Methods

EGFR Biochemical Assays

The protocol used for the continuous-read kinase assays to measure inherent potency of compounds against active forms of EGFR (WT) and EGFR (T790M/L858R) enzymes is described in detail below. The mechanics of the assay platform can be found at (Invitrogen, Carlsbad, CA) on their website at the following URL: http://www.invitrogen.com/.

Briefly, 10X stocks of EGFR-WT (PV3872) from Invitrogen and EGFR-T790M/L858R (40350) (BPS Bioscience, San Diego, CA), 1.13X ATP (AS001A) and Y12-Sox conjugated peptide substrate (KPZ3121C) were prepared in 1X kinase reaction buffer consisting of 20 mM Tris, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 5 mM β-glycerophosphate, 5% glycerol (10X stock, KB002A) and 0.2 mM DTT (DS001A). 5 μL of each enzyme were pre-incubated in a Corning (#3574) 384-well, white, non-binding surface microtiter plate (Corning, NY) for 30 min at 27°C with a 0.5 μL volume of 50% DMSO and serially diluted compounds prepared in 50% DMSO. Kinase reactions were started with the addition of 45 μL of the ATP/Y12-Sox peptide substrate mix and monitored every 71 seconds for 30-120 minutes at $\lambda_{ex}360/\lambda_{em}485$ in a Synergy^4 plate reader from BioTek (Winooski, VT). At the conclusion of each assay, progress curves from each well were examined for linear reaction kinetics and fit statistics (R^2, 95% confidence interval, absolute sum of squares). Initial velocity (0 minutes to +30 minutes) from each reaction was determined from the slope of a plot of relative fluorescence units *vs* time (minutes) and then plotted against inhibitor concentration to estimate apparent IC_{50} from log[Inhibitor] vs Response, Variable Slope model in GraphPad Prism from GraphPad Software (San Diego, CA). The following reagents were used for the EGFR-WT kinase assay: EGFR kinase (5 nM), ATP (15 μM) and Y12-Sox peptide (5 μM). For the EGFR-T790M/L858R kinase assay (3 nM), ATP (20-50 μM) and Y12-Sox peptide (5 μM).

Cell Culture

A431 human epidermoid carcinoma cells, H1975 human NSCLC cells and HCC827 human NSCLC adenocarcinoma cells were obtained from the American Type Culture Center (Manassas, VA). A431 cells were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (HyClone, South Logan, UT) and 1% Penicillin-Streptomycin (P/S, Lonza, Walkersville, MD). H1975 and HCC827 cells were grown in RPMI 1640 (Invitrogen) supplemented with 10% FBS and 1% P/S. All cells were maintained and propagated as monolayer cultures at 37°C in a humidified 5% CO_2 incubator.

Phospho-EGFR Assay in Cells

Cells were grown in 12-well plates (Corning, NY) to 90% confluence and then incubated in low-serum (0.1% FBS) media for 16-18 hr. Cells were then treated with 5, 1.25, 0.31, 0.078, 0.020 µM test compound or 0.5% DMSO in low-serum (0.1% FBS) media for 1 hr. A431 cells were then stimulated with 50 ng/ml EGF (Peprotech, Rocky Hill, NJ) for 15 min. After treatment, cell monolayers were washed with cold PBS (Invitrogen, Carlsbad, CA) and immediately lysed by scraping into 50 uL cold Cell Extraction Buffer (Biorad, Hercules, CA) supplemented with Complete Protease inhibitors (Roche, Indianapolis, IN) and PhosphoSTOP (Roche, Indianapolis, IN) phosphatase inhibitors.

Lysate protein concentrations were determined by BCA Assay (Pierce, Rockford, IL) and approximately 50 ug of each lysate were separated by 4-12% gradient SDS-PAGE (Invitrogen, Carlsbad, CA), transferred to nitrocellulose membrane (Biorad, Hercules, CA) and probed with specific antibodies. Phospho-protein signals were quantitated using Odyssey Infrared Imaging (Li-Cor Biosciences, Lincoln, NE).

To assess phospho-signaling, blots were immunoblotted with phospho and total antibodies for EGFR (Y1068), Akt, pS6RP and Erk1/2 (Cell Signaling, Danvers, MA). Phospho-signals were normalized to total protein expression for each biomarker. Results are indicated as % DMSO control. Normalized data were fitted using a sigmoidal curve analysis program (Graph Pad Prism version 5) with variable Hill slope to determine the EC_{50} values.

Antibodies

All primary antibodies were obtained from Cell Signaling (Danvers, MA) and used at 1:1000. Secondary antibodies were used at 1:20,000. Goat anti-mouse IgG IRDye 800CW antibody was obtained from LiCor Biosciences (Lincoln, NE) and goat anti-rabbit IgG Alexa Fluor 680 was obtained from Invitrogen (Carlsbad, CA).

Cell Proliferation

Cells were plated in growth media (described above) supplemented with 5% FBS and 1% P/S at a density of 3,000 cells per well in 96 well tissue culture plates (Corning, NY). Cells were allowed to adhere for 4 hr and then treated with 5, 1.25, 0.31, 0.078, 0.020 or 0.005 μM test compound for 72 hr. Cell viability was determined by CellTiter Glo (Promega, Madison, WI) and results were converted to cell numbers using a standard curve. Growth inhibition (GI_{50}) values were determined using Graph Pad Prism.

Washout Experiment for EGFR Activity

A431 human epidermoid carcinoma cells were grown in 6-well plates to 90% confluence and then incubated in serum-free media for 18 hr. Duplicate sets of cells were treated with 1 μM compound for 1 hr. One set of cells was then stimulated with 100 ng/ml EGF for 5 min, and extracts were made as described. The other set of cells was washed free of the compound with warmed compound-free medium, and continued incubating with repeated washes every 2 hours, before stimulation with EGF as described.

Proliferation assay

H1975 cells were plated at 10000 cells/96 well overnight in growth media. The next day media was changed to media with 1% serum . Serially diluted compound was added, not exceeding a final 0.1% concentration of DMSO. Plates were read after 72h of compound addition using CellTiter-Glo (Promega G7573, Madison, WI). HCC827 Cells were plated at 8000 cells/96 well overnight in growth media. The next day media was to media containing 1% serum . Serially diluted compound was added, not exceeding a final 0.1% concentration of DMSO. Plates were read after 72h of compound addition using CellTiter-Glo (Promega G7573, Madison, WI).

Mass Spectrometry

EGFR wild type was incubated with a 10-fold excess of CNX17 for 1 and 3 hrs. 1 μl aliquots of the samples (total volume 5-8 ul) were diluted with 10 ul of 0.1% TF A prior to micro C4 Zip Tipping directly onto the MALDI target using Sinapinic acid as the desorption matrix (10 mg/ml in 0.1% TFA:Acetonitrile 50:50).

Metabolism-Free GSH Reactivity

One μL of a 10 mM DMSO stock of either CNX17 or 70 were diluted into 999 μL of reaction mixture (pre-warmed to 37°C) containing the following; 5 mM GSH, PBS pH 7.4, and 10% acetonitrile, to provide a 10 μM final concentration for CNX-17 & 70. At the 0, 30, 60, 120, 180, 240, & 360 minute time points, 15 μL of sample was transferred to a 96-well plate and snap frozen. After the final time point was collected and frozen, 60 μL of stop solution (1 μg/mL of carbutamide and 0.1% formic acid in acetonitrile) and 75 μL of HPLC grade water were added to each well. The sample plate was then mixed at a medium speed for 10 minutes prior to analysis on the ABSciex 4000 quadrupole mass spectrometer.

Molecular modeling

CNX17 was discovered from an iterative computational design and computational testing paradigm to optimize reversible interactions within the EGFR ATP binding site while positioning an electrophilic functionality near Cys773 to enable the formation of a covalent bond

Compounds were designed to:

1) Preserve the key kinase hinge interactions of the pyrimidine ring of the quinazoline
2) Identify functionality to replace the phenyl ring of the quiinazoline ring system to :
    a. Enable further hydrogen-bonding interactions with the kinase hinge region
    b. Provide functionality to enable targeting of Cys773

The x-ray structure of EGFR with Tarceva (pdb code: 1m17) was used as a template in the modeling with the docking components in Discovery Studio (v2.1.0.8130, Accelrys Inc). Successive cycles of design and docking chemotypes led to the discovery of CNX17. Compounds were first docked into the WT EGFR protein as a reversible inhibitor, and then one of the best poses was chosen based on the distance and orientation of the electrophilic group (acrylamide). Consequently, the covalent bond between thiol (Cys773) and acrylamide was formed and the minimization of the additive product was performed using CHARMM in Discovery Studio. A comparison of the binding mode of CNX17 and the xray structure of a quinazoline irreversible inhibitor (pdbcode 2J5F) shows them to adopt similar binding and interactions (Supplementary Figure 7)