Chemical Proteomic Analysis Reveals Alternative Modes of Action for Pyrido[2,3-d]pyrimidine Kinase Inhibitors*

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Small molecule inhibitors belonging to the pyrido[2,3-d]pyrimidine class of compounds were developed as antagonists of protein tyrosine kinases implicated in cancer progression. Derivatives from this compound class are effective against most of the imatinib mesylate-resistant BCR-ABL mutants isolated from advanced chronic myeloid leukemia patients. Here, we established an efficient proteomics method employing an immobilized pyrido[2,3-d]pyrimidine ligand as an affinity probe and identified more than 30 human protein kinases affected by this class of compounds. Remarkably, in vitro kinase assays revealed that the serine/threonine kinases Rip-like interacting caspase-like apoptosis-regulatory protein kinase (RICK) and p38 were among the most potently inhibited kinase targets. Thus, pyrido[2,3-d]pyrimidines did not discriminate between tyrosine and serine/threonine kinases. Instead, we found that these inhibitors are quite selective for protein kinases possessing a conserved small amino acid residue such as threonine at a critical site of the ATP binding pocket. We further demonstrated inhibition of both p38 and RICK kinase activities in intact cells upon pyrido[2,3-d]pyrimidine inhibitor treatment. Moreover, the established functions of these two kinases as signal transducers of inflammatory responses could be correlated with a potent in vivo inhibition of cytokine production by a pyrido[2,3-d]pyrimidine compound. Thus, our data demonstrate the utility of proteomic methods employing immobilized kinase inhibitors for identifying new targets linked to previously unrecognized therapeutic applications. *Molecular & Cellular Proteomics 3: 1181–1193, 2004.

Protein kinases are key control elements of cellular signaling, which is de-regulated in various diseases such as human cancer. Therefore, the protein kinase family of enzymes has emerged as a major class of drug targets in recent years (1). Pharmacological inhibition of protein kinases can be achieved with small molecule inhibitors, which block the catalytic ac-

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substitution, which directly interferes with inhibitor binding at the ATP-binding pocket, rendered Bcr-Abl resistant to both imatinib and the pyrido[2,3-d]pyrimidine PD180970 (Fig. 1). But, remarkably, all other clinically relevant Bcr-Abl variants tested by La Rosée et al. had retained their sensitivity to inhibition by PD180970. These included the Y253F, E255K, and M351T mutants frequently detected in imatinib-insensitive patients, which either interfere with the distorted conformation of the ATP-binding loop or the closed conformation of the activation loop critical for imatinib binding (3–5, 11, 12). Thus, pyrido[2,3-d]pyrimidine-based drugs could show efficacy in many cases of imatinib-resistant CML, because constitutive Bcr-Abl activity remains causative for further disease progression. Previous in vivo testing of pyrido[2,3-d]pyrimidine kinase inhibitors has demonstrated inhibition of tumor growth, but, due to the limited solubility of these compounds, no consistent dose-dependent effect could be observed in mouse xenografts (8, 13, 14). Thus, additional efforts in medicinal chemistry are obviously needed to confer better drug-like properties to this class of compounds. Moreover, in accordance with nonspecific toxic effects previously attributed to pyrido[2,3-d]pyrimidine kinase inhibitors, selectivity remains a critical issue that has to be evaluated for derivatives of this versatile compound scaffold (9).

Inhibitor selectivity is usually examined against panels of protein kinases, in which the activities of various recombinant enzymes are tested for their sensitivities to an inhibitor (15, 16). Although this established strategy provides useful results about the relative selectivity of an inhibitor, it has several shortcomings because only a small subset of the more than 500 human protein kinases can be tested and alternative protein targets such as different types of enzymes are usually excluded altogether (17). A conceptually different approach to study inhibitor function employs suitable small molecule derivatives, which can be covalently immobilized on chromatography beads and then used for the affinity purification of cellular target proteins (18–21). In combination with sensitive MS analysis, this chemical proteomics strategy has the potential to reveal the cellular target proteins of small molecule inhibitors in extracts from cultured cells, tissues, or even whole organisms. The successful implementation of chemical proteomic techniques critically depends on the efficiency and performance of the affinity purification procedures. We have recently reported an affinity chromatography technique, which operates under optimized biochemical conditions and allowed the identification of several previously unknown cellular targets of the p38 inhibitor SB 203580 and bisindolylmaleimide-type protein kinase C inhibitors (22–24).

Here, we have adapted this proteomic approach for a pyrido[2,3-d]pyrimidine derivative and identified more than 30 new cellular protein kinase targets of this inhibitor class. Remarkably, both the in vitro and cellular activities of the identified serine/threonine kinases Rip-like interacting caspase-like apoptosis-regulatory protein kinase (RICK) and p38α were potently blocked by the pyrido[2,3-d]pyrimidine inhibitor used in this study. In agreement with their established roles during inflammatory cytokine biosynthesis, pyrido[2,3-d]pyrimidine inhibitor treatment interfered with various innate immune responses such as the lipopolysaccharide (LPS)-induced tumor necrosis factor-α (TNF-α) release from monocyctic cells. Thus, we demonstrate the utility of a protein kinase inhibitor-based proteomics strategy for the identification of novel targets linked to previously unrecognized therapeutic applications.

**EXPERIMENTAL PROCEDURES**

**Reagents and Plasmids—**Cell culture media and LipofectAMINE for transfection were purchased from Invitrogen (San Diego, CA). Radiochemicals, ECH Sepharose 4B, and poly(I:C) were purchased from Amersham Biosciences (Uppsala, Sweden). Histone was obtained from Roche (Basel, Switzerland). GST-activating transcription factor 2 (ATF2), GST-Crk, catalytically inactive GST-extracellular signal-regulated protein kinase 2 (ERK2), and kemptide were obtained from Upstate (Lake Placid, NY). All other reagents were obtained from Sigma (St. Louis, MO).

Antibodies used were mouse monoclonal anti-v-Src (Oncogene, San Diego, CA), mouse monoclonal anti-Abl (Pharmining, San Diego, CA), rabbit polyclonal anti-RICK (Affinity BioReagents, Golden, CO), mouse monoclonal anti-cyclin G-associated kinase (GAK) (MoBiTec, Gottingen, Germany), mouse monoclonal anti-Yes, mouse monoclonal anti-SR protein-specific kinase 1 (SRPK1), and mouse monoclonal anti-Aurora A (all three from BD Transduction Laboratories, Lexington, KY). Antibodies purchased from Cell Signaling Technology (Beverly, MA) were rabbit polyclonal anti-p38, rabbit polyclonal anti-mitogen-activated protein kinase-activated protein kinase 2 (MAPKAP-K2), rabbit polyclonal anti-phospho-MAPKAP-K2 (Thr344), rabbit polyclonal anti-phospho-ribosomal S6 protein kinase 1 (Rsk1) (Thr359/Ser363). Antibodies obtained from Santa Cruz Biotechnology (Santa Cruz, CA) were rabbit polyclonal anti-FGFR1, rabbit polyclonal anti-c-Jun N-terminal kinase (JNK)1/2, rabbit polyclonal anti-ERK1/2, rabbit polyclonal anti-focal adhesion kinase (FAK), rabbit polyclonal anti-C-terminal Src kinase (CSK), rabbit polyclonal anti-MAPK/ERK kinase 1 (MEK1), rabbit polyclonal anti-Wee1, rabbit polyclonal anti-Rsk1, and mouse monoclonal anti-Lyn.

Recombinant protein kinases from commercial sources were human p38α, human JNK2α2, human Aurora A, human MEK1, human EphB4, human Src (all from Upstate), and mouse Abl (New England Biolabs, Beverly, MA).

Site-directed mutagenesis of the previously described plasmid pGEX-4T1-GAK was performed according to the manufacturer’s protocol ( Stratagene, La Jolla, CA) (22). Recombinant wild-type and mutant GAK were expressed as GST fusion proteins in Escherichia coli as described (22). Recombinant RICK enzyme production using adenovirus-directed expression was performed as described (25). The RICK and FGFR1 plasmids for expression in mammalian cells have been described elsewhere (22, 26).

**Compound Synthesis and Covalent Coupling—**The pyrido[2,3-d]pyrimidine kinase inhibitors referred to as PP58 and PP58peg in this study were made by and purchased from Evotec-OAI. PP58 was synthesized as previously described (8). PP58peg was prepared by dissolving 0.44 mmol PP58, 0.44 mmol 1-bromo-2-[2-(2-ethoxy-ethoxy)-ethoxy]-ethane, and 0.44 mmol diisopropylamine in 10 ml of 1,4-dioxane and refluxing the reaction mixture for 36 h. The solution was cooled to room temperature, and the solvent was removed under vacuum. The residue was purified by column chromatography silica gel; dichloromethane/methanol (95:5) to yield PP58peg as a yellow solid.

For PP58 immobilization, 2.5 ml of drained ECH Sepharose 4B
were washed three times with 50 ml of 0.5 mM NaCl, twice with 50 ml of H₂O and finally once with 50 ml of 50% dimethylformamide (DMF)/50% ETOH. Then 2.5 ml of drained beads were resuspended in 5 ml of a 0.75 mM solution of PPS8 dissolved in 50% DMF/50% ETOH followed by the addition of 750 µl of 1 mM N-ethyl-N’-(3-dimethylaminomethyl) carbodiimide hydrochloride (EDC) in 50% DMF/50% ETOH. After incubation overnight at room temperature with continual agitation in the dark, beads were washed three times with 5 ml of 50% DMF/50% ETOH prior to the addition of 5 ml of 33% DMF/33% ETOH/34% 1 mM ethanolamine pH 8.0 and 650 µl of 1 mM EDC. After 2-h incubation at room temperature with continual agitation in the dark, beads were washed three times with 15 ml of 50% DMF/50% ETOH, twice with 15 ml of 0.5 mM NaCl and once with 15 ml of 20% ETOH. The obtained beads were stored in the dark at 4 °C as a suspension in 20% ethanol.

Cell Culture, Lysate Preparation, and in Vitro Association Experiments—COS-7, HeLa, U373, and human foreskin fibroblast (HFF) cells were maintained in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum (FBS). COS-7 cell transfections, [32P]orthophosphate labeling, and the analysis of cellular RICK kinase activity was performed as previously described (22, 27).

To examine the cellular p38 and ERK activities in the presence of different PPS8 concentrations, HeLa cells were lysed with buffer containing 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS plus additives (10 mM sodium fluoride, 1 mM orthovanadate, 10 µM aprotinin, 10 µM leupeptin, 1 mM PMSF, 1 mM DTT). After SDS-PAGE, immunoblotting with phospho-specific antiserum detecting the phosphorylation of either the cellular p38 substrate MAPKAP-K2 or the cellular ERK substrate Rsk1 provided a read-out for cellular p38 and ERK activities.

For in vitro association with inhibitor affinity beads, HeLa cells or transfected COS-7 cells were lysed in buffer containing 50 mM HEPS pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 1 mM EGTA plus additives. After centrifugation, lysates were adjusted to 1 mM NaCl prior to in vitro association of 200 µl of high salt lysate with either 20 µl of drained PPS8 matrix or control matrix for 3 h at 4 °C. Where indicated, 1 mM free PPS8 was added to the lysate. After three washing steps with lysis buffer containing 1 mM NaCl, the beads were eluted with 40 µl of 1.5× SDS sample buffer. After SDS-PAGE, proteins were transferred to nitrocellulose membrane and immuno-blotted with the indicated antibodies.

Affinity Chromatography and Preparative Gel Electrophoresis—Frozen HeLa cells (2.5×10⁶; Cilibiotech, Mons, Belgium) were lysed in 30 ml of the same Triton X-100-containing buffer plus additives used for sample preparation prior to the analytical in vitro association experiments. Affinity chromatography using PPS8 columns was then performed essentially according to the previously described protocol for the purification of cellular targets of immobilized SB 203580-related inhibitor (22). After elution of bound proteins with buffer containing 1 mM PPS8 and 10 mM ATP, a second elution step was performed using lysis buffer containing 0.5% SDS. Both elution fractions were concentrated and precipitated as described. Two-thirds of the first elution fraction were resolved by 16-benzylidimethyl-n-hexadecylammonium chloride (16-BAC)/SDS-PAGE, and the remaining material was used for LC-MS/MS analysis (22, 27).

Mass Spectrometry—Gel-excised protein spots and protein bands were trypsin-digested and then analyzed by MALDI mass mapping on a Bruker Ultraflex TOF/TOF mass spectrometer (Bruker, Billerica, MA) as described (22, 28). For nano-HPLC MS/MS, proteins eluted from PPS8 columns were resolved by SDS-PAGE over a separation distance of only about 12 mm. After staining with Coomassie blue, the gel was cut into slices and the protein content in each fraction was estimated using the Pharmacia (Uppsala, Sweden) low molecular weight protein standard and BSA as the reference protein at a concentration of 1 µg per lane and AIDA software (raytest, Straubenhardt, Germany) for quantification. Protein digest and peptide isolation was carried out as for MALDI samples.

The separation of the peptide samples was performed using a bioclear Ultimate nano-HPLC system (Dionex, Sunnyvale, CA). Then 10 µl of each sample (containing up to 1 µg peptides) were injected, and peptides were purified and concentrated on a C₁₈-PepMap pre-column (0.3 mm inner diameter × 5 mm, 100 Å pore size, 3 µm particle size) at a flow rate of 30 µl/min in 0.1% TFA. Subsequently, peptides were separated on an analytical 75 µm inner diameter × 150 mm C₁₈-PepMap column (Dionex; 100 Å pore size, 3 µm particle size) at a column flow rate of 200 nl/min. The gradient (Solution A: 0.1% formic acid, 5% ACN; solution B: 0.1% formic acid, 80% ACN) started at 5% and ended at 60% B after 90 min.

MS and MS/MS data were acquired using a Q-TOF II mass spectrometer (Waters Corp., Micromass, Manchester, United Kingdom). Doubly and triply charged peptide-ions were automatically chosen by the MassLynx software and fragmented for a maximum of 18 s for each peptide. MS data were automatically processed and peaklists for protein identifications by database searches were generated by the MassLynx software. Database searches were carried out with an in-house MASCOT server using the NCBI protein database.

In Vitro Kinase Assays—Substrate phosphorylation reactions were linear with respect to time in all kinase activity assays and therefore represented the initial velocities. Kinase assays of p38x, RICK, GAK, and JNK2 were performed as described in the presence of the indicated PPS8 concentrations and 50 µM cold ATP (22). For K, determination, p38x was also assayed at 100, 150, and 200 µM ATP, and JNK2 was also tested at 5, 10, and 25 µM ATP. PP58 inhibition of Abl, Src, and EphB4 activities were assayed for 30 min at 30 °C in a reaction buffer containing 20 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 0.8 mM MnCl₂, 1 mM DTT, 0.1 mM EDTA, 100 µM sodium orthovanadate, 50 µM PP58, 2 µCi [γ-32P]ATP, and 0.2 mg/ml GST-Crk, 0.4 mg/ml myelin basic protein (MBP), or 0.2 mg/ml glyceraldehyde-3-phosphate dehydrogenase as kinase substrates, respectively.

MEK1 and Aurora A activities were tested at 37 °C in a total volume of 30 µl. The kinases were assayed according to the manufacturer’s protocol using 50 µM ATP and 1 µCi [γ-32P]ATP in the presence of different PPS8 concentrations. Kinase substrate proteins included were 0.25 mg/ml inactive GST-ERK2 for MEK1 and 0.025 mg/ml kinetide for Aurora A, respectively. Reactions, in which protein substrates were used to measure kinase activities, were stopped by addition of SDS sample buffer. After gel electrophoresis, phosphorylated substrate proteins were visualized by autoradiography and quantified by phosphoimaging. For quantification of Aurora A kinase-mediated peptide substrate phosphorylation, peptides were bound to Whatman P51 paper, washed, and 32P incorporation was then measured in a scintillation counter. Determination of IC₅₀ [0–100%] values was performed using GraFit software (Erithacus, Horley, Surrey, United Kingdom).

Cytokine Assays—Frozen human peripheral blood mononuclear cells (PBMCs) were purchased from Cambrex Bio Science (East Rutherford, NJ). Thawed and washed cells were resuspended in RPMI 1640 medium containing 1% FBS and then seeded into 24 well dishes (1×10⁵ cells per well). After 1 h, the medium was aspirated, the cell culture plates were washed with medium to remove nonadherent cells and fresh medium was added. The medium was exchanged again after a further 3.5 h prior to addition of either Me₂SO or different amounts of PPS8 and subsequent LPS (Sigma; serotype 055:B5) stimulation of the human PBMCs. Sixteen hours later, supernatants were harvested and analyzed using ELISA kits from R&D Systems to quantify the levels of TNF-α, macrophage inflammatory protein-1α (MIP-1α), and IL-8 released into the medium.
inhibitor and subsequent LPS or poly(I:C) treatment, confluent HFF cells were incubated with fresh DMEM containing 1% FBS for 30 min. After 16 h of ligand stimulation, IL-8 levels in the supernatants were measured. All cytokine assays were performed according to the manufacturer’s instructions.

U373 cell stimulations were also performed in DMEM/1% FBS, and total RNA was isolated 2 h after poly(I:C) treatment using the Trizol method (Invitrogen) followed by a DNase I digest on RNeasy columns (Qiagen, Hilden, Germany). Interferon-β (IFN-β) mRNA levels were determined by quantitative RT-PCR based on the 5′ exonuclease activity of Taq polymerase on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). RNA was reverse transcribed with Superscript II (Invitrogen) and oligo-dT primers according to the manufacturer’s protocol. Gene-specific TaqMan probes were labeled with FAMTM at the 5′ end and TAMRATM at the 3′ end of the oligonucleotides. Glyceraldehyde-3-phosphate dehydrogenase (determined with pre-developed TaqMan assay reagents (Giagen, Hilden, Germany). Interferon-β (IFN-β) mRNA levels were determined by quantitative RT-PCR based on the 5′ exonuclease activity of Taq polymerase on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). RNA was reverse transcribed with Superscript II (Invitrogen) and oligo-dT primers according to the manufacturer’s protocol. Gene-specific TaqMan probes were labeled with FAM™ at the 5′ end and TAMRATM at the 3′ end of the oligonucleotides. Glyceraldehyde-3-phosphate dehydrogenase (determined with pre-developed TaqMan assay reagents from Applied Biosystems) served as a housekeeping gene. Primer sequences for IFN-β were: 5′-GACATCCCTGAGGAGATTAAGCA-3′ (forward), reverse: 5′-GGAGCATCTCATAGATGGTCAATG-3′ (reverse), 5′-FAM-CGTCCTCCTTCTGGAACTGCTGCAG-TAMRA-3′ (TaqMan probe).

**RESULTS**

**Generation of a Functional Pyrido[2,3-d]pyrimidine Inhibitor Affinity Matrix—** Derivatives from the pyrido[2,3-d]pyrimidine class of compounds were originally identified as potent inhibitors of several protein tyrosine kinases such as the FGFR1 and Src (8). PD166285, one of the most potent and soluble analogues of this initial series (Fig. 1), was characterized in a variety of cellular assays and later found to block Wee1 kinase activity at nanomolar concentrations (7, 29). The search for alternative inhibitors of cellular Bcr-Abl activity led to the identification of pyrido[2,3-d]pyrimidine derivatives such as PD180970, which potently interfered with the tyrosine kinase activities of Abl and c-kit in the low nanomolar range (Fig. 1) (6, 9). The crystal structure of the Abl kinase domain in complex with a similar pyrido[2,3-d]pyrimidine compound demonstrated exposure of the inhibitors 2-anilino substituent at the protein surface (30). Based on these structural data, we reasoned that a previously described analogue exposes a primary amino function into the solvent and should therefore retain its protein kinase binding properties after covalent immobilization at this substituent. This inhibitor is shown in Fig. 1 and has been described as derivative 58 out of a series of pyrido[2,3-d]pyrimidines (8). We therefore refer to this compound as PP58 in this study. PP58 was then pegylated at the primary amino position. The resulting compound PP58peg “topologically” mimics immobilized PP58, which was obtained by covalent coupling to the free carboxyl groups of ECH Sepharose in the presence of carbodiimide (Fig. 1). Both PP58 and PP58peg were then tested in in vitro assays of Src kinase activity using MBP as a substrate protein (31). Importantly, pegylation of PP58 did not have a significant effect on the inhibitor’s potency to suppress Src kinase activity (Fig. 2A). Thus, extension of PP58 at its primary amine did not interfere with kinase binding, a result that identified this moiety as ideal site for covalent immobilization of the inhibitor. Notably, PP58 inhibited Src with a subnanomolar IC_{50} value in our assays, which is more than an order of magnitude below the IC_{50} value of 13 nM reported earlier (8). This discrepancy is most likely due to the much lower Src enzyme concentrations present in our assays. In our experiments, we observed that PP58 behaved as a titration reagent at higher Src protein concentrations. To measure Src activities at low enzyme concentrations, it was also necessary to analyze the phosphorylation of a protein substrate, which could be quantified after gel electrophoretic separation. This experimental design features a more favorable ratio of measurable kinase activity to background signal compared with filter binding assays of kinase substrate phosphorylation.

To test whether endogenously expressed Src kinase specifically interacts with the pyrido[2,3-d]pyrimidine affinity matrix, we subjected total cell lysates from HeLa cells to in vitro association with either control beads or PP58 beads in the absence or presence of free PP58 compound. As analyzed by
immunoblotting with specific antiserum, the PP58 matrix specifically depleted Src from total lysate, whereas binding to the PP58 beads was prevented when free inhibitor was included (Fig. 2B). To verify the functionality of the pyrido[2,3-d]pyrimidine matrix with a second known inhibitor target, we prepared total cell lysate from FGFR1-expressing COS-7 cells for in vitro binding studies. As shown in Fig. 2C, the ectopically expressed FGFR1 receptor tyrosine kinase was specifically retained on PP58 beads. In conclusion, these experiments established the PP58 matrix as a novel affinity reagent for the purification of cellular pyrido[2,3-d]pyrimidine inhibitor targets.

Purification of Cellular Pyrido[2,3-d]pyrimidine Inhibitor Targets by Affinity Chromatography—To characterize the cellular targets of the pyrido[2,3-d]pyrimidine inhibitor PP58 on a proteome-wide scale, we loaded total cell extract from 2.5 × 10⁸ HeLa cells on a PP58 affinity column using a protocol adapted from our previously described procedures (22, 23). After extensive washing, bound proteins were specifically released from the inhibitor column with running buffer containing free PP58 and ATP. Eluted proteins were then precipitated and two-thirds of the purified material were resolved by preparative 16-BAC/SDS-PAGE. Subsequent Coomassie staining allowed the detection of more than 50 protein spots (Fig. 3A), which were cut out from the gel and analyzed by mass fingerprinting using a MALDI-TOF/TOF mass spectrometer. Selected peptides were further characterized by PSD fragmentation. Strikingly, the majority of the excised spots contained protein kinases and almost 25 different members of this enzyme class could be identified from the preparative 16-BAC/SDS gel (Fig. 3A). A list of the characterized protein spots is shown in Table I. Among the identified cellular targets were several cytoplasmatic tyrosine kinases such as FAK, TEC, JAK1, Fer, the Src-related kinase Yes, and, represented by one of the most prominent spots, CSK, which negatively regulates Src family enzymes through phosphorylation of a conserved C-terminal tyrosine residue (32). Although Src itself specifically bound to the PP58 matrix as shown above, it was not identified from the gel. Most likely, Src co-migrates with its more abundant relative Yes and is therefore covered by the rather prominent Yes protein spot. Interestingly, Abl tyrosine kinase, a known pyrido[2,3-d]pyrimidine inhibitor target, could be identified in the high molecular weight region of the gel. In addition to cytoplasmatic tyrosine kinases, the MS data also revealed the receptor tyrosine kinases EphB2, EphB4, and Ron as pyrido[2,3-d]pyrimidine-interacting proteins. Taken together, these identifications of various new tyrosine kinase targets of pyrido[2,3-d]pyrimidines support the concept that derivatives from this compound class are rather broadly active tyrosine kinase inhibitors.

Surprisingly, the perhaps most prominent protein spot on the gel was not a tyrosine kinase but instead represented the Ser/Thr protein kinase p38 (Fig. 3A). In addition to p38, we identified several other Ser/Thr kinases involved in mitogen-activated protein kinase (MAPK) signaling. Furthermore, MS revealed RICK (also known as Rip2 or CARDIAK), GAK and PKN3 as cellular targets. These Ser/Thr kinases were also identified in an earlier study, in which we used the same proteomic strategy to characterize the cellular targets of the p38 inhibitor SB 203580. From these results, it appears that
pyrido[2,3-d]pyrimidine and pyridinyl imidazole inhibitors have an overlapping set of cellular protein kinase targets. Most of the other analyzed protein spots represented abundant cellular protein species not belonging to the superfamily of protein kinases. Although several of these identified proteins also bind nucleotides such as ATP or NADH and could therefore be potential PP58 targets, their presence in the elution fraction was most likely due to their high cellular abundance that compensated for a rather weak affinity of these proteins for the PP58 matrix (Fig. 3A, Table I). To test this assumption for one of the nonprotein kinase targets, we analyzed the activity of lactate dehydrogenase in vitro and did not detect any significant inhibitory effect of 100 μM PP58 on this enzyme (data not shown).

In addition, we performed nanoflow reversed-phase HPLC-MS/MS analysis using a Q-TOF mass spectrometer. For this purpose, smaller aliquots of the cellular target protein fraction purified by PP58 affinity chromatography were resolved over a short distance in a SDS gel. After incubation of gel slices with trypsin, the eluted peptides were subjected to LC-MS/MS analysis. This approach revealed 10 additional cellular protein kinase targets of the pyrido[2,3-d]pyrimidine PP58 such as the tyrosine kinases Fyn, Lyn, BLK, DDR2, and EphA2. Protein kinase identifications from these experiments are shown in Table II. Interestingly, PP58 affinity chromatography led to the identification of protein kinases belonging to various different groups and families, indicating that the pyrido[2,3-d]pyrimidine inhibitor is not selective for a set of phylogenetically related members of the human kinome (Tables I and II). Moreover, several kinases found in the 16-BAC/SDS gel were not detected by LC-MS/MS and vice versa. Thus, even when highly enriched subfractions of the proteome are analyzed as in our study, these two different proteomic approaches delivered complementary sets of results. This observation is in accordance with various studies in which both two-dimensional gel-MS and LC-MS/MS identification strategies have been applied to more complex protein mixtures such as total cell extracts (33, 34).

Finally, we performed a second elution step using SDS-containing buffer to release any remaining bound proteins from the PP58 affinity column. Subsequent SDS-PAGE revealed a few Coomassie-stainable protein bands and the protein kinases Yes, RICK, Wee1, and p38α could be identified by MS (Fig. 3B). With the exception of Wee1, all of these...
| Spot no. | Protein name                                           | Gi no.  | $M_r$ [Da] | Group/family$^b$ |
|---------|--------------------------------------------------------|---------|------------|-----------------|
| 1       | Methylthioadenosine phosphorylase                      | 4505273 | 31,250     | –               |
| 2       | Not identified                                         | –       | –          | –               |
| 3       | HSP27                                                 | 662841  | 22,327     | –               |
| 4       | Lactate dehydrogenase                                  | 13788647| 36,507     | –               |
| 5       | Protein phosphatase 4                                   | 4506027 | 36,080     | –               |
| 6       | Pyridoxal kinase                                       | 13543317| 31,808     | –               |
| 7       | Glyceraldehyde-3-phosphate dehydrogenase               | 71645   | 36,054     | –               |
| 8       | p38                                                    | 4503069 | 41,493     | CMGC/MAPK       |
| 9       | ERK2                                                   | 20986531| 41,390     | CMGC/MAPK       |
| 10      | JNK1                                                   | 20986519| 42,022     | CMGC/MAPK       |
| 11      | ERK1                                                   | 186696  | 42,106     | CMGC/MAPK       |
| 12      | JNK2                                                   | 1463129 | 44,024     | CMGC/MAPK       |
| 13      | MEK1                                                   | 5579478 | 43,439     | STE/STE7        |
| 14      | Aurora A                                               | 27923855| 45,809     | Other/AUR       |
| 15      | Aurora A                                               | 27923855| 45,809     | Other/AUR       |
| 16      | Aurora A                                               | 27923855| 45,809     | Other/AUR       |
| 17      | Aurora A                                               | 27923855| 45,809     | Other/AUR       |
| 18      | CSK                                                    | 4758078 | 50,704     | TK/Csk          |
| 19      | Eukaryotic translation elongation factor 1α            | 4503471 | 50,140     | CMGC/MAPK       |
| 20      | JNK2                                                   | 1463129 | 44,029     | CMGC/MAPK       |
| 21      | MYT1                                                   | 2914671 | 53,992     | Other/WEE       |
| 22      | Yes                                                    | 4885661 | 60,801     | TK/Src          |
| 23      | Yes                                                    | 4885661 | 60,801     | TK/Src          |
| 24      | Yes                                                    | 4885661 | 60,801     | TK/Src          |
| 25      | Pyruvate kinase 3                                      | 3296418 | 57,937     | –               |
| 26      | RICK                                                   | 4506537 | 61,195     | TKL/RIPK        |
| 27      | Not identified                                          | –       | –          | –               |
| 28      | TEC                                                    | 4507429 | 73,629     | TK/Tec          |
| 29      | Heat shock 70-kDa protein 8                            | 5729877 | 70,898     | –               |
| 30      | Heat shock 70-kDa protein 9B                            | 24234688| 73,680     | –               |
| 31      | Moesin                                                 | 14625824| 61,872     | –               |
| 32      | Heat shock 70-kDa protein 5                            | 16507237| 72,333     | –               |
| 33      | Radixin                                                | 4506467 | 68,564     | –               |
| 34      | Ezrin                                                  | 21614499| 69,413     | –               |
| 35      | MARK2                                                  | 9845487 | 83,205     | CAMK/CAMKL      |
| 36      | Heat shock 90-kDa protein 1β                            | 20149594| 83,264     | –               |
| 37      | Heat shock 90-kDa protein 1β                            | 20149594| 83,264     | –               |
| 38      | HRI                                                    | 7839458 | 71,315     | Other/PEK       |
| 39      | Fer                                                    | 4885231 | 94,624     | TK/Fer          |
| 40      | KHS1                                                   | 30316147| 95,040     | STE/STE20       |
| 41      | Heat shock protein gp96                                | 15010550| 90,194     | –               |
| 42      | KHS2                                                   | 25987447| 94,954     | STE/STE20       |
| 43      | PKN3                                                   | 40254851| 99,421     | AGC/PKN         |
| 44      | PKN3                                                   | 40254851| 99,421     | AGC/PKN         |
| 45      | FAK                                                    | 24476013| 119,233    | TK/Fak          |
| 46      | JAK1                                                   | 4504803 | 131,957    | TK/JakA         |
| 47      | EphB4                                                  | 16209618| 108,270    | TK/Eph          |
| 48      | EphB2                                                  | 17975765| 117,493    | TK/Eph          |
| 49      | Abl                                                    | 514268  | 124,955    | TK/Abl          |
| 50      | GAK                                                    | 4885251 | 143,165    | Other/NAK       |
| 51      | GAK                                                    | 4885251 | 143,165    | Other/NAK       |
| 52      | Ron                                                    | 4505265 | 152,227    | TK/Met          |
| 53      | Carbamoylphosphate synthetase 1                        | 21361331| 164,939    | –               |
| 54      | GCN2                                                   | 7243057 | 169,235    | Other/PEK       |
| 55      | Not identified                                          | –       | –          | –               |
| 56      | Not identified                                          | –       | –          | –               |

$^a$ NCBI GenBank™ accession number.

$^b$ Classification of the identified protein kinases into groups and families according to Manning et al. (17).
Protein Kinase Targets of Pyrido[2,3-d]pyrimidine Inhibitors

Protein kinase targets identified by LC-MS/MS

| Protein name | Gi no. | Mr [Da] | Group/family |
|--------------|--------|---------|--------------|
| Abl          | 514268 | 124,955 | TK/Abl       |
| Arg          | 6382062| 128,343 | TK/Abl       |
| BLK          | 914204 | 57,757  | TK/Src       |
| CSK          | 4758078| 50,704  | TK/Csk       |
| DDR2         | 5453814| 96,752  | TK/DDR       |
| EphA2        | 125333 | 108,254 | TK/Eph       |
| EphB2        | 12644190| 117,507 | TK/Eph       |
| EphB4        | 16209618| 108,270 | TK/Eph       |
| ERK1         | 232066 | 43,136  | CMGC/MAPK    |
| ERK2         | 23879  | 40,420  | CMGC/MAPK    |
| Fer          | 4885231| 94,624  | TK/Fer       |
| Fyn          | 4503823| 60,762  | TK/Src       |
| GAK          | 4885251| 143,165 | TK/Src       |
| JNK1         | 20986519| 44,022 | CMGC/MAPK    |
| JNK2         | 1082266| 48,149  | CMGC/MAPK    |
| Lyn          | 187271 | 56,033  | TK/Src       |
| MEK1         | 5579478| 43,439  | STE/STE7     |
| MEK2         | 13489054| 44,424 | STE/STE7     |
| MYT1         | 2914671| 53,992  | Other/WEE    |
| NEK2         | 31807297| 37,956 | Other/NEK    |
| p38α         | 2499600| 41,293  | CMGC/MAPK    |
| p38β         | 20128774| 41,357 | CMGC/MAPK    |
| RICK         | 4506537| 61,195  | TKL/RICK     |
| Yes          | 4885661| 60,801  | TK/Src       |
| ZAK          | 7542537| 91,264  | TKL/MLK      |

Note: NCBI GenBank accession number.

Classification of the identified protein kinases into groups and families according to Manning et al. (17).

In Vitro Characterization of Pyrido[2,3-d]pyrimidine Inhibitor Targets—To verify the MS results with a secondary assay, total cell extracts from HeLa cells were used for in vitro association studies with either control matrix or PP58 matrix with or without free pyrido[2,3-d]pyrimidine compound added to the incubations. Total cell lysate, nonbound proteins from the supernatant fractions and proteins retained by the affinity beads were then resolved by gel electrophoresis and subjected to immunoblotting with specific antibodies against protein kinases identified by MS (Fig. 4). All of the analyzed kinases could be confirmed as specific binders of the PP58 matrix. As a negative control, we probed for the Ser/Thr kinase SRPK1, which is expressed in HeLa cells and did not interact with the PP58 affinity material (Fig. 4).

Although the different PP58 kinase targets were more or less quantitatively depleted from the lysate by the inhibitor beads, this type of assay does not yield quantitative data about their respective sensitivities to PP58. To address this important issue, we performed in vitro kinase assays for the three tyrosine kinases Src, Abl, and EphB4 and the six Ser/Thr kinases p38α, RICK, GAK, MEK1, JNK2, and Aurora A in the

Fig. 4. Confirmation of MS results by immunoblot analysis. Total cell lysate from HeLa cells was subjected to in vitro association with either control matrix or the PP58 matrix. Free PP58 was added to the incubations where indicated. Total lysate, nonbound proteins from the supernatants and bound proteins eluted from the beads were separated by SDS-PAGE and immunoblotted with specific antibodies against Abl, Yes, FAK, CSK, GAK, RICK, p38, JNK1/2, ERK1/2, MEK1, Wee1, Aurora A, Lyn, and SRPK1.
presence of different PP58 concentrations and 50 μM cold ATP. With the exception of the EphB4 receptor tyrosine kinase, we could determine the IC50 values of kinase activity inhibition by PP58 for all of the recombinant enzymes tested. In the case of EphB4, the IC50 for PP58 inhibition is below 10 nM, but could not be exactly determined due to the relatively low specific activity of the enzyme preparation used. Strikingly, we found that not only tyrosine kinases such as Src and Abl were potently inhibited by PP58 with subnanomolar IC50 values, but also identified the Ser/Thr kinases p38, RICK, and GAK as highly sensitive to inhibition by the pyrido[2,3-d]pyrimidine derivative (Fig. 5A). RICK showed the lowest IC50 value of only 0.18 nM with respect to PP58 inhibition. In stark contrast, 1,000- to 10,000-fold higher PP58 concentrations were required for half maximal inhibition of the Ser/Thr kinases MEK1, JNK2, and Aurora A (Fig. 5A). This dramatically lower sensitivity to PP58 correlated with the presence of a larger methionine or leucine side chain at a position where a smaller threonine residue is found in the potently inhibited kinases (Fig. 5A). As earlier studies demonstrated for Abl (10, 30) and was recently shown by us for tyrosine kinase targets of pyrido[2,3-d]pyrimidine inhibitors such as Src (26), substitution of this critical threonine with a larger amino acid apparently interferes with the extension of the inhibitor’s dichlorophenyl moiety into a hydrophobic cavity at the nucleotide-binding pocket and thereby strongly interferes with the binding of the ATP-competitive pyrido[2,3-d]pyrimidine inhibitors to these kinase targets. To test whether this structural determinant is also relevant for the highly sensitive Ser/Thr kinases, we mutated the equivalent threonine of GAK to methionine and performed kinase assays with both wild-type and mutant enzyme in the presence of PP58. As shown in Fig. 5B, the Thr-123 to methionine mutant of GAK was resistant to PP58 concentrations that completely suppressed the activity of wild-type enzyme. We further quantified the affinity of PP58 for p38 and JNK2 and therefore determined the respective Ki value of PP58 for each kinase. These PP58 targets are closely related kinases but differ in the amino acid residue critical for PP58 sensitivity (Thr-106 in p38 compared with Met-108 in JNK2). The Ki values of PP58 for p38 and JNK2 were 3.8 ± 1.9 nM and 0.32 ± 0.04 μM, respectively, indicating an about 100-fold higher affinity of the inhibitor for p38 than for JNK2. Taken together, our results indicate that the presence of a small residue such as threonine at the relevant site is a structural determinant conserved among high-affinity targets of pyrido[2,3-d]pyrimidine inhibitors. However, it is noteworthy that the PP58 affinity matrix also serves as an efficient purification reagent for a variety of protein kinases, which lack this structural feature and have much lower affinities for the pyrido[2,3-d]pyrimidine inhibitor PP58.

**Cellular Inhibition of p38 and RICK Kinase Activities by the Pyrido[2,3-d]pyrimidine PP58.** Pyrido[2,3-d]pyrimidine compounds were developed as tyrosine kinase inhibitors for cancer treatment. Our findings that a pyrido[2,3-d]pyrimidine derivative effectively targeted the Ser/Thr kinases p38 and RICK, which are both established signal transducers of inflammatory cellular responses.
Protein Kinase Targets of Pyrido[2,3-d]pyrimidine Inhibitors

responses, prompted us to study the effect of PP58 on their kinase activities in intact cells (35–37). To monitor cellular p38 activity, we analyzed the phosphorylation of its direct cellular substrate MAPKAP-K2 by immunoblotting with phospho-specific antiserum (38). Activation of p38 by anisomycin treatment triggered MAPKAP-K2 phosphorylation at Thr-344, and this stimulation was inhibited by PP58 in a dose-dependent manner with a cellular IC_{50} value somewhat below 10 nM (Fig. 6A). Immunoblot analysis with MAPKAP-K2 protein-specific antibody showed the anisomycin-induced band shift of MAPKAP-K2 due to phosphorylation of the protein at multiple sites. This apparent change in molecular weight was also reverted by PP58, albeit at slightly higher concentrations than phosphorylation at Thr-344. In contrast, up to 100 nM PP58 had no effect on the epidermal growth factor (EGF)-stimulated phosphorylation of Rsk1, which is a downstream signaling event mediated through the ERK MAPK cascade. This control experiment illustrates that PP58 can exhibit some degree of selectivity at low nanomolar concentrations in vivo.

To investigate the effect of PP58 on RICK activity in intact cells, we performed an assay in which a kinase-inactive RICK fragment serves as a cellular phosphorylation substrate for co-expressed, wild-type enzyme. As shown in Fig. 6B, dose-dependent in vivo inhibition of RICK activity by PP58 was observed, with as low as 30 nM of the inhibitor already reducing the cellular RICK substrate phosphorylation by more than 50%.

Cellular Inhibition of Cytokine Production by the Pyrido[2,3-d]pyrimidine Compound PP58—Our identification of p38 and RICK as cellular targets of pyrido[2,3-d]pyrimidine inhibitors raised the issue whether compounds such as PP58 might suppress inflammatory responses in addition to their cell growth-inhibitory properties. To test this, we first analyzed the effect of PP58 on LPS-induced TNF-α release in primary human PBMCs (39). As seen in Fig. 7A, LPS-stimulated TNF-α production was potently inhibited by PP58 with a cellular IC_{50} value of around 3 nM. This effect might be functionally linked to the PP58-mediated suppression of cellular MAPKAP-K2 phosphorylation shown in Fig. 6A, because genetic evidence has established this cellular target of p38 kinase as essential transcriptioner of LPS-stimulated TNF-α biosynthesis (40). In addition to TNF-α, we measured the levels of the chemokines MIP-1α and IL-8. LPS-regulated biosynthesis of MIP-1α was also efficiently suppressed by PP58 with an IC_{50} of about 15 nM in human PBMCs, whereas IL-8 production was less susceptible to the pyrido[2,3-d]pyrimidine and 100 nM of PP58 reduced the release of this cytokine by about 50%. Compared with PBMCs, the inhibitory effect of 100 nM PP58 on LPS-induced IL-8 production was somewhat more pronounced in human foreskin fibroblasts (Fig. 7B). As further shown in Fig. 7B, PP58 also interfered with IL-8 biosynthesis upon exposure of cells to the synthetic double-stranded RNA analogue poly(I:C), which acts on toll-like receptor 3 and triggers cellular signaling in a way similar to viral infection (41). The cellular effects of PP58 on cytokine secretion were not a result of general compound toxicity, because up to 100 nM of the inhibitor did not significantly affect cell viability in our assays (data not shown). One of the hallmarks of antiviral responses is the induction of interferons such as IFN-β. Using U373 astrocytoma cells, we investigated the effect of PP58 on poly(I:C)-triggered IFN-β gene induction by quantitative RT-PCR (42). Interestingly, pyrido[2,3-d]pyrimidine concentrations as low as 10 nM strongly impaired the synthesis of IFN-β mRNA. Taken together, our results establish the pyrido[2,3-d]pyrimidine PP58 as a small molecule antagonist of innate immune responses with potent anti-inflammatory properties.

DISCUSSION

Although inhibitors belonging to the pyrido[2,3-d]pyrimidine class of compounds were known to act on several protein kinases, all of these previously described targets belonged to
the tyrosine kinase family and it therefore appeared that pyrido[2,3-d]pyrimidine inhibitors are selective for this particular group of the human kinome (6–9). The results presented here identify several Ser/Thr kinases as highly sensitive pyrido[2,3-d]pyrimidine targets, and, moreover, these protein kinases belong to different groups of the human kinome. Thus, sensitivity to pyrido[2,3-d]pyrimidine inhibition does not closely correlate with phylogenetic relationship (17). Instead, a small residue at the ATP binding pocket, usually a threonine, is conserved in all of the highly sensitive kinases. This structural determinant permits the projection of the dichlorophenyl substituent of the inhibitor into a hydrophobic pocket adjacent to the ATP binding site. At the ATP binding site itself, other parts of the inhibitor such as its pyrido[2,3-d]pyrimidine core structure are bound to the same region as the adenine moiety of ATP (26). Importantly, the hydrophobic pocket does not interact with any part of a kinase-bound ATP molecule, explaining why the substitution of the critical threonine with a larger residue such as methionine sterically interferes with inhibitor binding, but does not abrogate protein kinase activity. The relevance of this structural motif was previously documented for tyrosine kinases and also applies to Ser/Thr kinases, as shown for GAK by mutational analysis (10, 26). However, our affinity method also resulted in the efficient purification of several targets, mostly Ser/Thr kinases, which had a large hydrophobic residue such as methionine at the critical position. Consistent with the sterical clash conferred by such a bulky side chain, these kinases were inhibited by the pyrido[2,3-d]pyrimidine PP58 with micromolar IC50 values and not with the low nanomolar or even subnanomolar IC50 values determined for kinases such as p38, RICK, and GAK. These secondary assays in combination with Ki determinations for p38 and JNK2 confirmed that the pyrido[2,3-d]pyrimidine inhibitor beads permitted the enrichment of protein kinases with dramatically different relative affinities to the inhibitor. The efficient purification of target kinases that are only affected in the presence of micromolar inhibitor concentrations in vitro further indicates that the covalent linkage of PP58 to the matrix did not result in steric hindrance with respect to kinase binding, and this conclusion is consistent with GraFit. Compared with control-treated monocytes, LPS stimulated the release of TNF-α, MIP-1α, and IL-8 by about 75-, 25-, and 5-fold, respectively. Cytokine levels in the supernatant from LPS-treated cells were 12 ng/ml (TNF-α), 70 ng/ml (MIP-1α), and 247 ng/ml (IL-8). B, HFF cells were pretreated with Me2SO or the indicated PP58 concentrations for 15 min and then stimulated with either 50 μg/ml poly(I:C) or 1 μg/ml LPS. After 16 h, IL-8 levels in the cell culture supernatants were measured by ELISA. C, following preincubation with the indicated PP58 concentrations for 15 min, U373 astrocytoma cells were stimulated for 2 h with 50 μg/ml poly(I:C) prior to lysis and RNA isolation. After reverse transcription, quantitative PCR was performed to measure IFN-β gene induction in control incubated and poly(I:C)-treated cells. Transcript levels in IFN-β-treated cells were set to 100% and the levels upon inhibitor treatment are shown relative to this value.
with the data from Src kinase assays obtained with the pegylated inhibitor derivative. To our knowledge, the pyrido[2,3-d]pyrimidine inhibitor matrix allowed the purification and identification of more human protein kinases than found in any previous proteomic study. Thus, the PP58 resin provides a useful reagent to compare kinase expression levels of a significant subset of the kinome. Potential applications of this new biochemical tool involve expression analysis of potential drug targets in different human tumor cells. Such an experimental approach could lead to important findings, as relevant oncogenic protein kinases are frequently present at elevated levels in human cancer (43, 44).

A significant outcome of the present study is the identification of p38 and RICK as pyrido[2,3-d]pyrimidine inhibitor targets in the context of the potent anti-inflammatory effects observed for the derivative PP58, for the following reasons: The pyrido[2,3-d]pyrimidine PD166285 was reported to have good oral bioavailability in rats by Klutschko et al. (8). Strikingly, PD166285 was converted via an intermediate into a final metabolite, which then was detected at stable plasma concentrations of more than 100 nm over at least 24 h. This stable metabolite was the PP58 derivative used in this study. Given the potent inhibition of LPS-induced TNF-α biosynthesis observed in primary monocytes at much lower PP58 concentrations in vitro, an obvious speculation is that similar anti-inflammatory effects should be seen in animals upon oral application of pyrido[2,3-d]pyrimidines such as PD166285. This remains to be tested. In a sense, positive results from such an animal study would validate our chemical proteomic approach as a valuable tool to link previously unrecognized therapeutic areas to existing drugs.

A major concern with the use of pyrido[2,3-d]pyrimidines such as PD166285 or the PP58 derivative is their apparent lack of specificity. However, there are data from earlier medicinal chemistry studies demonstrating that the selectivity of pyrido[2,3-d]pyrimidine-based compounds can be improved. For example, a 3,5-dimethoxyphenyl instead of the 2,6-dichlorophenyl functionality present in the 6-position was shown to dramatically increase the selectivity of pyrido[2,3-d]pyrimidines for the FGFR (45, 46). These derivatives were then inactive against other targets such as Src. Thus, there seems to be potential for optimizing this potent compound class for other targets such as p38 kinase, which could ultimately result into the development of novel drugs for the treatment of human disease.

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