

Quantification of Focal Adhesion Kinase Activation Loop Phosphorylation as a Biomarker of Src Activity

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ABBREVIATIONS: ACN, acetonitrile; c-Src, cellular-Src; DMEM, Dulbecco's Modified Eagle's Medium; FAK, focal adhesion kinase; FERM, protein 4.1, ezrin, radixin, and moesin; FAT, focal-adhesion targeting; LC-MRM/MS, liquid chromatography-multiple reaction monitoring/mass spectrometry; MEF, mouse embryonic fibroblast; NRTK, non-receptor tyrosine kinase; PRR, proline-rich region; SEM, standard error of the mean.

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ABSTRACT

A recently developed stable isotope dilution liquid chromatography-multiple reaction/mass spectrometry (LC-MRM/MS) method to quantify focal adhesion kinase (FAK) activation loop phosphorylation was utilized to study endogenous Src kinase activity. This revealed that *bis*-phosphorylated pY⁵⁷⁶/Y⁵⁷⁷-FAK was a biomarker of Src activity and inactivation *in vitro* and in cell culture. Mouse embryonic fibroblasts (MEFs) expressing endogenous Src-family kinases contained 65 % unmodified Y⁵⁷⁶/Y⁵⁷⁷, 33 % mono-phosphorylated-pY⁵⁷⁶-FAK, and 6 % *bis*-phosphorylated-pY⁵⁷⁶/pY⁵⁷⁷-FAK. In contrast, MEFs expressing oncogenic Y⁵²⁹FSrc contained 38 % unmodified-Y⁵⁷⁶/Y⁵⁷⁷-FAK, 29 % mono-phosphorylated-pY⁵⁷⁶-FAK, and 19 % *bis*-phosphorylated-pY⁵⁷⁶/pY⁵⁷⁷-FAK. This new method has made it possible to accurately determine the absolute amounts of FAK phosphorylation that occur following Src inhibition in cell culture and *in vitro* with increasing concentrations of the Src inhibitor AZD0530. Phosphorylation of FAK at Y⁵⁷⁶/Y⁵⁷⁷ was inhibited by AZD0530 in a dose-dependant manner both in cell culture and *in vitro*. However, there was a substantial difference in the ability of AZD0530 to inhibit Src that was constitutively activated in a cellular context (IC₅₀ = 2.12 μM) when compared with the isolated enzyme (IC₅₀ = 0.14 μM). When normal MEFs and Y⁵²⁹FSrc-expressing MEFs were treated with pervanadate (a global phosphatase inhibitor) pY⁵⁷⁶/pY⁵⁷⁷-FAK accounted for almost 60 % of total FAK present in the cells. This suggests that activation loop phosphorylation is regulated by tyrosine phosphatases. These results confirm that FAK phosphorylation is a useful biomarker of Src inhibition *in vivo*. The accuracy and specificity of stable isotope dilution LC-MS methodology offers significant advantages over current immunochemical approaches for monitoring Src activity.

Introduction

The Src family kinases comprise eleven structurally related, membrane-associated, non-receptor protein tyrosine kinases (NRTK) including cellular-Src (c-Src), c-Yes, Fyn, Lyn, Hck, Blk, Brk, Fgr, Frk, Srm, and Yrk (Summy and Gallick, 2003; Yeatman, 2004; Meyn, III and Smithgall, 2008). Although most Src family members are expressed primarily in cells of hematopoietic origin, c-Src, c-Yes and Fyn are expressed more ubiquitously with high levels of expression in some epithelial tissues, platelets, and neurons (Thomas and Brugge, 1997). They are rarely mutated in human tumors but are overexpressed or activated in a variety of tumors, including colon, breast, pancreatic, bladder, head and neck, ovarian, and brain (Summy and Gallick, 2003). Although Src is a protooncogene, its association with FAK (another NRTK), is critical for the formation of metastases (Basson, 2008). Therefore, Src is an emerging therapeutic target to prevent metastases from occurring (Sawyer, 2004; Rucci et al., 2008). Preclinical experiments indicate that small-molecule inhibitors of Src block tumor growth, metastasis, and angiogenesis (Hiscox and Nicholson, 2008). The binding of Src to FAK results in phosphorylation of FAK at multiple residues, critical among them being the Y⁵⁷⁶ and Y⁵⁷⁷ sites located on FAK's kinase activation loop (Fig. 1) (Calalb et al., 1995; Owen et al., 1999; Ruest et al., 2000; Ciccimaro et al., 2006). In a global phosphotyrosine proteome analysis, FAK Y⁵⁷⁶ emerged as the most readily detected Src target in cultured non-transformed fibroblasts (Luo et al., 2008). The interplay of Src and FAK is a vital convergence point, integrating signals of cell growth, survival, and migration, making the pharmacological inhibition of Src a unique target for cancer drug therapy (Rucci et al., 2008). The use of recently developed orally available Src kinase inhibitors to reduce tumor growth shows particular promise for preventing metastasis *in vivo* (Summy et al., 2005; Shor et al., 2007; Jallal et al., 2007; Ischenko et al., 2007;

Park et al., 2008). Although the inhibition of Src in the treatment of cancer represents an important molecular target, there remains a need to develop validated biomarkers to monitor the efficacy of this approach. It has been very challenging to accurately assess the function of Src in intact cells because of the complex signal transduction pathways that are involved (Mishra et al., 2005; Kansra et al., 2005; Vultur et al., 2008; Ozawa et al., 2008). Most current approaches employ biomarkers for Src activity that rely upon quantifying the effects of an isolated enzyme acting upon a synthetic peptide substrate (Hennequin et al., 2006) or the use of immunoprecipitation of a downstream protein target followed by Western blot analysis (Summy et al., 2005).

Several studies have been conducted to identify biomarkers of Src activity after treatment of cells with Src inhibitors including those employing bosutinib (SKI-606) (Vultur et al., 2008) and N-(5-Chloro-1,3-benzodioxol-4-yl)-7-[2-(4-methylpiperazin-1-yl)ethoxy]-5-(tetrahydro-2H-pyran-4-yloxy)quinazolin-4-amine (AZD0530; Serrels et al., 2006). These studies were able to correlate tyrosine phosphorylation of Src substrates with drug treatment; autophosphorylation of Src in the former, and paxillin and FAK phosphorylation in the latter. However, they were limited to conducting relative quantification and so normalization of phosphorylation to the amount of residual un-phosphorylated protein was not possible. Therefore, differences in protein expression, immunoprecipitation efficiency, or other inter-sample variability factors could not be addressed. FAK phosphorylation on Y^{576}/Y^{577} most accurately reflects Src activity (Ciccimaro et al., 2006) and so we have developed a new LC-MRM/MS method that quantifies FAK phosphorylation as a robust biomarker of Src activity *in vitro*, in cell culture, and ultimately *in vivo* (Ciccimaro et al., 2008).

This new method has made it possible to accurately determine the absolute amounts of FAK phosphorylation that occurs following Src inhibition *in vitro* and in cell culture with increasing concentrations of the Src inhibitor AZD0530 (Hennequin et al., 2006). Furthermore, because the technique is self-normalizing problems arising from inter-sample differences in FAK expression levels, immunoprecipitation efficiency, and assay sensitivity were eliminated.

Materials and Methods

Materials. The gene for human FAK (PTK2- α) in the Gateway® entry vector pENTER221 was from Invitrogen (Carlsbad, CA). The pDEST26 and pEXP1 plasmids, nickel-chelating resin (Probond™), the cell free expression system, and recombinant full-length c-Src and FAK were also from Invitrogen (Carlsbad, CA). Dialysis cassettes were from Pierce Biotechnology (Rockford, IL). Anti-FAK beads (clone 4.47, agarose conjugate beads) were from Millipore (Billerica, MA). Protease inhibitors were from Roche Applied Sciences (Indianapolis, IN). The Src inhibitor, AZD0530, was a kind gift from AstraZeneca (Macclesfield, Cheshire, UK). The autosampler was a CTC pal from Leap technologies (Carrboro, NC), while the LC pump was an ExpressLC100 system from Eksigent Technologies (Dublin, CA). LC grade water and CAN were from Burdick and Jackson (Muskegon, MI), while Suprapur® formic acid was from EMD Chemical (Gibbstown, NJ). The reversed phase column (50 x 1 mm internal diameter) was custom made using extended C18 (300 Å, 3.5 µm) by Agilent Technologies (Santa Clara, CA). All standard peptides were synthesized and quantified using amino acid analysis by AnaSpec, Inc. (San Jose, CA).

Cell-free synthesis of stable isotope labeled FAK protein ([¹³C¹⁵N]-FAK). A full-length FAK construct (from cDNA clone MGC: 34721) correlating to the human gene, protein tyrosine

kinase 2 alpha (PTK2- α), was obtained from commercial sources. Creation of an expression clone suitable for cell free production was accomplished following manufacturer's instructions using restriction free recombination technology (Gateway®) and plasmids pDEST26 then pEXP1. The resultant expression clone contained FAK N-terminally tagged with a 6XHIS epitope downstream of the T7 promoter and ribosomal binding site. FAK protein labeled with [$^{13}\text{C}_6$ $^{15}\text{N}_4$]-arginine, [$^{13}\text{C}_9$]-tyrosine, [$^{13}\text{C}_6$ $^{15}\text{N}_2$]-lysine, and [$^{13}\text{C}_6$ ^{15}N]-leucine was produced using the expressway cell free expression system, supplied with heavy isotope-labeled amino acids, similar to published techniques (Hanke et al., 2008; Torizawa et al., 2004). The cell free reaction was conducted according to the manufacturer's directions and the subsequent [$^{13}\text{C}^{15}\text{N}$]-labeled 6XHIS-FAK ([$^{13}\text{C}^{15}\text{N}$]-FAK) was purified on a 2 mL nickel-chelating resin under non-denaturing conditions. Following a wash step, [$^{13}\text{C}^{15}\text{N}$]-FAK was eluted and stored in the presence of 10 % glycerol at -80 °C until further use. A portion of this reaction mixture was resolved on an SDS-PAGE gel and stained with Coomassie blue. The prominent band corresponding to ~ 125 kDa (as compared to a protein mass marker) was picked for LC-MS analysis. In gel trypsin digestion was performed and the isotopic purity of [$^{13}\text{C}^{15}\text{N}$]-FAK was determined by LC-tandem MS (MS/MS).

***In vitro* tyrosine phosphorylation of [$^{13}\text{C}^{15}\text{N}$]-FAK.** In order to phosphorylate [$^{13}\text{C}^{15}\text{N}$]-FAK using purified recombinant Src in an *in vitro* kinase reaction, it was first necessary to denature [$^{13}\text{C}^{15}\text{N}$]-FAK and allow refolding during buffer exchange. To accomplish this, [$^{13}\text{C}^{15}\text{N}$]-FAK was mixed 1:1 with a denaturing buffer consisting of 6 M urea, 2 M thiourea, and 10 mM dithiothreitol (DTT), and heated at 37 °C for 30 min. Following heating, the solution containing denatured [$^{13}\text{C}^{15}\text{N}$]-FAK was dialyzed for 6 h at 4 °C against oxidizing dialysis buffer {1 mM oxidized glutathione, 40 mM Tris-HCl (pH = 7.5), 150 mM NaCl, 270 mM sucrose, 100

μM EGTA, 100 μM Na_3VO_4 , and 0.03 % Brij-35} using a 10,000 MWCO filter dialysis cassette. After 6 h, the dialysis buffer was replaced and dialysis was allowed to continue for an additional 6 h. The refolded and buffer exchanged [$^{13}\text{C}^{15}\text{N}$]-FAK was then split into portions, and each portion was adjusted to contain, 1 mM ATP, 1 X kinase buffer, and recombinant Src (33 nM of full length recombinant c-Src). Following incubation at 37 °C for 30 min, all portions were pooled and stored at -80 °C. Both full scanning LC-electrospray ionization (ESI)/MS/MS and LC-MRM/MS were used to ascertain the extent of Y⁵⁷⁶/Y⁵⁷⁷ phosphorylation on [$^{13}\text{C}^{15}\text{N}$]-FAK.

***In vitro* Src inhibition assay.** Recombinant full-length human c-Src (12 nM) was incubated in kinase buffer [25 mM Tris-HCl (pH 7.5), 5 mM β -glycerophosphate, 2mM dithiothreitol, 0.1 mM Na_3VO_3 , and 10 mM MgCl_2] containing vehicle (0.1 % DMSO) or AZD0530 (0.01, 0.1, 1 and 10 μM) at 4 °C for 30 min. Following incubation, samples were prepared with recombinant human FAK (120 nM) and 1 mM ATP except for the vehicle (- ATP) sample, which did not receive ATP. Samples were brought to 37 °C and allowed to incubate for 10 minutes, at which point samples were heat inactivated at 70 °C for 10 min and un-phosphorylated together with phosphorylated [$^{13}\text{C}^{15}\text{N}$]-FAK were added as recovery standards. All samples were then trypsin digested in the presence of 1 pmol [$^{13}\text{C}^{15}\text{N}$]-peptide standard mixture (Ciccimaro et al., 2008). Results were normalized to total FAK and represent the activation loop form amount per total FAK (pmol/pmol).

LC-MS conditions. Tryptic peptides were loaded onto a micro-analytical C18 column at 25 $\mu\text{L}/\text{min}$ using 100 % buffer A [acetonitrile (ACN)/ H_2O (0.1:20, v/v) with 0.1 % formic acid]. The mobile phase was diverted to waste for the first 5 min under these conditions in order to remove salts, following which, the flow rate was reduced to 14 $\mu\text{L}/\text{min}$ and peptides were eluted over a 30 min gradient from 0 % to 40 % B [ACN/ H_2O (19:1, v/v) with 0.1 % formic acid]. An

LTQ mass spectrometer (ThermoFisher Scientific, San Jose, CA) was operated in the positive electrospray ionization mode with helium as the collision gas.

Analysis of FAK activation loop phosphorylation status using LC-MRM/MS. Peptides spanning the tryptic region containing FAK activation loop Y⁵⁷⁶ and Y⁵⁷⁷ (Y⁵⁷⁰MEDSTYYK⁵⁷⁸) with the endogenous four amino acid overhangs at both the amino and carboxy terminus and their [¹³C¹⁵N]-analogs were synthesized as standards for unmodified FAK, pY⁵⁷⁶-FAK, and pY⁵⁷⁶pY⁵⁷⁷-FAK (Ciccimaro et al., 2008). In addition, a control segment of FAK, representing an unmodified tryptic peptide, was synthesized as labeled and unlabeled forms. Following trypsin digestion, LC-MRM/MS was employed to monitor a total of twelve peptides resulting from the hydrolysis of endogenous FAK, [¹³C¹⁵N]-FAK proteins, and [¹³C¹⁵N]-labeled peptide standards in a single LC-MRM/MS analysis. MRM transitions for the unmodified Y⁵⁷⁶/Y⁵⁷⁷ FAK tryptic peptide were as follows: YMEDSTYYK (*m/z* 600.25 to 905.40), [¹³C₉]YMEDST[¹³C₉]Y[¹³C₉]Y[¹³C₆¹⁵N₂]K (*m/z* 617.25 to 930.40), and YMEDSTYY[¹³C₆¹⁵N₂]K (*m/z* 604.25 to 913.40). MRM transitions for the mono-phosphorylated (pY⁵⁷⁶) peptide were as follows: YMEDSTpYYK (*m/z* 640.25 to 985.35 and 967.35), [¹³C₉]YMEDST[¹³C₉]pY[¹³C₉]Y[¹³C₆¹⁵N₂]K (*m/z* 657.25 to 1010.36 and 992.36), and YMEDSTpYY[¹³C₆¹⁵N₂]K (*m/z* 644.25 to 993.36 and 975.36). MRM transitions for the bis-phosphorylated (pY⁵⁷⁶/pY⁵⁷⁷) peptide were as follows: YMEDSTpYpYK (*m/z* 680.25 to 1047.31), [¹³C₉]YMEDST[¹³C₉]pY[¹³C₉]pY[¹³C₆¹⁵N₂]K (*m/z* 697.25 to 1072.32), and YMEDSTpYpY[¹³C₆¹⁵N₂]K (*m/z* 684.25 to 1055.32). MRM transitions for the control segment peptide were as follows: EVGLALR (*m/z* 379.23 to 359.24), EVG[¹³C₆¹⁵N]LA[¹³C₆¹⁵N]L[¹³C₆¹⁵N₄]R (*m/z* 382.23 to 359.24), and E[¹³C₅¹⁵N]VGLALR (*m/z* 391.23 to 376.24).

Preparation of standard curves, data processing, and normalization. Standard curves were prepared by mixing known amounts of unlabeled peptide standards, a known amount of [$^{13}\text{C}^{15}\text{N}$]-FAK, and fixed amounts of the [$^{13}\text{C}^{15}\text{N}$]-peptide standards. Concentrations were calculated using Excalibur (Version 2.2) software (ThermoFisher Scientific). For immunoprecipitation experiments, the [$^{13}\text{C}^{15}\text{N}$]-FAK standards were used to determine percent recovery over a range of 2 % to 50 %. Response ratios of endogenous and [$^{13}\text{C}^{15}\text{N}$]-FAK to their heavy isotope-labeled standards were calculated by interpolation using their respective standard curves to determine the pmol amount of endogenous FAK and immunoprecipitation recovery for [$^{13}\text{C}^{15}\text{N}$]-FAK. Two corrections for immunoprecipitation efficiency, an unmodified percent recovery and a phosphorylated percent recovery were calculated. These correction values were applied to correct for recovery of the relevant FAK form. Results are presented as means (\pm SEM).

Cell culture. MEF populations expressing murine Src Y⁵²⁹F or vector-only were used (Brabek et al., 2004). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10 % fetal bovine serum, and penicillin/streptomycin. Cultures were kept below 90 % confluence on 100 mm culture dishes until harvesting, at which point they were allowed to grow to full confluence. Pervanadate treatment was conducted by exposing cell monolayers to 3 mM H₂O₂/1 mM Na₃VO₄ for 10 min prior to harvesting. Src inhibition, using AZD0530 was performed by incubating cells in complete DMEM (as above) containing either vehicle (0.1 % DMSO) or AZD0530 (0.01, 0.1, 1, 10, or 50 μM) for 3 or 12 h.

Immunoprecipitation recovery of FAK from cell lysates. Recombinant FAK (258.8 nM) and c-Src (12.8 nM) in a total volume 200 μL was incubated in the presence of ATP (1 mM) at 37 °C for 5 min. The reaction mixture was then heated to 70 °C for 10 min. A 10 μL portion of

this reaction mixture together with 12 μL of [$^{13}\text{C}^{15}\text{N}$]-FAK solution were added to a lysing dish of FAK-/- MEFs, or a 10 μL portion of this reaction, together with 6 μL of the [$^{13}\text{C}^{15}\text{N}$]-FAK solution were added to two lysing dishes, and pooled. Immunoprecipitation was conducted as described above. In parallel, 12 μL of heat-inactivated [$^{13}\text{C}^{15}\text{N}$]-FAK solution was mixed with a 10 μL portion of the *in vitro* kinase reaction in a vial. Following the elution of FAK from the immune-complex, [$^{13}\text{C}^{15}\text{N}$]-Peptide standards were added, the mixtures were digested with trypsin, and the digests analyzed by LC-MRM/MS as described above.

Analysis of FAK activation loop phosphorylation following immunoprecipitation. The quantification of FAK Y⁵⁷⁶ and Y⁵⁷⁷ in the unmodified, mono-(pY576), and *bis*-phosphorylated-pY⁵⁷⁶/pY⁵⁷⁷ forms relative to total FAK was carried out as described previously (Ciccimaro et al., 2008). Briefly, Confluent plates of MEF cells were lysed in the presence of [$^{13}\text{C}^{15}\text{N}$]-FAK and total FAK was immunoprecipitated. [$^{13}\text{C}^{15}\text{N}$]-Peptide standards were added to the immunoprecipitate elution and the mixture was digested with trypsin. Tryptic digests were analyzed by LC-MRM/MS to quantify both endogenous FAK as well as [$^{13}\text{C}^{15}\text{N}$]-FAK. Endogenous and [$^{13}\text{C}^{15}\text{N}$]-FAK peptides were quantified using their respective calibration curves. Endogenous FAK peptide levels were then corrected using the calculated immunoprecipitation recovery value (using the [$^{13}\text{C}^{15}\text{N}$]-FAK standard) and normalized to total FAK (using the amount unmodified control peptide).

Analysis of FAK activation loop phosphorylation in MEFs following inhibition with pervanadate. Prior to cell lysis, confluent plates of normal and Y⁵²⁹FSrc expressing MEFs were treated with pervanadate for 10 min. Cells were then lysed in the presence of [$^{13}\text{C}^{15}\text{N}$]-FAK and total FAK was immunoprecipitated and processed as above.

Analysis of FAK activation loop phosphorylation in MEFs following Src inhibition.

Confluent plates of normal and Y⁵²⁹FSrc expressing MEFs were treated with 1 μM of the Src inhibitor AZD0530 for 3 h. Following this incubation, cells were treated with pervanadate for 10 min. Cells were then lysed in the presence of [¹³C¹⁵N]-FAK and total FAK was immunoprecipitated and processed as described above.

Dose response of FAK activation loop phosphorylation after Src inhibition in oncogenic Y529FSrc expressing MEFs. Confluent plates of normal and Y⁵²⁹FSrc expressing MEFs were treated with vehicle (0.1 % DMSO) or (0.01, 0.1, 1, 10 and 50 μM) the Src inhibitor AZD0530 for 12 h. Cells were then lysed in the presence of [¹³C¹⁵N]-FAK and total FAK was immunoprecipitated and processed as described above.

Results

***In vitro* inhibition of Src.** In order to evaluate FAK inhibition by AZD0530 in a controlled setting, Src was inhibited by the drug *in vitro* and then incubated with recombinant FAK. FAK incubated with vehicle treated Src in the presence of ATP showed 0.170 (± 0.016) pmol of unmodified-FAK. This represents consumption of 0.660 pmol or 78 % of the starting material, which was found to contain 0.832 (± 0.012) pmol unmodified-FAK. Additionally, this condition showed the production of 0.240 (± 0.005) mono-pY⁵⁷⁶-FAK, and 0.422 (± 0.018) pmol of *bis*-pY⁵⁷⁶/pY⁵⁷⁷-FAK above the starting material amount, which contained 0.132 (± 0.009) and 0.036 (± 0.004) pmol of each form respectively. The values recorded for FAK reacted with vehicle treated Src in the presence of ATP correspond to maximal kinase activity under these

experimental conditions and, therefore, all AZD0530 inhibited Src results are presented as percent maximal activity relative to these amounts.

Analysis of the unmodified activation loop region (YMEDSTYYK) showed the activity of Src under assay conditions was not significantly inhibited by 0.01 μM AZD0530. A 10 % reduction in Src activity to 89.9 % of the maximum was seen in the 0.1 μM AZD0530 treatment, with further reductions to 44.7 % and 4.9 % of maximum at 1 μM and 10 μM treatment with AZD0530, respectively (Fig. 2A). The prevention of Src's ability to modify the activation loop region coincided with a dose-dependant decrease in the production of FAK mono-pY⁵⁷⁶ and *bis*-pY⁵⁷⁶pY⁵⁷⁷ activation loop forms. At 0.1 μM , AZD0530 reduced the *bis*-phosphorylation of the activation loop to 64.8 % of the maximal level; however, this was concurrent with an increase in the amount of the mono-pY⁵⁷⁶ form to 34.0 % above the maximum. *Bis*-pY⁵⁷⁶/pY⁵⁷⁷ was further reduced with 1 μM and 10 μM AZD0530 to 12.5 % and 0.4 %, respectively. Mono-phosphorylation at Y⁵⁷⁶ was not inhibited at 1 μM but was reduced to 12.6 % of the maximal level at a concentration of 10 μM AZD0530 (Fig. 2B). A best fit IC₅₀ value using the quantification of *bis*-phosphorylated FAK activation loop domain was found to be 0.14 μM (95 % confidence 0.07 μM to 0.30 μM) (Fig. 2C).

Immunoprecipitation recovery of FAK from cell lysates. To confirm our methodology correctly quantified FAK phosphorylation following protein immunoprecipitation, we measured the amount of FAK phosphorylation on a known amount of recombinant FAK spiked into, and then immunoprecipitated from, a FAK null cell lysate. The amounts of unmodified, pY⁵⁷⁶, and pY⁵⁷⁶/pY⁵⁷⁷ FAK present in the kinase reaction mixture before the immunoprecipitation were 0.707 (\pm 0.029), 1.893 (\pm 0.025), and 1.080 (\pm 0.021) pmol, respectively. Following immunoprecipitation, the amounts quantified were 0.160 (\pm 0.011) pmol unmodified, 0.338 (\pm

0.018) pmol pY⁵⁷⁶, and 0.187 (± 0.011) pmol pY⁵⁷⁶/pY⁵⁷⁷ FAK. The immunoprecipitation recovery based on [¹³C¹⁵N]-FAK was 24.5 % for unmodified-FAK and 15.1 % for pY⁵⁷⁶-FAK. Applying these correction values for each sample to the initial immunoprecipitated amounts resulted in adjusted amounts of 0.669 (± 0.030) pmol for unmodified-FAK, 2.123 (± 0.191) pmol for pY⁵⁷⁶-FAK, and 1.221 (± 0.063) pmol for pY⁵⁷⁶/pY⁵⁷⁷-FAK. The total amount of activation loop peptides (corrected for losses during immunoprecipitation) was 4.025 (± 0.298) pmol and the amount control peptide was 3.891 (± 0.278) pmol. The total FAK amount based on the control peptide was therefore 3.891 (± 0.278) pmol (i.e. 486 ng FAK/1.65 mg total protein (single dish background) and 486 ng FAK/3.30 mg total protein (double dish background)).

Analysis of FAK activation loop phosphorylation in MEFs. Following *in vitro* experimentation, the ability of the assay to monitor Src activity was investigated in a cell culture system containing normal and oncogenic Src. MEFs expressing normal Src were found previously to contain 0.654 (± 0.034) pmol of unmodified-FAK, 0.331 (± 0.052) pmol of pY⁵⁷⁶-FAK, and 0.059 (± 0.010) pmol of pY⁵⁷⁶/pY⁵⁷⁷-FAK (Ciccimaro et al., 2008). The total amount of activation loop peptides normalized to control peptide for normal Src in the basal state was 1.044 (± 0.087) pmol. In contrast, MEFs expressing Y⁵²⁹FSrc were found to express 0.381 (± 0.026) pmol of unmodified-FAK, 0.287 (± 0.005) pmol of pY⁵⁷⁶-FAK, and 0.193 (± 0.006) pmol of pY⁵⁷⁶/pY⁵⁷⁷-FAK (Fig. 3). The total amount of FAK activation loop peptides in the Y⁵²⁹FSrc expressing MEFs was 0.836 (± 0.027) pmol (Fig. 3). These values were obtained by using calculated immunoprecipitation recoveries of 25 % for unmodified FAK and 14 % for phosphorylated FAK in the Y⁵²⁹FSrc expressing MEFs. The average total FAK amount (corrected for losses during immunoprecipitation) was 1.5 pmol/plate (i.e. 187.5 ng FAK/2.47 mg total protein).

Analysis of FAK activation loop phosphorylation in MEFs following inhibition with pervanadate. The ability of AZD0530 to inhibit Src phosphorylation of FAK's activation loop was studied under conditions of FAK hyperphosphorylation. This was accomplished by broadly inhibiting tyrosine phosphatases with pervanadate. A typical LC-MRM/MS chromatogram for digested endogenous and [$^{13}\text{C}^{15}\text{N}$]-FAK immunoprecipitated from the normal MEFs treated with pervanadate and their heavy isotope-labeled internal standards is shown in Fig. 4. $\text{Y}^{529}\text{FSrc}$ expressing MEFs treated with pervanadate gave similar chromatograms (data not shown). Following data normalization, as above, the pervanadate-treated normal MEFs contained 0.109 (± 0.008) pmol of unmodified-FAK, 0.242 (± 0.023) pmol of pY^{576} -FAK, and 0.504 (± 0.080) pmol of $\text{pY}^{576}/\text{pY}^{577}$ -FAK. Phosphatase inhibited $\text{Y}^{529}\text{FSrc}$ -expressing MEFs were found to express similar amounts, with 0.175 (± 0.026) pmol of unmodified-FAK, 0.132 (± 0.019) pmol of pY^{576} -FAK, and 0.440 (± 0.040) pmol of $\text{pY}^{576}/\text{pY}^{577}$ -FAK. The total amount of activation loop peptides (normalized to control peptide) for pervanadate treated normal and $\text{Y}^{529}\text{FSrc}$ expressing MEFs were 0.855 (± 0.106) pmol and 0.747 (± 0.081) pmol, respectively (Fig. 5). These values were corrected using calculated immunoprecipitation recoveries of 26.0 % for unmodified-FAK and 14.0 % phosphorylated FAK from normal MEFs and 28.0 % for unmodified-FAK and 16.0 % for phosphorylated from $\text{Y}^{529}\text{FSrc}$ -expressing MEFs. The average total FAK amounts (based on the immunoprecipitation corrected amount of control peptide) following pervanadate treatment were 2.70 pmol/plate (*i.e.* 337.5 ng FAK/2.62 mg total protein) for normal Src expressing MEFs and 1.05 pmol/plate (*i.e.* 125 ng FAK/2.62 mg total protein) for $\text{Y}^{529}\text{FSrc}$ expressing MEFs.

Analysis of FAK activation loop phosphorylation in MEFs following treatment with 1 μM Src inhibitor. To demonstrate the sensitivity and selectivity of the assay to monitor Src

activity, Src kinase function was inhibited in cell culture. MEFs expressing oncogenic Y⁵²⁹FSrc treated with Src inhibitor prior to pervanadate showed similar chromatograms to those recorded for fibroblasts treated with pervanadate alone (see Fig. 4). Following data normalization, the Src inhibited and pervanadate-treated normal MEFs contained 0.667 (\pm 0.074) pmol of unmodified-FAK and 0.302 (\pm 0.032) pmol of pY⁵⁷⁶-FAK, while pY⁵⁷⁶/pY⁵⁷⁷-FAK was not detected. Src inhibited and phosphatase treated Y⁵²⁹FSrc-expressing MEFs were found to contain, 0.332 (\pm 0.057) pmol of unmodified-FAK, 0.431 (\pm 0.021) pmol of pY⁵⁷⁶-FAK, and 0.392 (\pm 0.037) pmol of pY⁵⁷⁶/pY⁵⁷⁷-FAK. The total amount of activation loop peptides (normalized to control peptide) for Src inhibited and pervanadate treated normal and Y⁵²⁹FSrc expressing MEFs were 0.951 (\pm 0.065) pmol and 1.160 (\pm 0.047) pmol, respectively (Fig. 6). These values were corrected using calculated immunoprecipitation recoveries of 27.8 % unmodified and 20.5 % phosphorylated for normal MEFs and 27.3 % unmodified and 18.0 % phosphorylated for Y⁵²⁹FSrc-expressing MEFs on average. The average total FAK amounts (based on the immunoprecipitation corrected amount of control peptide) following pervanadate treatment were 2.00 pmol/plate for normal Src expressing MEFs and 1.15 pmol/plate for Y⁵²⁹FSrc expressing MEFs.

Dose response of FAK activation loop phosphorylation after Src inhibition in oncogenic Y529FSrc expressing MEFs. Treatment with 1 μ M Src inhibitor failed to significantly alter FAK activation loop phosphorylation in oncogenic Src expressing MEFs. Therefore, FAK activation loop was quantified in oncogenic Src expressing MEFs treated with a broad range of inhibitor concentrations. Culturing Y⁵²⁹FSrc expressing MEFs in the presence of vehicle, 0.01 μ M, or 0.1 μ M AZD0530 did not alter FAK activation loop phosphorylation levels as compared to those observed in the basal state. The amount of FAK activation loop forms observed in

vehicle treated Y⁵²⁹FSrc expressing MEFs, was considered the maximal level, and all other values are presented as a percentage of these levels. At 1 μ M, AZD0530 treatment reduced pY⁵⁷⁶/pY⁵⁷⁷-FAK amounts to 73.2 % (\pm 11.4 %), while it increased pY⁵⁷⁶-FAK amounts to 112.5 % (\pm 9.0 %) of maximum. At this treatment concentration, the amount of unmodified-FAK was approximately 100 % of the maximal level at 101.8 % (\pm 10.9 %). Treatment with 10 μ M AZD0530 further reduced pY⁵⁷⁶/pY⁵⁷⁷-FAK amounts to 5.9 % (\pm 8.3 %), while it reduced pY⁵⁷⁶-FAK amounts to 76.4 % (\pm 14.0 %) of maximum. Concurrently, this dose increased the amount of unmodified-FAK, and decreased the amount of phosphorylated-FAK activation loop to 47.5 % (\pm 4.5 %) of maximum. At 50 μ M AZD0530, no pY⁵⁷⁶/pY⁵⁷⁷-FAK was detected, and pY⁵⁷⁶-FAK was reduced to only 14.8 % (\pm 5 %) of maximum. At this dose, nearly all FAK recovered was in the unmodified form, reducing the amount of modified forms to 8.7 % (\pm 3.0 %) of their maximum levels (Figs. 7A and 7B). A best fit IC₅₀ value using the quantification of *bis*-phosphorylated FAK activation loop was found to be 2.12 μ M (95 % confidence 0.74 μ M to 6.07 μ M (Fig. 7C).

Discussion

Inhibition of Src with AZD0530 *in vitro* prevented modification on the activation loop region as reflected in both the amount of unmodified FAK preserved and the amount of pY⁵⁷⁶-FAK and pY⁵⁷⁶/pY⁵⁷⁷-FAK produced. Interestingly, the dose-response curve obtained for pY⁵⁷⁶-FAK showed an initial increase at 0.1 μ M AZD0530 (Fig. 2B). This result can be explained by the prevention of Src from converting the mono-phosphorylated activation loop domain into the *bis*-phosphorylated form at this concentration without preventing Y⁵⁷⁶-FAK phosphorylation.

Concurrently, there was a dose-dependant decrease in pY^{576}/pY^{577} -FAK with increasing AZD0530 concentrations with an IC_{50} of 0.14 μM (Fig. 2C). The initial description of AZD0530 inhibition of Src activity toward the poly(GluTyr) peptide by Hennequin *et al.* found an IC_{50} value of 0.0027 (\pm 0.0005) μM (Hennequin *et al.*, 2006). The discrepancy between IC_{50} values can be explained by differences between assay methodologies. Both Src concentrations and ATP concentrations could alter the amount of inhibitor required to inhibit Src activity, since AZD0530 will compete for binding at the ATP binding pocket of Src proteins. Assay conditions in the present study involved the use of 12 nM Src and 1 mM ATP, whereas studies by other investigators employed 2 nM Src and 0.04 mM ATP (Ple *et al.*, 2004). The differences in assay conditions reflect the goal of the respective experiments. Our studies will involve Src inhibition in cell culture systems; therefore, we chose to use 1 mM ATP as found in cell models (Beis and Newsholme, 1975). Conversely, the goal of previous studies was to develop small compound inhibitors of Src, which required the assay to be sensitive to even weakly inhibiting compounds (Hennequin *et al.*, 2006).

Two cell models were used in the present study; vector control MEFs that expressed only the endogenous Src-family kinases and oncogenic MEFs that expressed the $Y^{529}FSrc$ variant (Brabek *et al.*, 2004). The Y^{529} to F^{529} amino acid substitution in Src results in a constitutively active form due to the inability of the kinase to achieve its normal autoinhibited and inactive conformation. $Y^{529}FSrc$ extensively phosphorylates multiple substrate proteins (including FAK), resulting in cellular transformation (Cooper *et al.*, 1986; Okada and Nakagawa, 1989; Brabek *et al.*, 2004). Our results showed a distinct increase of pY^{576}/pY^{577} -FAK in the MEFs expressing $Y^{529}FSrc$ (0.193 pmol/pmol of total FAK), relative to that found previously in normal MEFs (0.059 pmol/pmol of total FAK) (Ciccimaro *et al.*, 2008). A large amount of unmodified-FAK

and pY⁵⁷⁶-FAK was present in the Y⁵²⁹FSrc-expressing MEFs, calculated at 0.381 pmol, and 0.287 pmol/pmol of total FAK respectively (Fig. 3). This indicates that a significant fraction of total FAK exists in a subcellular locale sequestered from the action of Src and/or that Y⁵⁷⁷ is de-phosphorylated by a phosphatase at a greater rate than Src phosphorylates it. To address these issues, the consequences of global tyrosine phosphatase inhibition using pervanadate were explored.

Pervanadate is known to broadly inhibit tyrosine phosphatases, thus dramatically increasing the extent of tyrosine phosphorylation on multiple proteins, and more relevantly, increasing the extent that FAK Y⁵⁷⁶ and Y⁵⁷⁷ are phosphorylated (Maa and Leu, 1998). Pervanadate treatment of either normal or Y⁵²⁹FSrc expressing MEFs led to the majority of total FAK in the cell existing as the pY⁵⁷⁶/pY⁵⁷⁷-FAK form, with approximately 0.4 - 0.5 pmol/pmol of total FAK, leaving only 0.1 - 0.2 pmol/pmol of total FAK in the unmodified form, regardless of which form of Src was present (Fig. 5). After the 10 min pervanadate treatment, almost 20 % of FAK remained in the unmodified form, indicating that a portion of the cellular protein was resistant to phosphorylation by Src; however, almost 60 % of FAK existed as the pY⁵⁷⁶/pY⁵⁷⁷-FAK form, suggesting that activation loop phosphorylation is potently regulated by a tyrosine phosphatase(s). We considered the possibility that FAK phosphorylation might be increased in pervanadate treated MEFs due to increased Src activity. This is unlikely because oncogenic Y⁵²⁹F Src lacks regulation by C-terminal tail phosphorylation. In addition, the observation that FAK activation loop phosphorylation were similar in both pervanadate treated normal MEFs and oncogenic Src expressing MEFs, confirmed that phosphatase regulation of the C-terminal tail of Src was not an important factor in these experiments. We cannot rule out the possibility that phosphatase inhibition caused a decrease in the de-phosphorylation of Y⁴¹⁶, which could then

have led to increased Src activity in both experimental cell models. However, it is clear that the methodology we have developed is able to accurately quantify changes in FAK activation loop phosphorylation.

The sum of activation loop forms following pervanadate treatment for normal and Y⁵²⁹FSrc expressing MEFs were 0.855 (\pm 0.106) and 0.744 (\pm 0.081) pmol/pmol of total FAK, respectively. This indicated that a small portion of this region was modified to a form(s) undetected in our assay. The most likely explanation for this observation is that the hydrogen peroxide exposure during the pervanadate treatment resulted in some endogenous methionine sulfoxide formation of M⁵⁷¹ within the activation loop segment tryptic peptide (YMEDSTYYK). Alternatively, it is possible that Y⁵⁷⁰ became partially phosphorylated during the pervanadate treatment. It is noteworthy that normal Src expressing MEFs were consistently found to have an almost three-fold increase in total FAK/plate compared with Y⁵²⁹FSrc expressing MEFs, while the total protein amounts per plate between the MEFs were equivalent.

To further investigate the relationship between Src activity and FAK activation loop phosphorylation, the small molecule AZD0530 was used to inhibit Src tyrosine kinases in cell culture. The recommended concentration of AZD0530 for inhibition of Src kinases in cell culture is 1 μ M. At this concentration, AZD0530 has been shown by Western blot to reduce the phosphorylation of Src substrates such paxillin (Hennequin et al., 2006; Jones et al., 2008).

AZD05030 is a potent inhibitor of FAK phosphorylation *in vitro*. However, it is a poor inhibitor of a number of other tyrosine- and serine/threonine protein kinases (Hannequin et al, 2006).

Furthermore, the specificity and potency of AZD0530 toward Src compares very favorably to other inhibitors such as the pyrazolo[3,4-*d*]pyrimidines PP1 and PP2, CGP-77675, Dasatinib, and Bosutinib (Hanke et al., 1996; Missbach et al., 1999; Serrels et al., 2006; Vultur, et al.,

2008). Although the phosphorylation of Src substrates is reduced by AZD0530 treatment, FAK autophosphorylation of Y³⁹⁷ is not altered, indicating that FAK kinase activity is unaffected by treatment with AZD0530. Our results showed that 1 μM AZD0530 reduced the amount of pY⁵⁷⁶/pY⁵⁷⁷-FAK to undetectable levels when normal fibroblasts were incubated in the presence of the drug for 3 h prior to pervanadate treatment. Surprisingly, the amount of pY⁵⁷⁶pY⁵⁷⁷-FAK in Src-transformed fibroblasts expressing Y⁵²⁹FSrc was not significantly affected by 1 μM AZD0530 pre-treatment. Although the amount of pY⁵⁷⁶/pY⁵⁷⁷-FAK in Src-transformed fibroblasts was unchanged when the pervanadate treatment was compared with Src inhibition and pervanadate treatment (Figs. 5 and 6), the amounts of unmodified and pY⁵⁷⁶-FAK forms were slightly increased. However, the sum of FAK activation loop forms for Src inhibited and pervanadate treated transformed fibroblasts was in agreement with calculated amounts of the control peptide. This suggests that oncogenic Y⁵²⁹FSrc expression can cause further post translational modifications to FAK's activation loop domain during pervanadate treatment and that these additional modifications are inhibited by 1 μM AZD0530 pre-treatment.

Src inhibitors will be targeted to cancers in which elevated Src activity is driving disease progression; therefore, the Src-transformed MEF model was chosen to conduct dose-response experiments. More importantly, pY⁵⁷⁶/pY⁵⁷⁷-FAK was found to contribute only 6.0 % to the total FAK in MEFs expressing normal Src, compared with 23.0 % in oncogenic Src expressing MEFS. This limited the possible observable response to Src inhibition in the normal MEFs. A dose-response curve to the Src inhibitor AZD0530 conducted in cell culture showed a similar trend (Fig. 7C) to that observed *in vitro* (Fig. 2C), although the dose response was shifted to the right by more than an order of magnitude. In comparison to *in vitro* Src inhibition, which showed a decrease in the amount of pY⁵⁷⁶/pY⁵⁷⁷-FAK with a drug concentration as low as 0.1

μM , a decrease in $\text{pY}^{576}/\text{pY}^{577}$ -FAK was not observed in Src-transformed fibroblasts until the cells had been treated with $1 \mu\text{M}$ of the drug (Figure 7C). A reduction of 50 % in *bis*-phosphorylation of $\text{Y}^{576}/\text{Y}^{577}$ -FAK was calculated to occur at $2.12 \mu\text{M}$ AZD0530 (IC_{50}).

Inhibition of paxillin (a substrate for Src phosphorylation at focal adhesions) phosphorylation by 70 % was observed with $1 \mu\text{M}$ AZD0530 when studies were conducted in the human lung tumor cell line (A549 NSCLC) using Western blot analysis (Hennequin et al., 2006). Therefore, these previous experiments showed a similar trend to those we have obtained using *bis*-phosphorylation of FAK as a biomarker of cellular Src activity.

In summary, our new stable isotope dilution LC-MRM/MS assay showed that the Src inhibitor AZD0530 caused inhibition of $\text{pY}^{576}/\text{pY}^{577}$ -FAK formation in a dose-dependent manner (Figs. 2B, 7B). From these data it was possible to determine AZD0530 IC_{50} values for Src inhibition both *in vitro* (Fig. 2C) and in cell culture (Fig. 7C). There was a substantial difference in the ability of AZD0530 to inhibit Src that was constitutively activated in a cellular context ($\text{IC}_{50} = 2.12 \mu\text{M}$) when compared with the isolated enzyme ($\text{IC}_{50} = 0.14 \mu\text{M}$). This suggests that higher concentrations of Src inhibitors such as AZD0530 might be required for *in vivo* efficacy than has been considered previously (Jones et al., 2008). Results from the present study can be considered as the proof-of-concept for using FAK phosphorylation as a biomarker to determine the efficacy of Src inhibition *in vivo*. The increased accuracy and specificity that can be obtained by quantitative stable isotope dilution LC-MRM/MS methodology to quantify FAK phosphorylation offers significant advantages over current immunochemical approaches. Furthermore, elaboration of this methodology to other kinase targets will make it possible to rigorously assess the efficacy and specificity of novel targeted kinase inhibitors that are developed in the future.

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FOOTNOTE

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FIGURE LEGENDS

Fig. 1. FAK domain structure. Shown is a schematic representation of FAK domains (see abbreviations section) indicating important sites of tyrosine phosphorylation (Y^{397} , Y^{407} , Y^{576} , Y^{577} , Y^{861} , and Y^{925}).

Fig. 2. Dose-response of Src inhibition on FAK activation loop Y^{576} and Y^{577} modification by Src *in vitro*. Following incubation with varying concentrations of AZD0530 (0.01, 0.1, 1 and 10 μ M), recombinant Src was incubated with recombinant FAK for 15 min at 37 °C. LC-MRM/MS was then used to absolutely quantify the amount of FAK activation loop modification. (A). The amount of modified Y^{576}/Y^{577} FAK activation loop (\diamond). (B) The amount of FAK activation loop in the mono-phosphorylated pY^{576} (\square) and *bis*-phosphorylated pY^{576}/pY^{577} (\triangle) forms. (C) An IC_{50} value of Src inhibition calculated from the amount of pY^{576}/pY^{577} (\triangle) determined at each dose. Data points represent the means of three separate experiments, where error bars are \pm S.E.M.

Fig. 3. Quantification of FAK activation loop Y^{576} and Y^{577} in MEFs expressing oncogenic Y^{529} FSrc. Shown are amounts of unmodified Y^{576}/Y^{577} (\square), mono- pY^{576} (\square), and *bis*-phosphorylated pY^{576}/pY^{577} (\blacksquare) activation loop peptides, and the sum amount of activation loop peptides (\blacksquare) from a FAK trypsin digest following immunoprecipitation from confluent monolayers of MEFs expressing oncogenic Y^{529} FSrc. The amounts of each peptide are shown after correction for FAK immunoprecipitation recoveries and normalization to the amount total FAK. Data points represent the means of three separate experiments, where error bars are \pm SEM.

Fig. 4. LC-MRM/MS analysis of activation loop and control peptides from digested endogenous and [$^{13}\text{C}^{15}\text{N}$]-FAK immunoprecipitated from pervanadate treated MEFs expressing normal Src, and their corresponding heavy isotope-labeled internal standards.

Shown are ion chromatograms for peptides resulting from the trypsin hydrolysis of immunoprecipitated endogenous (~) and [$^{13}\text{C}^{15}\text{N}$]-FAK (**), spiked with [$^{13}\text{C}^{15}\text{N}$]-labeled peptide standards (*). MRM transitions monitored the unmodified activation loop peptide (YMEDSTY 576 Y 577 K, t_{R} = 16.49 min) in the unlabeled (m/z 600.25 \rightarrow 905.40), [$^{13}\text{C}_{33}^{15}\text{N}_2$]-labeled (m/z 617.25 \rightarrow 930.40), and [$^{13}\text{C}_6^{15}\text{N}_2$]-labeled (m/z 604.25 \rightarrow 913.40) species. Mono-phosphorylated (pY 576) peptides (t_{R} = 15.58 min) were monitored by transitions for the unlabeled (m/z 640.25 \rightarrow 985.35 and 967.35), [$^{13}\text{C}_{33}^{15}\text{N}_2$]-labeled (m/z 657.25 \rightarrow 1010.36 and 992.36), and [$^{13}\text{C}_6^{15}\text{N}_2$]-labeled (m/z 644.25 \rightarrow 993.36 and 975.36) species. Bis-phosphorylated (pY 576 /pY 577) peptides (t_{R} = 14.11) were monitored by transitions for the unlabeled (m/z 680.25 \rightarrow 1047.31), [$^{13}\text{C}_{33}^{15}\text{N}_2$]-labeled (m/z 697.25 \rightarrow 1072.32), and [$^{13}\text{C}_6^{15}\text{N}_2$]-labeled (m/z 684.25 \rightarrow 1055.32) species. Lastly, the control segment peptides (E 956 VGLALR 962 , t_{R} = 18.62) were monitored by transitions for the unlabeled (m/z 379.23 \rightarrow 359.24), [$^{13}\text{C}_{18}^{15}\text{N}_6$]-labeled (m/z 382.23 \rightarrow 359.24), and [$^{13}\text{C}_5^{15}\text{N}$]-labeled (m/z 391.23 \rightarrow 376.24) species.

Fig. 5. Quantification of FAK activation loop Y 576 and Y 577 in MEFs expressing normal Src or oncogenic Y 529 FSrc after pervanadate treatment. Legends and experimental conditions are as for Fig. 3 except that both normal and Y 529 FSrc-expressing MEFs were used.

Fig. 6. Quantification of FAK activation loop Y⁵⁷⁶ and Y⁵⁷⁷ phosphorylation in MEFs expressing normal Src or oncogenic Y⁵²⁹FSrc after treatment with 1 μM Src inhibitor.

Legends and experimental conditions are as for Fig. 3 except that both normal and Y⁵²⁹FSrc-expressing MEFs were used.

Fig. 7. Dose-response of Src inhibitor AZD0530 on FAK activation loop Y⁵⁷⁶ and Y⁵⁷⁷

isolated from oncogenic Src expressing MEFs. Oncogenic Y⁵²⁹FSrc expressing MEFs were cultured in the presence of varying concentrations of AZD0530 (0.01, 0.1, 1 and 10, and 50 μM) for 12 h. FAK was isolated from cellular lysate by immunoprecipitation. LC-MRM/MS was then used to absolutely quantify the amount of FAK activation loop modification. **(A)** The amount of modified Y⁵⁷⁶/Y⁵⁷⁷ FAK activation loop (◊). **(B)** The amount of FAK activation loop in the mono-phosphorylated pY⁵⁷⁶ (◻) and bis-phosphorylated pY⁵⁷⁶/pY⁵⁷⁷ (◄) forms. **(C)** An IC₅₀ value of Src inhibition was calculated using the amount of pY⁵⁷⁶/pY⁵⁷⁷ (◄) determined at each dose. Data points represent the means of duplicate experiments, where error bars are ± standard deviation.

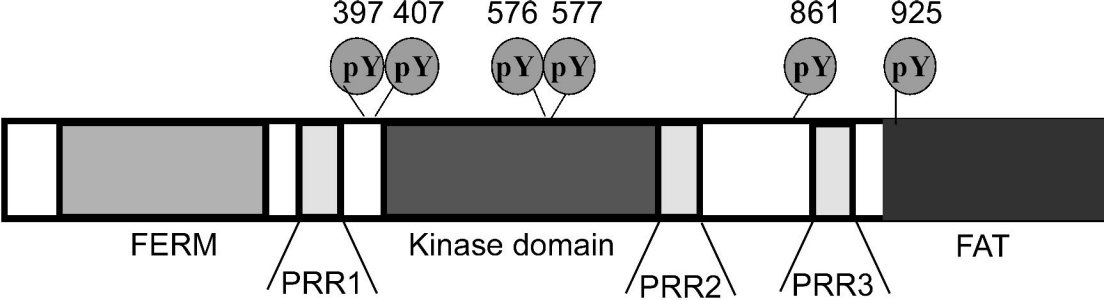


Figure 1

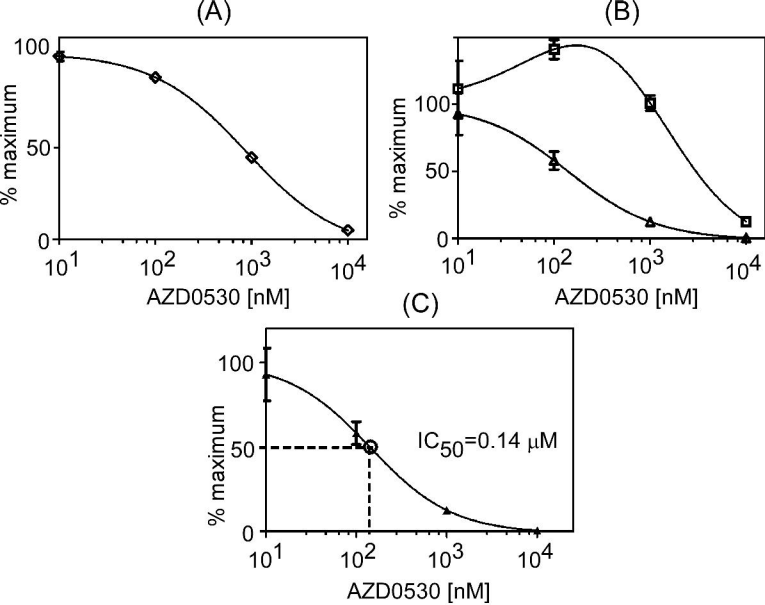


Figure 2

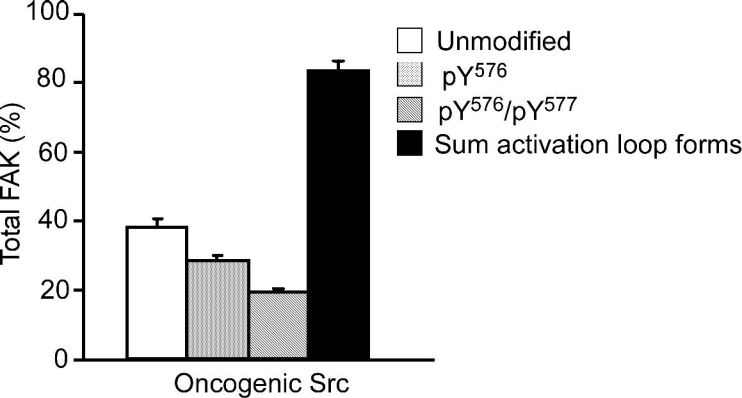


Figure 3

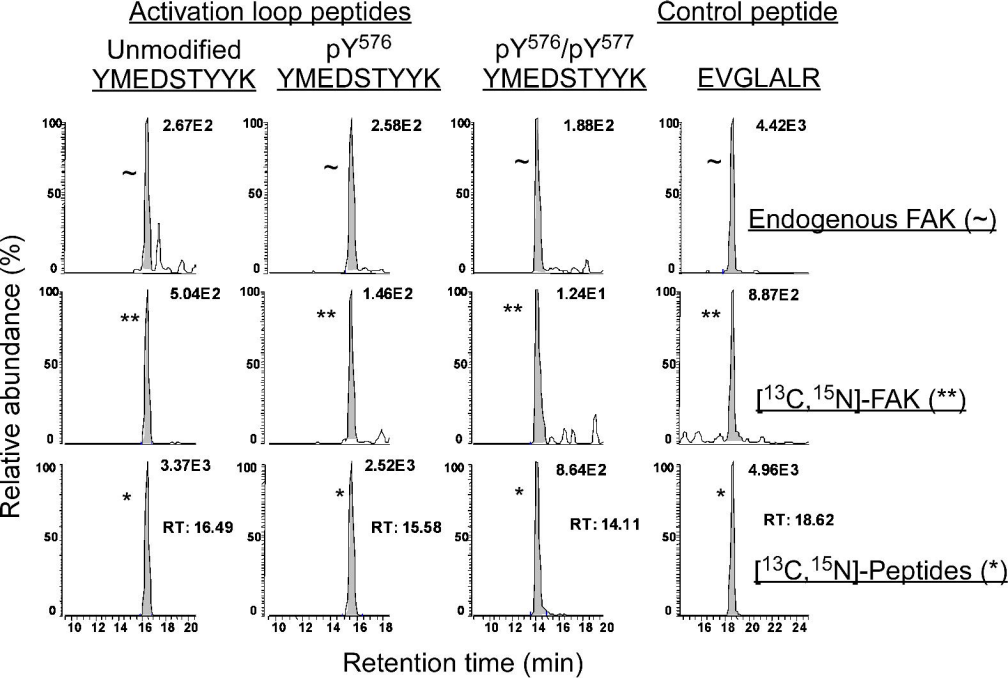


Figure 4

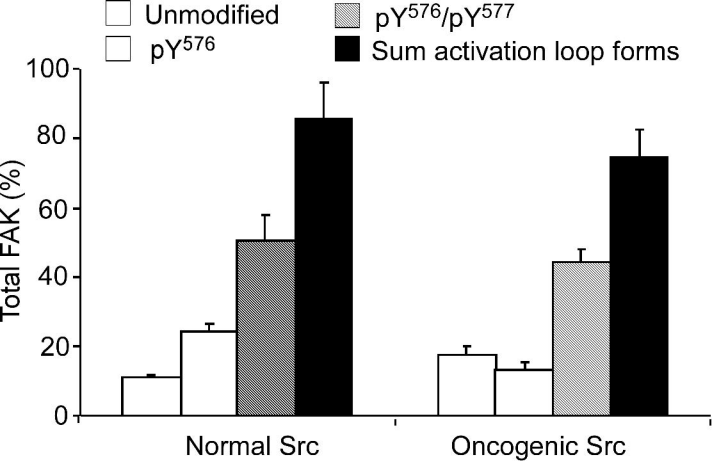


Figure 5

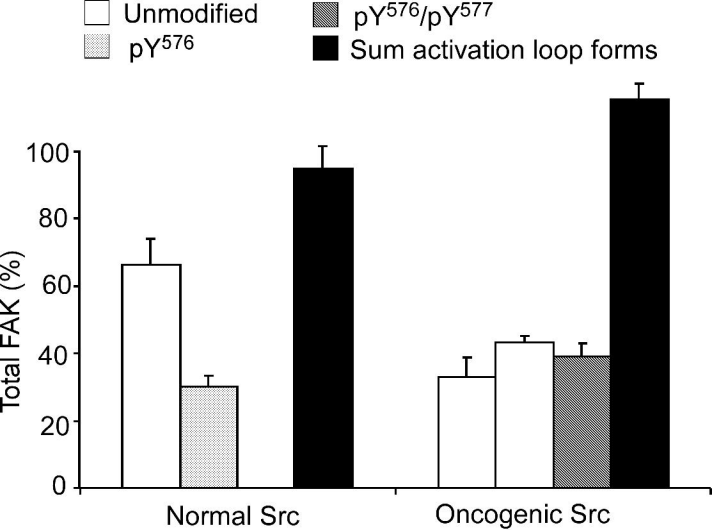


Figure 6

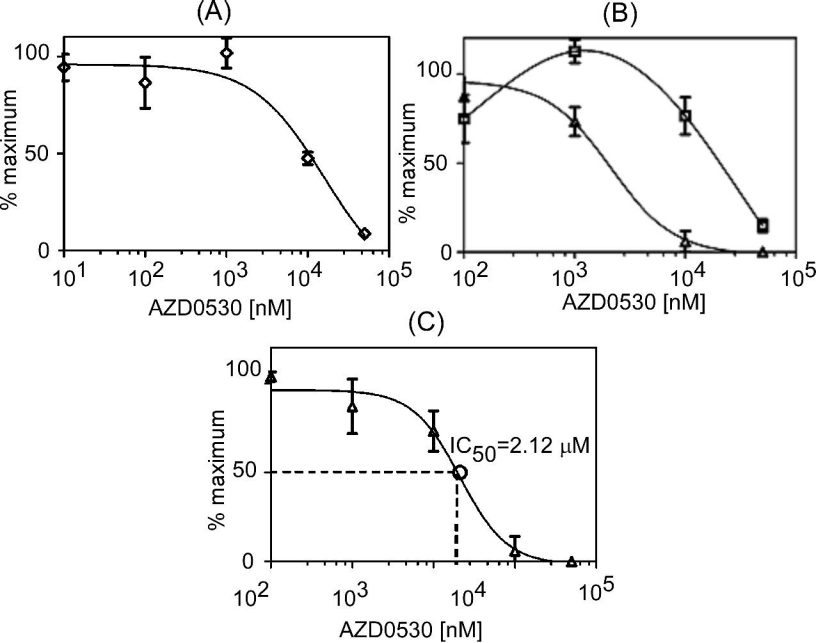


Figure 7