Chemistry & Biology, Vol. 11, 691–701, May, 2004, ©2004 Elsevier Science Ltd. All rights reserved. DOI 10.1016/j.chembiol.2004.02.029

Characterization of a Conserved Structural Determinant Controlling Protein Kinase Sensitivity to Selective Inhibitors

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Summary

Some protein kinases are known to acquire resistance to selective small molecule inhibitors upon mutation of a conserved threonine at the ATP binding site to a larger residue. Here, we performed a comprehensive mutational analysis of this structural element and determined the cellular sensitivities of several disease-relevant tyrosine kinases against various inhibitors. Mutant kinases possessing a larger side chain at the critical site showed resistance to most compounds tested, such as ZD1839, PP1, AG1296, STI571, and a pyrido[2,3-d]pyrimidine inhibitor. In contrast, indolinones affected both wild-type and mutant kinases with similar potencies. Resistant mutants were established for pharmacological analysis of βPDGF receptor-mediated signaling and allowed the generation of a drug-inducible system of cellular Src kinase activity. Our data establish a conserved structural determinant of protein kinase sensitivity relevant for both signal transduction research and drug development.

Introduction

Protein kinases control nearly all aspects of cellular signal transmission under both physiological and pathophysiological conditions [1]. In hyperproliferative diseases such as human cancer, deregulation of protein kinase activity often correlates with disease progression and poor prognosis. Therefore, various members of the protein kinase family of enzymes have moved into the focus of intense research efforts aiming at the development of target-selective drugs for anticancer therapy [2, 3]. Small molecule inhibitors of protein kinases are one major class of these molecularly targeted agents, and the first of them, STI571 (Gleevec, imatinib mesylate) and ZD1839 (Iressa, gefitinib), have already entered the market [4, 5]. STI571 is a potent inhibitor of the tyrosine kinases Abl, c-kit, and platelet-derived growth factor receptor (PDGFR) [6]. In normal cells, Abl kinase activity is tightly regulated through multiple control mechanisms, which are lost in chronic myeloid leukaemia (CML). In this malignancy, the Philadelphia chromosomal translocation leads to the creation of the BCR-ABL gene, which encodes the constitutively active Bcr-Abl fusion protein. Importantly, deregulation of Abl tyrosine kinase activity is sufficient to trigger CML pathogenesis [4]. The 2-phenylaminopyrimidine STI571 is highly effective in early phases of the disease, whereas resistance formation and subsequent therapy failure was observed in patients with advanced CML. Analysis of Bcr-Abl from relapsed patients revealed a variety of amino acid substitutions within the Abl kinase domain, and biochemical and biological assays established these mutations as molecular determinants for STI571 insensitivity [7, 8]. These results raise the critical issue of whether molecular resistance formation will emerge as a general drawback inherent to protein kinase-targeted anticancer therapy. For Bcr-Abl, some of the mutations, such as the Thr-315 to isoleucine substitution, directly interfere with STI571 interaction at the ATP binding pocket, whereas most of them induce structural changes within either the activation loop or the ATP phosphate binding loop regions and thereby prevent the Abl kinase domain from adapting the closed, inactive conformation required for the induced fit with STI571 [8, 9]. Remarkably, these conformation-dependent mechanisms of drug resistance were recently found to be STI571 specific and did not apply to Bcr-Abl inhibition by the pyrido[2,3-d]pyrimidine PD180970 [10]. But this study further highlighted the significance of the Thr-315 to isoleucine mutation in Bcr-Abl, which conferred resistance to inhibition by both STI571 and PD180970. Interestingly, Thr-315 of Abl corresponds to Thr-106 of p38 and Thr-766 of the epidermal growth factor receptor (EGFR), and these residues have also been identified as critical structural determinants for the sensitivities of these two kinases to the pyridinylimidazole inhibitor SB203580 and the 4-anilinoquinazoline PD153035, respectively [11, 12]. The same C to T single nucleotide change as found in codon 315 of Bcr-Abl from relapsed CML patients replaces Thr-766 of the EGFR by methionine and dramatically desensitizes its tyrosine kinase to inhibition by PD153035 [12]. Since PD153035 is related in structure to the recently approved drug ZD1839 (Iressa, gefitinib), our earlier results raised the issue of whether similar resistance formation will also be observed for the clinical EGFR inhibitor ZD1839 [5]. Moreover, it remains to be determined whether these known examples of inhibitor-resistant protein kinases are representative of a common theme of drug insensitivity acquired through substitutions at the conserved site

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corresponding to Thr-315 of Abl and Thr-766 of the EGFR.

To address these important questions, we introduced equivalent amino acid substitutions into three other protein tyrosine kinases. The sensitivities of the mutant kinases were determined for a selection of structurally distinct inhibitors. With one important exception, all of the inhibitor classes tested were prone to a conserved mechanism of molecular resistance formation. In addition, the drug-resistant kinase mutants provided useful tools for chemical-genetic analysis of cellular signaling.

Results

Protein Kinase Alignment and Inhibitor Selection

The side chain of Thr-315 in the Abl tyrosine kinase controls the access to a hydrophobic pocket at the ATP binding site [7, 9]. This cavity accommodates moieties of ATP-competitive inhibitors, such as STI571 and PD180970, but no functional groups of ATP itself [9, 13]. Thus, replacement of Thr-315 by larger residues interferes with inhibitor binding, while leaving the kinase activity intact. The smaller amino acids threonine or valine are present at the corresponding site in a subset of all human kinases, and an alignment of those belonging to this group and relevant for this study is shown in Figure 1A. For a comprehensive analysis of this structural feature with respect to resistance formation, we selected a variety of inhibitors belonging to distinct compound classes (Figure 1B). The EGFR inhibitor ZD1839 (Iressa, gefitinib) has recently received FDA approval for third-line treatment of non-small cell lung cancer [5, 14]. The pyrazolopyrimidine PP1 was originally described as a Src family kinase-specific inhibitor but was later found to block βPDGFR activity with similar potency [15]. The 2-phenylaminopyrimidine STI571 is a potent inhibitor of the tyrosine kinases Abl, c-kit, and PDGFR [6]. The quinoxaline compound AG1296 is used as a selective PDGFR inhibitor [16]. The pyrido[2,3-d]pyrimidine-based inhibitor used in this study has previously been described as compound 58 out of a series of pyrido[2,3-d]pyrimidines by Klutschko et al. and is referred to as PP58 [17]. PP58 is known to block the in vitro activities of PDGFR, fibroblast growth factor receptor (FGFR), and Src tyrosine kinases with nanomolar IC_{50} values. The indolinone SU6668 was developed as an angiogenesis inhibitor for anticancer therapy targeting three receptor tyrosine kinases (RTKs) involved in this process (PDGFR, FGFR1, and vascular endothelial growth factor receptor-2) [18]. Finally, the indolinones SU6656 and SU4984 have been described as inhibitors of Src family kinases and FGFR1, respectively [19, 20].

Substitution of Methionine for Thr-766 Desensitizes the EGFR to the Anticancer Drug ZD1839

In a previous report, we demonstrated that replacing Thr-766 with methionine dramatically reduced the sensitivity of the EGFR to inhibition by the specific 4-anilinoquinazoline PD153035 [12]. Importantly, structurally related quinazolines have been developed for target-selective inhibition of the EGFR in human cancer patients, and ZD1839 (Iressa, gefitinib) is the most advanced of these drugs [5]. To analyze potential EGFR resistance formation to ZD1839, we transiently expressed both wild-type EGFR and the EGFR-T766M mutant in CHO-K1 cells, which lack endogenous EGFR expression. As shown in Figure 2, pretreatment of cells with 25 nM ZD1839 strongly suppressed EGF-stimulated tyrosine phosphorylation of the wild-type RTK, whereas, in stark contrast, 100-fold higher concentrations were without effect on the EGFR-T766M mutant. Thus, molecular resistance formation of the EGFR is also observed for ZD1839 upon introduction of the equivalent nucleotide change that substitutes Thr-315 with isoleucine in Bcr-Abl from STI571-insensitive CML patients. Moreover, in case of the EGFR, the C to T transition replacing Thr-766 with methionine (ACG to ATG) would occur within a CpG dinucleotide sequence. Notably, due

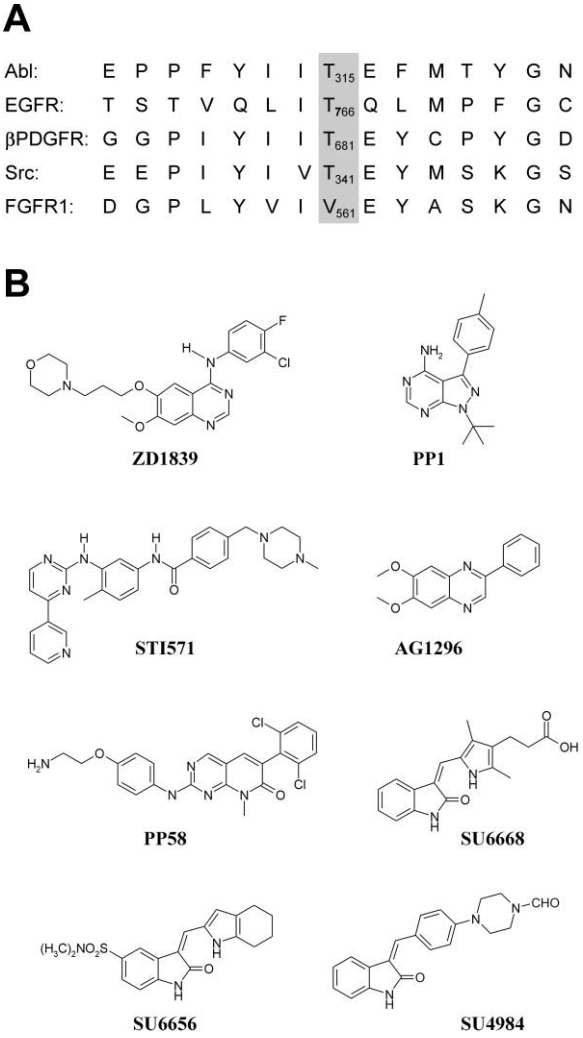

Figure 1. Protein Kinase Alignment and Inhibitor Structures

(A) Residues surrounding Thr-315 in Abl aligned with the corresponding sequences of EGFR, βPDGFR, Src, and the FGFR1. Thr-315 in Abl and the equivalent amino acids in the other protein kinases are highlighted in gray.

(B) The chemical structures of ZD1839, PP1, STI571, AG1296, a pyrido[2,3-d]pyrimidine-based compound (PP58), and three indolinones (SU6668, SU6656, SU4984) are shown.

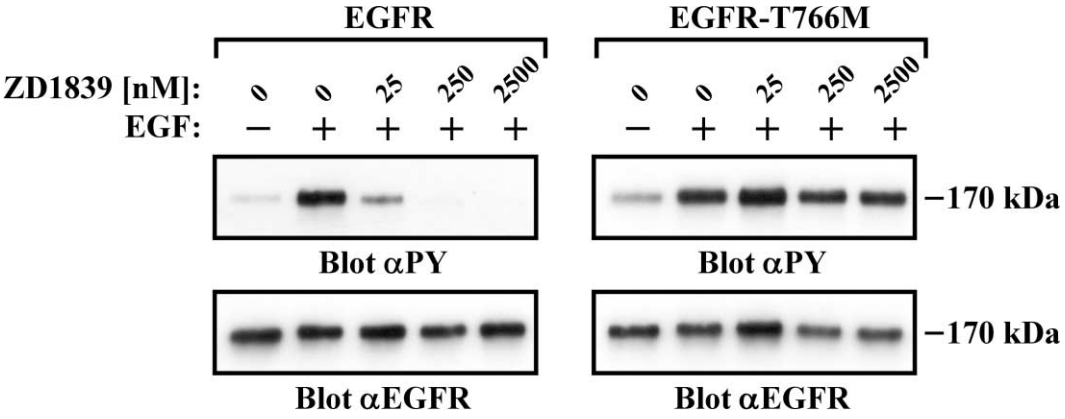

Figure 2. EGFR-T766M Is Resistant to ZD1839

CHO-K1 cells lacking endogenous EGFR expression were transiently transfected with pLXSN expression plasmids encoding either wild-type EGFR or the EGFR-T766M mutant. Serum-starved cells were preincubated with the indicated concentrations of ZD1839 or DMSO for 25 min prior to stimulation with 10 ng/ml EGF for 5 min. After cell lysis and immunoprecipitation, EGFR was analyzed by immunoblotting with anti-phosphotyrosine (αPY) antibody (upper panels). In parallel, the amount of EGFR in immunoprecipitates was detected using anti-EGFR antibody (lower panels).

to their susceptibility to methylation-deamination reactions, these sites are mutational hotspots within the human genome and therefore account for the most frequent forms of single-nucleotide substitutions [21]. It remains to be determined whether this particular type of mutation indeed occurs in ZD1839-treated patients. If so, the Thr-766 to methionine mutation in the EGFR would be a marker of significant diagnostic value.

Analysis of βPDGFR Sensitivity to Different Protein Kinase Inhibitors upon Replacement of Thr-681 with Isoleucine

Activation of the βPDGFR tyrosine kinase has been established as critical for the progression of various types of cancers such as glioblastoma, dermatofibrosarcoma protuberans, and chronic myelomonocytic leukemia [4]. STI571 (Gleevec) inhibits the βPDGFR as potently as the Abl tyrosine kinase, and exploratory clinical testing has already indicated efficacy of STI571 in hyperproliferative diseases with constitutive βPDGFR activation [4, 22, 23]. This raises the important issue of whether clinical resistance formation in these βPDGFR-driven malignancies might occur through similar mechanisms as demonstrated for Bcr-Abl in late-phase CML patients. Here, we focus on the single C to T nucleotide change known to confer STI571-resistance to Bcr-Abl by replacing Thr-315 with isoleucine. Analogous mutation of the equivalent codon 681 of the βPDGFR generated the βPDGFR-T681I mutant possessing the same amino acid substitution. We then transiently expressed both wild-type receptor and the βPDGFR-T681I mutant in COS-7 cells and measured the effect of STI571 treatment on PDGF-stimulated cellular RTK activity by immunoblot analysis of βPDGFR autophosphorylation on intracellular tyrosine residues. As shown in Figure 3A, ligand-triggered cellular activity of the wild-type βPDGFR was strongly suppressed by the 2-phenylaminopyrimidine STI571 with an IC_{50} value somewhat below 1 μM, whereas inhibitor concentrations of up to 25 μM did not affect the T681I mutant. Thus, the rather modest threonine to isoleucine conversion leads to dramatic resistance formation of the βPDGFR. This finding extends previous data from mutational analysis of this position in the βPDGFR. Böhmer et al. demonstrated STI571 resistance upon replacement of Thr-681 with a much bulkier phenylalanine residue, but this type of substitution is unlikely to occur in vivo, as it would require a double nucleotide change in codon 681 of the βPDGFR [24]. We then compared the sensitivities of wild-type and mutant receptors to various other βPDGFR tyrosine kinase inhibitors belonging to different compound classes. These experiments revealed that the Thr-681 to isoleucine mutation conferred resistance to PP1, AG1296, and the pyrido-[2,3-d]pyrimidine compound PP58 (Figures 3A–3C). Based on these results, βPDGFR resistance formation due to Thr-681 mutation emerges as a rather broad concept relevant for a variety of structurally unrelated βPDGFR kinase inhibitors.

Importantly, we found a notable exception to this rule, as Thr-681 was not critical for inhibition by the indolinone drug SU6668. As seen in Figure 3D, both wild-type and mutant βPDGFR were inhibited by this compound with an comparable IC_{50} of about 0.5 to 1 μM in intact cells.

Analysis of Resistance Formation for Src and FGFR1 Tyrosine Kinase Mutants

The βPDGFR mutant experiments established Thr-681 as a structural determinant critical for sensitivity to several distinct inhibitor scaffolds. We next asked whether these findings can be extended to other tyrosine kinase targets. For this purpose, we replaced the equivalent amino acids Thr-341 in the cytoplasmic Src tyrosine kinase and Val-561 in the FGFR1 RTK with larger methionine residues. To activate Src tyrosine kinase, the carboxy-terminal Tyr-530 residue, which negatively regulates Src upon C-terminal Src kinase (CSK)-mediated phosphorylation, was mutated to phenylalanine, and the resulting Src-Y530F and Src-T341M-Y530F mutants were transiently expressed in COS-7 cells [25]. Cellular

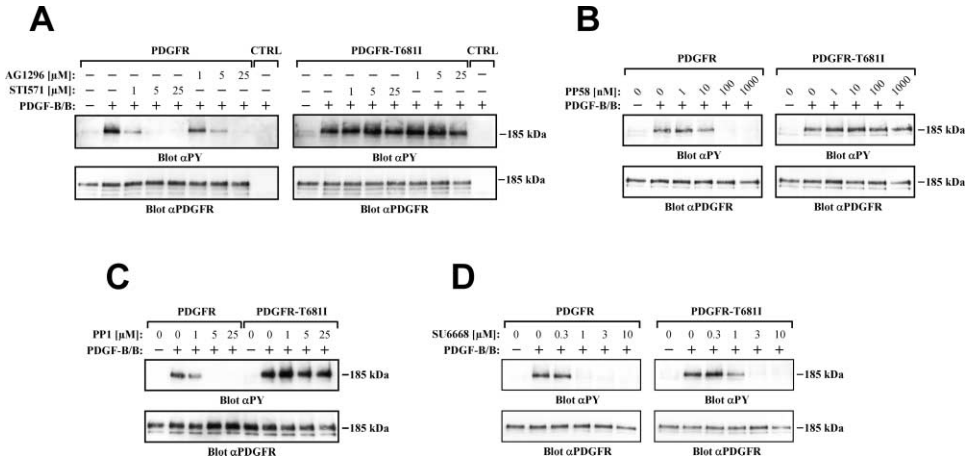

Figure 3. Mutant βPDGFR Sensitivity to Different Protein Kinase Inhibitors

COS-7 cells were transiently transfected with empty vector or pLXSN expression plasmids encoding βPDGFR or βPDGFR-T681I. Serum-starved cells were preincubated with the indicated inhibitor concentrations of STI571 or AG1296 (A), PP58 (B), PP1 (C), or SU6668 (D) for 30 min prior to stimulation with 30 ng/ml PDGF-B/B for 5 min. After cell lysis, βPDGFR was immunoprecipitated and analyzed by immunoblotting with anti-phosphotyrosine antibody (αPY, upper panels). In parallel, expression levels of transiently expressed βPDGFR in total cell lysates were measured with anti-βPDGFR antibody (lower panels).

Src kinase activity was then measured by immunoblot analysis with antiserum specifically recognizing Src-mediated autophosphorylation on its tyrosine residue 419. As seen in Figure 4A, the widely used inhibitor PP1 suppressed cellular autophosphorylation of activated Src tyrosine kinase with an IC_{50} value of about 5 μM, whereas full resistance at 25 μM PP1 was observed for the Src variant possessing the Thr-341 to methionine mutation. These results are consistent with previous in vitro studies by Shokat and colleagues and verify the in vivo relevance of their earlier findings [26]. When we tested the pyrido[2,3-d]pyrimidine inhibitor PP58 against the activities of either Src-Y530F or Src-T341M-Y530F in intact cells, dramatic resistance formation became apparent. The T341M mutation abrogated the sensitivity to PP58 inhibition by increasing the cellular IC_{50} value of about 10 nM by more than 1000-fold (Figure 4B). This finding was in stark contrast to results obtained with the Src kinase inhibitor SU6656 [19]. This indolinone compound inhibited both Src variants irrespective of a threonine or methionine residue present at the critical position 341 with an cellular IC_{50} in the range of 3 to 10 μM (Figure 4C). Moreover, we consistently observed an increased phospho-Tyr-419 signal for the Src-T341M-YF mutant, indicating that substitution of Thr-341 with methionine did not abrogate but instead somewhat enhanced the cellular Src kinase activity (Figures 4A–4C). A similar observation was made when the cellular autophosphorylation of wild-type FGFR1 was compared with mutant receptor, which possessed a methionine residue instead of the Val-561 corresponding to the equally sized Thr-341 of Src (Figures 4D and 4E). The cellular wild-type FGFR1 activity was potently inhibited by low nanomolar concentrations of the broadly active pyrido[2,3-d]pyrimidine tyrosine kinase inhibitior PP58, whereas dramatic resistance formation was detected for the FGFR1-V561M mutant (Figure 4D). Thus, FGFR1 mutant analysis yielded similar results for PP58 as obtained for the βPDGFR and Src tyrosine kinases. Conversely, the FGFR-specific indolinone inhibitor SU4984 inhibited both wild-type FGFR1 and the V561M mutant in a comparable dose-dependent manner with an IC_{50} around 10 μM (Figure 4E). Taken together, our data establish the concept that efficient inhibition by many kinase inhibitors requires a threonine or valine in a conserved position at the ATP binding site, where these smaller residues sterically control the interaction of inhibitor moieties with a hydrophobic pocket not involved in binding of ATP itself. But, as verified for three derivatives with three different kinase targets, inhibition by indolinone compounds occurs independently of this structural determinant.

Cellular Signaling Mediated by Inhibitor-Insensitive βPDGFR Tyrosine Kinase

PDGFR tyrosine kinases are expressed in most cell types. To investigate the signaling capacity of drug-resistant βPDGFR in intact cells, it was either necessary to introduce wild-type and mutant receptors into a PDGFR-deficient cell system or to specifically trigger signaling through an ectopically expressed receptor without activating its endogenous counterpart. We chose the second strategy and made use of immortalized EF1.1$^{-/-}$ fibroblasts derived from EGFR knockout mice, in which we expressed chimeric RTKs consisting of the extracellular domain of the human EGFR and the transmembrane and cytoplasmic domains of either wild-type (EPR) or T681I mutant βPDGFR (EPR-T681I) [27, 28]. In these cell lines, intracellular βPDGFR signaling could then be specifically induced upon extracellular addition of EGF. As shown in Figure 5A, both wild-type EPR and the T681I mutant were expressed at comparable levels and became tyrosine phosphorylated to a similar extent upon EGF stimulation. Moreover, in accor-

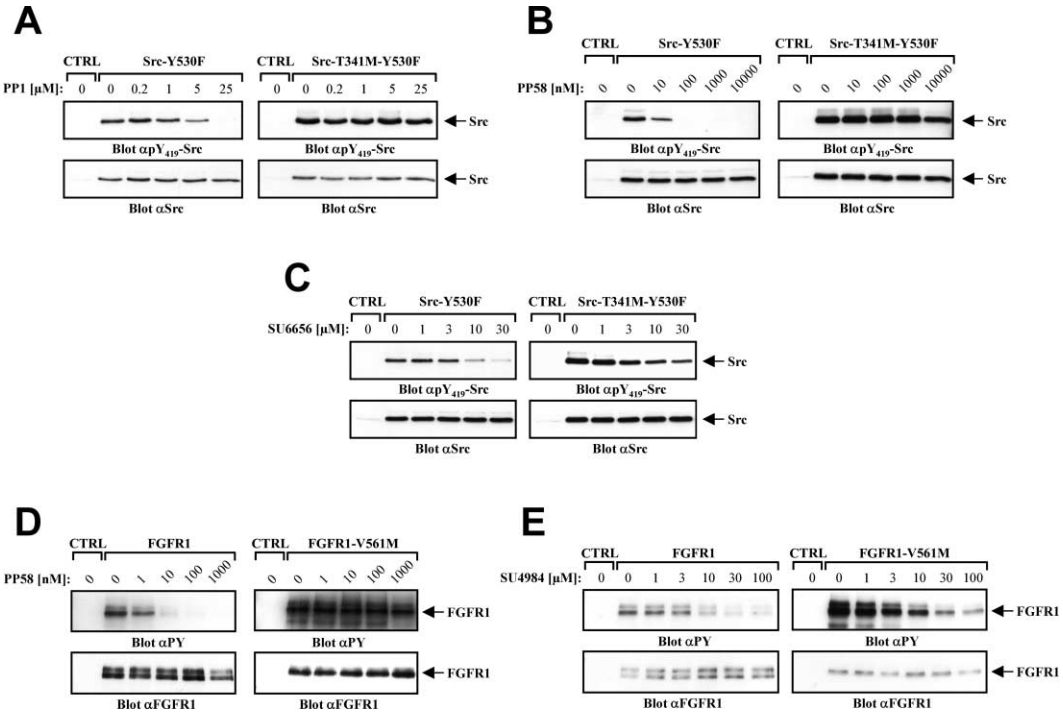

Figure 4. Cellular Resistance Formation of Src and FGFR1 Tyrosine Kinase Mutants

Control-transfected COS-7 cells or COS-7 cells transiently expressing human Src-Y530F, Src-T341M-Y530F, FGFR1, or FGFR1-V561M were serum starved for 24 hr. Prior to lysis, cells were treated with the indicated inhibitor concentrations of PP1 (A), PP58 (B and D), SU6656 (C), or SU4984 (E) for 30 min.

(A–C) Src tyrosine kinase in total lysates was analyzed by parallel immunoblotting with anti-phosphoTyr-419-Src family kinase specific antibody (upper panels) and anti-v-Src antibody (lower panels).

(D and E) FGFR1 was purified with WGA-Sepharose, and tyrosine-phosphorylated FGFR1 was detected by immunoblotting with anti-phosphotyrosine antibody (αPY, upper panels). In parallel, the amount of FGFR1 was analyzed using anti-FGFR1 antiserum (lower panels).

dance with the results presented above, 25 μM of either PP1 or STI571 abrogated wild-type EPR tyrosine phosphorylation, but was without effect on the activation of the EPR-T681I mutant. For these two compounds, we further analyzed the mitogenic responses through wild-type and inhibitor-resistant βPDGFR tyrosine kinases on the levels of mitogen-activated protein kinase (MAPK) activation, c-Fos protein expression, and DNA synthesis [12]. As shown in the time-course experiment in Figure 5B, STI571 pretreatment of EPR-expressing cells strongly interfered with the activation of the extracellular signal-regulated protein kinase (ERK) MAPKs and c-Fos protein production upon EGF addition. Protein levels of the c-Fos transcription factor were analyzed as a surrogate marker for c-*fos* immediate-early gene induction. In contrast, neither downstream signaling event was affected by STI571 when triggered through the STI571-resistant intracellular domain in EPR-T681I-expressing fibroblasts. Moreover, ligand-stimulated DNA synthesis was only slightly diminished by 25 μM STI571 in cells expressing the inhibitor-insensitive chimeric RTK, whereas thymidine incorporation induced through wild-type βPDGFR kinase was already reduced to basal levels by 5-fold lower STI571 concentrations (Figure 5C). These results demonstrate that the antiproliferative effect of STI571 on βPDGFR-mediated signaling is dramatically reduced upon introduction of the equivalent C to T single nucleotide mutation as previously found in codon 315 of Abl kinase from STI571-resistant CML patients.

Pharmacological analysis of PDGFR signal transduction employing the Src family kinase inhibitor PP1 has always been hampered by the fact that the PDGFR itself is targeted by this compound. Utilizing the EPR-T681I-expressing fibroblasts, we were now able to conduct this type of experiment and prepared total lysates from either wild-type or EPR-T681I-expressing cells after different times of EGF stimulation in the presence or absence of PP1. Surprisingly, although rapid induction of ERK activity was reconstituted through inhibitor-insensitive βPDGFR kinase in the presence of PP1, this compound abrogated the sustained ERK activity observed in control-treated cells as revealed by time-course analysis (Figure 5B). As further seen in Figure 5B, the less sustained ERK activation also resulted in a strongly reduced expression of c-Fos protein upon EPR-T681I stimulation. As analyzed by anti-phosphotyrosine immunoblots of the same lysates, the time course of EPR-T681I phosphorylation appeared to be unaffected by PP1 pretreatment (data not shown). Thus, PP1 interfered with a signaling step downstream of the βPDGFR and upstream of ERK activation. Moreover, these PP1-sensitive signal transducers are unlikely to be Src family kinases, as previous data have excluded a role for these kinases in ERK and c-Fos activation and instead impli-

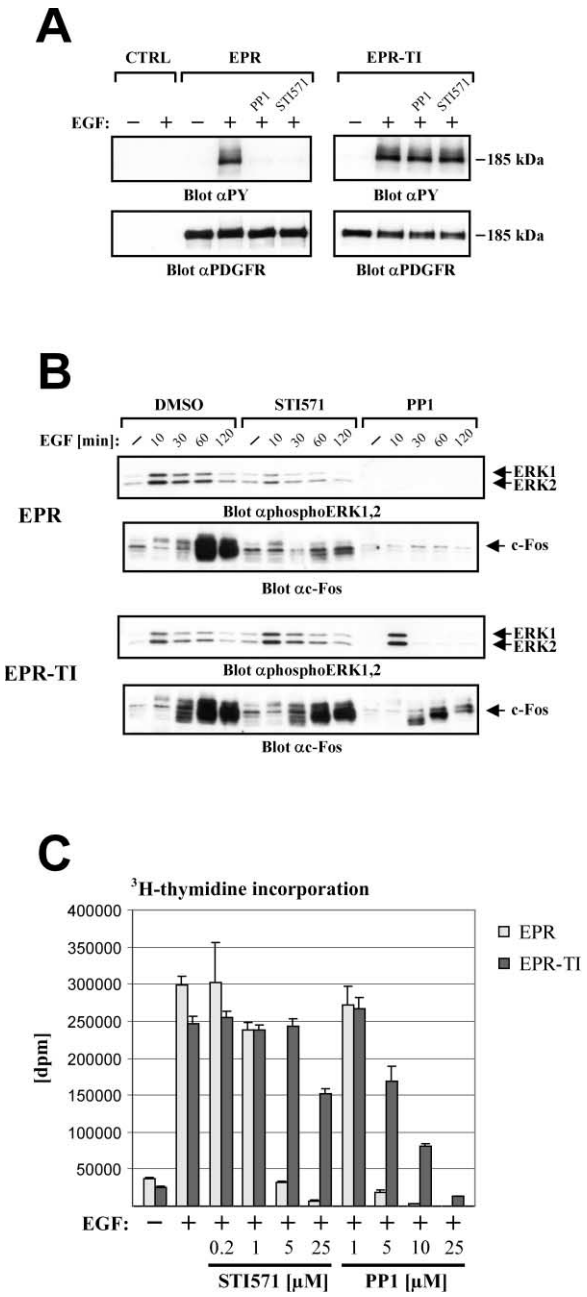

Figure 5. Inhibitor Effects on Cellular Signaling through Drug-Resistant βPDGFR

Control EF1.1$^{-/-}$ fibroblasts or cells stably expressing either chimeric EPR or the EPR-T681I mutant were serum-starved for 24 hr.

(A) After preincubation with 25 μM of either PP1 or STI571 for 30 min where indicated, cells were stimulated for 5 min with 50 ng/ml EGF prior to lysis. EPR was immunoprecipitated with mAb108.1 and analyzed by immunoblotting with anti-phosphotyrosine antibody (αPY) and anti-βPDGFR antibody.

(B) Following pretreatment with 25 μM STI571, 25 μM PP1, or DMSO for 30 min, cells were stimulated with 50 ng/ml EGF for the indicated times prior to lysis. Total cell extracts were immunoblotted with either anti-phospho-ERK1/2 antibody or anti-c-Fos antibody.

(C) EF1.1$^{-/-}$ fibroblasts were treated with the indicated concentrations of STI571 and PP1 for 30 min prior to stimulation with 50 ng/ml EGF. After 14 hr, cells were pulse labeled with methyl-[^{3}H]thymidine for 2 hr, and its incorporation into DNA was measured. The dpm values shown represent the mean ±SD for triplicate samples.

cated them in PDGFR-mediated c-Myc induction [19]. Therefore, the PP1 effect more likely relates an unrecognized element critically involved in βPDGFR signaling. As measured in thymidine incorporation experiments, the antiproliferative effect of PP1 was more pronounced in EPR- than in EPR-T681I-expressing cells (Figure 5C). From these results, we conclude that the βPDGFR itself is the most sensitive PP1 target required for βPDGFR-mediated cell cycle progression.

A Chemical-Genetic System to Trigger Cellular Src Kinase Signaling

Src family kinases are negatively regulated through CSK-mediated phosphorylation of a tyrosine residue at their C terminus. Therefore, pharmacological inhibition of CSK activity in intact cells would trigger Src kinase signaling. The pyrido[2,3-d]pyrimidine tyrosine kinase inhibitior PP58 is known to be effective against PDGFR, FGFR, and Src family kinases, and, in addition, related derivatives potently interfere with Abl tyrosine kinase activity in the low nanomolar range [10, 17]. Furthermore, we could identify CSK as a potential target of pyrido[2,3-d]pyrimidine-based compounds employing a chemical proteomics approach (H.D. and J. Wissing, unpublished data). In vitro kinase assays revealed that PP58 inhibited CSK activity with an IC_{50} value of around 100 nM (Figure 6A). Based on this result, we assumed that PP58 could activate Src in intact cells through CSK inhibition in case Src itself is not targeted by the pyrido[2,3-d]pyrimidine derivative. Our identification of Thr-341 as the residue critical for Src inhibition by PP58 allowed us to test this hypothesis. To verify the model for pharmacological Src activation shown in Figure 6B, we transiently expressed either wild-type Src or the inhibitor-insensitive Src-T341M mutant and then treated the transfected COS-7 with PP58. As shown in Figure 6C, cellular kinase activity of the PP58-resistant T341M mutant was indeed stimulated within only 5 min of PP58 incubation, as revealed by immunoblot analysis with antiserum specifically detecting Src autophosphorylation. Prolonged incubation with PP58 did not further increase Src phosphorylation on Tyr-419. In contrast, the PP58-sensitive wild-type kinase was not stimulated, although expressed at similar levels (Figure 6C). PP58-induced Src-T341M activity led to the tyrosine phosphorylation of various cellular proteins, and this effect was specific for the transfected Src mutant since PP58 even reduced the basal tyrosine phosphorylation in control-transfected and wild-type Src expressing cells (Figure 6C, lower panel). These experiments establish a new chemical-genetic strategy for small molecule-regulated stimulation of cellular Src kinase signaling. Importantly, this straightforward approach can be applied to any of the Src family members with minimal experimental efforts, which might be an advantage over the more complicated, small molecule-induced Src kinase membrane targeting system reported several years ago [29]. In this earlier work, a membrane-bound FK506 binding protein 12 (FKBP12) variant recruited coexpressed FKBP12-Src kinase fusion protein upon addition of a small molecule inducer of FKBP12 dimerization, and this membrane translocation event initiated Src kinase signaling. Compared to the

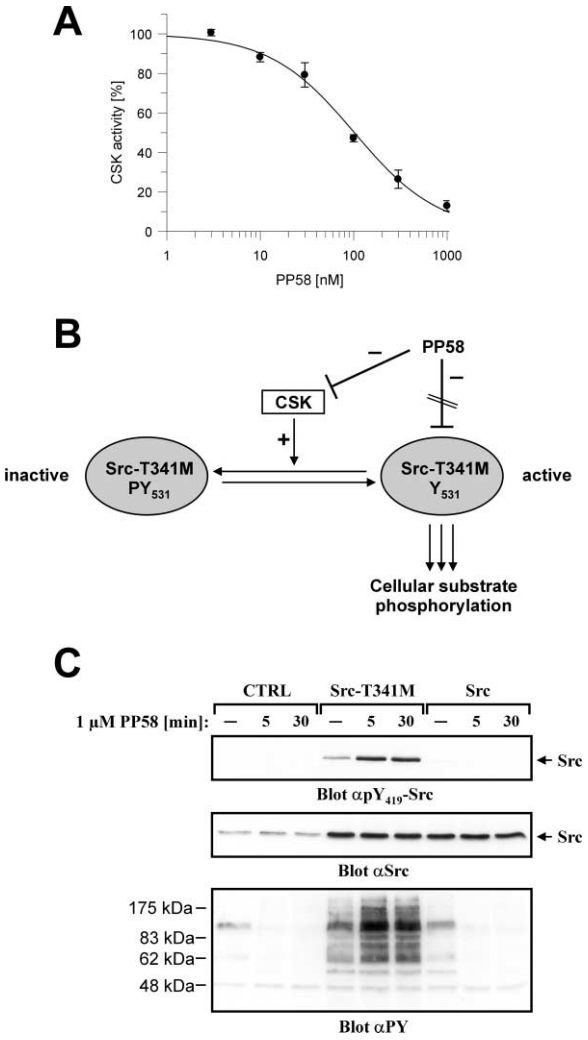

Figure 6. Pharmacological Induction of Src Kinase Signaling Employing an Inhibitor-Resistant Mutant

(A) Inhibition of CSK activity by PP58 was determined in vitro. Kinase activities in the absence of inhibitor were set to 100%, and remaining activities at different PP58 concentrations are shown relative to this value.

(B) Model for PP58-induced activation of Src-T341M through cellular inhibition of antagonizing CSK activity.

(C) COS-7 cells were either control transfected with 2 μg pLXSN vector or cotransfected with 1.5 μg of vector DNA plus the pLXSN expression plasmids encoding for Src or Src-T341M (0.5 μg/well each). After serum starvation, cells were incubated with 1 μM PP58 for the indicated times prior to lysis. Total cell lysates were subjected to immunoblotting with anti-phosphoTyr-419-Src family kinase specific antibody, anti-v-Src antibody, and anti-phosphotyrosine (αPY) antibody.

FKBP12-based dimerization technique, the chemical-genetic system presented here should more reliably reproduce the regulated cellular localization of Src family kinases, which is known to occur by mechanisms such as palmitoylation-dependent partitioning into lipid rafts and has functional consequences on substrate phosphorylation [30–32]. Thus, our approach might be particularly useful to characterize cellular substrate proteins of Src family members and thereby contribute to a better mechanistic understanding of Src kinase-mediated signal transduction in intact cells.

Structural Basis for Differential Inhibitor Sensitivity of Protein Kinase Mutants

To investigate the molecular basis of the differential inhibitor sensitivities measured for wild-type and mutated kinases, we docked the indolinone compound SU6656 and the pyrido[2,3-d]pyrimidine derivative PP58 into a Src homology model. Since our experimental data was obtained with constitutively active human Src, we decided to generate a model of active Src instead of using its crystallized inactive kinase structure [33]. The model was generated on active Lck (Protein Data Base ID code 3lck), which shows high sequence identity (67%) to human Src and is crystallized with a resolution of 1.7 Å [34].

Docking studies with both inhibitors to either wild-type or T341M mutant Src correlated with the experimental data. While SU6656 fit into both wild-type and mutant Src, PP58 could only be accommodated by the wild-type enzyme (Figure 7A, upper panels and lower left panel). These results can be explained by the orientations of both inhibitors in the ATP binding site. Both ligands form the classical H bonds to the hinge region, identical to those found in other crystal structures of similar inhibitors, indicating a correct binding mode [13, 18, 20]. SU6656 uses the carbonyl oxygen of Glu-342 and the backbone NH of Met-344 for H bonding and exclusively occupies the ATP binding pocket. PP58 establishes two H bonds to both the amide nitrogen and the carbonyl oxygen of Met-344 and further extends its 2,6-dichlorophenyl substituent into the hydrophobic back pocket adjacent to the ATP binding site. In contrast, the indolinone SU6656 does not interact with this hydrophobic cavity. While SU6656 can be accommodated in the model structure of the Src-T341M mutant (Figure 7A, upper right panel), our docking study did not suggest any low energy binding mode that PP58 could adapt in the mutant binding pocket. Consistent with these results, superimposition of the PP58 conformation derived from the wild-type model with the mutant Src structure resulted in a steric clash of the inhibitor with the methionine side chain (Figure 7A, lower left panel). Thus, mutation of Thr-341 to methionine appears to abrogate PP58 binding by blocking the access of the inhibitor's dichlorophenyl group to the hydrophobic back pocket.

To compare the binding modes of our docked ligands with publicly available X-ray structures of similar compounds, we superimposed both mutation-insensitive and mutation-sensitive ligands in the protein environment of the published structure of active Lck (PDB ID code 3lck). For clarity, only the surface of the Lck nucleotide binding cavity is shown. The indolinone compound SU6668 overlays nicely with the adenine part of ATP, and both ligands do not occupy the hydrophobic back pocket. The conformation of SU6668 derived from its FGFR cocrystal structure (PDB ID code 1fgi) is very similar to our docked SU6656 conformation (Figure 7B) [18]. In contrast, PP1 (PDB ID code 1qpe) and the PP58 analog PD173955 (PDB ID code 1m52) both target the

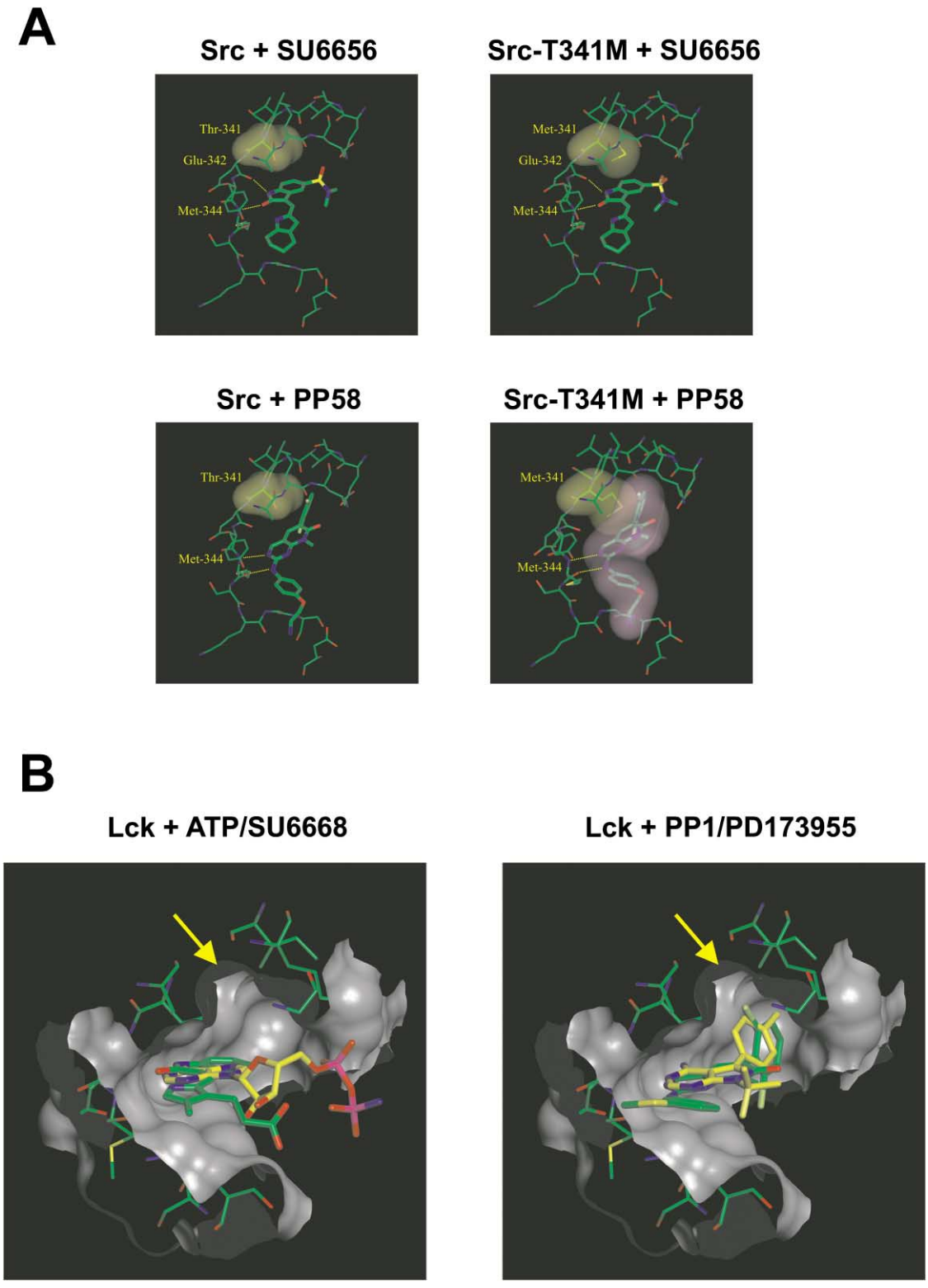

Figure 7. Structural Basis for Differential Inhibitor Sensitivities of Wild-Type and Mutant Kinases

The pictures were generated with WebLabViewer (A) and Insight II (B).

(A) SU6656 was docked into homology models of the activated forms of both wild-type human Src and the Src-T341M mutant (upper panels). PP58 was docked into wild-type human Src and manually overlaid with the Src-T341M mutant structure (lower panels). The solvent accessible surface of the critical residue in position 341 is shown in yellow in all panels. In the lower right panel, the surface of PP58 is shown in pink to visualize the steric clash of PP58 with the Met-341 side chain in the mutant structure.

(B) Ligand conformations derived from various crystal structures were inserted into the ATP binding pocket of Lck (PDB ID code 1qpe). The indolinone SU6668 (PDB ID code 1fgi; green/CPK) and ATP (PDB code 1qpc; yellow/CPK) are shown on the left. The inhibitors PP1 (PDB ID code 1qpe; yellow/CPK) and PD173955 (PDB ID code 1m52; green/CPK) are shown on the right. The yellow arrows point to the hydrophobic pocket, which accommodates moieties of PP1 and PD173955 but is not targeted by ATP and SU6668.

hydrophobic back pocket [13], further illustrating the structural basis for resistance formation defined for various protein kinases in this study.

Discussion

Protein kinase targets are prone to molecular resistance formation, which is of high clinical relevance as exemplified by a variety of mutations found in STI571-insensitive Bcr-Abl from relapsed CML patients [7, 8]. As shown for a βPDGFR-T681I mutant equivalent to the clinical T315I Abl kinase isolate, our results indicate that similar molecular insensitivity occurs in case of the βPDGFR and can further translate into STI571-resistant but still βPDGFR-driven biological responses such as cell proliferation. With respect to clinical resistance formation, these results could become a relevant feature for STI571 therapy of malignancies, such as dermatofibrosarcoma protuberans and chronic myelomonocytic leukemia, in which defined chromosomal translocations trigger constitutive βPDGFR signaling causative for disease progression [4, 22, 23]. The same conserved threonine might also be critical for the RTK c-kit, the cellular target of STI571 therapy in gastrointestinal stromal tumors, and a recent study indeed detected the corresponding mutation in the tyrosine kinase domain of the αPDGFR in an STI571-treated patient suffering from idiopathic hypereosinophilic syndrome [4, 35]. In addition, mutations analogous to the Y253F, E255K, M351T, H396P, and a variety of other substitutions in the Abl kinase domain associated with STI571 resistance in CML might also affect PDGFR and c-kit sensitivities [7, 8]. This remains to be tested. These amino acid replacements appear to affect the characteristic inactive conformation, which Abl has to adopt for high affinity interaction with STI571. Despite their relevance for STI571 sensitivity, these mutations did not affect Abl kinase inhibition by the pyrido[2,3-d]pyrimidine PD180970 [10, 13]. Thus, from these data, it appears that the Abl kinase domain mutations can be divided into two groups: some substitutions of general relevance for protein kinase inhibition, such as T315I, which directly interfere with inhibitor interaction, and the larger group of those, which are specific for STI571 due to the special conformational state of its targets required for efficient binding. These considerations imply that STI571 would be particularly vulnerable to resistance formation and that this risk can be minimized with target conformation-independent inhibitors. For these compounds, only the direct interaction sites, such as the residue corresponding to Thr-315 of Abl and Thr-766 of the EGFR, might be critical for protein kinase inhibition. We have therefore focused on the relevance of this structural feature in the present study [7, 12]. The equivalent threonines (or the equally sized valine, in case of the FGFR) determined the sensitivities for all inhibitor classes analyzed, with the exception of the indolinones. The structural basis of these experimental data could further be illustrated by molecular modeling studies in which the pyrido[2,3-d]pyrimidine PP58 and the indolinone SU6656 were docked into the wild-type and T341M mutant Src kinase structures. The presence of a long methionine side chain in position 341 of Src leads to sterical clash with the dichlorophenyl group of the pyrido[2,3-d]pyrimidine PP58, which is accommodated by a hydrophobic pocket at the ATP binding site in the wild-type enzyme. In contrast, the T341M substitution did not interfere with SU6656 binding, since no moiety of the indolinone extends into the corresponding cavity of human Src according to our model.

Inhibitor resistance cannot only be exploited for signal transduction analysis, as shown for PDGFR and Src signaling in this study, but also provides insights for drug development in the protein kinase field. About 75% of all protein kinases possess a larger, hydrophobic residue, such as methionine, leucine, or phenylalanine, in the position equivalent to the threonine or valine residues present in the kinases we investigated here [36]. Thus, compounds from a variety of inhibitor classes, such as 4-anilinoquinazolines, pyrazolopyrimidines, 2-phenylaminopyrimidines, quinoxalines, and pyrido[2,3-d]pyrimidines, might preferably target only a small subset of protein kinases possessing a small residue at the critical site corresponding to Thr-315 of Abl. In contrast, indolinones are not selective according to this criteria, at least not the derivatives we have tested. In addition to drug-resistance studies performed by others and our group, selectivity profiling of PP1 and SU6656 against a panel of recombinant kinases supports this concept [37]. These structural aspects might aid the design of compound libraries optimized for the screening of each individual protein kinase target and thereby help to develop more potent and selective drugs for the treatment of diseases with unmet medical needs.

Significance

Protein kinases are key control elements of cellular signaling and therefore represent a major family of drug targets. Numerous selective and potent small molecule inhibitors of protein kinases have been identified in recent years. These reagents are useful for both signal transduction research and therapeutic intervention in various diseases. The inhibition of some protein kinases, such as p38, the EGFR, or Bcr-Abl, by target-selective, ATP-competitive compounds was described to depend on the presence of a small threonine residue at a specific site near the nucleotide binding pocket. Substitutions with larger residues rendered mutant protein kinases insensitive to selective inhibitors without abrogating kinase activity, and this type of mutation was also prominent in leukemia patients who had developed resistance to treatment with the BCR-ABL kinase inhibitor STI571 (Gleevec) [7, 11, 12]. We show a similar mode of cellular resistance formation for the EGFR-selective drug ZD1839 (Iressa). To characterize whether this structural feature is of general relevance, we tested mutants of several tyrosine kinases possessing larger side chains at the critical site against a selection of structurally diverse small molecule inhibitors. With the exception of the indolinone class of compounds, molecular resistance formation was observed for all inhibitor scaffolds tested. These results provide a rationale for the generation of smaller, focused inhibitor libraries, depending on a

small residue like threonine or a larger one present at the critical position of the protein kinase target to be screened for potent inhibitors. In addition to these results relevant for drug development, inhibitor-resistant mutants were introduced into a cellular system that allows the pharmacological dissection of βPDGFR-mediated signaling with inhibitors previously not suitable for this approach. By employing an inhibitor targeting both Src tyrosine kinase and its negative regulator CSK, the expression of a drug-insensitive mutant further permitted the small molecule-induced activation of cellular Src kinase signaling.

Experimental Procedures

Cell Lines, Reagents, and Plasmids

CHO-K1 cells and COS-7 cells were from ATCC. Immortalized embryonic EF1.1$^{-/-}$ fibroblasts derived from EGFR knockout mice were a generous gift from Maria Sibilia and Erwin Wagner (Vienna, Austria). Cell culture media and Lipofectamine were purchased from Invitrogen. Radiochemicals were from Amersham Biosciences.

PP1 was from Alexis. AG1296, SU4984, SU6656, and human recombinant EGF were from Calbiochem. The pyrido[2,3-d]pyrimidine-based compound referred to as PP58 in this study was prepared by and purchased from Evotec-OAI. PP58 synthesis was performed as described [17]. ZD1839, STI571, and SU6668 were synthesized as described [14, 18, 38]. Recombinant CSK was from Upstate. Human PDGF-B/B was from Roche. All other reagents were obtained from Sigma.

Commercial antibodies were rabbit polyclonal anti-PDGFR type A/B (Upstate), rabbit polyclonal anti-PDGFR type B (Upstate), 4G10 mouse monoclonal anti-phosphotyrosine antibody (Upstate), mouse monoclonal anti-v-src antibody (Oncogene), rabbit polyclonal anti-phosphoSrc antibody recognizing phosphorylated Tyr-419 in human Src (Cell Signaling Technology), rabbit polyclonal anti-FGFR1 (Santa Cruz Biotechnology), rabbit polyclonal anti-phospho-Erk1/2 (Cell Signaling Technology), and rabbit polyclonal anti-c-Fos antibody (Santa Cruz Biotechnology). The mAb108.1 mouse monoclonal anti-EGFR antibody has been described previously [12].

Human cDNAs encoding for EGFR, βPDGFR, and Src kinase were cloned in the retroviral expression vector pLXSN, whereas the FGFR1 construct was a pRK5 expression plasmid [39, 40]. The pLXSN-EPR construct encodes a chimeric RTK consisting of the extracellular part of human EGFR and the transmembrane and intracellular domain of the murine βPDGFR [27]. All mutants were generated using a mutagenesis kit according to the manufacturer's instructions (Stratagene).

Cell Culture, Transfections, Cell Lysis, Immunoprecipitation, Immunoblotting, and [^{3}H]Thymidine Incorporation

All methods were performed essentially as previously described [12].

CSK In Vitro Kinase Assay

CSK was assayed for 30 min at 30°C in a total volume of 25 μl. Reactions were performed with 25 ng of enzyme and 10 μg glucose-6-phosphate dehydrogenase as substrate in 50 mM Tris-HCl (pH 7.5), 3 mM $MnCl_2$, 0.5 mM DTT, 0.1 mM EGTA, 50 μM sodium orthovanadate, 50 μM ATP, and [γ-^{32}P]ATP in the presence of indicated PP58 concentrations [12]. After gel electrophoresis, phosphorylated substrate protein was visualized by autoradiography and quantified by phosphoimaging. Determination of the IC_{50} value was performed using GraFit (Erithacus).

Molecular Modeling

A homology model of the active conformation of human Src was generated on activated Lck (PDB ID code 3lck) using the homology modeling package MODELLER implemented in Insight II (Version 2000.1, www.accelrys.com) [41] (www.salilab.org/modeller/modeller.html). The sequence identity between both kinase domains is 67% (homology 82%, no gaps). The quality check of the human Src tyrosine kinase model including a Ramachandran plot was performed with ProStat in Insight II. All nonglycine amino acids were in the allowed regions, and all amide bonds were in *trans* conformation. The overall rmsd of our active conformation of the human Src model compared to inactive X-ray structures of Src (PDB ID codes 2src and 1fmk) is 1.1 Å. The active site mutation of T341 to M was generated with the Biopolymer Module within Insight II.

The kinase inhibitors SU6656 and PP58 were docked with Moloc into wild-type Src and the Src-T341M mutant [42] (www.moloc.ch). Docked structures were manually inspected and pictures were generated with WebLabViewer (www.accelrys.com).

Acknowledgments

We are very grateful to M. Sibilia and E. Wagner for providing EF1.1$^{-/-}$ fibroblasts. This work was supported by a grant from the German Bundesministerium für Bildung und Forschung. All authors are shareholders in Axxima Pharmaceuticals AG.

Received: January 9, 2004
Revised: February 5, 2004
Accepted: February 23, 2004
Published: May 21, 2004

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