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Optimization of Diarylthiazole B-Raf Inhibitors: Identification of a Compound Endowed with High Oral Antitumor Activity, Mitigated hERG Inhibition, and Low Paradoxical Effect

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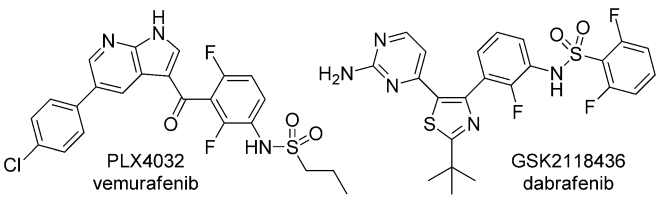
Aberrant activation of the mitogen-activated protein kinase (MAPK)-mediated pathway components, RAF-MEK-ERK, is frequently observed in human cancers and clearly contributes to oncogenesis. As part of a project aimed at finding inhibitors of B-Raf, a key player in the MAPK cascade, we originally identified a thiazole derivative endowed with high potency and selectivity, optimal in vitro ADME properties, and good pharmacokinetic profiles in rodents, but that suffers from elevated hERG inhibitory activity. An optimization program was thus undertaken, focused mainly on the elaboration of the R^1 and R^2 groups of the scaffold. This effort ultimately led to *N*-(4-{2-(1-cyclopropylpiperidin-4-yl)-4-[3-(2,5-difluorobenzenesulfonylamino)-2-fluorophenyl]thiazol-5-yl}-pyridin-2-yl)acetamide (**20**), which maintains favorable in vitro and in vivo properties, but lacks hERG liability. Besides exhibiting potent antiproliferative activity against only cell lines bearing B-Raf V600E or V600D mutations, compound **20** also intriguingly shows a weaker "paradoxical" activation of MEK in non-mutant B-Raf cells than other known B-Raf inhibitors. It also demonstrates very good efficacy in vivo against the A375 xenograft melanoma model (tumor volume inhibition $>90\%$ at 10 mg kg^{-1}); it is therefore a suitable candidate for preclinical development.

Introduction

The oncology research community has concentrated much of its effort over the past few years toward the development of novel, target-directed therapeutic agents. In this respect, the RAS/RAF/MEK/ERK (MAPK) signal transduction cascade[1] has attracted much attention, because aberrant activation of the pathway components is frequently found in human cancers and contributes to oncogenesis. B-Raf, one of the three isoforms of the Raf family of serine/threonine-specific protein kinases, is crucial to this pathway and presents activating somatic mutations[2] in approximately 50% of melanomas, 45% of thyroid, 10% of colon, and 8% of ovarian carcinomas. The most common B-Raf mutation, replacement of valine with glutamic acid at position 600 within the activation segment of the kinase domain, accounts for 90% of mutated B-Raf cases, and results in destabilization of the inactive conformation, as shown by the crystal structure of the B-Raf V600E mutant protein, elevated kinase activity, and enhanced promotion of cell survival and proliferation.[3] Selective targeting of V600E B-Raf with small-molecule inhibitors is an already proven valid therapeutic approach for the treatment of metastatic melanoma. Vemurafenib (PLX4032/RG7204, Plexxikon/Roche)[4,5] was the first entity to be approved for this specific indication by the US Food and Drug Administration (FDA) in 2011. A number of additional small-molecule inhibitors have shown preclinical[6] and clinical value.[7] Among these, dabrafenib (GSK2118436, GlaxoSmithKline),[8–10] which received FDA approval as a single agent in 2013 and in combination therapy in January 2014, deserves particular mention in the context of the present work. In fact, dabrafenib belongs to a chemical series—arylthiazole derivatives bearing a sulfonamide head group—closely related to the compound class we have independently developed and which is the object of the present disclosure.

Our initial efforts in the thiazole series originated from the previous identification of a class of potent and selective B-Raf inhibitors based on a substituted pyrazole template.[11] In carrying out diversification and scaffold-hopping efforts, we next



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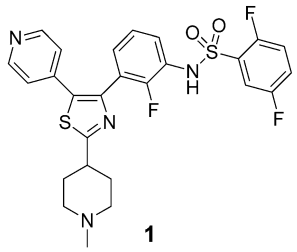
discovered the bioisosteric thiazole series, which had very similar in vitro activity, metabolic profile, and pharmacokinetics in mouse, but, as was the case with lead compound **1**, significantly improved pharmacokinetics in rat. Nevertheless, **1** suffered from other liabilities, namely a high level of hERG inhibition. Herein we present an account of the efforts undertaken during project advancement, which produced derivatives **2**–**19** and ultimately led to the identification of compound **20**, a candidate suitable for preclinical development.



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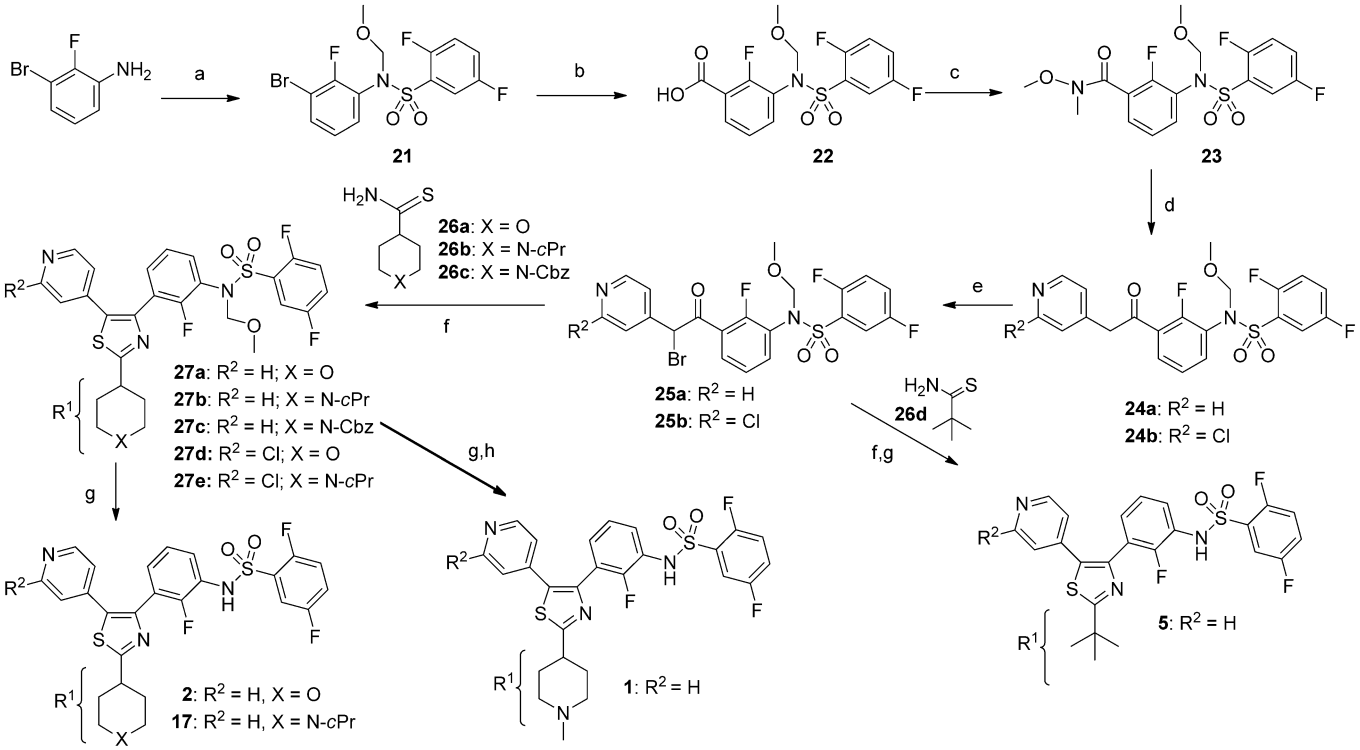
Results and Discussion

Chemistry

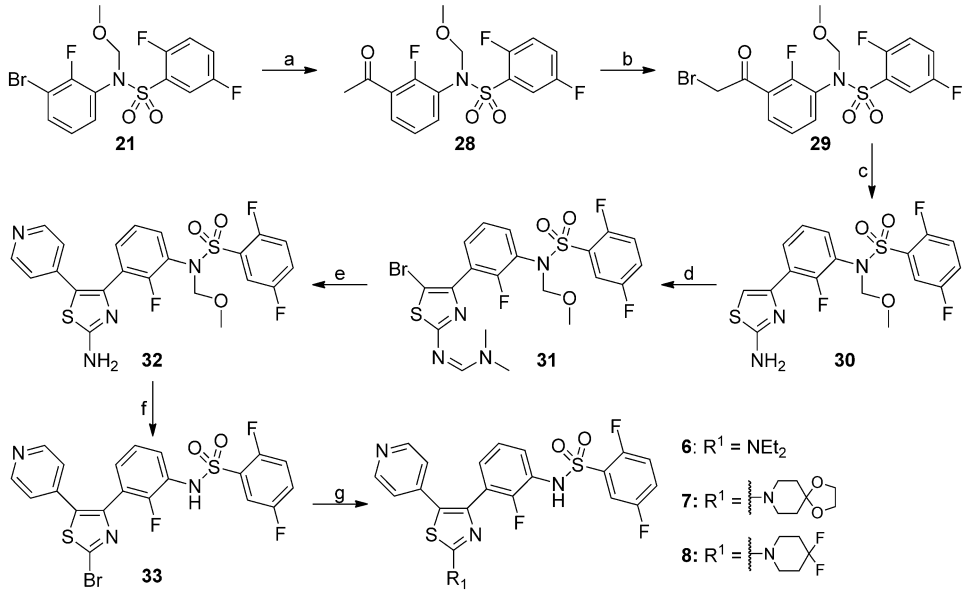
Two main strategies were pursued to access the thiazole derivatives. The first entails a convergent approach, whereby formation of the core thiazole ring bearing the appropriate substituent at position 2 (R^1 group) is achieved by reaction of a preformed thioamide with a suitable bromoketone (Scheme 1). The second approach involves formation of an aminothiazole intermediate, which is further elaborated by first introducing the pyridine moiety and then the R^1 group (Scheme 2).

In the first approach, 2-fluoro-3-bromoaniline was treated with 2,5-difluorobenzenesulfonyl chloride, leading to an intermediate sulfonamide that was protected with a methoxymethyl (MOM) group. Arylbromide **21** was converted into the corresponding Grignard reagent, which was quenched with carbon dioxide to afford the benzoic acid **22** in good yield. This compound was transformed into the corresponding Weinreb amide **23** which, in turn, was condensed with the carbanion of 4-picoline, providing the intermediate ketone **24a**. Bromination of the latter afforded the intermediate bromoketone **25a**. Condensation of this key derivative with thioamide **26c** led to thiazole **27c**, which was further elaborated by removing the protecting groups and methylating the piperidine nitrogen atom to afford the lead compound **1**. This synthetic pathway proved quite versatile and was also pursued to synthesize compounds **2**, **17**, and **5** (Scheme 1) through the analogous condensation of **25a** with **26a**, **26b**, and **26d**, respectively, followed by protecting group removal.

Aminothiazole derivatives **6**, **7**, and **8** were instead synthesized by following the second approach. Methylketone **28** was obtained from bromo derivative **21** by means of a Heck coupling reaction with *n*-butylvinyl ether and subsequent acidic hydrolysis of the corresponding enol ether. Bromination of **28** gave the α-bromoketone **29**, which was condensed with thiourea to give the aminothiazole derivative **30**. The thiazole moiety was then brominated at position 5, and the amino



Scheme 1. Synthesis of lead compound **1** and compounds **2**, **17**, and **5**. *Reagents and conditions*: a) 1. 2,5-difluorobenzensulfonylchloride, pyridine, CH_2Cl_2, RT, 2 h, 2. MOMCl, DIPEA, CH_2Cl_2, RT, 1.5 h; b) 1. *i*PrMgCl, THF, RT, 2 h, 2. CO_2, 0 °C, 20 min; c) *N*-methoxy-*N*-methylamine, EDCI, NMM, DMAP, CH_2Cl_2, DMF, RT, 4 h; d) 4-picoline or 2-chloro-4-picoline, LDA, THF, −78 °C→RT, 2.5 h; e) pyridinium bromide perbromide, DMF, RT, 50 min; f) thioamide, EtOH, 60 °C, 2 h; g) TFA/H_2O, 80 °C, 3 h; h) aq. formaldehyde 37%, AcOH, MeOH, $NaBH_3CN$, RT, 2 h.



Scheme 2. Synthesis of compounds **6**, **7**, and **8**. *Reagents and conditions*: a) 1. dppp, *n*-butylvinylether, $Pd(OAc)_2$, ethylene glycol, 120 °C, 6 h, 2. HCl 1 N in 1,4-dioxane, RT, 1 h; b) pyridinium bromide perbromide, THF, microwave, 80 °C, 15 min; c) thiourea, EtOH, 60 °C, 20 min; d) 1. NBS, CH_2Cl_2, RT, 30 min, 2. dimethylformamide dimethylacetal, DMF, RT, 16 h; e) 1. 4-pyridylboropinacolate, $PdCl_2(dppf)\cdot CH_2Cl_2$, Cs_2CO_3, DME, H_2O, microwave, 110 °C, 1 h, 2. ethylenediamine, EtOH, 8 h; f) 1. $CuBr_2$, *tert*-butyl nitrite, CH_3CN, 85 °C, 8 h, 2. TFA, H_2O, 80 °C, 6 h; g) secondary amine, DMA, microwave, 120 °C, 3 h.

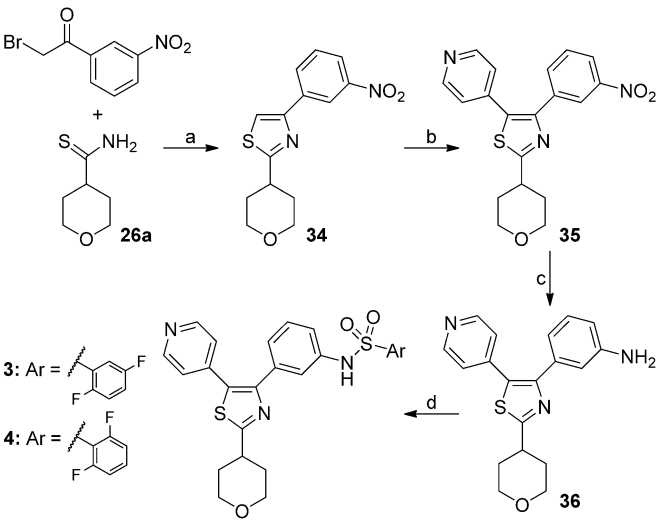
group was protected as the dimethylaminoimino derivative, leading to intermediate **31**. The latter was subjected to Suzuki reaction to introduce the pyridyl moiety, followed by removal of the amine protecting group, thus affording the aminothiazole **32**. Sandmeyer reaction and MOM removal gave 2-bromothiazole **33**, which was allowed to react with the suitable secondary amine to yield compounds **6**, **7**, and **8** (Scheme 2).

A simpler strategy was adopted to synthesize derivatives without the fluorine atom on the central phenyl ring. Thiazole **34** was prepared by condensation between thioamide **26a** and the commercially available 3-nitrobromoacetophenone. Bromination followed by Suzuki coupling allowed introduction of the pyridyl moiety to afford derivative **35**. Reduction of the nitro group provided intermediate **36**, which was in turn treated with the suitable sulfonyl chloride, leading to final compounds **3** and **4**, as depicted in the Scheme 3.

The compounds bearing a substituent at position 2 of the pyridine ring were obtained by elaborating either the 2-chloropyridine intermediates **27d** and **27e**, or the pyridine *N*-oxide derivative **38**, in turn obtained from **27a**, as outlined in Schemes 4 and 5, respectively. Compounds **27d** and **27e** were obtained from intermediate **25b** as illustrated in Scheme 1. Starting from **27d**, simple sulfonamide deprotection gave compound **15**, while palladium-catalyzed coupling with trimethylaluminum followed by MOM group removal afforded compound **16**. The amino group was introduced by Buchwald reaction with *tert*-butoxycarbamate, giving intermediates **37a** and **37b**, which could be either fully deprotected to give compounds **9** and **18**, respectively, or methylated with methyl iodide before deprotection to afford the respective compounds **10** and **19**. Acetylaminopyridine derivatives **14** and **20** were instead obtained by direct palladium-mediated coupling with acetamide followed by MOM removal. Activation of the pyridine *N*-oxide derivative **38** with PyBroP and reaction with the corresponding primary amines was exploited to prepare compounds **11**, **12**, and **13**.

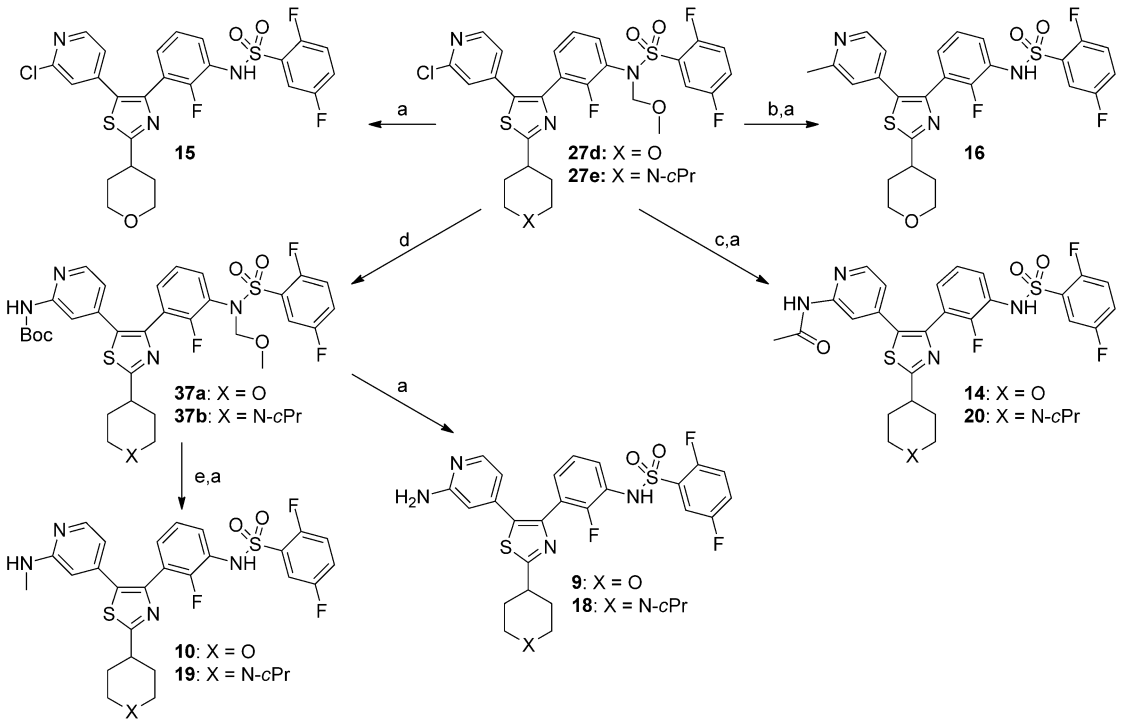
Optimization of compound 1

Compound **1** is a potent ATP-competitive, low-nanomolar B-Raf inhibitor with high selectivity against a panel of 53 kinases belonging to several families (see Supporting Information). It is endowed with nanomolar antiproliferative activity against cell lines harboring the V600E B-Raf mutation (e.g., melanoma A375) and high selectivity versus other tumor cell types expressing wild-type (wt) B-Raf (i.e., $IC_{50} > 10$ μM

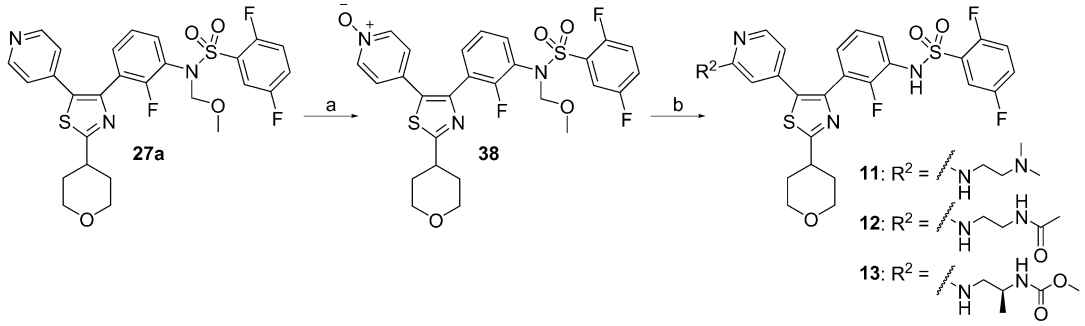


Scheme 3. Synthesis of difluorinated compounds **3** and **4**. *Reagents and conditions*: a) EtOH, reflux, 2 h; b) 1. Br_2, glacial AcOH, NaOAc, RT, 2 days, 2. 4-pyridylboropinacolate, $PdCl_2(dppf)$, Cs_2CO_3, dioxane, H_2O, 100 °C, 5 h; c) Zn, AcOH, NaOAc, RT, 3 h; d) difluorobenzenesulfonyl chloride, CH_2Cl_2, pyridine, RT, 2 h.

toward melanoma MEWO, Table 1, or ovarian A2780 cell lines). Compound **1** is characterized by good physicochemical and in vitro ADME properties, which correlate well with its favorable mouse and rat pharmacokinetics (Table 1), including low clearance, high exposure, and oral bioavailability. Further in vitro profiling, however, revealed that **1** is also a quite potent hERG inhibitor (96.7% at 10 μM), thereby posing a risk of cardiotoxic-



Scheme 4. Synthesis of 2-substituted pyridyl compounds **9**, **10**, **14**–**16**, and **18**–**20**. *Reagents and conditions*: a) TFA, H_2O, 70 °C, 5 h; b) $AlMe_3$, $Pd(PPh_3)_4$, dioxane, reflux, 16 h; c) CH_3CONH_2, $Pd(OAc)_2$, xantphos, Cs_2CO_3, THF, 120 °C, microwave, 30 min; d) *t*BuOCONH$_2$, $Pd(OAc)_2$, xantphos, Cs_2CO_3, THF, 120 °C, microwave, 30 min; e) 1. NaH, DMF, 0 °C, 16 h, 2. MeI, RT, 1 h.



Scheme 5. Synthesis of 2-alkylaminopyridyl compounds **11**, **12**, and **13**. *Reagents and conditions*: a) *m*-CPBA, CH_2Cl_2, RT, 5 h; b) 1. primary amine, PyBroP, DIPEA, CH_2Cl_2, RT, 18 h, 2. TFA, H_2O, 70 °C, 5 h.

ity. This finding prompted us to undertake a medicinal chemistry program aimed at overcoming the hERG liability while preserving the favorable in vitro potency and rodent pharmacokinetic profiles that characterize the series. Toward this goal we relied on cell proliferation assays as the primary screening method. Thus, we measured the potency against A375 and selectivity versus MEWO cells, which lack the V600E B-Raf mutation, and subsequently demonstrated the mode of inhibition in a fluorescence polarization competition binding assay for both wild-type and mutant B-Raf. We then further investigated the most interesting hits for inhibition of mutant B-Raf kinase activity and pERK inhibition in cells.

Several strategies have been reported to minimize hERG/Ikr inhibitory activity, including formation of zwitterions, decrease in pK_a, modulation of lipophilicity, and discrete (often peripheral) structural modifications.[12] Notably, it is often not possible to alter any of these parameters in isolation, thus making identification of the actual controlling factor difficult.

In the architecture of compound **1**, the nitrogen atom of the pyridine ring and the aromatic sulfonamide moiety represent the fundamental pharmacophores of the class, respectively expected to bind the kinase hinge region and the backbone of the kinase DFG motif in its "in" conformation[13] (see also crystallographic data below). In addition, compound **1** features two relatively lipophilic fluorophenyl moieties (compound **1** clog $P = 3.29$)[14] and a basic *N*-methylpiperidine ring that, incidentally, masks its otherwise partial zwitterionic nature stemming from the presence of the pyridine and the sulfonamide.[15] We reasoned that removal of the tertiary amine moiety would be a viable means to disrupt the potential key

Table 1. In vitro and in vivo data of compounds **1**–**8**.

Compd	W	X	Y	R¹	IC_{50} [nM][a] A375	IC_{50} [nM][a] MEWO	K_D [nM][b] wt-B-Raf	K_D [nM][b] mut-B-Raf	IC_{50} [nM][b] mut-B-Raf	IC_{50} [nM][a] A375 pERK	Sol. [μM] pH 7	HLM CL_{int} [mL min^{-1} kg^{-1}]	Rat hepat. CL_{int} [mL min^{-1} kg^{-1}]	hERG inhib. [%@10 μM][c]	Pharmacokinetics[d] V_{dss} [L kg^{-1}]	CL [mL min^{-1} kg^{-1}]	AUC p.o. [μM h]	*F* [%]
1	F	H	F		40	>10	<3	<3	2.4	<16	149	5	57	96.7	0.72[e] 0.87[f]	9.1[e] 12.6[f]	23.3[e] 11.4[f]	88.7[e] 46[f]
2	F	H	F		78	>10	<3	<3	5.8	<16	59	12	81	68.3	0.50[e]	3.98[e]	10.6[e]	16.8[e]
3	F	H	H		141	>10	8	12	ND	ND	22	6	264	ND	ND	ND	ND	ND
4	H	F	H		805	>10	164	127	ND	ND	30	71	290	ND	ND	ND	ND	ND
5	F	H	F		12	>10	4	6	ND	ND	6	163	451	ND	ND	ND	ND	ND
6	F	H	F		100	>10	17	25	ND	ND	1	196	123	ND	ND	ND	ND	ND
7	F	H	F		270	>10	14	27	ND	ND	3	52	80	71.1	ND	ND	ND	ND
8	F	H	F		127	>10	4	7	ND	ND	3	16	25	88.8	ND	ND	ND	ND

[a] Values are the mean of $n \geq 2$ experiments; individual values are within threefold of the reported mean value. [b] Experiments were performed in duplicate, and values are the average of three independent experiments; individual values are within twofold of the reported mean value. [c] Patch-clamp assay. [d] Determined at 10 mg kg^{-1}; i.v. formulation: 5% glucose solution containing 10% Tween 80; p.o. formulation: water containing 0.5% HPMC. [e] Mouse. [f] Rat. ND = Not determined.

pharmacophoric interactions with the hERG K^+ channel,[16,17] while also preserving kinase activity, as the piperidine is directed toward the kinase solvent-accessible region, and does not establish key interactions with the enzyme (see below).

As the first step, we substituted a tetrahydropyran for the piperidine ring, leading to compound **2**. The latter, as expected, performed reasonably well in terms of B-Raf inhibition and general in vitro properties (Table 1), and showed an improvement in limiting hERG inhibition (68.3% at 10 μM). Interestingly, compound **2** has a clog*P* value of 3.71, slightly higher than that of compound **1**, suggesting that lipophilicity plays a less relevant role and thus supporting the selected hERG-detuning strategy. Unfortunately, however, compound **2** was endowed with suboptimal mouse pharmacokinetics, having low oral bioavailability (16%). We tackled this problem by capitalizing on experience acquired with the analogous pyrazole series, the improvement in bioavailability of which correlated with changes in pK_a. The aim here was to increase the overall low pK_a of the molecule, considering that **1** displays a basic piperidine moiety that allows the compound to bear a net positive charge along the entire gastrointestinal tract. The weak basicity of pyridine in **2**, in contrast, only partially counteracts the sulfonamide acidity, implying that the net charge of this compound changes while passing through the gut,[18] and thus potentially affecting its ability to be absorbed. Accordingly, we planned to either decrease the sulfonamide acidity or increase, by other means, the compound's basicity to match a suitable overall pK_a window. However, removal of the fluorine atom from the central phenyl ring, the simplest step to increase the sulfonamide pK_a, actually led to a slight decrease in cellular potency, metabolic instability, and to a greater inhibition of cytochromes (compound **3**). The use of a different difluoro substitution pattern on the peripheral phenyl ring (compound **4**) further worsened the trend (Table 1).

The negative impact observed with these preliminary elaborations suggested that modifying either the central or peripheral phenyl ring was not a viable strategy to tune the overall compound basicity. Toward this end, we decided to pay attention to modulating the R^1 group (Table 1) on the thiazole scaffold and the R^2 substituent (Table 2) in the 2-position of the pyridine ring, while keeping the trifluorinated benzensulfonamido appendage unchanged.

Substituents other than a 4-heterocyclic group at R^1 were detrimental: a *tert*-butyl group gave rise to a compound that, while preserving the cellular potency, was quickly degraded by human liver microsomes (HLM) and rat hepatocytes (compound **5**), whereas compounds bearing an alkylamino substituent (**6**–**8**), although biochemically potent, constantly performed poorly in terms of antiproliferative activity and were no longer pursued (Table 1).

We therefore focused on the tetrahydropyranyl series and worked on the R^2 substituent with the aim of moderately increasing the compounds' overall basicity. We first prepared the 2-aminopyridine derivative **9**, which was found to be very potent and selective in cells. However, compared with **2**, compound **9** showed a higher intrinsic clearance, especially in the rat hepatocytes, thus preventing its further advancement. Despite a greater propensity toward metabolic instability, **9** revealed reasonable mouse pharmacokinetics, reporting encouraging oral absorption with 23% bioavailability. The modulation of pyridine basicity was pursued by preparing a series of additional amines (**10**–**13**). However, the metabolic liability against both HLM and rat hepatocytes proved intrinsic to the whole series. Interestingly, the acetamido compound **14** turned out to be extremely potent, with an optimal in vitro metabolic profile, but had a very low overall pK_a; not surprisingly, it was almost devoid of oral exposure. Other modifications to the *ortho* position of the pyridine ring (compounds **15** and **16**) were ineffective (Table 2).

It became clear that we could not match our bioavailability objectives by working on the R^2 substituent of the pyridine moiety in the tetrahydropyranyl series, and so we turned our attention back to the piperidine derivatives. However, instead of working on the very basic and problematic *N*-methylpiperidine derivatives, we focused our attention on the *N*-cyclopropylpiperidine compounds, the amine pK_a of which are supposed to be one to two orders of magnitude lower.[19]

The first compound synthesized, **17**, showed a similar in vitro potency as analogues **1** and **2**; however, it had some metabolic liabilities and proved to be a quite potent cytochrome inhibitor. The corresponding 2-aminopyridine **18** and 2-methylaminopyridine **19** derivatives also maintained some parallels with analogues of the tetrahydropyranyl series (**9** and **10**), being very potent, but suffering from metabolic liabilities. However, their mouse pharmacokinetics showed the viability of the series, and in particular compound **19** had a high exposure and an oral bioavailability approaching 60%. Finally, we prepared the acetamido derivative **20**, which displayed compelling inhibitory activity, was extremely potent and selective in antiproliferative tests, and exhibited a favorable in vitro ADME profile, with good permeability in Caco2 cells (A→B $P_{app} = 20.5 \times 10^{-6}$ cm s^{-1}, efflux ratio = 2.69), good metabolic stability, no significant inhibition of cytochromes (CYP 3A4 IC_{50} = 20 μM; CYP 2D6 and CYP 2C9 IC_{50} = 31 μM), and high solubility. Pharmacokinetic studies in mice showed compound **20** to have very low clearance and distribution volume, a half life of ~2 h, an extremely high exposure (AUC 206 μM h after oral treatment at 10 mg kg^{-1}) with satisfying oral bioavailability (40%). A similar profile was subsequently observed in the rat pharmacokinetic studies, in which a 35% oral bioavailability was obtained, associated with high exposure (AUC 408 μM h after oral treatment at 10 mg kg^{-1}) and very low clearance and distribution volume, therefore ensuring the possibility to perform rat toxicological studies. Gratifyingly, in the hERG inhibition test, compound **20** showed a limited inhibition of 57% at 10 μM (Table 3), and was therefore selected for additional studies.

Crystallographic data

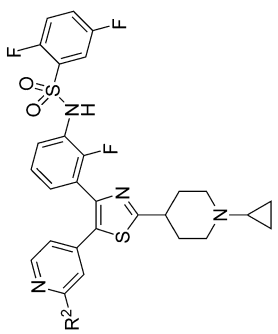
The 2.3 Å crystal structure of the V600E B-Raf kinase domain in complex with compound **20** was solved to characterize the interactions between the enzyme and the inhibitor and to better understand its potency and selectivity (Figure 1). The crystal re-

Table 2. In vitro and in vivo data of compounds **9-16**.

Compd	R^2	IC_{50} [nM][a]		K_D [nM][b]		IC_{50} [nM][b]	IC_{50} [nM][a]	Sol. [μM]	HLM CL_{int}	Rat hepat. CL_{int}	Pharmacokinetics[c]			
		A375	MEWO	wt-B-Raf	mut-B-Raf	mut-B-Raf	A375 pERK	pH 7	[mL min^{-1} kg^{-1}]	[mL min^{-1} kg^{-1}]	V_{dss} [L kg^{-1}]	CL [mL min^{-1} kg^{-1}]	AUC p.o. [μM h]	F [%]
9	NH_2	15	>10	<3	<3	6.1	18	121	18	230	0.34[d]	2.4[d]	26.7[d]	23.2[d]
10	NHMe	32	>10	10	9	7.2	20	66	80	280	0.23[d]	4.9[d]	4.0[d]	7.3[d]
11		221	>10	26	40	ND	ND	163	21	249	ND	ND	ND	ND
12		69	>10	34	33	ND	ND	143	66	138	ND	ND	ND	ND
13		8	>10	15	17	ND	ND	40	131	66	ND	ND	ND	ND
14	NHCOMe	<0.6	>10	<3	<3	4.3	<16	110	30	114	ND	ND	1.4[d]	ND
15	Cl	1020	>10	30	58	ND	ND	28	40	162	ND	ND	ND	ND
16	Me	383	>10	28	22	ND	ND	23	23	71	ND	ND	ND	ND

[a] Values are the mean of $n \geq 2$ experiments; individual values are within threefold of the reported mean value. [b] Experiments were performed in duplicate, and values are the average of three independent experiments; individual values are within twofold of the reported mean value. [c] Determined at 10 mg kg^{-1}; i.v. formulation: 5% glucose solution containing 10% Tween 80; p.o. formulation: water containing 0.5% HPMC. [d] Mouse. ND = Not determined.

Table 3. In vitro and in vivo data of compounds **17–20**.



Compd	R^2	IC_{50} [nM][a] A375	MEWO	K_D [nM][b] wt-B-Raf	mut-B-Raf	IC_{50} [nM][b] mut-B-Raf	IC_{50} [nM][a] A375 pERK	Sol. [μM] pH 7	HLM CL_{int} [mL min^{-1} kg^{-1}]	Rat hepat. CL_{int} [mL min^{-1} kg^{-1}]	hERG inhib. [%@10 μM][c]	Pharmacokinetics[d] V_{dss} [L kg^{-1}]	CL [mL min^{-1} kg^{-1}]	AUC p.o. [μM h]	F [%]
17	H	23	>10	<3	<3	5.2	40	102	38	282	ND	ND	ND	ND	ND
18	NH_2	27	>10	<3	<3	4.7	<16	114	43	400	ND	0.76	13.1	3.57	21.1
19	NHMe	92	>10	20	22	9.9	17	79	101	375	83.6	0.48	9.1	16.5	57.5
20	NHCOMe	3	>10	<3	<3	3.7	<16	128	37	112	57.1	0.11[e]	0.47[e]	206[e]	40.3[e]
												0.06[f]	0.24[f]	408[f]	35[f]

[a] Values are the mean of $n \geq 2$ experiments; individual values are within threefold of the reported mean value. [b] Experiments were performed in duplicate, and values are the average of three independent experiments; individual values are within twofold of the reported mean value. [c] Patch-clamp assay. [d] Determined at 10 mg kg^{-1}; i.v. formulation: 5% glucose solution containing 10% Tween 80; p.o. formulation: water containing 0.5% HPMC. [e] Mouse. [f] Rat. ND = Not determined.

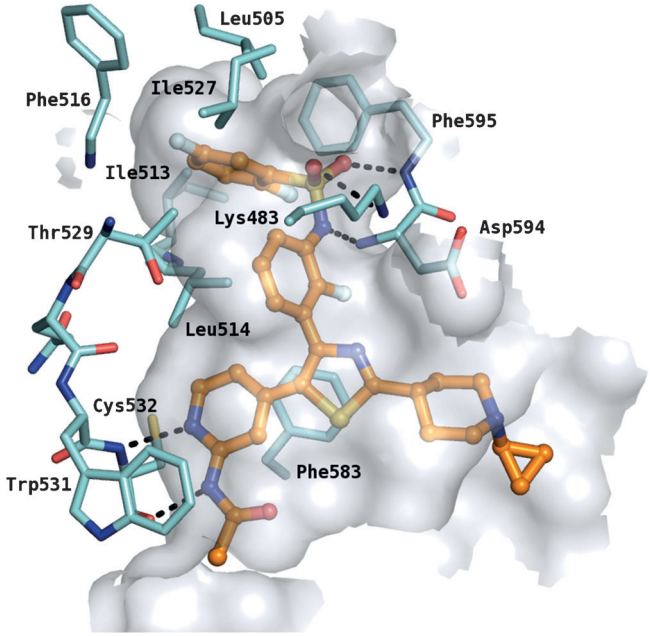


Figure 1. X-ray crystal structure of the complex between B-Raf V600E and compound **20**.

veals a dimeric X-ray structure in which compound **20** occupies the ATP binding site, stabilizes the DFG "in" conformation of the protein, and induces an outward shift of the regulatory αC-helix. As expected, the acetamidopyridine moiety of **20** is located in front of the hinge region and forms two significant hydrogen bonds between the backbone carbonyl group of Cys532 and the nitrogen atom of the acetamide group, and between the backbone amide of Cys532 and the pyridine nitrogen. In addition, the pyridine ring makes favorable π-stacking interactions with the side chains of Trp531 and Phe583. The thiazole core occupies the sugar region, and the *N*-cyclopropylpiperidine group extends into the solvent-accessible region located between the glycine loop and the kinase C lobe. The cyclopropyl ring, highly solvent exposed, is poorly resolved in the electron density, indicating high mobility. The *ortho*-fluorophenyl moiety at position 4 of the thiazole ring is positioned in the back pocket, sandwiched between the gatekeeper residue Thr529 and the catalytic Lys483. Similar to what was observed in the B-Raf–PLX4720 and B-Raf–vemurafenib structures, the sulfonamide nitrogen atom of **20** makes a hydrogen bond with the backbone nitrogen of the DFG motif Asp594, while the two oxygen atoms of the sulfonamide moiety interact with the backbone nitrogen atom of Phe595 (DFG) and the side chain nitrogen of Lys483. Analogously to PLX-4720 and vemurafenib, the sulfonamide nitrogen of **20** is deprotonated, and this interaction plays an important role for the compound's potency and selectivity, as confirmed by the fact that lowering the sulfonamide acidity negatively affects potency. The 2,5-difluorophenyl group is also critical for selectivity; in fact, this moiety binds to the so-called "Raf-selective pocket",[4] a mainly hydrophobic pocket formed by the outward movement of the αC-helix. The phenyl ring fits nicely

into the pocket and makes favorable hydrophobic interactions with Leu505, Leu514, Phe516, Ile527, and Phe595 which contribute to the binding. Comparison of crystal structures of compound **20**, PLX-4720, and PLX-4032 (PDB IDs 3OG7 and 3C4C) indicates that the residues forming the dimer interface adopt a similar conformation. Interestingly, however, closer inspection of the hydrophobic back pocket ("Raf-selective pocket") reveals that the fluorine atom at position 5 of the difluorophenyl group induces a slight outward rotation of the Phe516 side chain. This residue is directly engaged in a hydrogen bond network with Arg509 of the adjacent monomer,[20] which is known to be critical for the dimerization process, and is located in close contact with Trp450, another conserved amino acid that is an essential component of the dimer interface.[21] We speculate that the presence of this fluorine atom in compound **20** could play a role in the dimerization ability of the protein, thus providing a possible explanation for the peculiar behavior of this molecule (see below).

Table 4. Comparison of the antiproliferative activities of compound **20**, vemurafenib, and dabrafenib.

Cell line	B-Raf	IC_{50} [μM][a]		
		20	vemurafenib	dabrafenib
A375	V600E	0.003	0.074	0.004
COLO205	V600E	0.005	0.073	0.006
SW1417	V600E	0.208	1.825	>10
RKO	V600E	2.275	5.35	>10
WM115	V600D	0.148	1.710	0.680
MDA-MB-231	G464V	8.480	>10	>10
NCI-H1395	G469A	>10	>10	>10
NCI-H2087	L597V	>10	>10	>10
MEWO	wt	>10	>10	>10
MCF7	wt	>10	>10	>10

[a] Values are the mean of $n \geq 2$ experiments; individual values are within twofold of the reported mean value.

Biological profile of compound 20

Compound **20** inhibited both wild-type and mutant (V600E) B-Raf (IC_{50}: 4.8 and 3.7 nM respectively), as well as C-Raf (IC_{50} = 36.5 nM), with no other kinases inhibited below an IC_{50} value of 500 nM in a panel of 61 kinases (see Supporting Information).[22] As expected, it exhibited an antiproliferative activity in the low-nanomolar range against several cell lines bearing B-Raf V600E and also showed high potency against the melanoma WM115 cell line bearing the B-Raf V600D mutation, while it had IC_{50} values >10 μM in all non-mutant B-Raf cell lines. In some cell lines, such as the colon SW1417 B-Raf V600E and the melanoma WM115 B-Raf V600D lines, it was also more active than dabrafenib (Table 4). The observed selectivity in proliferation reflects the significant higher $K_{M(ATP)app}$ for the mutant B-Raf versus the wild-type enzyme that makes non-mutant B-Raf cell lines more resistant to inhibition under millimolar cellular ATP concentrations.[23]

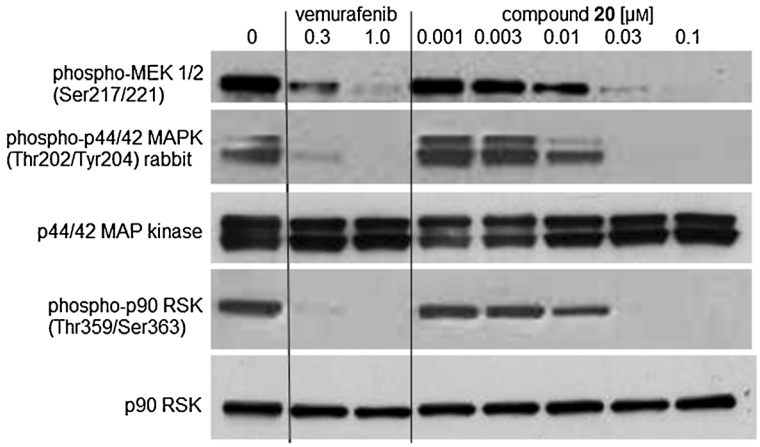


Figure 2. In vitro pathway inhibition of compound **20** and vemurafenib in A375 (B-Raf V600E) cells after 2 h treatment. Complete inhibition of MEK, MAPK, and p90 RSK phosphorylation is evident at very low concentrations of compound **20** (0.03 μM) and at a much higher concentration (1 μM) for vemurafenib.

The mechanism of action was confirmed in the A375 cell line, where a strong inhibition of the MAPK pathway was observed (Figure 2). Moreover, in B-Raf wild-type cells the paradoxical effect, consisting of activation of the downstream pathway in cells lacking the mutation as a result of Raf dimerization events,[24] was found very weak and less pronounced than with vemurafenib and dabrafenib (Figure 3). We hypothesize that this may arise from the observed shift of the Phe516 side chain caused by the fluorine atom at position 5 of the difluorophenyl ring of compound **20** that could partly impair the dimerization capacity of the protein.

Compound **20** was tested for efficacy on A375 B-Raf V600E human melanoma xenograft in comparison with vemurafenib and dabrafenib. All compounds were dissolved in water containing 0.5% (*w/v*) hydroxypropyl methylcellulose (HPMC) and orally administered twice a day for 10 consecutive days. Compound **20** demonstrated high, dose-dependent antitumor activity with strong and persistent tumor regression observed in all mice at the highest dose, whereas no toxicity in terms of body weight decrease was observed. Under the experimental conditions chosen, compound **20** was found to be more potent than both vemurafenib and dabrafenib, being more active at 10 mg kg^{-1} than vemurafenib at 50 mg kg^{-1} and equally active to dabrafenib at 30 mg kg^{-1} (Figure 4). The mechanism of action was also demonstrated ex vivo in A375-bearing mice: the MAPK pathway was found to be completely inhibited up to 6 h after a single treatment at 30 mg kg^{-1} (Figure 5).

Conclusions

Starting from lead compound **1**, which displayed good in vitro and pharmacokinetic profiles, but which has an unacceptable level of activity against hERG, a program aimed at overcoming

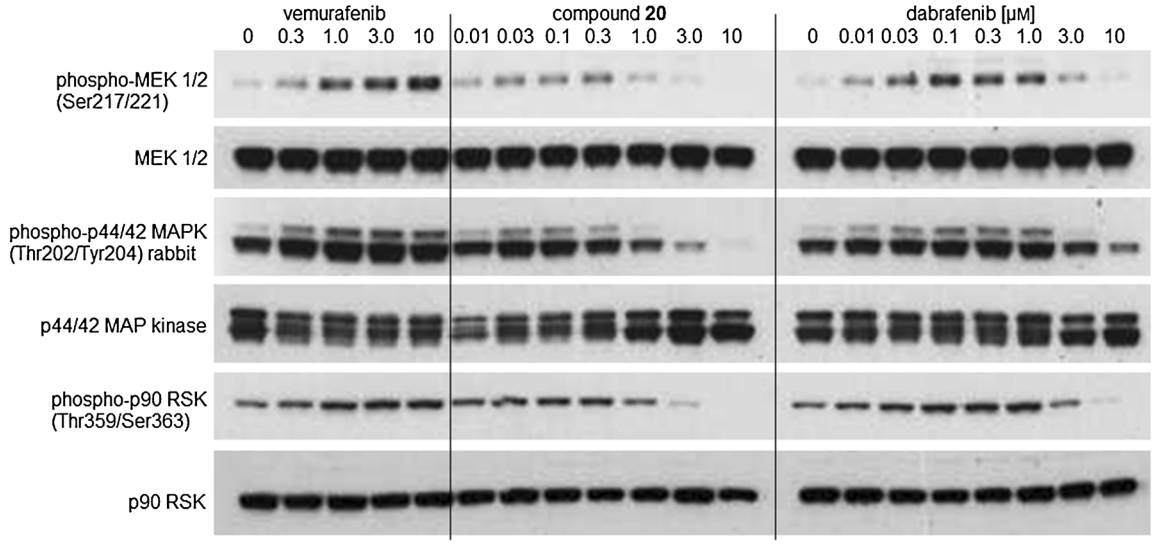


Figure 3. In vitro paradoxical effect of vemurafenib, compound **20**, and dabrafenib in MEWO (wt-B-Raf) cells after 2 h treatment. Activation of the B-Raf downstream pathway, i.e., phosphorylation of MEK, MAPK, and p90 RSK, is strong with vemurafenib (from the dose of 1 μM) and dabrafenib (in the dose range 0.01–3 μM). With compound **20** such activation is low and occurs only within a small dose range (0.03–0.3 μM).

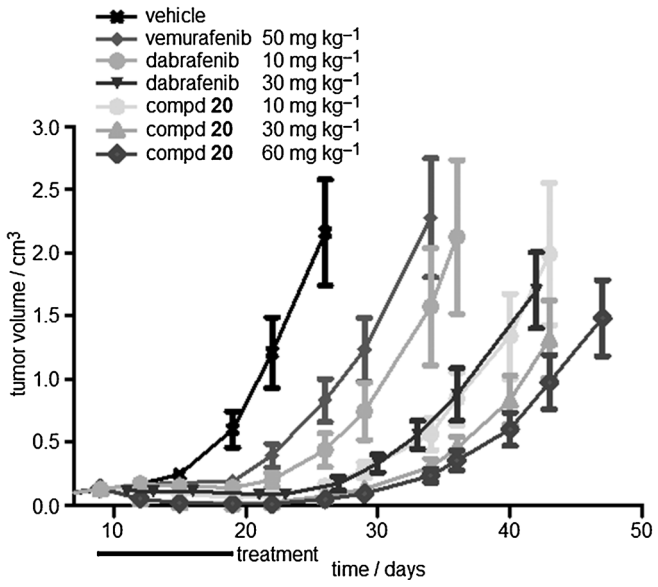


Figure 4. Efficacy of compound **20**, vemurafenib, and dabrafenib in the A375 (B-Raf V600E) human melanoma xenograft model. Compounds were dosed orally twice a day for 10 days ($n = 7$). The vehicle group received 0.5% HPMC. Tumor volume and body weights were measured regularly. No toxicity in terms of body weight decrease was observed in any group. A tumor volume inhibition of $> 90\%$ was maintained with all doses of compound **20** for at least 10 days after stopping treatment.

this liability was undertaken. The challenge consisted of modifying multiple sites of the lead molecule in a balanced fashion for effective optimization. The key polar interaction of the acidic sulfonamide with the kinase DFG motif had to be preserved, while properties such as oral absorption and low hERG inhibition required modulation of charge distribution and elimination of potential hERG liabilities, such as basic nitrogen centers flanked by aromatic or hydrophobic groups. The strategy involved mainly replacement of the *N*-methylpiperidine group with tetrahydropyran and, eventually, *N*-cyclopropylpiperidine moieties, and introduction of an *ortho* substituent on the pyridine ring. This led to the identification of the highly potent, selective, and orally available compound **20**, the efficacy of which is consistent with B-Raf inhibition. Compound **20** exhibited compelling antiproliferative activity only against cell lines bearing B-Raf V600E or V600D mutations, weak paradoxical effect in non-mutant B-Raf cells, and good efficacy in vivo on a A375 xenograft model. On the basis of the relevant profile, compound **20** was selected as a candidate suitable for preclinical development.

Experimental Section

Chemistry

General: Flash chromatography was performed with silica gel (Merck grade 9395, 60 A). HPLC was performed on a Waters X Terra RP18 (4.6×50 mm, 3.5 μm) column using a Waters 2790 HPLC system equipped with a 996 Waters PDA detector and Micromass model ZQ single quadrupole mass spectrometer, equipped with an electrospray (ESI) ion source. Mobile phase A was ammonium acetate buffer (5 mM, pH 5.5 with AcOH/CH_3CN 95:5), and mobile phase B was H_2O/CH_3CN (5:95). Gradient: 10→90% B in 8 min, hold 90% B 2 min; UV detection at λ 220 and 254 nm; flow rate: 1 mL min^{-1}; injection volume: 10 mL; full scan, mass range: 100–800 amu; capillary voltage: 2.5 kV; source temperature: 120 °C; cone voltage: 10 V. Retention times (HPLC t_R) are given in minutes at 220 nm or at 254 nm; MS data are given as *m/z* ratio.

When necessary, compounds were purified by preparative HPLC on a Waters Symmetry C_{18} (19×50 mm, 5 μm) column or on a Waters X Terra RP18 (30×150 mm, 5 μm) column using a Waters preparative HPLC 600 instrument equipped with a 996 Waters PDA detector and a Micromass model ZMD single quadrupole mass spectrometer, ESI, positive mode. Mobile phase A was H_2O/0.01% tri-

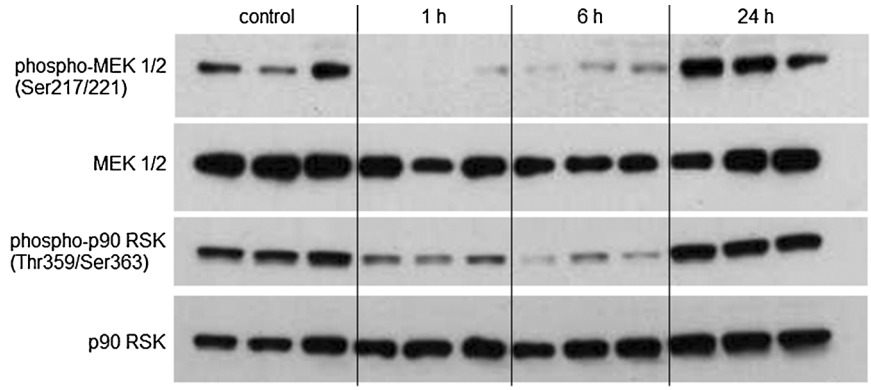


Figure 5. Pathway inhibition in the A375 (B-Raf V600E) human melanoma xenograft model after a single oral treatment with compound **20** (30 mg kg^{-1} p.o.). Inhibition of MEK and p90 RSK phosphorylation is still marked, 6 h after treatment.

fluoroacetic acid (TFA), and mobile phase B was CH_3CN. Gradient: 10→90% B in 8 min, hold 90% B 2 min; flow rate: 20 mL min^{-1}. As alternative, mobile phase A was H_2O/0.1% NH_4OH, and mobile phase B was CH_3CN. Gradient: 10→100% B in 8 min, hold 100% B 2 min; flow rate: 20 mL min^{-1}. HPLC–MS/UV analyses were performed on a LCQ DecaXP (Thermo, San Jose, CA, USA) ion-trap instrument, equipped with an ESI source. The mass spectrometer was connected to a Surveyor HPLC system (Thermo, San Jose, CA, USA) with a UV PDA detector (detection λ 215–400 nm). A Phenomenex Gemini C_{18} column 110 A 50×4.6 mm, 3 μm particle size was used. Mobile phase A was ammonium acetate buffer (5 mM, pH 4.5 with AcOH)/CH_3CN 95:5, and mobile phase B was ammonium acetate buffer (5 mM, pH 4.5 with AcOH)/CH_3CN 5:95. Gradient: 0→100% B in 7 min, hold 100% B 2 min; flow rate: 1 mL min^{-1}; injection volume: 10 μL. Retention times (HPLC t_R) are given in minutes; full scan, mass range: 50–1200 amu; heated capillary temperature: 275 °C; spray voltage: 4 kV; MS data are given as *m/z* ratio.

As formerly reported,[25] ESI(+) high-resolution mass spectra (HRMS) were obtained on a Q-ToF Ultima (Waters, Manchester, UK) mass spectrometer directly connected to an Agilent 1100 micro-HPLC system (Palo Alto, CA, USA). GC–MS analyses were performed on a GCQ (FinniganMAT) ion-trap instrument equipped with an electron ionization (EI) source. The mass spectrometer was connected to a Trace GC system (Thermo, San Jose, CA, USA) through a transfer line. A DB-5MS (J&W Scientific), 30 m×0.25 mm i.d., film 0.25 μm was used. Temperature gradient: from 60 °C (hold 1 min) to 280 °C (hold 10 min) in 12 min; injector temperature: 280 °C; transfer line temperature: 250 °C; full scan, mass range: 35–650 amu; source temperature: 200 °C; electron energy: 70 eV. ^{1}H NMR spectra were recorded at a constant temperature of 28 °C on three different spectrometers: Varian INOVA 400 (operating at 400.5 MHz for ^{1}H) and equipped with 5 mm ^{1}H{^{15}N-^{31}P} *z* axis PFG indirect detection probe; Varian INOVA 500 (operating at 499.75 MHz for ^{1}H) and equipped with 5 mm ^{1}H{^{13}C,^{15}N} *z* axis PFG indirect detection cold probe; Varian INOVA 600 (operating at 599.88 MHz for ^{1}H) and equipped with 5 mm ^{1}H/^{19}F *z* axis PFG probe. Spectra were all recorded in [D_6]DMSO unless otherwise stated. Chemical shifts were referenced with respect to the residual solvent signals. Data are reported as follows: chemical shift (δ), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br s = broad signal, td = triplet of doublet, dd = doublet of doublets, ddd = doublet of doublets of doublets, m = multiplet), coupling constants (Hz), and number of protons.

***N*-(3-Bromo-2-fluorophenyl)-2,5-difluoro-*N*-(methoxymethyl)benzenesulfonamide (21)**: 3-Bromo-2-fluoroaniline (10 g, 52.63 mmol) was dissolved in CH_2Cl_2 (100 mL) under nitrogen atmosphere. Dry pyridine was added (6 mL, 73.68 mmol), followed by 2,5-difluorobenzenesulfonyl chloride (7.08 mL, 52.63 mmol), and the mixture was stirred at room temperature for 2 h. It was then diluted with CH_2Cl_2 and washed with aqueous 0.5 N HCl (3×80 mL) and brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The solid was taken up with Et_2O and stirred for 30 min. It was then filtered and dried at 40 °C under reduced pressure to give 17.8 g of *N*-(3-bromo-2-fluorophenyl)-2,5-difluorobenzenesulfonamide as a pale-yellow solid (92%). HPLC: t_R = 6.28 min; ^{1}H NMR (400 MHz): δ = 10.86 (s, 1H), 7.50–7.73 (m, 4H), 7.23–7.31 (m, 1H), 7.12 ppm (dt, J = 1.3, 8.1 Hz, 1H). To a solution of this intermediate (17.8 g, 48.61 mmol) in anhydrous CH_2Cl_2 (160 mL) at 0 °C, DIPEA (12.5 mL, 73 mmol) was added followed by methoxymethyl chloride (5.7 mL, 73 mmol). The reaction mixture was stirred at 0 °C for 10 min and then allowed to warm to room temperature. After 2 h a saturated solution of ammonium chloride was added and the mixture was stirred at room temperature for 10 min. It was then diluted with CH_2Cl_2 and washed with H_2O and brine, dried over Na_2SO_4 and evaporated to dryness. The residue was treated with hexane and stirred for 30 min. The solid was filtered and dried to give the title compound (18.52 g, 93%) as a white powder. HPLC: t_R = 6.88 min; ^{1}H NMR (400 MHz): δ = 7.78 (ddd, J = 7.9, 6.4, 1.6 Hz, 1H) 7.69 (m, 1H) 7.64 (m, 1H) 7.51 (m, 1H) 7.31 (m, 1H) 7.22 (m, 1H) 5.06 (s, 2H) 3.35 ppm (s, 3H); HRMS-ESI *m/z* $[M+Na]^+$ calcd for $C_{14}H_{11}BrF_3NO_3SNa$ 431.9487, found 431.9487; GC–MS (EI) *m/z* 409 $[M]^{+\bullet}$.

3-{[(2,5-Difluorophenyl)sulfonyl](methoxymethyl)amino}-2-fluorobenzoic acid (22): *N*-(3-bromo-2-fluorophenyl)-2,5-difluoro-*N*-(methoxymethyl)benzenesulfonamide **21** (10.92 g, 26.62 mmol) was dissolved in anhydrous THF (53 mL) and cooled to 0 °C. A solution of isopropylmagnesium chloride (2 N in THF) (13.3 mL, 26.62 mmol) was added dropwise. At the end of the addition the reaction mixture was allowed to warm to room temperature and stirred for 2 h. The yellow solution was then cooled back to 0 °C and gaseous carbonic anhydride (generated from solid carbonic anhydride and dried through concentrated sulfuric acid) was bubbled through the solution for 20 min. A 0.5 N HCl solution (50 mL) was then added and the mixture was extracted with iPr_2O (3× 130 mL). The desired product was then extracted from the organic phase with 1 N NaOH (3×100 mL). Under vigorous stirring 2 N HCl (150 mL) was then added. The precipitate was collected by filtration, washed with H_2O and hexane and dried in the oven, giving the title compound as a white solid (9.07 g, 91%). HPLC: t_R = 3.67 min; ^{1}H NMR (600 MHz): δ = 7.89 (t, J = 6.5 Hz, 1H) 7.67 (m, 1H) 7.62 (td, J = 9.4, 4.0 Hz, 1H) 7.51 (m, 2H) 7.32 (t, J = 7.8 Hz, 1H) 5.06 (s, 2H) 3.35 ppm (s, 3H); LC–MS-ESI *m/z* (%): 376 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+Na]^+$ calcd for $C_{15}H_{12}F_3NO_5SNa$ 398.0280, found 398.0280.

3-{[(2,5-Difluorophenyl)sulfonyl](methoxymethyl)amino}-2-fluoro-*N*-methoxy-*N*-methylbenzamide (23): 3-{[(2,5-difluorophenyl)sulfonyl](methoxymethyl)amino}-2-fluorobenzoic acid **22** (8.86 g, 23.61 mmol) was dissolved in CH_2Cl_2 (73 mL) under nitrogen atmosphere. Dry DMF (14 mL) was added, followed by *N*-methoxy-*N*-methylamine hydrochloride (3.72 g, 38.13 mmol), *N*-methylmorpholine (4.1 mL, 37.3 mmol), and DMAP (293 mg, 2.4 mmol). The reaction mixture was then cooled to 0 °C, and EDCI (5.38 g, 28.06 mmol) was added portionwise. The mixture was allowed to warm to room temperature and stirred for 4 h. It was then cooled to 0 °C and cold 1 N HCl (100 mL) was added. The mixture was extracted with iPr_2O, and the organic phase was washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and evaporated to dryness. The crude product was purified by flash chromatography on silica gel (CH_2Cl_2/MeOH 98:2) to give the title compound as an amorphous colorless solid (8.97 g, 91%). HPLC: $t_R = 5.25$ min; ^{1}H NMR (600 MHz): $\delta = 7.67$ (m, 1H) 7.61 (td, $J = 9.4$, 3.9 Hz, 1H) 7.50 (m, 2H) 7.42 (t, $J = 7.2$ Hz, 1H) 7.31 (m, 1H) 5.07 (s, 2H) 3.36 (s, 3H) 3.16–3.31 ppm (br s, 6H); LC–MS-ESI *m/z* (%): 419 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{17}H_{18}F_3N_2O_5S$ 419.0883, found 419.0893.

2,5-Difluoro-*N*-{2-fluoro-3-[(pyridin-4-yl)acetyl]phenyl}-*N*-(methoxymethyl)benzenesulfonamide (24a): Dry *N*,*N*-diisopropylamine (0.7 mL, 5.09 mmol) was dissolved in dry THF (20 mL) under argon atmosphere and cooled to −78 °C. *n*-Butyl lithium 2.0 M in hexane (2.55 mL, 5.09 mmol) was then added, followed, after 5 min, by a solution of 4-methylpyridine (0.49 mL, 5.09 mmol) in THF (5 mL). The mixture was stirred at −78 °C for 1 h, then a solution of 3-{[(2,5-difluorophenyl)sulfonyl](methoxymethyl)amino}-2-fluoro-*N*-methoxy-*N*-methylbenzamide **23** (1.94 g, 4.63 mmol) in THF (15 mL) was added dropwise. After 10 min at −78 °C the deep-yellow mixture was warmed to 0 °C and stirred for 1 h. It was then quenched with saturated aqueous ammonium chloride and extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by flash chromatography on silica gel (hexane/EtOAc 1:1) to give the title compound (1.15 g, 55%) as a yellow oil. HPLC: $t_R = 5.31$ min; ^{1}H NMR (500 MHz, $CDCl_3$) $\delta = 8.57$ (m, 2H) 7.87 (m, 1H) 7.51 (m, 1H) 7.41 (m, 1H) 7.19–7.32 (m, 3H) 7.12 (m, 2H) 5.17 (s, 2H) 4.15 (d, $J_{H,F} = 2.2$ Hz, 2H) 3.53 ppm (s, 3H); LC–MS-ESI *m/z* (%): 451 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{21}H_{18}F_3N_2O_4S$ 451.0934, found 451.0922.

2,5-Difluoro-*N*-{2-fluoro-3-[(2-chloropyridin-4-yl)acetyl]phenyl}-*N*-(methoxymethyl)benzenesulfonamide (24b): Prepared by using the procedure described above with **23** and 2-chloro-4-picoline: (2.37 g, 68%). HPLC: $t_R = 6.04$ min; ^{1}H NMR (600 MHz): $\delta = 8.35$ (d, $J = 5.1$ Hz, 1H) 7.95 (m, 1H) 7.69 (m, 1H) 7.60 (m, 1H) 7.59 (m, 2H) 7.52 (ddd, $J = 7.8$, 5.0, 3.3 Hz, 1H) 7.49 (s, 1H) 7.40 (t, $J = 7.8$ Hz, 1H) 7.30 (m, 1H) 5.09 (s, 2H) 4.39 (s, 2H) 3.37 ppm (s, 3H); LC–MS-ESI *m/z* (%): 485 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{21}H_{17}ClF_3N_2O_4S$ 485.0544, found 485.0539.

2,5-Difluoro-*N*-{2-fluoro-3-[5-(pyridin-4-yl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-4-yl]phenyl}-*N*-(methoxymethyl)benzenesulfonamide (27a): 2,5-difluoro-*N*-{2-fluoro-3-[(pyridin-4-yl)acetyl]-phenyl}-*N*-(methoxymethyl)benzenesulfonamide **24a** (869 mg, 1.932 mmol) was dissolved in dry DMF (17 mL) under argon atmosphere. Pyridinium bromide perbromide (556 mg, 1.74 mmol) was added, and the mixture was stirred at room temperature to give *N*-{3-[bromo(pyridin-4-yl)acetyl]-2-fluorophenyl}-2,5-difluoro-*N*-(methoxymethyl)benzenesulfonamide **25a**. After 50 min, tetrahydro-2*H*-pyran-4-carbothioamide **26a** (305 mg, 2.1 mmol) was added, and the reaction mixture was heated at 60 °C and stirred for 2 h. The mixture was concentrated under reduced pressure, taken up with EtOAc and washed with saturated aqueous $NaHCO_3$. The aqueous phase was back extracted with EtOAc. The combined organic layers were washed with H_2O and brine, dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by flash chromatography on silica gel (CH_2Cl_2/MeOH 98:2) to give the title compound (996 mg, 89%). HPLC: $t_R = 5.83$ min; ^{1}H NMR (600 MHz): $\delta = 8.48$ (m, 2H) 7.57–7.66 (m, 2H) 7.53 (td, $J = 9.3$, 4.0 Hz, 1H) 7.46 (ddd, $J = 7.8$, 5.1, 3.2 Hz, 1H) 7.35 (m, 1H) 7.32 (m, 1H) 7.12 (m, 2H) 4.96 (s, 2H) 3.95 (m, 2H) 3.48 (td, $J = 11.6$, 2.0 Hz, 2H) 3.23 (s, 3H) 2.03 (m, 2H) 1.78 ppm (m, 2H); LC–MS-ESI *m/z* (%): 576 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{27}H_{25}F_3N_3O_4S_2$ 576.1233, found 576.1245.

Intermediates **27b**, **27c**, **27d**, and **27e** were prepared by using the procedure described above, starting from **24a** or **24b**, and using **26a**, **26b**, and **26c**.

***N*-{3-[2-(1-Cyclopropylpiperidin-4-yl)-5-(pyridin-4-yl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,5-difluoro-*N*-(methoxymethyl)benzenesulfonamide (27b)**: (823 mg, 80%). ^{1}H NMR (400 MHz): $\delta = 10.52$ (s, 1H), 8.48 (d, $J = 5.4$ Hz, 2H), 7.50–7.67 (m, 3H), 7.43 (m, 1H), 7.33 (d, $J = 5.4$ Hz, 2H), 4.95 (s, 2H), 3.23 (s, 3H), 3.03 (m, 3H), 2.32 (m, 2H), 2.08 (m, 2H), 1.68 (m, 3H), 0.42 (m, 2H), 0.31 ppm (m, 2H).

Benzyl 4-[4-(3-{[(2,5-difluorophenyl)sulfonyl](methoxymethyl)amino}-2-fluorophenyl)-5-(pyridin-4-yl)-1,3-thiazol-2-yl]piperidine-1-carboxylate (27c): (1.15 g, 95%). ^{1}H NMR (600 MHz): $\delta = 8.48$ (m, 2H), 7.60 (m, 2H), 7.53 (dt, $J = 9.4$, 4.0 Hz, 1H), 7.45 (m, 1H), 7.37 (m, 4H), 7.33 (m, 4H), 7.12 (m, 2H), 5.09 (s, 2H), 4.96 (s, 2H), 4.10 (m, 2H), 3.23 (s, 3H), 3.03 (br s, 2H), 2.11 (m, 2H), 1.65 ppm (dq, $J = 12.2$, 4.2 Hz, 2H); LC–MS-ESI *m/z* (%): 709 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{35}H_{32}F_3N_4O_5S_2$ 709.1761, found 709.1763.

***N*-{3-[5-(2-Chloropyridin-4-yl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,5-difluoro-*N*-(methoxymethyl)benzenesulfonamide (27d)**: (1.28 g, 46%). HPLC: $t_R = 7.14$ min; ^{1}H NMR (600 MHz): $\delta = 8.33$ (dd, $J = 5.1$, 0.5 Hz, 1H), 7.64 (m, 2H), 7.55 (dt, $J = 9.4$, 3.9 Hz, 1H), 7.45 (m, 1H), 7.38 (m, 2H), 7.24 (dd, $J = 1.6$, 0.6 Hz, 1H), 7.16 (dd, $J = 5.1$, 1.6 Hz, 1H), 4.98 (s, 2H), 3.95 (m, 2H), 3.49 (m, 2H),3.30 (m,1H), 2.03 (m, 2H), 1.78 ppm (m, 2H); LC–MS-ESI *m/z* (%): 610 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{27}H_{24}ClF_3N_3O_4S_2$ 610.0844, found 610.0860.

***N*-{3-[5-(2-Chloropyridin-4-yl)-2-(1-cyclopropylpiperidin-4-yl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,5-difluoro-*N*-(methoxymethyl)benzenesulfonamide (27e)**: (1.62 g, 59%). HPLC: $t_R = 7.74$ min ^{1}H NMR (400 MHz): $\delta = 8.32$ (d, $J = 5.2$ Hz, 1H), 7.63 (m, 2H), 7.55 (m, 1H), 7.46 (m, 1H), 7.33 (m, 2H), 7.22 (s, 1H), 7.15 (dd, $J = 5.1$, 1.3 Hz, 1H), 4.98 (s, 2H), 3.24 (s, 3H), 2.95–3.15 (m, 3H), 2.33 (m, 2H), 2.06 (d, $J = 11.7$ Hz, 2H), 1.67 (m, 3H), 0.43 (m, 2H), 0.32 ppm (br s, 2H); LC–MS-ESI *m/z* (%): 649 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{30}H_{30}N_4O_3F_3S_2Cl$ 649.1316, found 649.1317.

2,5-Difluoro-*N*-{2-fluoro-3-[2-(1-methylpiperidin-4-yl)-5-(pyridin-4-yl)-1,3-thiazol-4-yl]phenyl}benzenesulfonamide (1): Benzyl 4-[4-(3-{[(2,5-difluorophenyl)sulfonyl](methoxymethyl)amino}-2-fluorophenyl)-5-(pyridin-4-yl)-1,3-thiazol-2-yl]piperidine-1-carboxylate **27c** (200 mg, 0.28 mmol) was dissolved in a mixture TFA/H_2O 9:1 (15 mL) and the solution was stirred at 80 °C for 3 h. The solvent was then removed under vacuum, the residue was taken up with toluene, and the solvent evaporated again several times, to afford 2,5-difluoro-*N*-{2-fluoro-3-[2-(piperidin-4-yl)-5-(pyridin-4-yl)-1,3-thiazol-4-yl]phenyl}benzenesulfonamide. This intermediate (138 mg, 0.26 mmol) was dissolved in MeOH (15 mL) and glacial AcOH

(45 μL, 0.78 mmol), $NaBH_3CN$ (26 mg, 0.52 mmol) and 37% formaldehyde (20 μL, 0.39 mmol) were added. The resulting solution was stirred at room temperature for 2 h and then the solvent evaporated. The residue was taken up with CH_2Cl_2, washed with 15% NH_4OH and extracted several times with a mixture of CH_2Cl_2/MeOH 9:1. The organic layer was dried over Na_2SO_4 and evaporated, giving the title compound (130 mg, 92%). HPLC: $t_R = 4.57$ min; ^{1}H NMR (500 MHz): $\delta = 8.46$ (d, $J = 5.9$ Hz, 2H), 7.35–7.43 (m, 2H), 7.21–7.34 (m, 2H), 7.07–7.16 (m, 2H), 7.00 (t, $J = 7.9$ Hz, 1H), 6.80–6.95 (m, 1H), 2.97–3.17 (m, 4H), 2.42 (s, 4H), 2.10–2.21 (m, 2H), 1.68–1.96 ppm (m, 2H); LC–MS-ESI *m/z* (%): 545 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{26}H_{24}F_3N_4O_2S_2$ 545.1288, found 545.1286.

2,5-Difluoro-*N*-{2-fluoro-3-[5-(pyridin-4-yl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-4-yl]phenyl}benzenesulfonamide (2): A solution of 2,5-difluoro-*N*-{2-fluoro-3-[5-(pyridin-4-yl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-4-yl]phenyl}-*N*-(methoxymethyl)benzenesulfonamide **27a** (138 mg, 0.24 mmol) in a 9:1 mixture of TFA/H_2O (10 mL) was heated under stirring at 80 °C for 1.5 h. The solvent was then evaporated, the residue re-dissolved in CH_2Cl_2, washed with 15% NH_4OH and extracted several times with a mixture of CH_2Cl_2/MeOH 9:1. The organic layer was dried over Na_2SO_4 and evaporated. The product was finally purified by preparative RP-HPLC (0.05% NH_4OH/CH_3CN 95:5), affording, after trituration with Et_2O, the title compound (70 mg, 55%). HPLC: $t_R = 5.38$ min; ^{1}H NMR (600 MHz): $\delta = 10.83$ (br s, 1H), 8.48 (m, 2H) 7.57–7.66 (m, 2H) 7.53 (td, $J = 9.3$, 4.0 Hz, 1H) 7.46 (ddd, $J = 7.8$, 5.1, 3.2 Hz, 1H) 7.35 (m, 1H) 7.32 (m, 1H) 7.10 (m, 2H) 3.95 (m, 2H) 3.48 (m, 2H) 2.02 (m, 2H) 1.76 ppm (m, 2H); LC–MS-ESI *m/z* (%): 532 (100) $[M+H]^+$; HRMS-ESI *m/z* calcd for $C_{25}H_{21}F_3N_3O_3S_2$ $[M+H]^+$ 532.0971, found 532.0991.

Compounds **17** and **5** were prepared by using the above-mentioned procedure, starting from the corresponding protected sulfonamides.

***N*-{3-[2-(1-Cyclopropylpiperidin-4-yl)-5-(pyridin-4-yl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,5-difluorobenzenesulfonamide (17)**: (21 mg, 46%). HPLC: $t_R = 4.70$ min; ^{1}H NMR (600 MHz): $\delta = 10.53$ (br s, 1H), 8.45 (m, 2H), 7.54 (m, 1H), 7.44 (m, 1H), 7.40 (ddd, $J = 7.8$, 5.1, 3.2 Hz, 1H), 7.34 (m, 1H), 7.28 (m, 1H), 7.20 (m, 1H), 7.07 (m, 2H), 3.05–3.10 (m, 3H), 2.39 (m, 2H), 2.06 (m, 2H), 1.73 (br s, 1H), 1.68 (m, 2H), 0.45 (m, 2H), 0.35 ppm (m, 2H); LC–MS-ESI *m/z* (%): 571 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{28}H_{26}F_3N_4O_2S_2$ 571.1444, found 571.1463.

***N*-{3-[2-*tert*-Butyl-5-(pyridin-4-yl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,5-difluorobenzenesulfonamide (5)**: (51 mg, 60%). HPLC: $t_R = 7.16$ min; ^{1}H NMR (400 MHz): $\delta = 10.65$ (br s, 1H), 8.45 (m, 2H), 7.56 (m, 1H), 7.46 (m, 1H), 7.41 (m, 1H), 7.34 (m, 2H), 7.24 (m, 1H), 7.07 (m, 2H), 1.43 ppm (s, 9H); LC–MS-ESI *m/z* (%): 504 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{24}H_{21}F_3N_3O_2S_2$ 504.1022, found 504.1031.

***N*-(3-Acetyl-2-fluorophenyl)-2,5-difluoro-*N*-(methoxymethyl)benzenesulfonamide (28)**: To a solution of *N*-(3-bromo-2-fluorophenyl)-2,5-difluoro-*N*-(methoxymethyl)benzenesulfonamide **21** (6.15 g, 15 mmol) in ethylene glycol (30 mL), in a flask equipped with a rubber septum, through which nitrogen was fluxed via a needle, palladium acetate (37.5 mg, 0.15 mmol), DPPP (129 mg, 0.30 mmol), TEA (5.4 mL, 37.5 mmol) and *n*-butylvinyl ether (5.9 mL, 45 mmol) were added consecutively. The mixture was heated at 120 °C under stirring for 6 h and then diluted with CH_2Cl_2 and washed with brine. The organic layer was dried over Na_2SO_4 and evaporated, giving *N*-[3-(1-butoxyethenyl)-2-fluorophenyl]-2,5-difluoro-*N*-(methoxymethyl)benzenesulfonamide as a brown oil. This intermediate, without any further purification, was dissolved in a mixture of 1,4-dioxane (65 mL), and 1 N HCl (11 mL) was added to the resulting solution. After 1 h under stirring at room temperature the reaction was complete. The solvent was then removed, the residue re-dissolved with CH_2Cl_2 and washed with aqueous $NaHCO_3$. The organic layer was dried over anhydrous Na_2SO_4 and evaporated. After trituration with Et_2O the title compound (4.82 g, 86%) was collected by filtration. HPLC: $t_R = 6.28$ min; ^{1}H NMR (400 MHz): $\delta = 7.85$ (m, 1H) 7.47–7.73 (m, 5H) 7.36 (t, $J = 7.8$ Hz, 1H) 5.07 (m, 2H) 3.36 (s, 3H) 2.49 ppm (s, 3H); HRMS-ESI *m/z* $[M+Na]^+$ calcd for $C_{16}H_{14}F_3NO_4SNa$ 396.0488, found 396.0488. GC–MS-EI *m/z* 373 $[M]^{+\bullet}$.

***N*-[3-(Bromoacetyl)-2-fluorophenyl]-2,5-difluoro-*N*-(methoxymethyl)benzenesulfonamide (29)**: *N*-(3-acetyl-2-fluorophenyl)-2,5-difluoro-*N*-(methoxymethyl)benzenesulfonamide **28** (100 mg, 0.27 mmol) was dissolved in dry THF (10 mL) and pyridinium bromide perbromide (100 mg, 0.3 mmol) was added. The resulting solution was heated in a microwave apparatus at 80 °C for 15 min. The solvent was then evaporated, the residue taken up with CH_2Cl_2 and washed with 0.5 N HCl. The organic layer was then dried over Na_2SO_4 and evaporated to give the title compound as an oil (91 mg, 75%). HPLC: $t_R = 6.77$ min; ^{1}H NMR (400 MHz): $\delta = 10.54$ (s, 1H), 7.63 (m, 1H), 7.56 (m, 1H), 7.50 (m, 2H), 7.43 (m, 1H), 7.20 (m, 1H), 4.34 ppm (s, 2H).

***N*-[3-(2-Amino-1,3-thiazol-4-yl)-2-fluorophenyl]-2,5-difluoro-*N*-(methoxymethyl)benzenesulfonamide (30)**: Thiourea (21 mg, 0.27 mmol) was added to a solution of *N*-[3-(bromoacetyl)-2-fluorophenyl]-2,5-difluoro-*N*-(methoxymethyl)benzenesulfonamide **29** (122 mg, 0.27 mmol) in EtOH (5 mL). The mixture was heated at 60 °C for 20 min in a microwave apparatus. The solvent was then removed, the residue taken up with CH_2Cl_2 and washed with H_2O. The organic layer was separated, dried over Na_2SO_4 and evaporated. The crude was purified by flash chromatography on silica gel (CH_2Cl_2/CH_3OH 95:5), giving the title compound (92 mg, 80%). HPLC: $t_R = 6.26$ min; ^{1}H NMR (400 MHz): $\delta = 7.97$ (td, $J = 7.5$, 1.8 Hz, 1H) 7.57–7.76 (m, 2H) 7.49 (m, 1H) 7.24 (m, 1H) 7.16 (m, 1H) 7.11 (s, 2H) 6.82 (d, $J = 2.6$ Hz, 1H) 5.09 (s, 2H) 3.37 ppm (s, 3H); LC–MS-ESI *m/z* (%): 430 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{17}H_{15}F_3N_3O_3S_2$ 430.0501, found 430.0493.

***N*-[3-(5-Bromo-2-{[(*E*)-(dimethylamino)methylidene]amino}-1,3-thiazol-4-yl)-2-fluorophenyl]-2,5-difluoro-*N*-(methoxymethyl)benzenesulfonamide (31)**: *N*-[3-(2-amino-1,3-thiazol-4-yl)-2-fluorophenyl]-2,5-difluoro-*N*-(methoxymethyl)benzenesulfonamide **30** (1.26 g, 2.94 mmol) was dissolved in 30 mL dry CH_2Cl_2, and *N*-bromosuccinimide (523 mg, 2.94 mmol) was added. The resulting solution was stirred at room temperature for 30 min. The mixture was diluted with the same solvent and washed with aqueous $NaHCO_3$. The organic layer was dried over Na_2SO_4 and evaporated. The residue was triturated with iPr_2O and filtered to afford *N*-[3-(2-amino-5-bromo-1,3-thiazol-4-yl)-2-fluorophenyl]-2,5-difluoro-*N*-(methoxymethyl)benzenesulfonamide (1.4 g, 93%). HPLC: $t_R = 6.52$ min; ^{1}H NMR (400 MHz): $\delta = 7.57$–7.71 (m, 2H), 7.49 (m, 2H), 7.37 (m, 2H), 7.34 (s, 2H), 7.28 (m,1H) 5.08 (m, 2H), 3.37 ppm (m, 3H); LC–MS-ESI *m/z* (%): 508 $[M+H]^+$. This intermediate (1.4 g, 2.75 mmol) was dissolved in dry DMF (30 mL), and dimethylformamide dimethylacetale (448 μL, 2.75 mmol) was added to the mixture. The reaction was stirred at room temperature overnight, and then the solvent was removed under reduced pressure. The residue was taken up with CH_2Cl_2 and washed with brine. The organic layer was then dried over Na_2SO_4 and evaporated. The crude was finally purified by flash chromatography on silica gel (cyclohexane/EtOH 9:1), to

afford the title compound (0.8 g, 52%). HPLC: $t_R = 7.18$ min; ^{1}H NMR (400 MHz): $\delta = 8.25$ (s, 1H), 7.72–7.57 (m, 2H), 7.57–7.52 (m, 1H), 7.52–7.45 (m, 1H), 7.45–7.37 (m, 1H), 7.34–7.28 (m, 1H), 5.09 (s, 2H), 3.38 (s, 3H), 3.11 (s, 3H), 2.97 ppm (s, 3H); LC–MS-ESI *m/z* (%): 563 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{20}H_{19}BrF_3N_4O_3S_2$ 563.0029, found 563.0049.

***N*-{3-[2-Amino-5-(pyridin-4-yl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,5-difluoro-*N*-(methoxymethyl)benzenesulfonamide (32)**: To a solution of *N*-[3-(5-bromo-2-{[(*E*)-(dimethylamino)methylidene]amino}-1,3-thiazol-4-yl)-2-fluorophenyl]-2,5-difluoro-*N*-(methoxymethyl)-benzenesulfonamide **31** (342 mg, 0.61 mmol) in a mixture of DME (12 mL) and H_2O (2 mL), Cs_2CO_3 (596 mg, 1.83 mmol), $PdCl_2(dppf)_2{\cdot}CH_2Cl_2$ (160 mg, 0.2 mmol), and 4-pyridylboropinacolate (244 mg, 1.22 mmol) were added consecutively. The mixture was heated in a microwave oven at 110 °C for 1 h and then filtered through a Celite pad. The filtrate was evaporated under reduced pressure, the crude taken up with CH_2Cl_2 and washed with brine. The organic layer was separated, dried over Na_2SO_4 and the solvent removed. The product was purified by flash chromatography (CH_2Cl_2/MeOH 98:2) to give *N*-{3-[2-{[(*E*)-(dimethylamino)methylidene]amino}-5-(pyridin-4-yl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,5-difluoro-*N*-(methoxymethyl)benzenesulfonamide (240 mg, 70%). HPLC: $t_R = 6.30$ min; ^{1}H NMR (400 MHz): $\delta = 8.39$ (m, 3H) 7.62 (m, 1H) 7.55 (m, 2H) 7.48 (m, 1H) 7.25–7.38 (m, 2H) 6.97 (m, 2H) 4.98 (s, 2H) 3.27 (s, 3H) 3.14 (s, 3H) 3.01 ppm (s, 3H); LC–MS-ESI *m/z* (%): 562 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{25}H_{23}F_3N_5O_3S_2$ 562.1189, found 562.1183. This intermediate (550 mg, 0.98 mmol) was suspended in EtOH (15 mL) and ethylenediamine (460 μL, 6.86 mmol) was added. The mixture was maintained at reflux giving a clear solution. After 8 h the solvent was removed under reduced pressure, and the residue was re-dissolved in CH_2Cl_2 and washed with brine. The organic phase was dried over Na_2SO_4 and the solvent evaporated. The crude was triturated with Et_2O, giving, after filtration, the title compound (450 mg, 90%). HPLC: $t_R = 5.68$ min; ^{1}H NMR (400 MHz): $\delta = 8.33$ (m, 2H) 7.62 (m, 1H) 7.45–7.55 (m, 5H) 7.33 (m, 1H) 7.26 (m, 2H) 6.87 (m, 2H) 4.99 (s, 2H) 3.27 ppm (s, 3H); LC–MS-ESI *m/z* (%): 507 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{22}H_{18}F_3N_4O_3S_2$ 507.0767, found 507.0769.

***N*-{3-[2-Bromo-5-(pyridin-4-yl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,5-difluorobenzenesulfonamide (33)**: *N*-{3-[2-amino-5-(pyridin-4-yl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,5-difluoro-*N*-(methoxymethyl)-benzenesulfonamide **32** (450 mg, 0.89 mmol) was suspended in dry CH_3CN (45 mL) and $CuBr_2$ (298 mg, 1.34 mmol) and *tert*-butyl nitrite (2 mL, 16.8 mmol) were added. The mixture was stirred at 85 °C for 8 h. After this time the reaction was filtered through a Celite pad and the filtrate evaporated. The residue was taken up with CH_2Cl_2 and washed with aqueous $NaHCO_3$. The organic layer was separated, dried over Na_2SO_4 and evaporated. The crude was purified by flash chromatography, eluted with CH_2Cl_2/MeOH 98:2, affording *N*-{3-[2-bromo-5-(pyridin-4-yl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,5-difluoro-*N*-(methoxymethyl)benzenesulfonamide (350 mg, 69%). HPLC: $t_R = 7.14$ min; ^{1}H NMR (400 MHz): $\delta = 8.52$ (d, $J = 5.9$ Hz, 2H) 7.62 (m, 2H) 7.53 (m, 1H) 7.47 (m,1H) 7.39 (m, 1H) 7.33 (m, 1H) 7.14 (d, $J = 5.9$ Hz, 2H) 4.97 (s, 2H) 3.23 ppm (s, 3H); LC–MS-ESI *m/z* (%): 570 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{22}H_{16}BrF_3N_3O_3S_2$ 569.9763, found 569.9789. This intermediate (174 mg, 0.305 mmol) was dissolved in TFA (6 mL). H_2O (0.5 mL) was added, and the mixture was stirred at 80 °C for 6 h. The solvent was concentrated under reduced pressure and the residue was taken up with CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$ and brine. The organic phase was dried over Na_2SO_4 and evaporated to dryness. The crude product was treated with petroleum ether, filtered, and dried under high vacuum to afford the title compound (130 mg, 80%) as a colorless solid. HPLC: $t_R = 5.81$ min; ^{1}H NMR (400 MHz): $\delta = 10.71$ (s, 1H) 8.53 (d, $J = 4.7$ Hz, 2H) 7.32–7.72 (m, 5H) 7.26 (t, $J = 7.7$ Hz, 1H) 7.16 ppm (m, 2H); LC–MS-ESI *m/z* (%): 526 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{20}H_{12}BrF_3N_3O_2S_2$ 525.9501, found 525.9508.

***N*-{3-[2-(Diethylamino)-5-(pyridin-4-yl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,5-difluorobenzenesulfonamide (6)**: *N*-{3-[2-bromo-5-(pyridin-4-yl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,5-difluorobenzenesulfonamide **33** (50 mg, 0.1 mmol) was dissolved in dimethylacetamide (3 mL) and diethylamine (80 μL) was added to the mixture. The solution was heated in a microwave oven at 120 °C for 3 h. After that time the solvent was removed under vacuum, the residue was taken up with CH_2Cl_2 and washed with aqueous $NaHCO_3$. The organic phase was dried over Na_2SO_4 and evaporated. The crude was then purified by flash chromatography (CH_2Cl_2/MeOH 95:5) to afford the title compound (30 mg, 59%). HPLC: $t_R = 6.17$ min; ^{1}H NMR (400 MHz): $\delta = 10.66$ (br s, 1H) 8.31 (d, $J = 6.1$ Hz, 2H) 7.55 (m, 1H) 7.40–7.50 (m, 2H) 7.36 (m, 1H) 7.28 (m, 1H) 7.21 (m, 1H) 6.85 (m, 2H) 3.47 (q, $J = 7.0$ Hz, 4H) 1.18 ppm (t, $J = 7.0$ Hz, 6H); LC–MS-ESI *m/z* (%): 519 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{24}H_{22}F_3N_4O_2S_2$ 519.1131, found 519.1134.

Compounds **7** and **8** were prepared by using the procedure mentioned above, starting from **33** and using appropriate commercially available amino derivatives.

***N*-{3-[2-(1,4-Dioxa-8-azaspiro[4.5]dec-8-yl)-5-(pyridin-4-yl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,5-difluorobenzenesulfonamide (7)**: (14.7 mg, 25%). HPLC: $t_R = 5.76$ min; ^{1}H NMR (400 MHz): $\delta =$ 10.66 (br s, 1H) 8.32 (m, 2H) 7.56 (m, 1H) 7.40–7.48 (m, 2H) 7.36 (td, $J = 7.3$, 1.8 Hz, 1H) 7.28 (m, 1H) 7.21 (m, 1H) 6.87 (m, 2H) 3.93 (s, 4H) 3.55 (m, 4H) 1.78 ppm (m, 4H); LC–MS-ESI *m/z* (%): 589 (100) $[M+H]^+$; HRMS-ESI *m/z* calcd for $C_{27}H_{24}F_3N_4O_4S_2$ $[M+H]^+$ 589.1186, found 589.1193.

***N*-{3-[2-(4,4-Difluoropiperidin-1-yl)-5-(pyridin-4-yl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,5-difluorobenzenesulfonamide (8)**: (30 m, 59%) HPLC: $t_R = 6.1$ min; ^{1}H NMR (400 MHz): $\delta = 10.66$ (br s, 1H) 8.35 (d, $J = 5.4$ Hz, 2H) 7.55 (m, 1H) 7.39–7.49 (m, 2H) 7.35 (m, 1H) 7.10–7.30 (m, 2H) 6.89 (m, 2H) 3.64 (m, 4H) 2.12 ppm (m, 4H); LC–MS-ESI *m/z* (%): 567 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{25}H_{19}F_5N_4O_2S_2$ 567.0943, found 567.0963.

4-(3-Nitrophenyl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazole (34): To a solution of 2-bromo-1-(3-nitrophenyl)ethanone (245 mg, 1 mmol) in EtOH (25 mL), tetrahydro-2*H*-pyran-4-carbothioamide **26a** (144 mg, 1 mmol) was added. The reaction mixture was held at reflux with stirring for 2 h. The solvent was then removed, the residue taken up with CH_2Cl_2 and washed twice with a saturated aqueous solution of $NaHCO_3$. The organic layer was separated, dried over Na_2SO_4 and evaporated to give the title compound (285 mg, 98%). ^{1}H NMR (600 MHz): $\delta = 8.75$ (t, $J = 1.8$ Hz, 1H), 8.40 (d, $J = 7.7$ Hz, 1H), 8.33 (s, 1H), 8.19 (dd, $J = 8.1$, 1.4 Hz, 1H), 7.75 (t, $J = 7.9$ Hz, 1H), 3.95 (m, 1H), 3.50 (dt, $J = 11.6$, 1.8 Hz, 1H), 3.37 (m, 1H), 2.04 (m, 2H), 1.76 ppm (m, 2H); LC–MS-ESI *m/z* (%): 291 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{25}H_{19}F_5N_4O_2S_2$ 291.0798, found 291.0798.

4-[4-(3-Nitrophenyl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-5-yl]pyridine (35): 4-(3-nitrophenyl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazole **34** (113 mg, 0.39 mmol) was dissolved in glacial AcOH (10.5 mL) and NaOAc (71 mg, 0.87 mmol) was added. Bromine (44.4 μL, 0.89 mmol) was added as eight 5.5 μL portions over the

course of two days at room temperature. The mixture was then poured into 1 N NaOH (100 mL) and extracted with EtOAc. The organic phase was evaporated to afford 5-bromo-4-(3-nitrophenyl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazole. (141 mg, 97%). ^{1}H NMR (600 MHz): δ = 8.72 (t, J = 1.8 Hz, 1H), 8.39 (m, 1H), 8.30 (ddd, J = 8.2, 2.3, 1.0 Hz, 1H), 7.82 (t, J = 7.9 Hz, 1H), 3.95 (m, 2H), 3.47 (dt, J = 11.6, 1.9 Hz, 2H), 2.00 (m, 2H), 1.76 ppm (m, 2H); LC–MS-ESI *m/z* (%): 369 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{14}H_{14}BrN_2O_3S$ 369.9903, found 369.9894. To a solution of this intermediate (140 mg, 0.38 mmol) in a degassed mixture of 1,4-dioxane (8 mL) and H_2O (1.6 mL), 4-pyridylboropinacolate (85 mg, 0.41 mmol), Cs_2CO_3 (401 mg, 1.23 mmol), and $PdCl_2$(dppf) (33 mg, 0.041 mmol) were added consecutively. The reaction mixture was heated at 100 °C for 5 h. After cooling to room temperature the suspension was filtered through a Celite pad and the filtrate evaporated to dryness. The residue was re-dissolved in EtOAc and washed with brine. The organic layer was then dried over Na_2SO_4 and evaporated again. The crude was finally purified by flash chromatography (EtOAc/hexane, from 1:1 to 6:4) to give the title compound (89 mg, 70%). ^{1}H NMR (600 MHz): δ = 8.51–8.65 (m, 2H), 8.29 (t, J = 1.9 Hz, 1H), 8.21 (ddd, J = 8.2, 2.3, 1.0 Hz, 1H), 7.82 (td, J = 8.0, 1.2 Hz, 1H), 7.65 (t, J = 7.9 Hz, 1H), 7.37 (m, 2H), 3.96 (m, 2H), 3.50 (dt, J = 11.6, 1.8 Hz, 2H), 2.07 (m, 2H), 1.81 ppm (m, 2H); LC–MS-ESI *m/z* (%): 369 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{14}H_{14}BrN_2O_3S$ 369.9903, found 369.9894.

3-[5-(Pyridin-4-yl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-4-yl]aniline (36): 4-[4-(3-nitrophenyl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-5-yl]pyridine **35** (96 mg, 0.26 mmol) was dissolved in a mixture of EtOH (5 mL) and glacial AcOH (1 mL). Zn powder (300 mg) was added to the resulting solution that was held at reflux with stirring for 3 h. The solvent was removed under vacuum, and the residue taken up with 1 N HCl and extracted with CH_2Cl_2. The aqueous layer was adjusted to pH 14 and extracted again with CH_2Cl_2. The organic phase was then dried over Na_2SO_4 and evaporated. The residue was purified by flash chromatography on silica gel (EtOAc/hexane, from 1:1 to 4:1, then EtOAc/hexane/30% NH_3 4:1:0.1) to afford the title compound (48 mg, 59%). ^{1}H NMR (600 MHz): δ = 8.52 (m, 2H), 7.29 (m, 2H), 6.96 (t, J = 7.8 Hz, 1H), 6.74 (t, J = 1.8 Hz, 1H), 6.55 (m, 1H), 6.45 (m, 1H), 5.13 (s, 2H), 3.95 (m, 2H), 3.48 (dt, J = 11.7, 1.9 Hz, 2H), 3.30 (m, 1H), 2.02 (m, 2H), 1.74 ppm (m, 2H); LC–MS-ESI *m/z* (%): 338 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{19}H_{20}N_3OS$ 338.1322, found 338.1331.

2,5-Difluoro-*N*-{3-[5-(pyridin-4-yl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-4-yl]phenyl}benzenesulfonamide (3): To a solution of 3-[5-(pyridin-4-yl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-4-yl]aniline **36** (28 mg, 0.09 mmol) in CH_2Cl_2 (2 mL), pyridine (10 μL) and 2,5-difluorobenzensulfonyl chloride (20 μL, 0.1 mmol) were added. The resulting solution was stirred at room temperature for 2 h, then diluted with CH_2Cl_2, washed with aqueous $NaHCO_3$ and dried over Na_2SO_4. The solvent was finally removed, and the residue purified by flash chromatography (from CH_2Cl_2/MeOH 1% to CH_2Cl_2/MeOH/30% NH_4OH 100:2:0.2) to afford the title compound (23 mg, 50%), after trituration with Et_2O and filtration. ^{1}H NMR (600 MHz): δ = 10.83 (br s, 1H), 8.51 (m, 2H), 7.58 (m, 1H), 7.53 (m, 1H), 7.50 (m, 1H), 7.24 (m, 2H), 7.20 (m, 2H), 7.09 (m, 2H), 3.95 (m, 2H), 3.49 (dt, J = 11.6, 2.0 Hz, 2H), 2.02 (m, 2H), 1.76 ppm (m, 2H); LC–MS-ESI *m/z* (%): 514 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{25}H_{22}F_2N_3O_3S_2$ 514.1065, found 514.1075.

2,6-Difluoro-*N*-{3-[5-(pyridin-4-yl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-4-yl]phenyl}benzenesulfonamide (4): Prepared by using the procedure mentioned above, starting from **36** and using the appropriate commercially available sulfonyl chloride (26 mg, 70%). ^{1}H NMR (600 MHz): δ = 10.96 (s, 1H), 8.50 (m, 2H), 7.71 (m, 1H), 7.30 (s, 1H), 7.21–7.28 (m, 3H), 7.19 (m, 2H), 7.14 (dd, J = 8.1, 1.2 Hz, 1H), 7.06 (d, J = 7.5 Hz, 1H), 3.96 (m, 2H), 3.49 (dt, J = 11.6, 1.9 Hz, 2H), 3.32 (m, 1H), 2.02 (m, 2H), 1.77 ppm (m, 2H); LC–MS-ESI *m/z* (%): 514 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{25}H_{22}F_2N_3O_3S_2$ 514.1065, found 514.1074.

***N*-{3-[5-(2-Chloropyridin-4-yl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,5-difluorobenzenesulfonamide (15)**: *N*-{3-[5-(2-chloropyridin-4-yl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,5-difluoro-*N*-(methoxymethyl)benzenesulfonamide **27 d** (50 mg, 0.08 mmol) was dissolved in a mixture TFA/H_2O 9:1 (1 mL) and heated at 70 °C for 5 h. The solvent was removed under reduced pressure, and the residue was re-dissolved in CH_2Cl_2 and washed with aqueous $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4 and evaporated. The crude was then purified by flash chromatography (toluene/EtOAc 6:4) to give the title compound (38 mg, 82%). ^{1}H NMR (600 MHz): δ = 10.69 (s, 1H), 8.29 (d, J = 5.13 Hz, 1H), 7.57 (m, 1H), 7.46 (m, 1H), 7.43 (m, 1H), 7.37 (m, 2H), 7.26 (m, 1H), 7.22 (d, J = 0.9 Hz, 1H), 7.08 (dd, J = 5.2, 1.5 Hz, 1H), 3.93 (m, 2H), 3.48 (dt, J = 11.6, 1.9 Hz, 2H), 3.37 (m, 1H), 2.02 (m, 2H), 1.75 ppm (m, 2H); LC–MS-ESI *m/z* (%): 566 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{25}H_{20}ClF_3N_3O_3S_2$ 566.0581, found 566.0588.

2,5-Difluoro-*N*-{2-fluoro-3-[5-(2-methylpyridin-4-yl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-4-yl]phenyl}benzenesulfonamide (16): *N*-{3-[5-(2-chloropyridin-4-yl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,5-difluoro-*N*-(methoxymethyl)benzenesulfonamide **27 d** (150 mg, 0.25 mmol) was dissolved in dry 1,4-dioxane (2 mL), degassed under argon stream. Palladium tetrakis (6 mg, 0.005 mmol) and $AlMe_3$ 2 N in hexane (184 μL, 0.37 mmol) were added to the mixture. The reaction was heated in a close bottle at 105 °C under stirring then maintained at room temperature overnight. The mixture was diluted with EtOAc and washed with an aqueous saturated $NaHCO_3$ solution and with brine. The organic layer was dried over Na_2SO_4 and evaporated to give a residue that was purified by flash chromatography on a silica gel column (eluent: from CH_2Cl_2/MeOH 98:2 to 97:3) to afford 97 mg of 2,5-difluoro-*N*-{2-fluoro-3-[5-(2-methylpyridin-4-yl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-4-yl]phenyl}-*N*-(methoxymethyl)benzenesulfonamide. This intermediate was then dissolved in a mixture TFA/H_2O 9:1 (1 mL) and heated at 70 °C under stirring for 5 h. The solvent was evaporated and the residue taken up with CH_2Cl_2 and washed with aqueous $NaHCO_3$ and brine. The organic phase was separated, dried over Na_2SO_4 and evaporated. The crude was then purified by flash chromatography on silica gel (CH_2Cl_2/MeOH, from 97:3 to 96:4) to afford the title compound (76 mg, 57%), after trituration with Et_2O and filtration. HPLC: t_R = 6.23 min; ^{1}H NMR (600 MHz): δ = 10.67 (br s, 1H), 8.29 (d, J = 5.1 Hz, 1H), 7.56 (t, J = 8.3 Hz, 1H), 7.46 (dt, J = 9.2, 3.9 Hz, 1H), 7.39 (ddd, J = 7.8, 4.9, 3.3 Hz, 1H), 7.33 (m, 2H), 7.23 (m, 1H), 7.03 (s, 1H), 6.79 (dd, J = 5.2, 1.2 Hz, 1H), 3.93 (td, J = 9.6, 2.1 Hz, 2H), 3.47 (m, 2H), 2.39 (s, 3H), 2.01 (dd, J = 12.7, 1.9 Hz, 2H), 1.75 ppm (m, 2H); LC–MS-ESI *m/z* (%): 546 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{26}H_{23}F_3N_3O_3S_2$ 546.1128, found 546.1127.

***tert*-Butyl {4-[4-(3-{[(2,5-difluorophenyl)sulfonyl](methoxymethyl)amino}-2-fluorophenyl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-5-yl]pyridin-2-yl}carbamate (37 a)**: In a microwave tube *N*-{3-[5-(2-chloropyridin-4-yl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,5-difluoro-*N*-(methoxymethyl)benzenesulfonamide **27 d** (200 mg, 0.33 mmol) was dissolved in anhydrous THF (3 mL) and the solution was degassed by bubbling argon for 5 min. *tert*-Butyl carbamate (152 mg, 1.31 mmol) was then added,

followed by Cs_2CO_3 (212 mg, 0.66 mmol), palladium acetate (8 mg, 0.03 mmol) and xantphos (40 mg, 0.66 mmol) and the mixture was irradiated in the microwave oven at 120 °C for 30 min. The mixture was filtered on a Celite pad and the Celite was washed with EtOAc. The filtrate was washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4 and evaporated to dryness. The crude product was purified by flash chromatography on silica gel (EtOAc/hexane 6:4) to give the title compound (160 mg, 71%) as a pale-yellow solid. HPLC: $t_R = 7.57$ min; ^{1}H NMR (600 MHz): $\delta = 9.83$ (s, 1H), 8.08 (d, $J = 5.1$ Hz, 1H), 7.76 (s, 1H), 7.63 (m, 1H), 7.58 (m, 1H),7.56 (m, 1H) 7.45 (m, 2H), 7.25–7.36 (m, 2H), 6.60 (dd, $J = 5.1$, 1.5 Hz, 1H), 4.96 (s, 2H), 3.95 (m, 2H), 3.48 (dt, $J = 11.6$, 1.8 Hz, 2H), 3.34 (m, 1H) 3.23 (m, 3H), 2.04 (m, 2H), 1.78 (m, 2H), 1.44 ppm (m, 9H); LC–MS-ESI *m/z* (%): 691 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{26}H_{23}F_3N_3O_3S_2$ 691.1867, found 691.1866.

***tert*-Butyl {4-[2-(1-cyclopropylpiperidin-4-yl)-4-(3-{[(2,5-difluorophenyl)sulfonyl](methoxymethyl)amino}-2-fluorophenyl)-1,3-thiazol-5-yl]pyridin-2-yl}carbamate (37b)**: Prepared by using the procedure mentioned above, starting from **27e** (150 mg, 65%). ^{1}H NMR (400 MHz): $\delta = 10.52$ (s, 1H), 8.16 (d, $J = 5.4$ Hz, 1H), 8.01 (s, 1H), 7.50–7.67 (m, 3H), 7.43 (m, 1H), 7.29 (m, 2H), 6.73 (dd, $J = 5.1$, 1.3 Hz, 1H), 4.95 (s, 2H), 3.23 (s, 3H), 3.03 (m, 3H), 2.32 (m, 2H), 2.08 (m, 2H), 1.68 (m, 3H), 1,44 (s, 9H), 0.42 (m, 2H), 0.31 ppm (m, 2H).

***N*-{3-[5-(2-Aminopyridin-4-yl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,5-difluorobenzenesulfonamide (9)**: *tert*-Butyl {4-[4-(3-{[(2,5-difluorophenyl)sulfonyl](methoxymethyl)amino}-2-fluorophenyl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-5-yl]pyridin-2-yl}carbamate **37a** (58 mg, 0.084 mmol) was dissolved in a 9:1 TFA/H_2O mixture and stirred at 70 °C for 1 h. The mixture was evaporated to dryness, taken up with CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4 and concentrated under reduced pressure. The crude was purified by flash chromatography on silica gel (pure EtOAc) to give the product which was triturated with Et_2O, filtered and dried under high vacuum providing the title compound (32 mg, 70%), as a colorless solid. HPLC: $t_R = 5.86$ min; ^{1}H NMR (600 MHz) $\delta = 10.69$ (br s, 1H) 7.70 (d, $J = 5.3$ Hz, 1H) 7.56 (m, 1H) 7.46 (m, 2H) 7.33 (t, $J = 6.9$ Hz, 1H) 7.26 (m, 1H) 7.20 (m, 1H) 6.34 (s, 1H) 6.04 (br s, 2H) 5.98 (d, $J = 5.1$ Hz, 1H) 3.93 (m, 2H) 3.46 (td, $J = 11.6$, 1.8 Hz, 2H) 3.30 (m,1H) 2.01 (m, 2H) 1.73 ppm (m, 2H); LC–MS-ESI *m/z* (%): 547 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{25}H_{22}F_3N_4O_3S_2$ 547.1080, found 547.1092.

***N*-{3-[5-(2-Aminopyridin-4-yl)-2-(1-cyclopropylpiperidin-4-yl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,5-difluorobenzenesulfonamide (18)**: Prepared by using the procedure mentioned above, starting from **37b** (70 mg, 80%). HPLC: $t_R = 5.49$ min; ^{1}H NMR (600 MHz) $\delta = 10.56$ (m, 1H), 7.70 (d, $J = 5.3$ Hz, 1H), 7.54 (m, 1H), 7.42 (m, 2H), 7.31 (t, $J = 7.1$ Hz, 1H), 7.21 (br s, 1H), 7.16 (m, 1H), 6.33 (s, 1H), 6.01 (s, 2H), 5.98 (d, $J = 4.9$ Hz, 1H), 3.03 (m, 3H), 2.36 (m, 2H), 2.04 (m, 2H), 1.66 (m, 3H), 0.45 (m, 2H), 0.34 ppm (br s, 2H); LC–MS-ESI *m/z* (%): 586 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{28}H_{27}F_3N_5O_2S_2$ 586.1553, found 586.1556.

2,5-Difluoro-*N*-(2-fluoro-3-{5-[2-(methylamino)pyridin-4-yl]-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-4-yl}phenyl)benzenesulfonamide (10): *tert*-Butyl {4-[4-(3-{[(2,5-difluorophenyl)sulfonyl](methoxymethyl)amino}-2-fluorophenyl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-5-yl]pyridin-2-yl}carbamate **37a** (97 mg, 0.140 mmol) was dissolved in dry THF (1.5 mL) under argon atmosphere. The solution was cooled to 0 °C and methyl iodide (0.02 mL, 0.321 mmol) was added, followed by NaH (60% in mineral oil) (20 mg, 0.353 mmol) and the mixture was stirred at room temperature overnight. The mixture was then diluted with H_2O and EtOAc. The two phases were separated and the aqueous phase was extracted with EtOAc three times. The combined organic layers were washed with brine, dried over Na_2SO_4 and evaporated to dryness, to give *tert*-butyl {4-[4-(3-{[(2,5-difluorophenyl)sulfonyl]amino}-2-fluorophenyl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-5-yl]pyridin-2-yl}methylcarbamate. This compound was treated with a 9:1 TFA/H_2O mixture (2 mL) and stirred at 70 °C for 1 h. The mixture was evaporated to dryness, taken up with CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by flash chromatography on silica gel (EtOAc/hexane 3:1) to give 59 mg product, which was triturated with Et_2O, filtered and dried under high vacuum, to afford the title compound (36 mg, 46%) as a white solid. HPLC: $t_R = 6.24$ min; ^{1}H NMR (600 MHz): $\delta = 10.68$ (br s, 1H), 7.80 (d, $J = 5.3$ Hz, 1H), 7.56 (t, $J = 8.6$ Hz, 1H), 7.45 (m, 2H), 7.34 (m, 1H), 7.27 (m, $J = 6.0$ Hz, 1H), 7.19 (m, 1H), 6.54 (br s, 1H), 6.24 (s, 1H), 6.03 (d, $J = 4.4$ Hz, 1H), 3.93 (m, 2H), 3.46 (dt, $J = 11.6$, 1.9 Hz, 2H), 2.66 (d, $J = 4.7$ Hz, 3H), 2.204 (m, 2H), 1.75 ppm (m, 2H); LC–MS-ESI *m/z* (%): 561 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{26}H_{24}F_3N_4O_3S_2$ 561.1237, found 561.1223.

***N*-(3-{2-(1-Cyclopropylpiperidin-4-yl)-5-[2-(methylamino)pyridin-4-yl]-1,3-thiazol-4-yl}-2-fluorophenyl)-2,5-difluorobenzenesulfonamide (19)**: Prepared by using the procedure mentioned above, starting from **37b** (22 mg, 78%). HPLC: $t_R = 5.94$ min; ^{1}H NMR (600 MHz): $\delta = 10.56$ (br s, 1H), 7.80 (d, $J = 5.5$ Hz, 1H), 7.54 (m, 1H), 7.45 (m, 2H), 7.31 (m, 1H), 7.23 (m, 1H), 7.16 (m, 1H), 6.52 (q, $J = 4.6$ Hz, 1H), 6.23 (s, 1H), 6.04 (d, $J = 5.3$ Hz, 1H), 3.03 (m, 3H), 2.66 (d, $J = 4.7$ Hz, 3H), 2.36 (m, 2H), 2.05 (m, 2H), 1.60–1.76 (m, 3H), 0.45 (m, 2H), 0.34 ppm (br s, 2H); LC–MS-ESI *m/z* (%): 600 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{29}H_{29}F_3N_5O_2S_2$ 600.1709, found 600.1710.

***N*-{4-[4-(3-{[(2,5-Difluorophenyl)sulfonyl]amino}-2-fluorophenyl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-5-yl]pyridin-2-yl}acetamide (14)**: *N*-{3-[5-(2-chloropyridin-4-yl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,5-difluoro-*N*-(methoxymethyl)-benzenesulfonamide **27d** (100 mg, 0.164 mmol) was dissolved in dry THF (2 mL) and the solution was degassed by bubbling argon for 5 min. Acetamide (20 mg, 0.339 mmol) was then added, followed by Cs_2CO_3 (107 mg, 0.328 mmol, 2 equiv), palladium acetate (2 mg, 0.008 mmol, 0.05 equiv) and xantphos (10 mg, 0.016 mmol, 0.1 equiv) and the mixture was irradiated in the microwave oven at 100 °C for 30 min. The mixture was filtered on a Celite pad and the Celite was washed with EtOAc. The filtrate was washed with saturated aqueous $NaHCO_3$, H_2O and brine, dried over Na_2SO_4 and evaporated to dryness. The crude product was purified by flash chromatography on silica gel (CH_2Cl_2/EtOH 98:2) to yield *N*-{4-[4-(3-{[(2,5-difluorophenyl)sulfonyl](methoxymethyl)amino}-2-fluorophenyl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-5-yl]pyridin-2-yl}acetamide (50 mg, 50%). ^{1}H NMR (600 MHz): $\delta = 10.54$ (s, 1H), 8.08 (d, $J = 5.1$ Hz, 1H), 7.76 (s, 1H), 7.56–7.64 (m, 2H), 7.53 (m, 1H), 7.45 (m, 1H), 7.32 (m, 2H), 6.60 (dd, $J = 5.2$, 1.56, Hz, 1H), 4.96 (s, 2H), 3.95 (m, 2H), 3.48 (m, 2H), 3.22 (s, 3H), 2.01 (s, 3H) 2.02 (m, 2H), 1.78 ppm (m, 2H); MS (ESI) $[M+H]^+$ 633, $[M-H]^-$ 631. This intermediate (50 mg, 0.09 mmol) was treated with a 9:1 TFA/H_2O mixture (1 mL) and stirred at 70 °C for 1.5 h. The mixture was evaporated to dryness, taken up with CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by flash chromatography on silica gel (CH_2Cl_2/MeOH 95:5) to give 35 mg product, which was triturated with Et_2O filtered and dried under

high vacuum. The title compound (28 mg, 60%) was then obtained as an off-white solid. HPLC: $t_R = 5.84$ min; ^{1}H NMR (600 MHz): $\delta =$ 10.63 (s, 1H), 10.54 (s, 1H), 8.12 (d, $J = 5.3$ Hz, 1H), 8.04 (s, 1H), 7.55 (m, 1H), 7.45 (dt, $J = 9.3$, 3.9 Hz, 1H), 7.41 (ddd, $J = 7.8$, 4.9, 3.4 Hz, 1H), 7.28–7.37 (m, 2H), 7.22 (m, 1H), 6.62 (dd, $J = 5.1$, 1.6 Hz, 1H), 3.93 (td, $J = 9.4$, 2.2 Hz, 2H), 3.47 (dt, $J = 11.6$, 1.9 Hz, 2H), 3,38 (m, 1H) 2.07 (s, 3H), 2.01 (m, 2H), 1.76 ppm (m, 2H); LC–MS-ESI m/z (%): 589 (100) $[M+H]^+$; HRMS-ESI m/z $[M+H]^+$ calcd for $C_{27}H_{24}F_3N_4O_4S_2$ 589.1186, found 589.1187.

***N*-{4-[2-(1-Cyclopropylpiperidin-4-yl)-4-(3-{[(2,5-difluorophenyl)-sulfonyl]amino}-2-fluorophenyl)-1,3-thiazol-5-yl]pyridin-2-yl}acetamide (20)**: Prepared by using the procedure mentioned above, starting from **27e** (856 mg, 76%). HPLC: $t_R = 5.57$ min; ^{1}H NMR (400 MHz): $\delta = 10.54$ (s, 1H), 8.12 (d, $J = 5.2$ Hz, 1H), 8.04 (s, 1H), 7.52 (m, 1H), 7.40 (m, 2H), 7.31 (m, 1H), 7.27 (m, 1H), 7.17 (m, 1H), 6.62 (dd, $J = 5.2$, 1.6 Hz, 1H), 3.04 (m, 2H), 2.36 (t, $J = 11.3$ Hz, 2H), 2.07 (m, 5H), 1.68 (m, 3H), 0.43 (m, 2H), 0.34 ppm (m, 2H); LC–MS-ESI m/z (%): 628 (100) $[M+H]^+$; HRMS-ESI m/z $[M+H]^+$ calcd for $C_{30}H_{29}F_3N_5O_3S_2$ 628.1659, found 628.1659.

2,5-Difluoro-*N*-{2-fluoro-3-[5-(1-oxidopyridin-4-yl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-4-yl]phenyl}-*N*-(methoxymethyl)benzenesulfonamide (38): 2,5-Difluoro-*N*-{2-fluoro-3-[5-(pyridin-4-yl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-4-yl]phenyl}-*N*-(methoxymethyl)benzenesulfonamide **27a** (500 mg, 0.869 mmol) was dissolved in CH_2Cl_2 (9 mL) and 70% *m*-chloroperbenzoic acid was added (215 mg, 0.869 mmol). After stirring for 1 h an addition of *m*-chloroperbenzoic acid (215 mg, 0.869 mmol) was made, followed by a second addition of 190 mg after one more hour. After 5 h total, the mixture was diluted with CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4 and evaporated to dryness. The crude product was purified by flash chromatography on silica gel (CH_2Cl_2/MeOH 95:5) to give the title compound (420 mg, 82%) as an amorphous solid. HPLC: $t_R = 5.40$ min; ^{1}H NMR (600 MHz): $\delta = 8.11$ (m, 2H), 7.63 (m, 2H), 7.55 (m, 1H), 7.51 (m, 1H), 7.32 (m, 2H), 7.15 (m, 2H), 4.99 (s, 2H), 3.95 (m, 2H), 3.48 (dt, $J = 11.6$, 2.0 Hz, 2H), 3.26 (s, 3H), 2.02 (m, 2H), 1.76 ppm (m, 2H); LC–MS-ESI m/z (%): 592 (100) $[M+H]^+$; HRMS-ESI m/z $[M+H]^+$ calcd for $C_{27}H_{25}F_3N_3O_5S_2$ 592.1182, found 592.1188.

***N*-{3-[5-(2-{[2-(Dimethylamino)ethyl]amino}pyridin-4-yl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,5-difluorobenzenesulfonamide (11)**: To a solution of 2,5-Difluoro-*N*-{2-fluoro-3-[5-(1-oxidopyridin-4-yl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-4-yl]phenyl}-*N*-(methoxymethyl)benzenesulfonamide **38** (104 mg, 0.175 mmol) in CH_2Cl_2 (1.5 mL), DIPEA (0.112 mL, 0.656 mmol) was added, followed by PyBroP (106 mg, 0.228 mmol) and *N*,*N*-dimethylaminoethylamine (0.024 mL, 0.219 mmol) and the mixture was stirred at room temperature overnight. Further additions of both *N*,*N*-dimethylaminoethylamine (0.01 mL) and PyBroP (20 mg) were made and, after an addition 2 h stirring, the mixture was diluted with CH_2Cl_2 and washed with H_2O and brine, dried over Na_2SO_4 and evaporated to dryness. The crude product was purified by flash chromatography on silica gel (CH_2Cl_2/MeOH 96:4) to give *N*-{3-[5-(2-{[2-(dimethylamino)ethyl]amino}pyridin-4-yl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-4-yl]-2-fluorophenyl}-2,5-difluoro-*N*-(methoxymethyl)benzenesulfonamide (118 mg, 85%). This intermediate (100 mg, 0.15 mmol) was treated with a 9:1 TFA/H_2O mixture (2 mL) and stirred at 60 °C for 5 h. The mixture was evaporated to dryness, taken up with CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by flash chromatography on silica gel (CH_2Cl_2/MeOH 95:5) to give the title compound (71 mg, 66%) as an off-white solid. HPLC: $t_R = 5.33$ min; ^{1}H NMR (600 MHz): $\delta = 7.85$ (d, $J = 5.3$ Hz, 1H), 7.42 (ddd, $J = 8.2$, 5.3, 3.3 Hz, 1H), 7.36 (m, 1H), 7.31 (dt, $J = 8.9$, 4.2 Hz, 1H), 7.23 (dt, $J = 7.9$, 1.4 Hz, 1H), 6.96 (t, $J = 7.9$ Hz, 1H), 6.86 (brs, 1H), 6.66 (t, $J = 5.2$ Hz, 1H), 6.40 (s, 1H), 6.22 (dd, $J = 5.3$, 1.2 Hz, 1H), 3.93 (m, 2H), 3.47 (dt, $J = 11.6$, 1.8 Hz, 2H), 3.29 (m, 1H) 2.86 (m, 2H), 2.56 (s, 6H), 2.0 (m, 2H), 1.74 ppm (m, 2H); LC–MS-ESI m/z (%): 618 (100) $[M+H]^+$; HRMS-ESI m/z $[M+H]^+$ calcd for $C_{29}H_{31}F_3N_5O_3S_2$ 618.1815, found 618.1822.

Compounds **12** and **13** were prepared by using the procedure mentioned above, starting from **38** and the appropriate amino derivatives.

***N*-[2-({4-[4-(3-{[(2,5-Difluorophenyl)sulfonyl]amino}-2-fluorophenyl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-5-yl]pyridin-2-yl}amino)ethyl]acetamide (12)**: (55 mg, 78%). HPLC: $t_R = 5.47$ min; ^{1}H NMR (600 MHz): $\delta = 10.68$ (brs, 1H), 7.91 (t, $J = 5.4$ Hz, 1H), 7.78 (d, $J = 5.5$ Hz, 1H), 7.57 (m, 1H), 7.45 (m, 2H), 7.33 (m, 1H), 7.30 (m, 1H), 7.21 (m, 1H), 6.73 (brs, 1H), 6.38 (brs, 1H), 6.02 (brs, 1H), 3.93 (m, 2H), 3.46 (dt, $J = 11.6$, 1.8 Hz, 2H), 3.30 (m, 1H), 3.24 (m, 2H), 3.16 (m, 2H), 1.99 (m, 2H), 1.80 (s, 3H), 1.74 ppm (m, 2H); LC–MS-ESI m/z (%): 632 (100) $[M+H]^+$; HRMS-ESI m/z $[M+H]^+$ calcd for $C_{29}H_{29}F_3N_5O_4S_2$ 632.1608, found 632.1625.

Methyl [(2*S*)-1-({4-[4-(3-{[(2,5-difluorophenyl)sulfonyl]amino}-2-fluorophenyl)-2-(tetrahydro-2*H*-pyran-4-yl)-1,3-thiazol-5-yl]pyridin-2-yl}amino)propan-2-yl]carbamate (13): (20 mg, 20%). ^{1}H NMR (600 MHz): $\delta = 10.66$ (brs, 1H), 7.76 (d, $J = 5.5$ Hz, 1H), 7.57 (brs, 1H), 7.46 (m, 2H), 7.33 (t, $J = 6.8$ Hz, 1H), 7.27 (brs, 1H), 7.19 (m, 1H), 7.01 (d, $J = 7.3$ Hz, 1H), 6.63 (brs, 1H), 6.40 (s, 1H), 5.96 (d, $J = 4.9$ Hz, 1H), 3.93 (td, $J = 9.5$, 2.0 Hz, 2H), 3.64 (dq, $J = 13.9$, 6.8 Hz, 1H), 3.50 (s, 3H), 3.47 (dt, $J = 11.6$, 1.9 Hz, 3H), 3.19 (brs, 2H), 2.00 (m, 2H), 1.74 (m, 2H), 1.03 ppm (d, $J = 6.6$ Hz, 3H); LC–MS-ESI m/z (%): 662 (100) $[M+H]^+$; HRMS-ESI m/z $[M+H]^+$ calcd for $C_{30}H_{31}F_3N_5O_5S_2$ 662.1713, found 662.1714.

Tetrahydro-2*H*-pyran-4-carbothioamide (26a): A mixture of methyl tetrahydro-2*H*-pyran-4-carboxylate (7 g, 48.6 mmol) and 30% aqueous ammonia (20 mL) was stirred in a closed bottle at room temperature for 18 h. Excess ammonia was removed under reduced pressure, and the residue was crystallized from EtOH affording tetrahydro-2*H*-pyran-4-carboxamide (5.6 g, 89%). ^{1}H NMR (400 MHz): $\delta = 7.21$ (brs, 1H) 6.73 (brs, 1H) 3.80–3.90 (m, 2H) 3.27 (m, 2H) 2.30 (m, 1H) 1.47–1.66 ppm (m, 4H); LC–MS-ESI m/z (%): 130 $[M+H]^+$. Tetrahydro-2*H*-pyran-4-carboxamide (2 g, 15.5 mmol) was suspended in dry THF (20 mL) and Lawesson's reagent (3.13 g, 7.75 mmol) was added. After holding at reflux for 4 h, the mixture was poured into a saturated $NaHCO_3$ aqueous solution (200 mL) and then extracted with Et_2O (4×100 mL). The organic layer was dried over Na_2SO_4 and evaporated to dryness, to afford the title compound (1.2 g, 54%). HPLC: $t_R =$ min 2.79; ^{1}H NMR (600 MHz): $\delta = 9.38$ (brs, 1H) 9.08 (brs, 1H) 3.87 (dd, $J = 11.3$, 4.4 Hz, 2H) 3.31 (m, 2H) 2.71 (tt, $J = 11.7$, 3.7 Hz, 1H) 1.75 (qd, $J = 12.5$, 4.5 Hz, 2H) 1.56 ppm (m, 2H); LC–MS-ESI m/z (%): 146 (100) $[M+H]^+$; HRMS-ESI m/z $[M+H]^+$ calcd for $C_6H_{12}NOS$ 146.0634, found 146.0634.

1-Cyclopropylpiperidine-4-carbothioamide (26b): To a solution of piperidine-4-carboxamide (1 g, 7.8 mmol) in MeOH (80 mL) 1-ethoxy-1-trimethylsilyloxycyclopropane (2.35 mL, 11.7 mmol) was added, followed by AcOH (1.34 mL, 23.4 mmol) and $NaBH_3CN$ (923 mg, 12.48 mmol) and the mixture was stirred at 60 °C overnight. The solvent was then concentrated under reduced pressure, and the residue was purified by flash chromatography on silica gel (CH_2Cl_2/MeOH/NH_4OH 7 N in MeOH 90:9:1) affording 1-cyclopropylpiperidine-4-carboxamide. This intermediate was suspended in dry THF (20 mL) and Lawesson's reagent (2.7 g, 6.67 mmol) was added.

After holding at reflux for 6 h the solvent was concentrated under reduced pressure. The residue was dissolved in a EtOAc/MeOH mixture and washed with saturated aqueous $NaHCO_3$. The aqueous phase was back-extracted with EtOAc and evaporated to dryness. The crude product was treated with EtOH and filtered. The white solid was dried under high vacuum to afford the title compound (1.2 g, 86%). ^{1}H NMR (600 MHz): δ = 9.32 (br s, 1H) 9.04 (br s, 1H) 2.95 (m, 2H) 2.49 (m, 1H) 2.11 (m, 2H) 1.38–1.75 (m, 5H) 0.39 (m, 2H) 0.27 ppm (m, 2H); LC–MS-ESI *m/z* (%): 185 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_9H_{17}N_2S$ 185.1107, found 185.1104.

Benzyl-4-carbamothioylpiperidine-1-carboxylate (26c): To a solution of piperidine-4-carboxamide (5 g, 39 mmol) and benzylchloroformate (5.54 mL, 39 mmol) in a mixture of H_2O (30 mL) and acetone (40 mL), NaOH 1 N (39 mL, 39 mmol) was added dropwise, while maintaining pH 6–8. The mixture was stirred at room temperature for 3 h; then acetone was evaporated and the resulting precipitate filtered and dried at 70 °C under reduced pressure, giving benzyl-4-carbamoylpiperidine-1-carboxylate (7.75 g, 76%). HPLC: t_R = 4.54 min; ^{1}H NMR (600 MHz): δ = 7.29–7.41 (m, 5H), 7.25 (br s, 1H), 6.76 (br s, 1H), 5.07 (s, 2H), 4.00 (m, 2H), 2.72–2.92 (m, 2H), 2.27 (tt, *J* = 11.5, 3.7 Hz, 1H), 1.69 (m, 2H), 1.40 ppm (dq, *J* = 12.4, 4.3 Hz, 2H); LC–MS-ESI *m/z* (%): 263 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{14}H_{19}N_2O_3$ 263.1390, found 263.1390. This intermediate (7.5 g, 38.6 mmol) was dissolved in THF (160 mL) and Lawesson's reagent (6.9 g, 17.1 mmol) was added. After 4 h the solvent was evaporated and the residue purified by flash chromatography on silica gel (CH_2Cl_2/MeOH 95:5) to afford the title compound (1.8 g, 23%), which was recrystallized from MeOH. ^{1}H NMR (600 MHz): δ = 9.40 (br s, 1H), 9.11 (br s, 1H), 7.36 (m, 5H), 5.07 (s, 2H), 4.07 (m, 2H), 2.73–2.90 (m, 2H), 2.69 (tt, *J* = 11.6, 3.8 Hz, 1H), 1.67 (m, 2H), 1.59 ppm (m, 2H); LC–MS-ESI *m/z* (%): 279 (100) $[M+H]^+$; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{14}H_{19}N_2O_2S$ 279.1162, found 279.1163.

Crystallization, data collection, structure determination, and refinement

The protein construct used for structural studies contains the kinase domain of B-Raf V600E (residues 448–723) with 16 mutations that were introduced to improve the protein expression.[4] Crystals of B-Raf V600E in complex with compound **20** were grown using the sitting-drop vapor diffusion method from a solution of 20% PEG 6000, 0.2 M $MgCl_2$, 0.1 M HEPES (pH 7.0) at 4 °C. Before crystallization, the protein was concentrated to 10 mg mL^{-1} in 50 mM Tris·HCl pH 7.6, 150 mM NaCl, 10% glycerol, 2 mM DTT buffer and compound **20** was added to the protein solution to a final concentration of 0.5 mM. For data collection the crystals were transferred briefly to a solution containing 20% PEG 6000, 0.2 M $MgCl_2$, 0.1 M HEPES (pH 7.0) and 30% (*v/v*) glycerol and then flash-frozen in liquid nitrogen. Diffraction data were collected at the European Synchrotron Radiation Facility (Grenoble, France) on the beamline ID29. Indexing, integration, and scaling were performed using DENZO and SCALEPACK.[26] The structure was solved by molecular replacement using the MOLREP software[27] with the structure of B-Raf (PDB ID 3C4C) as the search model. The structure was refined with Refmac,[28] using the CCP4[29] suite and model building was done with Coot.[30] The final model has R_{factor} = 0.207 and R_{free} = 0.255. The structure has been deposited at the RCSB Protein Data Bank (PDB) with accession code 4CQE.

Biology

Cloning, expression, and purification of B-Raf, C-Raf, and MEK1: For in vitro kinase assays, full-length B-RAF cDNA, a fragment encoding amino acid residues 306–648 of C-RAF and full-length MEK1 were cloned in a baculovirus expression vector based on pVL1393 (Invitrogen) in frame with an N-terminal GST tag. To obtain a catalytically active protein, CRAF was mutagenized at residues Y340D and Y341D. The proteins were expressed in Sf21 (B-Raf) or High5 (C-Raf and MEK1) cells and purified via GSH affinity chromatography. Briefly, insect cell pellets were homogenized in lysis buffer (50 mM Tris·HCl pH 7.6, 150 mM NaCl, 0.2% CHAPS, 20 mM DTT, 10% glycerol, protease inhibitors) and cleared lysates were mixed with GSH Sepharose 4 Fast Flow resin (GE Life Sciences) for 3 h at 4 °C. Proteins were then cleaved with PreScission protease (GE Life Sciences) for N-terminal tag removal and eluted. The intrinsic ATPase activity of MEK1 was inactivated by covalent modification with 5′-*p*-fluorosulfonylbenzoyladenosine (FSBA) according to a modification of a reported method.[31] Purified MEK1 was incubated with a 15-fold molar excess of FSBA in the presence of 10 mM $MgCl_2$ for 1 h at 27 °C and then purified by size-exclusion chromatography. Complete derivatization of MEK1 with FSBA was confirmed by LC–MS-ESI.

For crystallization, the B-RAF cDNA (both wild-type and V600E) fragments corresponding to the kinase domain (residues 448–723) with 16 mutations to improve expression (I543A, I544S, I551K, Q562R, L588N, K630S, F667E, Y673S, A688R, L706S, Q709R, S713E, L716E, S720E, P722S, K723G) as previously described,[4] were cloned into a modified pGEX vector (GE Life Sciences), in frame with an N-terminal His–GST double tag. The proteins were expressed in *E. coli* cells with induction for 48 h at 16 °C. For purification, bacterial cells were homogenized in lysis buffer (50 mM Tris·HCl pH 7.6, 150 mM NaCl, 0.2% CHAPS, 1 mg mL^{-1} lysozyme, 10% glycerol, protease inhibitors) and cleared lysates were mixed with Ni Sepharose 6 Fast Flow resin (GE Life Sciences) for 3 h at 4 °C. Proteins were eluted with an imidazole-containing buffer and mixed with GSH Sepharose 4 Fast Flow resin (GE Life Sciences) for 2 h at 4 °C. Proteins were then cleaved with PreScission protease (GE Life Sciences) for N-terminal tag removal, eluted and purified by size-exclusion chromatography using a Superdex 200 16/60 equilibrated with 50 mM Tris·HCl pH 7.6, 150 mM NaCl, 10% glycerol, 2 mM DTT.

In vitro kinase assays:[22] B- and C-Raf enzymatic activities were quantified by measuring ADP formation upon phosphorylation of full-length human MEK covalently modified FSBA. Briefly, 1 nM of wild-type, V600E B-Raf or Y340D/Y341D C-Raf were incubated with 500 nM MEK-FSBA and 76 μM ATP in assay buffer containing 50 mM HEPES pH 7.9, 10 mM $MgCl_2$, 1 mM DTT, 0.2 mg mL^{-1} BSA, 3 μM Na_3VO_4. Inhibitors were serially diluted in 100% DMSO (dilution steps 1:3) and added to the sample in order to reach 1 μM as the highest compound concentration in the presence of 1% DMSO. IC_{50} values were determined by monitoring the enzymatic activity over 120 min at room temperature. Reactions were processed with ADP-Glo (Promega) following manufacturer's instructions, and luminescence was read with a Viewlux Plate reader (PerkinElmer). IC_{50} values were calculated by fitting a standard four-parameter logistic model to the dose–response curve. Experiments were performed in duplicate and IC_{50} values are the average of three independent experiments.

Fluorescence polarization (FP) assays: FP experiments were carried out at room temperature using no-binding black 384-well microplates (Corning). Unless otherwise specified, FP assays were per-

formed in 50 mM HEPES, pH 7.9, 1 mM CHAPS, 10 mM $MgCl_2$ (assay buffer), at a final volume of 21 μL. The plate reader was a Tecan Saphire2 (Tecan US Inc.). Polarization values were read using excitation and emission wavelengths of 590 and 630 nm, respectively. All FP values are expressed as mP units.[32] Displacement experiments were carried out at a constant concentration of probe and proteins, respectively 0.02 and 0.03 μM. Compounds were serially diluted in 100% DMSO, followed by an intermediate dilution step in water to reach 1% DMSO and 10 μM compound final concentration. Experimental data were analyzed using the Dynafit software package (BioKin Ltd.).[33] Reported values represent the average of at least two independent experiments performed in duplicate. Reported values, unless otherwise specified, represent the average of at least three independent experiments.

Cell culture: Human cancer cell lines were obtained either from the American Type Culture Collection (Manassas, VA, USA) or from NCI (Bethesda, MD, USA). Cells were maintained, routinely characterized using AmpFlSTR Identifiler PCR Amplification kit (Applied Biosystems, Paisley, UK).

Analysis of cell proliferation: Cells were seeded into 96- or 384-well plates at densities ranging from 10000 to 30000 cm^2 in appropriate medium plus 10% fetal calf serum (FCS). After 24 h, cells were treated in duplicate with serial dilutions of the compounds for 72 h. At the end of treatment, cell proliferation was determined by an intracellular ATP monitoring system (CellTiterGlo, Promega), using Envision software (PerkinElmer) as reader. Inhibitory activity was evaluated by comparing treated versus control data using Symix Assay Explorer (Symix Technology Inc.) software. IC_{50} values were calculated by a sigmoidal fitting algorithm.

Cellular ERK1/2 phosphorylation assays: A375 cells were seeded in Matrix 384-well black, clear-bottom poly-L-Lysine-coated plates (Thermo Fisher Scientific, Waltham, MA, USA) at a density of 1000 cells per well and incubated overnight in complete medium with 10% *v/v* FCS. Cells were treated with various doses of compounds for 90 min and fixed for 30 min with formaldehyde 3.7%. The fixative was removed, and cells were washed twice with PBS then saturated for 30 min with PBS containing 0.1% Triton X-100 and 1% BSA (staining solution). Primary anti-phospho-ERK (Thr202/Tyr204) antibody (Cell Signaling Technology, Danvers, MA, USA) was added at the recommended dilution in staining solution for 1 h at 37 °C. The solution was removed and cells were washed twice with PBS, and then an anti-mouse Cy2-conjugated secondary antibody (GE Healthcare, Little Chalfont, UK) was added in staining solution containing 1 mg mL^{-1} DAPI. Cells were incubated for 1 h at 37 °C then washed twice with PBS; 80 μL PBS were left in each well. Plates were sealed and read with the ArrayScan VTI HCS reader (Thermo Fisher Scientific), acquiring four fields per well with the CytoNuclTrans algorithm. The parameter "MEAN_RingAvgIntenCh2", which measures the mean cytoplasmatic fluorescence intensity associated with *p*-ERK staining, was reported as readout.

Western blot analysis: Effect of compound treatment on signaling pathways was evaluated after 2 h treatment. Cell extracts were prepared in lysis buffer containing 125 mM Tris·HCl (pH 6.8) and 5% (*w/v*) SDS. Samples were heated at 95 °C for 5 min and then sonicated. Protein extract was loaded and separated by SDS-PAGE in 8% bis/acrylamide gels. Immunoblotting was done according to standard procedures, and staining was done with the following antibodies: anti-phospho-MEK1/2 (Ser217/221), anti-MEK1/2, anti-phospho-p44/42 MAPK(Thr202/Tyr204), anti-p44/42 MAP kinase, anti-phospho-p90 RSK (Thr359/Ser363), and anti-p90 RSK, which were purchased from Cell Signaling (Danvers, MA, USA).

Pharmacokinetic profile evaluation: Pharmacokinetic properties were investigated in mouse (nu/nu) or rat after single intravenous (i.v.) and oral (p.o.) administration at a dose of 10 mg kg^{-1}. Compounds were dissolved at 1 mg mL^{-1} with glucose solution containing 10% (*w/v*) Tween 80 for i.v. administration and with water containing 0.5% (*w/v*) hydroxypropyl methylcellulose (HPMC) for p.o. administration. Plasma was collected at various time points, from 5 min to 24 h. Plasma levels were determined by protein precipitation in a 96-well plate format followed by LC–MS/MS (Waters Acquity-TQD system). Pharmacokinetic data analysis was carried out by using a noncompartmental approach with the aid of WinNonlin software version 5.2.1 (Pharsight Inc.).

Animal efficacy studies: All procedures adopted for housing and handling of animals were in strict compliance with European and Italian Guidelines for Laboratory Animal Welfare. Human melanoma A375 cells were transplanted subcutaneously (s.c.) into Balb/c athymic nu/nu mice (Harlan, S. Pietro al Natisone, Udine, Italy). Mice bearing a palpable tumor ($\geq$100 mm^3) were randomized into vehicle and treated groups. Treatments started the day after randomization. Tumor dimensions were measured regularly using Vernier calipers. Tumor volumes were calculated as follows: volume (mm^3) = length (mm) × width^2 (mm^2)/2. Tumor volume inhibition (TI) was determined according to the equation: %TI = 100 − [(mean tumor volume of treated group)/(mean tumor volume of control group)] × 100. Toxicity was evaluated based on body weight reduction. At the end of the experiment, mice were sacrificed, and gross autopsy findings were reported.

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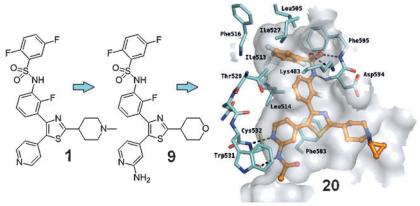
Curb the paradoxical effect! Optimization of the potent B-Raf inhibitor **1** led to the identification of compound **20**, which preserves the favorable in vitro and in vivo properties of **1**, but is devoid of hERG liability. Intriguingly, compound **20** shows a markedly decreased "paradoxical" effect.



Compd	IC_{50} [nM]		Rat hepat. CL_{int}	hERG inh.
	A375	m B-Raf	[mL min^{-1} kg^{-1}]	@ 10 mM [%]
1	40	2.4	57	96.7
9	15	6.1	230	
20	3	3.7	112	57.1

M. Pulici, G. Traquandi, C. Marchionni, M. Modugno, R. Lupi, N. Amboldi, E. Casale, N. Colombo, L. Corti, M. Fasolini, F. Gasparri, W. Pastori, A. Scolaro, D. Donati, E. Felder, A. Galvani, A. Isacchi, E. Pesenti, M. Ciomei*

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Optimization of Diarylthiazole B-Raf Inhibitors: Identification of a Compound Endowed with High Oral Antitumor Activity, Mitigated hERG Inhibition, and Low Paradoxical Effect