Supporting Information

PDK1 Scintillation Proximity (LEADseeker) Assays using AKT3 or PDKtide as the phosphoacceptor

Truncated-PDK1 – AKT3 assay (Fragment Screen – Table 1 Data)

The assay was performed using His-tagged-truncated hPDK1(52-556) enzyme and biotinylated-AKT3 (135-479) as the substrate in 384-well microtiter plates. The reaction volume (10 µl) contained, in final concentrations, 25mM MOPS (pH 7.5), 10mM MgCl2, 1µM ATP, 0.03 uCi/well ^{33}PγATP, 1mM DTT, 0.05% Tween20, 0.5µM AKT1 biotinylated protein, and 30 nM hPDK1. Compounds, titrated in DMSO, were evaluated at eleven concentrations ranging from 400 µM to 6.77 nM. Final assay concentrations of DMSO did not exceed 1%. hPDK1 enzyme was pre-incubated, 30 minutes at room temperature, with compounds before initiating phosphorylation of AKT3 by addition of substrate to the PDK1/compound mixture. Reactions were stopped after 3 hours at room temperature by the addition of 0.05 mg/well of LEADseeker beads (GE Healthcare) by a Titertek Multidrop. Beads were allowed to settle overnight, and samples were then imaged on a Viewlux (PerkinElmer). Dose response curves were generated and IC50s calculated using Activity Base/XC50 module.

fl-PDK1 – PDKtide assay (Table 2 Data)

Assays were performed in 384-well microtiter plates. The reaction volume (10 µl) contained, in final concentrations, 25mM MOPS (pH 7.5), 10mM MgCl2, 50mM KCl, 0.1mg/ml BSA, 1µM ATP, 3uCi/ml ^{33}PγATP, 5mM DTT, 0.5mM CHAPS, 1µM biotinylated-PDKtide peptide (Biotin-Ahx-KTFCGTPEYLAPEVRREPRILSEEEQEMFRDFDYIADWC) , and 0.5 nM fl-hPDK1. Compounds, titrated in DMSO, were evaluated at eleven concentrations ranging from 25 µM to 0.4 pM. Final assay concentrations of DMSO do not exceed 1%. fl-PDK1 was pre-incubated for 30 minutes at room temperature with compounds before initiating phosphorylation of biotinylated-PDKtide peptide by addition of substrate to the fl-PDK1/compound mixture. Reactions were stopped after 4 hours at room temperature by the addition of 50 mM EDTA, 5 mg/ml LEADseeker beads (GE Healthcare) in PBS (10 ul). Beads were allowed to settle overnight, and samples were imaged on a Viewlux (PerkinElmer). Dose response curves were generated and IC_{50}s calculated using Activity Base/XC50 module.

PDK1 Crystallography

hPDK1 catalytic domain was cloned, expressed, purified and crystallized in a manner similar to that previously described for PDK1(51-559).[1] Human PDK1 coding sequence (48-359) was amplified with KOD HiFi Hot start polymerase (Merck Biosciences Ltd.) from pFBHTB PDK1delta1-50 (obtained from the University of Dundee), incorporating a

tev protease cleavage site preceding G48 shown underlined in the forward primer sequence below. The forward primer was tailed with CACC for directional cloning into pENTR/D/Topo (Invitrogen). The truncated insert was subsequently transferred by *att* L x *att* R recombination into pDEST8His, a modification of pDEST8 (Invitrogen) encoding an N-terminal hexa histidine tag fused through the Gateway^{Tm} vector module. The resultant tag fusion encodes MGHHHHHHTSTSLYKKAGSAAAPFTENLYFQ/G with the tev protease cleavage site preceding the start of the PDK1 sequence (underline). The encoded PDK1 sequence following Tev cleavage is identical to the crystallography design described previously[1] except that alanine 50 is present.

Forward: 5'
…CACCGAGAACCTGTACTTCCAGGGCCCCGCCATGGACGGCACTGCAGCCGAGC
CTCGG…3'

Reverse: 5'…GGATCCTCAGGTGAGCTTCGGAGGCGTCTGCTGGTG…3'

Recombinant protein was generated using the bac-to-bac system (Invitrogen). Briefly, the *Spodoptera frugiperda* cell line, sf9 cells were routinely cultured in Ex420 media (SAFC Inc.) at 27°C using a WAVE Bioreactor (GE Healthcare Biosciences) at 10L scale at 24.5 rocks per minute. Cells were seeded into 25L WAVE one day prior to infection at 0.7 x 10^6 cells/ml. Recombinant HistevPDK1 (48-359) was expressed using exponentially growing sf9 cells at the density of 3.8e6 cells/mL (viable cell count) infected with baculovirus stocks at an MOI of 3. The infection was allowed to proceed at 27°C for 68hrs at 26 rocks per minute and an airflow rate of 0.25L/min. At the time of harvest, the average cell viability was approximately 80%. Cell paste was frozen and stored at -80°C until protein purification. Masses observed by LC/MS indicate loss of methionine (-131Da), phosphorylation (+80Da) and acetylation (+42Da).

The cells were re-suspended in 400 ml of lysis buffer A (25 mM Tris-HCl, pH 7.5, 0.5M NaCl) and broken by sonication. The whole cell lysate was centrifuged at 38,000 *g* for 1 h at 4°C and soluble cell extracts were mixed with 7 ml of NiNTA Superflow resin (Qiagen) for batch binding 2 hours. The Ni resin column was first washed with wash buffer B (25 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 30 mM imidazole) in order to remove any weakly bound protein, and HisPDK1 was eluted with 10 column volume gradient from wash buffer B to elution buffer C (25 mM Tris-HCl, pH 7.5, 0.5 M NaCl , 300 mM imidazole). The target protein was further purified by 5 ml HiTrap Q HP and 120 ml Superdex 200 columns (both from GE healthcare). All the PDK1 protein stayed in the flow through of the Q column under 25 mM Tris-HCl, pH 7.5, 200 mM NaCl buffer condition, and they were off the sizing column as single monomeric peak in the final buffer 25 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 1mM DTT. N-term sequencing and LCMS analysis showed that protein self-cleavage happened after the protein was eluted from Ni resin. The final protein produced was PDK1 (67-359) with S241 phosphorylation (confirmed by phosphorylation mapping). The protein was concentrated to 8.7 mg/ml.

Crystallization conditions were conducted and obtained at room temperature in 24-well sitting drops. 8-16 mM ATP was added to protein sample that was previously

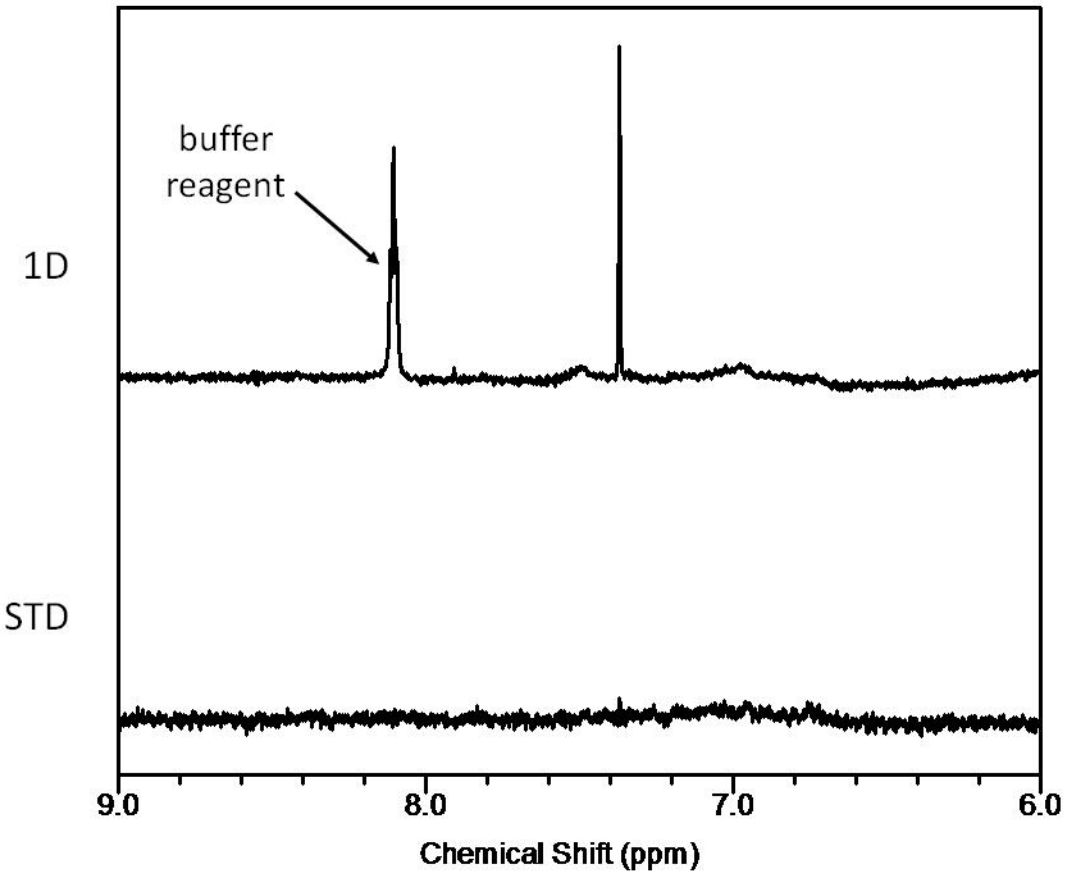
concentrated to 8.7 mg/ml then dispensed at a 1:1 ratio (2 ul protein + 2 ul well content). The well content varied from 1.9-2 M ammonium sulfate and 0.1 M tris pH 9. Crystals begin to grow within 24 hours with a thick, rod-shaped morphology and continued to slowly grow larger over a period of a week. After a 5-7 day incubation, several crystals are harvested and transferred into a drop containing mother liquor mixed with 0.0325 mM to 2 mM inhibitor for soaks. Depending on the inhibitor, some crystals can soak from 3 to 72 hours with minimal cracking. 15-30% glycerol plus mother liquor mixed with inhibitor served as the cryo-protectant before being frozen in liquid nitrogen. X-ray diffraction data were collected at the Advanced Photon Source, processed with HKL2000.[2] Model structures were refined with phenix.refine,[3] model building was done with COOT[4] and figures were generated with PYMOL.[5]

NMR Saturation Transfer Difference Experiments

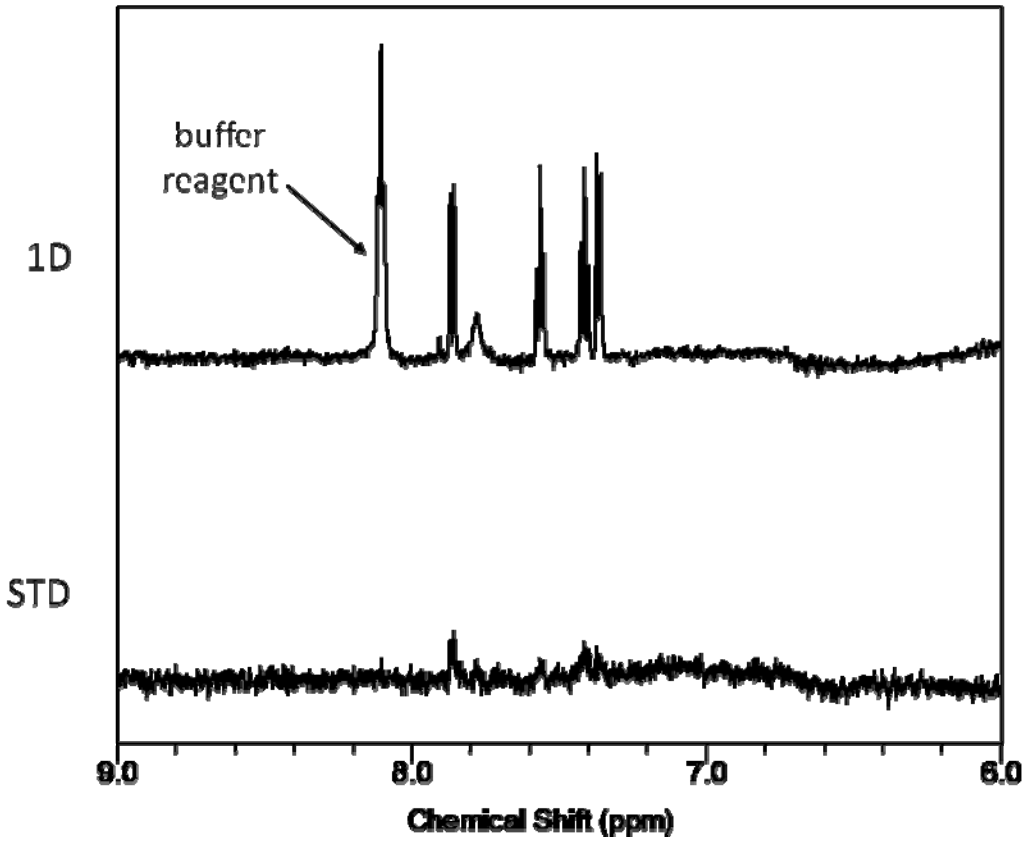
Saturation transfer difference (STD)[6] NMR experiments were carried out on a Varian Unity INOVA^{TM} operating at 600MHz. The NMR samples were prepared in a H_2O/D_2O (9:1) buffer solution consisting of 21.3 mM MOPS (pH 7.5), 8.5 mM $MgCl_2$, 42.6 mM KCl, 0.43 mM CHAPS, 1.0 mM DTT, 10% glycerol-d_5 and 1% DMSO-d_6. A ligand concentration of 200 uM with a protein concentration of 4 uM PDK1 (67-359) were used in a volume of 540 uL for all the experiments. The STD NMR spectra were acquired with a 5-mm inverse broadband probe regulated at 25° C, using 16K data points, a sweep width of 10,000 Hz, an acquisition time of 0.819 second, 1,280 scans, a protein pre-saturation time of 2 seconds and a short relaxation delay of 10 milliseconds (ms). Prior to Fourier transformation, the signal-to-noise ratio of the NMR signal was improved by using an exponential apodization with a line broadening of 2 Hz and resolution was enhanced by zero filling to 64K data points. A WATERGATE[7] technique with soft pulses and double gradients[8] was used to suppress the strong water signal in the pulse scheme[9] used for acquiring the STD NMR spectra. No $T_{1\rho}$ filter was employed and pre-saturation of the protein signal was achieved with a train of Gauss-shaped pulses of 25 ms each with an irradiation field strength of 50 Hz.
The detection strength of the STD signal was crudely divided into three categories by using the following signal-to-noise ratio (S/N) of the largest STD peak in the aromatic region of the NMR spectrum: S/N > 11:1 (+) STD observed; 4 < S/N < 11 (+/-) weak STD observed; S/N < 4 (-) no STD observed.

Representative NMR STD data for compounds **2**, **8**, and **11** are shown below.

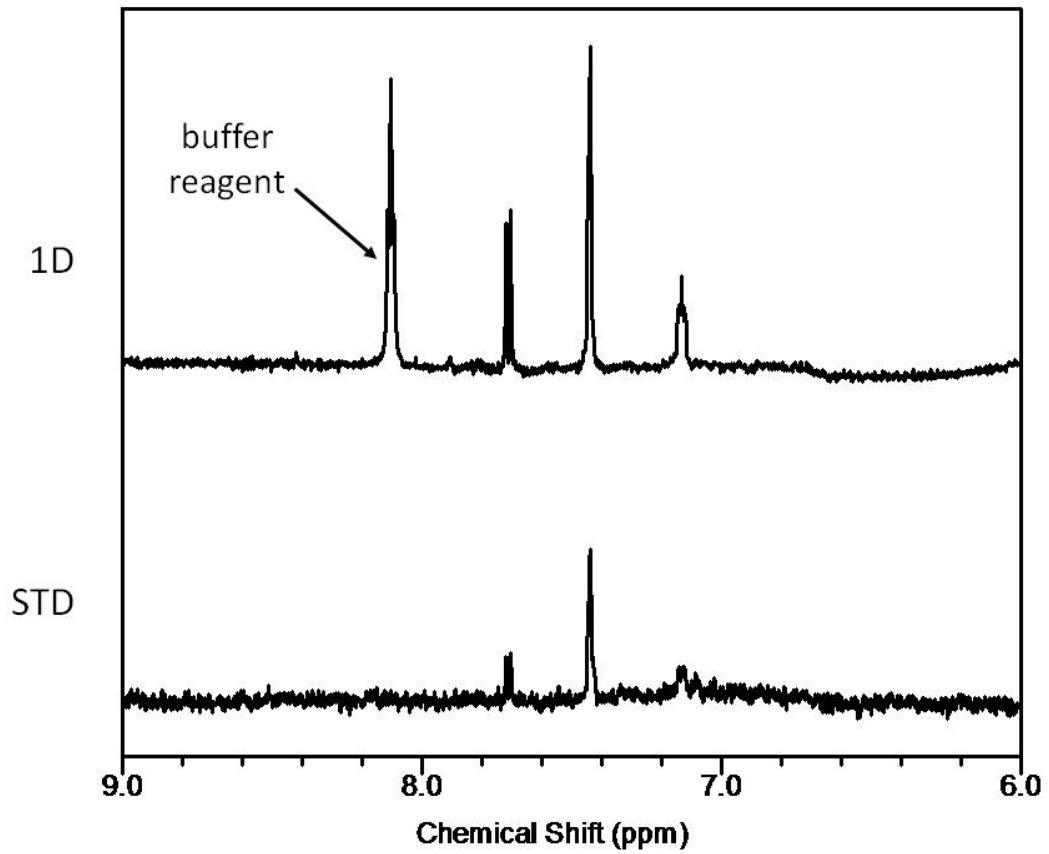
Compound **2** (STD = −) : S/N of aromatic peaks in STD spectrum < 4



Compound **11** (STD = +/−) : S/N of aromatic peaks in STD spectrum = 8.4



Compound **8** (STD = +) : S/N of aromatic peaks in STD spectrum = 27.5



Synthesis and Characterization of Compounds 19-25

Commercially available materials were used without further purification. All reactions were carried out under nitrogen atmosphere, with magnetic stirring unless noted otherwise. Analytical LCMS spectra were recorded on an automated Agilent 1100 instrument in Electrospray (ESI) mode fitted with a Zorbax 4.6 x 150 mm, 5u, SB-C18 analytical column using the following method: 15 minute run, 40˚C column temperature, 0-12.5 minute gradient of 5-100% acetonitrile-water containing 0.01% TFA, 12.5-15 minutes isocratic 100% acetonitrile containing 0.01% TFA. NMR spectra were recorded on a Bruker 400 MHz instrument using solvent or TMS as a reference. Chemical shifts (δ) are quoted in parts per million (ppm). Coupling constants (*J*) are recorded in Hertz. HRMS were acquired on a QTOF Premier (Waters) and the analysis was done via FIA using an Acquity Nano pump system (Waters).

6-(2-Amino-4-pyrimidinyl)-1H-indazol-3-amine (19)

4-Chloro-2-pyrimidinamine (100mg, 0.772 mmol) and (4-cyano-3-fluorophenyl)boronic acid (127 mg, 0.772 mmol) were combined in a 25mL sealed tube under argon with 1,4-dioxane (5 ml) and saturated sodium bicarbonate (1.25 ml). This mixture was degassed for 10 minutes with bubbling argon. Tetrakis(triphenylphosphine)palladium(0) (44.9 mg, 0.039 mmol) was then added. The reaction vessel was sealed and heated at 95ºC in an oil bath for 3 hours. After cooling, an aqueous workup using 50ml water and 50ml EtOAc was performed. The organics were dried with $MgSO_4$, filtered through a pad of Celite, and concentrated to give a light yellow solid. The crude material was dissolved in chloroform and purified on 34g of silica gel using a gradient of 0-100% (90/10/1 $CHCl_3$:MeOH:NH_4OH in $CHCl_3$). The product fractions were combined and concentrated to afford 4-(2-amino-4-pyrimidinyl)-2-fluorobenzonitrile as an off-white solid (138mg, 83%). LC/MS *m/z* 215.2 $(M+H)^+$. ^1H NMR (400 MHz, DMSO-d_6) δ ppm 6.88 (s, 2 H) 7.29 (d, *J*=5.05 Hz, 1 H) 8.05 - 8.14 (m, 2 H) 8.17 (dd, *J*=10.86, 1.26 Hz, 1 H) 8.43 (d, *J*=5.05 Hz, 1 H).

Hydrazine monohydrate (5.00 ml, 102 mmol) was added to a slurry of 4-(2-amino-4-pyrimidinyl)-2-fluorobenzonitrile (138mg, 0.644 mmol) in ethanol (10 mL) under argon. The mixture was stirred in a 70ºC oil bath for 3 hours. Cooling the reaction in an ice bath afforded a precipitate which was filtered, washed with 50/50 EtOH/hexanes followed by 100% hexanes to afford **19** as a yellow solid (81mg, 55%). Analytical LC/MS *m/z* 227.0 $(M+H)^+$, purity = 98.6% @ 254 nM. ^1H NMR (400 MHz, DMSO-d_6) δ ppm 5.43 (s, 2 H) 6.66 (s, 2 H) 7.16 (d, *J*=5.31 Hz, 1 H) 7.59 (dd, *J*=8.34, 1.26 Hz, 1 H) 7.76 (d, *J*=8.59 Hz, 1 H) 7.98 (s, 1 H) 8.30 (d, *J*=5.05 Hz, 1 H) 11.62 (s, 1 H). HRMS calculated for $C_{11}H_{11}N_6$ (M+H): 227.1045 Found: 227.1043.

6-(4-Pyridinyl)-1H-indazol-3-amine hydrochloride (25)

6-Bromo-1H-indazol-3-amine (2.00 g, 9.43 mmol) and DMAP (0.115 g, 0.943 mmol) were combined with acetic anhydride (8.90 mL, 94 mmol). This mixture was heated at 120ºC for 4 hours under nitrogen. Concentration afforded 3.19g (100%) of a light tan solid that was a mixture (~1.3:1 by NMR) of N-acetyl-N-(1-acetyl-6-bromo-1H-indazol-3-yl)acetamide and N-(1-acetyl-6-bromo-1H-indazol-3-yl)acetamide. LC/MS (ES) *m/z* 360.0 and 318.0 $(M+Na)^+$. This mixture was used without purification in the next step.

N-Acetyl-N-(1-acetyl-6-bromo-1H-indazol-3-yl)acetamide (0.264 g, 0.780 mmol), N-(1-acetyl-6-bromo-1H-indazol-3-yl)acetamide (0.347 g, 1.170 mmol), 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridine (0.400 g, 1.951 mmol), $PdCl_2$(dppf)-CH_2Cl_2 adduct (0.064 g, 0.078 mmol), and sodium bicarbonate (0.655 g, 7.80 mmol) were combined in a 25mL sealed tube. To this mixture was added 1,4-dioxane (9.75 mL) and water (3.25 mL). The vessel was sealed and heated in a 100ºC oil bath for 5 hours. After cooling to room temperature, the reaction was diluted with 50mL EtOAc and the aqueous layer was discarded. The organics were washed with 2x10mL water and dried with $MgSO_4$. After filtration through Celite, the organics were concentrated to dryness affording the mixture of products N-[1-acetyl-6-(4-pyridinyl)-1H-indazol-3-yl]acetamide and N-[6-(4-pyridinyl)-1H-indazol-3-yl]acetamide (330 mg, 34%) as a tan solid. LC/MS

(ES) *m/z* 295.2 and 253.2 $(M+H)^+$. This mixture was used without purification in the next step.

N-[1-Acetyl-6-(4-pyridinyl)-1H-indazol-3-yl]acetamide (.224 g, 0.761 mmol) and N-[6-(4-pyridinyl)-1H-indazol-3-yl]acetamide (.106 g, 0.420 mmol) were slurried in methanol (20 mL). 12M HCl (2.00 mL, 24.00 mmol) was added and the resulting mixture was heated in a 65ºC oil bath overnight. After cooling to room temperature, the reaction was diluted with 20mL Et_2O and filtered affording 6-(4-pyridinyl)-1H-indazol-3-amine hydrochloride **(25)** (90mg, 44%) as a tan solid after drying under high vacuum at 40ºC for 1 hour. Analytical LC/MS *m/z* 211.2 $(M+H)^+$, purity = 91.5% @ 254 nM. ^{1}H NMR (400 MHz, D_2O) δ ppm 7.54 (dd, *J*=8.59, 1.26 Hz, 1 H) 7.78 - 7.95 (m, 2 H) 8.23 (d, *J*=6.82 Hz, 2 H) 8.71 (d, *J*=6.82 Hz, 2 H). HRMS calculated for $C_{12}H_{11}N_4$ (M+H): 211.0984 Found: 211.0983.

6-(2-Amino-4-pyridinyl)-1H-indazol-3-amine hydrochloride (21) *(Representative 1-pot Procedure)*

A mixture of N-acetyl-N-(1-acetyl-6-bromo-1H-indazol-3-yl)acetamide (0.260 g, 0.768 mmol) and N-(1-acetyl-6-bromo-1H-indazol-3-yl)acetamide (0.344 g, 1.162 mmol), bis(pinacolato)diboron (0.500 g, 1.969 mmol), $PdCl_2$(dppf)-CH_2Cl_2 adduct (0.064 g, 0.079 mmol), and potassium acetate (0.386 g, 3.94 mmol) were combined with 1,4-dioxane (13mL) in a 25mL sealed tube under argon. The reaction was stirred in a 100ºC oil bath for 5 hours. The reaction was cooled to room temperature and unsealed. 1,1-Dimethylethyl (4-iodo-2-pyridinyl)carbamate (634 mg, 1.98 mmol), sodium bicarbonate (665 mg, 7.92 mmol), $PdCl_2$(dppf)-CH_2Cl_2 adduct (64.7 mg, 0.079 mmol), and water (4.38 mL) were added. The tube was flushed with argon and re-sealed. The reaction was stirred in a 100ºC oil bath for 5 hours and was then allowed to cooled to room temperature and stir overnight. After diluting the reaction with 50mL EtOAc, the resulting solids were filtered, washed with EtOAc and discarded. The filtrate was concentrated to dryness. The resulting solids were sonicated in 30mL of water and 1mL of TFA for 10 minutes. The solids were once again filtered, washed with water, and discarded. The filtrate was concentrated to an oil that was purified by automated reversed-phase HPLC (acetonitrile/water gradient containing 0.1% TFA). The product fractions containing both N-[1-acetyl-6-(2-amino-4-pyridinyl)-1H-indazol-3-yl]acetamide and N-[6-(2-amino-4-pyridinyl)-1H-indazol-3-yl]acetamide were combined and concentrated to dryness to afford 250mg of material which was dissolved in 10mL methanol under argon. 12M HCl (1.023 mL, 12.28 mmol) was added to the resulting suspension and the mixture was heated in a 60ºC oil bath overnight. After cooling to room temperature, the reaction was diluted with 30mL of Et_2O. The resulting solids were filtered and washed with Et_2O to afford 6-(2-amino-4-pyridinyl)-1H-indazol-3-amine hydrochloride **(21)** (115mg, 22%) as an off-white solid. Analytical LC/MS *m/z* 226.2 $(M+H)^+$, purity = 99.2% @ 254 nM. ^{1}H NMR (400 MHz, D_2O) δ ppm 6.99 (s, 1 H) 7.01 (dd, *J*=6.82, 1.77 Hz, 1 H) 7.33 (dd, *J*=8.72, 1.39 Hz, 1 H) 7.57 (s, 1 H) 7.70 (d, *J*=6.82 Hz, 1 H) 7.76 (d, *J*=8.59 Hz, 1 H). HRMS calculated for $C_{12}H_{12}N_5$ (M+H): 226.1093 Found: 226.1093.

The following compounds were prepared according to the above 1-pot procedure. Specific modifications are included.

6-(4-Pyrimidinyl)-1H-indazol-3-amine trifluoroacetate (20) (78mg, 14%) The crude HCl salt was dissolved in a minimal volume of water and treated with saturated sodium bicarbonate solution. The solution was extracted with EtOAc. The organics were dried with $MgSO_4$ and concentrated to an oil which was purified by automated reversed-phase HPLC (acetonitrile/water gradient containing 0.1% TFA). The product fractions were combined and concentrated to afford 6-(4-pyrimidinyl)-1H-indazol-3-amine **(20)** trifluoroacetate as a yellow solid. Analytical LC/MS *m/z* 212.2 $(M+H)^+$, purity = 94.3% @ 254 nM. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 7.83 - 7.87 (m, 1 H) 7.91 - 7.95 (m, 1 H) 8.17 (dd, *J*=5.56, 1.26 Hz, 1 H) 8.20 (s, 1 H) 8.89 (d, *J*=5.56 Hz, 1 H) 9.27 (s, 1 H). HRMS calculated for $C_{11}H_{10}N_5$ (M+H): 212.0936 Found: 212.0937.

6-(6-Amino-2-pyridinyl)-1H-indazol-3-amine (22) (51mg, 11%) The crude HCl salt was purified by automated reversed-phase HPLC (acetonitrile/water gradient containing 0.1% TFA). The product fractions were combined and concentrated to a white solid which was dissolved in a minimal volume of water and treated with saturated sodium bicarbonate solution. The resulting precipitate was filtered, washed with water and dried to afford 6-(6-amino-2-pyridinyl)-1H-indazol-3-amine **(22)** as a white solid. Analytical LC/MS *m/z* 226.2 $(M+H)^+$, purity = 99.7% @ 254 nM. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 5.34 (s, 2 H) 5.98 (s, 2 H) 6.41 (d, *J*=8.08 Hz, 1 H) 7.08 (d, *J*=7.58 Hz, 1 H) 7.45 (t, *J*=7.71 Hz, 1 H) 7.51 (dd, *J*=8.59, 1.26 Hz, 1 H) 7.69 (d, *J*=8.34 Hz, 1 H) 7.86 (s, 1 H) 11.45 (s, 1 H). HRMS calculated for $C_{12}H_{12}N_5$ (M+H): 226.1093 Found: 226.1094.

6-(3-Aminophenyl)-1H-indazol-3-amine (23) (97mg, 35%) The crude HCl salt was dissolved in a minimal volume of water and treated with saturated sodium bicarbonate solution. The resulting precipitate was filtered, washed with water and dried to afford 6-(3-aminophenyl)-1H-indazol-3-amine **(23)** as a white solid. Analytical LC/MS *m/z* 225.2 $(M+H)^+$, purity = 96.1% @ 254 nM. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 5.15 (s, 2 H) 5.34 (s, 2 H) 6.56 (dd, *J*=7.96, 1.39 Hz, 1 H) 6.80 (d, *J*=7.83 Hz, 1 H) 6.84 - 6.93 (m, 1 H) 7.04 - 7.16 (m, 2 H) 7.31 (s, 1 H) 7.70 (d, *J*=8.34 Hz, 1 H) 11.39 (s, 1 H). HRMS calculated for $C_{13}H_{13}N_4$ (M+H): 225.1140 Found: 225.1141.

6-(2-Pyridinyl)-1H-indazol-3-amine hydrochloride (24) (36mg, 7%) Analytical LC/MS *m/z* 211.2 $(M+H)^+$, purity = 95.9% @ 254 nM. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 7.52 - 7.64 (m, 1 H) 7.84 - 7.94 (m, 1 H) 8.05 - 8.15 (m, 3 H) 8.17 - 8.23 (m, 1 H) 8.78 (d, *J*=4.29 Hz, 1 H) 8.81 - 9.65 (br. s, 2 H) 13.12 (br. s., 1 H). HRMS calculated for $C_{12}H_{11}N_4$ (M+H): 211.0984 Found: 211.0984.

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