNulty, D., Gallagher, T. F., Fisher, S., McDonnell, P. C., Carr, S. A., Huddleston, M. J., Seibel, G., Porter, T. G., Livi, G. P., Adams, J. L. and Lee, J. C. (1997) Pyridinyl imidazole inhibitors of p38 mitogen-activated protein kinase bind in the ATP site. *J. Biol. Chem.* 272: 12116–12121.
- Zheng, J., Knighton, D. R., Ten Eyck, L. F., Karlsson, R., Zuong, N.-H., Taylor, S. S. and Sowadski, J. M. (1993) Crystal structure of the catalytic subunit of cAMP-dependent protein kinase complexed with MgATP and peptide inhibitor. *Biochemistry* 32: 2154–2161.