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

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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 indolinone 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

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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 covalently coupled to EAH Sepharose 4B (Amersham Biosciences, Buckinghamshire, United Kingdom) using carbodiimide coupling chemistry (5, 12). Lysis of frozen HeLa cell pellets (1×10^9 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 16-benzyldimethyl-*n*-hexadecylammonium chloride (16-BAC)/SDS-PAGE and mass spectrometry (MS) analysis were carried out essentially as described (10, 13).

Cell culture, transfections, adenovirus 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-4-[(*N*-succinylamino)carbonyl]-5-aminoimidazole ribonucleotide synthetase (AIRc-SAICARs) or IκB kinase–related enzyme TANK-binding kinase 1 (TBK1) fused to a NH_2-terminal FLAG tag. Human UNC-51-like kinase 3 (ULK3), interferon regulatory factor 3 (IRF3) and TBK1 cDNA were cloned into pPM7 vector encoding a COOH-terminal streptag epitope (16). The interferon-β (IFN-β) luciferase reporter plasmid was cloned as described (17).

For analysis of p53 expression, A549 cells were cultured in MEM supplemented with 10% fetal bovine serum and were seeded at 400,000 cells per six-well dish. Twelve hours later, cells were treated with different concentrations of SU6668. As a control for p53 up-regulation, cells were transduced with the indicated quantities of control adenovirus (AdJ5) or an adenovirus expressing a short interfering RNA (siRNA) hairpin targeted to p53 (AdH35). At 48 hours, the cells were washed twice with 150 mmol/L NaCl, 20 mmol/L HEPES (pH 7.5) and lysed in 1% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.005% bromophenol blue, and 40 mmol/L Tris (pH 6.8) prior to SDS-PAGE and immunoblotting. Control adenovirus AdJ5 is E1/E5-defective and bears no expression cassette. AdH35 is E1/E3-negative and bears the expression cassette for an H1 promoter driving the expression of a siRNA hairpin sequence targeted to p53 similar to that described by Brummelkamp et al. (18). The adenovirus were grown, purified by double CsCl density gradients and quantified as previously described (15).

For analytic *in vitro* association experiments with SU6668 beads, HFF, HeLa, or transfected COS-7 cells were lysed with the same buffer as used for the preparative protein work. Binding assays were done as previously described (10, 12). For subsequent immunoblotting, the following antibodies were used: rabbit anti-PDGFR type B, rabbit anti–AMP-activated protein kinase (AMPK; both from Upstate Biotechnology, Lake Placid, NY), mouse anti-TBK1 (Merck, Darmstadt, Germany), mouse anti-p53 (Oncogene Research Products, San Diego, CA), mouse anti-Yes, mouse anti-Aurora A (both from BD Transduction Laboratories, Lexington, KY), goat anti–ribosomal protein S6 kinase 3 (RSK3), mouse anti-Lyn (both from Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-FLAG antibody and mouse anti-α-tubulin antibody (both from Sigma, St. Louis, MO). ULK3-strep was detected with StrepTactin-HRP (IBA, Göttingen, Germany).

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, 10^4 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 Ser^{10} (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 TBK1-induced IFN-β reporter gene activity in 293 cells, 4×10^4 cells per 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′-GCATCTTCACTGATTCTT-GGATACC-3′ (forward), 5′-GTCTGGACCCCAAGGAAAACT-3′ (reverse), and 5′-TCTTCAAAAACTTCTCCACAACCCTCTGCA-3′ for gene-specific quantification. Human cytomegalovirus (HCMV) replication assays were done 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 carboxyl group could generate an affinity purification reagent with SU6668-like binding characteristics (5). Based on these considerations, we covalently linked the carboxyl 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. 1*A*. 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. 1*B*). 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 indolinone drug SU6668. Interestingly, in addition to tyrosine

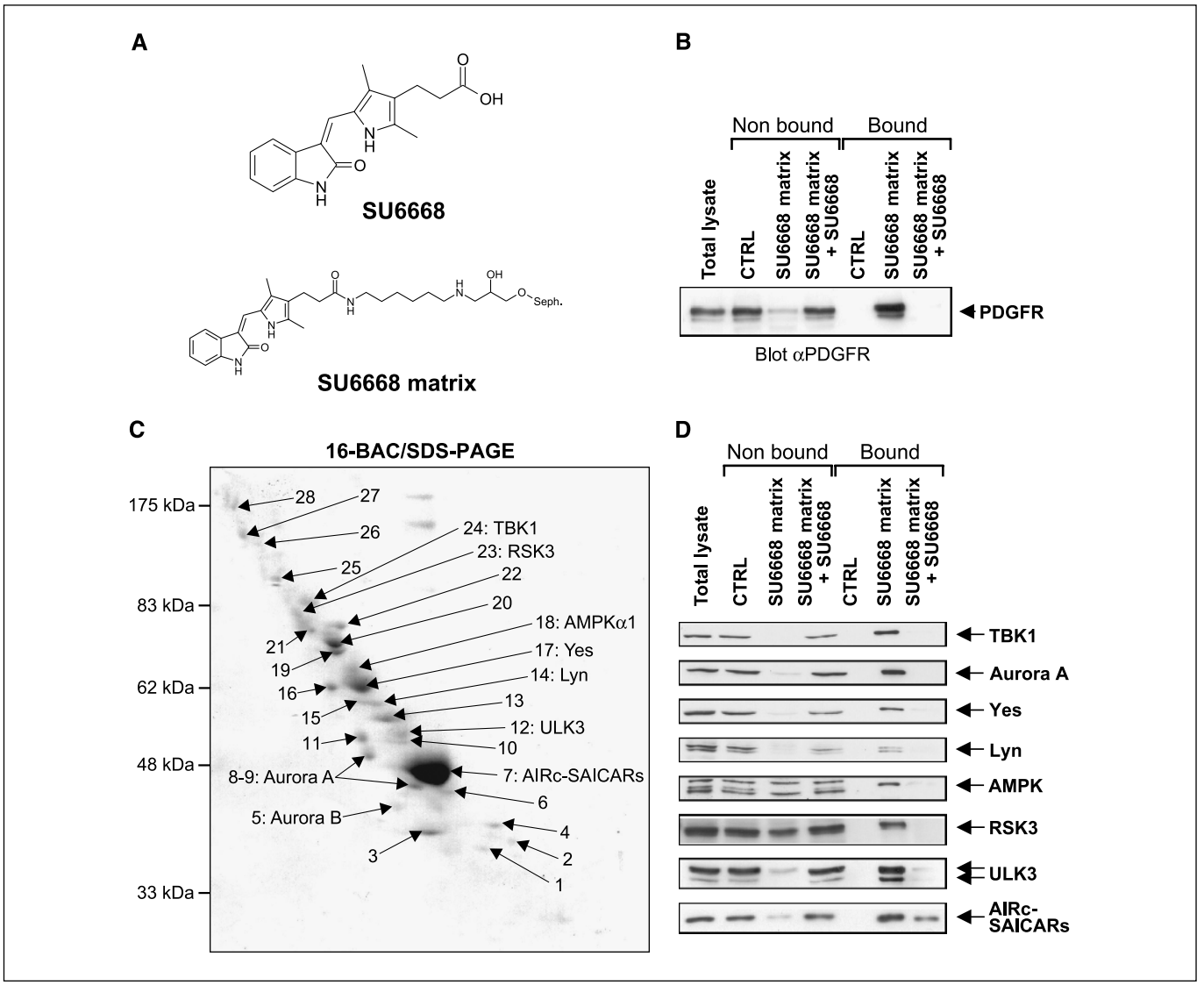

Figure 1. Identification and characterization of cellular SU6668 target proteins. *A,* chemical structures of SU6668 in its free and immobilized form. *B,* the SU6668 affinity matrix specifically retains βPDGFR from cellular lysate. HFF cell extracts were subjected to *in vitro* association with either 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 βPDGFR-specific antiserum. *C,* total lysate from 1.0×10^9 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.

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, AMPKα1, and ULK3 (Fig. 1*C*; Table 1). Known RTK targets of SU6668 such as βPDGFR 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. 1*D*). 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 IC_{50} 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

Table 1. Cellular protein targets of SU6668

Spot no.	Protein name	Gi no.*	MW (Da)	Score†	Peptides identified	Sequence coverage (%)‡
1	L-lactate dehydrogenase	13786849	36,558	69	8	34
2	L-lactate dehydrogenase	13786849	36,558	57	6	18
3	glyceraldehyde-3-phosphate dehydrogenase	31645	36,054	41	6	21
4	AMPKγ1	4506061	37,579	144	12	41
5	Aurora B	4759178	39,280	73	10	35
6	not identified	—	—	—	—	—
7	AIR carboxylase/SAICAR synthetase	5453539	47,079	65	20	49
8	Aurora A	7446411	45,790	91	11	45
9	Aurora A	7446411	45,790	97	12	54
10	enolase 1	4503571	47,169	72	11	29
11	eukaryotic translation elongation factor 1α	4503471	50,141	90	17	38
12	ULK3	39930361	53,444	100	12	38
13	aldehyde dehydrogenase X	399363	57,217	70	15	36
14	Lyn	187271	56,033	50	12	29
15	not identified	—	—	—	—	—
16	pyruvate kinase M2	125604	57,914	97	25	53
17	Yes	4885661	60,801	90	16	32
18	AMPKα1	5410312	62,808	73	11	31
19	heat shock 70 kDa protein 1 (HSP70.1)	462325	70,052	139	17	36
20	not identified	—	—	—	—	—
21	not identified	—	—	—	—	—
22	heat shock 70 kDa protein 5 (BiP)	16507237	72,333	181	17	35
23	RSK3	6166243	83,253	46	7	15
24	TBK1	7019547	83,642	77	14	30
25	eukaryotic translation elongation factor 2	181969	95,338		17	27
26	ATP citrate lyase	13623199	120,839	61	8	11
27	not identified	—	—	—	—	—
28	carbamoyl-phosphate synthetase 1	21361331	164,939	90	16	32

*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.

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. 2*A*). In control populations and in the presence of 10 μmol/L SU6668, most cells were found to be diploid (2 N DNA, indicative of G_1 phase) and a smaller fraction tetraploid (4 N DNA, indicative of G_2 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 G_2 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. 2*A*, 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 G_1 phase.

To study the cell cycle arrest in more detail, we analyzed cellular DNA as well as histone H3 phosphorylation on Ser^{10} in SU6668-treated cells by immunofluorescence microscopy. Histone H3 phosphorylation on Ser^{10} 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. 2*B*). Interestingly, many cells still showed condensation of chromatin in the presence of SU6668 (Fig. 2*B*). 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. 2*B*), 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. 2*C*).

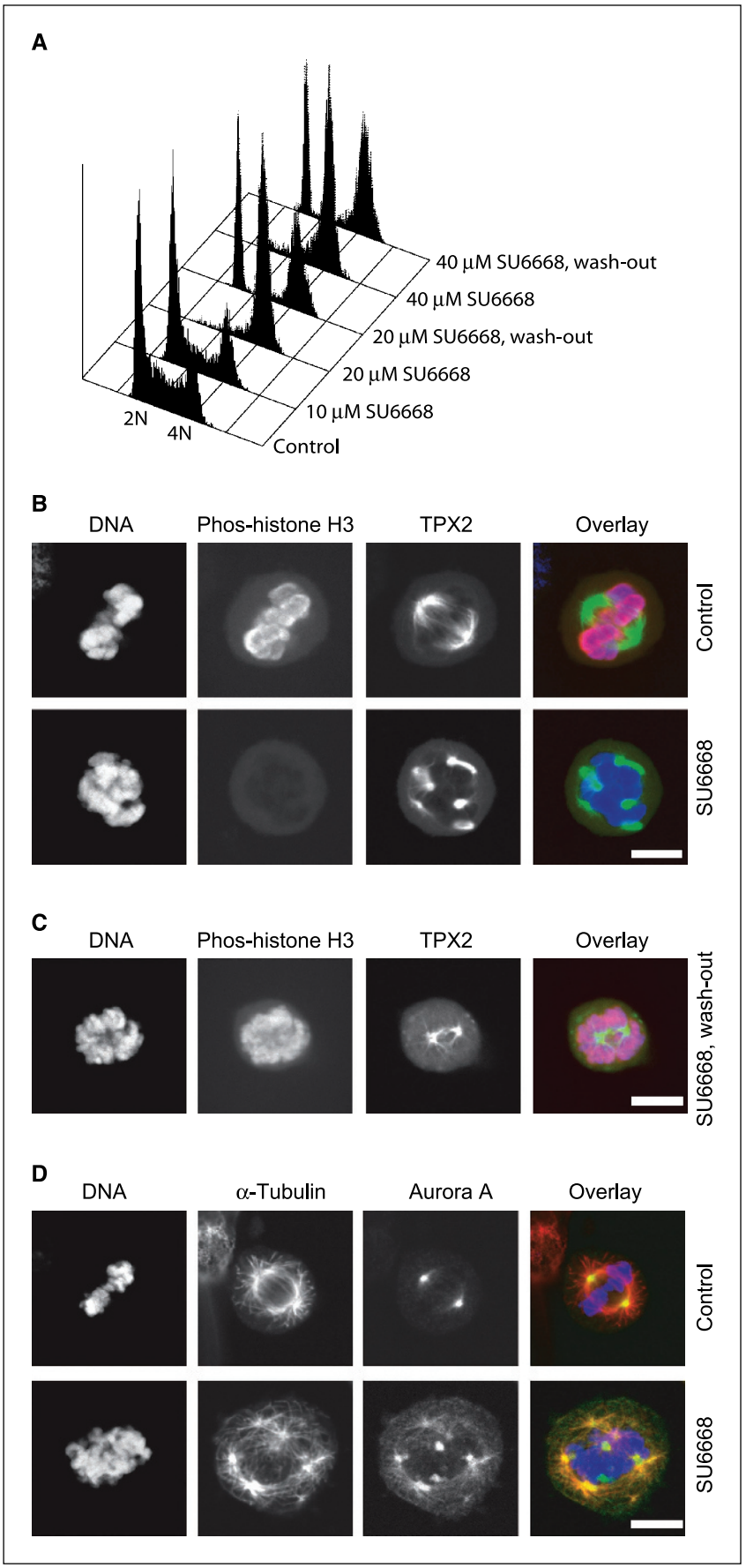

Figure 2. SU6668 inhibits Aurora kinase activity and cell cycle progression in human HeLa cells. *A,* distribution of the DNA content in control and SU6668-treated cells. HeLa cells were incubated with different concentrations of SU6668 or DMSO as a control for 15 hours and then stained with propidium 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 Ser^{10}; 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. 2*D*). In contrast, the presence of 20 μmol/L SU6668 Aurora A kinase localized to several spots confirming defects in centrosome organization (Fig. 2*D*). Consistent with these observations, the organization of mitotic microtubules was destroyed in SU6668-treated cells (Fig. 2*D*). 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 IRF3-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 TBK1 *in vitro* kinase activity raised the question of whether this inhibitor might also interfere with TBK1-mediated signaling in intact cells. As seen in Fig. 4*A*, TBK1-induced IFN-β reporter gene activity was effectively suppressed by low micromolar concentrations of SU6668 in transfected 293 cells. This result, together with the failure of kinase-inactive TBK1 to induce reporter gene expression strongly suggests that the kinase activity of TBK1 is critical for IFN-β promoter induction. Consistent with the described function of TBK1 as IRF3 kinase, SU6668 reversed the TBK1-induced increase in cellular phosphorylation of IRF3 in transfected COS-7 cells (Fig. 4*B*; refs. 31, 32). We next examined the cellular effects of SU6668 on endogenous signaling through TBK1. Exposure of primary HFF cells to poly(I:C), a double-stranded RNA agonist of toll-like receptor 3 mimicking viral infection, strongly activated transcription of the IFN-β and IL-8 genes. As monitored by quantitative RT-PCR, SU6668 concentrations as low as 3 μmol/L strongly impaired IFN-β induction, whereas 10-fold higher inhibitor doses were without effect on IL-8 gene expression (Fig. 4*C*). The specific abrogation 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 IKKε (33), which was inhibited by SU6668 *in vitro* with an IC_{50} 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. 4*D*). 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).

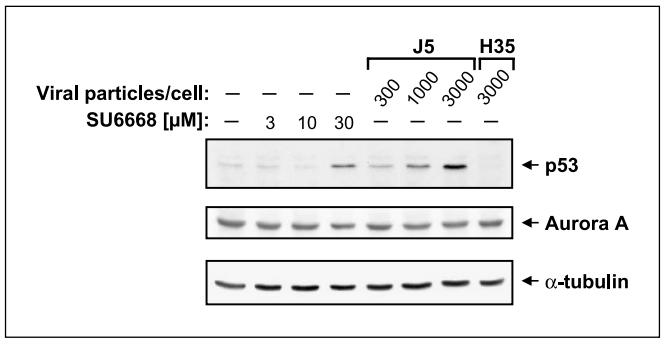

Figure 3. Exposure to SU6668 results in an increase of cellular p53 protein. A549 cells were seeded into six-well dishes and then either treated with different concentrations of SU6668 or infected with the indicated amounts of either control adenovirus (AdJ5) or adenovirus expressing an siRNA hairpin molecule targeted to p53 (AdH3). After 48 hours, cells were lysed followed by immunoblotting with p53- and Aurora A-specific antibodies. To control equal protein loading, the same lysates were further probed with antibody recognizing α-tubulin.

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_2-M block consistent with Aurora inactivation, the development of indolinone 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).

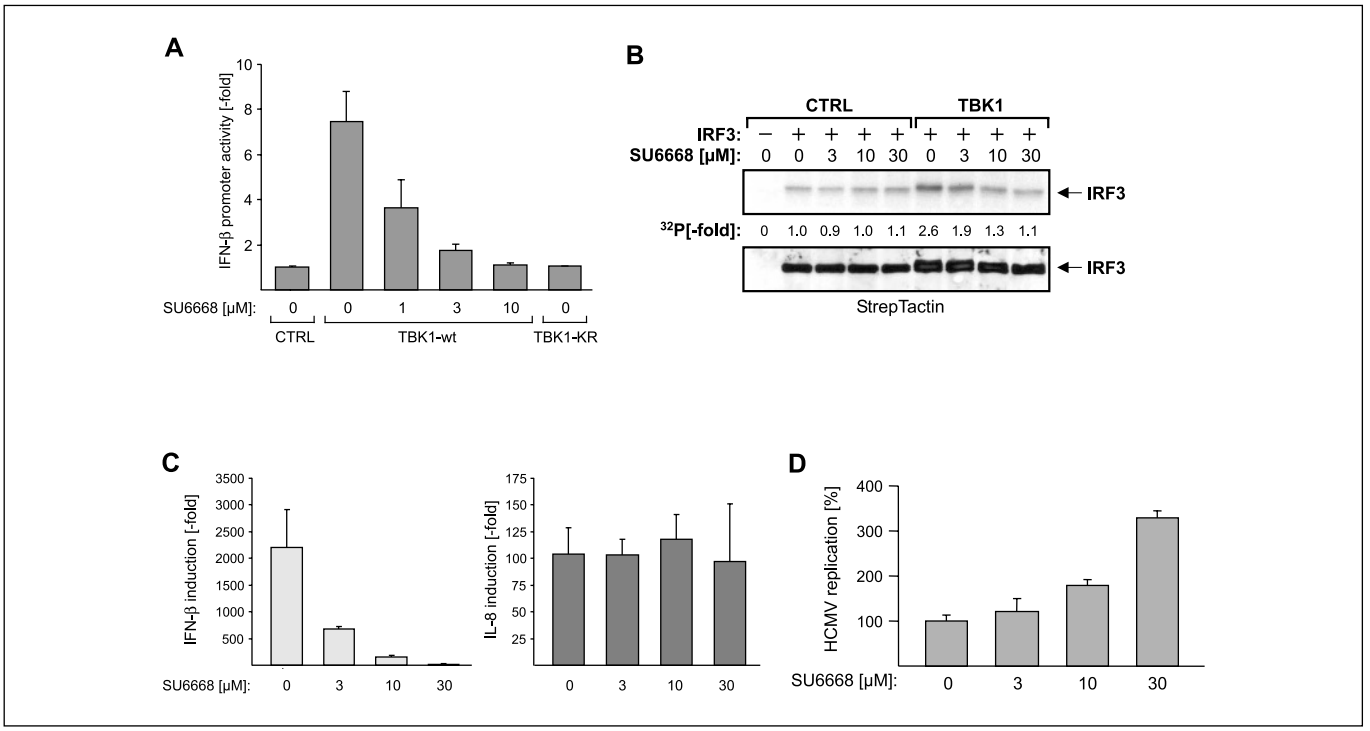

Figure 4. The indolinone drug SU6668 is a cellular inhibitor of TBK1-mediated signaling. *A,* SU6668 antagonizes TBK1-induced IFN-β reporter gene activity. 293 cells transiently cotransfected with an IFN-β luciferase reporter gene construct and either control, TBK1, or TBK1-K38R expression plasmid were incubated with medium containing the indicated SU6668 concentration for 6 hours prior to measurement of luciferase activity. *B,* effect of SU6668 on TBK1-mediated IRF3 phosphorylation in intact cells. COS-7 cells transiently expressing either strep-tagged IRF3, TBK1 or both proteins were treated with the indicated SU6668 concentrations prior to [^{32}P]orthophosphate labeling and precipitation of IRF3 using StrepTactin beads. IRF3 phosphorylation was detected by autoradiography and IRF3 protein expression was monitored by immunoblot analysis. *C,* SU6668 selectively blocks endogenous IFN-β gene induction. HFF cells were treated with the indicated SU6668 concentrations for 30 minutes and then stimulated for further 2 hours with 10 μg/mL poly(I:C) prior to RNA extraction. After reverse transcription, quantitative PCR was done to measure IFN-β and IL-8 mRNAs. Transcript levels in poly(I:C)-treated cells are displayed as fold induction. *D,* SU6668 accelerates HCMV replication. HFF cells maintained in medium supplemented with 10% fetal bovine serum were pretreated with SU6668 for 6 hours, infected with HCMV expressing enhanced green fluorescent protein, and then cultured for an additional 7 days prior to analysis of enhanced green fluorescent protein fluorescence as a marker for HCMV replication.

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.

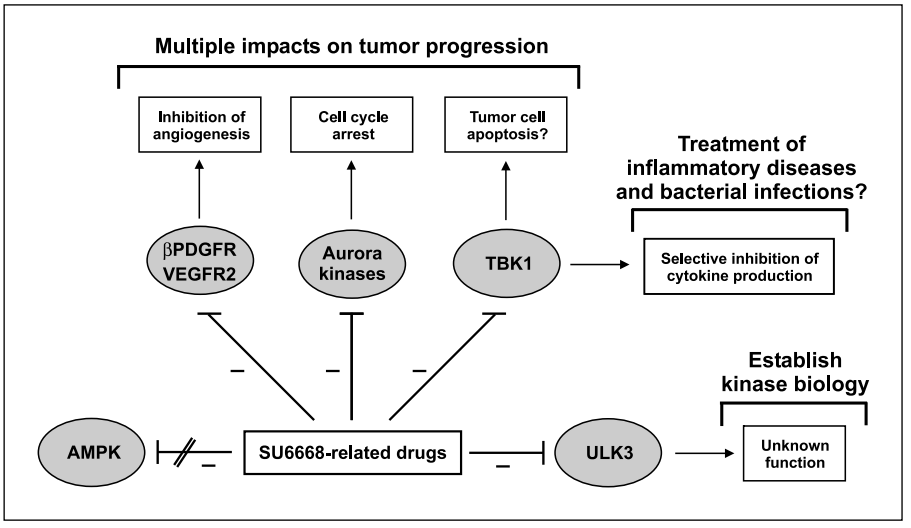

Figure 5. Schematic presentation illustrating how results from this study translate into rationales for further development of SU6668-related indolinone drugs. To increase the antineoplastic potency of SU6668-related indolinones, derivatives might be selected which are effective *in vivo* inhibitors of both RTKs and Ser/Thr kinases involved in various aspects of cancer development. In addition to Aurora kinases, the Ser/Thr kinase TBK1 might represent a molecular target in case it is validated as a tumor cell survival factor. Alternatively, TBK1 inhibition by indolinone compounds could be considered as a therapeutic approach for the treatment of certain inflammatory disorders or bacterial infections. The identification of ULK3 as a potential SU6668 target raises the issue whether this uncharacterized kinase plays physiologic roles related to human disease states. Moreover, SU6668-related development compounds should not affect AMPK, because inhibition of this critical regulator would adversely affect cellular glucose metabolism.

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 IKKβ-, 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 IKKβ 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 a targeted polypharmacology would also minimize the risk of drug resistance formation during antineoplastic 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.

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