Proteome-wide Identification of Cellular Targets Affected by Bisindolylmaleimide-type Protein Kinase C Inhibitors*

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Bisindolylmaleimide compounds such as GF109203X are potent inhibitors of protein kinase C (PKC) activity. Although bisindolylmaleimides are not entirely selective for PKC and are known to inhibit a few other protein kinases, these reagents have been extensively used to study the functional roles of PKC family enzymes in cellular signal transduction for more than a decade. Here, we establish a proteomics approach to gain further insights into the cellular effects of this compound class. Functional immobilization of suitable bisindolylmaleimide analogues in combination with specific purification of cellular binding proteins by affinity chromatography led to the identification of several known and previously unknown enzyme targets. Subsequent in vitro binding and activity assays confirmed the protein kinases Ste20-related kinase and cyclin-dependent kinase 2 (CDK2) and the non-protein kinases adenosine kinase and quinone reductase type 2 as novel targets of bisindolylmaleimide inhibitors. As observed specifically for CDK2, minor chemical variation of the ligand by immobilizing the closely related bisindolylmaleimides III, VIII, and X dramatically affected target binding. These observed changes in affinity correlated with both the measured IC_{50} values for in vitro CDK2 inhibition and results from molecular docking into the CDK2 crystal structure. Moreover, the conditions for affinity purification could be adapted in a way that immobilized bisindolylmaleimide III selectively interacted with either PKCα or ribosomal S6 protein kinase 1 only after activation of these kinases. Thus, we have established an efficient technique for the rapid identification of cellular bisindolylmaleimide targets and further demonstrate the comparative selectivity profiling of closely related kinase inhibitors within a cellular proteome. *Molecular & Cellular Proteomics 3:490–500, 2004.

The protein kinase C (PKC) family of serine/threonine kinases comprises at least 11 isozymes, which can be divided into three subgroups based on biochemical criteria such as differential co-factor requirements. Classical PKCs (α, β1, β2, and γ) depend on both diacylglycerol (DAG) and Ca^{2+} for kinase activation, novel PKCs (δ, ε, η, θ, and μ) require only DAG, and the activity of the atypical PKCs (ζ and η) is regulated by neither of the two co-factors (1). Individual PKC isozymes have been implicated in various aspects of cellular physiology involving biological processes as diverse as cell proliferation and differentiation, apoptosis, ion channel regulation, glycolysis, and protein secretion (2, 3). Moreover, different PKCs have been implicated as potential targets for therapeutic intervention in various diseases including several types of cancer and heart failure (4, 5).

In the majority of previous studies, analysis of PKC function in cultured cells has been performed with pharmacological tools that either stimulate or interfere with its cellular activity. Phorbol esters such as phorbol myristate acetate bind with high affinity to the C_{1}-domain of classical and novel PKC isozymes and thereby induce kinase activity through the same membrane translocation mechanisms as the endogenous C_{1}-interacting ligand DAG. Small molecule inhibitors of PKC either interfere with C_{1}-domain-mediated kinase activation (e.g. Calphostin C) or directly block PKC kinase activity through ATP-competitive interaction with the nucleotide-binding pocket. Inhibitors belonging to the bisindolylmaleimide class of compounds fall into the second category. GF109203X, the most popular of these bisindolylmaleimide inhibitors of PKC activity, has been used in numerous studies contributing to more than 1000 published articles about cellular PKC function (6). Due to this widespread application of PKC inhibitors such as GF109203X, their selectivity is a highly relevant issue for many experimental studies and has previously been examined by parallel activity measurements of different recombinant protein kinases in the presence of bisindolylmaleimide compounds (7). Importantly, as well documented in the comprehensive selectivity study by Cohen and colleagues, various...
bisindolylmaleimide derivatives blocked the activities of the ribosomal S6 protein kinase 2 (Rsk2) and its close relative mitogen and stress-activated protein kinase 1 as potently as PKCa in vitro. Moreover, some other kinases including glycogen synthase kinase 3 (GSK3) and p70 ribosomal S6 protein kinase (S6K) were also inhibited, albeit to a lesser extent. In addition to these alternative kinase targets, GF109203X was reported as potent inhibitor of voltage-dependent sodium channels and the 5-hydroxytryptamine3 receptor (8, 9). But, as these previous studies only covered a tiny fraction of the potential bisindolylmaleimide targets expressed in the proteome of a mammalian cell or organism, tools for a more comprehensive and less biased analysis of PKC inhibitor selectivity are needed.

To study kinase inhibitor selectivity on a proteome-wide scale, affinity purification methods combined with mass spectrometry have the potential to reveal the relevant cellular drug targets in cases where suitable compound derivatives can be obtained for immobilization on chromatography beads. In several earlier studies, this straightforward “chemical proteomics” approach has been employed and could deliver some additional information about the proteins targeted by small molecule inhibitors (10–13). We have recently established an efficient proteomics method to identify the cellular targets of the p38 kinase inhibitor SB 203580 (14). In this study, affinity chromatography was performed under highly optimized biochemical conditions and led to the identification of a set of previously unknown SB 203580 targets, demonstrating that this widely used reagent is far less selective for p38 than assumed (14).

Here, we have adapted this proteomics approach to identify the cellular targets of bisindolylmaleimides. In addition to protein kinases known to be affected by these compounds, we found various other specifically interacting enzymes and could verify them by in vitro binding and activity assays as novel targets of bisindolylmaleimide inhibitors. Interestingly, cyclin-dependent kinase 2 (CDK2) interaction with bisindolylmaleimides was dramatically affected through subtle chemical variation of the immobilized inhibitors. This differential binding could be correlated with the derivatives’ in vitro potency toward CDK2 kinase activity and their respective affinities according to molecular docking analysis.

**EXPERIMENTAL PROCEDURES**

**Reagents and Plasmids**—Cell culture media and LipofectAMINE were purchased from Invitrogen (San Diego, CA). Radiochemicals, epoxy-activated Sepharose 6B and protein G Sepharose were obtained from Amersham Biosciences (Uppsala, Sweden). 2-[1-3-Aminopropyl]-1H-indol-3-yl)maleimide (BisIII), 2-[1-3-amino(propyl)-1H-indol-3-yl)maleimide (BisVIII), 2-[1-3-amino(propyl)-1H-indol-3-yl)maleimide (BisXII), 2-[1-3-amino(propyl)-1H-indol-3-yl)maleimide (BisIII), 2-[1-3-amino(propyl)-1H-indol-3-yl)maleimide (BisVIII), 2-[1-3-amino(propyl)-1H-indol-3-yl)maleimide (BisXII), 2-[1-3-amino(propyl)-1H-indol-3-yl)maleimide (BisIII), and histone H1 were obtained from Calbiochem (La Jolla, CA). 3-(4,5-dimethoxyphenyl-2-yl)-2,5-diphenyltetrazolium (MTT) was obtained from Sigma (St. Louis, MO). NADH was purchased from Roche (Basel, Switzerland). Menadion was obtained from Calbiochem. All other reagents were obtained from Sigma. Antibodies used in this study were: rabbit polyclonal anti-Rsk1, mouse monoclonal anti-GSK3α/β, rabbit polyclonal anti-CDK2 (all from Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-p38 antibody (Cell Signaling Technology, Beverly, MA), rabbit polyclonal anti-RICK antibody (Affinity BioReagents, Golden, CO), mouse monoclonal anti-PKCα (Upstate Biotechnology, Lack Placid, NY), mouse monoclonal anti-FLAG clone M2 (Sigma), and mouse monoclonal anti-c-myc clone 9E10 (Upstate Biotechnology). Human CDK2/cyclinA was purchased from Upstate Biotechnology. The DNA sequences coding for full-length Ste20-related kinase (SLK) and full-length adenosine kinase (AK) were PCR-amplified from human spleen and liver cDNA libraries. The SLK cDNA was fused to a N-terminal FLAG-tag, whereas the AK cDNA was fused to a N-terminal myc-tag and cloned into a pRK5 expression vector (15–17).

**Normalisation of the BisIII, BisVIII, and BisX Concentrations**—All three bisindolylmaleimide compounds were dissolved in 100% dimethylsulfoxide. Stocks were spectrophotometrically normalized (Spectramax Plus 384; Molecular Devices, Sunnyvale, CA) using their absorption maxima at 372 nm and 460 nm and stored under argon at −20 °C in the dark.

**Immunoblotting of Bisindolylmaleimides**—For immobilization, drained epoxy-activated Sepharose 6B was resuspended in 2 volumes of 20 mM BisIII, BisVIII, or BisX dissolved in 50% dimethylformamide/0.1 M Na2CO3, pH 11. After adding of 10 mM NaOH, coupling was performed overnight at 30 °C in the dark. After three washes with 50% dimethylformamide/0.1 M Na2CO3, remaining reactive groups were blocked with 1 mM ethanolamine, pH 11. Subsequent washing steps were performed according to the manufacturer’s instructions. To generate the control matrix (Ctrl), epoxy-activated Sepharose 6B was directly reacted with 1 mM ethanolamine pH 11 and equally treated as described above. The matrices were stored at 4 °C in the dark.

**Cell Culture**—COS-7, HeLa, or HuH-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. COS-7 cells were transiently transfected as previously described (15). Epidermal growth factor (EGF) stimulation of starved HeLa cells was carried out as described (18).

**Cell Lysis and in Vitro Association Experiments**—COS-7 and HeLa cells were usually lysed in Triton X-100 lysis buffer (TL-buffer) containing 50 mM HEPS, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 1 mM EGTA, 3 mM CaCl2 plus additives (10 mM sodium fluoride, 1 mM orthovandate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM diithiothreitol) and co-factors (100 μg/ml phosphatidylserine (PS), 20 μg/ml DAGI). For analytical in vitro association experiments, lysates were preclarified by centrifugation and equilibrated to 1 mM NaCl. Then 300 μl of the high salt lysate were incubated together with 20 μl of drained bisindolylmaleimide matrix (Bis matrix) for 3 h at 4 °C. Afterward, the Bis matrices were washed twice with 500 μl of TL-buffer plus 1 mM NaCl and once with 500 μl of TL-buffer; both steps were performed without additives, and the co-factor concentrations were reduced to one-tenth. In the experiments designed to detect specific SLK and AK binding to BisIII beads, CaCl2 and the co-factors were not included in the lysis and wash buffers. When the stimulation-dependent binding of Rsk1 to BisIII beads was analyzed, CaCl2 and the co-factors were omitted from the lysis and wash buffers and the NaCl concentration was kept at 150 mM. For activation-dependent detection of PKCa binding to HuH-7 cells, the lysis buffer was 20 mM HEPS, pH 7.5, 150 mM NaCl, 0.25% Triton X-100, 0.1 mM EDTA, 0.2 mM EGTA plus additives. Where indicated, 5 mM CaCl2 plus co-factors were added. Co-factor concentrations were also reduced to one-tenth in the respective washing buffer, which was lysis buffer without additives. Bound proteins were eluted by boiling of the affinity beads in 1.5x SDS sample buffer. After SDS-PAGE, proteins were transferred to nitrocellulose membrane and immunoblotted with the corresponding anti-
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tibodies. The preparative binding experiments using 2.5 \times 10^9 cells, 16-benzyl(dimethyl-n-hexadecyl)ammonium chloride (16-BAC)/SDS-PAGE separation and mass spectrometric analysis were carried out essentially as described, with the modification that 3 mM CaCl_2, 100 µg/ml PS, and 20 µg/ml DAG were included and glycerol was omitted from the lysis buffer (14). Moreover, the BisIII column was washed with buffer containing 3 mM CaCl_2, 20 µg/ml PS, and 4 µg/ml DAG after sample loading.

K_i Determination of BisIII for the Human Oxidoreductase NQO2—
Enzyme activity and inhibition were determined spectrophotometrically (Spectramax Plus 384; Molecular Devices) by measuring the reduction of MTT at 610 nm and 30 °C. In this assay, we used NADH as an electron donor for the menadion reduction and MTT for the continuous re-oxidation of menadion. The reactions (200 µl) were performed in 96-well plates, containing 50 mM K_2H.PO_4, pH 7.5, 1 µl of quinone-reductase type 2 (NQO2), 40 µM menadion, 200 µM MTT, and increasing concentrations of NADH (0–1000 µM) in the presence of different fixed BisIII concentrations (0, 1, 30, 60 µM). Using a Lineweaver-Burk application, we determined the apparent Km,app values for the different BisIII concentrations. The K_i was calculated by plotting the different K_m(app) values for the different BisIII concentrations.

In Vitro Kinase Assays—Lysates from COS-7 cells transiently expressing full-length SLK fused to a N-terminal FLAG-tag were subjected to immunoprecipitation with protein G Sepharose-bound anti-FLAG antibodies for 3 h at 4 °C. After binding, the beads were washed three times with 500 µl of TL-buffer and once with 500 µl of kinase buffer (20 mM HEPES, pH 7.5, 15 mM MgCl_2, 80 mM KCl, 1 mM Na_3VO_4, and 0.1 mM DTT). SLK kinase activity assays were performed in a total volume of 60 µl. Immunoprecipitated SLK bound to 15 µl of drained beads was mixed with 35 µl of kinase buffer containing the indicated BisIII concentrations and incubated for 10 min at 4 °C. The kinase reactions were started by addition of 100 µCi [y-32P]ATP, 1 µCi [y-32P]ATP, and 20 µg of myelin basic protein and incubated for 10 min at 30 °C. CDK2 assays were performed in a total volume of 50 µl. Then 100 ng of CDK2/CyclinA and BisIII, BisVIII, and BisX concentrations were indicated were pre-incubated in kinase buffer for 10 min at 4 °C. The kinase reactions were started by adding 100 µM ATP, 2 µM [y-32P]ATP, and 10 µg of histone H1 and incubated for 20 min at 30 °C. All kinase reactions were stopped by adding 25 µl of 3X SDS sample buffer. Samples were analyzed by SDS electrophoresis and autoradiography using phosphoimaging for quantification. IC_{50} calculations were performed with GraFit 5.0 (Erithacus, Horley, Surrey, United Kingdom).

Molecular Docking—BisIII, BisVIII, and BisX were converted with Corina (www2.chemie.uni-erlangen.de/corina) into three-dimensional structures. The structures were docked using LigandFit 4.8 (www.accelrys.com) into CDK2 (1AQ1). The LigandFit settings were optimized by redocking staurosporine into 1AQ1. A piecewise linear potential function was used for docking, generating 15 poses per ligand. All poses were rescored with the empirical scoring function X-score (sw16.im.med.umich.edu/software/xtool/manual).

RESULTS

Generation of PKC Inhibitor Affinity Matrices—Prior to covalent coupling to chromatography beads, structural aspects of binding had to be considered to identify bisindolylmaleimide derivatives with suitable functional moieties that would allow inhibitor immobilization without abrogating protein kinase interaction. The scaffold of bisindolylmaleimides is structurally similar to the indolocarbazole kinase inhibitor staurosporine. We therefore assumed a binding mode for bisindolylmaleimides similar to the well-characterized staurosporine orientation known from a variety of reported protein kinase co-crystal structures (19–21). This comparison enabled us to identify the three commercially available bisindolylmaleimide derivatives III, VIII, and X (BisIII, VIII, and X) as interesting candidates for covalent immobilization on chromatography beads. They have a primary alkylamino moiety in common, which is most likely accessible from the outside solvent and should thereby permit functional immobilization on activated support materials. The structures of these derivatives in comparison with the closely related GF109203X (also known as bisindolylmaleimide I) used in most signaling studies are shown in Fig. 1. Equal quantities of each of the three derivatives were then covalently coupled to epoxy-activated Sepharose to generate the respective BisIII, BisVIII, and BisX affinity matrices.

Identification of Cellular BisIII Targets—As BisIII is the most closely related analogue of the widely used PKC inhibitor GF109203X, the corresponding BisIII matrix was used for the preparative purification of cellular targets relevant for pharmacological analysis employing bisindolylmaleimides. The only structural distinction between the two compounds is that Bis contains a dimethylaminepropyl group instead of the corresponding primary aminepropyl moiety of BisIII (Fig. 1). For the preparative target identification experiment, we loaded total lysate from 2.5 \times 10^9 HeLa cells on a column containing immobilized BisIII affinity material.

After several washing steps, bound proteins were specifically eluted by a combination of ATP and free BisIII in the elution buffer. Fractions containing eluted proteins were
pooled and separated by 16-BAC/SDS gel electrophoresis. Coomassie-stained protein spots were cut out and analyzed by mass spectrometry (Fig. 2). Several of the identified cellular binding proteins were protein kinases known to be inhibited by bisindolylmaleimide compounds such as different PKC isoforms, Rsk2, and both GSK3 isoforms. These results provided various positive controls for the functionality of the purification method (Fig. 2) (7). Notably, the Rho-dependent protein kinase 1 (ROCK1) was identified in the high-molecular-mass region, although the kinase activity of the closely related ROCK2 was previously shown to be rather insensitive to 1 µM BisIII in vitro (7). Therefore, ROCK1 is likely to represent a cellular protein with a relatively weak affinity for immobilized BisIII ligand. In addition, we identified SLK and calmodulin-dependent kinase IIγ (CaMKIIγ) as new putative protein kinase targets of bisindolylmaleimide inhibitors. Moreover, some of the most prominent protein spots contained two non-protein kinase targets, which were AK and NQO2. A complete list of all identified BisIII-interacting proteins is shown in Table I.

**Selectivity Profiling with Closely Related Bisindolylmaleimide Affinity Matrices**—To test whether small structural variations of the immobilized bisindolylmaleimides would affect the pattern of specifically retained cellular proteins, HeLa cell lysate from 1.5 × 10⁶ cells per sample was used for in vitro associations on an analytical scale with either control beads or the closely related BisIII, BisVIII, and BisX matrices as affinity purification reagents. After incubation and several washing steps, bound proteins were eluted from the beads, separated by SDS-PAGE, and visualized by silver staining (Fig. 3A). Strikingly, small structural changes on the bisindolylmaleimide scaffold of the immobilized ligand strongly affected the affinity matrices’ selectivity for cellular targets, as evident from the both qualitative and quantitative differences in specific protein binding (Fig. 3A). For example, the BisVIII matrix weakly and the BisX matrix strongly interacted with a prominent 32-kDa protein not retained by either BisIII or control beads. Subsequent batch purification of this protein using freshly prepared lysate from 4 × 10⁷ HeLa cells as starting material yielded Coomassie-stainable quantities of the BisVIII- and BisX-interacting protein, which were then excised from a one-dimensional SDS gel and identified as the serine/threonine kinase CDK2. In addition, this “semi-preparative” batch purification approach using either BisIII, BisVIII, or BisX beads also permitted the isolation and mass spectrometric detection of Rsk2, PKCα, PKCδ, GSK3, and NQO2, which were among the most prominent BisIII-interacting proteins purified by column chromatography and subsequent 16-BAC/SDS-PAGE from 2.5 × 10⁹ HeLa cells (compare Fig. 3A with Fig. 2). In addition, we analyzed the selectivities of the related bisindolylmaleimide affinity matrices by immunoblot analysis with antibodies specific for Rsk1, PKCα, GSK3α/β, CDK2, RICK,
and p38. Immunoblots against RICK and p38 were included as controls, and both kinases did not interact with any of the immobilized bisindolylmaleimide compounds (Fig. 3B). As further shown in Fig. 3B, none of the other kinases interacted nonspecifically with control beads. PKCα and GSK3α/β were depleted from the lysate and retained on the three different affinity matrices to a similar extent. In contrast, Rsk1 binding was more pronounced for the BisVIII and BisX matrices compared with the BisIII affinity reagent. CDK2 did not bind to immobilized BisIII, showed a rather weak affinity for BisVIII beads, and strongly interacted with the BisX matrix. Thus, the silver gel and Western blot analysis of the unbound and bound protein fractions of the Ctrl, BisIII, BisVIII, and BisX matrices allowed a fast and simple estimation of the selectivity and potency of the used bisindolylmaleimide compounds toward their cellular targets.

**BisIII Is a Competitive Inhibitor of Human NQO2**—The cytosolic oxidoreductase NQO2 is able to reduce quinone substrates by two-electron transfer, and, although its physiological role in vivo remains to be established, NQO2 was recently shown to mediate the activation of the cancer prodrug CB 1954 in tumor cells displaying high expression levels of this enzyme (22, 23). To verify that association with the BisIII matrix also translates into inhibition of NQO2 enzymatic ac-

| Spot no. | Protein name                  | gi no.       | Mr (kDa) |
|----------|-------------------------------|--------------|----------|
| 1        | NQO2                          | 5822324      | 25821    |
| 2        | Not identified                 |              |          |
| 3        | Lactate dehydrogenase B       | 4557032      | 36638    |
| 4        | Glycerinaldehyde-3-phosphate dehydrogenase | 31645      | 36054    |
| 5        | GSK3β                         | 20455502     | 46744    |
| 6        | GSK3β                         | 20455502     | 46744    |
| 7        | GSK3α                         | 11995474     | 50997    |
| 8        | Adenosine kinase               | 6840802      | 40545    |
| 9        | Adenosine kinase               | 6840802      | 40545    |
| 10       | GSK3β                         | 20455502     | 46744    |
| 11       | GSK3β                         | 20455502     | 46744    |
| 12       | Eukaryotic translation elongation factor 1α | 4503471 | 50141    |
| 13       | Enolase 1                      | 4503571      | 47169    |
| 14       | HSP90α                        | 13291950     | 84660    |
| 15       | GSK3α                         | 11995474     | 50997    |
| 16       | GSK3α                         | 11995474     | 50997    |
| 17       | β-Tubulin                     | 18088719     | 49672    |
| 18       | α-Tubulin                     | 14389309     | 49895    |
| 19       | Glucose-6-phosphate dehydrogenase | 1085314 | 59126    |
| 20       | Proline-4-hydroxylase         | 20070125     | 57116    |
| 21       | CaMKIIγ                       | 422769       | 58365    |
| 22       | Pyruvate kinase, M1 isoenzyme | 20178296     | 57937    |
| 23       | HSP70.1                       | 462325       | 70052    |
| 24       | HSP73                         | 5729877      | 70898    |
| 25       | PKCδ                          | 14735945     | 77505    |
| 26       | PKCα                          | 4506067      | 76764    |
| 27       | Rsk2                          | 4759050      | 83736    |
| 28       | β-tubulin                     | 18088719     | 49672    |
| 29       | HSP90α                        | 13139150     | 84660    |
| 30       | Eukaryotic translation elongation factor 2 | 4503478 | 95338    |
| 31       | Not identified                | –           | –        |
| 32       | α-Glucosidase II, α subunit   | 21361456     | 109438   |
| 33       | HSP70RY                       | 6226699      | 94300    |
| 34       | Not identified                | –           | –        |
| 35       | Leucine-rich protein          | 18959202     | 145201   |
| 36       | Valyl-tRNA synthetase 2       | 5454158      | 140476   |
| 37       | ROCK1                         | 6677759      | 158171   |
| 38       | Carbamoyl-phosphate synthase (ammonia) precursor | 87018       | 164830   |
| 39       | Not identified                | –           | –        |
| 40       | Glutamyl-prolyl tRNA synthetase | 16158948 | 162969   |
| 41       | Not identified                | –           | –        |
| 42       | SLK                            | 7611994      | 132797   |
| 43       | Myosin                        | 12667788     | 226532   |
| 44       | Not identified                | –           | –        |
tivity by BisIII in vitro, we performed a coupled redox reaction assay using NADH as an electron donor for NQO2 (24). In this assay, NQO2 uses NADH to reduce its substrate menadion. The resulting product menadiol then causes the reduction of MTT, which can be measured photometrically at 622 nm. NADH shows only a low affinity for NQO2. To compare our assay with previously reported activity measurements, we first determined the $K_m$ of NADH for NQO2 and determined a value of $324 \pm 11006 \mu M$, which is in good agreement with earlier results (Fig. 4) (24). By measuring the initial rates of enzymatic activity at different NADH concentrations and in the presence of different fixed BisIII concentrations of 0, 1, 30, or 60 $\mu M$, we could show that the BisIII-mediated inhibition of NQO2 activity is competitive with respect to NADH. The $K_i$ of BisIII with respect to NQO2 inhibition was $16.5 \pm 4 \mu M$, as shown in the inset of Fig. 4. Thus, we could verify NQO2 as a cellular bisindolylmaleimide target, albeit suppression of its catalytic activity occurred at higher BisIII concentrations than required for potent inhibition of known protein kinase targets.

**Human AK Is a Cellular Target of BisIII**—The non-protein kinase AK metabolizes adenosine to AMP and was one of the major cellular proteins purified with the BisIII matrix. Interestingly, AK is a pharmacologically important target protein. Inhibition of its activity enhances the release of adenosine from cells, and extracellular adenosine is known to act as a neuromodulator with potent anti-nociceptive and anti-inflammatory actions. Therefore, inhibition of AK activity is proposed as a therapeutic strategy for the treatment of pain, and inflammation and several potent inhibitors of AK activity have been reported (25–27). To analyze AK binding to BisIII beads, a myc-tagged version of AK was transiently expressed in COS-7 cells. Cell lysate was then incubated with either control or BisIII beads prior to immunoblotting of bound proteins with myc-epitope-specific antibody. As shown in Fig. 5, ectopically expressed AK specifically interacted with BisIII beads and was not retained by the control matrix. Moreover, BisIII inhibited the enzymatic activity of recombinant AK with an IC$_{50}$ value of around 1 $\mu M$ in the presence of 100 $\mu M$ ATP (data not shown). Thus, these data establish AK as a new cellular target of bisindolylmaleimide inhibitors.

**SLK Is Potently Inhibited by BisIII**—Our mass spectrometry analysis of BisIII-interacting proteins revealed human SLK, a member of the Ste20 family of serine/threonine kinases, as a potential protein kinase target of this inhibitor class (Fig. 2).
SLK possesses several domains including a N-terminal protein kinase module and was previously characterized as regulator of the actin cytoskeleton and apoptosis (28, 29). To confirm SLK as a new BisIII target, we investigated the binding of a transiently expressed, FLAG-tagged variant of SLK to the BisIII matrix. SLK retained by the affinity beads was detected by immunoblotting with FLAG epitope-specific antibody. As shown in Fig. 6A, SLK specifically interacted with the BisIII matrix, whereas no binding to the control matrix was observed. Similar specific binding was also observed for a N-terminal fragment of SLK that only contained the protein kinase domain (data not shown). Moreover, BisIII potently inhibited SLK activity with an IC50 of 170 nM in vitro in the presence of 100 μM ATP and histone H1 acting as a substrate (Fig. 6B). Because no pharmacological inhibitor of SLK has been described yet, our identification of BisIII as such a reagent provides a useful tool for further analysis of SLK function.

Minor Structural Variations at the Bisindolylmaleimide Scaffold Affect the Potency to Inhibit CDK2 Activity—As shown in Fig. 3B, dramatic differences in CDK2 binding were observed for the three different bisindolylmaleimide affinity matrices. To test whether these data correlate with the sensitivity of the CDK2 activity to inhibition in vitro, we performed kinase assays using histone H1 as substrate and determined the IC50 values for the closely related BisIII, BisVIII, and BisX analogues. In agreement with the results from the binding experiment, BisX was the most potent antagonist of CDK2, with only 200 nM of the inhibitor conferring 50% kinase inhibition in vitro (Fig. 7). BisVIII, which only weakly retains CDK2 in its immobilized form, suppressed CDK2 with a 3-fold higher IC50 value of 600 nM and, moreover, the lack of CDK2 binding to the BisIII matrix correlated with a further reduction of inhibitor potency resulting in an IC50 value of 2 μM for the BisIII deriv-
In vitro kinase assays were performed for comparison of the IC_{50} values of BisIII, BisVIII, and BisX with respect to CDK2/cyclinA-mediated histone H1 phosphorylation. Kinase activity in the absence of the inhibitor was set to 100%, and remaining activities at different inhibitor concentrations are shown relative to this value. A 50% inhibition of CDK2/cyclinA activity was observed for 2 μM BisIII (closed triangles), 0.6 μM BisVIII (closed squares), and 0.2 μM BisX (open circles) in the presence of 100 μM ATP, respectively.

**Fig. 7.** BisIII, BisVIII, and BisX differ in their potencies to inhibit CDK2/cyclinA activity. In vitro kinase assays were performed for comparison of the IC_{50} values of BisIII, BisVIII, and BisX with respect to CDK2/cyclinA-mediated histone H1 phosphorylation. Kinase activity in the absence of the inhibitor was set to 100%, and remaining activities at different inhibitor concentrations are shown relative to this value. A 50% inhibition of CDK2/cyclinA activity was observed for 2 μM BisIII (closed triangles), 0.6 μM BisVIII (closed squares), and 0.2 μM BisX (open circles) in the presence of 100 μM ATP, respectively.

**Fig. 8.** Differences in hydrophobicity and flexibility of BisIII, BisVIII, and BisX affect the affinity to CDK2. BisIII, BisVIII, and BisX were docked into the kinase structure derived from CDK2 co-crystallized with staurosporine (1AQ1) (15). A, standard view of the CDK2 kinase domain (ribbon, blue) in complex with BisX (surface, green). B, view of the nucleotide-binding cleft of CDK2 occupied by BisX (upper panel). The lower panel shows the BisX coordinating residues (stick, yellow). Essential H-bonds between the backbone amino acids that form the hinge region of CDK2 and the maleimide group are shown as green dashed lines. The primary alkylamine group that was used for coupling with epoxy-activated Sepharose is indicated as linker position. C, structure of CDK2 with the co-crystallized inhibitor staurosporine (1AQ1) (15).
lobes of the kinase domain. The two H-bonds were similar in orientation and length to those previously described for staurosporine (Fig. 8, B and C). The ranking of theoretical affinities for the three bisindolylmaleimides correlated with their measured in vitro potencies toward CDK2 (Table II). Moreover, the calculated X-score for staurosporine was even higher than for BisX, consistent with the reported inhibition of CDK2 by staurosporine with a low nanomolar IC<sub>50</sub> value (19). Moreover, ligand flexibility was reduced from BisIII to BisVIII to BisX, and, conversely, their hydrophobic interactions at the ATP-binding cavity became more pronounced in the same order. Importantly, both effects strengthen the binding by moving the ligand somewhat deeper into the nucleotide-binding pocket. Furthermore, the docking approach revealed that the free amine group of the bisindolylmaleimide compounds pointed toward the solvent-accessible protein surface and confirmed our assumption that the coupling to an epoxy-activated linker moiety did not abrogate kinase binding. A fortiori, bisindolylmaleimides that are connected via this position to a matrix can be used for binding and identification of cellular targets.

**Specific Binding of Activated Protein Kinases by Immobilized Bisindolylmaleimide**—Efficient affinity purification of cellular bisindolylmaleimide targets required the presence of high salt concentrations during binding to the matrices. Under these conditions, the PKC-specific co-factors DAG, Ca<sup>2+</sup>, and PS did not significantly affect binding to the BisIII matrix, as tested for PKCa. Interestingly, when in vitro association experiments were performed at a physiological ionic strength of 150 mM NaCl, PKCa specifically interacted with BisIII beads only when DAG, Ca<sup>2+</sup>, and PS were included but not in the absence of these co-factors (Fig. 9A). To test whether the BisIII affinity matrix can be used as a tool to follow the cellular activation of a target kinase, we lysed HeLa cells after different times of EGF stimulation. EGF is known to trigger cellular Rsk activity through the activation of the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway (30). Lysates were subjected to in vitro association with BisIII beads prior to SDS-PAGE and immunoblotting with Rsk1-specific antiserum. As seen in Fig. 9B, Rsk1 from unstimulated cells weakly bound to the BisIII beads, whereas, upon 5 min of EGF stimulation, Rsk1 binding was strongly increased and then slowly decreased in a time-dependent manner. Total cellular Rsk1 expression levels were constant for all stimulation times, indicating that the EGF-induced binding to BisIII beads indeed correlated with the regulation of cellular Rsk1 activity (Fig. 9B). From these experiments, it appears that the BisIII affinity reagent can also be adapted for analytical purposes and then allows the specific detection of protein kinases either activated in vitro or in intact cells.

**DISCUSSION**

Bisindolylmaleimide inhibitors of protein kinases have been widely used in signal transduction research, but the tools available to examine their target selectivity have been rather limited and biased (7). In this study, we have established a set of functional bisindolylmaleimide affinity matrices, which enabled us to identify previously unknown targets of this inhibitor class. To accomplish this task, we used a proteomics approach similar to our recently described affinity purification protocol for p38 inhibitor-interacting cellular proteins (14). Our results with the bisindolylmaleimide matrices now demonstrate that this optimized biochemical procedure can be adapted for scaffolds completely unrelated to the pyridinyl imidazole core structure of the p38 inhibitor SB 203580.

In addition, as established for CDK2 in our experiments, differential binding to closely related inhibitor matrices provides a measure for the relative potencies of the corresponding free inhibitor compounds toward a kinase target. Due to the possibility to identify and evaluate the cellular proteins targeted by a whole set of inhibitor analogues based on the same chemical scaffold, such a proteomics strategy might deliver alternative drug targets and compare their affinities relative to each other within the same experimental set-up. Therefore, this type of application has the potential to become a relevant feature in future drug development (31, 32).

The overall sensitivity of our proteomics method is high, and even protein kinases expressed at 500 to 1000 copies per cell can be expected to appear as Coomassie stainable spots when extracts from 2.5 × 10<sup>5</sup> mammalian cells are used (33). Moreover, it should be feasible to further increase the sensitivity of this affinity purification-based approach by a factor of

![Fig. 9. Selective binding of activated PKCa and Rsk1 to the BisIII matrix.](image-url)
5 to 10, for example by combining it with gel-free analysis of enriched protein fractions through liquid chromatography coupled to mass spectrometry (34). Based on these estimations, the detection and identification of kinase inhibitor targets of a very low abundance (100 or less copies per cell) is a realistic goal, which might be achieved in the near future.

In this work, we have used total cell extracts from HeLa cells as a protein source for the identification of bisindolylmaleimide-binding proteins. Importantly, all bisindolylmaleimide compounds used for immobilization are commercially available reagents. Thus, our established purification protocol can be easily applied to characterize PKC inhibitor-targeted subproteomes in other cell lines, tissue extracts, or even whole organisms and thereby provide a comprehensive picture how this widely used class of signal transduction inhibitors exerts its effects on the molecular level.

Acknowledgments—We thank Stephanie Blencze and Robert Brehm for excellent technical assistance.

* This work was supported by a grant from the German Bundesministerium für Bildung und Forschung (BMBF). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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