SUPPLEMENTAL MATERIALS

COMPLETE METHODS

Cell Lines and Reagents

Cell lines A204 (HTB-82), SaoS2 (HTB-85), Mg63 (CRL-1427), MNNG/HOS Cl#5 (CRL-1547), A673 (CRL-1598), RD-ES(HTB-166), U2OS (HTB-96), SK-LMS1 (HTB-88) and HT1080 (CCL-221) were purchased from American Type Culture Collection (Manassas, VA) and subcultured using their recommended conditions. ATCC performed STR (Short Tandem Repeat) analysis on cells which were used within six months of purchase. RD18 cells were from Dr. Jack Pledger (Moffitt Cancer Center, Tampa, FL) and maintained in DMEM/F12 (1:1) supplemented with 10% FBS. All cells were maintained at 37°C and 5% CO_2. Erlotinib was provided by Genentech (San Francisco, CA); Dasatinib by Bristol Myers Oncology; PHA665752 by Pfizer (Groton, CT); OSI868 by OSI Pharmaceuticals; Imatinib by Novartis. JAK inhibitor and SU5402 were purchased from EMD (Gibbstown, NJ). ZD6474 (vandetanib) was purchased by Huskerchem Inc.(Riverside, CA). BMS754807 was a gift from Dr. Uwe Rix (Moffitt Cancer Center, FL). Stock solutions of inhibitors were reconstituted in 100% DMSO and then were further diluted with culture media to working concentration.

Phosphopeptide Immunoprecipitation, Analysis and Data Processing

Phosphopeptide immunoprecipitation and purification were performed and analyzed by nano-LC-MS/MS as described previously using a total of 2×10^8 cells of each cell line(12). For the tissue samples, mouse xenograft tissues were powdered using the assembled BioPuverizer (BIOSPEC, Cat No. 59013N), and lysed in urea lysis buffer. Extracted proteins (30 mg to 80 mg) were reduced by dithiothreitol (DTT), alkylated by iodoacetamide and then digested by trypsin. Peptide purification was carried out using Sep-Pak C18 columns, and phosphotyrosine peptides were pulled down by phosphotyrosine $pTyr^{100}$ antibody beads. The eluted peptide

mixtures were analyzed by a nanoflow liquid chromatograph (U3000, Dionex, Sunnyvale, CA) coupled to a LTQ-Orbitrip hybrid mass spectrometer (Thermo, San Jose, CA) in a data-dependent manner for tandem mass spectrometry peptide sequencing experiments. MASCOT and SEQUEST searches were performed against the Swiss-Prot human database downloaded on April 03, 2008. Both MASCOT and SEQUEST search results were summarized with Scaffold 2.0 software. We counted the number of spectra observed for each peptide in a MS/MS run. Spectra were subjected to an 80% peptide identification probability and a 50% protein identification probability on each of the 2 total runs (1 biological, 2 technical) per cell line. This was to ensure that the False Discovery Rate (FDR) was < 2%. To calculate the FDR, peptide sequences were assigned to MS/MS spectra using Sequest software searching (v.27, rev.12) and a composite forward/reverse IPI human protein database. False positives identified were less that 2% of the total dataset. To calculate a peptide site spectrum count, each unique phosphotyrosine site was isolated and counted. To calculate a protein spectrum count, we summed the numbers for all the peptides assigned to each protein in that run.

Kinase inhibitor screen

Cytotoxicity assays were performed by following the protocols provided by the manufacturer (cell proliferation kit I (MTT), catalog no. 1465007, Roche). In brief, a total of 2×10^3 (MNNG, SK-LMS1), 3×10^3 (U2OS, MG63, RD18, A673, A204, HT1080), 8×10^3 (Saos2), and 8×10^3 (RD-ES) cells in medium containing 10% FBS were seeded into each well of a 96-well plates (BD Falcon, Franklin Lakes, NJ), then cells were exposed to indicated agents post 24 hours culture. Viability was assessed at 72 hours using MTT assay. The IC_{50} was defined as the drug concentration that induced 50% cell death in comparison with DMSO controls and was calculated by non-linear regression analysis (Prism, GraphPad 5.0).

Phospho-RTK Array Assay

42 human receptor tyrosine kinases phosphorylation level determination in sarcoma cell lines was performed using the Proteome Profiler Array Kit (R&D Systems, Minneapolis, MN). Briefly, Cells were lysed in NP-40 Buffer (1% NP-40,20mM Tris-HCl pH8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1mM sodium orthovanadate, 10 µg/mL Aprotinin, 10 µg/mL Leupeptin). Two-hundred micrograms of total cell lysate protein were incubated with RTK array membranes. Detection of proteins was accomplished using the mouse anti-phospho-tyrosine antibody conjugated to HRP and enhanced chemiluminescence.

Protein Expression Analysis

Cell lysates were prepared using lysis buffer (25 mM Tris, pH 7.5, 225 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 1 µg/ml leupeptin, with phosphatase inhibitor and cocktail proteinase inhibitor). Protein concentration were determined by using Bradford assay and then adjusted to the same amount in total protein content (80 µg). Samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred onto Nitrocellulose membrane (Bio-RAD, Hercules, CA). Primary antibodies used in these studies consisted of PDGFRα, pIGFR/INSR, IGFR, INSR, pEGFR, EGFR, pMet, Met, pAkt-Ser^{473}, Akt, pErk, ErK, FYN, LYN and PARP (Cell Signaling, Beverly, MA), pPDGFRα Tyr742 (Invitrogen ,Grand Island, NY), as well as β-actin (Sigma, St. Louis, MO). YES antibody was a gift from Dr. Alvaro Monteiro (Moffitt Cancer Center, FL). Detection of proteins was accomplished using horseradish-peroxidase conjugated secondary antibodies and enhanced chemiluminescence (ECL) purchased through Amersham Biosciences (Piscataway, NJ).

Transfection of Small Interfering RNA

The small interfering RNAs (siRNA) ON-TARGET plus SMART pool were used along with ON-TARGET plus Non-targeting pool as a negative control obtained from Dharmacon (Chicago,

IL). Transfection was performed with Lipofectamine™ RNAiMAX from Invitrogen (Carlsbad, CA) using reverse transfection procedure as recommended by manufacturer. Cells were seeded at 1500 cells per well in 96-well plate. After transfection with 20nM or 40nM of siRNA for 5 days, cell proliferation assays were performed using CellTiter-Glo Luminescent Cell Viability Assay from Promega (Madison, WI), according to the manufacturer's recommendations. Luminescence was recorded using a Victor Plate Reader from PerkinElmer (Waltham, MA). Data are expressed as mean ± SD that represents two separate experiments with 3 data points separating each siRNA.

Rescue experiments with gatekeeper mutant version of tyrosine kinases

Rescue experiments were performed as previously described (12). Briefly, cells were seeded in black wall 96-well plate from NUNC (Rochester, New York). After overnight incubation, cells were infected with 30µl of viruses plus polybrene per well for 48 hours, and then treated with a series dilution of dasatinib for 120 hours for CellTiter-Glo cell viability assay as described above.

Human Tumor Tissues

Patients with the diagnosis of rhabdomyosarcoma and sufficient archived tissue block were identified under an IRB-approved protocol. The original diagnoses were confirmed by a sarcoma pathologist (MMB). Four patients had multiple specimens available from surgical procedures at different time for the same tumor. When possible, at least two blocks of the same specimen were obtained, which resulted to 23 specimens and 32 tissue blocks. Samples, 5 µm in thickness, including formalin fixed and paraffin embedded tumor tissue sections, cellblock sections from cell line and control slides, were stained using a Ventana Discovery XT automated system (Ventana Medical Systems, Tucson, AZ) as per manufacture's protocol with proprietary reagents. Positive and negative control slides were chosen from representative samples of GIST tissues with the negative sample omitting the primary antibody and being

substituted with rabbit polyclonal immunoglobulin. Slides were deparaffinized on the automated system with EZ Prep solution (Ventana). Heat-induced epitope retrieval method was used in Cell Conditional solution (CC1, Ventana). Polyclonal rabbit antibody specific to PDGFR*α* (Cell Signaling Technology, Danvers, MA) was used at a 1:400 concentration in Dako antibody diluent (Carpenteria, CA) and incubated for 60 min. Ventana OmniMap Anti-rabbit Antibody was used for 20 min. The detection system used was the Ventana ChromoMap DAB Kit and slides were then counterstained with Hematoxylin. Slides were then dehydrated and coverslipped as per normal laboratory protocol. Primary sarcoma explant models were generated as described under a University of South Florida IACUC approved protocol(15).

SUPPLEMENTAL DATA

Supplemental Figure Legends

Supplemental Figure1. Receptor tyrosine kinases phosphorylation in sarcoma cell lines A204, MNNG, RD-ES and Saos2, cells were lysed and the relative level of 42 phosphorylated human receptor tyrosine kinases were determinated using human Phospho-RTK Array Kit as described in Materials and Methods. A. Lighter exposure B. Darker exposure. C. 42 RTK lists.

Supplemental Figure2. Knockdown efficacy of siRNA. A204(**A**), MNNG(**B**), RD-ES (**C**) and Soas2 (**D**) Cells were transfected with indicated siRNA for 48 hrs. Total protein lysates were collected and evaluated using indicated antibodies and western analysis. Equal protein loading was confirmed by evaluation of β-actin.

Supplemental Figure 3. Effects of BMS754807, a dual IGFR and INSR inhibitor, on cell proliferation in sarcoma cell lines.

A. Indicated sarcoma cell lines were exposed to increasing concentrations of BMS754807 and cell viability was assessed by CellTiter-Glo after 72 hours. Cell viability was normalized to DMSO treated cells.

B. IC_{50} of BMS754807 in 10 sarcoma cell line. The median inhibition concentration (IC_{50}) value was calculated by non-linear regression analyses using GraphPad software .

C. Direct comparison of RD-ES sensitivity to BMS754807 and OSI868. Cell viability after 72 hours was assessed by CellTiter-Glo and normalized to DMSO treated cells.

Supplemental Table Legends

Supplemental Table 1. Proteins with phosphorylation tyrosine sites identified by phosphoproteomics approach in ten sarcoma cell lines.

Mass spectrometry data from ten sarcoma cell lines as described in Materials and Methods. Identified proteins are listed and numbers represents the total spectral counts of phosphotyrosine peptides for each protein across the one biological sample and two technical runs of each sample.

Supplemental Table 2. Raw data of phosphoproteomics experiments in sarcoma cell lines and tissues.

Ten sarcoma cell lines and three xenograft human sarcoma tissues were lysed and phosphopeptides were purified and identified using phosphopeptide Immunoprecipitation and LC-MS/MS. pY sites, peptide sequence and associated with the corresponding proteins are list and total spectral counts for each peptides are summed from one biological sample and two MS/MS runs.

Supplemental Table 3. PDGFRα immunostaining of tissue blocks.

Immunohistochemical stain of the human tumor with PDGFRα immunoreactivity as described in Materials and Methods. The result represents negative with 'NEG' and the weak to strong stain from '1+' to '3+'.

Supplemental Table 4. Characteristics of primary xenograft human sarcoma tumor tissues for phosphotyrosine profiling.

SRBCT indicates small round blue tumor and MPNST indicates malignant peripheral nerve sheath tumor histology.

Supplemental Table 5. Proteins with tyrosine phosphorylation sites identified by phosphoproteomics in xenograft human sarcoma tissues.

Mass spectrometry data from three primary xenograft human sarcoma tissues were profiled for tyrosine phosphopeptides. Identified proteins are list and number represents the total spectral counts of phosphotyrosine peptides for each protein across the one biological sample and two technical runs of each sarcoma tissue.