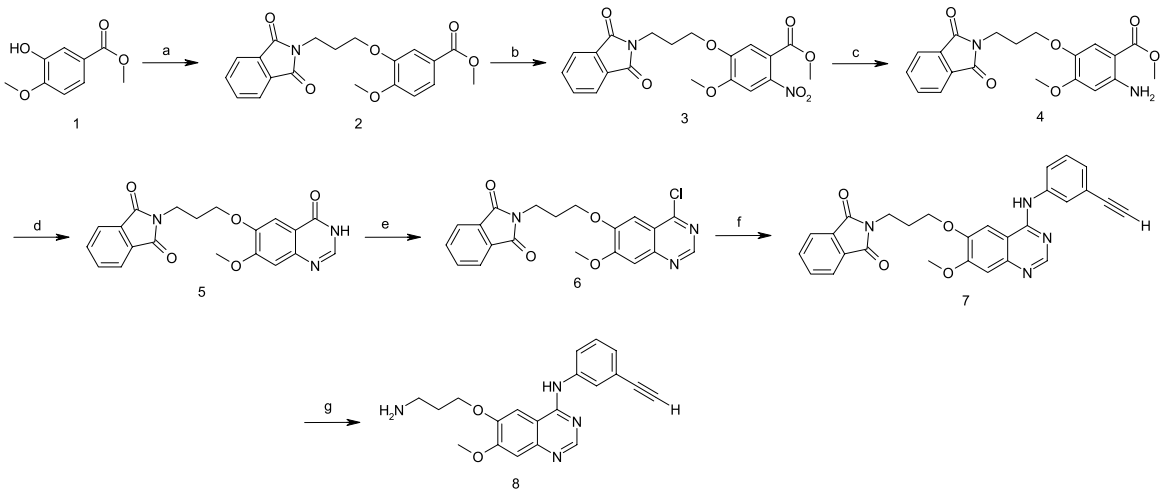
Supplemental Materials and Methods

Compound Synthesis and Immobilization

Erlotinib hydrochloride salt (Tarceva, OSI-774) and gefitinib free base (Iressa, ZD1839) were purchased from LC Laboratories (Woburn, MA). AX14596 (linker-gefitinib) was synthesized as described [1]. Synthesis of KX214 (6-amino-propoxy-linked 4-anilinoquinazoline) (linker-erlotinib):

The following schedule gives a short schematic overview about the synthetic route, which afforded the designated amino-propoxy–linked erlotinib-derivative **8**:



a) N -(3-bromopropyl)phthalimide, K_2CO_3, dimethylformamide (DMF), reflux; b) AcOH, HNO_3, H_2SO_4, room temperature; c) $SnCl_2$, ethyl acetate, H_2O, reflux; d) NH_4CHO_2, formamide, 140°C; e) $POCl_3$, *N*,*N*-dimethylaniline (DMA), 120°C; f) 3-aminophenylacetylene, chlorobenzene, 120°C; g) hydrazine hydrate, MeOH, room temperature.

The synthesis of compound **8** was performed by the application of a modified published synthetic procedure [2], starting with a base-promoted *O*-alkylation of methyl 3-hydroxy-4-methoxy-benzoate **1** with *N*-(3-bromopropyl)-phthalimide to give amino-protected intermediate **2**. Afterward, nitration and reduction of **2** afforded subsequently nitro-derivative **3** and methyl-2-amino-benzoate derivative **4**, respectively, and cyclization of **4** in the presence of formamide and ammonium formate yielded the key quinazoline-derivative **5**. Thereafter, **5** was treated with $POCl_3$ and a catalytical amount of *N*,*N*-dimethylaniline at 120°C and consecutive $S_{N,Ar}$ reaction with 3-aminophenylacetylene converted **6** to the amino-protected 4-anilinoquinazoline **7**. Finally, **7** underwent smoothly cleavage of the phthalimide functionality under basic conditions to release the free 6-amino-propoxy–linked 4-anilinoquinazoline **8**.

High-performance liquid chromatography–mass spectrometry (HPLC) and nuclear magnetic resonance spectroscopy (NMR) final analyses were performed with the following equipment and methods:

HPLC-MS.

Varian: 1200 L Triple Quadrupole mass spectrometer
Ionization mode: +ESI
UV-Detector: ProStar 325 (254 nm)
HPLC column: Varian Polaris C18 (length = 100 mm, diameter = 3 mm, particle size = 3 μm)
HPLC method:
Mobile phase: acetonitrile/water + 0.1% HCOOH
Run time: 9.50 minutes
Gradient: acetonitrile content was raised from 10% to 90% in 3 minutes

NMR.

Varian: Oxford NMR 400
^{1}H: 400.00 MHz
The samples were solved in $CDCl_3$ or dimethyl sulfoxide (DMSO)-d6.
Trimethylsilane was used as internal standard.

HPLC (preparative).

HPLC column: Varian RP Polaris C18 (length = 250 mm, diameter = 21.4 mm, particle size = 5 μm)
Mobile phase: acetonitrile/methanol + 0.1% HCOOH

*Synthesis of **2**.* A mixture of 0.2 g (0.0011 mol) of methyl 3-hydroxy-4-methoxy-benzoate, 0.45570 g (0.0017 mol) of *N*-(3-bromopropyl)-phthalimide, and 0.235 g (0.0017 mol) of sodium carbonate in 6 ml of DMF was stirred for 2 hours under reflux. The reaction mixture was

Table W1. Genotype of Murine Cell Lines.

Cell Line	Genotype
Murine PDAC cell lines	
W22	*Ptf1a$^{Cre/+}$;LSL-Kras$^{G12D/+}$;p53$^{loxP/lox}$;R26$^{LSL-TVA-LacZ/+}$*
PPT6554	*Ptf1a$^{Cre/+}$;LSL-Kras$^{G12D/+}$;p53$^{loxP/loxP}$;R26$^{LSL-TVA-LacZ/+}$*
PPT6556	*Ptf1a$^{Cre/+}$;LSL-Kras$^{G12D/+}$;p53$^{loxP/+;}$LSL-p53$^{R172H/+}$*
PPT6558	*Ptf1a$^{Cre/+}$;LSL-Kras$^{G12D/+}$;p53$^{loxP/+}$;LSL-p53$^{R172H/+}$;R26$^{LSL-TVA-LacZ/+}$*
W30	*Ptf1a$^{Cre/+}$;LSL-Kras$^{G12D/+}$;p53$^{loxP/+}$*
PPT15272	*Ptf1a$^{Cre/+}$;LSL-Kras$^{G12D/+}$*
PPT53268	*Ptf1a$^{Cre/+}$;LSL-Kras$^{G12D/+}$*
PPT16992	*Ptf1aCre/+;LSL-Kras$^{G12D/+}$;R26$^{LSL-TVA-LacZ/+}$*
PPT53631	*Ptf1a$^{Cre/+}$;LSL-Kras$^{G12D/+}$*
PPT6051	*PDX1-Cre;LSL-Kras$^{G12D/+}$;LSL-p53$^{R172H/+}$*
PPT3202	*Ptf1aCre/+;LSL-Kras$^{G12D/+}$;LSL-PCNA-Luc*
PPT5436	*Ptf1a$^{Cre/+}$;LSL-Kras$^{G12D/+}$;LSL-p53$^{R172H/R172H}$;R26$^{LSL-TVA-LacZ/LSL-TVA-LacZ}$*
PPT3107	*Ptf1a$^{Cre/+}$;LSL-Kras$^{G12D/+}$;LSL-p53$^{R172H/+}$;R26$^{LSL-TVA-LacZ/+}$*
ASC53909	*Ptf1a$^{Cre/+}$;LSL-Kras$^{G12D/+}$;R26$^{LSL-TVA-LacZ/+}$*

evaporated to dryness and the residue was dissolved in 20 ml of dichloromethane (DCM). The organic phase was washed with water and brine and finally dried over $MgSO_4$. Evaporation of the solvent under reduced pressure afforded the crude product, which was used for the following conversions without further purification.

Yield: 0.312 g (76.6%).

^{1}H NMR ($CDCl_3$, 400 MHz): 2.22–2.28 (m, 2H), 3.62 (s, 3H), 3.87 (s, 3H), 3.93 (t, ^3J = 8 Hz, 2H), 4.14 (t, ^3J = 2.4 Hz), 6.81 (d, ^3J = 4 Hz, 1H), 7.51 (d, ^4J = 2 Hz, 1H), 7.63–7.66 (m, 1H), 7.69–7.72 (m, 2H), 7.82–7.84 (m, 2H).

MS (ESI): m/z = 392 $[M + Na]^+$.

*Synthesis of **3**.* The amount of 10.56 ml of nitric acid and 12.31 ml of sulfuric acid at 0°C were dropwise added to a solution of 11,644 g (0.0315 mol) of **2** in 200 ml of acetic acid. After 1.5 hours of stirring at room temperature, the mixture was poured in 500 ml of H_2O at 0°C and extracted with 3 × 400 ml of DCM. The combined organic phases were washed with saturated Na_2CO_3(aq) and dried over $MgSO_4$. Evaporation of the solvent under reduced pressure afforded the crude product, which was used for the following conversions without further purification.

Yield: 10.48 g (80.2%).

^{1}H NMR ($CDCl_3$): 2.25–2.31 (m, 2H), 3.69 (s, 3H), 3.89 (s, 3H), 3.93 (t, ^3J = 6,4 Hz, 2H), 4.18 (t, ^3J = 5,6 Hz, 2H), 7.01 (s, 1H), 7.35 (s, 1H), 7.72–7.73 (m, 2H), 7.81–7.86 (m, 2H).

MS (ESI): m/z = 437 $[M + Na]^+$.

*Synthesis of **4**.* The amount 3.66 g (0,0193 mol) of tin chloride and 10 ml of water were subsequently added to a solution of 2 g (0.0048 mol) of **3** in 200 ml of ethyl acetate. After stirring for 1 hour under reflux, the heterogeneous mixture was diluted with 300 ml of ethyl acetate, and a resulting precipitate was removed by filtration. The organic phase was washed with saturated $NaHCO_3$(aq) and dried over $MgSO_4$. After removal of the solvent at reduced pressure, the desired product was purified and isolated by flash chromatography (silica, EE/H 1:1; 0.33).

Yield: 0.95 g (51.59%).

MS (ESI): m/z = 385 $[M + 1]^+$, 407 $[M + Na]^+$.

*Synthesis of **5**.* The amount of 0.237 g (0.0038 mol) of ammonium formate was added to a solution of 0.853 g (0.0022 mol) of **4** in 50 ml of formamide, and the mixture was stirred for 12 hours at 140°C. The reaction progress was monitored by HPLC, and after quantitative conversion, 100 ml of H_2O was poured to the organic solution, which conducted the formation of a beige precipitate. The solid was filtered off and washed subsequently with 50 ml of H_2O and 50 ml of ether. The isolated solid was dried in high vacuo, and the crude product was used in the following conversions without further purification.

Yield: 0.384 g (45.59%).

MS (ESI): m/z = 380 $[M + 1]^+$.

*Synthesis of **6**.* The amount of 1.93 ml (0,0027 mol) of *N*,*N*-dimethylaniline was dropwise added at 0°C to a solution of 2.127 g (0.0056 mol) of **5** in 10 ml of phosphorus oxychloride. After being stirred at room temperature for a further 5 minutes, the mixture was heated under reflux for additional 12 hours. Afterward, the mixture was poured into 50 ml of H_2O at 0°C, which induced precipitation of the demanded compound **6**. Filtration and washing with cold H_2O and finally with ether afforded compound **E** in satisfactory purity, and the crude product was used in the following conversions without further purification.

Yield: 1.77 g (79.53%).

MS (ESI): m/z = 398 $[M + 1]^+$.

*Synthesis of **7**.* A mixture of 1.774 g (0.0045 mol) of **6** and 0.783 g (0.0045 mol) of 3-aminophenylacetylene in 15 ml of chlorobenzene was stirred for 2 hours at 120°C. The maintaining precipitate was filtered off and subsequently washed with chlorobenzene, H_2O, and finally with ether. Removal of traces of solvent by high vacuo afforded the required compound **7**, which was applied in the following conversions without further purification.

Yield: 1.712 g (79.53%).

MS (ESI): m/z = 479 $[M + 1]^+$.

*Synthesis of **8**.* The amount of 0.020 g (0.0004 mol) g of hydrazine hydrate was added to a solution of 0.0654 g (0.00014 mol) of **7** in 4 ml of MeOH, and the mixture was stirred at ambient temperature. The reaction progress was monitored by HPLC, and after quantitative conversion, the reaction mixture was evaporated to dryness. The remaining residue was dissolved in 10 ml of DCM, washed with brine, and dried over $MgSO_4$. After removal of the solvent at reduced pressure, the product was purified by flash chromatography (silica, DCM/MeOH 4:1).

Yield: 20.7 mg (43.0%).

^{1}H NMR (D_2O): 2.19–2.22 (m, 2H), 3.30–3.31 (m, 2H), 3.51 (s, 1H), 3.98 (s, 3H), 4.30 (t, ^3J = 8,0 Hz, 2H), 7.10 (s, 1H), 7.23 (d, ^2J = 7,6 Hz, 1H), 7.35 (t, ^3J = 9,3 Hz, 1H), 7.68–7,77 (m, 2H), 7.87–8.0 (m, 1H), 8.41 (s, 1H).

MS (ESI): m/z = 349 $[M + 1]^+$.

For immobilization, drained epoxy-activated Sepharose 6B (GE Healthcare, Pittsburgh, PA) was resuspended in 2 volumes of 5 mM AX14596 or 5 mM linker-erlotinib dissolved in 50% DMSO/25 mM Na_2CO_3 and incubated with permanent agitation overnight at 30°C in the dark. Beads were washed with 50% DMSO/25 mM Na_2CO_3, and remaining active groups were blocked with 1 M ethanolamine. Subsequent washing steps were performed according to the manufacturer's instructions. To generate the control matrix, epoxy-activated Sepharose 6B was incubated with 1 M ethanolamine and treated as described above. The concentration of covalently immobilized inhibitor was determined spectrophotometrically by measuring the reduction of the inhibitor concentration in the soluble phase during the coupling reaction. EAH-Sepharose (GE Healthcare) was used as a negative control. The beads were stored at 4°C in the dark.

In Vitro *Association Experiments*

For SILAC, low-passaged (P4) primary dispersed murine pancreatic carcinoma cells from the primary PDAC of a *Ptf1a$^{Cre/+}$;LSL-Kras^{G12D};p53$^{lox/lox}$* mouse (#6554) (PPT6554) were cultivated as described previously [3,4]. For *in vitro* association experiments, PPT6554 cells were lysed in buffer containing 20 mM HEPES pH 7.5, 400 mM NaCl, 0.25% Triton X-100, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 80 U/ml Benzonase plus additives (10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM PMSF, 1 mM Na_3VO_4, 10 mM NaF). After centrifugation, lysates were adjusted to 1 M NaCl and filtered through a 0.45-µm cellulose acetate filter before *in vitro* association of 750 µl of lysate containing 3 mg of protein with either 30 µl of drained inhibitor

or control matrix for 2.5 hours at 4°C. Incubation procedures were performed essentially as described previously [4], with the addition that inhibitor beads representing five different compound densities were incubated with the cell extract to determine binding curves for each identified protein. For competition experiments, SILAC-encoded cell extracts were treated with different concentrations of erlotinib (0 nM, 10 nM, 100 nM, 300 nM, 1 μM, 10 μM, and 50 μM) or gefitinib (0 nM, 10 nM, 100 nM, 300 nM, 1 μM, 10 μM, and 100 μM) for 30 minutes before addition of inhibitor beads and incubation for an additional 2.5 hours at 4°C. In all *in vitro* association experiments, subsequent washing and elution steps including the separation of proteins by electrophoresis and the in-gel digest with trypsin were performed as described previously [4].

Mass Spectrometric Analysis

Mass spectrometric analysis of the labeled and combined peptide fractions was carried out by online nanoLC-MS/MS [4]. Samples were loaded directly by an Agilent 1200 nanoflow system (Agilent Technologies, Santa Clara, CA) on a 15-cm fused silica emitter (New Objective) packed in-house with reversed phase material (Reprusil-Pur C18-AQ, 3 μm; Dr. Maisch GmbH) at a flow of 500 nl/min. Bound peptides were eluted by a gradient from 2% to 40% solvent B (80% ACN and 0.5% HOAc) at a flow rate of 200 nl/min and sprayed directly into an LTQ-Orbitrap XL mass spectrometer (Thermo Fischer Scientific, Waltham, MA) at a spray voltage of 2 kV using a nanoelectrospray ion source (Proxeon Biosystems, Dreieich, Germany). The mass spectrometer was operated in the positive ion mode and a data-dependent switch between MS and MS/MS acquisition. To improve mass accuracy in the MS mode, the lock-mass option was enabled [5]. Full scans were acquired in the Orbitrap at a resolution $R = 60\,000$ and a target value of 1,000,000 charges. The five most intense ions detected in the MS were selected for collision-induced dissociation at a target value of 5000, and the resulting fragmentation spectra were recorded in the linear ion trap. Ions that were once selected for data-dependent acquisition were dynamically excluded for 30 seconds for further fragmentation.

Data Analysis

Mass spectra were processed using the MaxQuant software version 1.0.12.28 [6], using the Mascot search engine (version 2.2.0) for peptide and protein identification. A concatenated forward and reversed IPI mouse database (version 3.39) was used comprising 106,658 database entries. Regarding the search parameters, the minimal peptide length was set to six amino acids, trypsin was selected as the proteolytic enzyme, and maximally two missed cleavage sites were allowed. Carbamidomethylation of cysteine residues was selected as a fixed modification, whereas methionine oxidation and N-terminal protein acetylation were allowed as a variable modification. Because MaxQuant automatically extracts isotopic SILAC peptide triplets, the corresponding isotopic forms of lysine and arginine were automatically selected for database search as fixed modifications. The maximal mass deviation of precursor and fragment masses was set to 7 ppm and 0.5 Da. A false discovery rate of 0.01 was selected for proteins and peptides, and a posterior error probability (PEP) below or equal to 0.1 for each MS/MS spectrum was required. Target-specific dissociation constants for the tested free kinase inhibitors were calculated based on SILAC quantification data using the Cheng-Prusoff equation as described previously [7].

References

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