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Discovery and optimization of thieno[2,3-*d*]pyrimidines as B-Raf inhibitors

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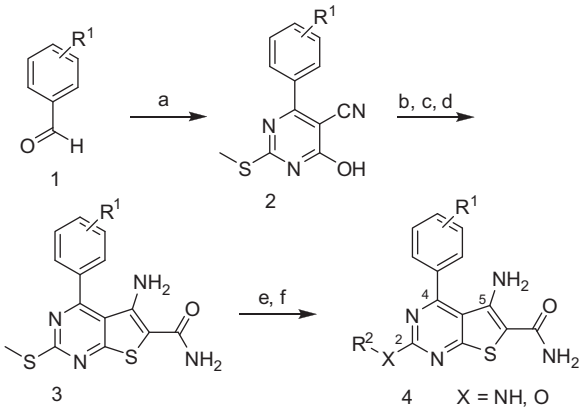
ABSTRACT

The serine/threonine specific protein kinase B-Raf is part of the MAPK pathway and is an interesting oncology target. We have identified thieno[2,3-*d*]pyrimidines as a core scaffold of small molecule B-Raf inhibitors. The SAR of analogs in this series will be described.

Raf proteins are components of the mitogen activated protein (MAP) kinase pathway, downstream of the membrane-bound small G-protein Ras. Upon activation by extracellular signals, Ras stimulates the activation of Raf protein kinases, which trigger a series of phosphorylation events to regulate cell proliferation, differentiation, senescence, and apoptosis.[1] Among the three Raf isoforms in humans (A-Raf, B-Raf, and C-Raf), B-Raf is the most critical isoform to mediate Ras activity and is important for tumor progression.[2] A significant fraction of melanoma, colorectal, and thyroid cancers have activating B-Raf mutations, particularly at valine 600.[3] A negatively charged amino acid substitution mimics the active form of the B-Raf enzyme thereby making it constitutively active.[4] The V600E B-Raf mutation is by far the most prevalent in human cancers.[3] In this publication, we present our efforts to discover V600E B-Raf inhibitors from a thieno[2,3-*d*]pyrimidine series.[5]

A general synthetic approach to the thieno[2,3-*d*]pyrimidine series is shown in Scheme 1. Condensation of an appropriately substituted benzaldehyde with 2-methyl isothiourea and ethyl cyanoacetate afforded intermediate **2**. Conversion of the 4-hydroxy substituent to a chloro followed by reaction and cyclization with 2-mercaptoacetamide gave intermediate **3**. The thiomethyl substituent at C-2 of the pyrimidine was oxidized with *m*-CPBA and then displaced using either an amine and diisopropylethylamine or an alcohol and sodium hydride to give the desired products **4**.

Our screening lead **5** was identified from a series of PDE4 inhibitors and is nearly equipotent against both B-Raf and PDE4. PDE4 inhibitors are known to induce emesis in animal species endowed with a vomiting reflex,[6] therefore we wished to identify potent B-Raf inhibitors with little or no PDE4 inhibitory activity. It was quickly determined that the C-5 amino and the C-6 carboxamide are both required for B-Raf inhibitory activity, therefore we



Scheme 1. Reagents and conditions: (a) 2-methyl-isothiouronium sulfate, ethyl cyanoacetate, K_2CO_3, EtOH, 80 °C; (b) $POCl_3$, 1,4-dioxane, reflux; (c) 2-mercaptoacetamide, Na_2CO_3, EtOH, 60 °C; (d) sodium ethoxide, EtOH, 75 °C; (e) *m*-CPBA, $CHCl_3$; (f) R^2NH_2, DIPEA, DMF, 90 °C, or R^2OH, NaH, THF.

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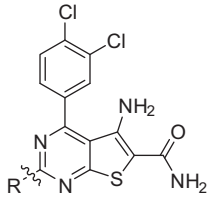
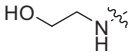
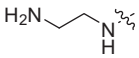
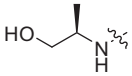
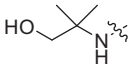
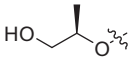
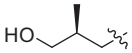
Table 1
C-2 SAR of the thienopyrimidine scaffold



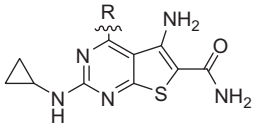
#	R	B-Raf IC_{50}[a] (μM)	PDE4 IC_{50}[b] (μM)	pMEK IC_{50}[c] (μM)	Hepat. stab.[d]
5		0.316 ± 0.059	0.261 ± 0.045	4.74 ± 1.01	8.8 ± 2.9
6		0.507 ± 0.103	11.8[e]	>60[e]	ND[f]
7		0.407	0.077[e]	17.6[e]	53 ± 10
8		0.718 ± 0.273	0.023[e]	ND	ND
9		0.275 ± 0.018	3.72[e]	7.91[e]	ND
10		0.110 ± 0.017	22.4[e]	9.75 ± 2.88	100 ± 27
11		0.067 ± 0.024	8.42[e]	3.82[e]	35 ± 2
12		0.112[e]	4.84[e]	9.64[e]	34 ± 5
13		0.050 ± 0.008	ND	6.88[e]	17 ± 2
14		0.219[e]	11.9[e]	14.5[e]	57 ± 4

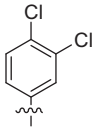
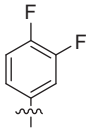
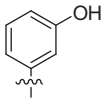
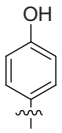
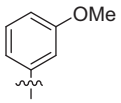
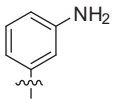
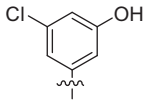
[a] In vitro enzyme activity (V600E B-Raf ^{33}P enzyme assay).[5]
[b] In vitro enzyme activity (U937 cell-derived PDE4 enzyme assay).[5]
[c] (Biomarker for B-Raf inhibition) In vitro cellular activity (A375 pMEK1/2 meso scale assay).[5]
[d] In vitro rat hepatocyte assay (% remaining after 1 h).[7]
[e] Compound was tested once in this assay.
[f] No data.

examined the SAR of substituents at C-2 and C-4 (Tables 1 and 2). A small amine substituent is required at C-2 for potent PDE4 inhibition (Table 1). Replacing the C-2 amine substituent with H (analog **6**) greatly reduced PDE4 inhibitory activity. Primary amine **7** and methyl amine **8** are both more potent inhibitors of PDE4 than B-Raf. An increase in B-Raf enzyme potency and a decrease in PDE4 enzyme potency were observed by extending the C-2 substituent from NHMe **8** to ethanolamine **9**. Further probing the amine linkers, we found variations of the ethanolamine **10–14** were well tolerated and offered improved rat hepatocyte stability compared to the cyclopropyl amine **5**. In general, oxygen-linked compounds were less stable in the rat hepatocyte assay than the corresponding amine-linked analogs (see analog **13** vs analog **11**). Carbon-linked analogs were generally less potent than both the corresponding amine and oxygen-linked analogs (see analog **14** vs analogs **13** and **11**). Table 2 details some of our initial efforts to optimize the C-4 aryl substituent. For SAR purposes, we chose to hold the cyclopropyl amine at C-2 constant while varying C-4 substituent. The 3,4-difluoro analog **15** is almost 10 times less potent than the 3,4-dichloro analog **5**. The 3-phenol analog **16** gave a significant boost in B-Raf potency. Close analogs of the 3-phenol such as 4-phenol **17**, 3-methoxy **18**, 3-pyridyl **19**, and 3-amino **20**, resulted in a dramatic drop in B-Raf potency. Combining the 3-chloro with the 3-hydroxy substituent resulted in analog **21** which afforded a ~10-fold boost in B-Raf and pMEK biomarker potency. This analog represents the most potent analog to date but suffers from poor hepatocyte stability correlating to rapid in vivo clearance.[8,9]

Our general strategy was guided by SBDD of compound **5** docked into the published crystal structure of B-Raf (Fig. 1).[10] The C-6 carboxamide of **5** acts as hydrogen bond donor and acceptor to CYS 531 in the hinge region of the kinase while the C-2 substituent occupies a solvent exposed region. The C-4 substituent of the pyrimidine occupies an interior pocket formed by residues Val470, Ala480, the backbone of Val481, Lys482, Leu513, Ile526, and the 'gatekeeper' residue Thr528. This pocket is fairly large owing to the small gatekeeper residue, Thr528, and it easily accommodates a 3,4-dichlorophenyl substituent at the C-4 position as in compound **5**. When a 3-phenol is at C-4, the OH group is modeled on the other side of the phenyl ring where it can form a key

Table 2
C-4 SAR of the thienopyrimidine scaffold



#	R	B-Raf IC_{50} (μM)	PDE4 IC_{50} (μM)	pMEK IC_{50} (μM)	Hepat. stab.
5		0.316 ± 0.059	0.261 ± 0.045	4.74 ± 1.01	8.8 ± 2.9
15		1.21[a]	ND	ND	ND
16		0.014 ± 0.001	0.982[a]	0.212[a]	7.5 ± 0.4
17		0.954[a]	0.850[a]	13.9[a]	ND
18		0.724 ± 0.367	0.342[a]	11.7[a]	ND
19		1.12[a]	2.30[a]	ND	ND
20		1.54[a]	4.34[a]	ND	ND
21		0.0020 ± 0.0002	0.469[a]	0.025 ± 0.005	7.8 ± 3.5

[a] Compound was tested once in this assay.

hydrogen bond to the backbone carbonyl of Asp593. The 3-Cl, 5-OH analog **21** combines both the lipophilic interactions and the phenol hydrogen bond; we postulate that this is the reason for its increased potency.

Compound **21** possesses significant pMEK biomarker potency making it useful as a proof-of-concept molecule. An in vivo biomarker study was performed in SCID mice bearing A375 melanoma tumors measuring the phosphorylation of both MEK and ERK.[11] After ip dosing (50 mg/kg) compound **21** demonstrated significant knockdown of both pMEK and pERK, 60% and 80%, respectively, at 30-min post dose.

From metabolite identification data, the C-4 phenol of compounds **16** and **21** was identified as the major site of metabolism (glucuronidation). Some of our efforts to stabilize or replace the phenol are presented in Table 3. Substituting ortho to the phenol was attempted to either sterically or electronically block glucuronidation. These analogs (**22–24**) maintain good B-Raf enzyme potency, but stability was not improved. Efforts to replace the phenol, such as benzyl alcohol **25**, cyanamide **26**, and benzimidazole analog **27**, were unsuccessful.

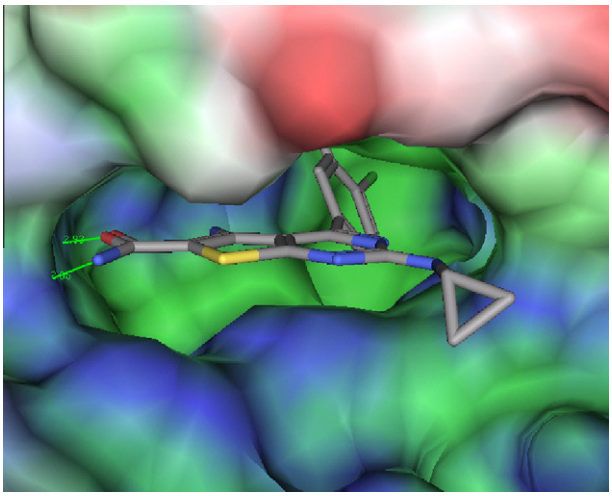
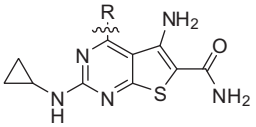
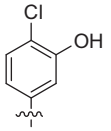
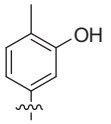
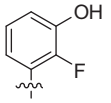
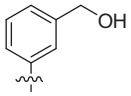
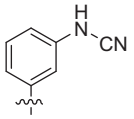
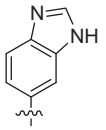
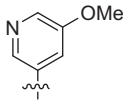


Figure 1. Compound **5** docked into the published crystal structure of B-Raf.

Table 3
C-4 phenol modifications



#	R	B-Raf IC_{50} (μM)	PDE4 IC_{50} (μM)	pMEK IC_{50} (μM)	Hepat. stab.
22		0.005 ± 0.001	1.30[a]	0.271[a]	8.4 ± 0.2
23		0.004[a]	1.31[a]	0.202 ± 0.011	11 ± 1
24		0.004 ± 0.001	3.77[a]	0.111 ± 0.077	17 ± 1
25		1.33[a]	ND	ND	ND
26		1.60[a]	ND	ND	ND
27		15.4[a]	ND	ND	ND
28		0.017 ± 0.006	1.06[a]	0.685 ± 0.167	22 ± 4

[a] Compound was tested once in this assay.

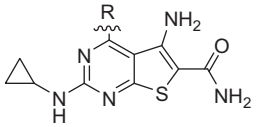
Interestingly, the 5-methoxypyridin-3-yl analog **28** proved to be a potent and moderately stable B-Raf inhibitor.

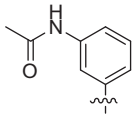
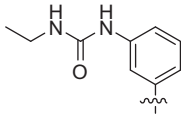
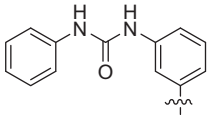
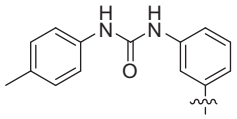
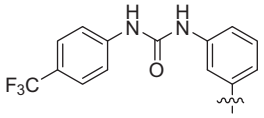
Further efforts to replace the phenol led us to explore a series of ureas at C-4 (Table 4). Replacing the phenol with an acetamide **29** and ethyl urea **30** resulted in a loss in potency. The addition of an aryl ring to the urea moiety **31** afforded a gain in enzyme potency, but the gain did not translate into acceptable cellular potency. Substituting the aryl urea with either a *para*-methyl or *para*-trifluoromethyl group, **32** and **33**, further improved both the enzyme and cellular potency of this series. The aryl urea analogs were much more potent in the B-Raf time-dependent assay, possibly indicating a slower on-rate binding event. This observation is consistent with movement of the activation loop of the B-Raf enzyme to the DFG-out conformation as described for 'second-generation type II' B-Raf inhibitors.[3]

Through our exploration of the C-4 position of the thienopyrimidine template we identified several different very potent substituents: phenols, methoxypyridine, and aryl ureas. Our exploration of the C-2 position identified ethanolamine derivatives with good potency and moderate stability. The ethanolamine substituents also generally improved the solubility of the template. The SAR of combinations of some of our best C-2 and C-4 substituents are presented in Table 5. Compounds bearing the aryl phenol at C-4, **34** and **35**, continued to suffer from rapid clearance.[9] The urea-containing C-4 analog **37** had slower clearance and good potency in both the enzyme and cellular assay. Unfortunately this analog exhibited poor exposure upon oral administration in rats.[12] Our most promising compound to date was obtained with the C-4 methoxypyridine analog **39** which has excellent hepatocyte stability and is cleared much more slowly than the C-4 phenols. In addition, further analysis of this compound showed it to be moderately orally bioavailable in rats (17%).[12]

In summary, a series of thieno[2,3-*d*]pyrimidines were identified as B-Raf inhibitors. Our research showed that small amine substituents at C-2 are essential for potent PDE4 inhibitory activity. Either removal of the C-2 substituent or use of larger substituents favored B-Raf activity. C-4 *meta*-phenol derivatives proved to be very potent B-Raf inhibitors and were selective against PDE4. Phenol derivative **21** proved to be a useful proof-of-concept compound showing significant knockdown of the biomarkers pMEK and pERK in vivo. Further research led to C-4 methoxypyridine and C-4 aryl urea analogs which proved to be potent and metabolically stable B-Raf inhibitors. Ultimately a combination of potent and stable C-2 and C-4 substituents led to analog **39** which proved to be a moderately bioavailable B-Raf inhibitor. The thieno[2,3-*d*]pyrimidine template described above is a novel addition to the list of

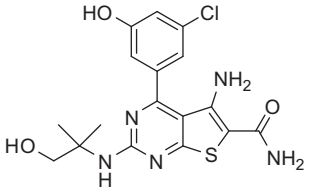
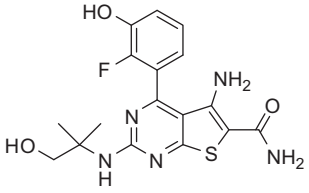
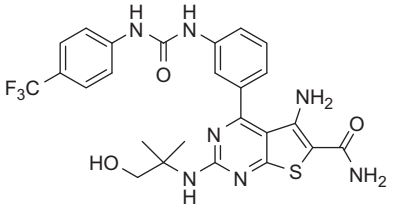
Table 4
C-4 urea SAR



#	R	B-Raf IC_{50} (μM)	B-Raf T-dep IC_{50}[a] (μM)	pMEK IC_{50} (μM)
29		2.45[b]	3.13[b]	ND
30		4.73[b]	>30[b]	ND
31		0.032 ± 0.019	0.038[b]	10.1[b]
32		0.028 ± 0.001	0.004[b]	2.03 ± 0.080
33		0.343 ± 0.032	0.003[b]	0.546 ± 0.184

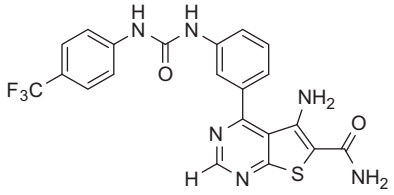
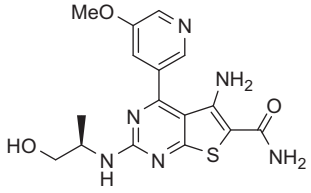
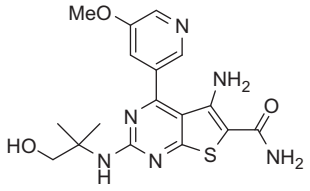
[a] B-Raf time-dependent assay (10× compound and 10× enzyme were allowed to pre-incubate for 1 h before initialization of the kinase reaction. Preincubation time for the normal assay is 15 min).
[b] Compound was tested once in this assay.

Table 5
C-2 and C-4 substituents pairing

#		B-Raf IC_{50} (μM)	pMEK IC_{50} (μM)	Hepat. stab.	Rat Cl[a]	Rat *F* (%)
34		0.003[b]	0.103[b]	23 ± 2	76 ± 14	ND
35		0.005 ± 0.001	0.332 ± 0.127	66 ± 1	119 ± 9	ND
36		0.014[c]	1.54 ± 0.44	111 ± 17	34 ± 4	ND

(*continued on next page*)

Table 5 (*continued*)

#		B-Raf IC_{50} (μM)	pMEK IC_{50} (μM)	Hepat. stab.	Rat Cl[a]	Rat *F* (%)
37		0.001[c]	0.327[b]	101 ± 11	12 ± 1	0.3 ± 0.1
38		0.040[b]	2.51[b]	ND	ND	ND
39		0.058 ± 0.006	1.01 ± 0.08	90 ± 5	32 ± 7	17 ± 5

[a] Rat CL_{iv} (mL/min/kg).[9]
[b] Compound was tested once in this assay.
[c] Compound was tested once using the B-Raf time-dependent assay.

known B-Raf inhibitors and may aid in the design of future useful templates.

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References and notes

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3. Li, H.; Chen, Y.; Rao, S.; Chen, X.; Liu, H.; Qin, J.; Tang, W.; Wang, Y.; Zhou, X.; Lu, T. *Curr. Med. Chem.* **2010**, *17*, 1618.
4. Mercer, K. E.; Pritchard, C. A. *Biochim. Biophys. Acta* **2003**, *1653*, 25.
5. For experimental details of compound synthesis and in vitro assays please see: McKenna, J.; Papa, P. W.; Sakata, S. T.; Erdman, P. E.; Packard, G. K. WO 2007/084560.
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7. Test compounds and control (7-ethoxycoumarin) were incubated in triplicate in cryopreserved male sprague–dawley rat hepatocytes (In Vitro Technologies). Thawed cryopreserved hepatocytes were re-suspended in Krebs-Henseleit Buffer (KHB), pH 7.4 at a density of 1×10^6 cells/mL (final cell density = 233,000 cells/well) and a final compound concentration of 3 μM. Percentage of parent disappearance was determined after 60 min of incubation at 37 °C by removing two aliquots (one at time zero and one at 60 min) and quenching the reaction with three volumes of 100% cold acetonitrile. Samples were mixed on a multi-tube vortexer for one minute and a volume of the mixture was filtered through a Captiva (Varian) 0.45 μm pore size polypropylene filter plate. Sample extracts were analyzed by LC–MS/MS to determine parent compound levels, reported as % remaining (mean ± SD).
8. Rat CL_{iv} = 116 mL/min/kg.
9. The total body clearance (CL) was assessed in male CD-IGS rats following a bolus intravenous (iv) injection via jugular vein catheter at 2 mg/kg in a vehicle containing 15% DMA/85% PEG 400. The clearance was calculated based on the following equation: $CL = dose/AUC_{(0-infinity)}$, where dose referred to the intravenous dose of the drug and $AUC_{(0-infinity)}$ was the area under the plasma drug concentration curve from time zero to time infinity following the iv dosing.
10. Initial molecular modeling coordinates of B-Raf came from the RCSB published crystal structure complex 1UWH containing wild-type B-Raf and sorafenib: Wan, P. T.; Garnett, M. J.; Roe, S. M.; Lee, S.; Niculescu-Duvaz, D.; Good, V. M.; Jones, C. M. Marshall, C. J.; Springer, C. J.; Barford, D.; Marais, R. *Cell* **2004**, *116*, 855. Protein chain A was used for modeling. All molecular modeling was performed using MOE software: Molecular Operating Environment (MOE), version 2001.01; Chemical Computing Group Inc., 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada H3A 2R7, 2005. Compounds were docked into the protein coordinates from 1UWH so that the carboxamide functionality formed hydrogen bonds to the hinge residue CYS 531. The inhibitor and all B-Raf residues within 6 Å of the inhibitor were allowed to minimize using the MMFF94× force field and a distance-dependent dielectric constant, while the rest of the protein was held fixed.
11. For a representative procedure see: Pratilas, C. A.; Taylor, B. S.; Ye, Q.; Viale, A.; Sander, C.; Solit, D. B.; Rosen, N. *Proc. Natl. Acad. Sci.* **2009**, *11*, 4519.
12. *Oral protocol*: Test compounds, prepared as a suspension in 0.5% carboxymethyl cellulose + 0.25% Tween80 in water, were dosed via oral gavage at 10 mg/kg (dose volume of 5 mL/kg) to non-fed male CD-IGS rats (n = 3 or 4). Blood samples were collected at 0.5, 1, 2, 3, 6, 9, and 26 h post dose via a jugular vein cannula and subsequently processed into plasma for analysis by LC–MS/MS. *IV protocol*: Test compounds, prepared as a solution in 15% DMA and 85% PEG 400, were dosed via jugular vein cannula as an iv bolus at 2 mg/kg (dose–volume of 2 mL/kg) to non-fasted male CD-IGS rats (n = 3). Blood samples were collected at 0.0833, 0.333, 0.75, 1.5, 3, 5, 7, 9, and 26 h post dose via a jugular vein cannula and subsequently processed into plasma for analysis by LC–MS/MS. Plasma concentration–time profiles for both oral and intravenous studies were constructed and the area under the curve (AUC) was calculated based on the 'linear trapezoidal–linear interpolation' method (using WINNONLIN software). The absolute bioavailability of a drug is defined by the AUC of a dose delivered by one method (e.g., oral) divided by the AUC of an iv bolus, corrected by different dose levels.