

## Supporting Information

# Discovery of Novel Dual Mechanism of Action Src Signaling and Tubulin Polymerization Inhibitors (KX2-391 and KX2-361)

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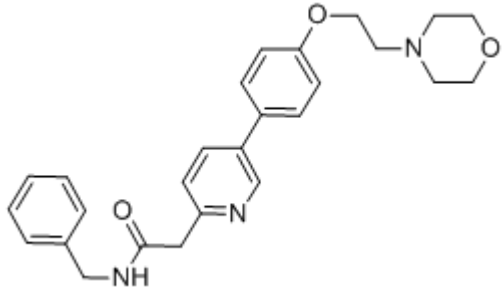
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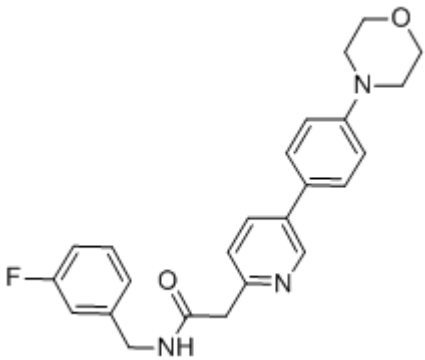
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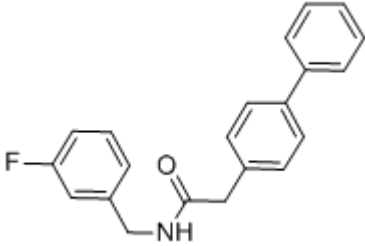
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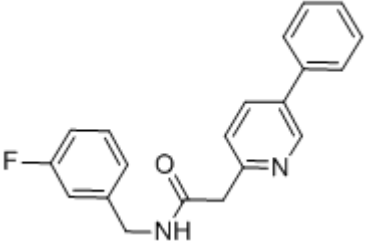
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# 1 CHEMICAL AND STRUCTURAL FORMULAS

<b>Compound 1</b>	
Chemical Structure	
Laboratory code	KX01; KX-01; KX2-391
CAS	897016-82-9 (free base)
CA Index name	2-Pyridineacetamide, 5-[4-[2-(4-morpholinyl)ethoxy]phenyl]-N-(phenylmethyl)-
Molecular weight	431.53
SMILES	<chem>O=C(CC1=NC=C(C2=CC=C(OCCN3CCOCC3)C=C2)C=C1)NCC4=CC=CC=C4</chem>

<b>Compound 2</b>	
Chemical Structure	
Laboratory code	KX02; KX-02; KX2-361
CAS	1571072-87-1 (besylate salt) 897016-26-1 (free base)
IUPAC name	2-Pyridineacetamide, N-[(3-fluorophenyl)methyl]-5-[4-(4-morpholinyl)phenyl]-, benzenesulfonate (1:1) 2-Pyridineacetamide, N-[(3-fluorophenyl)methyl]-5-[4-(4-morpholinyl)phenyl]-
Molecular weight	563.64 (KX2-361 besylate salt) 405.46 (KX2-361 free base)
SMILES	<chem>O=C(CC1=NC=C(C2=CC=C(N3CCOCC3)C=C2)C=C1)NC4=CC(F)=CC=C4</chem>

<b>Compound 7</b>	
Chemical Structure	
Laboratory code	-none-
CAS	897015-54-2
IUPAC name	2-([1,1'-biphenyl]-4-yl)-N-(3-fluorobenzyl)acetamide
Molecular weight	319.38
SMILES	<chem>O=C(CC1=CC=C(C2=CC=CC=C2)C=C1)NCC3=CC(F)=CC=C3</chem>

<b>Compound 8</b>	
Chemical Structure	
Laboratory code	KX1-329
CAS	897015-76-8
IUPAC name	N-(3-fluorobenzyl)-2-(5-phenylpyridin-2-yl)acetamide
Molecular weight	320.37
SMILES	<chem>O=C(CC1=NC=C(C2=CC=CC=C2)C=C1)NCC3=CC(F)=CC=C3</chem>

<b>Compound 9</b>	
Chemical Structure	
Laboratory code	KX2-377
CAS	897015-88-2
IUPAC name	N-(3-fluorobenzyl)-2-(5-(4-(2-morpholinoethoxy)phenyl)pyridin-2-yl)acetamide
Molecular weight	449.53
SMILES	<chem>O=C(CC1=NC=C(C2=CC=C(OCCN3CCOCC3)C=C2)C=C1)NCC4=CC(F)=CC=C4</chem>

<b>Compound 10</b>	
Chemical Structure	
Laboratory code	Photoaffinity analog; PAL2
CAS	Not available
IUPAC name	4-Benzoyl-N-(2-(4-(6-(2-(benzylamino)-2-oxoethyl)pyridin-3-yl)phenoxy)ethyl)-N-(pent-4-yn-1-yl)benzamide
Molecular weight	635.76
SMILES	<chem>O=C(CC1=NC=C(C2=CC=C(OCCN(C(C3=CC=C(C(C4=CC=CC=C4)=O)C=C3)=O)CCCC#C)C=C2)C=C1)NCC5=CC=CC=C5</chem>

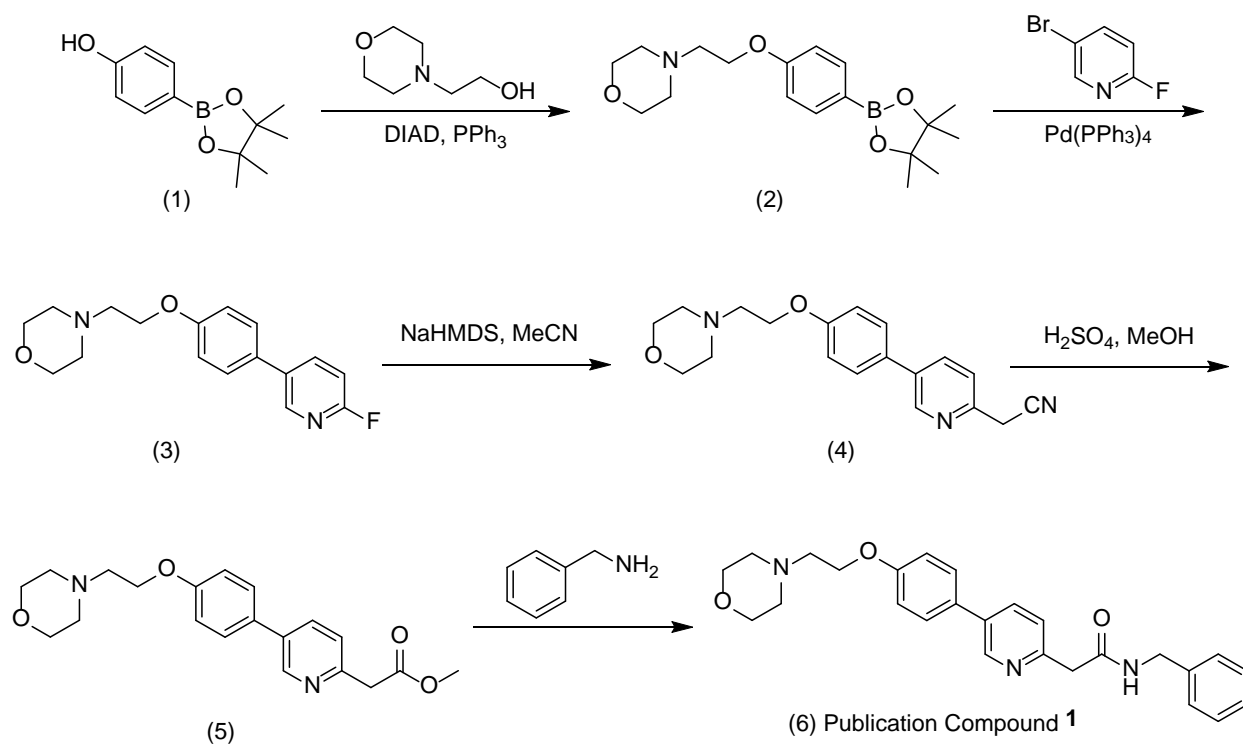
## 2 CHEMICAL SYNTHESIS

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Materials, reagents, solvents were obtained from commercial suppliers and used without further purification. Flash chromatography was performed manually or on a Teledyne Isco CombiFlash instrument. HPLC purification was performed on Shimadzu Preparative HPLC system with Phenomenex or Waters C18 columns using acetonitrile and 0.1% aqueous TFA as mobile phases. HPLC purity and identity by LCMS used a Thermo MSQ Plus instrument equipped with a Thermo Gold C18 column, PDA detector, and ESI mass detector, eluted with an acetonitrile and 1% acetic acid mobile phase gradient. <sup>1</sup>H NMR spectra recorded on Varian or Bruker 400 MHz instruments. NMR peak resonances were recorded in ppm relative to reference solvent. Compounds that were characterized at > 95% pure utilized the methods specified in their respective experimentals.

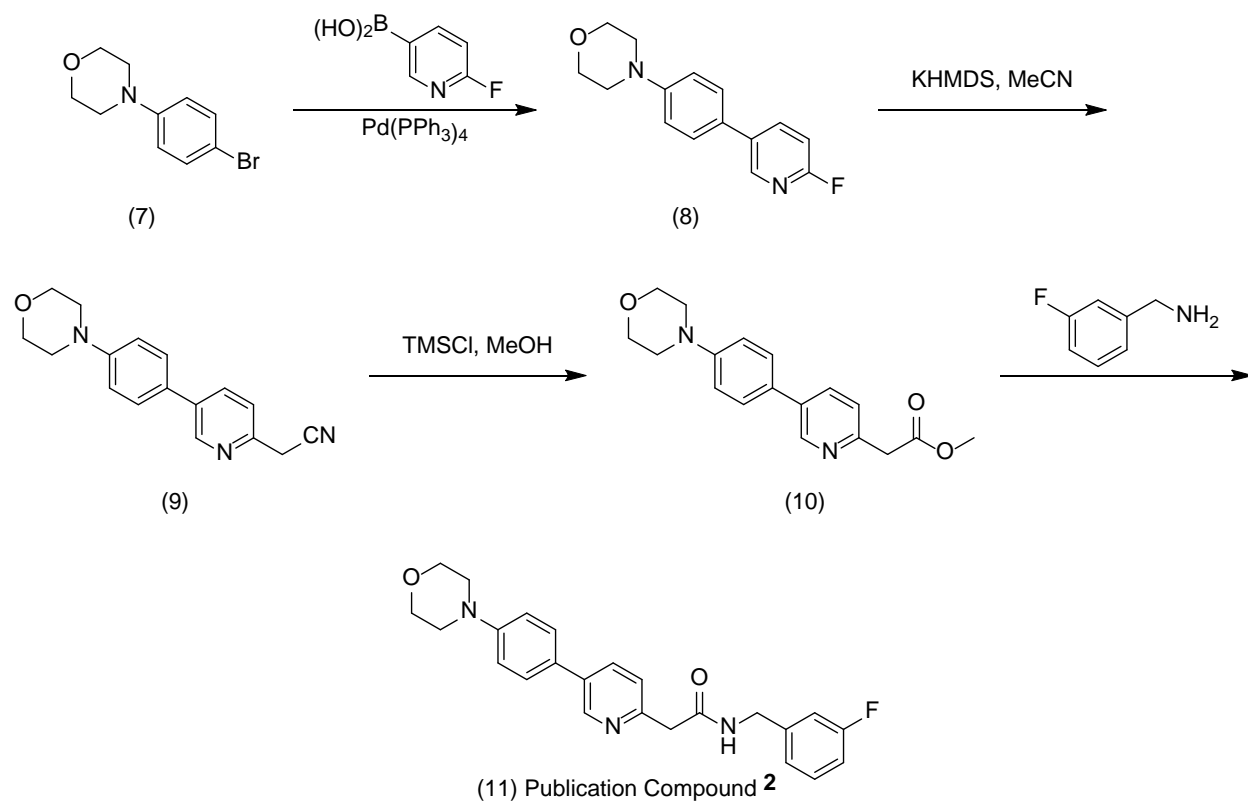
### 2.1 SYNTHESIS OF COMPOUND 1

Compound **1** (KX2-391) from the publication is synthesized by the following process. First, boronic ester (2) is formed by Mitsunobu coupling of (1) and 2-morpholinoethanol. This boronic ester is coupled with 2-fluoro-5-bromo pyridine via a Suzuki coupling to produce compound (3). Acetonitrile is combined with strong base, sodium bis(trimethylsilyl)amide (NaHMDS), in the presence of pyridyl fluoride (3) to yield nitrile (4) via nucleophilic aromatic substitution. The nitrile is then hydrolyzed with methanolic sulfuric acid to yield the methyl ester (5). In an excess of benzylamine, at high temperature in anisole, compound (5) is converted to Compound 1 which is isolated by crystallization. The synthesis has been published in several patents including; David G. Hangauer, Jr. Compositions for treating cell proliferation disorders. US patent 7,300,931, 2004. David G. Hangauer, Jr., Daniel Coughlin, Jonathan Gale, Jeremy A. Cody, Debasis Patra. Composition and methods for modulating a kinase cascade. US patent 7,851,470, 2006.



## 2.2 SYNTHESIS OF COMPOUND 2

Compound **2** (KX2-361) is synthesized by the following process. First the pyridyl boronic acid is coupled to aryl bromide (7) by Suzuki Coupling. Acetonitrile is combined with strong base, potassium bis(trimethylsilyl)amide (KHMDs), in the presence of pyridyl fluoride (8) to yield nitrile (9) via nucleophilic aromatic substitution. The nitrile is then hydrolyzed with methanolic hydrochloric acid, formed in-situ from the addition of trimethylsilyl chloride to methanol, to yield the methyl ester (10). In an excess of *meta*-fluorobenzylamine, at high temperature in anisole, compound (10) is converted to compound **2** which is isolated by crystallization. This synthesis has been published in a patent; David G. Hangauer, Jr. Compositions and methods for modulating a kinase cascade. US patent application 14,015,030, 2012.

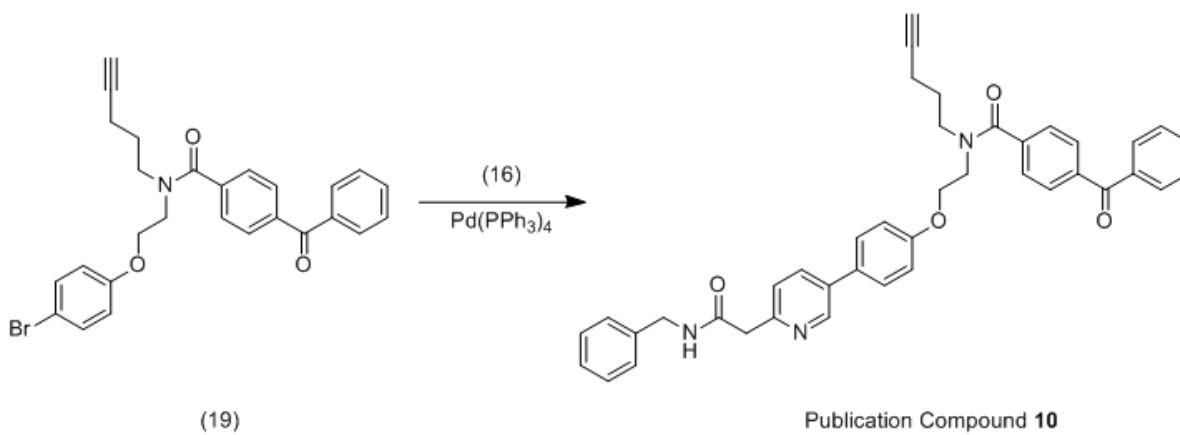
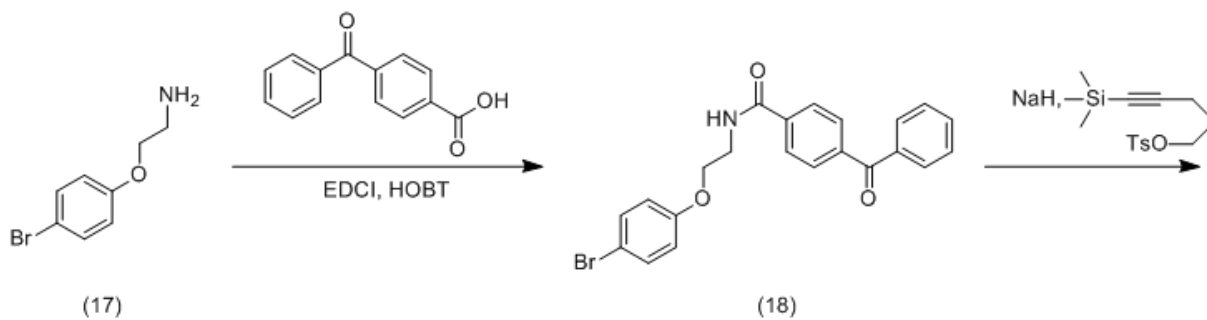
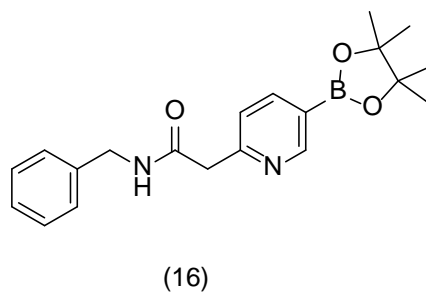
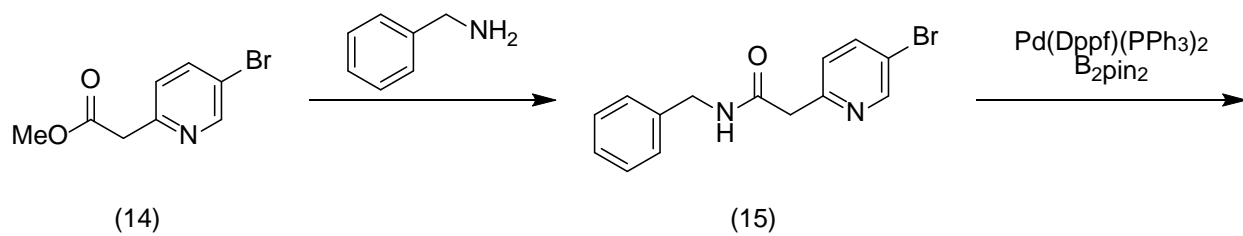
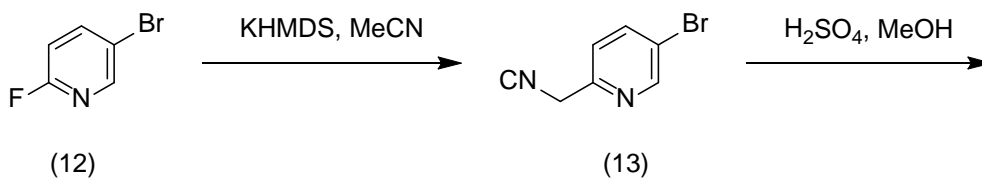


### 2.3 SYNTHESIS OF COMPOUNDS 7, 8 AND 9

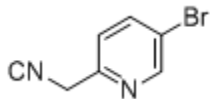
The synthesis of compounds **7**, **8** and **9** is described in David G. Hangauer, Jr. Compositions and methods of treating cell proliferation disorders. WO 2006071960 A2, 2006.

### 2.4 SYNTHESIS OF COMPOUND 10

The photoaffinity analog of KX2-391/361, compound **10**, was synthesized by a convergent process shown in schemes below.

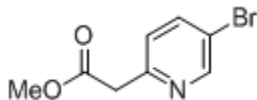


### Compound (13)



Under a rapid stream of nitrogen, a suspension of potassium bis(trimethylsilyl)amide (41.6 g, 0.208 mol) in 100 mL of anhydrous THF was prepared in a 500 mL round-bottomed flask equipped with a stir bar. The suspension was cooled using an ice/methanol bath and a solution of anhydrous MeCN (7.00 g, 0.170 mol) in anhydrous THF (11 mL) was slowly added to the suspension (over a period of 3-5 min). This was followed by the rapid addition of a solution of 2-fluoro-5-bromopyridine (4.93 g, 0.0280 mol) in anhydrous THF (40 mL). The reaction mixture was stirred under nitrogen for 2-3 hours, and checked by LC/MS for the complete consumption of the starting material. Upon reaction completion EtOAc (500 mL) was added. The solution was washed twice with saturated brine (250 mL), and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Anhydrous Na<sub>2</sub>SO<sub>4</sub> was filtered off, and the organic solution was concentrated *in vacuo* to give a red oily substance. This oily material was dissolved in EtOAc (25 mL), adsorbed on silica gel, and purified with flash chromatography using heptanes: EtOAc as a mobile phase to give compound (13) as a red oil (4.03 g, yield: 73%). LC/MS:  $m/z$  (M+H)<sup>+</sup> = 198/200 ([M+H]<sup>+</sup>). <sup>1</sup>H NMR (400MHz, CDCl<sub>3</sub>) δ 3.91 (s, 2H), 7.35 (d, 1H), 7.88 (dd, 1H), 8.64 (fine d, 1H).

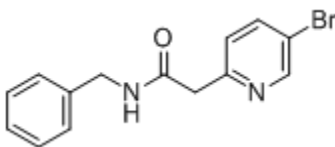
### Compound (14)



A single-necked round-bottomed flask was charged with compound (13) (4.55 g, 23.1 mmol) and MeOH (40 g) followed by the dropwise addition of 96% H<sub>2</sub>SO<sub>4</sub> (28 g). The resulting homogeneous solution was stirred at reflux in an oil bath (oil bath temperature: 115°C) until the reaction was complete by TLC. After brief cooling, MgSO<sub>4</sub> (9 g) was added and the mixture

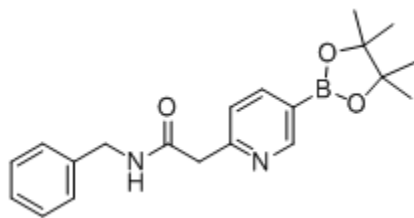
swirled and allowed to stand an additional 45 min. The reaction mixture was then added slowly to a rapidly stirred and cooled (ice-water bath) mixture of DCM (250 mL) and a solution of  $K_2CO_3$  (50 g) in  $H_2O$  (70 mL). The resulting emulsion was allowed to stand overnight. The clear portions of organic solution were siphoned off and the remainder portions were treated iteratively with water and DCM, the clear organics being combined with the original portion that was siphoned off. The combined organics were dried ( $Na_2SO_4$ ), filtered, concentrated, and purified by silica gel chromatography. The desired product, compound (14), was obtained as a colorless solid (3.82 g, 72% yield). LC/MS:  $m/z$  (ES+)  $(M+H)^+ = 230.7$ .

### Compound (15)



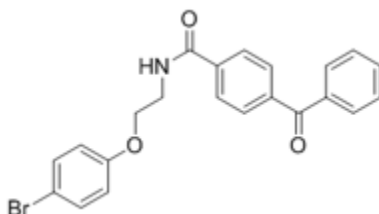
A single-necked round-bottomed flask was charged with compound (14) (1.80 g, 7.82 mmol), benzylamine (2.51 g, 23.5 mmol), and anhydrous anisole (15 g). The reaction was stirred at 150 °C until reaction was complete by LC/MS (approx. 24 h) and then allowed to cool to near RT. Crystals of the desired product, compound (15), formed during the cooling process and were collected by filtration and washed with toluene. Compound (15) was obtained as colorless crystalline solid (1.88 g, 79% yield).  $^1H$  NMR ( $DMSO-d_6$ )  $\delta$  3.68 (s, 2 H), 4.29 (d, 2 H), 7.2-7.4 (m, 6 H), 7.99 (dd, 1 H), 8.61 (overlapping fine d and an apparent broad t, 2 H).

### Compound (16)



In a single-neck round bottom flask, compound (15) (150 mg, 0.490 mmol), bis(pinacolato)diboron (374 mg, 1.47 mmol), [1,1'-bis(diphenylphosphino)ferrocene]dichloropalladium(II), complex with dichloromethane (40 mg, 0.049 mmol), and potassium acetate (144 mg, 1.47 mmol) were combined neat. The flask was equipped with a condenser, sealed, and purged with nitrogen. Anhydrous 1,4-dioxane was added and the reaction was refluxed for 24 hr. The reaction was cooled and solvent removed *in vacuo*. The residue was taken up in DCM and washed twice with 0.5N NaOH and twice with brine. The organic layer was dried over sodium sulfate and concentrated. This provided crude compound (16) (143 mg, 83% yield) as a dark brown tar. This material was progressed through to the next step without further purification. LC/MS:  $m/z$  (ES+) (M+H)<sup>+</sup> = 353.

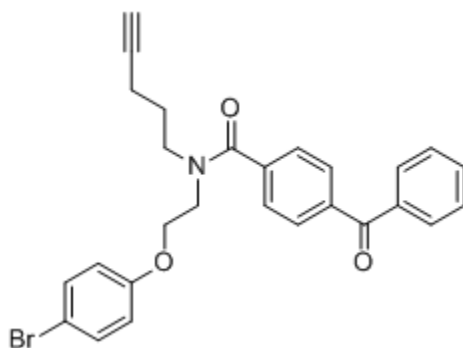
### Compound (18)



In a single-neck round bottom flask, 2-(4-bromophenoxy)ethan-1-amine hydrochloride (595 mg, 2.35 mmol), benzophenone-4-carboxylic acid (585 mg, 2.59 mmol), EDCI (497 mg, 2.59 mmol), HOBT (370 mg, 5.35 mmol), and diisopropylethylamine (818  $\mu$ L, 5.17 mmol) were combined in 4 mL DMF and stirred at room temperature for two hours before being taken up in ethyl acetate. Washed thrice each with 1N HCl, 1 N NaOH, and brine. The organic layer was dried over sodium sulfate, filtered, concentrated, and purified by flash silica chromatography. Compound (18) (690 mg, 69% yield) was afforded as a light brown solid. <sup>1</sup>H NMR (DMSO- $d_6$ )  $\delta$  3.64–3.68 (m, 2H), 4.14 (t, 2H), 6.95 (dd, 2H), 7.45 (dd, 2H), 7.58 (t, 2H), 7.65–7.77 (m, 3H), 7.80 (d, 2H), 8.02 (d,

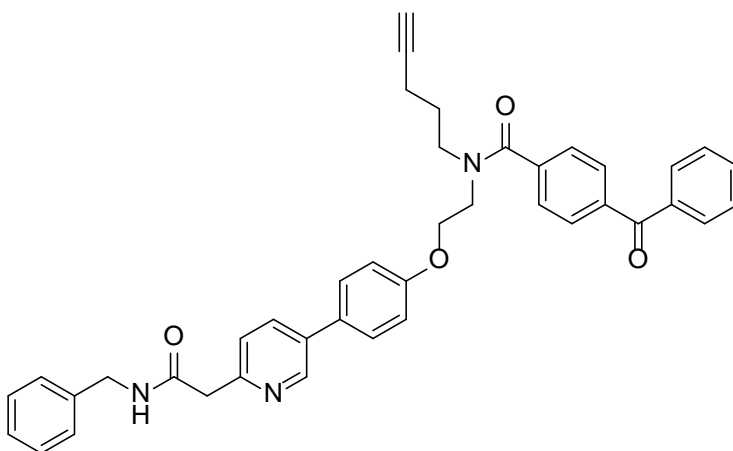
2H), 8.90 (t, 1H). Purity >95% by <sup>1</sup>H NMR. LCMS: *m/z* (ES+) (M+H)<sup>+</sup> = 424/426. Using the HPLC method described above, compound (18) was >95% pure by UV absorbance at 254 nm.

### Compound (19)



Compound (18) (100 mg, 0.235 mmol) and 5-(trimethylsilyl)pent-4-yn-1-yl 4-methylbenzenesulfonate (79 mg 0.235 mmol) were added, as a solution in THF, to a suspension of sodium hydride (20 mg, 0.471 mmol) in THF at 0 °C and allowed to warm to ambient temperature overnight. Reaction was quenched with water and extracted with ethyl acetate. After concentration, the residue was purified by reverse phase-HPLC (MeCN: 0.1% aqueous TFA) to afford compound (19) as a colorless oil (35 mg, 30% yield).

### Compound 10



Compound (19) (30.0 mg, 0.0611 mmol), compound (16) (115 mg, 0.122 mmol), Pd(PPh<sub>3</sub>)<sub>4</sub> (18.9 mg, 0.0061 mmol), and potassium carbonate (68 mg, 0.0183 mmol) were combined under an inert

atmosphere in 1,4-dioxane and refluxed for 4 h. Upon cooling, the reaction mixture was diluted with dichloromethane, and washed three times with brine. The organic layer was concentrated and purified by reverse phase-HPLC (MeCN: 0.1% aqueous TFA) to obtain compound **10** (7 mg, 18% yield) as an off white solid. LC/MS:  $m/z$  (ES+) (M+H)<sup>+</sup> = 636. Using the HPLC method described above, compound **10** was >95% pure by UV absorbance at 254 nm.

### 3 EVALUATION OF THE pKa OF COMPOUND 1 AND COMPOUND 2

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Compounds **1** and **2** were assayed with the co-solvent method. The co-solvent method is suitable for analysis of compounds possessing low aqueous solubility (typically a predicted intrinsic solubility of < 10 µg/ml). In this method, the 10 mM DMSO stock solutions were diluted 100-fold with 2 mM HCl and methanol, such that the final concentration was 100 µM, and DMSO concentration was 1%. The compounds were transferred into 24 consecutive wells of a 96-well plate for analysis using the co-solvent method (next paragraph). Both compounds yielded adequate data under the co-solvent method to extrapolate a pKa value.

All data was obtained using a pKa PRO Analyzer (AATI, Ames, IA). The average pH spacing between buffer points is 0.4 pH units covering a pH range of 1.7 – 11.2. Four consecutive CE runs are performed for each compound starting with 60% co-solvent buffers and decreasing to 30% co-solvent buffers. Norfloxacin is used as a performance-indicating standard. The total number of pKa values is predicted by relating mobility and compound molecular weight using pKa Estimator<sup>®</sup> software (AATI, Ames, IA).

**Table S1. Determined pKa's of Compound 1 and Compound 2**

	<b>Compound 1</b>	<b>Compound 2</b>
<b>pKa 1</b>	3.50	1.71
<b>pKa 2</b>	6.16	3.66

## 4 COMPUTATIONAL CHEMISTRY - HOMOLOGY MODELLING

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All modeling studies were carried out using Sybyl 6.9 from Tripos, Inc. that was available at the time this work was carried out. A model of c-Src in its active conformation, with the peptide substrate binding site partially formed, was generated through homology modeling of the c-Src structure and sequence onto the IRTK active conformation structure. This model aided the design of the peptide substrate targeted Src inhibitors, compounds **1** and **2**, as well as the intermediate compounds leading up to them, as described in the manuscript.

The homology modeling procedure used to generate a model of the active Src peptide substrate binding site is summarized as follows:

1. C-Src structure was obtained from the PDB (PDB code 2RSC) as well as the IRTK structure with a peptide substrate bound in the active site (PDB code 1IR3).
2. The residues forming the SH2 and SH3 domains, and the C-terminus, were removed from the c-Src protein (from the N-terminus through Ala 259 and from the C-terminus through Phe 520 were all deleted).
3. The water molecules that are no longer involved in any interactions were deleted.
4. The sequence of both IRTK (without the peptide as the fixed molecule) and c-Src (the kinase domain generated above was designated as the movable molecule) were compared using the command

```
Biopolymer>>
```

```
Align structures using homology.
```

5. The 2 proteins shared 106 residues out of about 260 forming the kinase domains. The sequence was analyzed to identify the important regions that are involved in interaction with the peptide substrate or inhibitor.
6. The residues of the identified important regions that interact with the peptide substrate or inhibitor in the IRTK were mutated to match the corresponding residues in c-Src using the command

Biopolymer>>

Modify>>

Mutate monomers.

7. The following residues were mutated in IRTK to match the corresponding residues in c-Src:

From 1222 to 1203

From 1181 to 1159

From 1153 to 1129

8. A total of 40 residues were mutated out of 68 residues involved (or in direct vicinity) of the catalytic site or the peptide-binding site. The resultant model active site sequence was a match to the active site sequence of c-Src except for residue 1162 (phosphotyrosine) which is present in the model but not in c-Src. This residue is located at the end of the peptide binding site in an area none of the compounds docked would contact and so it was not further modified.

9. The final model structure shares 146 residues out of 260 with c-Src and was used for the inhibitor docking studies since the residues lining the peptide substrate site are those from c-Src.

The candidate Src inhibitor compounds were docked into the homology built Src peptide substrate binding site as summarized below:

- 1) The compounds were drawn using the “Build edit >> sketch molecule” function of Sybyl.
- 2) The atom charges of the compounds were calculated using MMFF94.
- 3) The compounds were docked manually into the active site guided by the analogous positioning of a peptide substrate from the IRTK structure.
- 4) The compounds were minimized within the active site using the Sybyl dock function while holding the active site fixed (typically about 100 iterations).

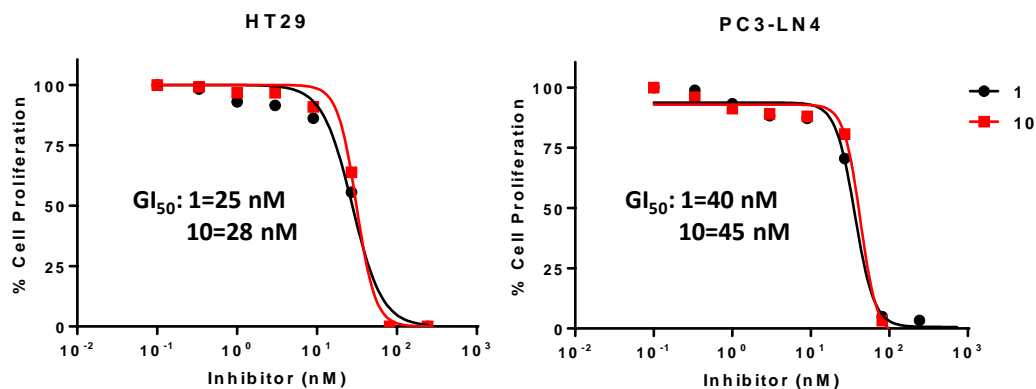
## 5 CONDITIONS OF THE *IN VITRO* STUDIES

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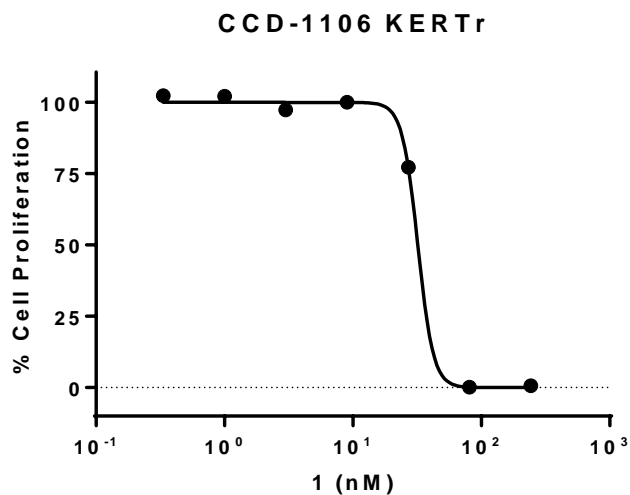
### 5.1 CELL PROLIFERATION ASSAY.

All cell lines procured from ATCC (Manassas, VA). Cell proliferation was assessed by using MTT assay. Cells seeded in 96-well plate ( $2-5 \times 10^3$  cells per well) were treated with test compounds at varying concentrations for 72h. 10  $\mu$ l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml in PBS) was added to each well and incubated for 3 h. After removal of the medium, the purple formazan dye was dissolved in 100  $\mu$ l of isopropanol containing 40 mM HCl followed by measurement of absorbance at 570 nm using a BioTek microplate reader (BioTek, Winooski, VT). The cell growth curve and  $GI_{50}$ 's were obtained using the GraphPad Prism 5 software (GraphPad Software Inc; La Jolla, CA).

**Figure S1.  $GI_{50}$  of Compounds 1 and 10 in HT29 and PC3-LN4 cell lines.**



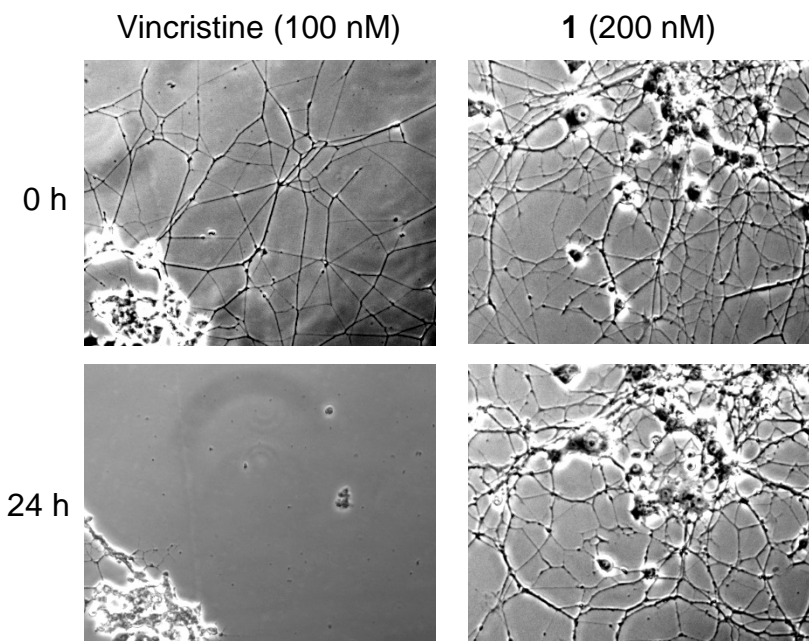
**Figure S2. GI<sub>50</sub> of Compound 1 in CCD1106 KERTr keratinocytes.**



## 5.2 NEURITE OUTGROWTH ASSAY.

Regular PC-12 rat pheochromocytoma cells (ATCC) were maintained in ATCC-formulated F-12K medium supplemented with 2.5% FBS and 15% horse serum (Invitrogen). Neuronal PC-12 cells were generated by growing on collagen-coated 12-well plates (1 X 10<sup>3</sup> cells per well) in the complete medium containing 200 ng/ml of NGF-S7 (Sigma). Media (including NGF) were changed every other day. After 10 days incubation, the representative field of neurite network in each well was imaged using bright-field microscopy. Vincristine or compound **1** was added to each well and the neurites in the same field as prior to addition of the compound were photographed after 24h incubation.

**Figure S3. Compound 1 does not alter neurite morphology.**



### 5.3 IMMUNOBLOTTING.

Cell lines were cultured in 6-well dishes at  $\times 10^6$  and treated for 24 h with 0.2X, 1X, 5X or 25X the  $GI_{50}$  drug concentrations. Cells were washed once in PBS and then lysed in RIPA buffer containing a protease inhibitor tablet (Roche Applied Science). The cell lysates were cleared by centrifugation and 50  $\mu$ g of total protein was separated by SDS-PAGE followed by blotting onto PVDF membrane. The membranes were blocked with 5% Bovine serum albumin in TBST buffer (50 mM Tris, pH 7.5, 180 mM NaCl, 0.05% Tween 20), then probed with primary antibodies specific to phospho-Src (Tyr416), Src (Cell Signaling Technology) or GAPDH (Santa Cruz Biotechnology) overnight in TBST, washed thrice in TBST, then incubated for 1 h with AlexaFluor680-labeled secondary antibody. After washing, the blots were imaged directly on a Li-Cor Infra BioImager. The protein bands identified were quantified using GeneTools software (Syngene) and the relative level of protein phosphorylation was determined by normalizing the

phospho-protein signal to that of the total protein, then normalizing to the GADPH, which served as a loading control.

#### 5.4 PHOTOAFFINITY LABELING AND CLICK CHEMISTRY.

HT29 cells seeded in 6-well plate were incubated with compound **10** in the presence or absence of excess of compound **1** for 2h in 37 °C incubator. After UV irradiation at 365 nm for 10 min at room temperature (RT), cells were collected in PBS containing protease inhibitor cocktail (Roche Applied Science) and lysed by sonication. Porcine brain tubulin (cytoskeleton) was incubated with compound **10** alone (500 nM) or in the presence of a molar excess (100x or 500x) of competitors under UV irradiation (365 nM) for 1 hour on ice. After photoaffinity labeling, the cell lysates or purified tubulin were subjected to copper catalyzed click chemistry reaction by incubating with 20 µM rhodamine-azide (Invitrogen, Carlsbad, CA), 100 µM ligand (Sigma-Aldrich), 10 mM TCEP (Sigma-Aldrich) and 1 mM CuSO<sub>4</sub> for 1h at room temperature. Samples were denatured, resolved by SDS-PAGE and visualized by in-gel fluorescent scanning using a Molecular Dynamics Typhoon 9410 molecular imaging system (GE Healthcare, Pittsburgh, PA).

#### 5.5 2-D GEL ELECTROPHORESIS.

A 1 mg aliquot of HT29 cell lysate prepared by sonication in PBS with protease inhibitor cocktail were incubated with 2 µM compound **10** in the absence or presence of 400 µM compound **1** on ice for 1 h with UV irradiation at 365 nm. Click chemistry was performed as described above. The proteins were pelleted by centrifugation at 13,000 rpm for 5 min at RT and resuspended in 500µl sample buffer (30 mM Tris, 7 M urea, 2 M thiourea, 4% CHAPS, 5 mM Magnesium acetate), followed by desalting using 2-D clean-up kit (GE Healthcare). The samples were then applied onto immobilized pH 3-10 non-linear gradient strips (GE Healthcare). The strips were then focused using the following program: 150 V for 1hr, 1000 V for 1hr, gradient 3000 V for 1 hr, gradient

8000 V for 3 hr and 8000 V for 45,000 V hrs. The second dimensional separation was performed on 12.5% SDS-PAGE. The Compound 10-labeled proteins were detected by in-gel fluorescent scanning using Typhoon 9410 scanner (GE Healthcare).

#### 5.6 IN-GEL DIGESTION AND MASS SPECTROMETRY.

Protein spots of interest were excised with an Ettan SpotPicker (GE Healthcare) and placed in a 96-well plate. The tryptic in-gel digestion was performed by the Ettan Digester (GE Healthcare). The tryptic digests were then analyzed by LC nanoelectrospray-tandem mass spectrometry (LC-ESI-MS/MS) using a nanoACQUITY UPLC (Waters) coupled through a nebulization-assisted nanospray ionization source to a Q-ToF Premier mass spectrometer (Waters/Micromass). The LC consisted of a trap column (Symmetry C18, 5  $\mu$ m, 180  $\mu$ m x 20 mm, Waters), followed by separation on an analytical column (Atlantis C18, 3  $\mu$ m, 100  $\mu$ m x 10 cm, Waters). Samples were loaded, trapped, and washed at a flow rate of 3  $\mu$ L/min with 98% solvent A (water containing 0.1% formic acid)/2% solvent B (acetonitrile containing 0.1% formic acid) for 5 min. Peptides were eluted with a gradient of 98% A/2% B to 65% A/35% B for 60 min at 0.3  $\mu$ L/min, 10% A/90% B for 15 min at 0.6  $\mu$ L/min, and then 10% A/90% B at 1.5  $\mu$ L/min for 10 min. Throughout the gradient, the mass spectrometer was programmed (Data Dependent Acquisition experiment, DDA) to monitor ions with m/z in the range of 300-1500, and ions with +2 to +5 charges only were selected for MS/MS experiments using the preset DDA collision energy parameters.

#### 5.7 DATABASE SEARCH AND PEPTIDE AND PROTEIN IDENTIFICATION.

MS/MS spectra were processed and transformed to the PKL file format using Proteinlynx Global Server v2.3 (Waters/Micromass) and the default parameters of MaxEnt3 (Waters/Micromass). The PKL files were used to search against the *Homo sapiens* subset of the Swiss-Prot database (release 55.2, containing 19,117 sequences) using a locally installed version of MASCOT (Matrix Science,

v 2.1). The search parameters were as follows: trypsin as the proteolytic enzyme with 2 possible missed cleavages, carboxyamidomethylation of cysteine as a fixed modification, oxidation of methionine as a variable modification, the allowable mass error was 100 ppm for peptides and 100 mDa for fragment ions, peptide charge was set to 2+ and 3+, the instrument was set to ESI-QUAD-TOF. The Mascot default significance threshold of  $p < 0.05$  for assignments was used in the searches and a minimum of two unique peptides were used as a criteria for a match.

### 5.8 IMMUNOFLUORESCENCE MICROSCOPY.

Human PBMCs were isolated by using ACCUSPIN™ System-Histopaque®-1077 (Sigma) according to manufacturer's instruction. PBMCs seeded on 22 mm coverslips were incubated with compound **1** at 125, 250 or 500nM for 4 hours at 37 °C. Cells were fixed with cold methanol for 20 min, immersed in blocking buffer (3% BSA in PBS) for 30 min, and then incubated with  $\alpha$ -tubulin antibody (Sigma-Aldrich) for 2 hours at RT in blocking buffer (1:500 dilution). Cells were washed three times with PBS and incubated with Alexa 594 anti-mouse IgG (Invitrogen) for 1 hour at RT in blocking buffer (1:500 dilution). Following three PBS washes, coverslips were mounted on glass slides in ProLong anti-fade reagent (Invitrogen). Fluorescent images were captured using a Nikon TE2000-E inverted microscope.

### 5.9 *IN VITRO* KINASE ACTIVITY ANALYSIS.

*In vitro* kinase activity against 16 kinases for compound **1** and AZ28 was assessed using Upstate's KinaseProfiler Express Service. Stock solutions at 5 mM in DMSO compound **1** and AZ28 were sent to Upstate's Drug Discovery Service department (Dundee, Scotland). From this sample, a single point kinase activity analysis was performed at 10  $\mu$ M. The relative activity of each kinase following treatment with the compounds was reported as a percentage of the activity relative to DMSO control.

**Table S2. Selectivity of compound 1 compared to AZ28 across a range of isolated kinases. The data are shown as % of control kinase activity, so low numbers represent significant inhibition.**

<b>Target</b>	<b>AZ28 @ 10 <math>\mu</math>M</b>	<b>1 @ 10 <math>\mu</math>M</b>
ABL (h)	1	120
CHK1 (h)	NT	105
EGFR (h)	3	134
FGFR2 (h)	94	94
FYN (h)	2	85
IGF-1R (h)	110	101
IR (h)	125	112
LCK (h)	1	109
LYN (h)	0	113
MAPK2 (h)	105	112
PDGFR $\beta$ (h)	98	110
PKC $\alpha$ (h)	111	111
PYK2 (h)	43	97
YES (h)	1	92
ZAP-70 (h)	97	108
PI3 kinase	99	100

## 6 PHARMACOKINETIC STUDIES

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### 6.1 COMPOUND 1 MOUSE PHARMACOKINETICS

Single dose mouse pharmacokinetics were determined in male CD-1 mice (Harlan, Indianapolis IN). Animals were not fasted for dose administration. During the test period, animals were individually housed in suspended, stainless steel wire-mesh cages. Water was provided *ad libitum*. Testing substance is dissolved in 0.9% sodium chloride for injection (saline). The oral doses of 4 mg/kg were administered via a ball-tipped gavage needle, and the intravenous doses were of 5 mg/kg administered as a bolus via a tail vein. For all groups, blood was collected via exsanguination (cardiac puncture) under carbon dioxide anesthesia using a needle and syringe at the following time points; 0.083, 0.25, 0.5, 1, 2, 4, 8, and 12 hours for IV and 0.5, 1, 2, 3, 4, 8, and 10 hours post-dose for oral. Three animals were used for each time point. The compound plasma concentrations were determined using liquid chromatography tandem mass spectrometry (LC-MS/MS). PK parameters calculated using standard non-compartmental analysis. Study was conducted at Covance (Madison, WI).

**Table S3. Pharmacokinetics of Compound 1 in Mouse**

Dosing route	Dose (mg/kg)	T <sub>max</sub> (hr)	C <sub>max</sub> (ng/L)	AUC <sub>last</sub> (hr-ng/mL)	t <sub>1/2</sub> (hr)	F%
IV	5	0.083	3220	2012	0.50	-
Oral	4	0.50	524	800	1.10	49

Single dose mouse pharmacokinetics were determined from compound 1 in male CD-1 mice to determine brain-to-plasma ratio. The animals were fasted overnight prior to dose administration through approximately 4 hours post dose. Water was provided *ad libitum*. Compound 1 is dissolved in purified water for oral administration at 10 mg/kg. Animals were sacrificed at time points of 0, 0.5, 1, 2, and 5 hours, three animals per time point, when blood and brain samples

were obtained for bioanalytical assessment. The compound **1** plasma concentrations were determined using liquid chromatography tandem mass spectrometry (LC-MS/MS). PK parameters calculated using standard non-compartmental analysis. Study was conducted at Xenobiotic Laboratories (Plainsboro, NJ)

**Table S4. Pharmacokinetics of Compound 1 in Mouse Brain and Plasma**

Sample ID	T <sub>max</sub> (hr)	C <sub>max</sub> (ng/mL)	AUC <sub>last</sub> (ng-hr/mL)	Brain/Plasma AUC <sub>last</sub> ratio
Plasma	0.50	871	1503	0.42
Brain	0.50	398	631	
Note: Brain C <sub>max</sub> and AUC <sub>last</sub> are ng/g and ng-hr/g, respectively.				

## 6.2 COMPOUND 1 RAT PHARMACOKINETICS

Single dose rat pharmacokinetics were determined in male Sprague Dawley Rats. The animals were fasted overnight prior to dosing through 4 hours post dose. Water was provided *ad libitum*. Compound **1** was formulated in 0.9% Sodium Chloride for Injection (saline) for IV and oral administration. For intravenous dosing, blood was collected from each animal at 0.083, 0.25, 0.5, 1, 2, 4, 8, and 12 hours post dose. For oral dosing, blood (approximately 0.35 mL) was collected from each animal at 0.5, 1, 2, 3, 4, 8, 12, and 24 hours post dose. All blood samples were collected via a jugular vein using a syringe and needle followed by transfer into tubes containing sodium heparin anticoagulant. Three animals were used for dosing group. The compound **1** concentrations were determined using liquid chromatography tandem mass spectrometry (LC-MS/MS). PK parameters calculated using standard non-compartmental analysis. Study was conducted at Covance (Madison, WI).

**Table S5. Pharmacokinetics of Compound 1 in Rat**

Dosing route	Dose (mg/kg)	T <sub>max</sub> (hr)	C <sub>max</sub> (ng/L)	AUC <sub>last</sub> (hr-ng/mL)	t <sub>1/2</sub> (hr)	%F
IV	1	0.083	609	321	0.44	-
Oral	12.5	0.50	2310	2226	1.91	55
Oral	25	0.67	3970	4339	2.09	54

**6.3 COMPOUND 1 DOG PHARMACOKINETICS**

Single dose dog pharmacokinetics were determined in male beagles. All animals were fasted overnight through approximately 4 hours post dose. Water was provided *ad libitum*. Compound 1 was formulated in 0.9% Sodium Chloride for Injection (saline) for oral administration and in 0.5% methylcellulose with 0.1% Tween 80 solution vehicle for IV dosing. For intravenous dosing, blood was collected via a jugular vein at 0.083, 0.25, 0.5, 1, 2, 3, 4, 6, 8, and 12 hours post dose. For oral dosing, blood was collected via a jugular vein into tubes at 0.5, 1, 2, 3, 4, 6, 8, 12, 16, 24, 36, and 48 hours post dose. Three animals were used for dosing group. The compound 1 concentrations were determined using liquid chromatography tandem mass spectrometry (LC-MS/MS). PK parameters calculated using standard non-compartmental analysis. Study was conducted at Covance (Madison, WI).

**Table S6. Pharmacokinetics of Compound 1 in Dog**

Dosing route	Dose (mg/kg)	T <sub>max</sub> (hr)	C <sub>max</sub> (ng/L)	AUC <sub>last</sub> (hr-ng/mL)	t <sub>1/2</sub> (hr)	%F
IV	2.5	0.139	1620	1929	8.25	-
Oral	12.5	1.00	3320	8581	8.20	89
Oral	25	0.833	3590	10834	6.54	56

**6.4 COMPOUND 2 MOUSE PHARMACOKINETICS**

Single dose mouse pharmacokinetics were determined in male CD-1 mice. The animals were fasted for 12 hours prior to dose administration and until 4 hours post-dose. Water was provided *ad libitum*. Testing substance was formulated in 10% ethanol : 30% saline : 60% PEG200 for IV

administration at 5mg/kg and in simulated gastric fluid for oral administration at 20 mg/kg. For all groups, blood was collected via exsanguination (cardiac puncture) using a needle and syringe at the following time points; 0.083, 0.25, 0.5, 1, 2, 4, 8 hours for IV dosing group and 0.25, 0.5, 1, 2, 4, 6, 8 hours for oral dosing group. For the oral dosing group, animal brains were also collected for bioanalytical assessment. Three animals were used for each time point. The compound 2 concentrations were determined using liquid chromatography tandem mass spectrometry (LC-MS/MS). PK parameters calculated using standard non-compartmental analysis. Study was conducted at Absorption Systems (Exton, PA)

**Table S7. Pharmacokinetics of Compound 2 in Mouse Brain and Plasma**

Dosing route (Tissue)	Dose (mg/kg)	T <sub>max</sub> (min)	C <sub>max</sub> (mg/L)	AUC <sub>last</sub> (hr-ng/mL)	t <sub>1/2</sub> (hr)	%F	Brain/plasma AUC <sub>last</sub> ratio
IV	5	0	4620	2764	0.36	-	NA
Oral (Plasma)	20	0.25	2593	4347	ND	39.3	1.16
Oral (Brain)	20	0.25	4025	5044	ND	ND	
ND=Not Determined; NA=Not Applicable							

#### 6.5 COMPOUND 8 MOUSE PHARMACOKINETICS

Single dose mouse pharmacokinetics were determined in male ICR mice. The animals are fasted for 16 hours prior to use. During the experiment, the animals are kept in sterile polycarbonate cage, maintained in controlled temperature (22~23°C) and humidity (60~70%) laboratory with 12-hour light-dark cycles. Water was provided *ad libitum*. Testing substance is dissolved in 2% Tween 80 as vehicle and administered as a bolus injection through the tail vein at a dose of 10 mg/kg or at 30 mg/kg by oral gavage. Following compound 8 administration, blood is drawn from the retro-orbital sinus at assigned time points; 0, 0.083, 0.25, 0.5, 1, 3, 6, 12 and 24 hours for the IV dosing group and at 0, 0.083, 0.25, 0.5, 1, 3, 6, 12 and 24 hours for the oral dosing group. The mice are

sacrificed sequentially, with three mice for individual time points. The compound **8** concentrations were determined using liquid chromatography tandem mass spectrometry (LC-MS/MS). PK parameters calculated using standard non-compartmental analysis. Study was conducted at MDS Pharma.

**Table S8. Pharmacokinetics of Compound 8 in Mouse**

Dosing route	Dose (mg/kg)	T <sub>max</sub> (min)	C <sub>max</sub> (mg/L)	AUC <sub>last</sub> (min-mg/L)	%F
IV	10	5	2770	53400	-
Oral	30	30	70.6	5720	3.6

#### 6.6 COMPOUND 9 MOUSE PHARMACOKINETICS

Single dose mouse pharmacokinetics were determined in male CD-1 mice (Harlan, Indianapolis IN). The animals were fasted approximately 10 hours prior to dose administration through approximately 2 hours post dose. During the test period, animals were individually housed in suspended, stainless steel wire-mesh cages. Water was provided *ad libitum*. Testing substance is dissolved in 0.9% sodium chloride for injection (saline). The oral doses of 50 mg/kg were administered via a ball-tipped gavage needle, and the intravenous doses were of 5 mg/kg administered as a bolus via a tail vein. For all groups, blood was collected via exsanguination (cardiac puncture) under carbon dioxide anesthesia using a needle and syringe at the following time points; 0.083, 0.25, 0.5, 1, 2, 4, 8, and 12 hours for the IV dosing group and 0.5, 1, 2, 3, 4, 8, 12, and 24 hours for the oral dosing group. Three animals were used for each time point. The compound **9** concentrations were determined using liquid chromatography tandem mass spectrometry (LC-MS/MS). PK parameters calculated using standard non-compartmental analysis. Study was conducted at Covance (Madison, WI)

**Table S9. Pharmacokinetics of Compound 9 in Mouse**

<b>Dosing route</b>	<b>Dose (mg/kg)</b>	<b>T<sub>max</sub> (min)</b>	<b>C<sub>max</sub> (ng/L)</b>	<b>AUC<sub>last</sub> (hr-ng/mL)</b>	<b>t<sup>1/2</sup> (hr)</b>	<b>%F</b>
IV	5	0.083	3030	1609	2.71	-
Oral	50	0.50	6920	10287	3.53	64

## 7 *IN VIVO* EVALUATION OF COMPOUND 1 DISTRIBUTION IN A HT29 XENOGRAFT MODEL

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All animal experiments were approved by the Institutional Animal Care and Use Committee and conducted in male nude BALB/c mice. After suitable time for acclimatization, 6- to 8-wk-old mice were subcutaneously injected with  $10 \times 10^6$  HT-29 cells in 0.1 mL of phosphate-buffered saline. Tumor growth was monitored by caliper measurements. When tumors reached a pre-determined size, mice were administered Compound 1 orally 5 mg/kg, formulated in saline. Individual animals were sacrificed to harvest plasma and tumor samples at time points of 0, 0.5, 2, 4, 8, 12 and 24 hours post dose. Compound 1 levels were determined in plasma or tumor homogenates and used to calculate standard pharmacokinetic parameters including maximal concentration ( $C_{max}$ ) and exposure as a function of time (AUC).

**Table S10. Compound 1 Drug Levels in Plasma and HT29 Tumor Samples**

<b>Time (hour)</b>	<b>Plasma Concentration of Compound 1 (ng/mL)</b>	<b>Tumor Concentration of Compound 1 (ng/mL)</b>
0	0	0
0.5	512	768
2	199	288
4	80.8	118
8	4.33	18.6
12	1.31	4.37
24	0	0

**Table S11. Pharmacokinetics of Compound 1 in Plasma and HT29 Tumor Sample**

	<b>Plasma</b>	<b>Tumor</b>	<b>Tumor/Plasma AUC<sub>0-12</sub> Ratio</b>
<b>AUC<sub>0-12</sub> (hr-ng/mL)</b>	1130	1740	1.52