Published in final edited form as:

Mol Cancer Ther. 2014 November ; 13(11): 2751–2762. doi:10.1158/1535-7163.MCT-14-0152.

Identification of kinase inhibitor targets in the lung cancer microenvironment by chemical and phosphoproteomics

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Abstract

A growing number of gene mutations, which are recognized as cancer drivers, can be successfully targeted with drugs. The redundant and dynamic nature of oncogenic signaling networks and complex interactions between cancer cells and the microenvironment, however, can cause drug resistance. Whereas these challenges can be addressed by developing drug combinations or polypharmacology drugs, this benefits greatly from a detailed understanding of the proteome-wide target profiles. Using mass spectrometry-based chemical proteomics, we report the comprehensive characterization of the drug-protein interaction networks for the multikinase inhibitors dasatinib and sunitinib in primary lung cancer tissue specimens derived from patients. We observed in excess of 100 protein kinase targets plus various protein complexes involving, for instance, AMPK, TBK1 (sunitinib) and ILK (dasatinib). Importantly, comparison with lung cancer cell lines and mouse xenografts thereof showed that most targets were shared between cell lines and tissues. Several targets, however, were only present in tumor tissues. In xenografts, most of these proteins were of mouse origin suggesting that they originate from the tumor microenvironment. Furthermore, intersection with subsequent global phosphoproteomic analysis identified several activated signaling pathways. These included MAPK, immune and integrin signaling, which were affected by these drugs in both cancer cells and the microenvironment. Thus, the combination of

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The authors declare no conflict of interest.

chemical and phosphoproteomics can generate a systems view of proteins, complexes and signaling pathways that are simultaneously engaged by multi-targeted drugs in cancer cells and the tumor microenvironment. This may allow for the design of novel anticancer therapies that concurrently target multiple tumor compartments.

Keywords

Kinase inhibitor; chemical proteomics; phosphoproteomics; non-small cell lung cancer; microenvironment

Introduction

Over the past years targeted drugs have profoundly changed the field of cancer therapy, particularly in chronic myeloid leukemia, melanoma and non-small cell lung cancer (NSCLC), which are often driven by oncogenic mutations in kinases. For instance, activating mutations in the epidermal growth factor receptor (EGFR) and fusions of the anaplastic lymphoma kinase (ALK) to echinoderm microtubule-associated protein-like 4 (EML4) in NSCLC have led to FDA approval of targeted therapies with erlotinib and crizotinib, respectively, which confer significant survival benefits to patients with these mutations. In addition, the discovery of various other oncogenic kinase drivers, such as BRAF, HER2, AKT, MEK, ROS1 and RET, (1, 2) has created a tremendous interest in the development of kinase inhibitors as promising novel options for targeted therapies in NSCLC. Oncogenic signaling networks, however, are often highly complex and redundant. Thus, it has been proposed that in order to elicit sufficient and durable clinical responses it may be necessary to target several signaling nodes simultaneously. At the same time, small molecule drugs in general, and kinase inhibitors in particular, are increasingly recognized as being unselective. As off-targets can cause toxic side effects, this may have important therapeutic implications. (3) Conversely, through concurrent targeting of important nodes within complex signaling networks, such off-target effects can also enhance the anticancer activity of kinase inhibitors and lead to entirely novel therapeutic applications, (4, 5) as shown in NSCLC for dasatinib and crizotinib. (6–9) Given that many of these findings originate from studies with cancer cell lines and considering the controversial discussion regarding differences between *in vitro* model systems and patient tumors, (10) it is necessary to determine, if off-targets that are functionally relevant in cancer cell lines are also expressed and engaged by the respective drugs in primary tumor tissues. Adding further complexity to the problem, several recent studies illustrated the significant effects that the tumor microenvironment can have on modulating drug sensitivity of cancer cells. (11–13) It is therefore important to also extend target profiling studies into the tumor microenvironment.

We have recently reported the comprehensive target profile and functional dissection of the mechanism of action of the multikinase inhibitor dasatinib in lung cancer cell lines. (4) To determine how different (or similar) drug target profiles are between cell lines and primary tumor tissues, we here expanded these studies to include lung tumor tissues from human patients and mouse xenografts. Using a combination of mass spectrometry (MS)-based

chemical and phosphoproteomics (Figure 1), we observed that the majority of targets were conserved between tissues and cell lines. Several other targets, however, some of which mapped to activated signaling pathways, were only present in tumor tissues. Interestingly, comparison with mouse xenograft tissues suggested that most of these additional targets originated from the tumor microenvironment. In summary, we demonstrate here that kinase inhibitors have complex off-target profiles that encompass both cancer cells and the surrounding tumor microenvironment. In addition, to the best of our knowledge we show for the first time that these drugs simultaneously engage activated signaling pathways in both compartments, and that these can be identified and differentiated by an integrated functional proteomic approach. These findings may have important implications for developing novel therapeutic approaches with kinase inhibitors that incorporate targeting of the tumor microenvironment.

MATERIAL AND METHODS

Biological Material

H292 and H23 cells were cultured in RPMI 1640 medium and 10% fetal calf serum (Invitrogen). Cell line authentication was done by STR analysis. Human lung cancer specimens were obtained from the Moffitt Tissue Procurement Core Facility and were treatment-naïve. The study was conducted in accordance with the Declaration of Helsinki and was approved by the institutional review board (University of South Florida). Written informed consent was obtained from each patient. For generating H292 and H23 mouse xenograft tumor samples, CD-1 female nude mice (Charles Rivers) were subcutaneously injected with 5×10^6 cells in 100 μ l of RPMI and Matrigel (1:1 ratio). After tumor sizes reached 50–100 mm³ (10–14 days) mice were sacrificed and tumors collected.

Compounds, Immobilization and Affinity Purification

c-Dasatinib and c-sunitinib were immobilized on NHS-activated Sepharose 4 Fast Flow resin (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) as reported previously (14, 15) with the exception that the final drug concentration was 25 nmol/50 μ l beads. Cell lysis of cell line pellets was performed as previously reported, (15) whereas lysis of primary xenograft and patient samples was achieved by pulverizing the flash-frozen tissue samples and resuspension with lysis buffer containing the Complete Protease Inhibitor Cocktail (Roche) in addition to the standard protease inhibitors. The targeted protein amount was 5 mg per experiment; all protein obtained from patient tumor tissues and xenografts was utilized (Table S1). Affinity chromatography and elution with formic acid were performed as described. (16) After incubation, drug affinity beads were washed with 100 bed volumes of lysis buffer and subsequently with 50 bed volumes of HEPES-NaOH buffer. The HEPES-NaOH buffer was composed of 50 mM HEPES (pH 8.0), 0.5 mM EDTA and 100 mM NaCl. All mass spectrometry analyses were performed in duplicate. In addition, biological duplicates of cell line and xenograft samples were generated.

Liquid Chromatography and Mass Spectrometry for Protein Identification

Sample preparation was done as previously described. (16) Mass spectrometry was performed on a hybrid linear trap quadrupole (LTQ) Orbitrap XL mass spectrometer

(ThermoFisher Scientific, Waltham, MA) using the Xcalibur version 2.0.7 coupled to an Agilent 1200 HPLC nanoflow system (dual pump system with one precolumn and one analytical column) (Agilent Biotechnologies, Palo Alto, CA) via a nanoelectrospray ion source using liquid junction (Proxeon, Odense, Denmark).(17) HPLC solvents were as follows: solvent A consisted of 0.4% formic acid in water and solvent B consisted of 0.4% formic acid in 70% methanol and 20% isopropanol. From a thermostatted microautosampler, 8 μ L of the tryptic peptide mixture were automatically loaded onto a trap column (Zorbax 300SB-C18 5 μ m, 5 $\times$ 0.3 mm, Agilent Biotechnologies, Palo Alto, CA) with a binary pump at a flow rate of 45 μ L/min. 0.1% TFA was used for loading and washing the pre-column. After washing, the peptides were eluted by back-flushing onto a 16 cm fused silica analytical column with an inner diameter of 50 μ m packed with C18 reversed phase material (ReproSil-Pur 120 C18-AQ, 3 μ m, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). The peptides were eluted from the analytical column with a 27 minute gradient ranging from 3 to 30% solvent B, followed by a 25 minute gradient from 30 to 70% solvent B and, finally, a 7 minute gradient from 70 to 100% solvent B at a constant flow rate of 100 nL/min. The analyses were performed in a data-dependent acquisition mode using a top 6 collision-induced dissociation (CID) method. Dynamic exclusion for selected ions was 60 seconds. No lock masses were employed. Maximal ion accumulation time allowed on the LTQ Orbitrap XL was 150 ms for MSⁿ in the LTQ and 1,000 ms in the C-trap. Automatic gain control was used to prevent overfilling of the ion traps and were set to 5,000 in MSⁿ mode for the LTQ, and 10⁶ ions for a full FTMS scan. Injection waveforms were activated for both LTQ and Orbitrap. Intact peptides were detected in the Orbitrap at 100,000 resolution and the threshold for switching from MS to MSMS was 2,000 counts.

Data Analysis for Protein Identification

The acquired data were processed with Bioworks v3.3.1 SP1 (ThermoFisher, Waltham, MA, USA), dta files merged with an internally-developed program, and searched against the human and/or mouse SwissProt databases version v2010.09 and v2011.06 (including isoforms and appended with common contaminants) with the search engines Mascot (v2.2.03, MatrixScience, London, UK) and Phenyx (v2.5.14, GeneBio, Geneva, Switzerland).(18) Submission to the search engines was achieved via a Perl script that performs an initial search with broader mass tolerances (Mascot only) on both the precursor and fragment ions (± 10 ppm and ± 0.6 Da, respectively). High-confidence peptide identifications were used to recalibrate all precursor and fragment ion masses prior to a second search with narrower mass tolerances (± 4 ppm and ± 0.3 Da). One missed tryptic cleavage site was allowed. Carbamidomethyl cysteine and oxidized methionine were set as fixed and variable modifications, respectively. To validate proteins, Mascot and Phenyx output files were processed by internally-developed parsers. Proteins with 2 unique peptides above a score T1, or with a single peptide above a score T2, were selected as unambiguous identifications. Additional peptides for these validated proteins with score >T3 were also accepted. For Mascot and Phenyx, T1, T2 and T3 were equal to 12, 45, 10 and 5.5, 9.5, 3.5, respectively (P -value $< 10^{-3}$). Following the selection criteria, proteins were grouped on the basis of shared peptides, and only the group reporters were considered in the final output of identified proteins. Spectral conflicts between Mascot and Phenyx peptide identifications were discarded. A false positive detection rate (FDR) of <1% and <0.1% was

determined for proteins and peptides, respectively, including the peptides exported with lower scores, by applying the same procedure against a reversed database. Comparison of the data sets was achieved by an internally-developed program that simultaneously computes the protein groups in all samples and extracts statistical data such as the number of distinct peptides, number of spectra, and sequence coverage. Protein abundance was estimated based on spectral counts using the distributed normalized spectral abundance factor (dNSAF), which adjusts spectral counts for protein length, differentially weighs spectra of unique and shared peptides and normalizes the dSAF values of an individual protein against the sum of all dSAFs in the experiment. (19)

For the xenograft samples, the validated results of Mascot and Phenyx and human and mouse searches were merged for each sample group, any spectral conflicts discarded, and grouped according to shared peptides using isobar version 1.5.3.(20)

Phosphoproteomic Analysis

Proteins were reduced with 10 mM dithiothreitol for 30 min at 56°C and alkylated with 22.5 mM iodoacetamide for 1 hr at room temperature in the dark. Eluates were digested with trypsin at 37°C overnight with end-over-end rotation. The resulting peptide solutions were acidified and desalted on a C18 column and lyophilized. Phosphopeptides were enriched with Fe³⁺-activated magnetic NTA beads (Qiagen) and analyzed by LC-MS/MS on an LTQ-Orbitrap Velos (ThermoFisher Scientific, Waltham, MA) coupled to a nano-Acquity UPLC (Waters, Milford, MA) as described previously. (21) Data files were processed using multiplier^z software to provide separate peak lists (.mgf files) for the CID and HCD MS/MS spectra; precursor *m/z* values were recalibrated using (Si(CH₃)₂O)₆ reference ions. (22) The two files were submitted to Mascot (v2.2.06) and searched against a concatenated reverse-forward human NCBI RefSeq database (released on 11/08/2010) appended with ~600 cRAP proteins (common repository of adventitious proteins). The mass tolerances were set at 10 ppm precursor, 0.6 Da product (CID) and 10 ppm precursor, 25 mmu product (HCD). Other search parameters included maximum of two missed cleavages, fixed modifications for cysteine (carbamidomethyl), variable oxidation on methionine and phosphorylation on tyrosine, serine and threonine. HCD spectra were deconvoluted prior to searching. CID and HCD data were separately filtered to 1% FDR using appropriate mascot cutoffs before combination.

Molecular Modeling

Interactions of sunitinib and dasatinib with NQO2 and PDXK were modeled through molecular dynamics simulations using the Schrodinger 2014 software suite, SiteMap and Desmond MD. The NQO2/imatinib (pdb: 3FW1) and the PDXK/theophylline (pdb: 4EOH) co-crystal structures served as modeling templates and co-crystallized ligands were deleted. Induced fit docking was implemented using Glide and Prime to find optimal binding sites and ligand conformations.

RESULTS

Dasatinib and sunitinib target complementary kinome portions in human lung cancers

Following up our previous study in NSCLC cell lines, (4) we selected dasatinib as the drug of choice to also probe primary tumor tissues. To further broaden the scope of our work, we included sunitinib as another multikinase inhibitor with a different *in vitro* kinase target profile from dasatinib. (23) Importantly, like dasatinib, sunitinib is in advanced clinical development for NSCLC. (7, 24, 25) For both drugs, we have recently described the design, synthesis and validation of close structural analogues suitable for chemical proteomics (c-dasatinib and c-sunitinib, Figure 1B). These drugs retain activity for the primary kinase targets c-ABL and PDGFR α , respectively, as well as for a plethora of other validated targets. (14, 15)

We next assembled a panel of 10 flash-frozen, non-macrodissected NSCLC tissue samples histologically classified as adenocarcinoma. Genotyping for *KRAS* and *EGFR* revealed that none of the samples had *EGFR* mutations, whereas six featured various activating *KRAS* mutations in codons 12 or 13 (Table S1). Total cell lysates of each individual tumor sample were generated and probed with affinity matrices of c-dasatinib and c-sunitinib using gel-free chemical proteomics (Figure 1A). (16) Mass spectrometry analysis showed that both drugs interacted with a large number of kinase targets in these patient-derived samples (Table S2). Across all tumor samples, a total of 79 tyrosine and serine/threonine kinases were observed. c-Dasatinib (Figure 2A) and c-sunitinib (Figure 2B) interacted with 52 and 42 protein kinases, respectively. The overlap between dasatinib and sunitinib consisted of only 15 kinases (Figure 2C). Most of these were SRC family kinases (SFKs), such as YES1 and SRC, and mitogen-activated protein kinases, namely MAP2K1/2 (MEK1/2), MAP3K2 and MAP4K5. Although YES1 was similarly strongly enriched with both compounds, CSK and MAP2K2 were much more substantially recovered with c-dasatinib and c-sunitinib, respectively. As reflected by the distributed normalized spectral abundance factor (dNSAF) values, (19) the most prominent differences between the dasatinib and sunitinib target profiles in these tumor samples were p38 α (MAPK14), BTK and several proteins mapping to the tyrosine-kinase like (TKL) branch of the kinome, in particular the specific dasatinib targets ILK and ZAK (MLTK). The strongest sunitinib-specific targets observed were TBK1, AMPK α 1 (PRKAA1) and several calcium/calmodulin-dependent protein kinases, particularly CAMK2G and CAMK2D, the latter constituting the most prominent sunitinib target in these samples. In summary, we identified close to 80 protein kinases that interact with dasatinib and sunitinib in primary lung adenocarcinoma tumors. Both drugs displayed largely complementary target profiles with little overlap.

Dasatinib and sunitinib engage protein kinase complexes in human lung cancers

In addition to the protein kinases mentioned above, c-dasatinib and c-sunitinib affinity chromatography recovered a large number of non-kinase proteins. Many of these are likely to interact with the kinases that are direct drug targets. For each tumor sample, we observed about 100 proteins after drug affinity enrichment (Table S2). In order to filter non-specific or background proteins, we determined the statistical significance of each protein across all samples to be more specific for dasatinib or sunitinib using a t-test (based on dNSAF values)

(Table S2). Selecting all proteins with a p-value below 0.05, this analysis highlighted 54 non-(protein) kinases, of which 21 and 19 exclusively interacted with the dasatinib and the sunitinib matrices, respectively. Using the MiMI Cytoscape plugin, (26, 27) which queries 10 different protein-protein interaction databases, subsequent network analysis identified various protein complexes that were enriched either by c-dasatinib or c-sunitinib (Figure 3). For instance, dasatinib engaged the well-characterized IPP complex, which involves integrin-linked kinase (ILK), parvin and pinch (LIMS1), as well as RSU1, and plays an important role in integrin signaling and focal adhesions. (28) Dasatinib also strongly interacted with several 14-3-3 proteins (YWHAB-Z) that partake in numerous protein-protein interactions with kinases. The sunitinib sub-network featured fewer 14-3-3 proteins. Instead, sunitinib targeted several other protein complexes that involve TBK1, IKBKE and AMPK. TBK1 and IKBKE have been reported to participate in various protein complexes with each other and with shared binding partners, such as AZI2 (NAP1) and TANK. (29–31) AMPK1 and 2 form tight multi-component kinase complexes consisting of the catalytic subunits AMPK α 1 or AMPK α 2 and several regulatory β and γ subunits (*e.g.*, PRKAG1, PRKAB1, PRKAB2), (32) all of which were enriched with c-sunitinib. In addition to known kinase interacting proteins, dasatinib and sunitinib matrices recovered several other prominent non-protein kinases. Some of these proteins, particularly the metabolic kinases, may also represent novel direct drug targets. For instance, ADK and PPAT, which are involved in purine metabolism, and FECH and TTR interacted specifically with the dasatinib matrix. PAICS, VAT1, NQO2 and PDXK were selectively purified by the sunitinib matrix. Considering that NQO2 is inhibited by several other kinase inhibitors, (15, 33, 34) it is possible that it is also a direct target of sunitinib. Pyridoxal kinase (PDXK) may be a novel anti-target of sunitinib as it plays an important role in vitamin B6 metabolism and has been recently proposed to be a positive prognostic marker for response of lung cancer to chemotherapy. (35) Molecular docking studies for NQO2 and PDXK support a potential direct binding of sunitinib as compared to dasatinib (Figure S1). In summary, dasatinib and sunitinib target several lung cancer signaling sub-networks through drug-protein and protein-protein interactions.

Kinase targets from cell lines are largely conserved across human tumor tissue samples

In order to develop an appreciation of the suitability of cell line models to reflect primary tissue samples for chemical proteomics, we next asked, if the observed drug-protein interactions in lung tumor samples can be also retrieved from NSCLC cell lines. In our previous study, conventional low-throughput, gel-based fractionation of drug affinity elution samples was utilized instead of the current, one-dimensional gel-free method. (4, 16) To enable a just comparison of the data sets, chemical proteomic experiments with both drugs were also performed in NSCLC cell lines using the same methodology as for the tumor tissue samples. Our panel of tumor tissues featured no *EGFR* mutations, but several samples with activating *KRAS* mutations. Therefore, the *EGFR*-wt/*KRAS*-mutant H23 (*KRAS* G12C) and *EGFR*-wt/*KRAS*-wt H292 NSCLC cell lines were selected as model systems to reflect the oncogenic background of the patient-derived material. Notably, these mutational profiles represent the majority of NSCLC cases. (1, 2)

As expected, blood components, such as hemoglobin, albumin, complement C3 and antibodies observed in the tumor tissue samples were naturally absent in the cell lines. Interestingly, the comparison of the combined analysis of all *KRAS*-wild-type and all *KRAS*-mutant tissue samples with the appropriate cell line target profiles showed that the majority of kinases that are engaged by dasatinib and sunitinib in the tissue samples were also enriched from the H23 and H292 cells (Table S3). Based on the dNSAF values, the most prominent targets in the cell lines were similar to those observed in the primary samples (Figure 4). For instance, CSK, p38 α , ILK and ZAK were amongst the strongest dasatinib targets in both cell lines and the patient samples. Likewise, sunitinib interacted strongly with AMPK α 1, several CAMK2s, MAP2K1, MAP2K2 and TBK1 across all samples examined. The latter, however, was less prominent in *KRAS*-mutant patient samples compared to *KRAS*-wt tissues and the cell lines. The most notable exceptions to this general trend were EPHA2, AURKA and STK3, STK4, AURKA/B for dasatinib and sunitinib, respectively. These kinases were much more prominent in the cell lines than in the patient samples. Most other kinases were similarly abundant as in the tissue samples. This was also the case for many of the kinase complex components although some differences were apparent. For instance, several members of the IPP complex (i.e., PARVG and LIMS2/3) were more evident in the tissue samples, whereas AMPK and TBK1 binding partners were only weakly observed or absent in *KRAS*-mutant tissue samples compared to *KRAS*-wt tumors and cell lines.

Interestingly, several kinases enriched by the drug affinity matrices from the lung cancer tissues were completely absent in the cell lines. These included several SFKs, such as FGR, LCK, HCK and LYN, which interacted with both drug affinity matrices. RSK1–2 (RPS6KA1/3), MARK2 and PYK2 (PTK2B; in *KRAS*-mutant samples) were detected as sunitinib-specific targets only in the patient samples. Similarly, the dasatinib targets SYK, IRAK3, RIPK3, STRADA, EGFR, KIT and PDGFRB were only observed in the tissue samples. BTK, one of the most potent dasatinib targets, (36) was particularly strongly enriched from patient material, but only weakly from cell lines. Several of these kinases, particularly BTK, IRAK3, RIPK3, SYK, LCK, HCK, FGR, etc., are known to be highly expressed in hematopoietic cells, such as B-, T- and myeloid cells (Figure S2).(37) Thus, it is possible that these targets are not derived from the actual lung cancer cells, but rather from tumor-infiltrating immune cells. Also, PDGFRB has been described to be expressed by cancer-associated fibroblasts. (38, 39) Consistently, comparison with previous studies highlights several of these kinases, such as BTK, PDGFRB, LCK and HCK, as some of the most differentially expressed targets between the NSCLC cell lines investigated here and leukemia or melanoma cell lines, whereas most other interactions were maintained (Table S4).(5, 14, 18) Additional differences were observed for instance for BCR-ABL, ephrin receptors, CAMK2B, FER and PTK2B, which is likely due to cell type-specific expression or post-translational modification patterns. Taken together, the NSCLC cell line target profiles of both kinase inhibitors are strongly conserved between primary lung cancer tissues and cell lines. Several kinase targets, however, are unique to NSCLC tissue samples and potentially originate from various cell types within the tumor microenvironment.

Differences in drug target profiles between primary and cell line samples can be mostly attributed to the microenvironment

To assess the hypothesis that several prominent dasatinib and sunitinib kinase targets in NSCLC tissue originate from the tumor microenvironment, mouse xenograft models were generated from the human H23 and H292 cell lines. After tumors had developed, the mice were sacrificed, tumors collected and utilized for chemical proteomic experiments with c-dasatinib and c-sunitinib. Exploiting species differences in the amino acid sequences of the proteomes (40, 41) sequential searching of the data against both the human and mouse protein databases allowed the identification of peptides specific to human or mouse, as well as peptides that are indistinguishable due to identical sequences of mouse and human orthologues. This analysis led to the assignment of kinases to both species (Tables S5, S6). Albeit not exclusively, the majority of the identified proteins that were common to cell lines and tumor tissues were also identified in the xenograft samples as human. Supporting the concept of exploiting species-specific sequences to determine origin, proteins like albumin and complement C3, which were observed in human tissues, but not cell lines, were of mouse origin in the xenograft samples. This was also the case for various antibodies. Interestingly, dasatinib kinase targets, such as Btk, Pdgfrb and Ripk3, which were present in the tissue, but not the cell line datasets, matched to mouse proteins in the xenograft samples (Figure 4A). Similarly, several sunitinib targets, *e.g.* Fgr, Hck and in the *KRAS*-mutant sample series also Ptk2b, Ikbke and Rps6ka4, originated from mouse (Figure 4B). These observations were particularly apparent for kinases usually expressed in hematopoietic cells or fibroblasts, *e.g.* Btk, Lck, Fgr, Pdgfrb. In addition, some kinases were only observed in the xenograft samples and only matched to the mouse protein database. Most notable were Pdgfrb (sunitinib), Fyn (dasatinib) and Csf1r (both drugs). This is likely to be attributable to either varying extents of stroma present in the individual samples or to differences between the human and mouse tumor microenvironment. Conversely, some dasatinib targets, such as DDR1, EPHA1, EPHB4 (*KRAS*-wild-type) and EGFR (Figure 4A), were identified as “human” in the xenograft samples, but were not identified in the cell lines. This may indicate that expression of some proteins in the cancer cells is induced in a paracrine fashion by stromal cells. In summary, analysis of drug target profiles in NSCLC mouse xenograft samples allowed distinction of human and mouse, *i.e.* host microenvironment-derived proteins. As the latter also accounted for the major differences between cell line and patient tissue target profiles, this suggested that these drug targets originated also from the tumor microenvironment in the patient tissue samples.

Phosphoproteomics reveals activated proteins and signaling pathways in tumor and microenvironment cells that are targeted by dasatinib and sunitinib

We next investigated the differences between the drug target profiles derived from cell line, tumor tissue and xenograft samples by global phosphoproteomics. In many cases such an approach allows conclusions about the activation status of proteins and signaling pathways. Chemical proteomics requires proteins to be in a native state; but proteins are preferentially denatured for phosphoproteomics. Thus we performed phosphoproteomics *subsequent* to the drug affinity purification experiments. At the stage of eluting proteins from the drug affinity matrices, proteins were denatured and split into two aliquots. One was analyzed according to

the chemical proteomic workflow whilst the other was processed for phosphopeptide enrichment. Despite the low amount of sample available from the drug-binding sub-proteomes, we identified in total 229 phosphoserine, -threonine or -tyrosine phosphosites that mapped to 101 different proteins across both species (Table S7). Amongst these, c-dasatinib and c-sunitinib purified 18 phosphorylated protein kinases each; plus 48 and 47 non-kinase phosphoproteins, respectively. Cross-sample comparison showed that phosphorylation of several sites was widely conserved. For instance, phosphorylation of the direct and indirect dasatinib targets p38 α , parvin A and ZAK at Y182, S14 and S637 (corresponding to S638 of murine Zak), respectively, was detected in cell line, xenograft and tumor tissue samples. RIPK2 was also phosphorylated at various sites in many samples. Importantly, phosphorylation of p38 α on Y182, ZAK on S637 (autophosphorylation) and RIPK2 on S176 are positively correlated with kinase activity. For sunitinib, phosphorylation of AAK1 particularly at T606 and T620 (corresponding to T523 and T537 of murine Aak1) and MEK2 (MAP2K2) at T394 were observed across most samples. In addition, some phosphorylation events were only detected in samples with *KRAS* wild-type background, but nonetheless across different sample types. These include AMPK α 1 T488, SIK1 S575 (mouse: S577) and STK4 S320. Although the functions of these phosphorylation sites are unknown, this suggests that they may denote more general differences between *KRAS*-mutant and -wild-type NSCLC. The sites discussed above represent cases in which cell lines, xenografts and patient tissue samples were similar. In addition, some prominent phosphorylation sites (particularly on SIK2, SRSF1 and CAMK2B and D) were exclusively observed in the human tumor tissues. Several of the observed CAMK2D sites represent autophosphorylation events and therefore indicate kinase activity. Phosphorylation of TENC1, a phosphatase that negatively regulates AKT signaling, (42) was only observed in primary human and mouse tissues, but was absent in the cell lines. In the xenograft, this TENC1 phosphopeptide by itself did not allow distinction between human and mouse origin. However, the observation of *unphosphorylated* Tenc1 peptides with mouse sequences in the *KRAS*-mutant xenograft samples suggests the possibility that Tenc1 is expressed and phosphorylated in the tumor microenvironment (Figure 5). This may be the case also for several other proteins, most prominently AAK1, RIPK2, ZAK, parvin A, TANK, MEK2 and p38 α , which feature unique (unphosphorylated) mouse peptides in the xenograft samples. In support of this, phosphopeptides from Zak, parvin A and Aak1 in the xenograft samples were unambiguously assigned to mouse sequences thereby providing direct evidence that these phosphorylations occur in the tumor microenvironment (Table S7). Mapping of all phosphoproteins to signaling pathways revealed that dasatinib and sunitinib engage several prominently phosphorylated pathways in human lung cancer tissues (Table S8). When focusing on the phosphorylation events that correlate with protein activity, this highlights in particular MAPK, integrin and immune signaling as activated pathways that are targeted by the drugs. Interestingly, comparison with mouse-derived drug targets and phosphopeptides suggests that some of these pathways are both activated and targeted in the tumor microenvironment. In summary, phosphoproteomic analysis shows that dasatinib and sunitinib concomitantly target several activated signaling proteins and pathways in tumor cells and the microenvironment.

DISCUSSION

It is becoming widely appreciated that small molecule kinase inhibitors are often non-specific and serendipitously target a wide range of kinases. (43) This makes such drugs potentially useful tools for polypharmacology approaches that aim at overcoming the intrinsic resilience of oncogenic signaling networks towards drug therapy and the support that the tumor microenvironment lends to cancer cells. Selecting the correct pleiotropic kinase inhibitor for a particular tumor, however, still remains a significant challenge. *In vitro* drug profiling using kinase binding or activity assays provides a wealth of information, (23, 44) but does not cover the entire kinome or account for differential protein expression, protein-protein interactions, (most) gene mutations or post-translational modifications that can modulate kinase activity. We therefore applied a mass spectrometry-based chemical proteomic approach, (45) to determine the unbiased and native protein binding profile across lung cancer patient tumor specimens, cell lines and xenografts. It is important to note that for the targets that are also covered by *in vitro* profiling panels, we observed good consistency between data from the different technologies, which cross-validates the approaches (Table S9). (23, 44, 46) In addition, chemical proteomics showed that dasatinib and sunitinib engage several protein complexes through their kinase targets, some of which have been described to play important roles in lung cancer. For instance, sunitinib specifically interacted with TBK1, IKBKE and AMPK complexes. TBK1 has been described to be synthetically lethal with *KRAS* mutations and is, based on our data, one of the most prominent sunitinib targets. (47) However, this is apparently mostly attributable to relatively high TBK1 expression levels, as *in vitro* kinase assays, while confirming this interaction, do not suggest that sunitinib would cause sufficiently potent TBK1 inhibition at physiologically relevant concentrations. Furthermore, it is curious that TBK1, IKBKE and their interaction partners were recovered much more weakly particularly from *KRAS*-mutant than from *KRAS*-wt patient tumor or cell line samples. Dasatinib prominently enriched the ILK-PINCH-PARVIN complex, (28) which has been shown recently to play an important role in epithelial-to-mesenchymal transitions in lung cancer cells. (48) It is interesting to note that the composition of this protein complex with respect to PINCH and PARVIN proteins seems to be more heterogenous in primary tumor tissue. In particular, PARVG and PINCH3 (LIMS3) were not observed at all in the cell lines. Consistently, PARVG is mainly expressed in hematopoietic cells. (28) In general, the drug target interaction profiles between cell lines and primary tissue samples were highly similar. This is consistent with the conclusion that biological observations from lung cancer cell lines generally translate well into *in vivo* and patient scenarios. (10) Similar observations as with PARVG, however, were made with other proteins including various kinases, *e.g.* BTK, PDGFRB, LCK and FGR, suggesting that some drug targets and their protein binding partners may be derived from the tumor microenvironment, such as cancer-associated fibroblasts or tumor-infiltrating immune cells. Although chemical proteomics is at best only semi-quantitative and it is not possible to assign the exact cell type within the microenvironment, from which these proteins originate, this interpretation is supported by the fact that many of these differentially observed targets feature peptides that match to the mouse orthologue sequences in the samples derived from human lung cancer cell lines grown as mouse xenografts. The observation that anticancer drugs engage targets not just in cancer cells themselves, but also in the microenvironment, is

not unexpected. However, the unbiased and proteome-wide determination of small molecule drug-protein interaction networks in the tumor microenvironment has to the best of our knowledge not been reported before. Through integration of chemical proteomics with a global phosphoproteomic approach, our data furthermore suggest that some of the drug targets in the microenvironment partake in activated signaling pathways, including for instance integrin and MAPK signaling. (49) Considering the complex bidirectional interactions between tumor and stromal cells, (50) modulation of these and other affected signaling pathways may therefore either enhance or decrease the desired anticancer activity of kinase inhibitors. (11–13) We believe these observations will have profound implications for drug development as they illustrate the importance to consider these drug effects and to determine drug target profiles in the tumor microenvironment early on. This furthermore harbors the exciting possibility to rationally design synergistic drug combinations based on simultaneous targeting of cancer and microenvironment cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial Support

U. Rix and E.B. Haura received funds from the Moffitt Cancer Center NIH/NCI SPORE in Lung Cancer (P50-CA119997) and the Moffitt Cancer Center. K.L. Bennett and J. Colinge were funded by the Austrian Federal Ministry for Science and Research (Gen-Au BIN). J. Colinge was furthermore supported by the Austrian Science Fund FWF (grant No P 24321-B21). G. Superti-Furga was funded by the Austrian Academy of Sciences and the Austrian Federal Ministry for Science and Research (Gen-Au APP). J.A. Marto received funding from the Dana-Farber Strategic Research Initiative and the NIH (P01NS047572 and R21CA178860). We also wish to acknowledge the Moffitt Chemical Biology Core Facility, which is supported by the NCI as a Cancer Center Support Grant (P30-CA076292).

We thank the patients who so graciously provided data and tissue for this study.

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Figure 1. Project outline

A. Schematic representation of chemical proteomics. Incubation of a cell lysate with a drug affinity matrix enriches for drug-binding proteins, which are proteolytically digested. Protein identification is achieved by analysis of the resulting peptide sequences with high resolution tandem MS and subsequent protein databases searching. LC-MS/MS: liquid chromatography coupled tandem mass spectrometry. B. Chemical structures of dasatinib, sunitinib and their coupleable analogues c-dasatinib and c-sunitinib. c-Dasatinib and c-sunitinib are immobilized on solid support via the terminal amino group, which is marked with an arrow. C. Project workflow scheme. Chemical proteomics experiments were performed for the multikinase inhibitors dasatinib and sunitinib using 10 primary NSCLC tumor tissue samples, as well as H292 and H23 NSCLC cell line and mouse xenograft samples of these cell lines. Drug affinity eluates were concurrently processed for identification of target proteins and phosphoproteomics. The datasets were subsequently

combined to generate a proteome-wide view of the signaling pathways engaged by dasatinib and sunitinib. White background of biological samples indicates *KRAS* wild-type, grey background *KRAS*-mutant status.

Figure 2. Dasatinib and sunitinib kinase targets in NSCLC patient tumor tissues mapped to the human kinome

A. Dasatinib protein kinase targets identified across all 10 human NSCLC tumor tissues. Color intensity depicts average dNSAF values. B. Sunitinib protein kinase targets identified across all 10 human NSCLC tumor tissues. Color intensity depicts average dNSAF values. C. Overlap between the dasatinib (blue) and sunitinib (red) protein kinase target profiles in human NSCLC tumor tissues. The Human Kinome Map was adapted with permission from Cell Signaling Technology (www.cellsignal.com). Kinases with less than 2 unique peptides across the sample set were discarded.

Figure 3. Global drug-protein interaction networks for dasatinib and sunitinib across NSCLC patient tumor tissues

Depicted are all protein kinases and non-kinase proteins, which were identified by drug affinity chromatography with c-dasatinib (blue) and c-sunitinib (red) across all human NSCLC patient tumor tissue samples. Non-protein kinases were submitted to the additional condition of being c-dasatinib or c-sunitinib specific ($P < 0.05$). Node color intensities indicate relative abundance of a protein as determined by the average dNSAF (see key). Proteins with dNSAF values below 0.001 are depicted in white. Node border colors correspond to the respective drug. Proteins identified with both drugs feature a grey border. Protein kinases feature a bold border. Protein-protein interactions between observed proteins were retrieved from various databases and are depicted as grey edges. Drug-kinase interactions are depicted by black edges. Known multiprotein complexes are highlighted in the color according to the respective kinase inhibitor, which is interacting with the primary kinase target within these complexes. HSP90 and 14-3-3 proteins partake in a variety of complexes and are highlighted in grey.

Figure 4. Comparison of dasatinib and sunitinib protein kinase target profiles across NSCLC tumor, cell line and xenograft samples depending on *KRAS* mutational status

Kinase target profiles of dasatinib (A) and sunitinib (B) across NSCLC cell lines, patient tumor (PT) samples and NSCLC cell line-derived mouse xenograft samples. Samples are grouped by *KRAS* mutational status. H292 cells are compared with *KRAS*-wild-type (wt) patient tissues and H292 mouse xenografts (X-H292). H23 cells are compared with *KRAS*-mutant (mut) patient tissues and H23 mouse xenografts (X-23). Kinases in xenografts are distinguished by species, i.e. human (hs: homo sapiens) or mouse (mus musculus: mm). Color intensities (blue for dasatinib, red for sunitinib) correspond to average dNSAF values

and fall into four categories: white: not detected, light: <0.001 , medium: $0.001-0.01$, dark: >0.01 . Grey tones depict proteins in the xenograft experiments, which due to identical sequences of mouse and human peptides cannot be unambiguously assigned to one species. Red arrows indicate the most noteworthy kinases that are likely of tumor microenvironment origin. Kinases with less than 2 unique peptides in a sample set were discarded unless they were observed in a different sample set with at least 2 unique peptides.

A KRAS mut **B KRAS wt**

Figure 5. Phosphorylation, signaling pathway and origin information mapped onto drug-protein interaction networks for dasatinib and sunitinib in primary human NSCLC tissue samples

Depicted are all proteins identified by drug affinity chromatography after background subtraction (as described for Fig. 3) supplemented with all phosphoproteins identified by phosphoproteomics. Protein-protein interactions between observed proteins were retrieved from public databases. Each phosphosite is indicated as a red circle around the respective protein node. Red protein nodes indicate proteins that are (at least in part) of “mouse” origin in the respective xenograft samples and thus may originate from the tumor microenvironment, grey nodes indicate proteins never observed with any mouse-specific

peptides. Dark blue pie slices: proteins involved in the MAPK signaling pathway, light blue pie slices: proteins involved in the integrin signaling pathway.