Cellular Targets of Gefitinib

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Abstract

Targeted inhibition of protein kinases with small molecule drugs has evolved into a viable approach for anticancer therapy. However, the true selectivity of these therapeutic agents has remained unclear. Here, we used a proteomic method to profile the cellular targets of the clinical epidermal growth factor receptor kinase inhibitor gefitinib. Our data suggest alternative cellular modes of action for gefitinib and provide rationales for the development of related drugs. (Cancer Res 2005; 65(2): 379-82)

Introduction

Aberrant activation of the epidermal growth factor receptor (EGFR) tyrosine kinase has been implicated in several key aspects of human neoplasia such as increased proliferation, survival, and invasiveness of cancer cells. In addition, signaling through the EGFR promotes tumor neovascularization and induces resistance to cytotoxic chemotherapy (1). Based on these multiple impacts on cancer cell physiology, the EGFR tyrosine kinase has been recognized as an attractive molecular target for selective treatment of solid tumors with increased EGFR expression levels. The ATP-competitive kinase inhibitor gefitinib (Iressa, ZD1839) was the first EGFR-directed small-molecule drug that received approval for the treatment of non–small cell lung cancer and is now being used as third-line treatment upon failure of established chemotherapies (2). However, an objective tumor response is only observed in a rather small fraction of gefitinib-treated patients. Recently, clinical responses to gefitinib could be correlated with acquired somatic mutations in the kinase domain-encoding region of the *EGFR* gene, which resulted in enhanced EGFR activity and sensitivity to gefitinib on the target protein level (3, 4). These data indicate that gefitinib is highly efficacious in a subset of patients with lung cancer but have further raised concerns that signaling through overexpressed, nonmutated EGFR in epithelial cancers might not be effectively suppressed up to the maximum tolerated gefitinib dose in a clinical setting (3). In this context, it is also noteworthy that dose escalation from 250 to 500 mg gefitinib per day even somewhat reduced efficacy in patients (2). Furthermore, it is also unclear whether dose-limiting side effects of gefitinib or occasional severe complications such as interstitial lung disease are related to inhibition of either EGFR or unknown secondary targets of the drug (1, 2).

To address the unresolved issue of possible alternative modes of action and to identify secondary gefitinib targets in addition to the EGFR, we adapted a recently described proteomic approach to study gefitinib selectivity in a cellular system (5, 6). In this report, we describe the identification and characterization of more than 20 previously unknown kinase targets of this clinical EGFR inhibitor.

Materials and Methods

Compound Synthesis and Immobilization. Gefitinib (Iressa, ZD1839) and 4-(3-chloro-4-fluoro-phenylamino)-7-methoxy-quinazolin-6-ol were synthesized as described (7). To prepare AX14596, 0.32 g (1.00 mmol) 4-(3-chloro-4-fluoro-phenylamino)-7-methoxy-quinazolin-6-ol was refluxed in 20 mL acetonitrile with 0.26 g (1.10 mmol) *N*-BOC-3-propyl-bromide and 0.15 g (1.10 mmol) potassium carbonate for 8 hours. The reaction mixture was cooled to room temperature, and the solvent was evaporated under reduced pressure. The residue was stirred in a mixture of 15 mL water and 15 mL ethyl acetate for 30 minutes at 0°C. The resulting [3-(4-(3-chloro-4-fluoro-phenylamino)-7-methoxy-quinazolin-6-yloxy)-propyl]-carbamicacid *tert*-butyl ester was filtered off, washed with 5 mL cold ethyl acetate, and air-dried. Subsequently, 0.25 g (0.50 mmol) of the product were suspended in 20 mL methanol, and 1.0 mL ethyl acetate saturated with hydrochloric acid was added to the reaction mixture. After stirring for 2 hours at room temperature and 30 minutes at 0°C, AX14596 (3-[4-(3-chloro-4-fluoro-phenylamino)-7-methoxy-quinazolin-6-yloxy]-propyl-ammonium chloride) was filtered off, washed with 15 mL diethyl ether, and air-dried. The chemical structures of gefitinib and AX14596 were confirmed by nuclear magnetic resonance and the purity of both compounds was more than 95% according to high-performance liquid chromatography-mass spectrometry (MS) analysis.

Covalent coupling of AX14596 to epoxy-activated Sepharose 6B (Amersham, Uppsala, Sweden) and the generation of control beads were done in 50% DMSO/0.05 mol/L Na_2CO_3 as previously described (5).

Affinity Chromatography and Mass Spectrometry. Affinity chromatography on AX14596 columns was done essentially as previously described for a different kinase inhibitor resin (5). Bound proteins were eluted with lysis buffer containing 1 mmol/L gefitinib, 10 mmol/L ATP, and 20 mmol/L $MgCl_2$ prior to precipitation and subsequent separation by preparative 16-benzyldimethyl-*n*-hexadecylammonium chloride (16-BAC)/SDS-PAGE followed by matrix-assisted laser desorption ionization MS analysis (5).

For liquid chromatography (LC)-MS/MS analysis, proteins bound to the AX14596 column were first released from the affinity matrix as described above and collected in three fractions followed by a second elution step with buffer containing 0.5% SDS (fraction 4). All fractions were precipitated and resolved by SDS-PAGE over a separation distance of about 20 mm. The gel was stained with Coomassie blue and each of the four lanes of the gel was cut into slices containing no more than 15 visible protein bands. Subsequent tryptic digests, peptide purification, LC-MS/MS analysis, and database searches were done as described (8).

Transfection and *In vitro* Association Experiments. Cell culture, transient transfection of COS-7 and 293 cells, [^{32}P]P_i labeling, and analysis of cellular Rip-like interacting caspase-like apoptosis-regulatory protein kinase (RICK) activity was done as previously described (5, 9). Breast tumor kinase (BRK) mutants were generated in a pRK-FLAG expression vector (10, 11). Transient transfection of BRK expression constructs into COS-7 cells and

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subsequent cell lysis and analysis of cellular protein tyrosine phosphorylation was done as described (12).

***In vitro* Kinase Assays.** All kinases were tested in the presence of different gefitinib concentrations, 100 μmol/L ATP, and 1 μCi [γ-^{32}P]ATP at 30°C and substrate phosphorylation was linear with respect to time in all assays. *In vitro* assays of RICK, p38, cyclin G–associated kinase (GAK), c-*jun* NH_2 terminal kinase 2 (JNK2), casein kinase 1δ, and carboxyl-terminal Src kinase (CSK)activities were done as described (5, 12). Activity assays of immunoprecipitated EGFR were done for 10 minutes at the temperature and ATP concentration described above using buffer conditions reported previously (9). Kinase reaction times for the other enzymes tested were 10 [Aurora A, Lyn, Yes, calcium/calmodulin-dependent protein kinase II (CaMKII), EGFR, IκB kinase ε (IKKε)], 20 (Met, B-lymphoid tyrosine kinase, Aurora B), or 30 minutes (EphB4) at 30°C. IKKε and BRK were transiently expressed from pRK5-FLAG plasmids in 293 cells and COS-7 cells, respectively (9–11). After cell lysis and immunoprecipitation with anti-FLAG antibody bound to protein G-Sepharose (11), beads were washed thrice with 500 μL lysis buffer without additives and once with 500 μL of the corresponding buffer system [IKKε: 50 mmol/L Tris-HCl (pH 7.5), 10 mmol/L $MgCl_2$, 0.1 mmol/L dithiothreitol, 0.1 mmol/L EGTA; BRK: 50 mmol/L Tris-HCl (pH 7.5), 10 mmol/L $MgCl_2$, 0.1 mmol/L EGTA, 0.8 mmol/L $MnCl_2$, 0.1 mmol/L Na_3VO_4]. All reactions were done in a final volume of 50 μL using 40 μg/mL *E. coli*–expressed glutathione *S*-transferase-interferon regulatory factor 3 as kinase substrate for IKKε or 0.5 mg/mL myelin basic protein for BRK. EphB4 (Upstate, Lake Placid, NY) was assayed in the same buffer as BRK with 0.3 mg/mL glyceraldehyde-3-phosphate dehydrogenase as protein kinase substrate. Reactions were stopped by adding SDS sample buffer, samples were separated by SDS-PAGE, and specific substrate phosphorylation was quantified by phosphoimaging. Aurora A (Upstate) and Aurora B (ProQinase, Freiburg, Germany) were assayed in a final volume of 50 μL containing 14 mmol/L 4-morpholinepropanesulfonic acid (pH 7.2), 15 mmol/L $MgCl_2$, 1 mmol/L EGTA, 0.3 mmol/L EDTA, 0.001% Brij-35, 0.5% glycerol, 0.01% 2-mercaptoethanol, 0.1 mg/mL bovine serum albumin, 5 mmol/L -glycerophosphate, 0.2 mmol/L Na_3VO_4, 0.2 mmol/L dithiothreitol, and Kemptide (200 μmol/L) as substrate peptide. B-lymphoid tyrosine kinase and Met (both from Upstate) assays were done in a final volume of 50 μL containing 20 mmol/L Tris-HCl (pH 7.5), 10 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol, 0.01% Brij-35, 0.1 mmol/L Na_3VO_4, 0.1 mmol/L EGTA and 10 μg poly(Glu_4-Tyr). Lyn, HCK, Yes and CaMKII (all from Upstate) were assayed according to the manufacturer's protocols prior to quantification of substrate phosphorylation using Whatman P81 paper. Calculations of all IC_{50} values were carried out with GraFit software (Erithacus, Horley, Surrey, United Kingdom).

Results and Discussion

Based on earlier structural and medicinal chemistry data, we reasoned that the morpholino group of gefitinib is exposed at the surface of the EGFR kinase domain and therefore synthesized the gefitinib derivative AX14596 with a primary amine at the position of the morpholino function for directed covalent immobilization and the generation of an affinity purification resin with intact, gefitinib-like binding properties (Fig. 1*A*; refs. 7, 14). Using total cell lysate from 2.5 × 10^9 HeLa cells as starting material, we then did affinity chromatography on an AX14596 column under optimized biochemical conditions. After sample loading and extensive washing of the column to remove nonspecifically interacting proteins, cellular drug targets were eluted with a combination of ATP and free gefitinib. Subsequently, we separated the ~10,000-fold enriched fraction of cellular gefitinib-binding proteins by two-dimensional 16-BAC/SDS-PAGE (Fig. 1*B*). MS analysis of the visualized protein spots not only permitted the detection of the EGFR at the expected molecular weight, but, in addition to this internal control, we could also identify additional, putative gefitinib targets such as the protein tyrosine kinases BRK, Yes, CSK, and EphB4 and the serine/threonine kinases RICK (also known as RIPK2, RIP2, and CARDIAK), GAK, CaMKII, Aurora A, JNK2 and p38 (Fig. 1*B*; Supplementary Table 1). Parallel to the gel-based proteomic approach, we directly digested the purified protein fraction with trypsin and subjected the resulting peptide mixture to LC-MS/MS analysis. The protein identifications from this experimental technique not only included all protein kinases detected in 16-BAC/SDS gels but further added a variety of potential gefitinib targets that included Bub1, integrin-linked kinase, Src family kinases such as Lyn, as well as the receptor tyrosine kinase (RTK) Met (see Supplementary Table 2). In total, more than 20 different protein kinases and various other cellular proteins were identified as putative gefitinib targets by MS analysis (Table 1). Moreover, despite the consistent high gefitinib selectivity for EGFR over closely related RTKs from the same family *in vitro*, our analysis did not reveal other EGFR-related RTKs such as HER2/ErbB2 as potential gefitinib targets (15). To validate the MS results, we did *in vitro* binding experiments with control and AX14596 beads in the absence or presence of free gefitinib to verify the specific binding of the identified kinase targets by immunoblot analysis (Table 1; Supplementary Fig. 1). In addition, the sensitivities of the protein kinase targets to gefitinib were assessed in various *in vitro*

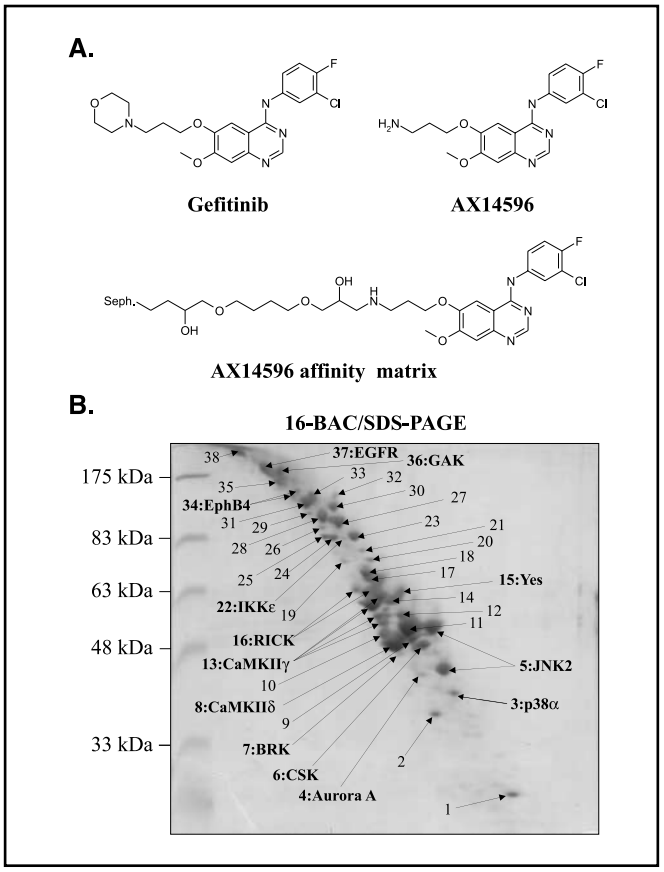

Figure 1. Identification of cellular gefitinib targets. *A,* chemical structures of gefitinib compared with AX14596 in its free and immobilized form. *B,* cellular gefitinib targets from HeLa cells were isolated by affinity chromatography on an AX14596 column and then resolved by 16-BAC/SDS-PAGE. Coomassie-stained protein spots were analyzed by mass spectrometry. The positions of identified protein kinases are indicated. See Supplementary Table 1 for a full list of all identified proteins.

Table 1. Identification and *in vitro* characterization of protein kinases targeted by gefitinib

Protein kinase	Gi no.	MW (kDa)	Gel identification*	LC-MS/MS identification †	Binding assay ‡	Kinase assay IC_{50} (μmol/L)
Aurora A	7446411	45,790	+	+	+	60 ± 7
Aurora B	4759178	39,280		+	ND	11.1 ± 2.9
BLK	914204	57,757		+	ND	3.1 ± 0.4
Bub1	2981233	122,389		+	+	ND
BRK	5174647	51,834	+	+	+	0.82 ± 0.06
CaMKII§						13.5 ± 1.4
CaMKIIα	26251712	54,088		+	ND	ND
CaMKIIβ	4139268	57,914		+	ND	ND
CaMKIIγ	26667199	58,365	+	+	ND	ND
CaMKIIδ	26667183	54,128	+	+	ND	ND
CK1δ	7512331	47,374		+	ND	61 ± 7
CK1ε	4503093	47,315		+	+	ND
CSK	4758078	50,704	+	+	+	41 ± 17
EGFR	29725609	134,277	+	+	+	$0.014 \pm 0.5 \times 10^3$
EphB4	495473	108,334	+	+	ND	1.22 ± 0.49
Fyn	4503823	60,762		+	+	ND
GAK	4885251	143,165	+	+	+	0.090 ± 0.018
HCK	306832	57,296	+	+	+	2.09 ± 0.35
ILK	4758606	51,419		+	+	ND
IKKε	7288878	80,462	+	+	+	35% at 100 μmol/L‖
JNK2	1082266	48,149	+	+	+	39% at 100 μmol/L‖
Lyn	4505055	58,574		+	+	0.95 ± 0.46
Met	37928127	35,259		+	+	3.2 ± 1.1
p38α	2499600	41,293	+	+	+	1.19 ± 0.03
RICK	4506537	61,195	+	+	+	0.049 ± 0.001
Tnk1	4092079	71,926		+	+	ND
Yes	4885661	60,801	+	+	+	1.75 ± 0.13

NOTE: Abbreviations: *BLK,* B-lymphoid tyrosine kinase; *CK1δ,* casein kinase 1δ; *HCK,* hemopoietic cell kinase; *ILK,* integrin-linked kinase; *ND,* not determined.
*Identified protein spots from Fig. 1 that contained protein kinases are included; for a full list of all analyzed protein spots, see Supplementary Table 1.
†LC-MS/MS data of all identified proteins are listed in Supplementary Table 2.
‡*In vitro* binding assays are shown in Supplementary Fig. 1.
§CaMKII activity was analyzed with an enzyme preparation that contains a mixture of different isoforms.
‖Percent inhibition of kinase activity in the presence of 100 μmol/L gefitinib is shown.

kinase activity assays with recombinant enzymes in the presence of different inhibitor concentrations. The calculation of the drug concentrations required for half-maximal inhibition of kinase activities (IC_{50}) provided a quantitative measure of the differential sensitivities to gefitinib. The IC_{50} values for all tested kinases are also displayed in Table 1. Interestingly, gefitinib inhibited the serine/threonine kinases RICK and GAK with respective IC_{50} values of about 50 and 90 nmol/L, showing that these previously unknown gefitinib targets are almost as potently affected by the drug as the tyrosine kinase activity of wild-type EGFR *in vitro.* In this context, it is noteworthy that gefitinib-responsive lung tumors were found to express EGFR variants with even higher sensitivity for the drug than the wild-type receptor (3, 4). Moreover, we measured IC_{50} values around 1 μmol/L for various protein tyrosine kinases such as EphB4, BRK, and Lyn, whereas half-maximal inhibition of several other protein kinases tested occurred at higher gefitinib concentrations (Table 1).

To test the potential *in vivo* significance of our identification of RICK as a highly sensitive gefitinib target, we did a transfection assay in which a kinase-deficient fragment of RICK served as cellular substrate for coexpressed, catalytically active RICK enzyme (5). Remarkably, pretreatment of cells with 0.3 μmol/L gefitinib already reduced cellular RICK activity by about 50%, indicating that RICK might represent a secondary target during gefitinib therapy (Fig. 2*A*). Genetic evidence has previously established RICK protein as a mediator of various innate and adaptive immune responses (16). However, it remains to be clarified to which extent RICK kinase activity is involved in these processes and whether RICK kinase inhibition by gefitinib or related inhibitors might have beneficial therapeutic effects in certain states of inflammatory disease. In a second cellular assay, we found that BRK-mediated protein tyrosine phosphorylation was strongly suppressed by low micromolar concentrations of gefitinib in intact cells (Fig. 2*B*). Importantly, the cytoplasmic tyrosine kinase BRK is frequently overexpressed in carcinomas of the breast and colon and represents a potential target for the treatment of these epithelial cancers (17, 18). Therefore, gefitinib derivatives with increased potency toward BRK could serve as antineoplastic agents directed against a primary molecular target distinct from the EGFR. As previously shown for the EGFR, mutation of a conserved threonine

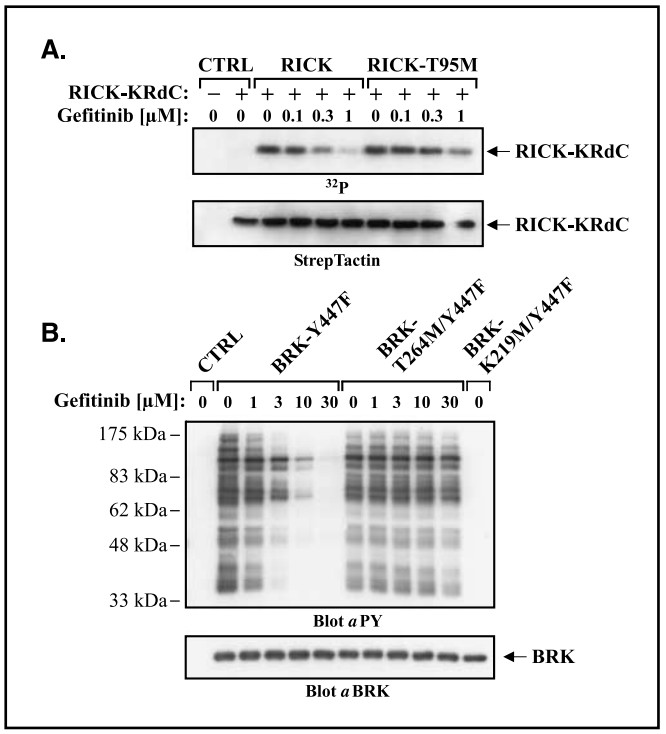

Figure 2. Characterization of alternative gefitinib targets in intact cells. *A,* effect of gefitinib on the kinase activities of wild-type RICK and a partially inhibitor-insensitive RICK-T95M mutant in COS-7 cells, as measured by [^{32}P]P_i incorporation into a cotransfected, kinase-inactive RICK fragment serving as cellular kinase substrate (5). *B,* COS-7 cells were transiently transfected with constitutively active BRK-Y447F or the derived gefitinib-resistant (BRK-T264M/Y447F) and kinase-deficient (BRK-K219M/ Y447F) mutants as indicated and treated with different gefitinib concentrations for 2 hours prior to cell lysis. Cellular protein tyrosine phosphorylation and BRK expression levels were detected by immunoblot analysis. *CTRL,* control.

residue at the ATP-binding pocket to methionine also strongly reduced the sensitivities of RICK and BRK to gefitinib inhibition (Fig. 2; ref. 12).

Furthermore, our identification of the serine/threonine kinase GAK as one of the most sensitive gefitinib targets points to a previously unrecognized molecular aspect of gefitinib therapy. Recent evidence indicates that GAK acts as a negative regulator of EGFR signaling (19). Although the precise role of GAK kinase activity for the process of EGFR down-modulation remains to be determined, gefitinib-mediated inactivation of a negative regulator would antagonize the inhibitory effect of the drug on EGFR signaling. Therefore, it will be important to find cellular readouts for GAK kinase activity, which permit the quantification of the gefitinib effect on GAK in comparison with dose-dependent EGFR inhibition in the biologically relevant context. In fact, observations consistent with such an undesirable effect of gefitinib downstream of the EGFR have been recently reported (20). In case further investigations establish GAK as a cellular gefitinib target that promotes EGFR downstream signaling in the presence of the drug, it would be highly favorable to select future EGFR-directed drugs for minimal interference with cellular GAK kinase activity.

In conclusion, we have introduced a proteomic method to identify the cellular targets of gefitinib in HeLa cells. The approach is generally applicable and can be expected to reveal even more gefitinib targets when other biological extracts are used as starting materials. The results reported here provide new insights into potential cellular modes of action of gefitinib, which are unrelated to EGFR inhibition, but are relevant for further optimization of gefitinib-like drugs with either the EGFR or various other protein kinases as primary disease targets.

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