Proteomic Characterization of the Angiogenesis Inhibitor SU6668 Reveals Multiple Impacts on Cellular Kinase Signaling

Klaus Godl,1 Oliver J. Gruss,2 Jan Eickhoff,1 Josef Wissing,1,3 Stephanie Blencke,1 Martina Weber,1 Heidrun Degen,1 Dirk Brehmer,1 László Örﬁ,1 Zoltán Horváth,1 György Kéri,1 Stefan Müller,1 Matt Cotten,1 Axel Ulrich,4,5 and Henrik Daub1

1Axxima Pharmaceuticals AG, Munich, Germany; 2ZMBH, Heidelberg, Germany; 3Department of Cell Biology, GBF, Braunschweig, Germany; 4Vichem Chemie Ltd., Budapest, Hungary; 5Department of Molecular Biology, Max Planck Institute of Biochemistry, Martinsried, Germany; and 6Centre for Molecular Medicine, Agency for Science, Technology, and Research, Proteos, Singapore

Abstract
Knowledge about molecular drug action is critical for the development of protein kinase inhibitors for cancer therapy. Here, we establish a chemical proteomic approach to profile the anticancer drug SU6668, which was originally designed as a selective inhibitor of receptor tyrosine kinases involved in tumor vascularization. By employing immobilized SU6668 for the affinity capture of cellular drug targets in combination with mass spectrometry, we identified previously unknown targets of SU6668 including Aurora kinases and TANK-binding kinase 1. Importantly, a cell cycle block induced by SU6668 could be attributed to inhibition of Aurora kinase activity. Moreover, SU6668 potently suppressed antiviral and inflammatory responses by interfering with TANK-binding kinase 1–mediated signal transmission. These results show the potential of chemical proteomics to provide rationales for the development of potent kinase inhibitors, which combine rather unexpected biological modes of action by simultaneously targeting defined sets of both serine/threonine and tyrosine kinases involved in cancer progression. (Cancer Res 2005; 65(15): 6919-26)

Introduction
The targeted inactivation of protein kinases implicated in tumor progression is rapidly emerging as a major new concept in antineoplastic therapy (1, 2). Protein kinases play central roles in cancer cell proliferation, survival, and metastasis. In addition, signaling through receptor tyrosine kinases (RTK) such as the vascular endothelial growth factor receptor 2 (VEGFR2) in endothelial cells and the β-platelet-derived growth factor receptor (βPDGFR) in endothelial cell–associated pericytes is required for the vascularization of tumor tissue (3). The formation of new blood vessels is mainly triggered by hypoxia-induced VEGF secretion from tumor cells and ensures the sustained growth and subsequent dissemination of cancer. VEGF activation of its cognate receptor on endothelial cells is particularly important for angiogenesis early in tumor development, whereas βPDGFR signaling in pericytes plays a critical role for the maintenance of established blood vessels in late-stage tumors (3). Moreover, fibroblast growth factor receptor (FGFR)–mediated VEGF biosynthesis in endothelial cells has been reported as an autocrine mechanism that further augments angiogenesis (4).

The knowledge about RTK function in the process of tumor vascularization has provided rationales for the development of antiangiogenic small molecule drugs such as the indolizine compound SU6668, which was characterized as an ATP-competitive inhibitor of the PDGFR, VEGFR2, and FGFR1 RTKs in vitro (5). In cell-based assays, low micromolar concentrations of SU6668 effectively blocked βPDGFR and VEGFR2 autophosphorylation on tyrosine residues. In comparison, cellular inhibition of FGFR signaling required relatively high SU6668 concentrations, arguing against a role of the FGFR as a physiologic target of the drug (5). The administration of SU6668 led to both βPDGFR and VEGFR2 inhibition in vivo and resulted in the disruption of tumor vasculature and the regression of established tumors in various mouse models (3, 6). Despite these promising results from mouse studies and the fact that some clinical benefit was seen upon SU6668 monotherapy, the pharmacokinetic data from patients indicated that the effective plasma concentrations could not be maintained during prolonged therapy at levels required for the drug to be effective as a single agent (7). However, recent reports indicate that SU6668 is significantly more efficacious when combined with either tumor irradiation or immune therapy and it remains to be determined whether these promising results eventually translate into clinical success of the drug (8, 9).

The selectivity of small molecule drugs targeting protein kinases is usually assessed by parallel activity assays employing a panel of recombinant kinases. The obvious shortcoming of this approach is its limitation to a relatively small fraction of the human kinome, which does not match the protein kinase complement of the biological system used to study the molecular mechanisms of drug action. In this study, we address the issue of inhibitor selectivity in a way that we employ a recently developed proteomic technique to map the cellular targets of SU6668 (10, 11). This chemical-biological approach relies on the use of immobilized inhibitor analogues as capture reagents for the selective isolation of drug-interacting protein species in the biological system under study. Importantly, we further show that SU6668 exerts pharmacologically relevant cellular effects, which are related to inhibition of previously unknown Ser/Thr kinase targets of the drug. Thus, this study shows that a functional proteomic approach can substantially extend the knowledge...
about the target-related, potentially physiologic effects of a clinical cancer drug and thereby provide valuable data for further drug optimization.

**Materials and Methods**

**Affinity purification and protein identification.** SU6668 was synthesized as described and coupled to EAH Sepharose 4B (Amersham Biosciences, Buckinghamshire, United Kingdom) using carbodiimide coupling chemistry (5, 12). Lysis of frozen HeLa cell pellets (1 × 10⁶ cells, Cilbiotech, Mons, Belgium) was done with 15 mL of buffer containing 20 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 0.25% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA plus additives (10 mmol/L sodium fluoride, 1 mmol/L orthovanadate, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L DTT). Affinity chromatography with SU6668 columns, preparative benzylidimethyl-n-hexadecylammonium chloride (16-BAC)/SDS-PAGE and mass spectrometry (MS) analysis were carried out essentially as described (10, 13).

**Cell culture, transfections, adenosivirus and in vitro association experiments.** HeLa, COS-7 and 293, and human foreskin fibroblast (HFF) cells were cultured in DMEM supplemented with 10% fetal bovine serum. Transient transfections of COS-7 and 293 cells were done as previously described (14, 15). Plasmids used for transfection experiments were pRK5 constructs expressing human 5-aminoimidazole ribonucleotide carboxylase-1/[Su5cycinamino]carbonyl]-5-aminoimidazole ribonucleotide synthetase (AMPK) or pRK5-FLAG-TBK1 or pRK5-FLAG-TBK1-K38R and 5 ng pGFP for normalization. One day later, cells were transferred into serum-free medium cultured for an additional 6 hours in the presence of DMSO or different concentrations of SU6668. Luciferase activity was then detected as described (15).

**Results**

**Identification and in vitro characterization of cellular protein targets of the angiogenesis inhibitor SU6668.** The crystal structure of the FGFR1 kinase domain in complex with SU6668 reveals that the propionic acid side chain of the inhibitor extends to the protein surface, suggesting that covalent immobilization at the terminal carbonyl group could generate an affinity purification reagent with SU6668-like binding characteristics (5). Based on these considerations, we covalently linked the carbonyl moiety of SU6668 to the free amino groups of EAH Sepharose in a carbodiimide-mediated coupling reaction. The chemical structures of SU6668 and the derived SU6668 affinity matrix are shown in Fig. 1A. To establish the SU6668 resin as a functional affinity reagent for a known inhibitor target, we prepared total cell lysate from HFF cells and tested the interaction of endogenous βPDGFR with either control or SU6668 beads. Specific βPDGFR binding to the SU6668 affinity matrix correlated with its depletion from the cell lysate and was prevented in the presence of free inhibitor (Fig. 1B). This result showed that covalent immobilization of SU6668 did not interfere with its kinase-binding properties. Similar results were obtained for the known SU6668 target VEGFR2, which was also specifically retained by the inhibitor beads (data not shown). Because other kinase targets are likely to bind the drug in the same spatial orientation, we reasoned that the affinity matrix might serve as a biochemical tool for the selective purification of additional SU6668-interacting proteins from cellular extracts. To characterize the SU6668 targets within a cellular proteome, we subjected total lysate from HeLa cells to affinity chromatography on a SU6668 column employing a purification protocol similar to our recently described procedures (10, 12). Specifically retained proteins were released from the inhibitor column using a combination of 10 mmol/L ATP and 100 μmol/L SU6668 as eluting agents. The highly enriched fraction of potential SU6668 target proteins was then resolved by 16-BAC/SDS-PAGE. Coomassie staining of the gel visualized about 30 protein spots, which were excised from the gel and digested with trypsin. Subsequent MS analysis revealed eight previously unknown protein kinase targets of the indoline drug SU6668. Interestingly, in addition to tyrosine phosphorylation on Ser²⁰⁰ (Cell Signaling Technology, Beverly, MA) and against Aurora A (Abcam, Cambridge, MA, all used at 1:500). Respective secondary antibodies were purchased from Amersham.

**Reporter gene, cytokine, and viral replication assays.** To measure TK1-induced IFN-β reporter gene activity in 293 cells, 4 × 10⁶ cells per well 96-well were cotransfected with 40 ng IFN-β luciferase reporter plasmid, 30 ng pRK-FLAG-TBK1 or pRK-FLAG-TBK1-K38R, and 5 ng pGFP for normalization. One day later, cells were transferred into serum-free medium cultured for an additional 6 hours in the presence of DMSO or different concentrations of SU6668. Luciferase activity was then detected as described (15).

To determine the induction of endogenous IFN-β and interleukin-8 (IL-8) genes, HFF cells were incubated with fresh, serum-free DMEM for 1 hour and then treated with the indicated SU6668 concentrations for 30 minutes prior to poly(I:C) stimulation. Total RNA was isolated 2 hours after poly(I:C) treatment (Roche, Basel, Switzerland) and subsequent analysis of IFN-β mRNA levels by quantitative RT-PCR was done as described (12). In parallel, IL-8 gene induction was determined from the same samples using the following primer sequences: 5'-GCACTCTCAGTCTTGGATACC-3' (forward), 5'-GTCTGGACCCCAAGGAAACCT3' (reverse), and 5'-TCTTAAAAATCTTCCACACACCTTCGCA-3' for gene-specific quantification. Human cytomegalovirus (HCMV) replication assays were done as described (15).

**Flow cytometry and immunofluorescence.** For flow cytometry and immunofluorescence analysis, treatment of cells with SU6668 or DMSO was done in OPTI-MEM serum-free medium (Invitrogen, San Diego, CA). Flow cytometry was done with cells that had been fixed in ice-cold methanol and stained with 40 μg/mL propidium iodide (Sigma) after a 30-minute RNase A (0.1 mg/mL) treatment. Subsequently, 10⁵ stained cells were analyzed on a Becton Dickinson FACScan. Cells were fixed for 10 minutes in 3% paraformaldehyde for immunofluorescence analysis. The following antibodies were used for immunofluorescence: against human full-length TPX2 protein (1:10,000; ref. 19), against α-tubulin (Sigma), against histone H3 phosphorylated on Ser²⁰ (Cell Signaling Technology, Beverly, MA) and against Aurora A (Abcam, Cambridge, MA, all used at 1:500). Respective secondary antibodies were purchased from Amersham.

**Cancer Research**

February 2005: 65 (15). August 1, 2005

6920

www.aacrjournals.org

Downloaded from cancerres.aacrjournals.org on May 1, 2017. © 2005 American Association for Cancer Research.
kinases such as the Src-family members Yes and Lyn, we also identified a variety of potential Ser/Thr kinase targets including TBK1 (also known as NAK or T2K), two Aurora kinases, RSK3, AMPK and ULK3 (Fig. 1C; Table 1). Known RTK targets of SU6668 such as hPDGFR or VEGFR2 were not detected due to their very low protein levels or lack of expression in HeLa cells. Strikingly, the most prominent spot on the preparative 16-BAC/SDS gel did not represent a protein kinase, but could be attributed to a different type of ATP-utilizing enzyme involved in de novo purine biosynthesis, the bifunctional AIRc-SAICARs (20, 21).

To validate the MS data, we investigated the interaction of several of the putative SU6668 target proteins with either control or SU6668 beads in vitro. Immunoblot analysis confirmed that the SU6668 matrix efficiently bound the protein kinases TBK1, Aurora A, Yes, Lyn, AMPK, and RSK3 from HeLa cell lysate as well as transiently expressed ULK3 and AIRc-SAICARs from COS-7 cell extracts (Fig. 1D). Furthermore, target binding was abrogated or largely reduced in the presence of soluble SU6668, verifying the specific interactions with the immobilized drug.

Because in vitro association experiments cannot provide quantitative information about the relative potency of SU6668 towards the identified protein kinase targets, we next did in vitro kinase assays in the presence of different inhibitor concentrations and determined the SU6668 concentrations required for half maximal kinase inhibition (Supplementary Fig. S1). All kinases tested were potently inhibited by low micromolar SU6668 concentrations, and the lowest IC50 values were determined for Aurora B (0.047 μmol/L), Aurora A (0.85 μmol/L) and TBK1 (1.4 μmol/L). Half maximal in vitro inhibition of Aurora B kinase occurred at SU6668 concentrations similar to those measured for

Figure 1. Identification and characterization of cellular SU6668 target proteins. A, chemical structures of SU6668 in its free and immobilized form. B, the SU6668 affinity matrix specifically retains hPDGFR from cellular lysate. HFF cell extracts were subjected to in vitro association with either control matrix or SU6668 beads in the absence or presence of 200 μmol/L free inhibitor. Total lysate, the supernatants and 5 × aliquots of the bound protein fractions were analyzed by immunoblotting with hPDGFR-specific antiserum. C, total lysate from 1 × 10⁷ HeLa cells was fractionated by SU6668 affinity chromatography. Cellular SU6668 targets were separated by 16-BAC/SDS-PAGE prior to Coomassie staining and MS analysis. D, total lysates from HeLa cells or COS-7 cells expressing epitope-tagged ULK3 or AIRc-SACAIRs were subjected to in vitro association with SU6668 beads as described under (B) followed by immunoblotting with specific antibodies for TBK1, Aurora A, Yes, Lyn, AMPK, RSK3, or epitope tag detection of ULK3 or AIRc-SACAIRs.
the βPDGFR and c-kit (22). SU6668 inhibited the kinase activities of Aurora A and TBK1 with IC$_{50}$ values quite similar to those previously determined for the SU6668 tyrosine kinase targets VEGFR2 and FGFR1 (5, 22). Thus, our results of potent Ser/Thr kinase inhibition show that SU6668 is not a selective antagonist of certain RTKs as previously assumed.

**SU6668 inhibits entry into M phase by interfering with Aurora kinase function.** Next, we wanted to directly test whether Aurora kinases are inhibited by SU6668 in intact cells. Experiments in different systems indicate that Aurora A functions early in mitosis in centrosome maturation and the establishment of the mitotic spindle. Aurora B is needed for bipolar attachment of microtubules to kinetochores and proper cytokinesis. Interfering with Aurora functions thus leads to defects in mitosis and blocks cell cycle progression (23). To analyze whether SU6668 induces similar effects in human HeLa cells, we first monitored cell cycle stages by flow cytometry after 15 hours of incubation with different concentrations of SU6668 (Fig. 2A). In control populations and in the presence of 10 μmol/L SU6668, most cells were found to be diploid (2 N DNA, indicative of G1 phase) and a smaller fraction tetraploid (4 N DNA, indicative of G2 or M phase), as expected for an exponentially growing cell population. In contrast, in the presence of 20 μmol/L or 40 μmol/L SU6668, the vast majority of cells was found with tetraploid DNA content consistent with a cell cycle arrest in G2 or M phase. To analyze whether this effect of SU6668 was reversible, the drug was washed out and cell cycle progression was analyzed 4 hours later (Fig. 2A, wash-outs). Indeed, inhibition of cell cycle progression by either 20 or 40 μmol/L SU6668 was relieved in a significant proportion of cells, which subsequently reentered the next G1 phase.

To study the cell cycle arrest in more detail, we analyzed cellular DNA as well as histone H3 phosphorylation on Ser10 in SU6668-treated cells by immunofluorescence microscopy. Histone H3 phosphorylation on Ser10 is a direct measure for Aurora kinase activity in living cells and indicates the presence of mitotic chromosomes (24). Although histone H3 phosphorylation was readily detectable in control cells, it was completely abolished in the presence of 20 μmol/L SU6668 (Fig. 2B). Interestingly, many cells still showed condensation of chromatin in the presence of SU6668 (Fig. 2B). However, apparently no functional spindles were formed as judged by staining of TPX2, a microtubule-associated protein essential for spindle formation, which localizes to the two spindle poles in control cells (19, 25). In SU6668-treated cells, TPX2 was often found in several aggregates per cell (Fig. 2B), which suggests defects in spindle pole or centrosome organization. The observed effects of SU6668 were readily reversible as shown by histone H3 phosphorylation and TPX2 staining in cells from which the drug had been removed for 30 minutes (Fig. 2C).

**Table 1. Cellular protein targets of SU6668**

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Protein name</th>
<th>Gi no.*</th>
<th>MW (Da)</th>
<th>Score†</th>
<th>Peptides identified</th>
<th>Sequence coverage (%)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>l-lactate dehydrogenase</td>
<td>13786849</td>
<td>36,558</td>
<td>69</td>
<td>8</td>
<td>34</td>
</tr>
<tr>
<td>2</td>
<td>l-lactate dehydrogenase</td>
<td>13786849</td>
<td>36,558</td>
<td>57</td>
<td>6</td>
<td>18</td>
</tr>
<tr>
<td>3</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
<td>31645</td>
<td>36,054</td>
<td>41</td>
<td>6</td>
<td>21</td>
</tr>
<tr>
<td>4</td>
<td>AMPKγ1</td>
<td>4506061</td>
<td>37,579</td>
<td>144</td>
<td>12</td>
<td>41</td>
</tr>
<tr>
<td>5</td>
<td>Aurora B</td>
<td>4759178</td>
<td>39,280</td>
<td>73</td>
<td>10</td>
<td>35</td>
</tr>
<tr>
<td>6</td>
<td>not identified</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>AIR carboxylase/SAICAR synthetase</td>
<td>5453539</td>
<td>47,079</td>
<td>65</td>
<td>20</td>
<td>49</td>
</tr>
<tr>
<td>8</td>
<td>Aurora A</td>
<td>7446411</td>
<td>45,790</td>
<td>91</td>
<td>11</td>
<td>45</td>
</tr>
<tr>
<td>9</td>
<td>Aurora A</td>
<td>7446411</td>
<td>45,790</td>
<td>97</td>
<td>12</td>
<td>54</td>
</tr>
<tr>
<td>10</td>
<td>enolase 1</td>
<td>4503571</td>
<td>47,169</td>
<td>72</td>
<td>11</td>
<td>29</td>
</tr>
<tr>
<td>11</td>
<td>eukaryotic translation elongation factor 1α</td>
<td>4503471</td>
<td>50,141</td>
<td>90</td>
<td>17</td>
<td>38</td>
</tr>
<tr>
<td>12</td>
<td>ULK3</td>
<td>39993036</td>
<td>53,444</td>
<td>100</td>
<td>12</td>
<td>38</td>
</tr>
<tr>
<td>13</td>
<td>aldehyde dehydrogenase X</td>
<td>399363</td>
<td>57,217</td>
<td>70</td>
<td>15</td>
<td>36</td>
</tr>
<tr>
<td>14</td>
<td>Lyn</td>
<td>187271</td>
<td>56,033</td>
<td>50</td>
<td>12</td>
<td>29</td>
</tr>
<tr>
<td>15</td>
<td>not identified</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>pyruvate kinase M2</td>
<td>125604</td>
<td>57,914</td>
<td>97</td>
<td>25</td>
<td>53</td>
</tr>
<tr>
<td>17</td>
<td>Yes</td>
<td>4885661</td>
<td>60,801</td>
<td>90</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>18</td>
<td>AMPKα1</td>
<td>5410312</td>
<td>62,808</td>
<td>73</td>
<td>11</td>
<td>31</td>
</tr>
<tr>
<td>19</td>
<td>heat shock 70 kDa protein 1 (HSP70.1)</td>
<td>462325</td>
<td>70,052</td>
<td>139</td>
<td>17</td>
<td>36</td>
</tr>
<tr>
<td>20</td>
<td>not identified</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>not identified</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>heat shock 70 kDa protein 5 (BiP)</td>
<td>16507237</td>
<td>72,333</td>
<td>181</td>
<td>17</td>
<td>35</td>
</tr>
<tr>
<td>23</td>
<td>RSK3</td>
<td>6166243</td>
<td>83,253</td>
<td>46</td>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>24</td>
<td>TBK1</td>
<td>7019547</td>
<td>83,642</td>
<td>77</td>
<td>14</td>
<td>30</td>
</tr>
<tr>
<td>25</td>
<td>eukaryotic translation elongation factor 2</td>
<td>181969</td>
<td>95,338</td>
<td>17</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>ATP citrate lyase</td>
<td>13623199</td>
<td>120,839</td>
<td>61</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>27</td>
<td>not identified</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>carbamoyl-phosphate synthetase 1</td>
<td>21361331</td>
<td>164,939</td>
<td>90</td>
<td>16</td>
<td>32</td>
</tr>
</tbody>
</table>

*National Center for Biotechnology Information Genbank accession number.
† Total search score calculated by Mascot from Matrix Science.
‡ Percentage of the entire protein sequence represented in the identified peptides.
Figure 2. SU6668 inhibits Aurora kinase activity and cell cycle progression in human HeLa cells. A, distribution of the DNA content in control and SU6668-treated cells. HeLa cells were incubated with different concentrations of SU6668 or DMSO as a control for 15 hours and then stained with propidium iodide for flow cytometry analysis. Where indicated, inhibitor-treated cells were washed and then cultured for a further 4 hours in fresh medium without SU6668 (wash-out). B, after 15 hours of treatment with 20 μmol/L SU6668 or DMSO as a control, HeLa cells were subjected to immunofluorescence analysis using the indicated antibodies (bar, 10 μm; Overlays: blue, DNA; red, histone H3 phosphorylated on Ser10; green, TPX2). C, after 15 hours of treatment with 20 μmol/L SU6668, cells were released into fresh medium without SU6668 for 30 minutes prior to immunofluorescence analysis as described in (B). D, HeLa cells treated with SU6668 as described in (B) and then stained with antibodies specific for α-tubulin and Aurora A.
To further analyze this phenotype, cells were directly stained for Aurora A protein. Aurora A was clearly detectable at the two separated centrosomes of the bipolar spindle in control cells (Fig. 2D). In contrast, the presence of 20 μmol/L SU6668 Aurora A kinase localized to several spots confirming defects in centrosome organization (Fig. 2D). Consistent with these observations, the organization of mitotic microtubules was destroyed in SU6668-treated cells (Fig. 2D). Taken together, these data strongly suggest that Aurora kinases are the SU6668 targets relevant for the observed cell cycle block. Treatment with SU6668 induces defects in centrosome organization, spindle assembly and histone modification and, as a consequence, leads to an arrest in cell cycle progression.

Elevated Aurora A kinase activity has been associated with aneuploidy and the disruption of chromosomal events in tumor cells (26, 27). More recently, an additional aspect of Aurora A biology was revealed when the kinase was found to directly phosphorylate p53, thus influencing the protein’s stability and activity (28). Depletion of Aurora A results in increased p53 protein levels and downstream gene expression. Thus, a primary driving force for amplification of Aurora A expression in many tumor cell types might be the need for down-regulation of p53 activity that occurs when the Aurora A kinase activity is elevated. Because Aurora A kinase was found to be sensitive to SU6668, we monitored the levels of p53 in A549 tumor cells upon treatment with the kinase inhibitor. If Aurora A kinase activity is important in determining p53 levels and function, then inhibition of the kinase with SU6668 should result in an increased expression of this central tumor suppressor protein. Indeed, we found that exposure of cells to 30 μmol/L SU6668 resulted in p53 elevation, but did not significantly affect the expression of Aurora A protein (Fig. 3). In agreement with the dose-dependent effects seen in HeLa cells, no significant p53 change is observed at lower inhibitor concentrations. As a control for changes in p53, cells were transduced with an adenovirus vector, which induces increased p53 protein levels in infected cells (Fig. 3). However, if transduction is done with a similar adenovirus expressing a hairpin siRNA to the p53 sequence, the p53 elevation was blocked (Fig. 3).

**Cellular TBK1 inhibition by SU6668 selectively interferes with antiviral responses.** The IκB kinase (IKK)-related Ser/Thr kinase TBK1 was originally implicated in certain aspects of nuclear factor κB (NF-κB) activation (29, 30). In addition, recent reports established a crucial role of TBK1 downstream of toll-like receptors as a regulator of IRAK-activated antiviral and inflammatory genes encoding cytokines such as IFN-β and regulated upon activation, normal T cell expressed and secreted (RANTES; refs. 17, 31, 32). Our identification of SU6668 as the first small molecule antagonist of IFN-β gene activity by SU6668 is consistent with recent genetic evidence from TBK1-deficient cells and further establishes a critical function of TBK1 kinase activity in this antiviral signaling pathway (17, 32). Furthermore, SU6668 reduced poly(I:C)-induced RANTES production and, at somewhat higher concentrations, also affected IL-6 biosynthesis (Supplementary Fig. S2). The latter effect could be attributable to inhibition of the TBK1-related kinase IKKα (33), which was inhibited by SU6668 in vitro with an IC<sub>50</sub> value of 5.2 μmol/L (data not shown). Our identification of SU6668 as a potent antagonist of IFN-β induction prompted us to examine whether this TBK1-related suppression of an antiviral response would accelerate virus production as a physiologic consequence. To test this, we measured human HCMV replication in the presence of increasing amounts of the inhibitor (Fig. 4D). SU6668 indeed enhanced HCMV replication in a dose-dependent manner, which might be linked to a pharmacologic blockade of HCMV-triggered IFN-β biosynthesis (15).

**Discussion**

The anticancer agent SU6668 was developed as a small molecule inhibitor with selectivity for RTKs involved in tumor angiogenesis and proliferation. Our proteomics approach markedly extends earlier knowledge about this compound and identifies both Ser/Thr and cytoplasmatic tyrosine kinases as additional targets of SU6668. Interestingly, SU6668 potently interfered with the kinase activities of Aurora A and Aurora B in vitro. Due to their essential roles at various stages of mitosis, Aurora kinases are regarded as potential cancer drug targets. The therapeutic concept of Aurora kinase inhibition has recently been validated with an ATP-competitive inhibitor, which effectively caused tumor regression in mouse xenograft models (34). In the context of these results and our observation that SU6668 treatment of HeLa cells leads to a reversible G<sub>2</sub>-M block consistent with Aurora inactivation, the development of indolione inhibitors targeting both RTKs involved in tumor vascularization and Aurora kinases critical for mitotic progression emerges as a possible strategy for more efficient therapeutic intervention in cancer. Interestingly, the previously described Aurora kinase inhibitor Hesperadin is also based on the same core structure as SU6668, further exemplifying the potential of this compound class with respect to Aurora kinase targeting (35).
In addition, the observation from our experiments that treatment of tumor cells with an Aurora kinase inhibitor can result in elevated p53 levels has important implications for cancer biology and may provide a novel strategy for inhibition of malignant cell growth.

We further characterized SU6668 as an inhibitor of the Ser/Thr kinase TBK1 in various cellular assays. In agreement with earlier evidence from TBK1-deficient mice, SU6668 potently suppressed poly(I:C)-induced IFN-β induction and RANTES biosynthesis. Moreover, recent data implicate the cellular TBK1 substrate IRF3 as essential host factor for *Listeria monocytogenes* infection, indicating that TBK1 could serve as a drug target for the treatment of bacterial disease (36). However, our HCMV experiments with SU6668 also exemplify proviral effects as a potential risk of therapeutic TBK1 inhibition.

In addition, the observation from our experiments that treatment of tumor cells with an Aurora kinase inhibitor can result in elevated p53 levels has important implications for cancer biology and may provide a novel strategy for inhibition of malignant cell growth.

We further characterized SU6668 as an inhibitor of the Ser/Thr kinase TBK1 in various cellular assays. In agreement with earlier evidence from TBK1-deficient mice, SU6668 potently suppressed poly(I:C)-induced IFN-β induction and RANTES biosynthesis. Moreover, recent data implicate the cellular TBK1 substrate IRF3 as essential host factor for *Listeria monocytogenes* infection, indicating that TBK1 could serve as a drug target for the treatment of bacterial disease (36). However, our HCMV experiments with SU6668 also exemplify proviral effects as a potential risk of therapeutic TBK1 inhibition.
In addition to mediating IRF3-regulated gene expression, TBK1 also contributes to NF-κB-dependent transcription via mechanisms, which are rather incompletely understood. Disruption of the TBK1 gene in mice led to massive liver degeneration before birth. Remarkably, this phenotype is highly reminiscent of those observed in IκκB-, NEMO- or RelA-deficient animals and is apparently linked to the loss of antiapoptotic NF-κB function (29). Because constitutive NF-κB signaling protects from apoptosis, TBK1 inhibition by SU6668 or related compounds could also thwart cancer cell survival in certain types of tumors as observed upon Iκκκ(3) inactivation (37).

We also identified protein kinase targets of SU6668 such as AMPK and ULK3, which were not further followed up in this study. Inhibition of AMPK activity should be avoided, because this enzyme is a key regulator of cellular glucose homeostasis (38). The function of mammalian ULK3 is not known, but our identification of SU6668 as a ULK3 inhibitor warrants further investigation of the biological roles of this kinase. Figure 5 summarizes how the findings from this study define objectives for the further optimization and development of SU6668-related small molecule drugs. Our results indicate unexpected opportunities for the development of indolinone-based drugs as multitargeted inhibitors, which not only oppose tumor angiogenesis by blocking selected RTK activities in endothelial cells and pericytes, but also directly interfere with certain Ser/Thr kinases essential for tumor cell proliferation and survival. In addition to increased therapeutic potency, such an approach of targeted polypharmacology would also minimize the risk of drug resistance formation during antiangiogenic therapy (39).

In conclusion, our study shows the utility of affinity-based proteomic approaches to evaluate the target selectivity of clinical kinase inhibitors. In combination with target-related biological assays, chemical proteomics can be instrumental in unraveling unknown cellular modes of action of the investigated compound. These data define new opportunities for drug development. Using previously established chemistries, rationales for the generation of potent multitarget inhibitors directed against phylogenetically unrelated kinases could be implemented.

Acknowledgments

Received 2/18/2005; revised 4/15/2005; accepted 5/16/2005.

Grant support: German Bundesministerium für Bildung und Forschung.

Cancer Research. 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.

We thank Michael and Karen, Ben Klebl, and Gerhard Müller for stimulating discussions and encouragement; Allegra Ritscher and Robert Brehm for excellent technical assistance; Achim Freisleben for analytical chemistry support; and Alexander Backes and Andrea Missio for their help and suggestions.

References


Proteomic Characterization of the Angiogenesis Inhibitor SU6668 Reveals Multiple Impacts on Cellular Kinase Signaling

Klaus Godl, Oliver J. Gruss, Jan Eickhoff, et al.


Updated version
Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/65/15/6919

Supplementary Material
Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2005/07/26/65.15.6919.DC1

Cited articles
This article cites 38 articles, 20 of which you can access for free at: http://cancerres.aacrjournals.org/content/65/15/6919.full.html#ref-list-1

Citing articles
This article has been cited by 11 HighWire-hosted articles. Access the articles at: /content/65/15/6919.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.