Discovery of a Highly Specific and Potent Pan-RAF Inhibitor

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We describe the structure-based design and synthesis of *N*-(2,6-difluorophenyl)-3-(9*H*-purin-6-yl)pyridine-2-amine derivatives as a selective pan-RAF kinase inhibitor. The synthesized compounds showed highly potent and specific inhibition of the $BRAF^{V600E}$ mutant cell line. Among them, *N*-(3-((3-(9*H*-purin-6-yl) pyridine-2-yl)amino)-2,4-difluorophenyl)furan-3-sulfonamide (**4b**) exhibited the most potent inhibitory activities against protein kinase enzymes $BRAF^{V600E}$, $BRAF^{WT}$, and CRAF (IC_{50} of 2, 2, and 1 nM, respectively) and a mutant cell line bearing a $BRAF^{V600E}$ mutation, A375P (GI_{50} of 7 nM).

Keywords: RAF, Pan-RAF inhibitor, Structure-based drug design, Melanoma, Anticancer

Introduction

RAFs are serine/threonine protein kinases. They comprise three evolutionarily conserved isoforms: ARAF, BRAF, and CRAF. RAF kinases are core members of the RAS-RAF-MEK-ERK (MAPK) signal transduction cascade. The upstream RAS protein is activated by extracellular stimuli, and then it recruits RAF, which is phosphorylated by adaptor proteins of RAS. The phosphorylated RAF activates MEK which signals forwards to its downstream transcription effectors that have cellular proliferation and survival activities.[1] The dimerization of RAF is a main RAS-regulated event in RAF activation.[2] BRAF could stimulate the catalytic activity of CRAF independently of the intrinsic kinase activity of BRAF.[3]

The aberrantly activated MAPK pathway results in carcinogenesis. Pathway mutations can be found in approximately 30% of all cancers. The *BRAF* mutation causes 8% of the MAPK pathway mutations. *BRAF* mutants are found in various cancers, such as melanoma (40–70%), papillary thyroid (36–53%), ovarian serous cancer (~30%), and colorectal cancer (5–22%). The most frequently detected mutations (>90%) in BRAF are the V600E type which leads to constitutive kinase activity greater than the BRAF wild type.[4]

The first clinically approved $BRAF^{V600E}$ kinase inhibitor, vemurafenib (PLX4032) was developed as a melanoma target drug. The inhibition of BRAF activity using this selective BRAF target drug can result in the diminished proliferation and survival of $BRAF^{V600E}$ melanoma.[5] After the development of the drug for the treatment of melanoma, there have been numerous efforts to develop therapeutic agents to $BRAF^{V600E}$ kinases as melanoma target drugs. Another $BRAF^{V600E}$ kinase drug (dabrafenib) has been approved, and some inhibitors have entered clinical trials.[6] However, melanoma rapidly develops resistance to selective BRAF inhibitors after a median duration of response of 6–7 months. The main reason for resistance is that drug-bound BRAF activates CRAF and causes the activation of the MAPK pathway.[3] Therefore, the development of a more effective pan-RAF inhibitor is needed to reduce resistance.

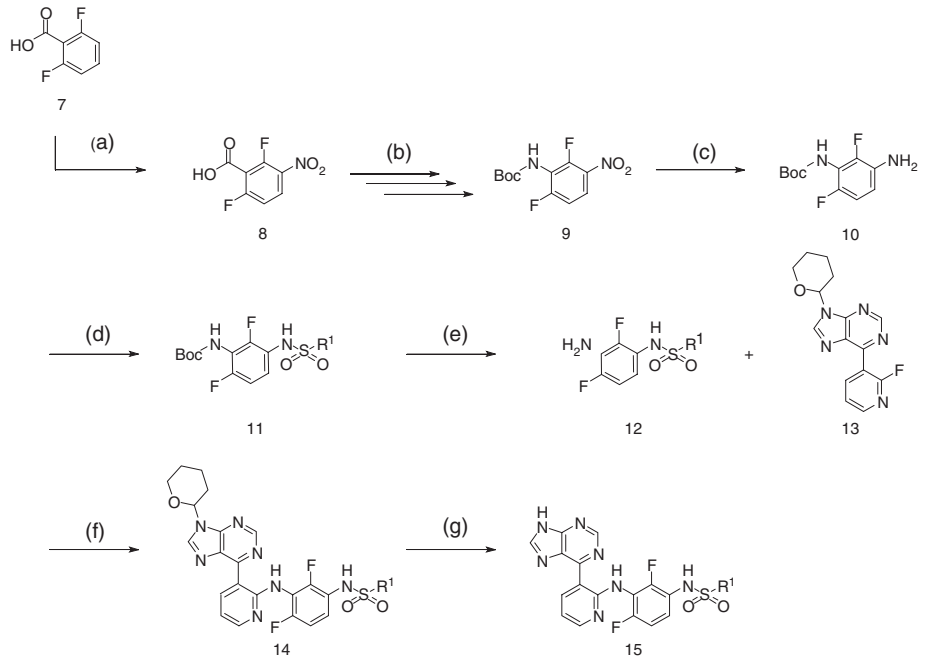
Herein, we report the design and synthesis of an *N*-(2,6-difluorophenyl)-3-(9*H*-purin-6-yl)pyridine-2-amine derivative as a selective pan-RAF kinase inhibitor.

Experimental

All derivatives were synthesized as shown in Scheme 1. Commercially available 2,6-difluorobenzoic acid **7** was converted to a 3-nitro substituent **8**. A boc-protected amine **9** was synthesized in three steps from the carboxylic acid **8** through Curtius rearrangement. The sulfonyl amide derivatives **11** were synthesized by coupling with aliphatic or arylsulfonyl chloride after the nitro reduction of compound **9**. Following the Boc-deprotection, compound **12** was coupled with a tetrahydropyran (THP)-protected purine compound **13**, which was made following the method described by Amgen group.[7] The final compounds were obtained after THP-deprotection (Scheme 1).[8]

Results and Discussion

Several BRAF inhibitors have an *N*-(2,4-difluorophenyl) propane-1-sulfonamide **1** functional group including



Scheme 1. Reagents and conditions: (a) KNO_3, H_2SO_4, r.t., 0.5 h; (b) (1) oxalyl chloride, DMF, 20°C; (2) NaN_3, DCM, 20°C, 0.5 h; (3) *t*-BuOH, DCM, reflux, 3 h; (c) H_2, Pd/C, MeOH, r.t., overnight; (d) sulfonyl chloride derivatives, pyridine, DCM, r.t., 2 h; (e) 4N HCl in 1,4-dioxane, EA, r.t., 2 h; (f) LiHMDS, THF, 0°C, 1 h; (g) HCl, water, reflux, 1 h.

PLX4032.[5,9–13] We superimposed all of the released BRAF X-ray crystal structures with the PLX4032 complex structure (3OG7.pdb) for the structure-based design of $BRAF^{V600E}$ kinase inhibitors. The difluorophenyl ring and isopropyl groups occupy the hydrophobic pockets adjacent to the Thr529 and Leu505 residues, respectively. Those binding interactions create a BRAF αC-helix-out conformation in which the highly conserved salt bridge between Lys483 and Glu501 is broken. Wang *et al.* classified the characteristic inhibitors as type IIB kinase inhibitors. Additionally, the authors explained that type IIB inhibitors had minimal preclinical toxicity by lacking significant MAPK activation or inhibition in $BRAF^{WT}$ cells.[14] A research group from Amgen determined that the hinge binding group **2** was a BRAF kinase inhibitor. Their reported compounds had selective BRAF kinase inhibitory activity and good bioavailability. However, they did not introduce an *N*-(2,4-difluorophenyl)propane-1-sulfonamide functional group. Furthermore, the compounds showed type IIA characteristics that were confirmed by an X-ray crystal complex structure (3IDP.pdb).[7]

We designed a novel chimeric compound for a BRAF inhibitor that had a previously known hinge binder **2** and a compound **1** group based on structure analysis as mentioned earlier (Figure 1). The initial chimeric compound **3a** bound well in the ATP binding pocket of $BRAF^{V600E}$ with type IIB characteristics based on a docking study (Figure 2).[15] The synthesis and cell-based *in vitro* assay of the compound **3a** was carried out using a $BRAF^{V600E}$ mutant melanoma cell line (A375P). The compound **3a** had significant antiproliferative effects (GI_{50}: 130 nM) using an MTT assay. The activity of compound **3a** was comparable to that of the reference compound, PLX4032 (GI_{50}: 90 nM).

We tried to obtain more potent chimeric preclinical candidates that contained the aforementioned hinge binder and type IIB characteristics of a hydrophobic binding group. Recently, Array BioPharma attempted an approach that was similar to our study. They changed the hinge binding group that retained the *N*-(2,4-difluorophenyl) propane-1-sulfonamide moiety. The compound exhibited desirable physicochemical properties and potent efficacy in a $BRAF^{V600E}$ mutant mouse xenograft model.[13]

Based on this information, we expected that our chimeric compounds would have good selectivity and activity to the enzyme. Therefore, we conducted an MTT cell proliferation assay against BRAF mutant (A375P) and wild-type (SK-MEL-2) cells to optimize the inhibitors more rapidly and effectively.[16] We first attempted to optimize the activity by changing the *n*-propyl group to other aliphatic functional groups (Table 1). An isopropyl derivative **3b** was shown to have comparable activity with *n*-propyl. However, other

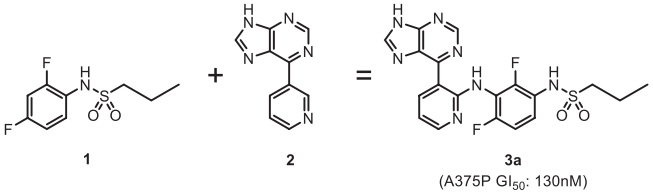
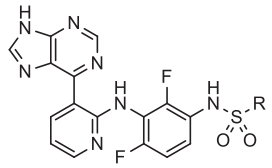


Figure 1. Design of a novel chimeric compound **3a**.

Table 1. Anti-proliferative activity of alkyl sulfonamide of *N*-(2,6-difluorophenyl)-3-(9*H*-purin-6-yl)pyridine-2-amine derivatives against A375P ($BRAF^{V600E}$) and SK-MEL-2 ($BRAF^{WT}$) cells.



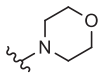
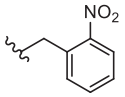
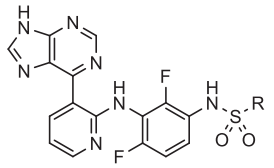
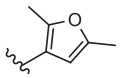
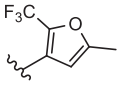
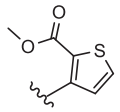
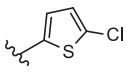
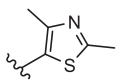
Compound	R	Cell viability GI_{50} (μM) A375P($BRAF^{V600E}$)	SK-MEL-2 ($BRAF^{WT}$)
3a		0.13	>10
3b		0.123	>10
3c		0.66	>10
3d		0.25	>10
3e		0.59	>10
3f		3.24	>10

Table 2. Anti-proliferative activity of five-membered heterocyclic sulfonamide derivatives of *N*-(2,6-difluorophenyl)-3-(9*H*-purin-6-yl) pyridine-2-amine against A375P ($BRAF^{V600E}$) and SK-MEL-2 ($BRAF^{WT}$) cells.



Compound	R	Cell viability GI_{50} (μM) A375P($BRAF^{V600E}$)	SK-MEL-2 ($BRAF^{WT}$)
4a		0.04	>10
4b		0.007	>10
4c		4.09	>10
4d		>15	>10
4e		0.03	>10
4f		0.02	>10
4g		2.25	>10
4h		0.22	>10
4i		3.82	>10
4j		7.84	>10
4k		1.37	>10

alkyl derivatives **3c-3f** showed slightly decreased A375P cell anti-proliferation activity.

Next, we evaluated the effect of the five-membered heterocyclic groups of the compounds on their inhibitory effects (Table 2).

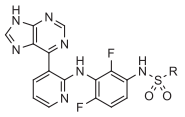
A couple of furan **4a** and **4b** and thiophene analogs **4e** and **4f** exhibited more potent cell inhibitory activity than an *n*-propyl derivative **3a**. In particular, *N*-(3-((3-(9*H*-purin-6-yl)pyridine-2-yl)amino)-2,4-difluorophenyl)furan-3-sulfonamide **4b** exhibited 1–9 nanomolar half-growth inhibitory activity against A375P cells. Substituted furan **4c** and **4d**, thiophene **4g** and **4h**, thiazole **4i**, imidazole **4j**, and pyrazole **4k** derivatives demonstrated 1000-fold less inhibitory activity than compound **4b**.

We also prepared several benzene derivatives (Table 3). Most of the phenyl substituted compounds and pyridinyl derivatives **5b** were equally potent to *n*-propyl compound **3a**. Finally, we prepared several bicyclic sulfonamide derivatives (Table 4). All of the bicyclic sulfonamide analogs showed decreased inhibitory activity compared to *n*-propyl compound **3a**.

The anti-proliferative effect against SK-MEL-2 ($BRAF^{WT}$) was examined to confirm the cell line selectivity. All of the test compounds displayed poor activity against the *BRAF* wild-type cell line. This result was anticipated from the type IIB characteristics of the synthesized compounds. The compound did not completely abolish MEK-ERK phosphorylation signaling in SK-MEL-2 cells, unlike in the A375P cells which was also observed in other BRAF inhibitors.[17]

Figure 3 represents the docking model of the most active compound **4b**. As previously demonstrated, the purine ring interacted with the amide backbone of C532 which was a hinge amino acid.[7] The purine and pyridine rings had π–π stacking interactions with W531 and F583 side chains. An

Table 3. Anti-proliferative activity of substituted benzene sulfonamide derivatives of *N*-(2,6-difluorophenyl)-3-(9*H*-purin-6-yl) pyridine-2-amine against A375P ($BRAF^{V600E}$) and SK-MEL-2 ($BRAF^{WT}$) cells.



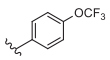
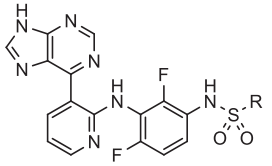
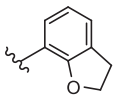
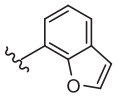
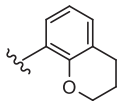
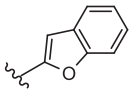
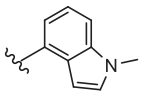
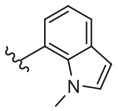
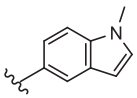
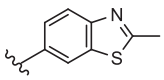
Compound	R	Cell viability GI_{50}(μM) A375P($BRAF^{V600E}$)	SK-MEL-2 ($BRAF^{WT}$)
5a		0.14	>10
5b		0.07	>10
5c		0.67	>10
5d		0.43	>10
5e		3.5	>10
5f		0.66	>10
5g		>100	>10
5h		54.62	>10
5i		0.76	>10
5j		0.16	>10
5k		0.59	>10
5l		0.23	>10
5m		1.00	>10
5n		1.05	>10
5o		21.03	>10
5p		0.97	>10
5q		5.91	>10

Table 4. Anti-proliferative activity of bicyclic sulfonamide derivatives of *N*-(2,6-difluorophenyl)-3-(9*H*-purin-6-yl)pyridine-2-amine against A375P ($BRAF^{V600E}$) and SK-MEL-2 ($BRAF^{WT}$) cells.



Compound	R	Cell viability GI_{50}(μM) A375P($BRAF^{V600E}$)	SK-MEL-2 ($BRAF^{WT}$)
6a		8.37	>10
6b		0.81	>10
6c		7.19	>10
6d		12.87	>10
6e		0.58	>10
6f		0.68	>10
6g		2.22	>10
6h		2.93	>10

additional two hydrogen bonding interactions existed between the sulfonyl oxygen of compound **4b** and the hydrogen of the F595 and G596 backbone amides. There were two van der Waals interactions sites. One was the difluorophenyl group with T529 and I527. The other site is near the L505 residue where it interacts with the furan ring. As shown in our binding model and the released X-ray crystal structure (3IDP.pdb), the intramolecular hydrogen bonding between the nitrogen of the purine and the hydrogen of the amine linker constrains the hinge binder conformation (Figure 3).[15]

We checked the protein kinase activities and selectivity of the most potent compound **4b**. The kinase inhibitory activities (IC_{50}) of compound **4b** against mutant BRAF, wild-type BRAF, and CRAF were 2, 2, and 1 nM, respectively (Table 5).[18] The cell line growth inhibitory activities were 7 and >10 000 nM against A375P ($BRAF^{V600E}$) and SK-MEL-2 ($BRAF^{WT}$), respectively. While the ATP-competitive BRAF inhibitors turn off the MAPK pathway

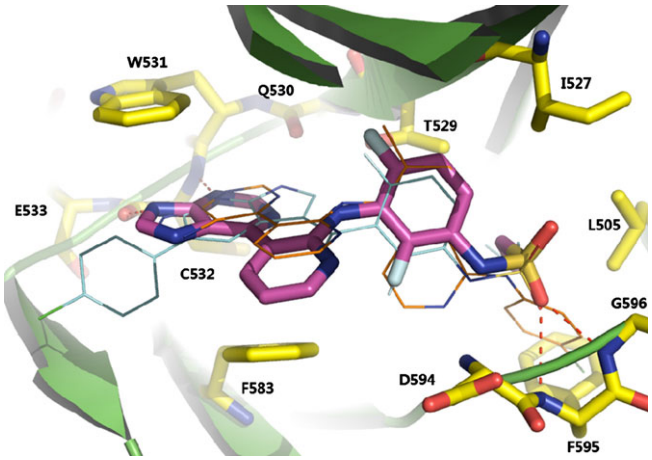


Figure 2. The superimposed model of BRAF inhibitors, PLX4032 (green) based on an X-ray crystal structure (3OG7.pdb) and the Amgen compound (cyan) and compound **3a** from docking using PyMol. The PLX4032 and Amgen compounds are depicted by lines with yellow sticks indicating the binding site residues. The compound **3a** is represented by a magenta stick. The dashed line indicates the hydrogen bonding interactions.

in cells bearing BRAF mutations, they lead to conformational changes to the wild-type BRAF kinase domain inducing heterodimerization with CRAF causing a paradoxical activation of MAPK pathway in cells bearing BRAF wild-type cells with RAS mutations.[16,19] Compound **4b** also showed selectivity against various other protein kinases. It demonstrated weak inhibition against the selected 29 kinases at a concentration of 10 μM (Table 6).[20]

Conclusion

In summary, compound **4b**, which has type IIB characteristics, was structure-based designed and synthesized. It exhibited a high degree of kinase selectivity and had more potent inhibitory activities against pan-RAF kinases and cell lines bearing a $BRAF_{V600E}$ mutation than vemurafenib (PLX4032). Preclinical development is ongoing for compound **4b**, and the design and synthesis of type IIB pan-RAF inhibitors are also in progress.

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Table 5. The IC_{50} profiles of compound **4b** against three RAF protein kinases.

	RAF kinase IC_{50}(nM)		
	$BRAF^{WT}$	$BRAF^{V600E}$	CRAF
Compound **4b**	2	2	1
Staurosporine	61	80	150

Table 6. Percentages of enzymatic inhibition by compound **4b** at a concentration of 10 μM on 30 selected protein kinases.

Kinase	% Inhibition
ABL1	16
AKT1	1
ALK	2
c-Kit	47
c-Src	43
CDK2/cyclin E	12
CDK5/p25	12
CHK1	<0
DMPK	<0
EGFR	<0
ERK2/MAPK1	<0
FAK/PTK2	8
FLT3	39
GSK3b	36
IGF1R	<0
JAK2	<0
JNK3	<0
KDR/VEGFR2	9
MEK1	<0
mTOR/FRAP1	4
P38a/MAPK14	4
p70S6K/RPS6KB1	<0
PAK2	4
PDGFRa	9
PKA	6
PKCa	2
PLK1	<0
RAF1/CRAF	99
SYK	8
TIE2/TEK	38
TRKB	6

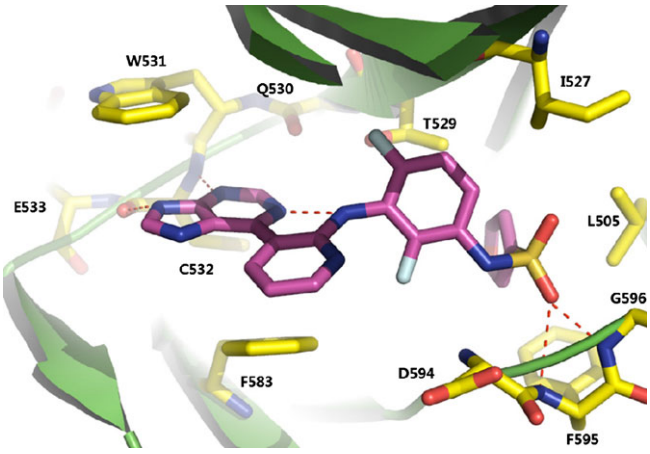

Figure 3. Compound **4b** docking pose is represented by a magenta stick with the yellow stick binding site residues of $BRAF^{V600E}$. The dashed line indicates hydrogen bonding interactions. Intramolecular hydrogen bonding between the purine and linker amine is also shown.

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