SUPPORTING INFORMATION:

THE DISCOVERY OF DABRAFENIB: A SELECTIVE INHIBITOR OF RAF KINASES WITH ANTI-TUMOR ACTIVITY AGAINST B-RAF DRIVEN TUMORS.

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MATERIALS AND METHODS:

Biochemical and Cellular Assays. Details of the B-Raf biochemical enzyme and cellular assays have been reported in manuscript Reference 6. The inhibition of 61 protein kinases by **12** was characterized at GlaxoSmithKline. In general, the assays were configured so that the IC_{50} values approximate the intrinsic binding constant (K_i or K_d) of **12** to each enzyme and can therefore be compared for selectivity against these kinases. A375P-F11 assay: A375P cells were plated in 96-well plates by limiting dilution and single cell-derived clones were harvested and tested for sensitivity to B-Raf inhibitors. The F11 clone was selected for future studies and was named A375P-F11. Cellular pSmad Assay to Measure Anti-TGF-β Activity: Activity of compounds was tested in a mechanistic assay in HepG2 cells. Cells were seeded in 12-well plates at a density of 500,000 cells/well and allowed to adhere overnight at 37°C/5% CO_2. Media (BME+10% serum) was removed and compound in serum free media was added to the cells for 45 minutes at 37°C/5% CO_2. Cells were stimulated with 1 ng/ml TGF-β (R&D systems) for 60 minutes. Cells were lysed in buffer (25 mM Tris-HCl ph: 7.5, 2 mM EDTA, 2 mM EGTA,1% Triton X-100, 0.1 % SDS, 50 mM sodium-β-glycerophosphate, 2 mM sodium orthovanadate, 12.5 mM sodium pyrophosphate, protease and phosphatase inhibitor cocktails) for 30 minutes, scraped, collected, clarified by centrifugation and prepared for western blots in LDS/reducing reagent (Invitogen). Samples were resolved on 4-12% Bis-Tris gels, transferred to PVDF, and probed for total and phospho-Smad2 using antibodies from Cell Signaling. Gels were imaged using the odyssey blot scanner (Licor) and quantified using Licor software. Phospho:total Smad2 ratios were determined and the IC_{50} was defined as the concentration of compound which decreased the phospho:total ratio by 50%.

Metabolite Identification. Metabolite identification study in dog liver microsomes: Dog liver microsomes were purchased from Xenotech (Lenexa, Kansas). The incubation mixture (800 μL) containing 50 mM potassium phosphate buffer, hepatic microsomal fraction (1.0 mg/mL protein) and 10 μM study compound in a 1.5 mL Eppendorf tube was pre-warmed to 37 °C. The cofactor was preincubated at 37 °C for 5 minutes. For 0 minute time point, aliquots of incubation mixture (200 μL) and cofactor solution (50 μL) were removed and crashed with stop solution [250 μL, (80/20/1, acetonitrile/ethanol/acetic acid, v/v/v)]. The reaction was started by addition of 150 μL NADPH generating system [2.2 mM NADP, 28 mM glucose-6-phosphate and glucose-6-phosphate dehydrogenase (6 units/mL) and 4.0 mM UDPGA in 2 % sodium bicarbonate] and the samples (final volume 750 μL) were incubated at 37 °C. No drug control incubations were conducted in parallel using blank incubation medium with an appropriate amount of methanol in place of the compound for liver microsomes. After a 30 minute incubation the reaction was terminated by the addition of one volume of stop solution. Samples were centrifuged at 34000 rpm for 5 min and 50 μL supernatants were injected to LC/MS for metabolite identification. Metabolite identification study in dog hepatocytes: Dog hepatocytes (Db157) were purchased from CellzDirect (Tucson, AZ). The incubation mixtures consisting of William's medium E (pH = 7.4), hepatocytes suspension (0.7 million cells/mL) and 10 μM study compound in a total volume of 600 μL were placed into a single well of 12-well culture plate and incubated for 4 h at 37 °C. No drug control incubations were conducted in parallel using blank incubation medium with an appropriate amount of methanol in place of active treatment for liver hepatocytes. After incubation, the cells were scraped from the bottom of each well and mixed with 600 μL of stop solution. The mixture was then stored in -80 °C freezer. Samples were centrifuged at 34000 rpm for 5 min and 50 μL supernatants were injected to LC/MS for metabolite identification.

A375P F11 Melanoma Xenograft Studies.

Cells were implanted in nude mice and grown as tumor xenografts. Dosing began when tumors achieved ~150-200mm^3 volume. GSK2118436 was administered by oral gavage at a dose volume of 0.2 mL/20 gram body weight in 0.5% hydroxypropylmethylcellulose (Sigma) and 0.2% Tween-80 in distilled water pH 8.0. Dosing was daily for duration stipulated. Results are reported as mean tumor volume for n=7-8 mice/group. Tumors were measured twice weekly with Vernier calipers, and tumor volume was estimated from two-dimensional measurements using a prolate ellipsoid equation (Tumor volume mm^3 = (length x width^2) x 0.5). Complete tumor regression was defined as a >93% decrease in an individual tumor volume for at least 1 week.

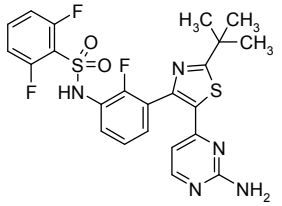
Pharmacokinetic (PK) Analysis.

Blood was drawn and hemolyzed immediately with an equal volume of water. Concentrations of compound in tumor were determined on polytron homogenized tissues in 4 volumes of water per volume of frozen tissue. Aliquots of the homogenized tumor and blood were flash frozen and subsequently evaluated for compound concentration by HPLC/MS/MS analysis.

Pharmacodyamic measurement of pERK levels in tissues.

Tissues were harvested and homogenized using Medimachine (BD Bioscience) with 1 mL of lysis buffer (25 mM Tris-HCl (pH 7.5), 2 mM EDTA (pH 8.0), 2 mM EGTA (pH 8.0), 1% Triton X-100, 0.1% SDS, 50 mM sodium glycerol phosphate, 2 mM Na_3VO_4, 4 mM Na-pyrophosphate, 2x phosphatase inhibitor cocktail), and kept on ice. Following homogenization of all samples, homogenates were centrifuged at 14,000 rpm for 15 min at 4°C and flash frozen for later analysis. All samples were analyzed with a duplex ELISA (MescoScale Discovery) measuring total ERK1/2 and phospho-ERK1/2 according to manufacturer's instructions. Plates were read on MSD.SI6000.

Table S1. Activity of GSK2118436 against selected kinases



Kinase Family	Assay	IC_{50} (nM)[a]
TKL	B-Raf^{V600E}	0.7
	B-Raf WT	5.2
	c-Raf	6.3
	Alk5	12[b]
	LRRK2	>10,000
TK	Lck	632
	VEGFR2	632
	EGFR	>10,000
	Syk	>10,000
STE	PAK1&4	>10,000
AGC	AurB	5,000
	ROCK1	>10,000
	Akt1&2	>10,000
CAMK	SIK2	76[b,c]
	MAPKAPK2	>10,000[c]
CMGC	GSK3	794[b]
	JNK1	>10,000
	P38α	>10,000

[a]IC_{50} values are means of at least 2 experiments; individual values are within 2-fold of the reported mean value. [b]Data from fluorescence polarization binding assay. All other data are from activity assays.[c] IC_{50} values are n=1.

Binding Mode:

GSK2118436 is an ATP-competitive inhibitor of B-Raf[11], and is postulated to bind to an inactive-like conformation of the kinase (Figure S1), based on a model of GSK2118436 docked into a recently reported crystal structure of B-Raf^{V600E} bound to other small molecule ATP-binding site inhibitors.[1] In this model, the αC helix 'shifts out' relative to an active-like conformation and a salt bridge between the conserved lysine and glutamic acid is broken. While Phe595 is not in a 'DFG-out' conformation, it is rotated to form the floor of a pocket similar to that observed in a reported lapatinib/EGFR co-structure.[2] The pyrimidine N1 forms the classic hinge interaction with Cys532 in the ATP-binding pocket of the enzyme. The *t*-butyl group and thiazole core bind underneath the P-loop leaving only a relatively small portion of the inhibitor as solvent exposed. The arylsulfonamide headgroup of GSK2118436 is predicted to bind into the lipophilic back pocket allowing the sulfonamide to form two hydrogen bonds with the backbone NHs of Asp594 and Phe595. The sulfonamide NH is depicted in a deprotonated form, leaving the nitrogen to participate as a hydrogen bond acceptor. The significant improvement in potency observed with compounds bearing a fluorine substitution at R^2 is hypothesized to be a result of modulating the pKa of the sulfonamide NH which could allow both increased ionization of **12** at cellular pH and a more favorable conformation for headgroup binding. Conversely, compounds containing fluorine substitution at R^1 (compounds **4** and **9**), may induce an unfavorable conformation of the benzenesulfonamide moiety in the binding pocket, potentially accounting for the reduced target affinity observed with those analogs.

Analogous models of aniline-tail containing molecules (like **1**) show that the aromatic ring in the tail group resides largely within the B-Raf binding pocket while the morpholino ring extends into the solvent exposed region. Comparing **1** and **2**, after complete truncation of the tail to the "naked" aminopyrimidine and a loss of 13 heavy atoms, a 10 fold drop in potency was observed. Despite the small drop in potency, **2** actually exhibits a higher ligand efficiency compared to **1** (LE = 0.30 for **2** vs 0.25 for **1**), as the all of the remaining heavy atoms in **2** are contained fully within the binding pocket.

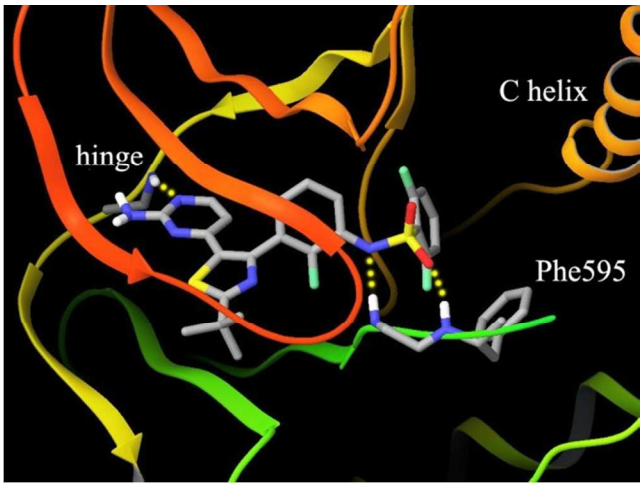


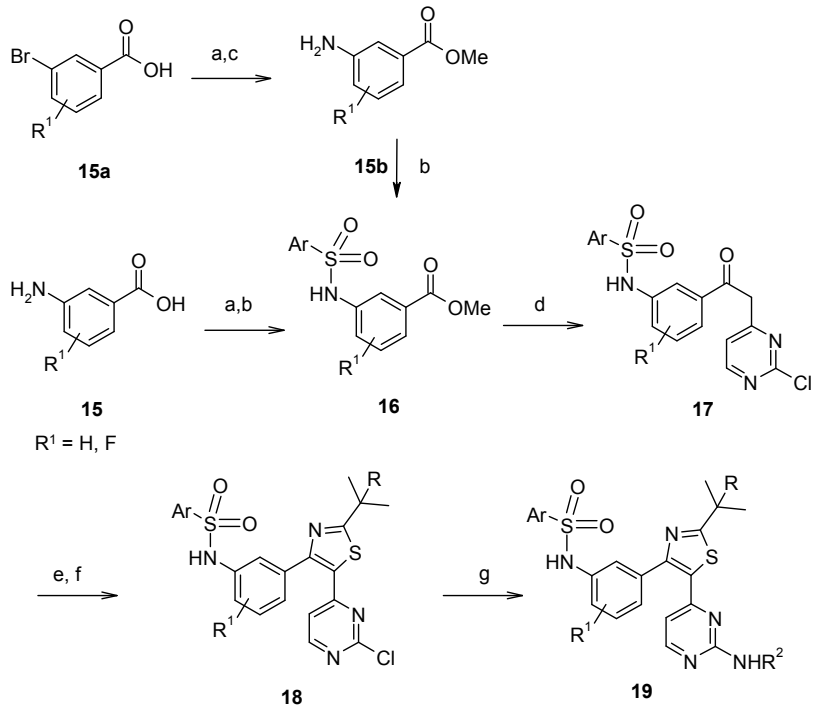
Figure S1. Model of GSK2118436 bound to B-Raf^{V600E} as seen from above the P-loop. Hydrogen bonds are shown in yellow. Image produced with Maestro (Schrodinger, LLC).

1. Tsai, J.; Lee, J.; Wang, W.; Zhang, J.; Cho, H.; Mamo, S.; Bremer, R.; Gillette, S.; Kong, J.; Haass, N.; Sproesser, K.; Li, L.; Smalley, K.; Fong, D.; Zhu, Y.-L.; Marimuthu, A.; Nguyen, H.; Lam, W.; Liu, J.; Cheung, I.; Rice, J.; Suzuki, Y.; Luu, C.; Settachatgul, C.; Shellooe, R.; Cantwell, J.; Kim, S.-H.; Schlessinger, J.; Zhang, K.; West, B.; Powell, B.; Habets, G.; Zhang, C.; Ibrahim, P.; Hirth, P.; Artis, D.; Herlyn, M.; Bollag, G. Discovery of a selective inhibitor of oncogenic B-Raf kinase with potent antimelanoma activity. *Proc. Nat. Acad. Sci*. **2008**, *105*, 3041-3046.

2. Wood, E.; Truesdale, A.; McDonald, O.; Yuan, D.; Hassell, A.; Dickerson, S.; Ellis, B.; Pennisi, C.; Horne, E.; Lackey, K.; Alligood, K.; Rusnak, D.; Gilmer, T.; Shewchuk, L. A unique structure for epidermal growth factor receptor bound to GW572016 (lapatinib): relationships among protein conformation, inhibitor off-rate, and receptor activity in tumor cells. *Cancer Res.* **2004**, *64*, 6652-6659.

Synthesis and characterization:

Scheme S1. General synthetic route for thiazole inhibitors.

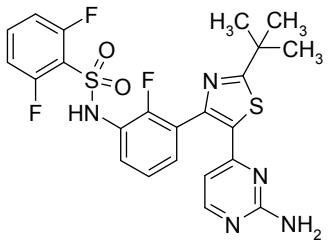


Reagents and conditions: a) H_2SO_4, MeOH; b) $ArSO_2Cl$, pyridine, DCM; c) 1,1-dimethylethyl carbamate, $Pd_2(dba)_3.CHCl_3$, xantphos, Cs_2CO_3 d) 2-chloro-4-methylpyrimidine, LiHMDS; e) NBS, DCM then 2-aminopyridine, dioxane; f) NBS, DMF then alkyl thioamide; g) 7N ammonia in methanol, sealed tube 100 ^oC or R^2NH_2 amine, HCl, 2,2,2-trifluoroethanol, microwave, 180^oC.

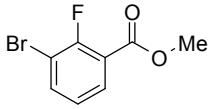
Analogs described herein were generally prepared according to Scheme S1. When the desired benzoic acid precursors were unknown, the synthetic scheme began with esterification of bromo-acids **15a**. Subsequent palladium-catalyzed amination with *t*-butyl carbamate afforded anilino esters **15b**. After esterification of benzoic acids **15**, or amination of bromo-esters leading to **15b**, the anilino esters were reacted with an arylsulfonyl chloride to form the sulfonamide headgroup. Ester **16** was then condensed with the lithium anion of 2-chloro-4-methylpyrimidine to generate ketone intermediate **17**. Bromination of **17** with NBS followed by cyclization with isopropyl or *t*-butyl thioamide afforded the desired thiazole core **18**. The tail was then installed by S_NAr displacement at the chloropyrimidine in **18** with either methanolic ammonia or a primary basic amine to generate the desired analogues **19**. An example of the synthesis of **12** is described below and other analogs were prepared in an analogous fashion and described in detail in the following reference: Adams, J.L., Dickerson, S. H.,

Johnson, N.W., Kuntz, K., Petrov, K., Ralph, J.M., Rheault, T.R., Schaaf, G., Stellwagen, J., Tian, X, Uehling, D.E., Waterson, A.G., Wilson, B. WO 2009137391 A2, 2009.

(**12**): *N*-{3-[5-(2-Amino-4-pyrimidinyl)-2-(1,1-dimethylethyl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,6-difluorobenzenesulfonamide

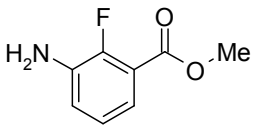


Step a: Methyl 3-bromo-2-fluorobenzoate



To a 100 mL round bottom flask was added 3-bromo-2-fluorobenzoic acid (10.4 g, 47.5 mmol), MeOH (100 mL, 2472 mmol) and sulfuric acid (6 mL, 113 mmol). The reaction mixture was refluxed for 1 hr. After cooling to rt, the MeOH was removed under reduced pressure and the acidic residue was poured into cold water and EtOAc, the layers were separated and the aqueous layer was extracted with EtOAc. The organic layers were combined, washed with brine, dried over $NaSO_4$ and concentrated under reduced pressure to afford 10.02 g of methyl 3-bromo-2-fluorobenzoate. ^{1}H-NMR (400 MHz, *DMSO-d*$_6$) δ 7.95 (ddd, *J* = 8.1, 6.4, and 1.7 Hz, 1 H), 7.82 - 7.87 (m, 1 H), 7.26 (t, *J* =7.9 Hz, 1 H), and 3.86 (s, 3 H).

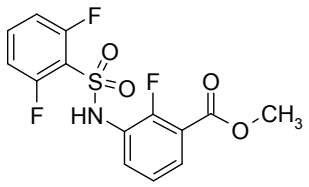
Step c: Methyl 3-amino-2-fluorobenzoate



In a 500 mL flask was placed 1,1-dimethylethyl carbamate (6.03 g, 51.5 mmol), methyl 3-bromo-2-fluorobenzoate (10 g, 42.9 mmol), $Pd_2(dba)_3.CHCl_3$ (0.89 g, 0.86 mmol), xantphos (1.49 g, 2.57 mmol) and ce-

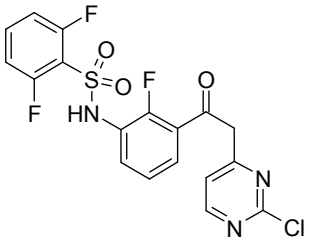
sium carbonate (16.8 g, 51.5 mmol). The flask was sealed with a rubber septum, placed under high vacuum, and toluene (200 mL) was added. Three cycles of high vacuum/N_2 were performed and the reaction mixture was stirred at 90 °C overnight. The reaction was filtered through a pad of celite with EtOAc washing and concentrated. To the residue was added DCM (200 mL) followed by TFA (50 mL, 649 mmol), and the mixture was stirred at rt for 1 h. The volatiles were removed under reduced pressure and the residue was taken up in EtOAc and washed with saturated $NaHCO_3$ and brine. The organic layer was dried over $NaSO_4$, stripped onto silica and column chromatographed on silica with 5% to 50% EtOAc:Hexane to give 5.53 g (76%) of the title compound of Step B. ^{1}H-NMR (400 MHz, *DMSO-d*$_6$) δ 6.92 - 7.01 (m, 3 H), 5.37 (s, 2 H), and 3.81 (s, 3 H). MS (ESI): 170 $[M+H]^+$.

Step b: Methyl 3-{[(2,6-difluorophenyl)sulfonyl]amino}-2-fluorobenzoate



In a 500 mL flask was placed methyl 3-amino-2-fluorobenzoate (5.5 g, 32.5 mmol) and DCM (100 mL), and pyridine (2.9 mL, 35.8 mmol) was added. 2,6-Difluorobenzenesulfonyl chloride (7.6 g, 35.8 mmol) in DCM (50 mL) was added dropwise via addition funnel and the reaction mixture was allowed to stir at rt overnight. The reaction mixture was stripped onto silica and column chromatographed on silica with 5% to 100% EtOAc:Hexane to give 9.75 g (87%) of the title compound of Step C. ^{1}H-NMR (400 MHz, *DMSO-d*$_6$) δ 10.98 (s, 1 H), 7.64 - 7.82 (m, 3 H), 7.46 - 7.61 (m, 1 H), 7.29 (t, *J* = 8.8 Hz, 2 H), and 3.81 (s, 3 H). MS (ESI): 346 $[M+H]^+$.

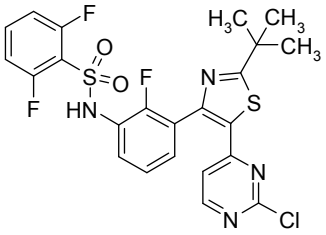
Step d: *N*-{3-[(2-Chloro-4-pyrimidinyl)acetyl]-2-fluorophenyl}-2,6-difluorobenzenesulfonamide



In a 1000 mL flask was placed methyl 3-{[(2,6-difluorophenyl)sulfonyl]amino}-2-fluorobenzoate (9.64 g, 27.9 mmol) and THF (200 mL) was added. The flask was placed in an ice/water bath and LiHMDS (90 mL, 90 mmol) was added. 2-Chloro-4-methylpyrimidine (4.5 g, 35.0 mmol) in THF (60 mL) was added dropwise via addition funnel. After the addition was complete, the reaction was allowed to warm to 20 °C over 1 h. The THF volume

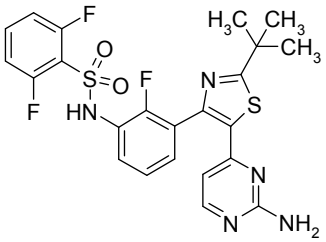
was reduced to half under reduced pressure and then treated with 6 N HCl. EtOAc was added and the layers were separated. The aqueous layer was extracted twice with EtOAc and the combined organic layer was washed once with brine, dried over $NaSO_4$, and concentrated. The residue was triturated with EtOAc/ether to afford 8.71 g (71%) of the title compound of Step D as a mixture of keto-enol. ^{1}H NMR (400 MHz, DMSO-d6) δ ppm 13.40 (s, 1 H) 10.82 - 11.12 (m, 2 H) 8.48 - 8.87 (m, 2 H) 7.67 - 7.80 (m, 3 H) 7.61 - 7.69 (m, 1 H) 7.52 - 7.61 (m, 2 H) 7.50 (d, J=5.31 Hz, 1 H) 7.43 (td, J=7.60, 1.28 Hz, 1 H) 7.21 - 7.38 (m, 6 H) 6.13 (s, 1 H) 4.49 (s, 2 H). MS (ESI): 442 $[M+H]^+$.

Steps e and f: *N*-{3-[5-(2-Chloro-4-pyrimidinyl)-2-(1,1-dimethylethyl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,6-difluorobenzenesulfonamide



To a solution of *N*-{3-[(2-chloro-4-pyrimidinyl)acetyl]-2-fluorophenyl}-2,6-difluorobenzenesulfonamide (2.0 g, 4.53 mmol) in 40 mL DMA, 1.0 eq. NBS (0.806 g, 4.53 mmol) was added and the solution was allowed to stir 15 min at rt. 2,2-dimethylpropanethioamide (0.531 g, 4.53 mmol) was then added at rt. The reaction was heated to 60 ºC for 2 hours. The reaction was not complete by LC-MS. The reaction mixture was then heated to 80 ºC for an additional hour. The reaction mixture was diluted with water and extracted x 2 with EtOAc. The combined EtOAc washings were washed with water x 3 to remove DMA, dried over $MgSO_4$, filtered and concentrated onto silica gel. The crude material was chromatographed in 10-80% EtOAc in Hexanes to give the desired product, 1.6 g (64%). ^{1}H NMR (400 MHz, DMSO-d6) δ ppm 10.92 (s, 1 H) 8.54 (d, J=5.31 Hz, 1 H) 7.60 - 7.79 (m, 1 H) 7.39 - 7.50 (m, 2 H) 7.29 - 7.40 (m, 1 H) 7.24 (t, J=9.16 Hz, 2 H) 6.86 (d, J=5.31 Hz, 1 H) 1.43 (s, 9 H). MS (ESI): 539.1 $[M+H]^+$.

Step g: *N*-{3-[5-(2-Amino-4-pyrimidinyl)-2-(1,1-dimethylethyl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,6-difluorobenzenesulfonamide



A suspension of *N*-{3-[5-(2-chloro-4-pyrimidinyl)-2-(1,1-dimethylethyl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,6-difluorobenzenesulfonamide (196 mg, 0.364 mmol) and 7M ammonia in MeOH (8 mL, 56.0 mmol) was heated in a sealed tube to 90 °C for 24 hours. The reaction was diluted with DCM and added silica gel and concentrated. The crude product was chromatographed on silica gel eluting with 100% DCM to 1:1 [DCM:(9:1 EtOAc:MeOH)]. The clean fractions were concentrated to yield the crude product as a yellow solid (62 mg). The crude product was repurified by reverse phase HPLC (a gradient of acetonitrile:water with 0.1%TFA in both). The combined clean fractions were concentrated then partitioned between DCM and saturated $NaHCO_3$. The DCM layer was separated and dried over Na_2SO_4. The title compound, *N*-{3-[5-(2-amino-4-pyrimidinyl)-2-(1,1-dimethylethyl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,6-difluorobenzenesulfonamide was obtained (94 mg, 47% yield). ^{1}H NMR (400 MHz, DMSO-*d*6) δ ppm 10.83 (s, 1 H), 7.93 (d, *J*=5.2 Hz, 1 H), 7.55 - 7.70 (m, 1 H), 7.35 - 7.43 (m, 1 H), 7.31 (t, *J*=6.3 Hz, 1 H), 7.14 - 7.27 (m, 3 H), 6.70 (s, 2 H), 5.79 (d, *J*=5.13 Hz, 1 H), 1.35 (s, 9 H). MS (ESI): 519.9 $[M+H]^{+}$. ^{13}C NMR (100 MHz, DMSO-*d*6) δ ppm 182.1, 164.0, 160.6, 159.4, 158.0, 154.9, 152.4, 145.8, 136.6, 135.1, 130.0, 128.4, 125.6, 124.7, 114.1, 113.9, 105.7, 38.3, 31.0.