Supplemental Materials

Oral delivery of PND-1186 FAK inhibitor decreases spontaneous breast to lung metastasis in pre-clinical tumor models

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Supplemental Materials and Methods

High-Performance Liquid Chromatography assay for measuring PND-1186 in mouse plasma. Fifty microliters of the mouse plasma samples were extracted using Methyl Tertiary Butyl Ether (MTBE) on Supported Liquid Extraction (SLE)+plates (Biotage, Charlottesville, VA). A known amount of a proprietary standard was added prior to extraction and served as an internal standard. The solvent was evaporated to dryness under nitrogen and reconstituted for analysis. The samples were analyzed by high-performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS) analyses. The HPLC mobile phase consisted of Solvent A (20 mM ammonium formate, 0.2% formic acid in water) and Solvent B (0.2% formic acid in acetonitrile) in a 6-minute gradient. The column was an XBridge Phenyl, 100 x 2.1 mm, 5 µm, (Waters, Milford, MA) with a flow rate of 0.300 mL/min maintained at 40°C. Retention time for PND-1186 was 3.00 ± 0.3 min. The mobile phase was nebulized using heated nitrogen in a Z-spray source/interface and the ionized compounds were detected using MS/MS. Mass spectrometer conditions were as follows: source temperature: 130°C; desolvation temperature: 350°C. The scan settings for PND-1186 included a cone energy of 30 V, a collision energy of 35 eV, and a mass transition of 501.7 > 193.04. This method can measure concentrations ranging from 10.0 to 4,000 ng/mL using 50.0 µL of plasma.

Peak heights of PND-1186 were acquired using MassLynx (Waters, Milford, MA). Calibration curves were obtained by fitting the peak height ratios of PND-1186/(internal standard) and the standard concentrations in mouse plasma to a power equation in MassLynx. The equations of the calibration curves were then used to interpolate the PND-1186 concentrations in plasma samples using peak height ratios.
**Supplemental Figure 1.** Inhibition of tumor-associated FAK and p130Cas tyrosine phosphorylation at 6h post PND-1186 administration. Subcutaneously grown 4T1 tumors from mice treated with a single 100 mg/kg PND-1186 i.p. dose. Tumors were collected at 6h (n=5 tumors per time point) and immunblots performed. (A) Phosphorylated FAK Tyr-397 (pY397 FAK) and total FAK in vehicle and PND-1186-treated tumors. Lane 1 (POS) is a positive control of 4T1 cell lysate. (B) Phosphorylated p130Cas Tyr-410 (pY410 Cas) and total p130Cas in vehicle and PND-1186-treated tumors. Lane 1 (POS) is a positive control of 4T1 cell lysate.

**Supplemental Figure 2.** Time course of 30 mg/kg PND-1186 inhibition of FAK pY397 phosphorylation in tumors. Subcutaneously grown 4T1 tumors from mice treated with a single 30 mg/kg PND-1186 i.p. dose were collected and lysed at the times indicated (n=5 tumors per time point). (A) Representative immunoblots for phosphorylated FAK Tyr-397 (pY397 FAK) and total FAK in vehicle and PND-1186-treated tumors. Lane 1 (POS) is a positive control of 4T1 cell lysate. (B) FAK pY397 to total FAK ratio in PND-1186 treated tumors over time. Box-and-whisker diagrams show the distribution of the data: square, mean; bottom line, 25th percentile; middle line, median; top line, 75th percentile; and whiskers, 5th or 95th percentiles. Significant differences between groups was ascertained using ANOVA followed by the Tukey post hoc test, p-values of < 0.05 were considered significant (* p = 0.018, ** p = 0.0301).
Supplemental Figure 3. Oral PND-1186 administration is not toxic. 4T1 tumor cells were implanted in the fat pad of BALB/c mice. Mice were treated with vehicle (water) or 150 mg/kg PND-1186 orally (p.o.) twice-daily (b.i.d.) for 15 days (n=12 per group). Treatment began 24 h after cell implantation. Body weights were recorded prior to the start of treatment (Day 0) and again on days 6, 8, 10, 12 and 16. Error bars represent standard deviation. Significant differences were ascertained using an unpaired two-tailed student's t-test, p-values of <0.05 were considered significant (*p < 0.05).

4T1 Tumor CD45 Staining

Supplemental Figure 4. PND-1186 reduces inflammatory cell infiltration in primary 4T1 tumors. Orthotopic mCherry-4T1 tumors in Balb/c mice were treated with vehicle (water) or 150 mg/kg PND-1186 as described in Figure 2. Primary tumors were sectioned and stained for anti-CD45 macrophage-associated marker (FITC, green) and with Hoechst 33342 (blue). Representative merged images are shown. Scale bar is 0.5 mm.
Supplemental Figure 5. Low-level *ad libitum* PND-1186 administration inhibits paxillin but not Src, Pyk2, or Akt phosphorylation in tumors. mCherry-4T1 tumor cells were implanted in the fat pad of BALB/c mice. After 48 h, mice were provided 5% sucrose (control) or 0.5 mg/kg PND-1186 in 5% sucrose as drinking water. Administration was *ad libitum*. (A) Representative immunoblots for phosphorylated paxillin Tyr-118 (pY118 Paxillin) and total Paxillin in vehicle and PND-1186-treated tumors. (B) Representative immunoblots for phosphorylated Src Tyr-416 (pY416 Src) and total c-Src in vehicle and PND-1186-treated tumors showed no detectable inhibition of Src pY416 phosphorylation after PND-1186 treatment. (C) Representative immunoblots for phosphorylated Pyk2 Tyr-402 (pY402 Pyk2) and total Pyk2 in vehicle and PND-1186-treated tumors showed no detectable inhibition of Pyk2. (D) Representative immunoblots for phosphorylated Akt Ser-473 (pS473 Akt) and total Akt in vehicle and PND-1186-treated tumors showed no detectable inhibition of Akt. (E) Representative immunoblots for phosphorylated Akt Thr-308 (pT308 Akt) and total Akt in vehicle and PND-1186-treated tumors showed no detectable inhibition of Akt. In all samples, Lane 1 (POS) is a positive control of 4T1 cell lysate.