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1,4-Dihydropyrazolo[4,3-*d*]imidazole phenyl derivatives: A novel type II Raf kinase inhibitors

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ABSTRACT

The synthesis of a novel series of 1,4-dihydropyrazolo[4,3-*d*]imidazole phenyl derivatives **1a**–**b**, **2a**–**v** and their antiproliferative activities against A375P and WM3629 human melanoma cell line were described. Most compounds showed competitive antiproliferative activities to sorafenib, the reference standard. Among them, pyrazoloimidazole phenyl urea compounds **2a**, **2d**, **2g**, **2i**, **2t** exhibited potent activities on WM3629 cell lines (IC_{50} = 0.56–0.86 μM). Especially, **2t** was found to be a potent and selective C-Raf inhibitor, showing a possibility as melanoma therapeutics.

The understanding of molecular mechanism for cancer has been dramatically progressed over the last decade. Since it is known that cancer is mainly caused by abnormal increased expression or activation of oncogenes and the corresponding proteins, it is rational to target a protein that is a key component of the oncogenic pathway as protein-targeted cancer therapies.

The Ras-Raf-Mek-Erk signal transduction cascade is well-known protein pathway that regulates cell growth, differentiation and proliferation.[1] Like other oncogenic pathway, overexpression or mutation of protein members of this pathway is a driving factor for numerous cancers.[2–4]

Especially mutations of the Raf protein have been found in approximately 7% of human cancers[5,6] with particulary high frequency in melanoma (50–70%), ovarian (35%), thyroid (30%), and colorectal (10%) cancers. There have been 40 reported B-Raf mutations, and the most frequent mutation of Raf protein (>85%) is valine substitution by glutamic acid at position 600 (V600E), which shows a 500-fold increase in catalytic activity, providing cancer cells with both proliferation and survival signals.[7] Therefore, B-Raf V600E is a high-interest therapeutic target for the treatment of human cancers. However, there is now a growing consensus being made that C-Raf is also associated significantly with disease progression and cell proliferation in a subset of melanoma.[8–10]

A number of inhibitors, designed to target Raf family proteins (A, B, and C) are known. These include benzylidene oxindoles,[11] PLX4720,[12] and sorafenib[13–16] (Fig. 1: Launched for RCC). The bi-aryl urea sorafenib is a potent inhibitor of preactivated C-Raf and B-Raf as well as oncogenically activated B-Raf kinases (V600E B-Raf: IC_{50} = 43 nM).

Crystal structure of V600E B-Raf kinase domains in complex with sorafenib showed that this inhibitor is namely *type II* kinase inhibitor, having the activation segment in an inactive conformation.[17] Most of known kinase inhibitors belong to *type I* inhibitors, which bind to the ATP binding site through the formation of hydrogen bonds to the kinase 'hinge' residues and through hydrophobic interactions in and around the region occupied by the adenine ring of ATP. However, type II inhibitors typically use the ATP binding site, but they also exploit unique hydrogen bonding and hydrophobic interactions made possible by the DFG residues of the activation loop being folded away in an inactive conformation.

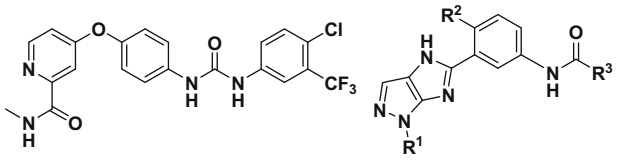

Figure 1. Structures of sorafenib and imidazolopyrazole derivatives.

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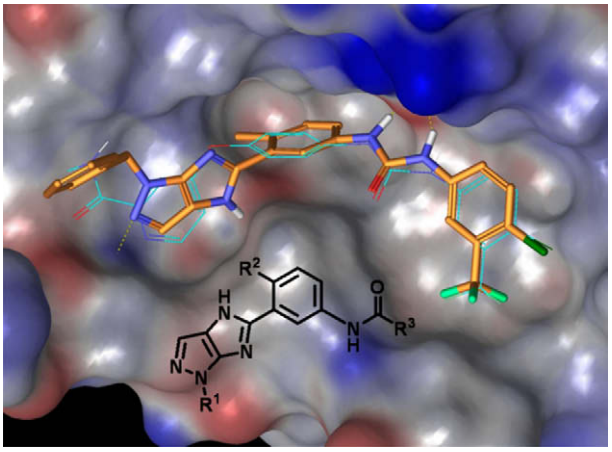


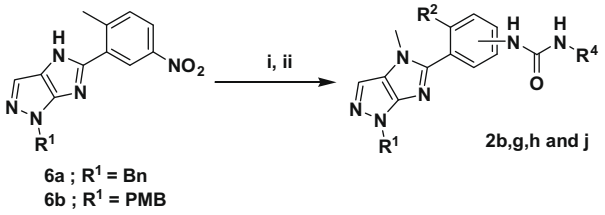
Figure 2. Docking structure of designed 1,4-dihydropyrazolo [4,3-*d*] imidazole (bold, orange) overlayed with sorafenib (thin, cyan) in V600E B-Raf.

The ATP binding and subsequent kinase reaction is prevented in this inactive kinase conformation, and the binding feature of sorafenib to B-Raf is interesting enough to prompt us to design a novel scaffold for Raf inhibitor. Since the amino acids surrounding this secondary hydrophobic pocket are less conserved relative to those in the ATP binding pocket, it has been proposed that it may be easier to achieve kinase selectivity with type II inhibitors.

Type II inhibitors typically have potent cellular activity, presumably because they recognize (or induce) the DFG-out conformation, which has a lower affinity for ATP than for the active kinase. Here we wish to report the initial SAR studies of a novel scaffold, 1,4-dihydropyrazolo[4,3-*d*]imidazole derivatives as type II inhibitor for Raf.

To design a new chemical entity (NCE), we need to complement the size, shape, and electronic properties of molecular targets. Designing a new *type II inhibitor* for V600E B-Raf, the hinge binder was still considered to be the primary pharmacophore, and the imidazolopyrazole bicyclic ring has attracted considerable attention of ours as a new hinge binder in the terms of novelty, and chemical modification accessibility (Fig. 2).

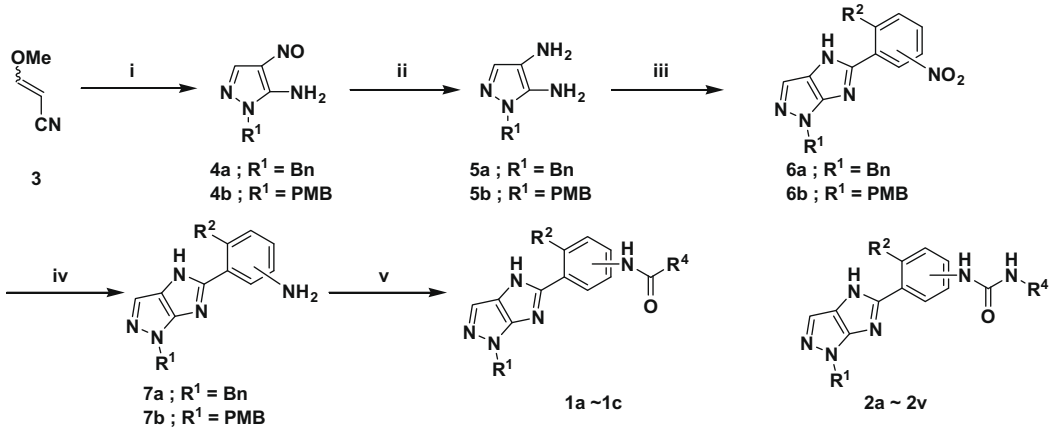
The structure of this series comprises 1,4-dihydropyrazolo[4,3-*d*]imidazole part, the middle phenyl ring moiety and various aromatic tail part connected by amide or urea linkage. Specifically, we modified the structures by (i) replacing hydrogen in pyrazole N-2 position with aromatic rings to get extended structural information, (ii) introducing methyl group in nitrogen of imidazole ring or/and in the middle phenyl moiety looking for a proper conformation, and (iii) introducing the various direction of connectivity (*m*-, *p*-) and changing the spacer to the aromatic tail part to understand the relative spatial arrangements for the two aromatic rings.



Scheme 2. Reagents and conditions: (i) KO^{t}Bu, MeI THF, rt; (ii) R^{4}NCO, THF, rt.

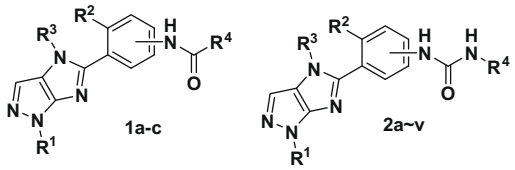
The general synthesis of 1,4-dihydropyrazolo[4,3-*d*]imidazole derivatives is shown in Scheme 1. The core pyrazole moiety with two nitrogen functionalities was made from protected hydrazine and 3-methoxyacrylonitrile in one pot process based on the literature.[18] Then, the nitroso group on pyrazole was reduced to give diamine[19] for subsequent imidazole cyclization. The diaminopyrazole **5** was reacted with corresponding nitro benzoic acid in $POCl_3$ to provide 1,4-dihydropyrazolo[4,3-*d*]imidazole skeleton **6**.[20] The resulting nitro group in **6** was reduced to amino group and coupled with various aromatic acids under EDCI/HOBt conditions or directly aromatic isocyanate to give amide (**1a–b**) or urea (**2a–v**) analogues. To investigate an optimal conformation of middle phenyl ring, we synthesized several compounds with additional methyl group on imidazole nitrogen. N-methylated analogues at R^3 position (**2b**, **2g**, **2h**, **2j**) were synthesized as shown in Scheme 2. The methylation was performed just after the second cyclization and the rest of reaction was followed to yield the desired analogues.

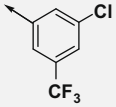
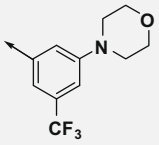
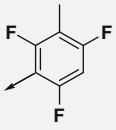
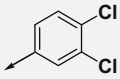
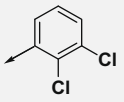
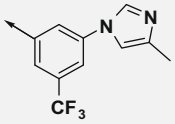
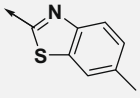
Table 1 shows the antiproliferative activitiy[21,22] (GI_{50} values) of those analogues **1a**, **2a**–**h** against A375P[21] and WM3629[22] human melanoma cell line together with that of sorafenib as a reference compound. The initial compounds with 3-chloro-5-trifluoromethyl benzoyl group as a tail (**1a**, **2a**–**h**) showed competitive antiproliferative activities against A375P and WM3629 human melanoma cell line. While an amide linkered compound **1a** showed a competitive activity, urea-linker compounds **2a**, **2g** exhibited better potency than reference sorafenib. Especially, the *p*-directed urea **2g** with extra R^3 methyl group possessed the best potency on WM3629 cell line. However, the free pyrazole or *p*-methoxybenzyl moiety on



Scheme 1. Reagents and conditions: (i) R^{1}HNNH$_2$, $NaNO_2$, EtOH, 50 °C; (ii) $SnCl_2{\cdot}2H_2O$, EtOH, 80 °C; (iii) 2-R^2-4-NO_2–C_6H_3–CO_2H, $POCl_3$, DMAP reflux; (iv) $SnCl_2{\cdot}2H_2O$, EtOH, 80 °C; (v) R$^4CO_2H$, EDCI, TEA, HOBt, DMF or R^{4}NCO, THF.

Table 1
Antiproliferative activity of pyrazoloimidazole phenyl amide/urea derivatives **1a–c**, **2a–v**



Compd	Orientation	R^1	R^2	R^3	R^4	GI_{50}[a] (μM)	
						A375P	WM3629
1a[24]	*m*-Phenyl	Bn	Me	H		2.06	1.14
2a	*m*-Phenyl	Bn	Me	H		3.79	0.85
2b	*m*-Phenyl	PMB	Me	H		>100	>100
2c	*m*-Phenyl	H	Me	H		>100	>100
2d	*p*-Phenyl	Bn	H	H		6.65	0.75
2e	*p*-Phenyl	H	Me	H		>100	14.4
2f	*m*-Phenyl	Bn	Me	Me		56.3	5.85
2g	*p*-Phenyl	Bn	Me	Me		4.16	0.56
2h	*p*-Phenyl	PMB	Me	Me		51.5	3.77
1b	*m*-Phenyl	H	Me	H		NA	NA
2i	*m*-Phenyl	Bn	Me	H		4.29	0.72
2j	*m*-Phenyl	Bn	Me	Me		>100	>100
2k	*p*-Phenyl	H	Me	H		>100	NA
2l	*p*-Phenyl	PMB	Me	H		>100	>100
2m	*m*-Phenyl	PMB	Me	H		>100	>100
2n	*m*-Phenyl	Bn	Me	H		7.73	1.29
2o	*p*-Phenyl	Bn	H	H		51.7	0.98
2p	*m*-Phenyl	PMB	Me	H		15.3	1.35
2q	*p*-Phenyl	Bn	H	H		>100	42.8
2r	*m*-Phenyl	H	Me	H		NA	NA
2s	*p*-Phenyl	H	Me	H		NA	NA
2t[24]	*p*-Phenyl	H	Me	H		2.24	0.86
2u	*p*-Phenyl	H	Me	H		NA	NA
2v	*p*-Phenyl	PMB	Me	H		NA	NA
Sorafenib						5.58	0.65

[a] Values are average of two or more experiments; NA: not active.

Table 2
Enzymatic activities of selected compounds

Compd	IC_{50} (nM) on C-Raf
1a	146
2a	76
2d	370
2g	NA
2i	156
2p	839
2t	50

pyrazole ring with this tail did not seem to be active for cellular activity (**2c**, **2e**).

Further, we continued the syntheses of more compounds with various hydrophobic tail groups, focusing on urea-linkered derivatives. The analogues with *m*-orientation of middle benzene ring were preferred in case of urea derivatives (**2i** >> **2l**, **2n** > **2o**, **2p** > **2q**) than *p*-orientation. It was interesting to see that the *ortho* substitution of fluorine in benzoic acid tail (**2n**) was tolerated but the chlorine substitution (**2r**, **2s**) was not, implying the secondary hydrophobic pocket is sensitive to the conformation of tail group. Some of pyrazoloimidazole phenyl urea derivatives (**2i**, **2t**) showed superior antiproliferative activities to sorafenib in both cell lines (4.29 μM, 720 nM and 2.68 μM, 860 nM, respectively). Especially, it was surprising that the free pyrazoloimidazole urea compound **2t** showed the best potency toward A375P cell line in these series. However, the flat bicyclic tail group such as methyl benzothiazole (**2u**, **2v**) did not seem to be proper for secondary hydrophobic pocket.

For several compounds with competitive cellular potency, C-Raf inhibitory activities (IC_{50}) were determined as shown in Table 2.[23] Most compounds tested are potent C-Raf inhibitors confirming that the primary cell line test was valuable. Assuming they are all *type II inhibitors* and the contact of hydrophobic tail group is important, it seems urea-linkered compounds seems more effective (**1a** < **2a**)

Table 3
Percentages of enzymatic inhibitions by compound **2t** (10 μM) on selected 30 Protein kinases

Kinase	% Inhibition
Abl(h)	37
ALK(h)	61
AMPK(r)	5
Aurora-A(h)	10
Axl(h)	6
Bmx(h)	16
BTK(h)	10
CDK2/cyclinE(h)	33
CDK5/p35(h)	0
CK2(h)	8
cKit(h)	0
C-Raf(h)	96
cSRC(h)	20
EphA1(h)	30
FGFR1(h)	26
Flt3(h)	53
Fms(h)	42
IGF-1R(h)	35
IKKβ(h)	16
IR(h)	0
JAK2(h)	0
KDR(h)	8
Met(h)	0
p70S6 K(h)	4
PDGFRα(h)	0
Plk1(h)	8
Syk(h)	16
Tie2(h)	82
TrkB(h)	62

than the amide. When the tail group becomes bulkier, then the *p*-orientation of middle phenyl ring seems preferred as C-Raf inhibitor (**2a** < **2t**). The best compound **2t** has showed an IC_{50} value of 50 nM, while the IC_{50} value for the GW5074 was 2.32 nM.

We further tried kinase panel screening of the best compound **2t** over 30 different kinases at a single dose concentration of 10 μM[23] (Table 3) and it was revealed that the compound has a decent selectivity profile. While this compound has inhibitory activity of 96% on C-Raf at this concentration, the inhibition exerted in most other kinases tested in activity was below 50%.

In conclusion, a series of novel scaffold, 1,4-dihydropyrazolo[4,3-*d*]imidazole phenyl derivatives based on the structural features of sorafenib has been synthesized and showed potent antiproliferative activities against A375P human and WM3629 melanoma cell line. Furthermore, one of the best compound **2t** in this series has been confirmed as a potent and selective C-Raf kinase inhibitor. These results suggest that the more development of pyrazoloimidazole phenyl scaffold is a very promising way for new therapeutics for melanoma, especially non-V600E classes.

Acknowledgments

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21. A375P cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, US) and maintained in DMEM medium (Welgene, Daegu, Korea) supplemented with 10% FBS (Welgene) and 1% penicillin/streptomycin (Welgene) in a humidified atmosphere with 5% CO_2 at 37 °C. A375P cells were taken from culture substrate with 0.05% trypsin–0.02% EDTA and plated at a density of 5×10^3 cells/well in 96 well plates and then incubated at 37 °C for 24 h in a humidified atmosphere with 5% CO_2 prior to treatment of various concentrations (threefold serial dilution, 12 points) of test compounds. The A357P cell viability was assessed by the conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. MTT assays were carried out with CellTiter 96® (Promega) according to the manufacturer's instructions. The absorbance at 590 nm was recorded using EnVision 2103 (Perkin Elmer; Boston, MA, US). The IC_{50} was calculated using GraphPad Prism 4.0 software.
22. WM3629 cell line was supplied from Dr. Merlyn lab at Wistar Institute (Philadelphia, PA, US) and maintained in Tu2% medium according to the literature.[10] The procedure for GI_{50} determination (MTT assay) was the same as in A375P cell line.
23. We used Millipore KinaseProfilerTM service (www.millipore.com) for screening of **2t** and IC_{50}ProfilerExpress for IC_{50} measurement. Assay protocol: In a final reaction volume of 25 μL, C-Raf (h) (5–10 mU) is incubated with 25 mM Tris pH 7.5, 0.02 mM EGTA, 0.66 mg/mL myelin basic protein, 10 mM Mg acetate and [^{33}P-ATP] (specific activity approx. 500 cpm/pmol, concentration as required). The reaction is initiated by the addition of the Mg–ATP mix. After incubation for 40 min at room temperature, the reaction is stopped by the addition of 5 μL of a 3% phosphoric acid solution. Ten microliters of the reaction is then spotted onto a P30 filter mat and washed three times for 5 min in 75 mM phosphoric acid and once in methanol prior to drying and scintillation counting.
24. *Selected data* **1a**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.17 (1H, s), 10.61 (1H, s), 8.41 (1H, d), 8.27 (1H, dd, J = 8.41 Hz, 1.92 Hz), 8.03 (1H, d, J = 2.22 Hz), 7.93 (1H, d, J = 8.39 Hz), 7.79 (1H, dd, J = 8.30 Hz, 2.26 Hz), 7.43 (1H, s), 7.35 (1H, d, J = 8.22 Hz), 7.32–7.24 (5H, m), 5.42 (2H, s), 3.37 (3H, s); MS m/z 481 $(M+H)^+$. **2t**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.14 (1H, s), 9.24 (1H, s), 8.24 (1H, s), 8.14 (1H, s), 7.71 (1H, d, J = 0.99 Hz), 7.66 (1H, d, J = 7.11 Hz), 7.60 (1H, d, J = 8.4 Hz), 7.51 (1H, s), 7.41 (1H, d, J = 8.32 Hz), 7.29 (1H, s), 3.3 (3H, s), 2.49 (3H, s); MS m/z 510 $(M+H)^+$.