Supporting Information

INCB24360 (epacadostat), a Highly Potent and Selective Indoleamine-2,3-dioxygenase 1 (IDO1) Inhibitor for Immuno-oncology

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IDO1 Enzyme Assay. Human IDO1 with an *N*-terminal His tag was expressed in *E.coli* and purified to homogeneity. IDO1 catalyzes the oxidative cleavage of the pyrrole ring of the indole nucleus of tryptophan to yield *N*'-formylkynurenine. The assays were performed at room temperature as described in the literature using 20 nM IDO1 and 2 mM D-Trp in the presence of 20 mM ascorbate, 3.5 μM methylene blue and 0.2 mg/mL catalase in 50 mM potassium phosphate buffer (pH 6.5). The initial reaction rates were recorded by continuously following the absorbance increase at 321 nm due to the formation of *N*'-formlylkynurenine. The percent inhibition at individual concentrations was determined. The data was processed using nonlinear regression to generate IC_{50} values (Prism Graphpad). Values are given as the mean of $N \geq 2$. See: Sono, M., Taniguchi, T., Watanabe, Y., and Hayaishi, O. *J. Biol. Chem.* (**1980**), *255*, 1339-1345. The use of D-Trp was a technical choice. The Km value is 100-fold higher, but the kcat value is nearly identical to the natural substrate. This allowed for the use of higher substrate concentrations in assays which afforded better linearity and higher signals relative to L-Trp. Additionally, as reported in the literature L-Trp causes inhibition at higher concentrations possibly due to a second L-Trp binding site (Lu et al., *J. Am. Chem. Soc.* **2009**, *131*, 12866-67; Weber et al, *J. Phys. Chem. Lett.* **2014**, *5*, 756-761). With respect to being a worse competitor, these relations are governed by the ratio of substrate concentration to the Km value. Since it was possible to reach D-Trp concentrations significantly above the Km value without inhibitory effects, proper competition kinetics were experimentally reachable. Similar experiments with L-Trp would be confounded by the inhibitory activity noted above. Additionally, it should be noted that confirmation of inhibitor potency in cell assays confirmed activity against the natural substrate L-Trp.

The IDO1 enzyme assay strongly correlated with the potency of the HeLa cellular assay, but we and others have observed less potency (5-10 fold typically) in the IDO1 enzyme assay than in the HeLa cellular assay. This is unusual for most medicinal chemistry programs, since one observes the opposite

trend due to protein binding and permeability issues lowering the potency within the cellular context compared to the enzyme potency. This phenomenon is thought to be due to multiple factors within the enzyme assay that do not accurately recapitulate the native IDO1 protein environment *in vivo* (oxidation state, conformation, catalytic activity). The Rohrig review and references within explain this challenge and others in greater detail. Rohrig, U. F.; Majjigapu, S. R.; Vogel, P.; Zoete, V.; Michielin, O., Challenges in the Discovery of Indoleamine 2,3-Dioxygenase 1 (IDO1) Inhibitors. *J. Med. Chem.* **2015,** *58*, 9421-9437.

Determination of Inhibitor Activity in HeLa Cell-Based IDO1/Kynurenine Assay. HeLa cells (#CCL-2) were obtained from the American Type Tissue Culture Collection (ATCC, Manassas, VA) and routinely maintained in minimum essential medium (eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 10 % fetal bovine serum (all from Invitrogen). Cells were kept at 37 ^{o}C in a humidified incubator supplied with 5% CO_2. The assay was performed as follows: HeLa cells were seeded in a 96 well culture plate at a density of 5 x 10^3 per well and grown overnight. On the next day, human IFN-γ (50 ng/mL final concentration) and serial dilutions of compounds in a total volume of 200 μL culture medium per well were added into cells. After an additional 48 hours of incubation, 140 μL of the supernatant per well was transferred to a new 96 well plate. Ten microliters of 6.1 N trichloroacetic acid (#T0699, Sigma) were mixed into each well and incubated at 50 °C for 30 min to hydrolyze *N*-formylkynurenine produced by IDO1 to kynurenine. The reaction mixture was then centrifuged for 10 min at 2500 rpm to remove sediments. One hundred microliters of the supernatant per well were transferred to another 96 well plate and mixed with 100 μL of 2% (w/v) *p*-dimethylaminobenzaldehyde (#15647-7, Sigma-Aldrich) in acetic acid. The yellow color derived from kynurenine was measured at 480 nm using a SPECTRAmax 250 microplate reader (Molecular Devices). L-Kynurenine (#K8625, Sigma), used as the standard, was prepared in a series of concentrations (240, 120, 60, 30, 15, 7.5, 3.75, 1.87 μM) in 100 μL HeLa cell culture media and analyzed in the same procedure. The percent

inhibition at individual concentrations was determined. The data was processed using nonlinear regression to generate IC_{50} values (Prism Graphpad). Values are given as the mean of $N \geq 3$. See: Takikawa O, et al. *J. Biol. Chem.* (**1988**), *263*(4), 2041-2048.

Whole Blood Assay. Heparin treated fresh blood samples from normal healthy human donors was aliquoted into Costar polypropylene tubes (300 μL/ tube) (Corning Inc., Corning, NY). INCB024360 at different concentrations in PBS was added to the blood and incubated at room temperature for 15 minutes. The whole blood was then stimulated with 100 ng/mL LPS (*Salmonella typhimurium*) (Sigma) and 100 ng/mL human recombinant IFN-γ (R&D System) for overnight at 37 °C before being spun down for plasma collection. The tryptophan/kynurenine levels in the plasma samples were determined by LC/MS/MS analysis following a protein-precipitation extraction using trichloracetic acid. This assay has a linear range from 20 to 10000 nM for kynurenine and 200 to 100000 nM for tryptophan. Each experiment was done in duplicate. Percent inhibition at individual compound concentrations was calculated relative to the DMSO control and analyzed by nonlinear regression (GraphPad Prism 3.0) to determine the concentration required for IC_{50}. Values are given as the mean of $N \geq 2$.

Cell-Based IDO2 and TDO Assays. The description of the study has been published. See: Liu, X., et al., *Blood* **2010**, *115*, 3520-3530.

hERG Patch Clamp Assay. The hERG patch clamp assay was performed by Charles River Laboratories.

Cyp Inhibition. The potential for INCB024360 to inhibit CYP1A2, CYP2C8, CYP2C9, CYP2D6 and CYP3A4 activity was determined using recombinant human cytochrome P-450 isozymes purchased from Gentest-BD Biosciences. For CYP1A2 and CYP3A4 isozyme activity, the hydroxylation of 7-benzyloxy-4-trifluoromethyl-coumarin was measured using fluorescence detection. To measure CYP2C9 and CYP2D6 activity, the hydroxylation of 7-methoxy-4-(trifluoromethyl)-coumarin and 7-methoxy-4-methylcoumarin were measured fluoremetrically, respectively. To measure

CYP2C8 activity, the metabolism of dibenzylfluoresein to fluorescein was measured fluoremetrically. Substrates were incubated at Km concentrations with INCB024360 at ranging from 0-25 μM.

The samples were read using the fluorescence detector on the Perkin Elmer Fusion α-Fp at the appropriate wavelengths. The excitation wavelength of 405 nm and emission wavelength of 535 nm were used for CYP1A2, CYP2C9, and CYP3A4. The excitation wavelength of 405 nm and emission wavelength of 465 nm were used for CYP2D6. For CYP2C8, the excitation wavelength of 485 nm and emission wavelength of 538 nm were used. Values are given as the mean of $N \geq 2$.

Transient transfection assay for PXR/cyp3A4 induction (luciferase assay) in HepG2 cells. The human or rat PXR/cyp3A4 transfected Hep G2 cells were plated in 96-well culture plates (Thermofisher) at 100 μL per well in plating medium (MEM with Earle's without phenol red (Thermofisher) supplemented with 2 mM L-glutamine, 10% charcoal stripped FBS (Hyclone)). Cells were kept at 37 °C in humidified incubator supplied with 5% CO_2 and grew for 24 hours. 1.5 μL test compounds in DMSO were first transferred to the 384 REMP plate well (Thermofisher) by PlateMate Plus (Thermofisher) followed with addition of 125 μL plating medium. 100 μL compounds in the plating medium were transferred to the cells with 0.6% DMSO in the final assay at 10 μM. After 24 hours cell growth, culture medium was removed with PlateMate Plus. Cells were then washed with 100 μL phosphate buffered saline (PBS, Thermofisher) followed with addition of 100 μL PBS with 100 mg/mL Calcium/Magnesium and 100 μL Dual-Glo® luciferase assay lysis buffer (Promega). 150 μL of the mixture were then transferred to a 384 white plate (Greiner Bio-One) and kept at room temperature for 10 minutes before firefly luminescence was read on TopCount (Perkin Elmer). After the first reading, the Dual-Glo® Stop & Glo® substrate was diluted 1:100 into Dual-Glo® Stop & Glo® buffer and 75 μL of the stop and glow reagent were added. Renilla luminescence was then read on TopCount (Perkin Elmer) after 10 minutes incubation at room temperature. The percentage of induction was reported based on the ratio of firefly/Renilla luminescence normalized to the control compound wells within each assay plate. Values are given as the mean of $N \geq 2$.

***In vivo* Biology.**

The PK/PD data (Figure 3) has been published with full description of the study. See: Koblish, H. K., et al., *Mol. Cancer Ther.* **2010,** *9*, 489-498. The *in vivo* efficacy study shown in Figure 2 for **4g** and **4f** is the original head-to-head study of the two lead compounds and is unpublished. The protocol is nearly identical to a different study of **4g** alone in the same in vivo efficacy model which was published in the article above.

Pharmacokinetic Assays

Protein Binding. The *in vitro* protein binding of INCB024360 was determined using plasma from humans. The Multi-Equilibrium Dialyzer System™ and diachema membranes from Harvard Apparatus (Holliston, MA) were used for the experiment. Equilibrium dialysis was carried out in teflon cells separated by a dialysis membrane with a molecular weight cut-off of 10,000 daltons. A plasma sample (1 mL) containing INCB024360 was added to one side of the membrane, while 1 mL of 0.133 M phosphate buffer (pH 7.4) was added to the other side. The cells were rotated at a speed of 12 rpm in a 37 °C water bath for 2 hours. Buffer and matrix samples were drained separately into empty pre-weighed tubes. The tubes were reweighed and the exact volume of sample recovered from each side of the dialysis cell was calculated. The same volume of either buffer or matrix was then added to the samples to ensure a final 1:1 buffer/matrix mixture was obtained for all the samples. An equal aliquot from each sample was protein precipitated with acetonitrile. The resulting supernatants were collected after centrifugation for analysis by LC/MS/MS.

Caco-2. Caco-2 cells were grown at 37 ^{o}C in an atmosphere of 5% CO_2 in DMEM growth medium supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) non-essential amino acids, penicillin (100 Unit/ml), and streptomycin (100 mg/ml). Confluent cell monolayers were subcultured every seven days by treatment with 0.25% trypsin containing 1 mM EDTA. Caco-2 cells were seeded at a density of 4,000 cells/well in 24-well plates on transwell PET (Polyethylene Terephthalate) membrane filters. The cells were grown until fully differentiated after 21 days and all experiments were conducted between 21 and 26 days. Cells of passage numbers 21 to 45 were used in these studies.

Cell culture media was removed and replaced with Hank's balanced salt solution. Cell membrane confluence was confirmed by measuring transepithelial electrical resistance (TEER) using a Millicell-ERS Voltohmmeter. Caco-2 cell monolayers with TEER values ≥ 300 $\Omega \cdot cm^2$ were used for transport experiments. For permeability studies in the absorptive direction (A-B), solutions of INCB024360 (50 μM) in 1% (v/v) DMSO/HBSS were added to the donor compartment (apical side).

HBSS solution with 4% BSA was added in the receiver compartment (basolateral side). The apical volume was 0.2 mL and the basolateral volume was 0.6 mL. Studies were of 120 minutes incubation, and 0.1 mL of sample was removed from the receiver side at the end of the 120 minute incubation and the same volume of acetonitrile was added for protein precipitation. The supernatants were collected after centrifugation for analysis by LC/MS.

(P1 + P2) Human Clearance. The intrinsic clearance of INCB024360 was determined *in vitro* using hepatic S9 fractions from rats. INCB024360 (5 μM) was incubated with Tris-HCl buffer (50 mM, pH 7.5), magnesium chloride (8mM), NADPH (2 mM), UDPGA (2 mM), alamethicin (12.7 μM) and S9 fractions (2 mg/mL of protein). The reactions were initiated by the addition of S9 fractions and incubated at 37 °C. Aliquots taken from 0 to 30 minutes and stopped by protein precipitation using acetonitrile. The resulting supernatants were analyzed by LC/MS.

Pharmacokinetic Studies. Male rats were given either a 5 mg/kg intravenous or 5 mg/kg oral dose. The vehicle for IV dosing was 10% dimethylacetamide (DMAC) in saline. The vehicle for oral dosing was a 0.5% methylcellulose (MC) aqueous solution. Male beagle dogs were given either a 5 mg/kg intravenous or 10 mg/kg oral dose. The vehicle for IV dosing was 10% DMAC in saline. The vehicle for oral dosing was 5% DMAC in 0.5% MC aqueous solution. Male cynomolgus monkeys were given either a 4 mg/kg intravenous or 10 mg/kg oral dose. The vehicle for IV dosing was 5% DMAC and 40% propylene glycol in saline. The vehicle for oral dosing was 0.5% MC aqueous solution.

Blood samples from each study were collected using EDTA as the anticoagulant at pre-dose until 24 hrs post-dose and centrifuged to obtain plasma. An analytical method for the quantification of INCB024360 in plasma was developed and utilized for non-GLP studies. The method uses a protein-precipitation extraction of samples using acetonitrile followed by LC/MS/MS analysis. The assay demonstrated a linear assay range from 2 to 10000 nM, utilizing 0.1 mL of plasma. The plasma concentration-time data was used to determine the pharmacokinetic parameters by standard non-compartmental methods.

Pharmacodynamic Studies. To determine the effect of IDO inhibition on plasma kynurenine, feasted C57BL/6 mice (Charles River) were administered a single dose of **4f** and **4g**, at which point food was removed from the cages. At various timepoints after dosing, mice were euthanized and blood was collected by cardiac puncture. Plasma was analyzed for the presence of **4f** and **4g**, tryptophan and kynurenine according to the methods below.

An analytical method for the quantification of tryptophan, kynurenine, and **4f** and **4g** was developed and utilized for non-GLP studies. The method combined a protein-precipitation extraction using trichloracetic acid and LC/MS/MS analysis. It demonstrated a linear assay range from 20 to 10,000 nM for kynurenine and **4f** and **4g** and 200 to 100,000 nM for tryptophan, analyzing 0.1 mL samples. Plasma samples were diluted 10 fold in water. Tissues are homogenized in 5% acetonitrile in water with 0.1% formic acid. The tissue dilution depended upon the mass of tissue (i.e. weight to volume ratio of 3 for tumors and 10 for lymph nodes). The homogenates are spun to allow for sampling of the supernatant. Aqueous standards are prepared to alleviate the need for adjustment of endogenous tryptophan and kynurenine present in biological matrices.

General Experimental Procedures and Analytical Characterization Methods

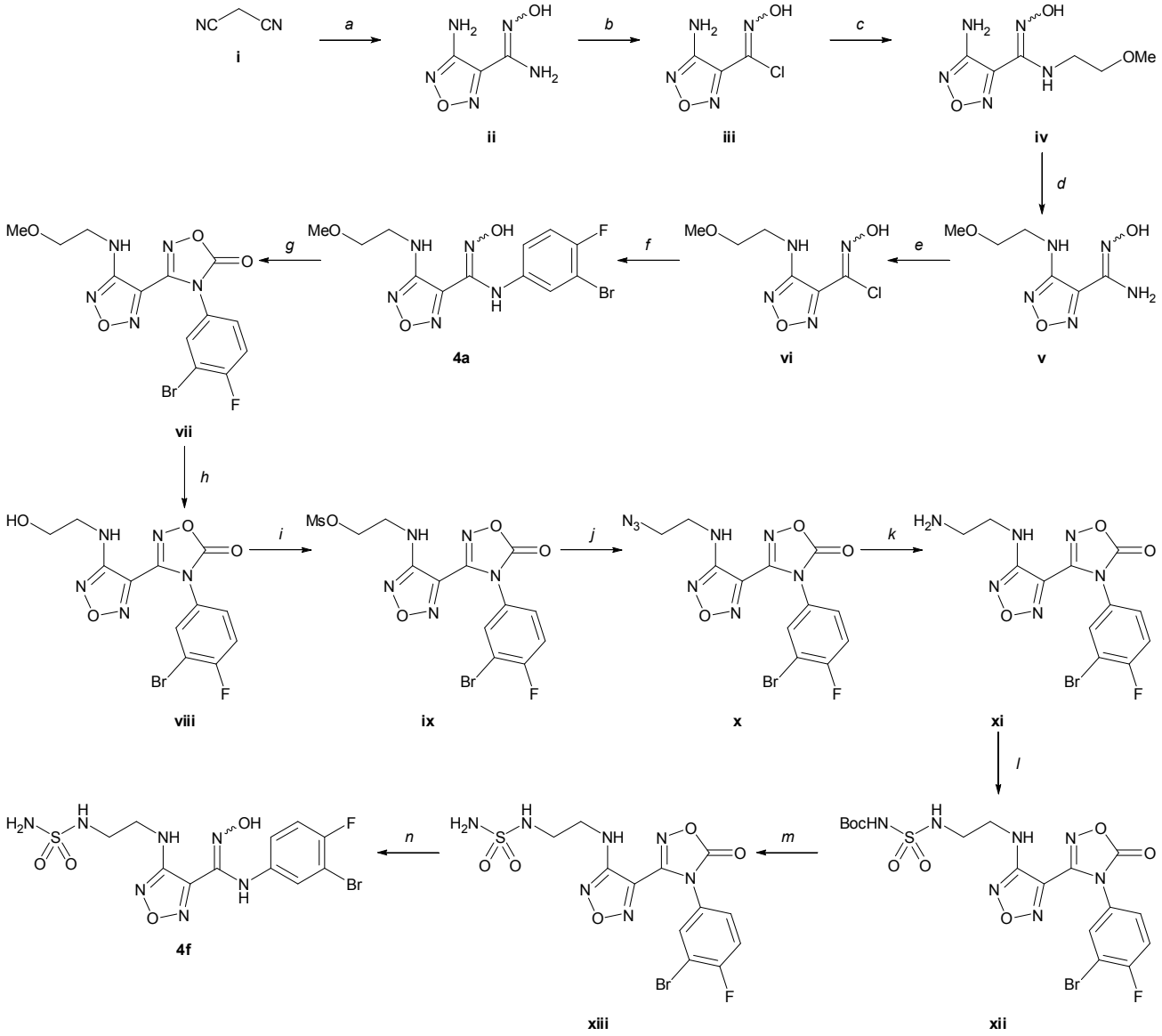
All reactions were run under an atmosphere of dry nitrogen. Unless otherwise noted, all reactions were performed at ambient temperature which averaged 20 °C. All solvents were used without further purification as acquired from commercial sources. NMR spectra were obtained using either a Varian Mercury-300, Mercury-400, or Inova-500 spectrometer. Chemical shifts are reported in parts per million relative to tetramethylsilane (TMS) as internal standard. All final products were characterized by ^{1}H NMR, HRMS or LCMS, and HPLC methods.

In general, ^{1}H NMR of some of the final compounds showed a small amount of the oxime isomer indicated by a large shift of the oxime proton. The oxime isomer could not be detected in HPLC or LCMS analysis and could only be detected with ^{1}H NMR, however, the amount of the minor isomer was dependent on the concentration and solvent used in the ^{1}H NMR analysis.

Purifications by flash chromatography were performed on RediSep columns using an Isco CombiFlash SG100c. Preparative LC purifications were performed on a Waters FractionLynx system using UV-triggered or mass directed fractionation and compound-specific method optimization (K. Blom, B. Glass, R. Sparks, A. Combs, "Preparative LC-MS Purification: Improved Compound Specific Method Optimization", *J. Combi. Chem.* (**2004**), *6*, 874-883).

Synthetic Schemes

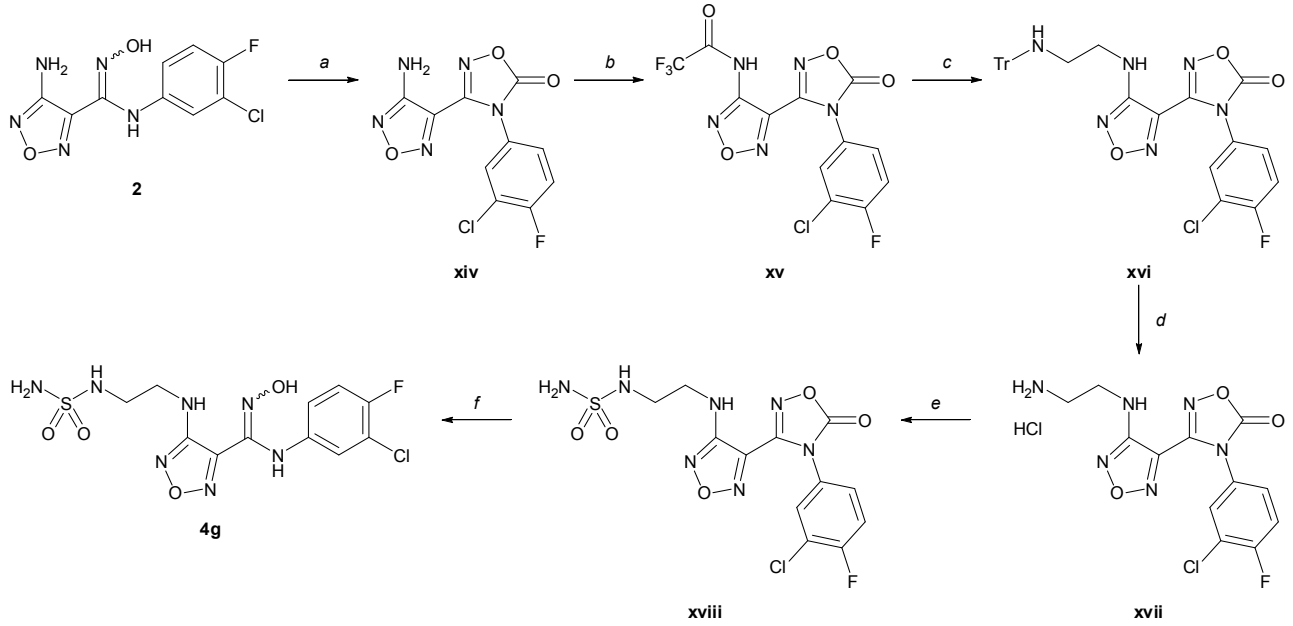
Scheme S1. Synthesis of 4a and epacadostat (4f).[a]



[a]Reagents: (a) $NaNO_2$, 6 N HCl; then NH_2OH, Δ, 2 h, 90%; (b) $NaNO_2$, AcOH, 6 N HCl, H_2O, NaCl, 0 °C, 1.5 h, 53%; (c) $MeOCH_2CH_2NH_2$, Et_3N, EtOAc, 0 °C, 5 min; (d) KOH, H_2O, 100 °C, overnight 81%; (e) $NaNO_2$, 6 N HCl, NaCl, H_2O, 0 °C, 2 h; (f) 3-bromo-4-fluoroaniline, $NaHCO_3$, H_2O, 60 °C, 20 min, 98%; (g) CDI, EtOAc, 60 °C, 20 min, 98%; (h) BBr_3, CH_2Cl_2, -67 °C to -10 °C, 30 min, 99%; (i) $MeSO_2Cl$, Et_3N, EtOAc, 2 h, quant.; (j) NaN_3, DMF, 50 °C, 6 h, 77%; (k) i: TMSCl, NaI, MeOH, 3.5 h; ii: Boc_2O, K_2CO_3 (83%, 2 steps); iii: 4 N HCl, 1,4-dioxane, 95%; (l) $ClSO_2NCO$,

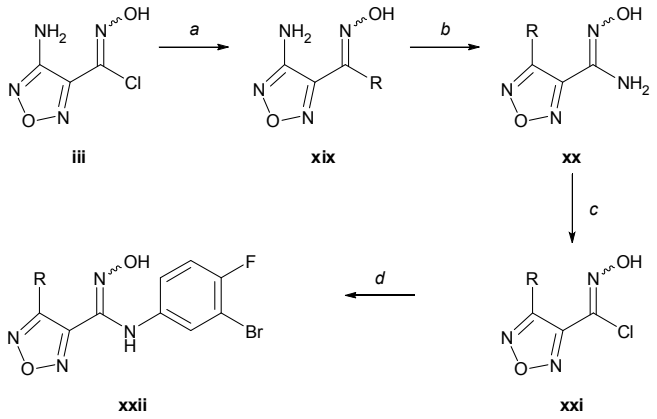
CH_2Cl_2, *t*-BuOH, 0 °C, 2 h; then Et_3N, -5 °C to 10 °C, 98%; (m) TFA, H_2O, 40-50 °C, 1 h, quant.; (n) 2 N NaOH, 3 h, 78%.

Scheme S2. Synthesis of 4g.[a]



[a]Reagents: (a) CDI, THF, Δ, 1 h, quantitative; (b) $(CF_3CO)_2O$, pyridine, CH_2Cl_2, 0 °C to 20 °C, 10 min, 99%; (c) $HOCH_2CH_2NHTr$, DIAD, Ph_3P, THF, 0 °C to 20 °C, 16 h, 74%; (d) TFA, *i*Pr_3SiH, 25 °C, 30 min; then 4 N HCl in dioxane, MeOH, 25 °C, 15 min, 98%; (e) $ClSO_2NCO$, CH_2Cl_2, *t*-BuOH, 0 °C to 25 °C, 1 h; then Et_3N, 0 °C to 25 °C, 3 h, 78%; (f) 2 N NaOH, MeOH, 25 °C, 2 h, 92%.

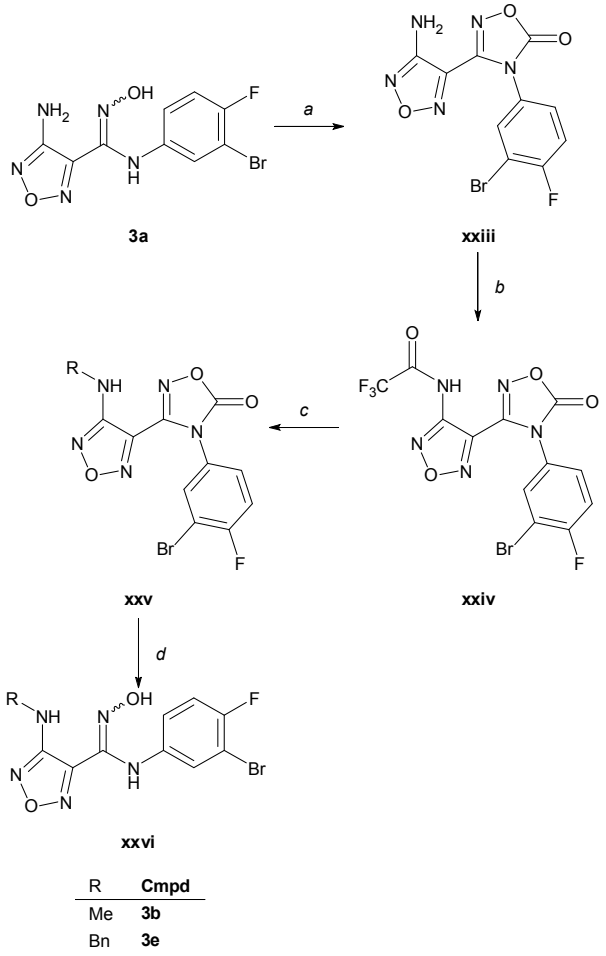
Scheme S3. Synthesis of 3c, 3d, 4b, 4d and 4e.[a]



R	Cmpd
NMe_2	**3c**
NHEt	**3d**
$NHCH_2CH_2OH$	**4b**
$NHCH_2CH_2NHAc$	**4d**
$NHCH_2CH_2NHSO_2Me$	**4e**

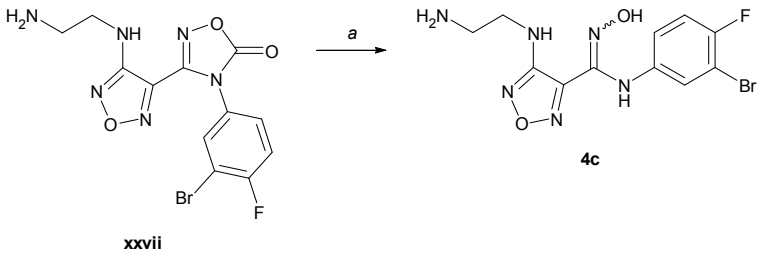
[a]Reagents: (a) R = Me_2NH or $EtNH_2$ or $NH_2CH_2CH_2OH$ or $NH_2CH_2CH_2NHAc$ or $NH_2CH_2CH_2NHSO_2Me$, Et_3N, EtOH, 0 → 25 °C, 56-58%; (b) KOH, 100 °C, overnight, 20-100%; (c) $NaNO_2$, 6 N HCl, NaCl, HCl, H_2O, 0 °C, 1.5 h, 60-100%; (d) 3-bromo-4-fluoroaniline, Et_3N, EtOH, 0 → 20 °C, 7-100%.

Scheme S4. Synthesis of 3b and 3e.[a]



[a]Reagents: (a) CDI, THF, Δ, 1 h, 96%; (b) $(CF_3CO)_2O$, pyridine, 20 °C, 3 h, 76%; (c) MeI or BnBr, K_2CO_3, 20 °C, overnight, 45-77%; (d) 2 N NaOH, 30 min, 70%.

Scheme S5. Synthesis of 4c.[a]



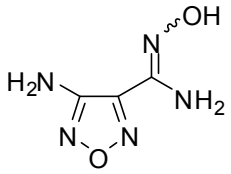
[a]Reagents: (a) 2 N NaOH, 1 h, 74%.

Experimental Procedures and Analytical Data for Compounds 3-4

The syntheses of **4a** and **4f** are illustrated in **Scheme S1** and detailed below.

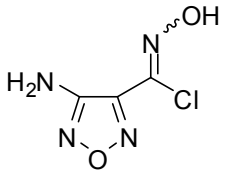
4-({2-[(Aminosulfonyl)amino]ethyl}amino)-*N*-(3-bromo-4-fluorophenyl)-*N′*-hydroxy-1,2,5-oxadiazole-3-carboximidamide (4f).

Step A: 4-Amino-*N′*-hydroxy-1,2,5-oxadiazole-3-carboximidamide



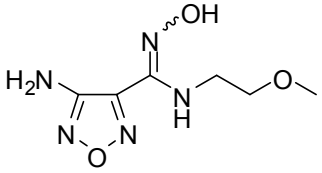
Malononitrile [Aldrich, product # M1407] (320.5 g, 5 mol) was added to water (7 L) preheated to 45 °C and stirred for 5 min. The resulting solution was cooled in an ice bath and sodium nitrite (380 g, 5.5 mol) was added. When the temperature reached 10 °C, 6 N hydrochloric acid (55 mL) was added. A mild exothermic reaction ensued with the temperature reaching 16 °C. After 15 min the cold bath was removed and the reaction mixture was stirred for 1.5 hrs at 16-18 °C. The reaction mixture was cooled to 13 °C and 50% aqueous hydroxylamine (990 g, 15 mol) was added all at once. The temperature rose to 26 °C. When the exothermic reaction subsided the cold bath was removed and stirring was continued for 1 hr at 26-27 °C, then it was slowly brought to reflux. Reflux was maintained for 2 hrs and then the reaction mixture was allowed to cool overnight. The reaction mixture was stirred in an ice bath and 6 N hydrochloric acid (800 mL) was added in portions over 40 min to pH 7.0. Stirring was continued in the ice bath at 5 °C. The precipitate was collected by filtration, washed well with water and dried in a vacuum oven (50 °C) to give the desired product (644 g, 90%). LCMS for $C_3H_6N_5O_2$ $(M+H)^+$: m/z = 144.0. ^{13}C NMR (75 MHz, CD_3OD): δ 156.0, 145.9, 141.3.

Step B: 4-Amino-*N*-hydroxy-1,2,5-oxadiazole-3-carboximidoyl chloride



4-Amino-*N*′-hydroxy-1,2,5-oxadiazole-3-carboximidamide (422 g, 2.95 mol) was added to a mixture of water (5.9 L), acetic acid (3 L) and 6 N hydrochloric acid (1.475 L, 3 eq.) and this suspension was stirred at 42 - 45 °C until complete solution was achieved. Sodium chloride (518 g, 3 eq.) was added and this solution was stirred in an ice/water/methanol bath. A solution of sodium nitrite (199.5 g, 0.98 eq.) in water (700 mL) was added over 3.5 hrs while maintaining the temperature below 0 °C. After complete addition stirring was continued in the ice bath for 1.5 hrs and then the reaction mixture was allowed to warm to 15 °C. The precipitate was collected by filtration, washed well with water, taken in ethyl acetate (3.4 L), treated with anhydrous sodium sulfate (500 g) and stirred for 1 hr. This suspension was filtered through sodium sulfate (200 g) and the filtrate was concentrated on a rotary evaporator. The residue was dissolved in methyl *t*-butyl ether (5.5 L), treated with charcoal (40 g), stirred for 40 min and filtered through Celite. The solvent was removed in a rotary evaporator and the resulting product was dried in a vacuum oven (45 ^{o}C) to give the desired product (256 g, 53.4%). LCMS for $C_3H_4ClN_4O_2$ $(M+H)^+$: m/z = 162.9. ^{13}C NMR (100 MHz, CD_3OD): δ 155.8, 143.4, 129.7.

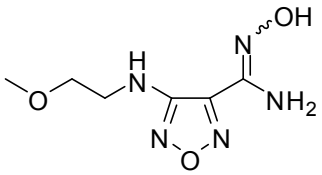
Step C: 4-Amino-*N*′-hydroxy-*N*-(2-methoxyethyl)-1,2,5-oxadiazole-3-carboximidamide



4-Amino-*N*-hydroxy-1,2,5-oxadiazole-3-carboximidoyl chloride (200.0 g, 1.23 mol) was mixed with ethyl acetate (1.2 L). At 0-5 °C 2-methoxyethylamine [Aldrich, product # 143693] (119.0 mL, 1.35 mol) was added in one portion while stirring. The reaction temperature rose to 41 °C. The reaction was cooled to 0 - 5 °C. Triethylamine (258 mL, 1.84 mol) was added. After stirring 5 min, LCMS indicated reaction completion. The reaction solution was washed with water (500 mL) and brine (500 mL), dried

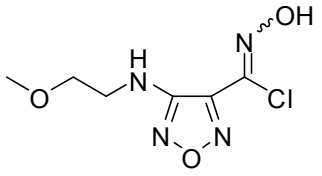
over sodium sulfate, and concentrated to give the desired product (294 g, 119%) as a crude dark oil. LCMS for $C_6H_{12}N_5O_3$ $(M+H)^+$: m/z = 202.3. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.65 (s, 1 H), 6.27 (s, 2 H), 6.10 (t, J = 6.5 Hz, 1 H), 3.50 (m, 2 H), 3.35 (d, J = 5.8 Hz, 2 H), 3.08 (s, 3 H).

Step D: *N′*-Hydroxy-4-[(2-methoxyethyl)amino]-1,2,5-oxadiazole-3-carboximidamide



4-Amino-*N′*-hydroxy-*N*-(2-methoxyethyl)-1,2,5-oxadiazole-3-carboximidamide (248.0 g, 1.23 mol) was mixed with water (1 L). Potassium hydroxide (210 g, 3.7 mol) was added. The reaction was refluxed at 100 °C overnight (15 hours). TLC with 50% ethyl acetate (containing 1% ammonium hydroxide) in hexane indicated reaction completed (product Rf = 0.6, starting material Rf = 0.5). LCMS also indicated reaction completion. The reaction was cooled to room temperature and extracted with ethyl acetate (3 x 1 L). The combined ethyl acetate solution was dried over sodium sulfate and concentrated to give the desired product (201 g, 81%) as a crude off-white solid. LCMS for $C_6H_{12}N_5O_3$ $(M+H)^+$: m/z = 202.3 ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.54 (s, 1 H), 6.22 (s, 2 H), 6.15 (t, J = 5.8 Hz, 1 H), 3.45 (t, J = 5.3 Hz, 2 H), 3.35 (m, 2 H), 3.22 (s, 3 H).

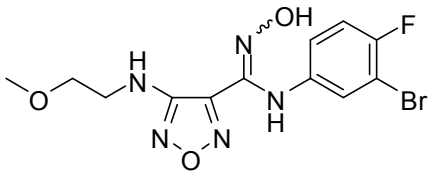
Step E: *N*-Hydroxy-4-[(2-methoxyethyl)amino]-1,2,5-oxadiazole-3-carboximidoyl chloride



At room temperature *N′*-hydroxy-4-[(2-methoxyethyl)amino]-1,2,5-oxadiazole-3-carboximidamide (50.0 g, 0.226 mol) was dissolved in 6.0 M hydrochloric acid aqueous solution (250 mL, 1.5 mol). Sodium chloride (39.5 g, 0.676 mol) was added followed by water (250 mL) and ethyl acetate (250 mL). At 3-5 °C a previously prepared aqueous solution (100 mL) of sodium nitrite (15.0 g,

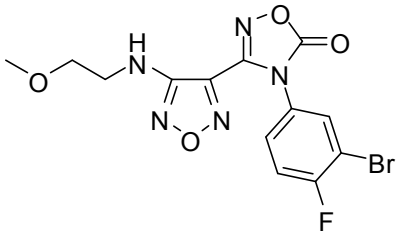
0.217 mol) was added slowly over 1 hr. The reaction was stirred at 3 - 8 °C for 2 hours and then room temperature over the weekend. LCMS indicated reaction completed. The reaction solution was extracted with ethyl acetate (2 x 200 mL). The combined ethyl acetate solution was dried over sodium sulfate and concentrated to give the desired product (49.9 g, 126%) as a crude white solid. LCMS for $C_6H_{10}ClN_4O_3$ $(M+H)^+$: m/z = 221.0. ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.43 (s, 1 H), 5.85 (t, J = 5.6 Hz, 1 H), 3.50 (t, J = 5.6 Hz, 2 H), 3.37(dd, J = 10.8, 5.6 Hz, 2 H), 3.25 (s, 3 H).

Step F: *N*-(3-Bromo-4-fluorophenyl)-*N*′-hydroxy-4-[(2-methoxyethyl)amino]-1,2,5-oxadiazole-3-carboximidamide (**4a**)



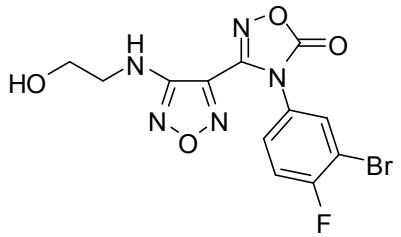
N-Hydroxy-4-[(2-methoxyethyl)amino]-1,2,5-oxadiazole-3-carboximidoyl chloride (46.0 g, 0.208 mol) was mixed with water (300 mL). The mixture was heated to 60 °C. 3-Bromo-4-fluoroaniline [Oakwood products, product # 013091] (43.6 g, 0.229 mol) was added and stirred for 10 min. A warm sodium bicarbonate (26.3 g, 0.313 mol) solution (300 mL water) was added over 15 min. The reaction was stirred at 60 °C for 20 min. LCMS indicated reaction completion. The reaction solution was cooled to room temperature and extracted with ethyl acetate (2 x 300 mL). The combined ethyl acetate solution was dried over sodium sulfate and concentrated to give the desired product (76.7 g, 98%) as a crude brown solid. LCMS for $C_{12}H_{14}BrFN_5O_3$ $(M+H)^+$: m/z = 374.0, 376.0. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.55 (s, 1 H), 8.85 (s, 1 H), 7.16 (t, J = 8.8 Hz, 1 H), 7.08 (dd, J = 6.1, 2.7 Hz, 1 H), 6.75 (m, 1 H), 6.14 (t, J = 5.8 Hz, 1 H), 3.48 (t, J = 5.2 Hz, 2 H), 3.35 (dd, J = 10.8, 5.6 Hz, 2 H), 3.22 (s, 3 H).

Step G: 4-(3-Bromo-4-fluorophenyl)-3-{4-[(2-methoxyethyl)amino]-1,2,5-oxadiazol-3-yl}-1,2,4-oxadiazol-5(4*H*)-one



A mixture of *N*-(3-bromo-4-fluorophenyl)-*N*′-hydroxy-4-[(2-methoxyethyl)amino]-1,2,5-oxadiazole-3-carboximidamide (76.5 g, 0.204 mol), 1,1'-carbonyldiimidazole (49.7 g, 0.307 mol), and ethyl acetate (720 mL) was heated to 60 °C and stirred for 20 min. LCMS indicated reaction completed. The reaction was cooled to room temperature, washed with 1 N HCl (2 x 750 mL), dried over sodium sulfate, and concentrated to give the desired product (80.4 g, 98%) as a crude brown solid. LCMS for $C_{13}H_{12}BrFN_5O_4$ $(M+H)^+$: m/z = 400.0, 402.0. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.94 (t, *J* = 8.2 Hz, 1 H), 7.72 (dd, *J* = 9.1, 2.3 Hz, 1 H), 7.42 (m, 1 H), 6.42 (t, *J*= 5.7 Hz, 1 H), 3.46 (t, *J* = 5.4 Hz, 2 H), 3.36 (t, *J* = 5.8 Hz, 2 H), 3.26 (s, 3 H).

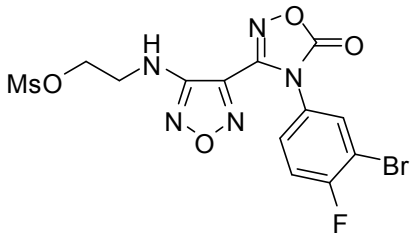
Step H: 4-(3-Bromo-4-fluorophenyl)-3-{4-[(2-hydroxyethyl)amino]-1,2,5-oxadiazol-3-yl}-1,2,4-oxadiazol-5(4*H*)-one



4-(3-Bromo-4-fluorophenyl)-3-{4-[(2-methoxyethyl)amino]-1,2,5-oxadiazol-3-yl}-1,2,4-oxadiazol-5(4*H*)-one (78.4 g, 0.196 mol) was dissolved in dichloromethane (600 mL). At -67 °C boron tribromide (37 mL, 0.392 mol) was added over 15 min. The reaction was warmed up to -10 °C in 30 min. LCMS indicated reaction completed. The reaction was stirred at room temperature for 1 hour. At 0 - 5 °C the reaction was slowly quenched with saturated sodium bicarbonate solution (1.5 L) over 30 min. The reaction temperature rose to 25 °C. The reaction was extracted with ethyl acetate (2 x 500 mL, first extraction organic layer is on the bottom and second extraction organic lager is on the top).

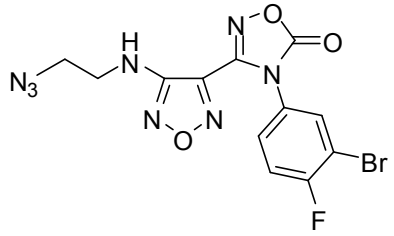
The combined organic layers were dried over sodium sulfate and concentrated to give the desired product (75 g, 99%) as a crude brown solid. LCMS for $C_{12}H_{10}BrFN_5O_4$ $(M+H)^+$: m/z = 386.0, 388.0. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.08 (dd, J = 6.2, 2.5 Hz, 1 H), 7.70 (m, 1 H), 7.68 (t, J = 8.7 Hz, 1 H), 6.33 (t, J = 5.6 Hz, 1 H), 4.85 (t, J = 5.0 Hz, 1 H), 3.56 (dd, J = 10.6, 5.6 Hz, 2 H), 3.29 (dd, J = 11.5, 5.9 Hz, 2 H).

Step I: 2-({4-[4-(3-Bromo-4-fluorophenyl)-5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl]-1,2,5-oxadiazol-3-yl}amino)ethyl methanesulfonate



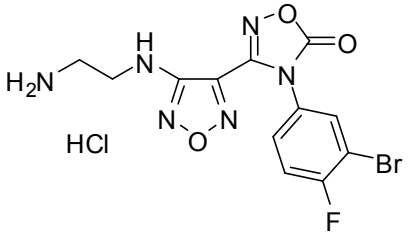
To a solution of 4-(3-bromo-4-fluorophenyl)-3-{4-[(2-hydroxyethyl)amino]-1,2,5-oxadiazol-3-yl}-1,2,4-oxadiazol-5(4*H*)-one (1.5 kg, 3.9 mol, containing also some of the corresponding bromo-compound) in ethyl acetate (12 L) was added methanesulfonyl chloride (185 mL, 2.4 mol) dropwise over 1 h at room temperature. Triethylamine (325 mL, 2.3 mol) was added dropwise over 45 min, during which time the reaction temperature increased to 35 °C. After 2 h, the reaction mixture was washed with water (5 L), brine (1 L), dried over sodium sulfate, combined with 3 more reactions of the same size, and the solvents removed *in vacuo* to afford the desired product (7600 g, quantitative yield) as a tan solid. LCMS for $C_{13}H_{11}BrFN_5O_6SNa$ $(M+Na)^+$: m/z = 485.9, 487.9. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.08 (dd, J = 6.2, 2.5 Hz, 1 H), 7.72 (m, 1 H), 7.58 (t, J = 8.7 Hz, 1 H), 6.75 (t, J = 5.9 Hz, 1 H), 4.36 (t, J = 5.3 Hz, 2 H), 3.58 (dd, J = 11.2, 5.6 Hz, 2 H), 3.18 (s, 3 H).

Step J: 3-{4-[(2-Azidoethyl)amino]-1,2,5-oxadiazol-3-yl}-4-(3-bromo-4-fluorophenyl)-1,2,4-oxadiazol-5(4*H*)-one



To a solution of 2-({4-[4-(3-bromo-4-fluorophenyl)-5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl]-1,2,5-oxadiazol-3-yl}amino)ethyl methanesulfonate (2.13 kg, 4.6 mol, containing also some of the corresponding bromo-compound) in dimethylformamide (4 L) stirring in a 22 L flask was added sodium azide (380 g, 5.84 mol). The reaction was heated at 50 °C for 6 h, poured into ice/water (8 L), and extracted with 1:1 ethyl acetate:heptane (20 L). The organic layer was washed with water (5 L) and brine (5 L), and the solvents removed *in vacuo* to afford the desired product (1464 g, 77%) as a tan solid. LCMS for $C_{12}H_8BrFN_8O_3Na$ $(M+Na)^+$: m/z = 433.0, 435.0. ^1H NMR (400 MHz, DMSO-d_6): δ 8.08 (dd, J = 6.2, 2.5 Hz, 1 H), 7.72 (m, 1 H), 7.58 (t, J = 8.7 Hz, 1 H), 6.75 (t, J = 5.7 Hz, 1 H), 3.54 (t, J = 5.3 Hz, 2 H), 3.45 (dd, J = 11.1, 5.2 Hz, 2 H).

Step K: 3-{4-[(2-Aminoethyl)amino]-1,2,5-oxadiazol-3-yl}-4-(3-bromo-4-fluorophenyl)-1,2,4-oxadiazol-5(4*H*)-one hydrochloride

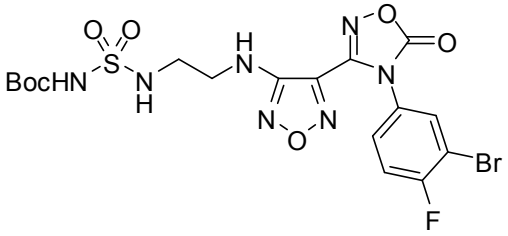


Sodium iodide (1080 g, 7.2 mol) was added to 3-{4-[(2-azidoethyl)amino]-1,2,5-oxadiazol-3-yl}-4-(3-bromo-4-fluorophenyl)-1,2,4-oxadiazol-5(4*H*)-one (500 g, 1.22 mol) in methanol (6 L). The mixture was allowed to stir for 30 min during which time a mild exotherm was observed. Chlorotrimethylsilane (930 mL, 7.33 mol) was added as a solution in methanol (1 L) dropwise at a rate so that the temperature did not exceed 35 °C, and the reaction was allowed to stir for 3.5 h at ambient temperature. The reaction was neutralized with 33 wt% solution of sodium thiosulfate pentahydrate in

water (~1.5 L), diluted with water (4 L), and the pH adjusted to 9 carefully with solid potassium carbonate (250 g – added in small portions: watch foaming). Di-*tert*-butyl dicarbonate (318 g, 1.45 mol) was added and the reaction was allowed to stir at room temperature. Additional potassium carbonate (200 g) was added in 50 g portions over 4 h to ensure that the pH was still at or above 9. After stirring at room temperature overnight, the solid was filtered, triturated with water (2 L), and then MTBE (1.5 L). A total of 11 runs were performed (5.5 kg, 13.38 mol). The combined solids were triturated with 1:1 THF:dichloromethane (24 L, 4 runs in a 20 L rotary evaporator flask, 50 °C, 1 h), filtered, and washed with dichloromethane (3 L each run) to afford an off-white solid. The crude material was dissolved at 55 °C tetrahydrofuran (5 mL/g), treated with decolorizing carbon (2 wt%) and silica gel (2 wt%), and filtered hot through celite to afford the product as an off-white solid (5122 g). The combined MTBE, THF, and dichloromethane filtrates were concentrated *in vacuo* and chromatographed (2 kg silica gel, heptane with a 0-100% ethyl acetate gradient, 30 L) to afford more product (262 g). The combined solids were dried to a constant weight in a convection oven (5385 g, 83%).

In a 22 L flask was charged hydrogen chloride (4 N solution in 1,4-dioxane, 4 L, 16 mol). *tert*-Butyl [2-({4-[4-(3-bromo-4-fluorophenyl)-5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl]-1,2,5-oxadiazol-3-yl}amino)ethyl]carbamate (2315 g, 4.77 mol) was added as a solid in portions over 10 min. The slurry was stirred at room temperature and gradually became a thick paste that could not be stirred. After sitting overnight at room temperature, the paste was slurried in ethyl acetate (10 L), filtered, re-slurried in ethyl acetate (5 L), filtered, and dried to a constant weight to afford the desired product as a white solid (combined with other runs, 5 kg starting material charged, 4113 g, 95%). LCMS for $C_{12}H_{11}BrFN_6O_3$ $(M+H)^+$: m/z = 384.9, 386.9. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.12 (m, 4 H), 7.76 (m, 1 H), 7.58 (t, J = 8.7 Hz, 1 H), 6.78 (t, J = 6.1 Hz, 1 H), 3.51 (dd, J = 11.8, 6.1 Hz, 2 H), 3.02 (m, 2 H).

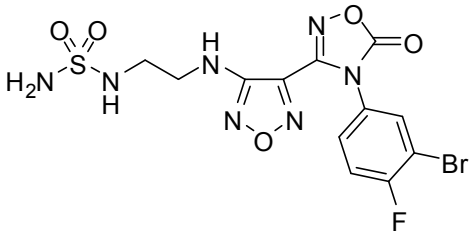
Step L: *tert*-Butyl ({[2-({4-[4-(3-bromo-4-fluorophenyl)-5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl]-1,2,5-oxadiazol-3-yl}amino)ethyl]amino}sulfonyl)carbamate



A 5 L round bottom flask was charged with chlorosulfonyl isocyanate [Aldrich, product # 142662] (149 mL, 1.72 mol) and dichloromethane (1.5 L) and cooled using an ice bath to 2 °C. *tert*-Butanol (162 mL, 1.73 mol) in dichloromethane (200 mL) was added dropwise at a rate so that the temperature did not exceed 10 °C. The resulting solution was stirred at room temperature for 30-60 min to provide *tert*-butyl [chlorosulfonyl]carbamate.

A 22 L flask was charged with 3-{4-[(2-aminoethyl)amino]-1,2,5-oxadiazol-3-yl}-4-(3-bromo-4-fluorophenyl)-1,2,4-oxadiazol-5(4*H*)-one hydrochloride (661 g, 1.57 mol) and 8.5 L dichloromethane. After cooling to -15 °C with an ice/salt bath, the solution of *tert*-butyl [chlorosulfonyl]carbamate (prepared as above) was added at a rate so that the temperature did not exceed -10 °C (addition time 7 min). After stirring for 10 min, triethylamine (1085 mL, 7.78 mol) was added at a rate so that the temperature did not exceed -5 °C (addition time 10 min). The cold bath was removed, the reaction was allowed to warm to 10 °C, split into two portions, and neutralized with 10% conc HCl (4.5 L each portion). Each portion was transferred to a 50 L separatory funnel and diluted with ethyl acetate to completely dissolve the white solid (~25 L). The layers were separated, and the organic layer was washed with water (5 L), brine (5 L), and the solvents removed *in vacuo* to afford an off-white solid. The solid was triturated with MTBE (2 x 1.5 L) and dried to a constant weight to afford a white solid. A total of 4113 g starting material was processed in this manner (5409 g, 98%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.90 (s, 1 H), 8.08 (dd, J = 6.2, 2.5 Hz, 1 H), 7.72 (m, 1 H), 7.59 (t, J = 8.6 Hz, 1 H), 6.58 (t, J = 5.7 Hz, 1 H), 3.38 (dd, J = 12.7, 6.2 Hz, 2 H), 3.10 (dd, J = 12.1, 5.9 Hz, 2 H), 1.41 (s, 9 H).

Step M: *N*-[2-({4-[4-(3-Bromo-4-fluorophenyl)-5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl]-1,2,5-oxadiazol-3-yl}amino)ethyl]sulfamide



To a 22 L flask containing 98:2 trifluoroacetic acid:water (8.9 L) was added *tert*-butyl ({[2-({4-[4-(3-bromo-4-fluorophenyl)-5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl]-1,2,5-oxadiazol-3-yl}amino)ethyl]amino}sulfonyl)carbamate (1931 g, 3.42 mol) in portions over 10 minutes. The resulting mixture was stirred at room temperature for 1.5 h, the solvents removed *in vacuo*, and chased with dichloromethane (2 L). The resulting solid was treated a second time with fresh 98:2 trifluoroacetic acid:water (8.9 L), heated for 1 h at 40-50 °C, the solvents removed *in vacuo*, and chased with dichloromethane (3 x 2 L). The resulting white solid was dried in a vacuum drying oven at 50 °C overnight. A total of 5409 g was processed in this manner (4990 g, quant. yield). LCMS for $C_{12}H_{12}BrFN_7O_5S$ $(M+H)^+$: m/z = 463.9, 465.9. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.08 (dd, J = 6.2, 2.5 Hz, 1 H), 7.72 (m, 1 H), 7.59 (t, J = 8.7 Hz, 1 H), 6.67 (t, J = 5.9 Hz, 1H), 6.52 (t, J = 6.0 Hz, 1 H), 3.38 (dd, J = 12.7, 6.3 Hz, 2 H), 3.11 (dd, J = 12.3, 6.3 Hz).

Step N: 4-({2-[(Aminosulfonyl)amino]ethyl}amino)-*N*-(3-bromo-4-fluorophenyl)-*N*′-hydroxy-1,2,5-oxadiazole-3-carboximidamide (**4f**)

To a crude mixture of *N*-[2-({4-[4-(3-bromo-4-fluorophenyl)-5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl]-1,2,5-oxadiazol-3-yl}amino)ethyl]sulfamide (2.4 mol) containing residual amounts of trifluoroacetic acid stirring in a 22 L flask was added THF (5 L). The resulting solution was cooled to 0 °C using an ice bath and 2 N NaOH (4 L) was added at a rate so that the temperature did not exceed 10 °C. After stirring at ambient temperature for 3 h (LCMS indicated no starting material remained), the pH was adjusted to 3-4 with concentrated HCl (~500 mL). The THF was removed *in vacuo*, and the

resulting mixture was extracted with ethyl acetate (15 L). The organic layer was washed with water (5 L), brine (5 L), and the solvents removed *in vacuo* to afford a solid. The solid was triturated with MTBE (2 x 2 L), combined with three other reactions of the same size, and dried overnight in a convection oven to afford a white solid (3535 g). The solid was recrystallized (3 x 22 L flasks, 2:1 water:ethanol, 14.1 L each flask) and dried in a 50 °C convection oven to a constant weight to furnish the title compound as an off-white solid (3290 g, 78%). HRMS (ESI/QTof) m/z: $[M+H]^+$ Calcd for $C_{11}H_{14}BrFN_7O_4S$ 437.9990, 439.9969; Found 437.9977, 439.9956. ^1H NMR: please see Table S1.

Figure S1. NMR Numbering Scheme for INCB024360 (4f).

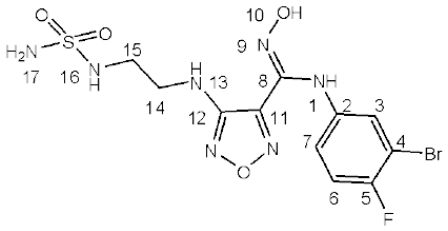


Figure S2. ^1H NMR Spectrum for INCB024360 (4f).

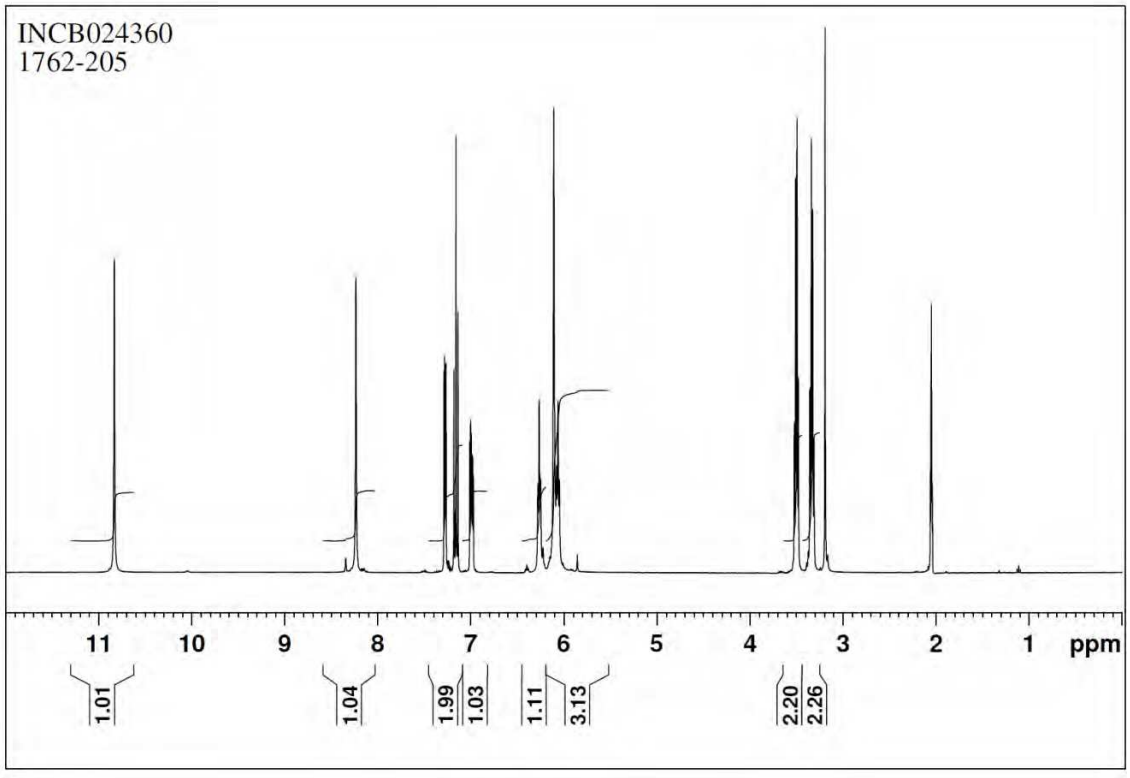


Figure S3. ^{13}C NMR Spectrum for INCB024360 (4f).

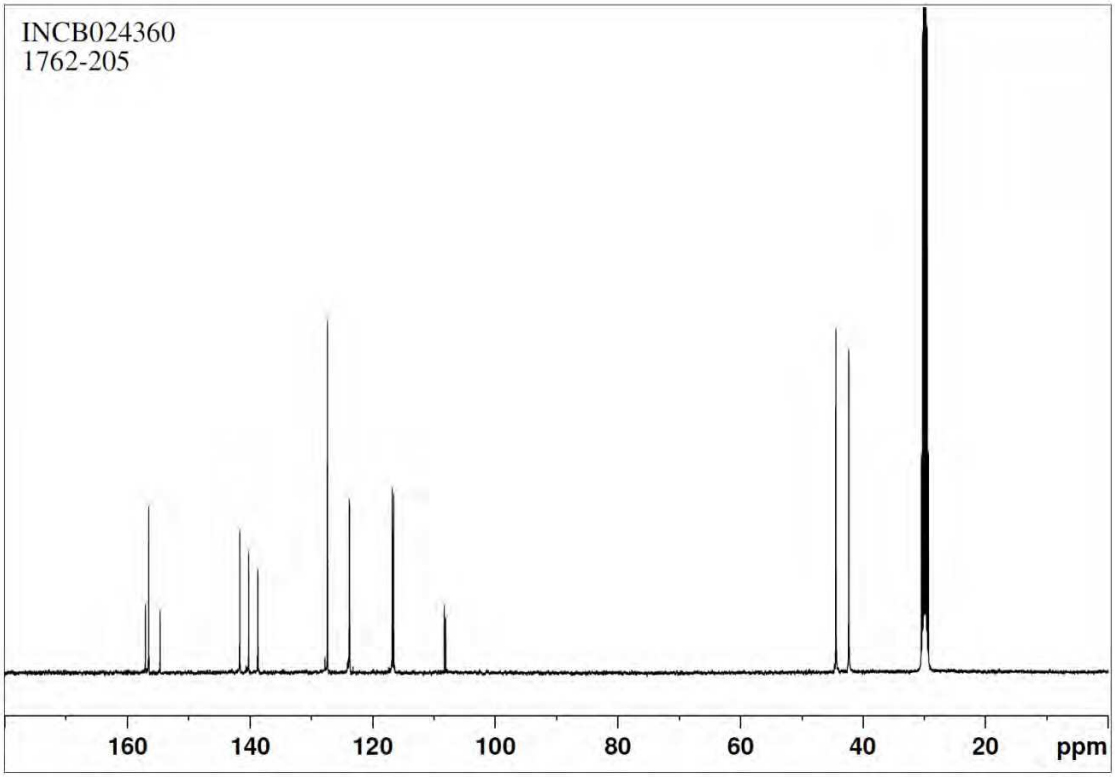


Table S1. ^{1}H and ^{13}C NMR Spectral Assignments for INCB024360 (4f).

Position	^{1}H Chemical Shift (ppm)	Multiplicity	Coupling Constant(s) (Hz)	Relative Intensity	^{13}C Chemical Shift (ppm)	Coupling Constants J_{CF} (Hz)
1	8.24	s	--	1H	--	--
2	--	--	--	--	141.7	--
3	7.28	dd	6.1, 2.7	1H	127.3	--
4	--	--	--	--	108.2	22.1
5	--	--	--	--	155.9	241.1
6	7.16	dd	8.7, 8.6	1H	116.7	23.5
7	6.99	ddd	8.8, 4.1, 2.8	1H	123.8	7.1
8	--	--	--	--	138.7	--
10	10.84	s	--	1H	--	--
11	--	--	--	--	140.2	--
12	--	--	--	--	156.5	--
13	6.07	t	6.0	1H	--	--
14	3.50	m	--	2H	44.4	--
15	3.34	m	--	2H	42.3	--
16	6.27	t	6.0	1H	--	--
17	6.11	s	--	2H	--	--

Structure Proof for INCB24360 (4f) using 2D NMR

Complete structure assignment and proof of bond connectivity was performed using multiple 2D NMR experiments.

Figure S4. Gcosy (Gradient cosy (Correlation spectroscopy)) - ^{1}H-^{1}H coupling.

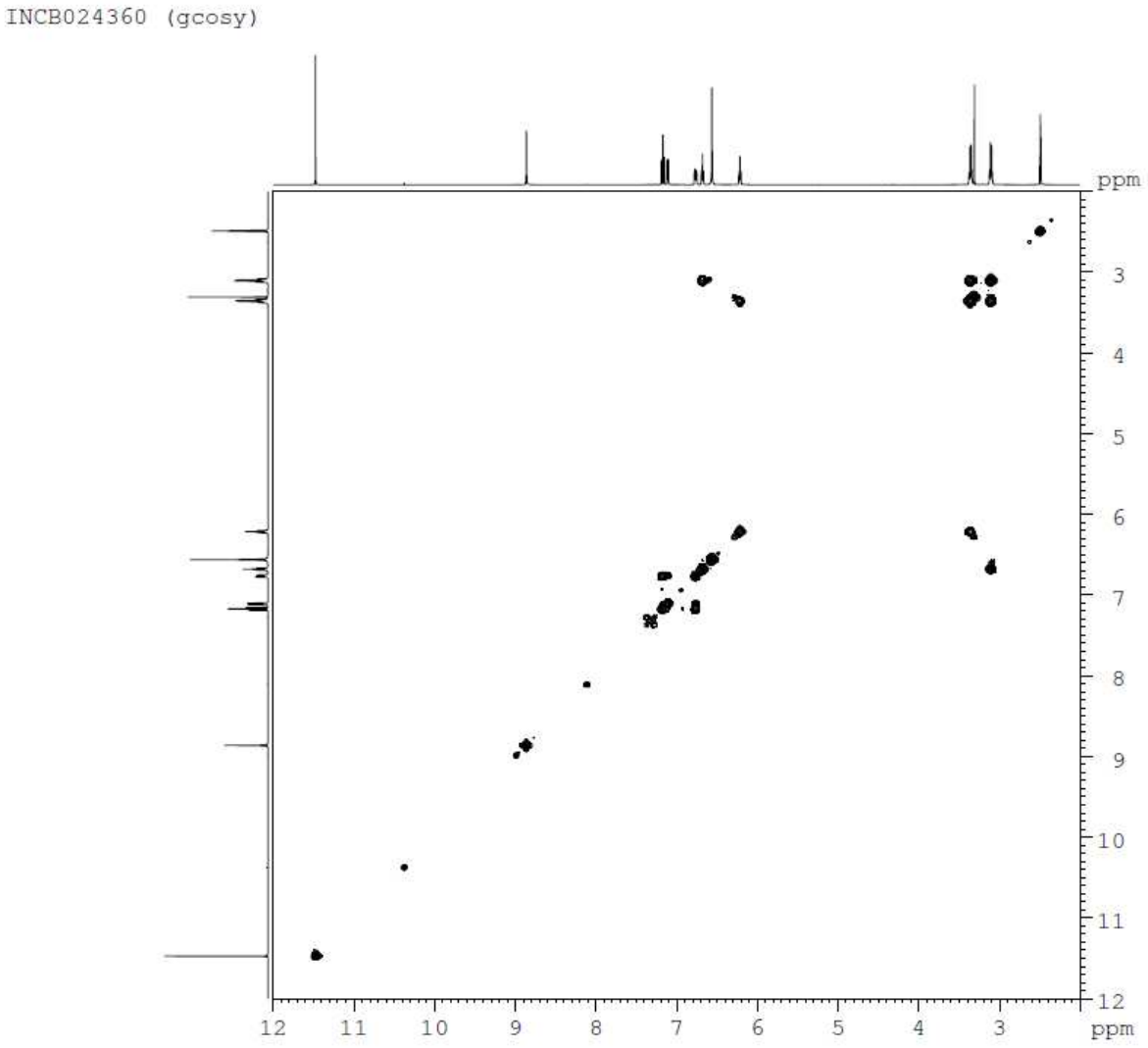


Figure S5. Ghsqc (Gradient Heteronuclear Single Quantum Correlation) - directly bonded C-H correlations.

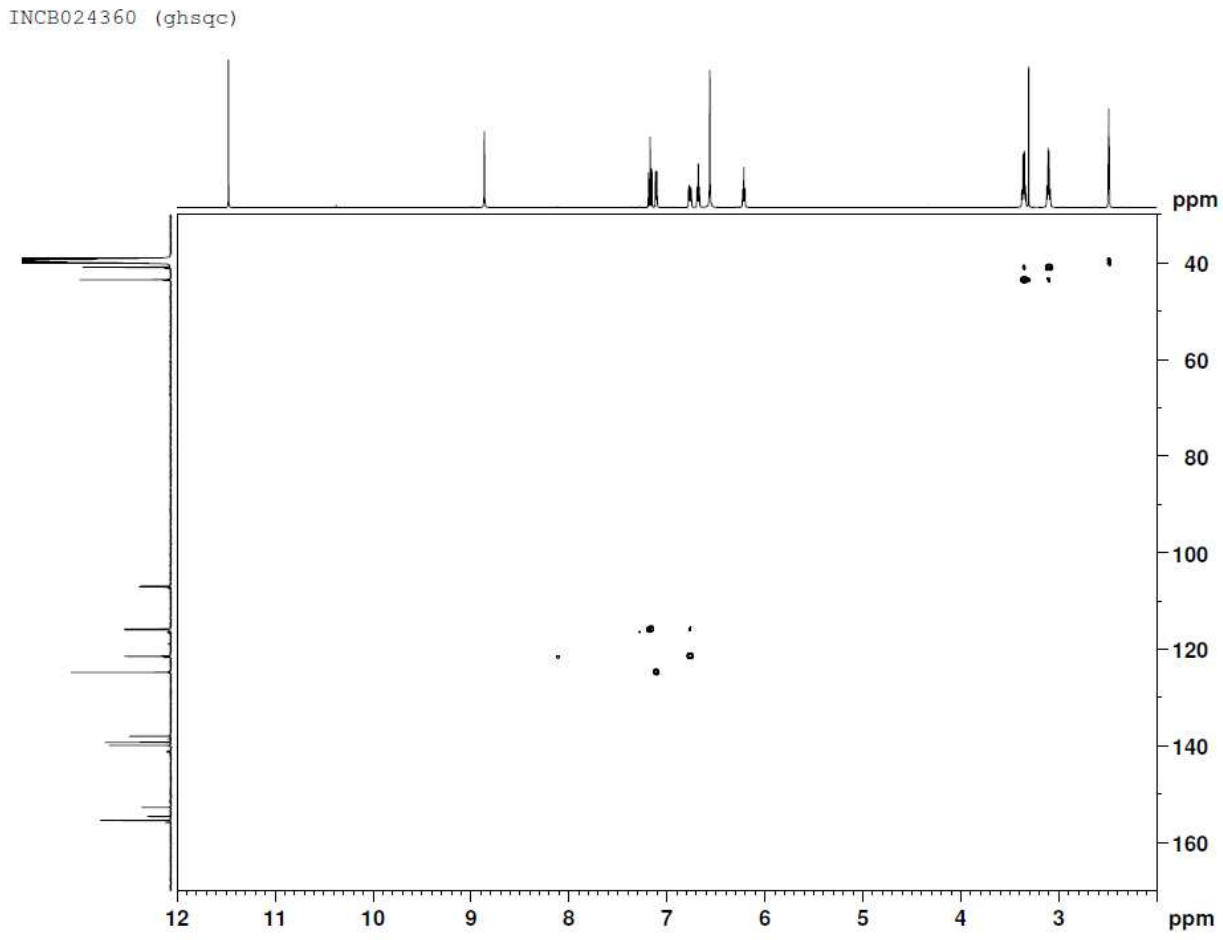


Figure S6. Ghmbc (Gradient Heternuclear Multiple Bond Correlation – long range C-H correlations 2-4 bonds between the H and C.

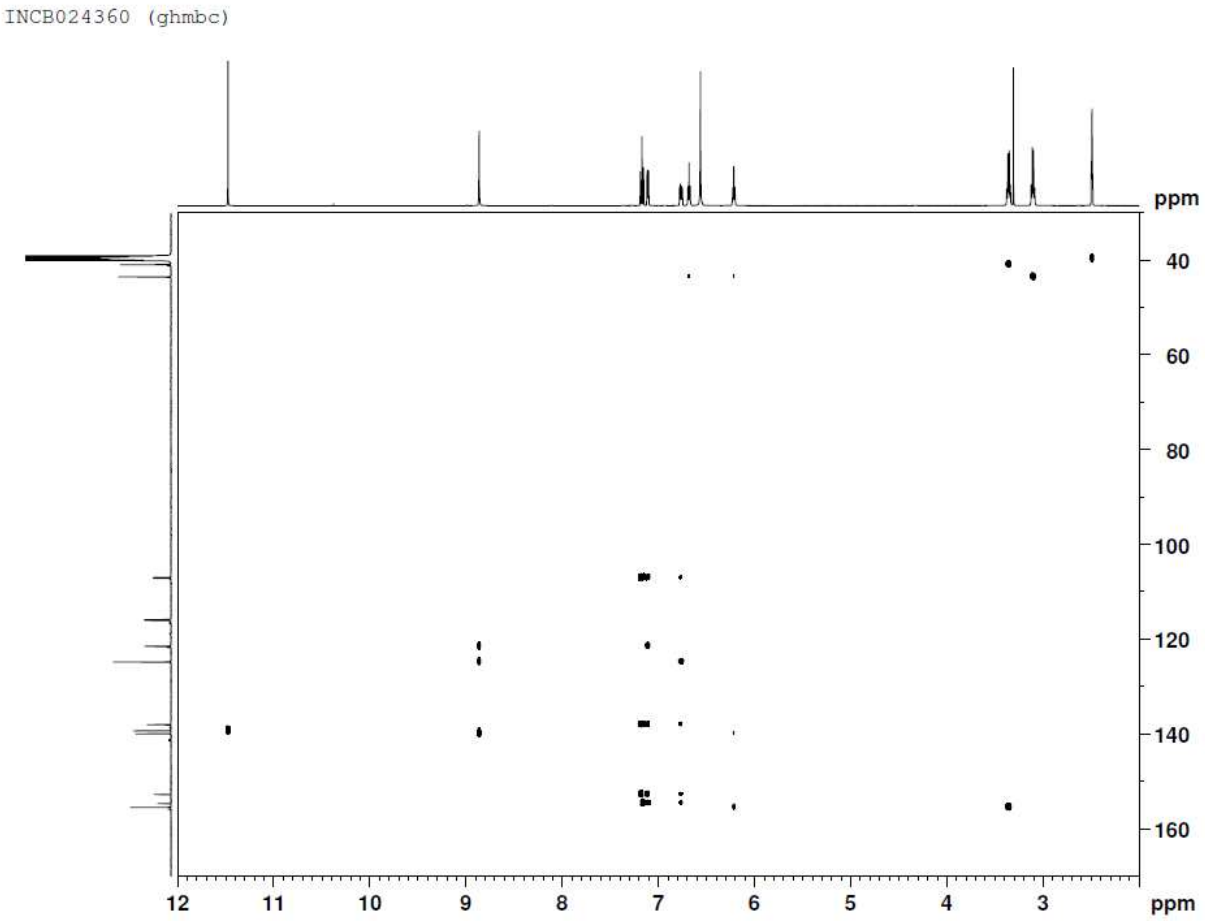
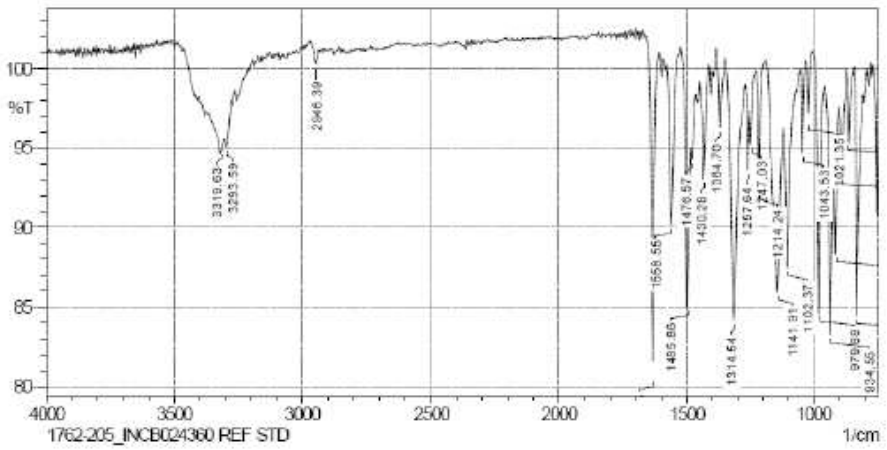


Table S2. Elemental Analysis Results for INCB024360 (4f).

Analysis	Calculated Value (wt %)	Found Value (wt %)
Carbon	30.15	30.20
Hydrogen	2.99	2.62
Nitrogen	22.37	22.24
Bromine	18.23	18.13
Fluorine	4.34	4.21
Sulfur	7.32	7.60

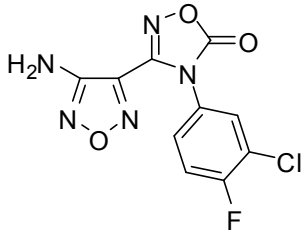
Figure S7. Infrared Absorption Spectrum of INCB024360 (4f).



The synthesis of **4g** is illustrated in **Scheme S2** and detailed below.

4-({2-[(Aminosulfonyl)amino]ethyl}amino)-*N*-(3-chloro-4-fluorophenyl)-*N′*-hydroxy-1,2,5-oxadiazole-3-carboximidamide (4g).

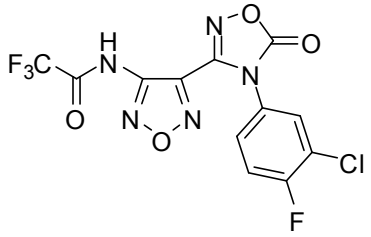
Step A: 3-(4-Amino-1,2,5-oxadiazol-3-yl)-4-(3-chloro-4-fluorophenyl)-1,2,4-oxadiazol-5(4*H*)-one



A solution of 4-amino-*N*-(3-chloro-4-fluorophenyl)-*N′*-hydroxy-1,2,5-oxadiazole-3-carboximidamide (80 g, 0.29 mol) [see US Pat. App. Pub. No. 2006/0258719] in tetrahydrofuran (500 mL) was treated with a solution of 1,1'-carbonyldiimidazole (53 g, 0.32 mol) in tetrahydrofuran (200 mL) and heated at reflux for 1 h. The reaction mixture was cooled to 25 °C and concentrated to the point where a large amount of solid precipitated. The heterogeneous mixture was diluted with ethyl acetate (1.5 L) and washed with 1 N HCl (2 x 300 mL), water (300 mL), and brine (200 mL). The organic layer was separated, dried over anhydrous sodium sulfate, filtered, and concentrated to give the desired product (88 g, quantitative) as an off-white solid. This material was used without further

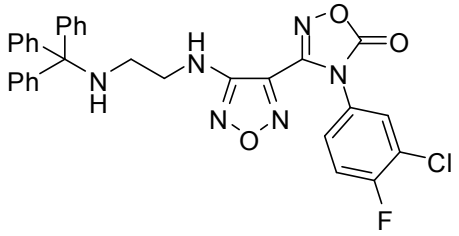
purification. LCMS for $C_{10}H_6ClFN_5O_3$ $(M+H)^+$: m/z = 298.0. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.96 (dd, J = 6.6, 2.3 Hz, 1 H), 7.69 - 7.60 (m, 2 H), 6.60 (s, 2 H).

Step B: *N*-{4-[4-(3-Chloro-4-fluorophenyl)-5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl]-1,2,5-oxadiazol-3-yl}-2,2,2-trifluoroacetamide



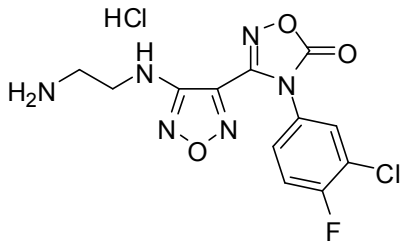
A solution of 3-(4-amino-1,2,5-oxadiazol-3-yl)-4-(3-chloro-4-fluorophenyl)-1,2,4-oxadiazol-5(4*H*)-one (15 g, 50 mmol) in dichloromethane (120 mL) was treated with trifluoroacetic anhydride (14 mL, 100 mmol), cooled to 0 °C, and treated with pyridine (8.2 mL, 100 mmol). The reaction mixture was stirred at 25 °C for 10 min, cooled to 0 °C, and quenched with water (10 mL). The reaction mixture was diluted with ethyl acetate (500 mL) and washed with 1 N HCl (300 mL), water (2 x 200 mL), and brine (200 mL). The organic layer was separated, dried over anhydrous sodium sulfate, filtered, and concentrated to ~50 mL volume. This solution was warmed (~40-50 °C) and treated with hexanes (600 mL) under vigorous stirring, followed by petroleum ether (200 mL). The mixture was stirred at 0 °C for 30 min and the solid was collected by filtration, washed with hexanes, and dried to give the desired product (19.7 g, 99%) as a white solid. LCMS for $C_{12}H_5ClF_4N_5O_4$ $(M+H)^+$: m/z = 394.0. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.82 (dd, J = 6.6, 2.5 Hz, 1 H), 7.59 (dd, J = 9.0, 9.0 Hz, 1 H), 7.52 - 7.47 (m, 1 H).

Step C: 4-(3-Chloro-4-fluorophenyl)-3-(4-{[2-(tritylamino)ethyl]amino}-1,2,5-oxadiazol-3-yl)-1,2,4-oxadiazol-5(4*H*)-one



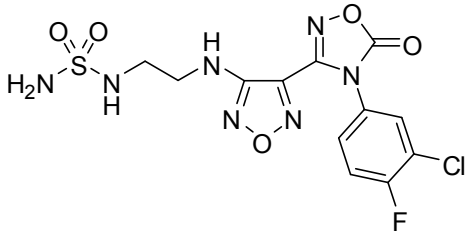
A solution of 2-(tritylamino)ethanol (10 g, 33 mmol) [EP599220 and *J. Org. Chem.* (2001), *66*, 7615] and triphenylphosphine (8.7 g, 33 mmol) in tetrahydrofuran (65 mL) at 0 °C was treated with diisopropyl azodicarboxylate (7.0 mL, 35 mmol) dropwise. The reaction mixture was stirred at 0 °C for 15 min, treated with a solution of *N*-{4-[4-(3-chloro-4-fluorophenyl)-5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl]-1,2,5-oxadiazol-3-yl}-2,2,2-trifluoroacetamide (9.3 g, 24 mmol) in tetrahydrofuran (28 mL), and stirred at 25 °C for 16 h. The reaction mixture was concentrated, diluted with ethyl acetate (350 mL), cooled to 0 °C, treated with 1 N HCl (200 mL), and stirred at 25 °C for 1 h. The reaction mixture was treated with additional 1 N HCl (150 mL) and stirred at 25 °C for 3 h. The organic layer was separated, washed with saturated sodium bicarbonate (200 mL) and brine (100 mL), dried over anhydrous sodium sulfate, filtered, and concentrated to a yellow foam which was reconcentrated from hexanes to give an oily solid. The oily solid was treated with methyl *tert*-butyl ether (50 mL) and stirred to give a heterogeneous mixture. The solid was filtered, washed with methyl *tert*-butyl ether (30 mL), and dried to give the desired product (10 g, 74%) as a white solid. LCMS for $C_{31}H_{24}ClFN_6O_3Na$ $(M+Na)^+$: m/z = 605.2. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.97 (dd, *J* = 6.7, 2.6 Hz, 1 H), 7.71 - 7.66 (m, 1 H), 7.60 (dd, *J* = 9.1, 8.8 Hz, 1 H), 7.40 - 7.37 (m, 6 H), 7.28 - 7.23 (m, 6 H), 7.18 - 7.12 (m, 3 H), 6.59 (dd, *J* = 5.9, 5.6 Hz, 1 H), 3.37 - 3.31 (m, 2 H), 2.96 (dd, *J* = 7.6, 7.6 Hz, 1 H), 2.27 - 2.19 (m, 2 H).

Step D: 3-{4-[(2-Aminoethyl)amino]-1,2,5-oxadiazol-3-yl}-4-(3-chloro-4-fluorophenyl)-1,2,4-oxadiazol-5(4*H*)-one hydrochloride



A premixed solution of triisopropylsilane (3.4 mL, 17 mmol) and trifluoroacetic acid (44 mL, 570 mmol) was added to 4-(3-chloro-4-fluorophenyl)-3-(4-{[2-(tritylamino)ethyl]amino}-1,2,5-oxadiazol-3-yl)-1,2,4-oxadiazol-5(4*H*)-one (6.5 g, 11 mmol) and the resulting suspension was stirred at 25 °C for 30 min. The reaction mixture was filtered and washed with trifluoroacetic acid. The filtrate was concentrated to an oil which was diluted with methanol (25 mL), cooled to 0 °C, treated with 4 M HCl in 1,4-dioxane (14 mL), and stirred at 25 °C for 15 min. The mixture was concentrated to a solid that was treated with diethyl ether (50 mL) and filtered. The solid was washed with diethyl ether (50 mL) and dried to give the desired product (4.1 g, 98%) as a white solid. LCMS for $C_{12}H_{11}ClFN_6O_3$ $(M+H)^+$: m/z = 341.1. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.05 - 8.00 (m, 4 H), 7.75 - 7.69 (m, 1 H), 7.64 (dd, *J* = 9.1, 8.8 Hz, 1 H), 6.77 (dd, *J* = 5.9, 5.9 Hz, 1 H), 3.54 - 3.47 (m, 2 H), 3.04 - 2.99 (m, 2 H).

Step E: *N*-[2-({4-[4-(3-Chloro-4-fluorophenyl)-5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl]-1,2,5-oxadiazol-3-yl}amino)ethyl]sulfamide



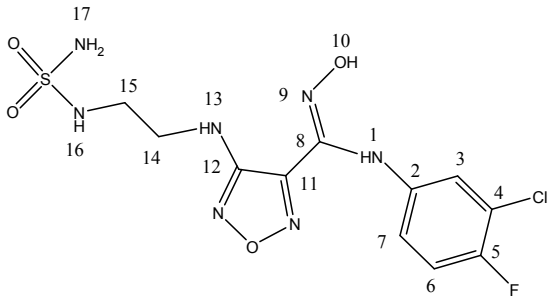
A solution of chlorosulfonyl isocyanate (2.0 mL, 23 mmol) in dichloromethane (70 mL) was treated with *t*-butyl alcohol (2.2 mL, 23 mmol) at 0 °C and stirred at 25 °C for 1 h. This mixture was added to a suspension of 3-{4-[(2-aminoethyl)amino]-1,2,5-oxadiazol-3-yl}-4-(3-chloro-4-fluorophenyl)-1,2,4-oxadiazol-5(4*H*)-one hydrochloride (4.3 g, 11 mmol) in dichloromethane (70 mL). The reaction mixture was treated with a solution of triethylamine (6.3 mL, 45 mmol) in dichloromethane

(20 mL) at 0 °C and stirred at 25 °C for 3 h. The reaction mixture was diluted with 0.1 N HCl and extracted with ethyl acetate (2 x 100 mL). The combined organic layers were washed with brine (100 mL), dried over anhydrous sodium sulfate, filtered, and concentrated to a white solid. The white solid was diluted with dichloromethane (100 mL), treated with trifluoroacetic acid (20 mL), and stirred at 25 °C for 3 h. The reaction mixture was concentrated to a crude residue that was purified by silica gel chromatography to give the desired product (3.7 g, 78%) as a white solid. LCMS for $C_{12}H_{12}ClFN_7O_5S$ $(M+H)^+$: m/z = 420.0. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.98 (dd, J = 6.4, 2.1 Hz, 1 H), 7.70 - 7.60 (m, 2 H), 6.66 (t, J = 5.9 Hz, 1 H), 6.57 (s, 2 H), 6.52 (t, J = 5.9 Hz, 1 H), 3.42 - 3.35 (m, 2 H), 3.13 - 3.06 (m, 2 H).

Step F: 4-({2-[(Aminosulfonyl)amino]ethyl}amino)-*N*-(3-chloro-4-fluorophenyl)-*N*′-hydroxy-1,2,5-oxadiazole-3-carboximidamide (**4g**)

A solution of *N*-[2-({4-[4-(3-chloro-4-fluorophenyl)-5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl]-1,2,5-oxadiazol-3-yl}amino)ethyl]sulfamide (3.7 g, 8.8 mmol) in methanol (70 mL) was treated with 2 M NaOH (18 mL, 35 mmol) and stirred at 25 °C for 2 h. The reaction mixture was quenched with 6 N HCl to pH~7 and the methanol was removed under reduced pressure. The solid that precipitated was filtered and washed with water to give the desired product (3.2 g, 92%) as a white solid. HRMS (ESI/QTof) m/z: $[M+H]^+$ Calcd for $C_{11}H_{14}ClFN_7O_4S$ 394.0495; Found 394.0498. ^{1}H NMR: please see below.

^{1}H and ^{13}C NMR Spectral Assignments for **4g** based on 2D NMR spectra (HMBC and HSQC).



Position	^{1}H Chemical Shift	Multiplicity	Relative Intensity	Coupling Constant(s) (Hz)	^{13}C Chemical Shift	C-F Coupling Constant (Hz)	HMBC Correlations
1	8.86	s	1H	--	--	--	H1: C3, C7, C8,C11
2	--	--	--	--	137.8	--	C2: H3, H6, H7
3	6.98	dd	1H	6.5, 2.7	122.0	--	H3: C2, C4, C5, C7
4	--	--	--	--	118.6	--	C4: H3, H6, H7
5	--	--	--	--	152.7	248.8	C5: H3, H6, H7
6	7.20	dd	1H	9.1, 9.1	116.1	--	H6: C2, C4, C5
7	6.74	ddd	1H	8.9, 4.0, 2.8	120.8	--	H7: C2, C3, C5
8	--	--	--	--	139.3	--	C8: H1, H10
10	11.47	s	1H	--	--	--	H10: C8
11	--	--	--	--	139.8	--	C11: H1, H13
12	--	--	--	--	155.4	--	C12: H13, H14
13	6.21	t	1H	6.0	--	--	H13: C11, C12, C14, C15
14	3.36	dt	2H	6.0, 6.3	43.6	--	H14: C12, C15
15	3.11	dt	2H	6.0, 6.2	40.9	--	H15: C14
16	6.67	t	1H	6.0	--	--	H16: C14
17	6.55	s	2H	--	--	--	--

The syntheses of **3c**, **3d**, **4b**, **4d** and **4e** are illustrated in **Scheme S3** utilizing experimental procedures similar to the synthesis of **4a**. The data for **3c**, **3d**, **4b**, **4d** and **4e** are included below.

***N*-(3-Bromo-4-fluorophenyl)-4-(dimethylamino)-*N′*-hydroxy-1,2,5-oxadiazole-3-carboximidamide (3c).** The starting material used in Step a was dimethylamine. ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.16 (s, 0.5 H), 10.34 (s, 0.5 H), 9.21 (s, 0.5 H), 9.15 (s, 0.5 H), 8.07 (dd, J = 6.2, 2.5 Hz, 0.5 H), 7.41 – 7.24 (m, 1 H), 7.23 – 7.07 (m, 1 H), 6.78 – 6.62 (m, 0.5 H), 2.91 (s, 3 H), 2.82 (s, 3 H). LCMS for $C_{11}H_{12}BrFN_5O_2$ $(M+H)^+$: m/z = 344.1, 346.1.

***N*-(3-Bromo-4-fluorophenyl)-4-(ethylamino)-*N′*-hydroxy-1,2,5-oxadiazole-3-carboximidamide (3d).** The starting material used in Step a was ethylamine. ^{1}H NMR (400 MHz, CD_3OD): δ 7.12 (dd, J = 6.0, 2.7 Hz, 1 H), 7.05 (dd, J = 8.7, 8.7 Hz, 1 H), 6.87 – 6.78 (m, 1 H), 3.40 – 3.18 (m, 2 H), 1.25 (t, J = 7.2 Hz, 3 H). LCMS for $C_{11}H_{12}BrFN_5O_2$ $(M+H)^+$: m/z = 344.0, 346.0.

***N*-(3-Bromo-4-fluorophenyl)-*N′*-hydroxy-4-[(2-hydroxyethyl)amino]-1,2,5-oxadiazole-3-carboximidamide (4b).** The starting material used in Step a was ethanolamine. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.17 (t, J = 8.8 Hz, 1 H), 7.08 (dd, J = 6.1, 2.7 Hz, 1 H), 6.83 – 6.69 (m, 1 H), 6.22 (dd, J = 5.7, 5.7 Hz, 1 H), 3.65 – 3.50 (m, 2 H), 3.30 – 3.00 (m, 2 H). LCMS for $C_{11}H_{12}BrFN_5O_3$ $(M+H)^+$: m/z = 359.9, 361.9.

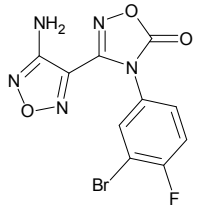
***N*-[2-({4-[(*Z*)-[(3-Bromo-4-fluorophenyl)amino](hydroxyimino)methyl]-1,2,5-oxadiazol-3-yl}amino)ethyl]acetamide (4d).** The starting material used in Step a was *N*-(2-aminoethyl)-acetamide. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.00 (s, 1 H), 7.17 (t, J = 8.8 Hz, 1 H), 7.09 (dd, J = 6.1, 2.7 Hz, 1 H), 6.81 – 6.70 (m, 1 H), 6.31 – 6.23 (m, 1 H), 3.29 – 3.17 (m, 4 H), 1.79 (s, 3 H). LCMS for $C_{13}H_{15}BrFN_6O_3$ $(M+H)^+$: m/z = 401.0, 403.0.

***N*-(3-Bromo-4-fluorophenyl)-*N′*-hydroxy-4-({2-[(methylsulfonyl)amino]ethyl}amino)-1,2,5-oxadiazole-3-carboximidamide (4e).** The starting material used in Step a was *N*-(2-aminoethyl)-methanesulfonamide. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.50 (s, 1 H), 8.91 (s, 1 H), 7.23 – 7.14 (m, 2 H), 7.10 (dd, J = 6.1, 2.7 Hz, 1 H), 6.79 – 6.71 (m, 1 H), 6.27 (t, J = 6.1 Hz, 1 H), 3.33 (s, 2 H), 3.19 – 3.10 (m, 2 H), 2.90 (s, 3 H). LCMS for $C_{12}H_{15}BrFN_6O_4S$ $(M+H)^+$: m/z = 436.9, 438.9.

The synthesis of **3b** is illustrated in **Scheme S4** and detailed below.

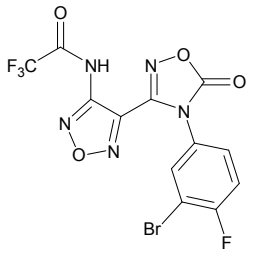
***N*-(3-Bromo-4-fluorophenyl)-*N′*-hydroxy-4-(methylamino)-1,2,5-oxadiazole-3-carboximidamide (3b).**

Step A: 3-(4-Amino-1,2,5-oxadiazol-3-yl)-4-(3-bromo-4-fluorophenyl)-1,2,4-oxadiazol-5(4*H*)-one



A solution of 4-amino-N-(3-bromo-4-fluorophenyl)-*N′*-hydroxy-1,2,5-oxadiazole-3-carboximidamide (3.44 g, 0.0109 mol) in tetrahydrofuran (33.0 mL) was treated with *N*,*N*-carbonyldiimidazole (1.941 g, 0.01197 mol) and heated to reflux for 1 h. The reaction mixture was concentrated and diluted with ethyl acetate (250 mL), washed with 0.1 M HCl (3 x 75 mL) and brine, dried with sodium sulfate, filtered, and concentrated to give the desired product (3.57g, 96%) as a tan solid. LCMS for $C_{10}H_6BrFN_5O_3$ $(M+H)^+$: m/z = 342.1, 344.0.

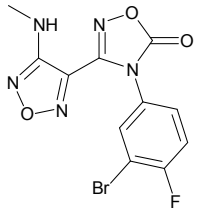
Step B: *N*-{4-[4-(3-Bromo-4-fluorophenyl)-5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl]-1,2,5-oxadiazol-3-yl}-2,2,2-trifluoroacetamide



A solution of 3-(4-amino-1,2,5-oxadiazol-3-yl)-4-(3-bromo-4-fluorophenyl)-1,2,4-oxadiazol-5(4*H*)-one (863.0 mg, 0.002523 mol) in pyridine (10.0 mL, 0.124 mol) was treated with trifluoroacetic anhydride (0.89 mL, 0.0063 mol) and stirred at 20 °C for 3 h. The reaction mixture was concentrated to remove most of the pyridine and diluted with ethyl acetate (~200 mL). The organic layer was washed with water (3X) and brine, dried with sodium sulfate, filtered, and concentrated to give a residue.

Purification via flash column chromatography (100% hexanes to 50% ethyl acetate/hexanes over 25 min) gave the desired product (841 mg, 76%) as a solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.21 – 7.78 (m, 1H), 7.70 – 7.28 (m, 2H). LCMS for $C_{12}H_5BrF_4N_5O_4$ $(M+H)^+$: m/z = 438.0, 440.0.

Step C: 4-(3-Bromo-4-fluorophenyl)-3-[4-(methylamino)-1,2,5-oxadiazol-3-yl]-1,2,4-oxadiazol-5(4*H*)-one



A solution of *N*-{4-[4-(3-bromo-4-fluorophenyl)-5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl]-1,2,5-oxadiazol-3-yl}-2,2,2-trifluoroacetamide (0.585 g, 0.00134 mol) in *N*,*N*-dimethylformamide (3.0 mL) was treated with potassium carbonate (277 mg, 0.00200 mol) followed by methyl iodide (125 µL, 0.00200 mol) dropwise and stirred at 20 °C for 2 h. The reaction mixture was treated with additional methyl iodide (200.0 µL, 0.003213 mol) and stirred at 20 °C overnight. The reaction mixture was diluted with water (100 mL) and brine (~25 mL) and extracted with ethyl acetate (2x100 mL). The combined organic layers were washed with water (3x100 mL) and brine, dried with sodium sulfate, filtered, and concentrated to give a residue. Purification via flash column chromatography (100% hexanes to 40% ethyl acetate/hexanes over 25 min) to gave the desired product (366 mg, 77%) as a solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.09 (dd, J = 6.2, 2.5 Hz, 1H), 7.85 – 7.67 (m, 1H), 7.60 (dd, J = 8.7, 8.7 Hz, 1H), 6.52 (q, J = 4.7 Hz, 1H), 2.87 (d, J = 4.9 Hz, 3H). LCMS for $C_{11}H_8BrFN_5O_3$ $(M+H)^+$: m/z = 355.9, 358.0.

Step D: *N*-(3-Bromo-4-fluorophenyl)-*N*′-hydroxy-4-(methylamino)-1,2,5-oxadiazole-3-carboximidamide (**3b**)

A solution of 4-(3-bromo-4-fluorophenyl)-3-[4-(methylamino)-1,2,5-oxadiazol-3-yl]-1,2,4-oxadiazol-5(4*H*)-one (20.0 mg, 0.0000562 mol) in ethanol (0.50 mL) was treated with 2.0 M Sodium

hydroxide in water (140 μL, 0.00028 mol) and stirred at 20 °C for 30 min. Purification via preparative LCMS gave the desired product (13 mg, 70%) as a solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.4 (s, 1 H), 8.88 (s, 1 H), 7.18 (dd, J = 8.8 Hz, 1 H), 7.09 (dd, J = 6.1, 2.7 Hz, 1 H), 6.74 (ddd, J = 8.9, 4.1, 2.8 Hz, 1 H), 6.11 (q, J = 5.0 Hz, 1 H), 2.84 (d, J = 5.1 Hz, 3 H). LCMS for $C_{10}H_{10}BrFN_5O_2$ $(M+H)^+$: m/z = 329.9, 331.9.

The synthesis of **3e** utilized the experimental procedures similar to the synthesis of **3b**. The data for **3e** are included below.

4-(Benzylamino)-*N*-(3-bromo-4-fluorophenyl)-*N′*-hydroxy-1,2,5-oxadiazole-3-carboximidamide (3e). The starting material used in Step c was iodomethane. ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.43 (s, 1 H), 8.93 (s, 1 H), 7.37 – 7.21 (m, 5 H), 7.21 – 7.08 (m, 2 H), 6.80 – 6.72 (m, 1 H), 6.71 – 6.62 (m, 1 H), 4.38 (d, J = 6.0 Hz, 2 H). LCMS for $C_{16}H_{14}BrFN_5O_2$ $(M+H)^+$: m/z = 406.1, 408.0.

The synthesis of **4c** is illustrated in Scheme S5 utilizing an experimental procedure similar to the synthesis of **4f**. The data for **4c** are included below.

4-[(2-Aminoethyl)amino]-*N*-(3-bromo-4-fluorophenyl)-*N′*-hydroxy-1,2,5-oxadiazole-3-carboximidamide (4c). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.87 (br s, 1 H), 7.17 (dd, J = 8.8, 8.8 Hz, 1 H), 7.10 (dd, J = 6.1, 2.7 Hz, 1 H), 6.81 – 6.72 (m, 1 H), 6.25 (t, J = 5.7 Hz, 1 H), 3.27 – 3.03 (m, 2 H), 2.82 – 2.62 (m, 2 H). LCMS for $C_{11}H_{13}BrFN_6O_2$ $(M+H)^+$: m/z = 359.0, 361.0.

HPLC Purity Analysis

HPLC purity (see below) was determined using a Waters SunFire column (30 x 100 mm, 5 μM particle size), with a water/0.1% TFA and acetonitrile/0.1% TFA gradient at a flow rate of 60 mL/min over a total run time of 5 min. All compounds (**3-4**) were analyzed by these methods and determined to be >90% purity.

Compound	HPLC Analysis Data t_R (min)
3b	1.83
3c	1.87
3d	2.56
3e	2.35
4a	2.31
4b	1.69
4c	1.13
4d	1.65
4e	1.73
4f	1.65
4g	1.60

Molecular Modelling of IDO1 complexed with INCB24360 (4f)

Modelling of IDO1 complexed to **4f** and was performed using the program MOE (Chemical Computing Group, Montreal, 2004). The published IDO1 crystal structure (PDB entry 4PK5; ACS Med. Chem. Lett. 2014, 5, 1119) was used as a basis set for the IDO1 structure. In our model, the hydroxyl group of the hydroxyamidine forms a dative bond with the iron of the heme. The *meta*-bromo aryl moiety is deep in the hydrophobic pocket. The 3- substituent of the furazan protrudes out of the active site into a solvent exposed region of IDO1 (See Figures S8 and S9). This model is consistent with all of our enzyme kinetics, SAR and the small molecule structure of INCB24360 (**4f**) (see Enzyme Kinetics section).

Figure S8. Model of IDO1 complexed to INCB24360 (4**f**) showing the sulfamide sidechain protruding from the active site.

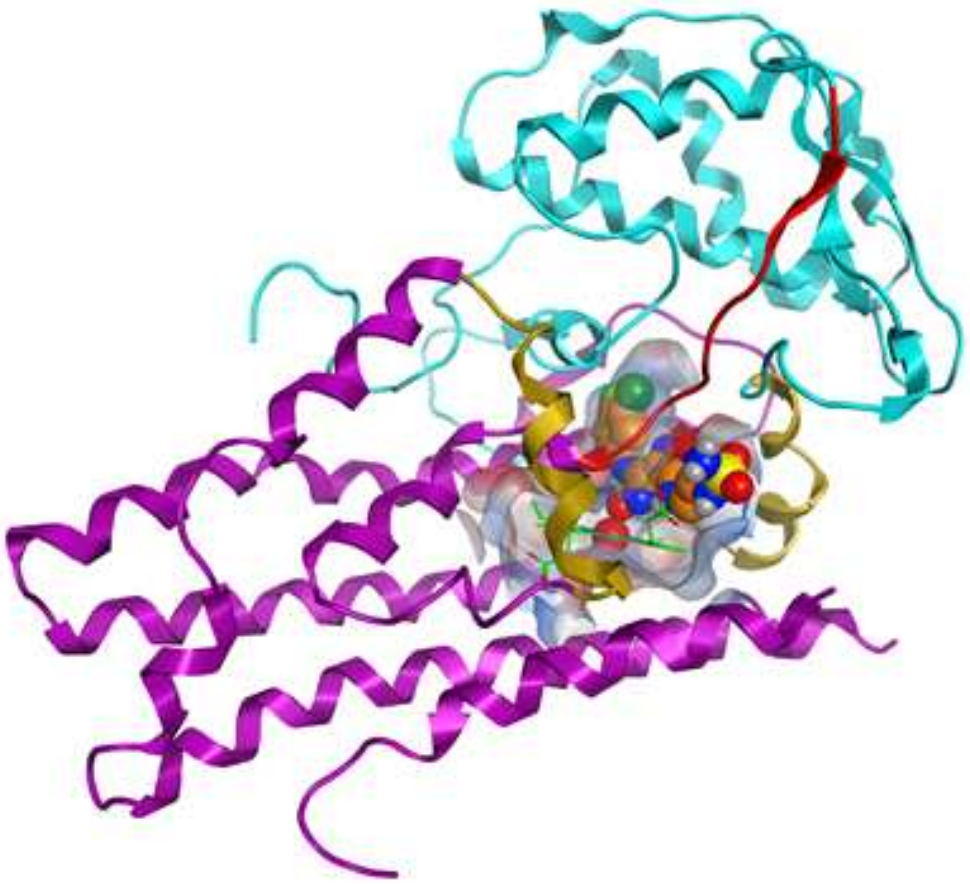
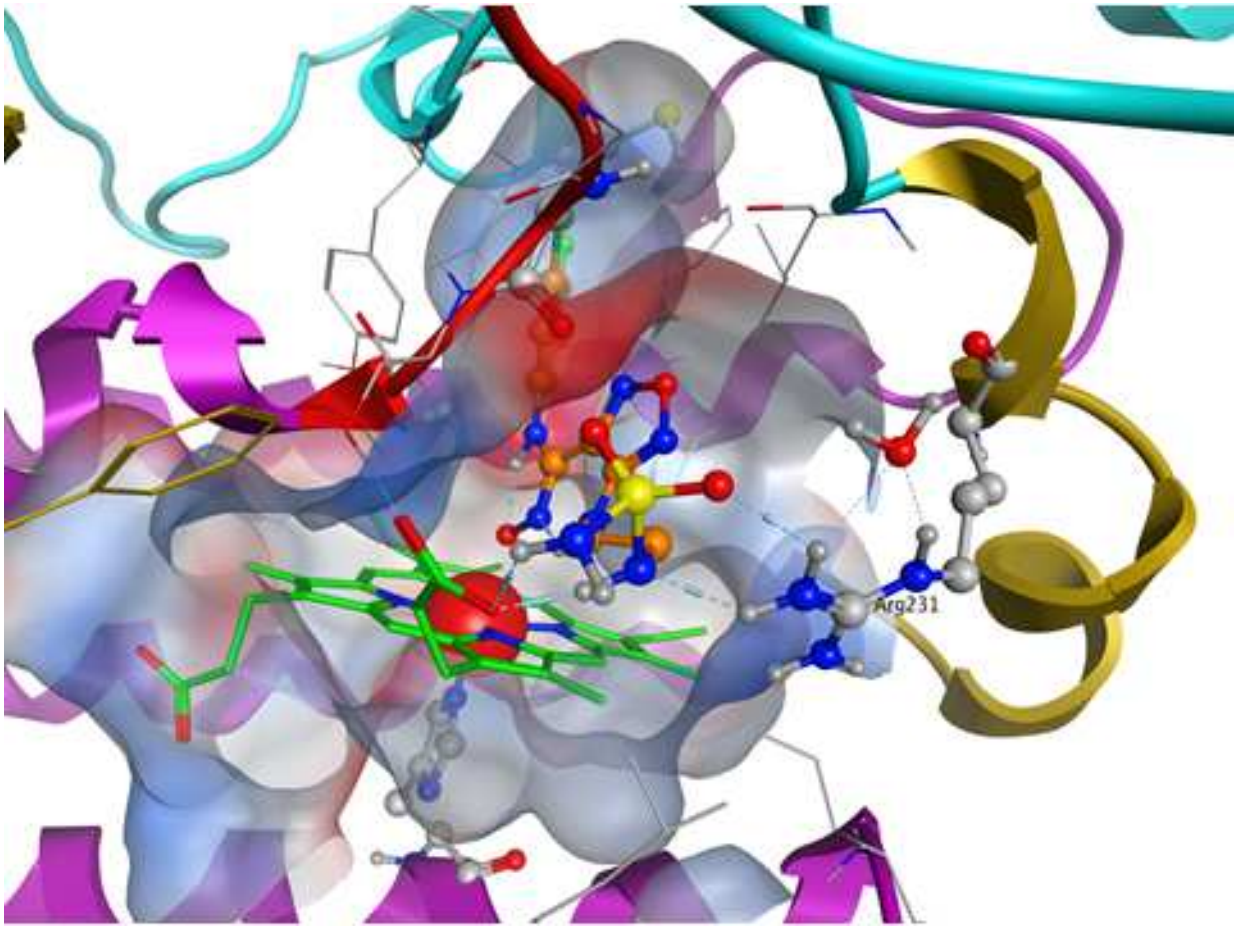


Figure S9. Model of IDO1 complexed to INCB24360 (4**f**) showing both nitrogens of sulfamide forming a bi-dentate interaction with propionic acid of Heme. Also, these interactions may be stabilized by hydrogen bonding interaction of sulfonamide with Arg231.



X-ray Crystal Structure of INCB024360 (4f)

Intramolecular hydrogen bonds stabilize the *cis*-conformation in the crystal form. We hypothesize these internal hydrogen bonds improve the membrane permeability of the compound in solution.

X-RAY CRYSTAL STRUCTURE ANALYSIS of **INCB24360 (4f)**

(C11,H13,N7,O4,Br1,S1,F1)

CRYSTAL DATA: C11 H13 Br F N7 O4 S , from EtOAc/MTBE, colorless, square plate, ~0.130 x 0.130 x 0.020mm, orthorhombic , Iba2 , a = 15.554(3) Å, b = 24.400(5) Å, c = 8.3412(18) Å, Vol = 3165.8(12) $Å^3$, Z = 8 , T = -100.°C, Formula weight = 438.25, Density = 1.839g/cm^3, μ(Mo) = 2.78mm^{-1}

DATA COLLECTION: Bruker SMART APEX-II CCD system, MoKalpha radiation, standard focus tube , anode power = 50kV x 42mA, crystal to plate distance = 5.0cm, 512 x 512 pixels/frame, beam center = (256.63,253.13), total frames = 923, oscillation/frame = 0.50°, exposure/frame = 60.7 sec/frame, SAINT integration, hkl min/max = (-20 , 20 , -32 , 29 , -11 , 11), data input to shelx = 13318 , unique data = 3918 , two-theta range = 3.10 to 56.60°, completeness to two-theta 56.60 = 100.00%, R(int-xl) = 0.0442, SADABS correction applied.

SOLUTION AND REFINEMENT: Structure solved using XS(Shelxtl), refined using shelxtl software package, refinement by full-matrix least squares on F^2, scattering factors from Int. Tab. Vol C Tables 4.2.6.8 and 6.1.1.4, number of data = 3918 , number of restraints = 1 , number of parameters = 251 , data/parameter ratio = 15.61, goodness-of-fit on F^2 = 1.00, R indices[I>4sigma(I)] R1 = 0.0354, wR2 = 0.0667, R indices(all data) R1 = 0.0502, wR2 = 0.0718, max difference peak and hole = 0.418 and -0.370 e/$Å^3$, refined flack parameter = 0.016(8) , All of the hydrogen atoms except the NH, NH2 and OH hydrogens have been idealized using a riding model. The NH, NH_2 and OH hydrogens were found from a difference map and fully refined.

RESULTS: This study determines the structure of **4f**. The asymmetric unit contains one molecule as shown in Figure S10 with thermal ellipsoids drawn to the 50% probability level. The Flack parameter refined to 0.016(8) indicating the correct enantiomeric setting.

Figure S10.

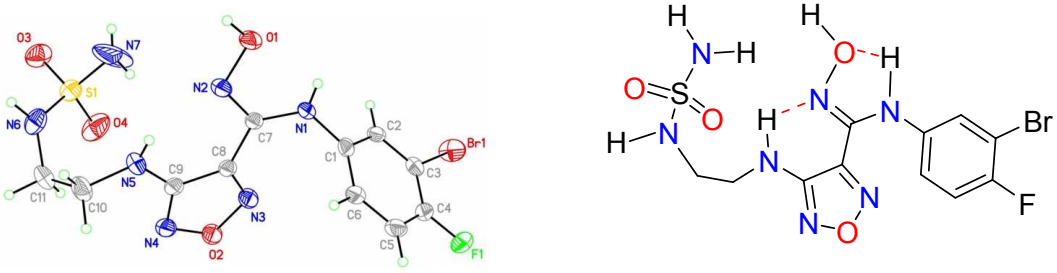


Table S3. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters (A^2 x 10^3) for **4f**. U(eq) is defined as one third of the trace of the orthogonalized Uij tensor.

	x	y	z	U(eq)
Br(1)	-1898(1)	9268(1)	1647(1)	45(1)
S(1)	1108(1)	5599(1)	5362(1)	36(1)
F(1)	-902(1)	9892(1)	4112(3)	39(1)
O(1)	-1584(1)	6849(1)	7292(3)	31(1)
O(2)	1572(1)	7747(1)	5067(3)	35(1)
O(3)	1063(2)	5047(1)	4760(3)	45(1)
O(4)	1479(2)	6005(1)	4359(3)	55(1)
N(1)	-1147(2)	7710(1)	5778(3)	25(1)
N(2)	-690(1)	6905(1)	7060(3)	25(1)
N(3)	692(1)	7809(1)	5033(3)	29(1)
N(4)	1820(1)	7327(1)	6114(3)	31(1)
N(5)	1038(2)	6720(1)	7768(3)	27(1)
N(6)	1659(2)	5601(1)	6961(3)	36(1)
N(7)	143(2)	5760(2)	5768(8)	109(3)
C(1)	-1046(2)	8270(1)	5359(4)	22(1)
C(2)	-1432(2)	8449(1)	3941(3)	23(1)
C(3)	-1382(2)	9001(1)	3542(4)	25(1)
C(4)	-948(2)	9356(1)	4525(4)	27(1)
C(5)	-560(2)	9185(1)	5904(4)	31(1)
C(6)	-616(2)	8637(1)	6343(4)	27(1)
C(7)	-530(2)	7359(1)	6324(3)	20(1)
C(8)	391(2)	7445(1)	6006(4)	21(1)
C(9)	1097(2)	7138(1)	6713(4)	24(1)
C(10)	1791(2)	6470(1)	8503(4)	34(1)
C(11)	2219(2)	6050(1)	7439(4)	34(1)

Table S4. Bond lengths [A] and angles [deg] for **4f**.

Br(1)-C(3)	1.889(3)
S(1)-O(4)	1.418(3)
S(1)-O(3)	1.439(2)
S(1)-N(6)	1.585(3)
S(1)-N(7)	1.587(4)
F(1)-C(4)	1.356(3)
O(1)-N(2)	1.412(3)
O(2)-N(3)	1.377(3)
O(2)-N(4)	1.400(3)
N(1)-C(7)	1.365(4)
N(1)-C(1)	1.418(4)
N(2)-C(7)	1.289(3)
N(3)-C(8)	1.291(4)
N(4)-C(9)	1.315(4)
N(5)-C(9)	1.349(4)
N(5)-C(10)	1.455(4)
N(6)-C(11)	1.456(5)
C(1)-C(6)	1.387(4)
C(1)-C(2)	1.396(4)
C(2)-C(3)	1.388(4)
C(3)-C(4)	1.370(4)
C(4)-C(5)	1.364(5)
C(5)-C(6)	1.389(4)
C(7)-C(8)	1.472(4)
C(8)-C(9)	1.455(4)
C(10)-C(11)	1.511(4)
O(4)-S(1)-O(3)	117.78(16)
O(4)-S(1)-N(6)	105.91(16)
O(3)-S(1)-N(6)	108.78(16)
O(4)-S(1)-N(7)	109.8(3)
O(3)-S(1)-N(7)	105.05(18)
N(6)-S(1)-N(7)	109.4(3)
N(3)-O(2)-N(4)	111.6(2)
C(7)-N(1)-C(1)	127.6(2)
C(7)-N(2)-O(1)	109.8(2)
C(8)-N(3)-O(2)	105.8(2)
C(9)-N(4)-O(2)	105.0(2)
C(9)-N(5)-C(10)	122.5(3)
C(11)-N(6)-S(1)	123.8(2)
C(6)-C(1)-C(2)	120.4(3)
C(6)-C(1)-N(1)	121.9(3)
C(2)-C(1)-N(1)	117.6(3)
C(3)-C(2)-C(1)	118.8(3)
C(4)-C(3)-C(2)	119.8(3)
C(4)-C(3)-Br(1)	119.4(2)
C(2)-C(3)-Br(1)	120.7(2)
F(1)-C(4)-C(5)	119.1(3)
F(1)-C(4)-C(3)	118.9(3)
C(5)-C(4)-C(3)	121.9(3)
C(4)-C(5)-C(6)	119.3(3)
C(1)-C(6)-C(5)	119.7(3)
N(2)-C(7)-N(1)	124.3(2)
N(2)-C(7)-C(8)	113.3(2)
N(1)-C(7)-C(8)	122.3(2)
N(3)-C(8)-C(9)	109.6(2)
N(3)-C(8)-C(7)	124.4(3)
C(9)-C(8)-C(7)	126.1(3)
N(4)-C(9)-N(5)	124.8(3)
N(4)-C(9)-C(8)	108.1(3)
N(5)-C(9)-C(8)	127.0(2)
N(5)-C(10)-C(11)	113.0(3)

N(6)-C(11)-C(10)	114.1(3)

Symmetry transformations used to generate equivalent atoms:

Table S5. Anisotropic displacement parameters (A^2 x 10^3) for **4f**. The anisotropic displacement factor exponent takes the form:

-2 pi^2 [h^2 a*^2 U11 + ... + 2 h k a* b* U12]

	U11	U22	U33	U23	U13	U12
Br(1)	52(1)	44(1)	38(1)	17(1)	-15(1)	-9(1)
S(1)	37(1)	28(1)	42(1)	-5(1)	-2(1)	0(1)
F(1)	42(1)	22(1)	52(1)	5(1)	-3(1)	-4(1)
O(1)	21(1)	33(1)	41(1)	11(1)	2(1)	-5(1)
O(2)	21(1)	30(1)	53(2)	6(1)	9(1)	-1(1)
O(3)	46(1)	35(1)	54(2)	-12(1)	10(1)	-2(1)
O(4)	90(2)	41(2)	35(2)	12(1)	-12(1)	-12(1)
N(1)	16(1)	24(1)	36(2)	4(1)	-2(1)	-1(1)
N(2)	19(1)	29(1)	28(2)	1(1)	-1(1)	-2(1)
N(3)	21(1)	30(1)	36(2)	2(1)	7(1)	1(1)
N(4)	23(1)	27(1)	42(2)	-6(1)	0(1)	-1(1)
N(5)	21(1)	32(2)	28(1)	2(1)	-1(1)	5(1)
N(6)	52(2)	29(2)	29(2)	6(1)	1(1)	1(1)
N(7)	31(2)	81(3)	215(7)	-102(4)	-11(3)	-4(2)
C(1)	18(1)	23(1)	26(2)	-1(1)	5(1)	4(1)
C(2)	18(1)	25(2)	26(2)	-4(1)	-1(1)	-2(1)
C(3)	19(1)	31(2)	26(2)	4(1)	-1(1)	2(1)
C(4)	23(1)	22(2)	35(2)	1(1)	2(1)	0(1)
C(5)	25(1)	32(2)	36(2)	-9(1)	-5(1)	-4(1)
C(6)	22(1)	27(2)	32(2)	1(1)	-4(1)	-2(1)
C(7)	19(1)	20(1)	21(2)	-5(1)	1(1)	-1(1)
C(8)	20(1)	20(1)	24(1)	-5(1)	1(1)	0(1)
C(9)	25(1)	20(1)	28(1)	-10(1)	-2(2)	0(1)
C(10)	32(2)	40(2)	31(2)	-3(2)	-13(1)	8(1)
C(11)	23(1)	44(2)	37(2)	1(2)	-5(1)	7(1)

Table S6. Hydrogen coordinates (x 10^4) and isotropic displacement parameters (A^2 x 10^3) for **4f**.

	x	y	z	U(eq)
H(2A)	-1717	8204	3275	27
H(5A)	-263	9432	6544	37
H(6A)	-365	8516	7292	32
H(10A)	1621	6296	9499	41

H(10B)	2203	6755	8761	41
H(11A)	2428	6232	6482	41
H(11B)	2712	5900	7999	41
H(1')	-1630(20)	6567(14)	7780(40)	27(9)
H(5)	560(20)	6691(12)	8130(40)	25(9)
H(1)	-1610(20)	7625(15)	5920(50)	42(11)
H(6)	1570(30)	5322(18)	7510(60)	64(14)
H(7)	-180(20)	5569(15)	5600(50)	45(12)
H(7')	160(30)	6040(18)	6140(70)	100(20)

Table S7. Torsion angles [deg] for **4f**.

N(4)-O(2)-N(3)-C(8)	-0.2(3)
N(3)-O(2)-N(4)-C(9)	-0.2(3)
O(4)-S(1)-N(6)-C(11)	18.2(3)
O(3)-S(1)-N(6)-C(11)	145.7(3)
N(7)-S(1)-N(6)-C(11)	-100.0(3)
C(7)-N(1)-C(1)-C(6)	-47.5(4)
C(7)-N(1)-C(1)-C(2)	135.3(3)
C(6)-C(1)-C(2)-C(3)	-0.5(4)
N(1)-C(1)-C(2)-C(3)	176.8(2)
C(1)-C(2)-C(3)-C(4)	1.0(4)
C(1)-C(2)-C(3)-Br(1)	-179.52(19)
C(2)-C(3)-C(4)-F(1)	179.7(2)
Br(1)-C(3)-C(4)-F(1)	0.2(4)
C(2)-C(3)-C(4)-C(5)	-0.3(4)
Br(1)-C(3)-C(4)-C(5)	-179.8(2)
F(1)-C(4)-C(5)-C(6)	179.1(3)
C(3)-C(4)-C(5)-C(6)	-0.9(4)
C(2)-C(1)-C(6)-C(5)	-0.7(4)
N(1)-C(1)-C(6)-C(5)	-177.8(3)
C(4)-C(5)-C(6)-C(1)	1.4(4)
O(1)-N(2)-C(7)-N(1)	-3.6(4)
O(1)-N(2)-C(7)-C(8)	-178.9(2)
C(1)-N(1)-C(7)-N(2)	161.6(3)
C(1)-N(1)-C(7)-C(8)	-23.4(4)
O(2)-N(3)-C(8)-C(9)	0.6(3)
O(2)-N(3)-C(8)-C(7)	-179.0(3)
N(2)-C(7)-C(8)-N(3)	166.1(3)
N(1)-C(7)-C(8)-N(3)	-9.3(4)
N(2)-C(7)-C(8)-C(9)	-13.4(4)
N(1)-C(7)-C(8)-C(9)	171.1(3)
O(2)-N(4)-C(9)-N(5)	179.0(3)
O(2)-N(4)-C(9)-C(8)	0.5(3)
C(10)-N(5)-C(9)-N(4)	7.1(5)
C(10)-N(5)-C(9)-C(8)	-174.8(3)
N(3)-C(8)-C(9)-N(4)	-0.7(3)
C(7)-C(8)-C(9)-N(4)	178.9(3)
N(3)-C(8)-C(9)-N(5)	-179.1(3)
C(7)-C(8)-C(9)-N(5)	0.4(5)
C(9)-N(5)-C(10)-C(11)	-81.8(4)
S(1)-N(6)-C(11)-C(10)	92.3(3)
N(5)-C(10)-C(11)-N(6)	-58.4(4)

Symmetry transformations used to generate equivalent atoms:

Enzyme Kinetics for INCB24360 (4f)

Enzyme assay of Indoeamine 2,3-Dioxygenase 1 (IDO1)

Human indoleamine 2, 3-dioxgenase 1 (IDO1) with an N-terminal his tag was expressed in *E.coli* and purified to homogeneity. It catalyzes the oxidative indole ring cleavage of tryptophan to yield *N*-formylkynurenine. The standard assay was performed as described by Shimizu et. al. (Shimizu, T., Nomiyama, S., Hirata, F., and Hayaishi, O., **1978** *J. Biol. Chem*) with the following modifications. For IC_{50} measurement, the assay mixture contained in a total volume of 90 μL, 20 nM active IDO1, 2 mM D-Trp, 20 mM ascorbate 3.5 μM methylene blue and 0.2 mg/ml catalase in 50 mM potassium phosphate buffer (pH 6.5 at room temperature, buffer A). Reaction was initiated by the addition of IDO1. The increase in absorbance at 321 nm was continuously followed. The reaction rates were fit to the sigmoidal dose response equation to obtain IC_{50} values for the compounds (GraphPad Software Prism 3.0). For mode of inhibition analysis, the final D-Trp concentrations varied between 0.31 mM and 15 mM and the apparent Km values of D-Trp in the presence of 0, 20, 40, 80, 120, 150 nM INCB024360 were determined. All samples were tested in hexplicate. The initial reaction rates of these reactions were fit to the Michaelis-Mention equation by nonlinear regression analysis (Graphpad Prism). A competitive K_i was determined by linear regression of a plot of Km vs. [inhibitor], such that K_i = -(x-intercept).

Reversibility of IDO1 inhibition by INCB024360

2 μM IDO1 was incubated with 3.5 μM INCB024360 or equal amount of DMSO in 100 μL buffer A. 10 μL of samples were taken out and directly diluted into 500 μL buffer A (50X dilution) at 0 and 1 hours. IDO1 activities were measured for each sample in octuplicate (IDO1 20 nM; INCB024360 35 nM in the final assay, the concentrations of the rest components in the reaction are the same as the standard IDO1 assay as described above). The remaining 70 μL samples were diluted into 4200 μL buffer A first (60X dilution) and subjected to concentration with 10k cut off micro-concentrator (Amicon). About 120 μL sample was maintained after concentration, which was further diluted into 2800 μL buffer A (23.3X dilution, total 1400X dilution). The diluted samples were tested for IDO1

activities (IDO1 20 nM; INCB024360 1.25 nM in the final assay, the concentrations of the rest components in the reaction are the same as the standard IDO1 assay as described above).

HPLC analysis of INCB024360 as potential IDO1 substrate

To explore the possibility of INCB024360 is a substrate of IDO1, 100 μM INCB024360 was incubated with buffer A or 200 nM IDO1 in the presence of 20 mM ascorbate, 3.5 μM methylene blue and 0.2 mg/ml catalase in total 500 μLfor 2 hrs at RT. IDO1 was precipitated by addition of 1:1 volume of acetonitrile at 0 hr and 2 hr. After centrifugation the supernatants were collected and subject to HPLC analysis. Samples were then quantitated by HPLC analysis to determine the remaining INCB024360 and compared with control sample in the absence of IDO1. As shown in Figure S11 for INCB024360 HPLC peak area comparison, at 254 nm, 101.1% and 100.9% of INCB024360 were found for the samples incubated with IDO1 for 0 hr and 2 hr respectively, were comparable to the sample with INCB024360 alone. At 220 nm 101.3% and 101.1% of INCB024360 was found for the samples incubated with IDO1 for 0 hr and 2 hr respectively. There is no sign of a decrease in the concentration of INCB024360 after two hours of incubation with IDO1 and therefore we concluded that INCB024360 is not substrate of IDO1.

Kinetic Characterization of IDO1 inhibition by INCB024360

In Figure S12, the reaction products were measured at various times for 7 different substrate concentrations. All the curves are linear up to 20 min, indicating that initial velocity conditions (<10% of substrate conversion) have been met. INCB024360 is a competitive inhibitor of IDO1 with respect to the substrate D-Trp, as shown in Figure S13. The apparent affinity of the substrate to the binding site is decreased with increasing inhibitor concentrations, while the Vmax is nearly unchanged. K_i was determined to be 21.2 nM.

Reversibility of INCB024360 inhibition

To rule out irreversible inhibition, we carried out a dilution experiment described in the experimental section. 2 μM of IDO1 was incubated with 3.5 μM INCB024360 (K_i = 21.2 nM) or 3.5% DMSO only, samples aliquots were directly diluted 50 fold with assay buffer and assayed at 0 and 1 hr after incubation. Assuming an irreversible binding mode, one could expect to see significant inhibition of IDO1. However, as shown in Figure S14, compared to the control sample, about 46 and 46% IDO1 activities were observed after the direct dilution at 0 and 1 hr respectively. The final concentration of INCB024360 in the assay was 35 nM assuming reversible binding mode. The inhibition percentage is similar to the IDO1 inhibition in the presence of 35 nM INCB024360 without preformation of enzyme inhibitor complex (59%), which indicated INCB024360 as a reversible inhibitor. After 2 hr pre-incubation period, the remaining samples were first diluted and followed with concentration and redilution resulting in 1400 fold dilution of INCB024360 assuming reversible binding mode with IDO1. Figure S15 showed that after 1400 fold dilution, the INCB024360 treated IDO1 sample exhibited 85% activities of DMSO treated IDO1 control sample, suggesting the reversible IDO1 inhibition behavior of INCB024360.

Conclusion

In summary, INCB024360 is a reversible and competitive inhibitor of IDO1 with respect to the substrate D-Trp and is not a substrate of IDO1.

Figure S11. HPLC analysis of INCB024360 as potential IDO1 substrate. INCB024360 and IDO1 were incubated at room temperature for 2 hrs in the presence of all IDO1 assay components except the substrate D-Trp. INCB024360 only incubated in the assay buffer was used as control. The INCB024360 in the samples were determined by HPLC. HPLC peak areas for these samples were compared and the percentage of remaining INCB024360 for samples with 0 hr and 2 hr IDO1 incubation were calculated based on the corresponding INCB024360 peak area of the control sample (100%) at 254 and 220 nm.

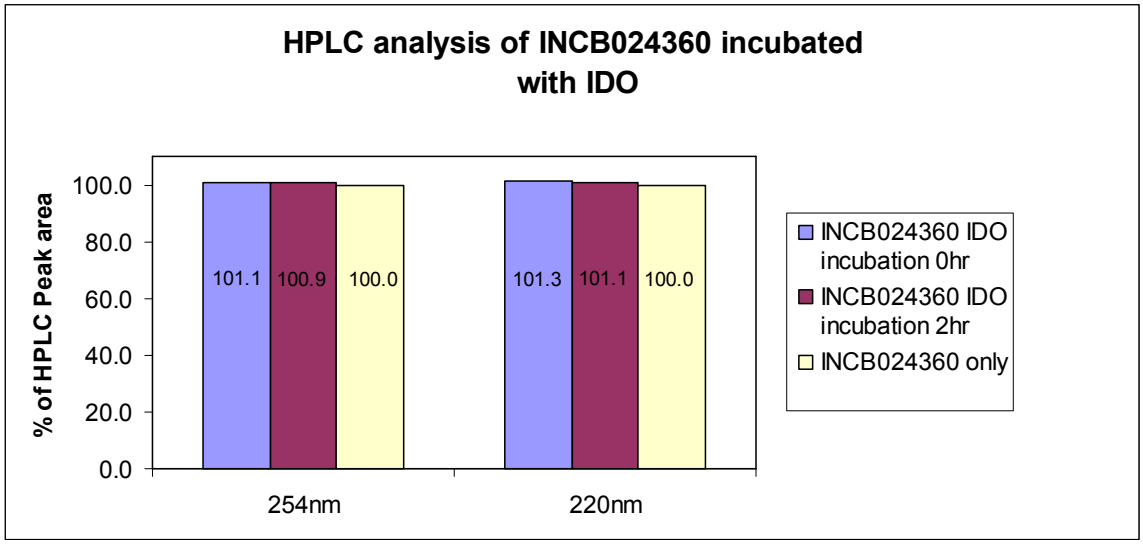


Figure S12. Progress curves for the reaction of IDO1.

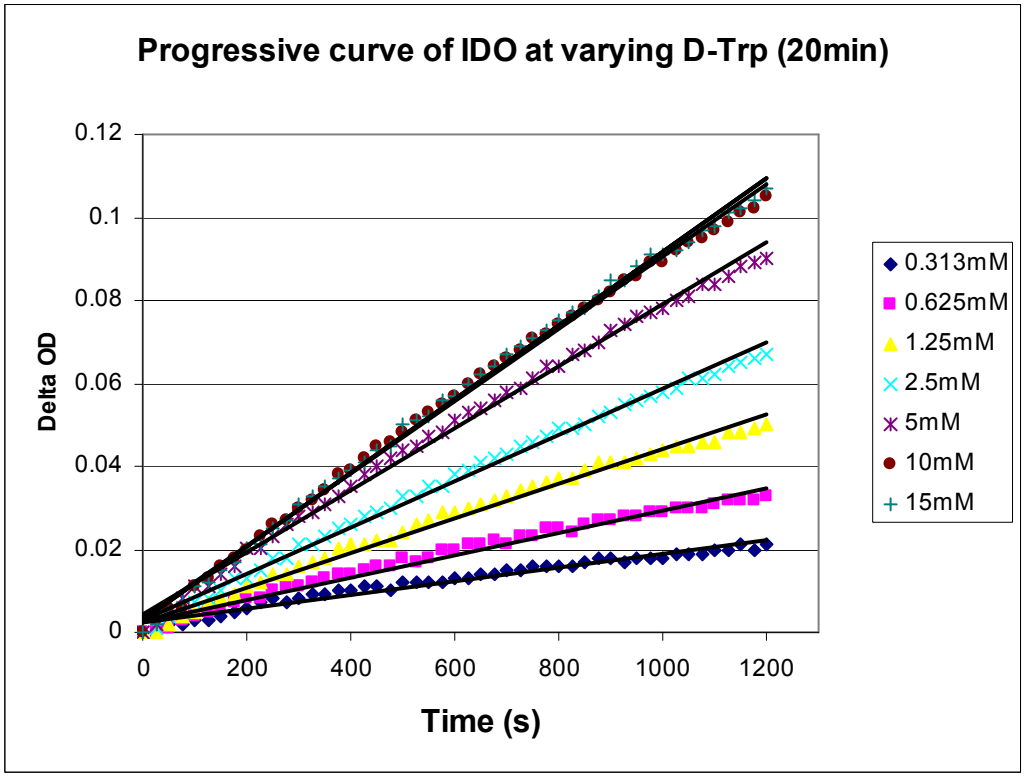


Figure S13. Mode of inhibition analysis of IDO1 inhibition by INCB024360.

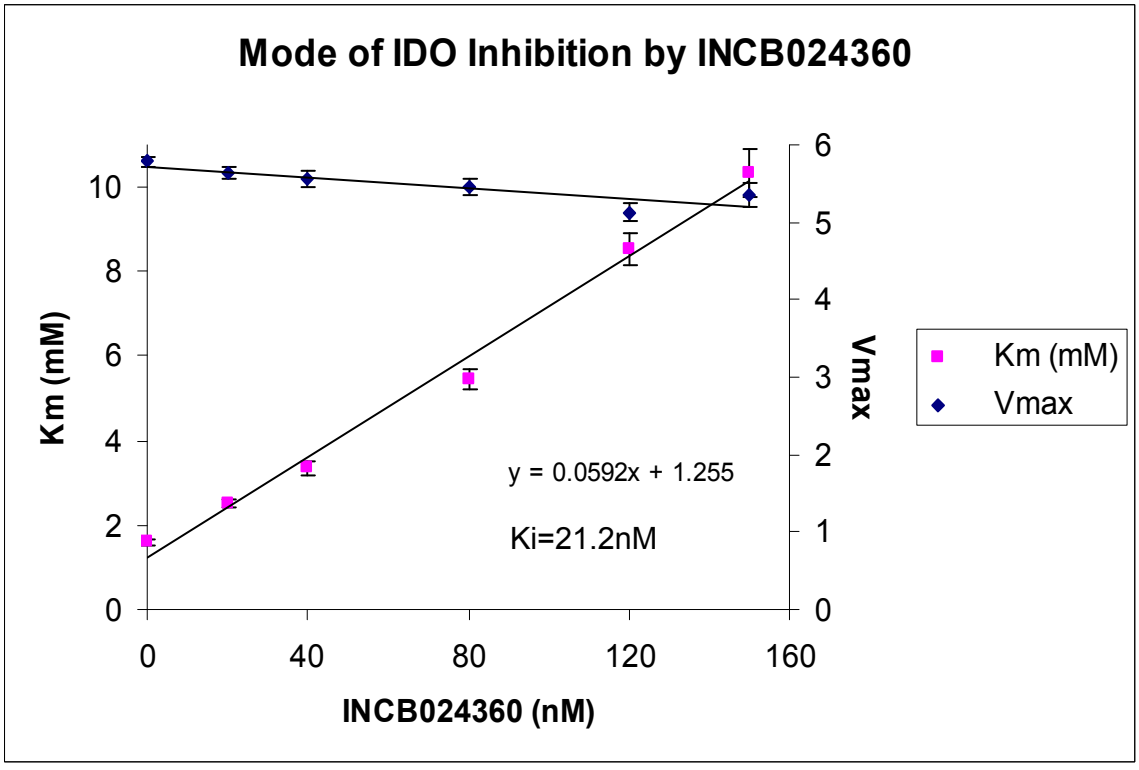


Figure S14. The reversible IDO1 inhibition by INCB024360 upon direct dilution. 2 μM IDO1 was mixed with DMSO or 3.5 μM INCB024360. Aliquots were taken from preincubated samples at 0, 1 hr and directly diluted 50 fold with assay buffer and subject to the enzymatic assay (in the final assay, IDO1 20 nM; INCB024360 35 nM assuming reversible binding mode). Percentage of IDO1 activity in the presence of INCB024360 compared to the corresponding DMSO sample were calculated as 46, 46% for 0, 1 hr after preincubation respectively.

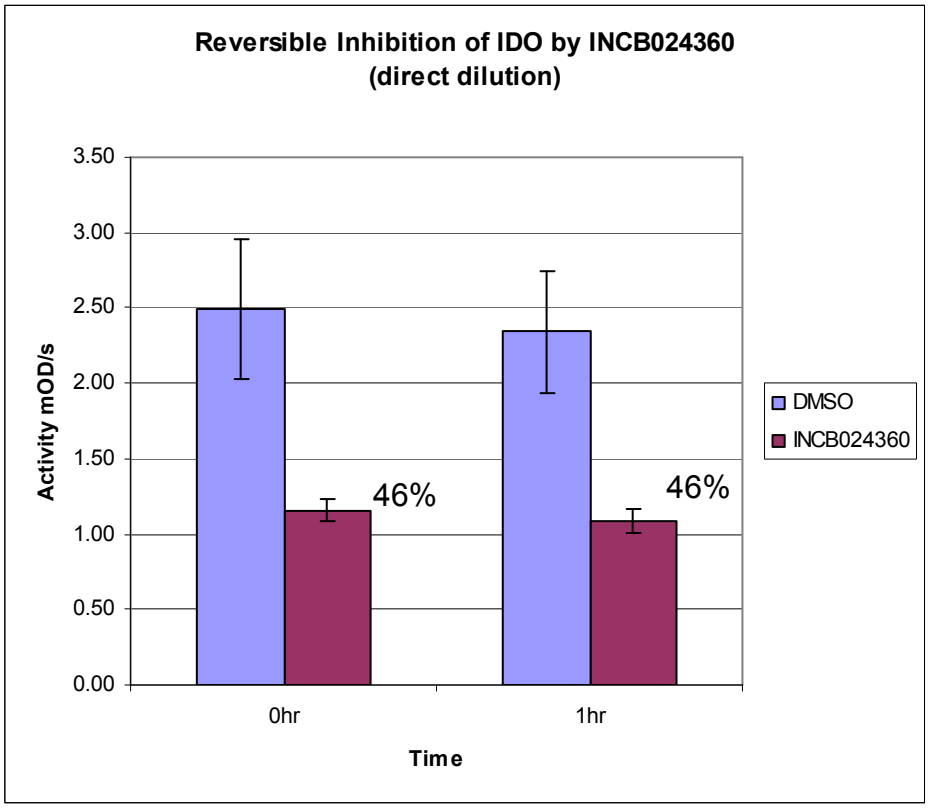
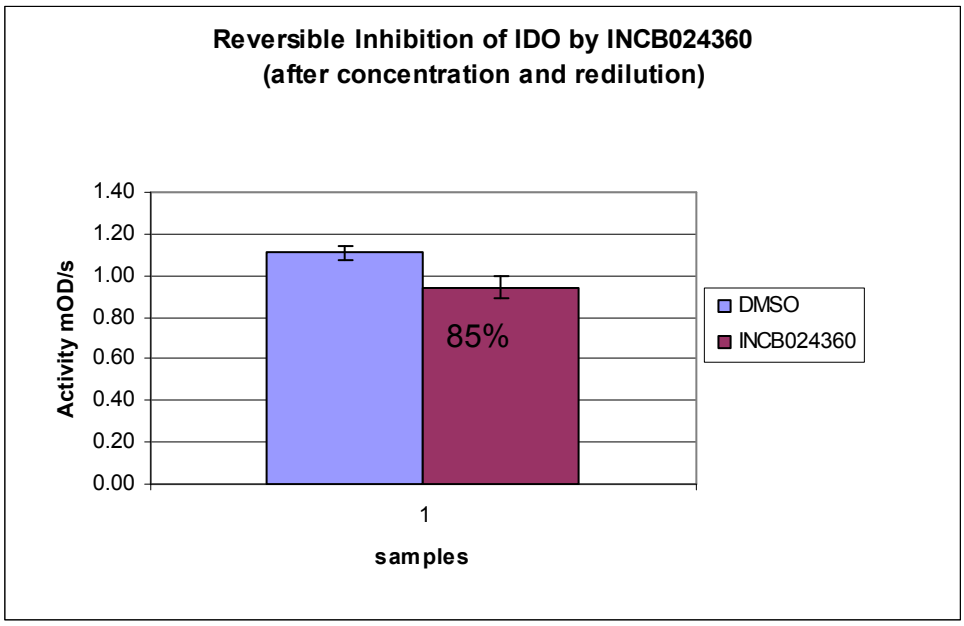


Figure S15. The IDO1 activities recovery from reversible inhibition by INCB024360. The 2 hr pre-incubated sample were diluted, concentrated and re-diluted leading to 1400 fold dilution of the INCB024360 in the sample. The further diluted sample (1.25 nM INCB024360 in the final assay) displayed 85% of the IDO1 activity compared to the control sample with DMSO only treatment.



In Vitro Profile and Physiochemical Properties of INCB24360 (4f)

The calculated properties (cLogP, PSA, MW) were generated from ChemDraw 10.

In Vitro Potency	Result	N
IDO1 IC_{50} (nM)	73 ± 19	28
HeLa IC_{50} (nM)	7.4 ± 2.4	91
WB IC_{50} (nM)	125 ± 43	4
TDO IC_{50} (nM)	>50,000	1
IDO2 -transfected HEK293 cells (nM)	>10,000	1
hERG patch IC_{50} (nM)	219,000	1
Cyp Inh. (1A2, 2C8, 2C9, 2D6, 3A4) IC_{50} (nM)	>10,000	2
PXR IC_{50} (nM)	>10,000	2
In Vitro ADME		
% free, f_u	3.1	
Caco-2 P_{app} ($x10^{-6}$) (cm/s)	4.0	
(P1 + P2) Human Clearance (L/h/kg)	0.2	
Physiochemical and Calculated Properties		
MW	438.2	
cLogP	1.5	
LogD at pH 7.4 (measured)	2.3	
HBD	6	
HBA (Total N + O)	11	
PSA ($Å^2$)	163	
LE (HeLa)	0.46	
LLE (HeLa)	6.6	
Solubility at pH 7.4 (μg/mL)	72	

In Vitro Potency of compounds **2-4**

Cmpd	IDO IC_{50} (nM)	N	HeLa IC_{50} (nM)	N
2	75 ± 36	14	19 ± 7	32
3a	50 ± 25	3	10 ± 5	11
3b	100 ± 60	3	14 ± 6	6
3c	>20000	3	>5000	6
3d	180 ± 62	3	35 ± 10	4
3e	230 ± 46	3	280 ± 40	4
4a	200 ± 110	3	100 ± 12	4
4b	170 ± 50	3	22 ± 3	4
4c	290 ± 110	4	98 ± 52	4
4d	210 ± 95	4	17 ± 5	4
4e	100 ± 19	4	16 ± 5	8
4f	73 ± 19	28	7.4 ± 2.4	91
4g	120 ± 30	17	11 ± 4	32