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Pyrazolopyridine inhibitors of B-Raf^{V600E}. Part 2: Structure–activity relationships

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ABSTRACT

Structure–activity relationships around a novel series of B-Raf^{V600E} inhibitors are reported. The enzymatic and cellular potencies of inhibitors derived from two related hinge-binding groups were compared and 3-methoxypyrazolopyridine proved to be superior. The 3-alkoxy group of lead B-Raf^{V600E} inhibitor **1** was extended and minimally affected potency. The propyl sulfonamide tail of compound **1**, which occupies the small lipophilic pocket formed by an outward shift of the αC-helix, was expanded to a series of aryl-sulfonamides. X-ray crystallography revealed that this lipophilic pocket unexpectedly enlarges to accommodate the bulkier aryl group.

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The Ras/Raf/MEK/ERK (MAPK) signaling pathway transduces signals from cell surface receptors to the nucleus leading to cellular proliferation, differentiation and survival.¹ Mutations in the *BRAF* gene may lead to constitutive activation of B-Raf kinase which results in amplification of the MAPK pathway. Mutated B-Raf is present in approximately 7% of all cancers and is most frequently associated with melanoma.² Over 90% of the detected mutations in B-Raf are a glutamic acid for valine substitution at residue 600 (V600E).² This mutation leads to constitutive kinase activity 500-fold greater than B-Raf^{WT} and correlates with increased malignancy and decreased response to chemotherapy.³ Thus, the development of small-molecule inhibitors of B-Raf^{VGODE} is a promising strategy for cancer therapy, particularly in melanoma.⁴

Using structure-based design our laboratory discovered a series of ATP-competitive B-Raf^{V600E} inhibitors which utilized 3-methoxy pyrazolopyridine as a novel hinge-binding group.⁵ Initial structure-activity relationships demonstrated that bicyclic hinge-binders such as pyrrolo- and pyrazolopyridine were more potent than either pyridine or aminopyridine, and that a 3-methoxy substituted pyrazolopyridine was more potent than 3-alkyl, 3-halo and 3-amino. Optimization led to compounds **1** and **2**, potent and selective inhibitors of B-Raf^{V600E} which were highly active against a broad panel of melanoma and colon cancer cell lines driven by this

* Corresponding author. *E-mail address:* wenglo1056@yahoo.com (S. Wenglowsky). activating mutation (Fig. 1). Compounds **1** and **2** possessed favorable ADME and pharmacokinetic profiles and displayed significant antitumor activity in the Colo205 mouse xenograft model.⁵ On the basis of their in vivo efficacy and preliminary safety profiles, **1** and **2** were selected for further preclinical evaluation.⁶

During the course of this work inhibitor **3** was also prepared. Although compound **3** utilizes a pyrrolopyridine hinge-binder common to inhibitors of several kinases^{4,7} it was notably less active than compounds **1** and **2** against B-Raf^{V600E} in the enzymatic and cellular assays.⁵ To further elucidate the nature of their binding to B-Raf^{V600E}, and as a broader comparison between these two hinge-binders, thorough structure-activity relationships around compounds **1** and **3** have been accomplished. Changes included several central phenyl ring substitutions and replacement of the propylsulfonamide tail with alkyl sulfonamides and sulfamides. Additionally, 3-substituents of both hinge-binding cores



Figure 1. B-Raf^{V600E} inhibitors **1–3**.

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Table 1

B-RafV^{600E} activity of central phenyl ring analogs **1–21**



R	Compd	B-Raf IC ₅₀ ^a (nM)	pERK IC ₅₀ ^a (nM)	Compd	B-Raf IC_{50}^{a} (nM)	pERK IC ₅₀ ^a (nM)
2,6-diF	1	4.8	19	3	38	94
2-F.6-CI	2	1.7	20	13	17	190
2,6-diCI	4	1.4	40	14	8.3	206
2-F	5	13	38	15	87	290
6-CI	6	61	250	16	88	2100
6-F	7	150	350	17	210	_
2,5-diF	8	7.5	80	18	76	400
2,5,6-triF	9	2.3	100	19	19	410
2-0Me,6-F	10	110	180	-	_	_
2-F,6-Me	-	_	_	20	170	1400
2-Me,6-F	-	_	_	21	680	_
2-F,6-OMe	11	2600	_	-	_	-
2-CF ₃ ,6-F	12	530	-	-	-	-

^a Values are means of at least two experiments.

Table 2

B-Raf^{VG00E} activity of sulfonamides and sulfamides **22–43**



R	Compd	B-Raf IC ₅₀ ^a (nM)	pERK IC ₅₀ ^a (nM)	Compd	B-Raf IC ₅₀ ^a (nM)	pERK IC ₅₀ ^a (nM)
n-Pr	1	4.8	19	3	38	94
Et	22	25	62	-	_	-
<i>n</i> -Bu	-	_	_	34	40	120
CH ₂ CH ₂ CH ₂ F	23	1.3	13	35	19	75
<i>n</i> -Bu	24	4.5	11	36	140	200
CH ₂ -cyclopropyl	25	17	31	37	29	230
CH ₂ CH ₂ CF ₃	-	_	_	38	33	270
CH ₂ CF ₃	-	_	_	39	38	2600
Cyclopropyl	26	63	170	40	99	710
Bn	27	190	830	_	_	-
NHCH ₂ CH ₃	28	18	180	_	_	-
NHCH(CH ₃) ₂	29	110	630	_	_	-
NHCH ₂ CHF ₂	30	13	500	_	_	-
$N(CH_3)_2$	31	18	63	41	200	260
N(CH ₃)CH ₂ CH ₃	32	29	28	42	400	340
N-Pyrrolidyl	33	8.6	18	43	120	170

^a Values are means of at least two experiments.

were compared, and aryl sulfonamides in the pyrazolopyridine series were examined. Details of this evaluation are provided herein.

The dihalo aryl ring of compounds **1–3** resides in the hydrophobic pocket adjacent to the gatekeeper residue Thr529. Molecular modeling indicated that there was scope for varying the substitutions at the 2- and 6-positions of this aryl ring, as well as space for a substituent at the 5-position. Halogen, alkyl and alkoxy groups were examined at these positions, and these changes were compared between the pyrrolo- and 3-methoxypyrazolopyridine hinge-binding groups. Table 1 provides the enzymatic and cellular activities of these inhibitors.⁸

While both chloro and fluoro were well-tolerated in the 2- and 6-positions of the central phenyl ring, 2,6-difluoro was the optimal combination for cellular activity across both series (**1–4**, **13**, **14**). The 2-fluoro substitution was only 2–3-fold less potent than 2,6-

difluoro (5 vs 1 and 15 vs 3), while a mono-halo substitution at the 6-position resulted in a significant loss of potency (6, 7, 16 and 17), which indicates that a 2-substituent is critical for binding. Addition of a fluorine at the 5-position of the central phenyl ring (8, 9, 18 and 19) resulted in a 2–5-fold loss in cellular activity. Other substituents were not well tolerated, including Me-, MeO- and F₃C- (10–12, 20 and 21). Overall, the trends observed within each series correlated closely to each other. A comparison between the 3-methoxy pyrazolopyridine and pyrrolopyridine hinge-binders revealed that the former is consistently more active in both the enzymatic and cellular assays regardless of the substitution on the central ring, which corroborates our earlier studies.⁵

The X-ray crystal structure of **1** in complex with B-Raf reveals that the kinase adopts the DFG-in conformation, but that the propyl sulfonamide tail occupies the small lipophilic pocket formed by

an outward shift of the α C-helix.⁵ Few kinases are known to achieve this peculiar binding mode,^{9,10} which likely contributes to the outstanding selectivity of these inhibitors for the Raf kinases.⁵ To further elucidate the nature of this particular binding mode this group was modified on both hinge-binding cores. Table 2 summarizes these results.

Decreasing (22) or increasing (34) the length of the alkyl group resulted in a loss of potency versus 1 or 3. In contrast, 3-fluoropropyl led to a slight increase in activity over propyl (23 vs 1 and 35 vs 3). The bulkier *iso*-butyl group (24 and 36) slightly improved potency with the 3-methoxy pyrazolopyridine hinge-binder, but not with pyrrolopyridine. Other alkyl and fluorinated alkyl groups resulted in a loss of potency with both cores (25, 26, 37-40), while a benzyl group resulted in a significant loss of potency (27). Sulfamides were also prepared (28-33 and 41-43) and, for good cellular activity, disubstituted amine inputs were determined to be essential. In particular, N-pyrrolidylsulfamide **33** was superior within this sub-series and equipotent to the lead compound 1. Again, the trends observed within the two series correlated closely to each other, and a comparison between the two hinge-binding groups revealed that 3-methoxy pyrazolopyridine still consistently produced more active inhibitors in the enzymatic and cellular assavs.

The superior potency observed for the 3-methoxy pyrazolopyridine hinge-binder may be explained by the X-ray crystal structure of **1** in which the methoxy group makes Van der Waals contacts with several residues of the kinase binding domain, including Ile463, Trp531, Ser535, Ser536, and Phe583.⁵ Based on this observation, attempts were made to duplicate these interactions with a 3-substituent on the pyrrolopyridine. The enzyme and cellular activities of the resulting compounds were compared to the corresponding analogs in the pyrazolopyridine series and these data are provided in Table 3.¹¹

Small, lipophilic substituents at the 3-position improved the potency of the pyrrolopyridine series (**50–52** vs **3**) as expected, but groups larger than methyl lost cellular activity (**53** and **54**). The SAR for the pyrazolopyridine hinge-binder was similar (**44–49**). Between the two hinge-binding groups, the potency of analogs bearing the same 3-substituent were within two-fold of each other, with the exception of 3-ethyl. Attempts to prepare 3-methoxy substituted pyrrolopyridine, the direct analog of **1**, failed and were attributed to insufficient chemical stability. Because the desired level of potency with the pyrrolopyridine hinge-binder could not be achieved, this group was not pursued further.

Due to the superiority of the 3-methoxy substitution on the pyrazolopyridine towards providing cell-potent inhibitors of

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Table 3

B-Raf^{V600E} activity of 3-substituted pyrazolo- and pyrrolopyridines 44–54

Table 4 B-Raf^{VG00E} activity of 3-alkoxy pyrazolopyridines 55-63



	0		
R	Compd	B-Raf IC ₅₀ ^a (nM)	pERK IC_{50}^{a} (nM)
Me	1	4.8	19
Et	55	2.4	12
$CH_2(CH_3)_2$	56	3.6	20
CHCH ₂ F	57	2.5	26
CH ₂ CF ₃	58	5.3	28
₩	59	3.5	76
-	60	4.2	43
	61	6.9	62
CHCH ₂ OCH ₃	62	6.3	70
CHCH ₂ CH ₂ OH	63	5.6	74

^a Values are means of at least two experiments.

B-Raf^{VGODE}, additional 3-alkoxy groups were installed on this core (Table 4). Analogs were prepared that incorporated both lipophilic (**55–59**) and polar groups (**60–63**). While 3-ethoxy (**55**) slightly improved the enzymatic and cellular activity compared to compound **1**, all other analogs in this series were similarly potent and within a narrow window. The flat SAR within the 3-alkoxy series correlated with the observation from X-ray crystallography that, while the methoxy group itself makes several contacts with the kinase binding domain, it is directed away from the pocket toward a solvent-exposed region.⁵

The SAR of the sulfonamide tail of **1** was further explored with a series of aryl and heteroaryl sulfonamides (Table 5). The X-ray crystal structure of **1** suggested that the lipophilic pocket occupied by the propyl group would not likely accommodate an aromatic ring; surprisingly, phenyl sulfonamide **64** was equipotent to **1**. Additional analogs indicated that *ortho* and *meta* fluorines were well tolerated (**65–67**), while *para* fluoro resulted in a significant loss of potency (**68**). Pyridine (**69–70**) and imidazole (**71**) sulfonamides lost cellular activity, possibly due to their polarity, while lipophilic heterocycles retained good cellular activity (**72–73**).

Н		F	
	O F H		O F H
N=	1, 44-49	B	3, 50-54

R	Compd	B-Raf IC_{50}^{a} (nM)	pERK IC ₅₀ ^a (nM)	Compd	B-Raf IC_{50}^{a} (nM)	pERK IC ₅₀ ^a (nM)
OMe	1	4.8	19	_	_	_
Н	44	17	62	3	38	94
Br	45	4.0	44	50	3.4	39
I	46	1.8	36	51	2.2	60
Me	47	12	86	52	16	62
Et	48	9.2	84	53	19	290
CF ₃	49	5.8	280	54	6.2	230

^a Values are means of at least two experiments.

Table 5

B-Raf^{V600E} activity of aryl- and heteroaryl sulfonamides **64-73**



R	Compd	B-Raf IC ₅₀ ^a (nM)	pERK IC ₅₀ ^a (nM)
X	64	1.6	17
F	65	1.8	9.1
F	66	0.7	12
F F	67	1.0	20
× F	68	46	340
X N	69	16	280
X	70	0.5	83
	71	320	>10,000
X_s	72	0.2	30
× o	73	0.7	66

^a Values are means of at least two experiments.

A comparison of the X-ray crystal structures of compounds **1** and **64** led to a rationale for the unexpected potency of the aryl sulfonamide series (Fig. 2).¹² While the α C-helix is similarly positioned in both structures, the orientation of Phe595 from the DFG sequence is shifted. This movement enlarges the pocket by \sim 1 Å, enough to accommodate a phenyl group as well as the substitution of *ortho* and *meta* fluorines.

Given the importance of the propyl sulfonamide for the selective inhibition of B-Raf^{V600E}, the kinase selectivity profile of two diverse, cell potent sulfonamide analogs was obtained and compared to that of compound **1** (Table 6). Each compound was screened against a panel of 65 kinases at 1 μ M and those with >40% inhibition are presented in Table 6. The selectivity profile for sulfamide **33** and aryl sulfonamide **66** was similar to lead compound **1** although they demonstrated increased activity against AuroraA, FLT3, LCK and SRC. Nonetheless, the overall effects of these propyl sulfonamide alternatives were minimal, and these compounds are still very selective inhibitors of B-Raf^{V600E}. These data, coupled with the crystal structure of compound **64**, suggest that selectivity for this kinase via a shift of the α C-helix can be achieved for a broad range of sulfonamide substitutions.

Pyrrolo- and 3-alkoxy pyrazolopyridines were generally prepared via an amide bond coupling between the hinge-binding core anilines and the fully elaborated benzoic acids, and these synthetic methods have been described elsewhere.^{5,13} The aryl sulfonamide subseries (compounds **64–73**) was an exception to this general synthetic route and utilized the incorporation of the sulfonamide in the final step (Scheme 1). Pyrazolopyridine-5-amine **74**⁵ was



Figure 2. Overlay of the X-ray crystal structures of compounds **1** (blue) and **64** (orange). Surfaces are shaded correspondingly to the inhibitor. Both the propyl and phenyl groups occupy a pocket that is enlarged by a shift of the α C-helix, while the DFG sequence resides in its active conformation (DFG-in) (3.2 Å resolution).

Table 6Kinase selectivity of compounds 1, 33 and 66^{a,b}

Compd	Aur A	FGR	FLT3	LCK	PTK6	SRC	SRMS
1	1	88	52	56	83	22	94
33	47	101	85	90	105	52	105
66	52	98	77	96	101	77	98

^a Percent inhibition at 1 μM concentration of test compound.
 ^b Values are means of at least two experiments.



Scheme 1. Reagents and conditions: (a) EDCI, HOBt, DMF, 25 °C, 69%; (b) SnCl₂·2H₂O, EtOAc, 77 °C, 84%; (c) CISO₂Ar, 4:1 DCM/pyridine, 15–58%.

coupled to commercially available 2,6-difluoro-3-nitrobenzoic acid **75**, and the product was reduced to aniline **76** with tin chloride. Aryl sulfonamides were then prepared in the final step with the appropriate sulfonyl chloride in a 4:1 mixture of DCM and pyridine. This route allowed for rapid and efficient preparation and screening of this class of B-Raf^{VG00E} inhibitors.

In summary, thorough structure–activity relationships were developed around the 3-methoxy pyrazolopyridine and pyrrolopyridine series of B-Raf^{V600E} inhibitors. Although the pyrrolopyri-

dine hinge-binder is a commonly utilized motif in kinase inhibitor development, 3-methoxy pyrazolopyridine consistently produced more active inhibitors in the enzymatic and cellular assays. Furthermore, attempts to discover equipotent pyrrolopyridines by incorporating substituents at the 3-position were unsuccessful. Based on these results, 3-alkoxypyrazolopyridines were further explored and as a class were determined to be broadly active.

Lastly, it was discovered that the small pocket formed by an outward shift of the α C-helix can expand to accommodate sulfonamides as large as a substituted phenyl, and that this group still maintained excellent selectivity toward B-Raf^{V600E}. This observation provides additional scope to pursue alternative groups that may achieve this particular B-Raf binding mode, but possess distinct properties from a sulfonamide. These efforts will be reported in due course.

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