Full Paper

Design, Synthesis, and Antiproliferative Activity of 3,4-Diarylpyrazole-1-carboxamide Derivatives Against Melanoma Cell Line

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Synthesis of a new series of 3,4-diarylpyrazole-1-carboxamide derivatives is described. Their antiproliferative activity against A375P human melanoma cell line was tested and the effect of substituents on the diarylpyrazole scaffold was investigated. The biological results indicated that five synthesized compounds (**Ig**, **Ii**, **IIc**, **IIg**, and **IIh**) exhibited similar activity to Sorafenib. In addition, three compounds (**IIa**, **IIb**, and **IIi**) were more potent than Sorafenib. Among all of these derivatives, compound **IIa** which has dimethylamino and phenolic moieties showed the most potent antiproliferative activity against A375P human melanoma cell line. Virtual screening was carried out through docking of the most potent compound **IIa** into the domain of V600E-b-Raf and the binding mode was studied.

Keywords: A375P / Antiproliferative activity / 3,4-Diarylpyrazole / 1*H*-Pyrazole-1-carboxamide / Melanoma

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Introduction

Melanoma is the most aggressive form of skin cancer and is the fastest growing cancer in the United States [1, 2]. Early stage melanoma can be cured surgically. However, melanoma metastasizing to major organs (stage IV) is virtually incurable [1]. Patients with advanced melanoma have a median survival time of less than one year, and the estimated 5-year survival time is less than 15% [1, 3]. With the incidence of melanoma rapidly rising in the United States and other developed countries, there is an urgent need to develop more effective drugs [4–6].

The current treatments involve surgical removal of the tumor, immunotherapy, radiotherapy, chemotherapy, various combinations, or the use of new treatments in clinical trials. As for immunotherapy, interferon alfa-2b (Intron-A) [7] has been approved by both the FDA and EMEA for adjuvant treatment of melanoma patients, and aldesleukin (Proleukin) [8, 9] has been also approved for the treatment of metastatic melanoma in the USA.

There are many promising, potent, and selective antiproliferative agents for treatment of melanoma. Sorafenib [10–15] is an oral multikinase inhibitor that targets 2 classes of kinases which are known to be involved in both tumor proliferation and angiogenesis [16]. It inhibits Raf kinases (Raf-1 and b-Raf), as well as proangiogenic receptor tyrosine kinases of the PDGFR and VEGFR family [12]. The antiproliferative activity of Sorafenib against melanoma is assumed to be due to b-Raf inhibition and induction of apoptosis in a caspase-independent manner [17]. Sorafenib demonstrated high antiproliferative activity against different melanoma xenografts and cell lines [17], but not in case of advanced metastatic melanoma (stage IV) [16]. In addition, sorafenib has been implicated in the development of reversible posterior leukoencephalopathy syndrome and secondary erythrocytosis [18]. These side effects together with the poor

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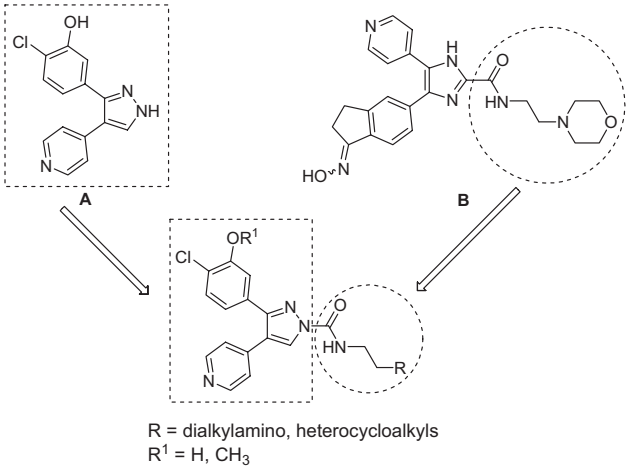


Figure 1. Structures of the parent compounds A and B, and target compounds.

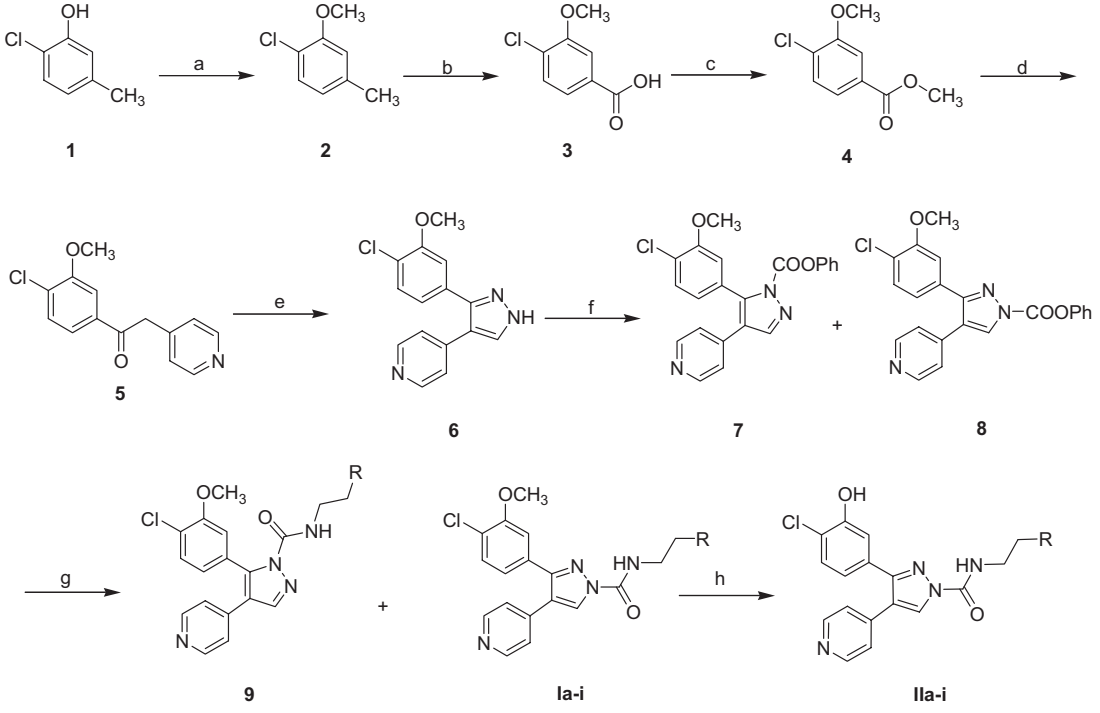
efficiency of sorafenib in case of advanced metastatic melanoma encourage the search for new antiproliferative agents for treatment of melanoma.

Moreover, antiproliferative agents with 3,4-diarylpyrazole scaffold targeting b-Raf kinase that can be efficient for treatment of melanoma have been identified [19, 20]. In the present study, compounds A [19] and B [21] (Fig. 1) were considered as the parent compounds for design of our target compounds. In our efforts in order to develop new antiproliferative agents for treatment of melanoma, we designed and synthesized new 3,4-diarylpyrazole derivatives possessing an amide moiety at position 1 of the pyrazole ring. The synthesized compounds **Ia–i** and **IIa–i** were designed by introduction of derivatives of the carboxamide side chain of compound B into position 1 of the pyrazole ring of compound A. The phenolic hydroxy group of A was retained in compounds **IIa–i** and modified into methoxy group in compounds **Ia–i** in order to examine its effect on the activity (Fig. 1). Herein, we report the synthesis and antiproliferative activity against A375P human melanoma cell line of these compounds. *In-silico* and molecular docking studies are also reported.

Results and discussion

Chemistry

3,4-Diarylpyrazole derivatives **Ia–i** and **IIa–i** with amide moiety at position 1 of the pyrazole ring were prepared according to the sequence of reactions, illustrated in Scheme 1. Methylation of the phenolic hydroxyl group of



Reagents and conditions: (a) $(CH_3)_2SO_4$, K_2CO_3, acetone; (b) $KMnO_4$, C_5H_5N, H_2O; (c) acetyl chloride, MeOH; (d) 4-picoline, LHMDS, THF; (e) (i) DMF-DMA, (ii) hydrazine monohydrate, EtOH; (f) phenyl chloroformate, TEA, THF; (g) substituted ethanamines, K_2CO_3, CH_2Cl_2; (h) $BF_3 \cdot Me_2S$,CH_2Cl_2.

Scheme 1. Synthesis of the target compounds **Ia–i** and **IIa–i**.

2-chloro-5-methylphenol (**1**) using dimethyl sulfate gave 1-chloro-2-methoxy-4-methylbenzene (**2**) [22]. Oxidation of the methyl group of **2** using $KMnO_4$ produced 4-chloro-3-methoxybenzoic acid (**3**) [23], which upon esterification with methanol in the presence of acetyl chloride afforded the corresponding methyl ester **4**. The pyridyl derivative **5** was obtained by treatment of **4** with 4-picoline in the presence of lithium bis(trimethylsilyl)amide (LHMDS). Cyclization to the pyrazole compound **6** was carried out by treatment of **5** with dimethylformamide dimethyl acetal (DMF-DMA), and subsequent treatment with hydrazine monohydrate. Interaction of **6** with phenyl chloroformate in the presence of triethylamine gave a mixture of pyrazole-2-carboxylate derivative **7** and pyrazole-1-carboxylate derivative **8** in an approximate ratio of 1:4∼1:5. The mixture was then reacted with the appropriate ethanamines to produce the target methoxy compounds **Ia–i** in combination with their regioisomers **9**. Compounds **Ia–i** with lower R_f values on TLC were obtained in the pure form after purification by flash column chromatography. Demethylation of the methoxy group of **Ia–i** using boron trifluoride–methyl sulfide complex afforded the corresponding hydroxy derivatives **IIa–i**.

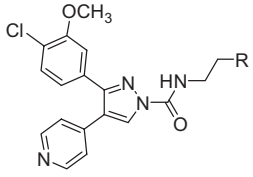
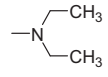
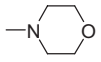
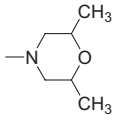
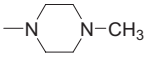
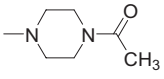
Antiproliferative activity and discussion

The antiproliferative activity of the synthesized compounds against A375P human melanoma cell line was tested. The ability of the 1*H*-pyrazole-1-carboxamide derivatives to inhibit the growth of A375P cell line is summarized in Tables 1 and 2. The results are expressed as IC_{50} values. Sorafenib was selected as a reference standard.

As listed in Tables 1 and 2, some of the compounds showed moderate activity, while compounds **Ig**, **Ii**, **IIc**, **IIg**, and **IIh** having IC_{50} values ranging from 11.8 to 12.7 μM exhibited similar activity to that of sorafenib (IC_{50} = 12.5 μM). In addition, compounds **IIa**, **IIb**, and **IIi** with IC_{50} values of 4.5, 8.1, and 8.3 μM, respectively, showed more potent antiproliferative activity than that of sorafenib. Compounds **Ig** and **Ii** possess a *meta*-methoxy group on the benzene ring while the other six potent compounds possess a hydroxy group. And the R moiety of compounds **Ig** and **IIg** is piperidinyl moiety and that of compounds **Ii** and **IIi** is pyrrolidinyl. The R moieties of compounds **IIa**, **IIb**, **IIc**, and **IIh** are dimethylamino, diethylamino, morpholino, and 2-methylpiperidinyl, respectively.

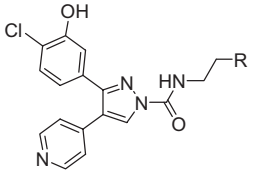
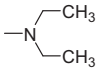
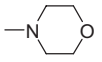
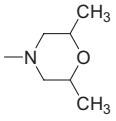
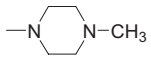
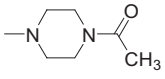
Most of hydroxyl compounds were generally more potent than the corresponding methoxy derivatives, which suggests that the *m*-hydroxy group on the benzene ring is optimal for the activity. This may be attributed to hydrogen bond formation at the receptor site. Docking of **IIa** structure into the domain of V600E-b-Raf kinase crystal structure revealed the formation of two hydrogen bonds by the hydroxyl group at the binding site (molecular docking part). Or the *o*-chlorophenolic moiety may induce additional DNA damage effect in the presence of copper and oxygen.

Table 1. Antiproliferative activity of methoxy compounds **Ia–i** against A375P cell line.

Structure	Comp. No.	R	IC_{50} (μM)
	**Ia**		14.8
	Ib		>20
	Ic		>20
	Id		>20
	Ie		15.1
	If		18.8
	Ig		12.7
	Ih		>20
	Ii		12.5
	Sorafenib		12.5

The effect of the terminal substituents of the tail at position 1 of the pyrazole ring was also investigated. Compounds **Id** and **IId** having a 2,6-dimethylmorpholine ring showed diminished activity. We can conclude that this moiety is unfavorable for antiproliferative activity against melanoma of this series. This may be due to the steric and/or electronic effect(s) of this moiety at the receptor site. In

Table 2. Antiproliferative activity of hydroxy compounds **IIa–i** against A375P cell line.

Structure	Comp. No.	R	IC_{50} (μM)
	**IIa**		4.5
	IIb		8.1
	IIc		12.5
	IId		>20
	IIe		>20
	IIf		>20
	IIg		12.2
	IIh		11.8
	IIi		8.3
Sorafenib			12.5

general, it was found that dialkylamino and pyrrolidinyl derivatives were more potent than other derivatives with 6-membered rings. This may be attributed to steric effect of the bulkier substituents at the receptor site. By comparing the activities of compounds **Ia,b** with those of **IIa,b**, we find that smaller alkyl groups, two methyl groups, are more optimal for activity than the slightly longer groups, ethyl groups. Upon comparing the activity of the piperazinyl derivatives, it was found that the *N*-methyl derivative **Ie** was more potent than that of *N*-acetyl derivatives **If**. These results may be rationalized by the steric and/or electronic effect(s) of the acetyl group, compared with the methyl group. Introduction of a methyl group on the piperidine ring (compound **Ih**) diminished the activity compared with unsubstituted piperidine ring (compound **Ig**). Similarly, introduction of two methyl groups on the morpholine ring (compound **IId**) diminished the activity, compared with unsubstituted morpholine derivative (compound **IIc**).

In conclusion, a new series of 3,4-diaryl-1*H*-pyrazole-1-carboxamide derivatives was synthesized based on our previous literature studies. Among all of these derivatives, compound **IIa** with substituted *m*-hydroxyphenyl and dimethylamino moieties showed the most potent antiproliferative activity against A375P human melanoma cell line. We can conclude that these moieties are optimal for antiproliferative activity of this series of compounds. Further modification of these compounds in order to improve their potency is currently in progress.

Lipinski's rule of five and drug-likeness profile

In this work, the bioavailability of the most active compounds **Ig**, **Ii**, **IIa–c**, and **IIg–i** was assessed using ADME (absorption, distribution, metabolism, elimination) prediction methods. In particular, we calculated the compliance of compounds to the Lipinski's rule of five [24]. This approach has been widely used as a filter for substances that would likely be further developed in drug design programs. In addition, we calculated the total polar surface area (TPSA) since it is another key property that has been linked to drug bioavailability. Thus, passively absorbed molecules with a TPSA > 140 are thought to have low oral bioavailability [25]. Molecules violating more than one of these rules may have problems with bioavailability. Predictions of ADME properties for the studied compounds are given in Table 3. The results showed that all the potent compounds comply with these rules and even sorafenib showed no violation. Theoretically, these compounds should present good passive oral absorption and differences in their bioactivity cannot be attributed to this property.

Currently, there are many approaches to assess a compound drug-likeness based on topological descriptors, fingerprints of molecular drug-likeness structure keys or other properties such as clog*P* and molecular weight [26]. In this work, we used the Osiris program [27] for calculating the fragment-based drug likeness of the most potent compounds and comparing them with sorafenib. Interestingly, all the potent compounds **Ig**, **Ii**, **IIa–c**, and **IIg–I** demonstrated better drug-likeness values (from 7.72 to 2.69) than sorafenib (−4.2). The drug-scores of the potent compounds have also been determined in the present study. The results showed that the eight potent compounds demonstrated higher drug-score

Table 3. Solubility and calculated Lipinski's rule of five for the most active compounds over human melanoma (A375P) cell line.

Compd. No.	IC_{50}[a]	log*S*[b]	Parameter					
			clog*P*[c]	TPSA[d]	MW[e]	*n* ON[f]	*n* OHNH[g]	*n* violations
Ig	12.7	−5.22	3.81	72.29	439.95	7	1	0
Ii	12.5	−4.95	3.49	72.29	425.92	7	1	0
IIa	4.5	−3.95	2.49	83.28	385.86	7	2	0
IIb	8.1	−4.55	3.36	83.28	413.91	7	2	0
IIc	12.5	−4.02	2.41	92.52	427.89	8	2	0
IIg	12.2	−4.91	3.61	83.28	425.92	7	2	0
IIh	11.8	−5.28	3.90	83.28	439.95	7	2	0
IIi	8.3	−4.64	3.30	83.28	411.89	7	2	0
Sorafenib	12.5	−6.69	4.27	92.35	464.82	7	3	0

[a] Data taken from Tables 1 and 2. [b] Solubility parameter. [c] Calculated lipophilicity. [d] Total polar surface area (Å^2). [e] Molecular weight. [f] Number of hydrogen bond acceptor. [g] Number of hydrogen bond donor.

values than sorafenib (Fig. 2). Moreover, we used the Osiris program for prediction of the overall toxicity of the most active derivatives as it may point to the presence of some fragments generally responsible for the irritant, mutagenic, tumorigenic, or reproductive effects in these molecules. Interestingly, most of the active compounds presented a low *in-silico* toxicity risk profile, similar to sorafenib (Fig. 2). These theoretical data reinforced the cytotoxicity experimental data described in this work pointing these compounds as lead compounds with low toxicity risk profile.

Molecular docking

The level of antiproliferative activity of the synthesized compounds over melanoma cells, in which b-Raf kinase is overexpressed and mutated, promoted us to perform molecular docking into the domain of b-Raf kinase. Compound **IIa** which is the most potent derivative of this series was used for docking study as a representative example. All calculations were performed using MOE 2008.10 software [28] installed on 2.0G Core 2 Duo. The crystal structure of V600E-b-Raf Kinase in complex with PLX4032 (PDB code: 3OG7) was obtained from protein data bank (PDB) [29]. The automated docking program of MOE 2008.10 was used for docking of **IIa** into the domain of V600E-b-Raf kinase. The complex was energy-minimized with a MMFF94× force-field [30] till the gradient convergence 0.01 kcal/mol was reached. The docking study has revealed that the ligand has bound in the active site of one of the protomers in the protein dimer through the formation of four strong hydrogen bonds between the binding site and the ligand. These hydrogen

Compd. No.	Toxicity effects			
	M	T	I	R
Ig	Low	Low	Low	Low
Ii	Low	Low	Medium	Low
IIa	High	Low	Low	Low
IIb	Low	Low	Low	Low
IIc	Low	Low	Low	Low
IIg	Low	Low	Low	Low
IIh	Low	Low	Low	Low
IIi	Low	Low	Medium	Low
Sorafenib	Low	Low	Low	Low

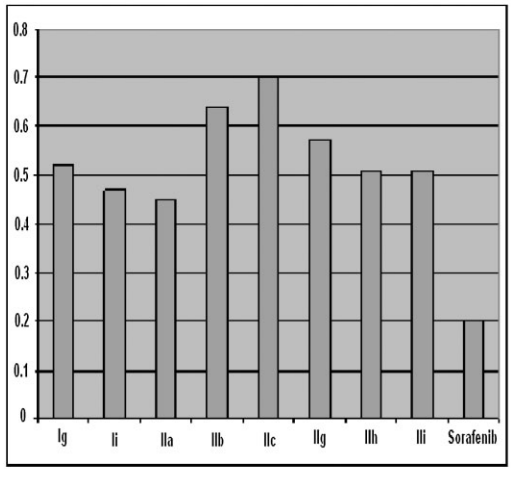


Figure 2. *In-silico* toxicity risks (left panel) and drug-score (right panel) of sorafenib and the potent antiproliferative pyrazole derivatives over melanoma cancer (M, mutagenic; T, tumorigenic; I, irritant; R, reproductive).

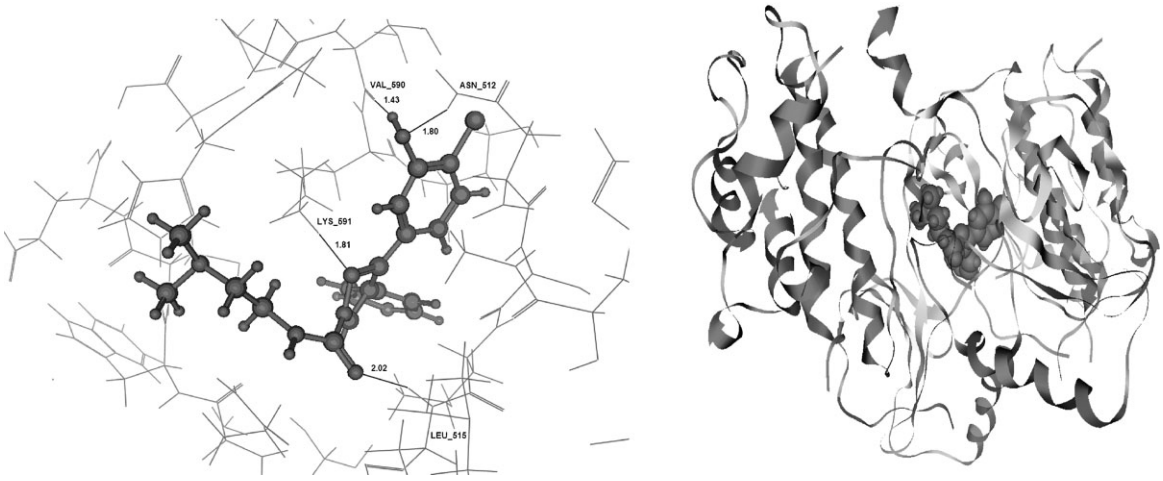


Figure 3. Interaction between compound **IIa** and V600E-b-Raf, hydrogen bonds are shown as black lines (left panel). The ligand **IIa** (space filling) is embedded into the kinase domain (right panel).

bonds have been formed between the phenolic hydroxyl hydrogen atom and Val-B590 (1.43 Å); phenolic hydroxyl oxygen atom and Asn-B512 (1.80 Å); pyrazole *N*2 atom and Lys-B591 (1.81 Å); and carbonyl oxygen atom and Leu-B515 (2.02 Å). Figures 3 and 4 demonstrate the binding model of the most potent compound **IIa** with the binding site of V600E-b-Raf. The results of this molecular docking study can support the postulation that our active compounds may act on the same enzyme target, b-Raf, whose inhibition can lead to antiproliferative effect against melanoma cells.

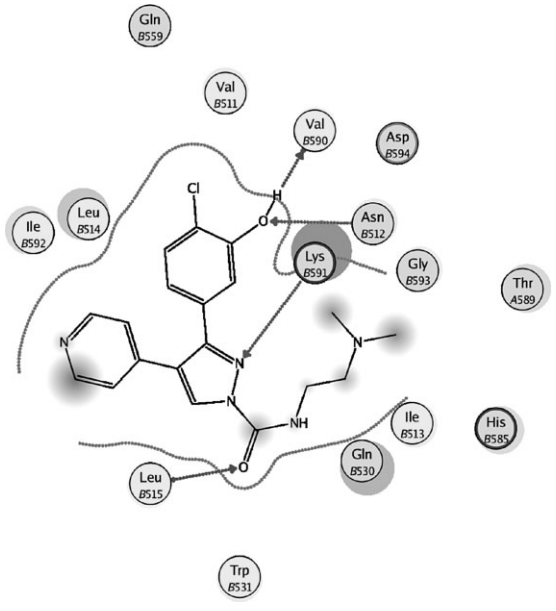


Figure 4. 2D-presentation for the binding interactions of compound **IIa** with V600E-b-Raf kinase domain.

Experimental

Chemistry

All melting points were obtained on a Walden Precision Apparatus Electrothermal 9300 apparatus and are uncorrected. Mass spectra (MS) were taken in ESI mode on a Waters 3100 Mass Detecter (Waters, Milford, MA, USA). Nuclear magnetic resonance (NMR) spectroscopy was performed using a Bruker ARX-400, 400 MHz spectrometers (Bruker Bioscience, Billerica, MA, USA) with TMS as an internal standard. IR spectra (KBr disks) were recorded with a Bruker FT-IR instrument (Bruker Bioscience, Billerica, MA, USA). %Purity of the target compounds (>95%) were determined by LC-MS analysis. All reagents and solvents were purchased from Aldrich chemical Co. and Tokyo Chemical Industry (TCI) Co., and used without further purification.

*1-Chloro-2-methoxy-4-methylbenzene **2***

A mixture of 2-chloro-5-methylphenol (**1**, 21.5 g, 150 mmol), dimethyl sulfate (20.8 g, 165 mmol), and anhydrous K_2CO_3 (51.8 g, 375 mmol) in acetone (250 mL) was heated under reflux for 1 h. The mixture was filtered, and the filtrate was evaporated under reduced pressure. Water (150 mL) was added to the residue and the resulting mixture was carefully extracted with Et_2O. The organic layer was separated and the aqueous layer was extracted with Et_2O (3×50 mL). The combined Et_2O extracts were washed with brine, dried over anhydrous Na_2SO_4, and filtered. The organic solvent was evaporated under reduced pressure, and the residue was used in the next step without further purification. MS *m/z*: 159.5 ($M^+ + 3$), 158.5 ($M^+ + 2$), 157.5 ($M^+ + 1$).

*4-Chloro-3-methoxybenzoic acid **3***

A stirred mixture of compound **2** (11.17 g, 71 mmol), potassium permanganate (35.0 g, 221 mmol), pyridine (36 mL), and water (107 mL) was heated at 50°C for 24 h. The mixture was then stirred at room temperature for 13 h. The mixture was filtered and MnO_2 was suspended in hot water and again filtered off. The combined aqueous filtrates were washed with ethyl acetate (3×75 mL) and

then acidified with 2 N H_2SO_4. The precipitate was filtered off, washed with water, and dried to give the title compound (11.98 g, 90%). M.p.: 215–216°C (lit. m.p.: 217–219°C [23]).

*Methyl 4-chloro-3-methoxybenzoate **4***

Acetyl chloride (1.9 mL, 28.1 mmol) was added dropwise to a solution of **3** (1.0 g, 5.4 mmol) in MeOH (40 mL) at 0°C and the reaction mixture was then stirred at room temperature for 15 h. After evaporation of the organic solvent, the residue was purified by flash column chromatography (silica gel, hexane/ethyl acetate 5:1 v/v) to give **4** (0.91 g, 85%) as a crystalline solid. M.p.: 49–50°C; ^{1}H-NMR (DMSO-d_6) δ 3.88 (s, 3H), 3.94 (s, 3H), 7.55–7.62 (m, 3H); MS *m/z*: 204.0 ($M^+ + 3$), 203.0 ($M^+ + 2$), 202.0 ($M^+ + 1$).

*1-(4-Chloro-3-methoxyphenyl)-2-(pyridin-4-yl)ethanone **5***

To a solution of compound **4** (1.0 g, 5.0 mmol) and 4-picoline (0.5 mL, 5.6 mmol) in THF (5 mL) in a cooled bath at −25°C, LHMDS (3.7 mL, 1.0 M solution in THF, 19.9 mmol) was slowly added to maintain the temperature at −25°C. The resulting mixture was stirred overnight at room temperature. The mixture was quenched with saturated aqueous NH_4Cl. Ethyl acetate was added and the organic layer was separated. The aqueous layer was extracted with ethyl acetate (3 × 10 mL). The combined organic layer extracts were washed with brine and dried over anhydrous Na_2SO_4. The organic solvent was evaporated under reduced pressure and the residue was purified by flash column chromatography (silica gel, hexane/ethyl acetate 1:1 v/v then switching to hexane/ethyl acetate 1:5 v/v) to yield compound **5** (0.58 g, 45%). M.p.: 85–88°C; ^{1}H-NMR (DMSO-d_6) δ 3.96 (s, 3H), 4.53 (s, 2H), 7.30 (d, 2H, J = 4.1 Hz), 7.64–7.69 (m, 3H), 8.53 (d, 2H, J = 4.5 Hz); MS *m/z*: 265.0 ($M^+ + 3$), 264.0 ($M^+ + 2$), 263.0 ($M^+ + 1$).

*4-(3-(4-Chloro-3-methoxyphenyl)-1H-pyrazol-4-yl) pyridine **6***

Compound **5** (1.0 g, 3.8 mmol) was added to DMF-DMA (5.14 mL, 38.2 mmol) and the mixture was stirred at room temperature for 18 h. The resulting solution was concentrated to dryness to furnish an oil which was used in the next step without purification. To a portion of the oil from the previous step (0.137 g, 0.457 mmol) in EtOH (3 mL) was added hydrazine monohydrate (0.04 mL, 0.76 mmol) and the reaction mixture was stirred overnight at room temperature. Water (5 mL) was added to the reaction mixture and the organics were extracted with ethyl acetate (3 × 5 mL). The combined organic layer extracts were washed with brine and dried over anhydrous Na_2SO_4. After evaporation of the organic solvent, the residue was purified by column chromatography (silica gel, hexane/ethyl acetate 1:1 v/v then switching to hexane/ethyl acetate 1:5 v/v) to yield compound **6** (0.11 g, 81%). M.p.: 248–251°C; ^{1}H-NMR (DMSO-d_6) δ 3.77 (s, 3H), 6.97 (dd, 1H, J = 1.5 Hz, J = 1.6 Hz), 7.18 (s, 1H), 7.28 (d, 2H, J = 5.9 Hz), 7.45 (d, 1H, J = 8.0 Hz), 8.14 (brs, 1H), 8.48 (d, 2H, J = 6.0 Hz), 13.39 (brs, 1H); MS *m/z*: 289.0 ($M^+ + 3$), 288.0 ($M^+ + 2$), 287.0 ($M^+ + 1$).

*Phenyl 3-(4-chloro-3-methoxyphenyl)-4-(pyridin-4-yl)-1H-pyrazole-1-carboxylate **8***

To a solution of compound **6** (0.1 g, 0.35 mmol) in anhydrous THF (10 mL), triethylamine (0.112 g, 1.1 mmol) was slowly added at 0°C. Phenyl chloroformate (0.165 g, 1.05 mmol) was slowly added to the above solution at 0°C. The reaction mixture was stirred at the same temperature for 2 h. The mixture was diluted with H_2O (10 mL) and CH_2Cl_2 (15 mL). The organic layer was separated, and the aqueous layer was extracted again with CH_2Cl_2 (2 × 15 mL). The combined organic layer extracts were washed with brine and dried over anhydrous $MgSO_4$. The organic solvent was evaporated under reduced pressure and the residue (a mixture of compounds **7** and **8**) was used in the next step without further purification.

*General procedure for preparation of compounds **Ia–i***

To a solution of the crude product of the previous step (0.1 g, 0.246 mmol) in dry CH_2Cl_2 (3 mL), a solution of the appropriate ethanamine derivative (0.738 mmol) in dry CH_2Cl_2 (2 mL) and anhydrous K_2CO_3 (68 mg, 0.492 mmol) were added. The reaction mixture was stirred at room temperature for 1 h. Water (5 mL) was added to the reaction mixture and the organic layer was separated. The aqueous layer was extracted with CH_2Cl_2 (2 × 3 mL) and the combined organic layer extracts were washed with brine and dried over anhydrous $MgSO_4$. The organic solvent was evaporated under reduced pressure and the residue was obtained.

*3-(4-Chloro-3-methoxyphenyl)-N-(2-(dimethylamino)-ethyl)-4-(pyridin-4-yl)-1H-pyrazole-1-carboxamide **Ia***

It was purified by flash column chromatography (silica gel, ethyl acetate then switching to ethyl acetate/methanol 4:1 v/v). Yield 72%; mp: 247–248°C; IR (KBr) [cm^{-1}]: 3436, 3093, 3000, 1729, 1603, 1543, 1522, 1478, 1410, 1262, 1245, 1140, 1059, 1035; ^{1}H-NMR (CD_3OD) δ 2.36 (s, 6H), 2.65 (t, 2H, J = 6.5 Hz), 3.57 (t, 2H, J = 6.5 Hz), 3.75 (s, 3H), 7.02 (d, 1H, J = 2.0 Hz), 7.22 (dd, 1H, J = 2.0 Hz, J = 8.1 Hz), 7.33 (d, 1H, J = 8.1 Hz), 7.38 (d, 2H, J = 6.0 Hz), 8.52 (d, 2H, J = 6.1 Hz), 8.61 (s, 1H); ^{13}C-NMR (CD_3OD) δ 154.9, 150.3, 149.7, 141.8, 141.1, 134.8, 134.2, 130.5, 122.8, 121.6, 113.0, 111.8, 112.7, 56.4, 53.5, 43.4, 37.3; MS *m/z*: 402.93 ($M^+ + 3$), 401.96 ($M^+ + 2$), 400.95 ($M^+ + 1$).

*3-(4-Chloro-3-methoxyphenyl)-N-(2-(diethylamino)ethyl)-4-(pyridin-4-yl)-1H-pyrazole-1-carboxamide **Ib***

It was purified by flash column chromatography (silica gel, ethyl acetate then switching to ethyl acetate/methanol 6:1 v/v). Yield 58%; mp: 115–116°C; IR (KBr) [cm^{-1}]: 3439, 3093, 3000, 1729, 1603, 1522, 1497, 1410, 1245, 1140, 1059, 1035; ^{1}H-NMR (DMSO-d_6) δ 0.99 (t, 6H, J = 6.4 Hz), 2.49 (q, 4H, J = 6.3 Hz), 2.63 (t, 2H, J = 5.9 Hz), 3.16 (t, 2H, J = 5.1 Hz), 3.73 (s, 3H), 7.02 (d, 1H, J = 2.0 Hz), 7.22 (dd, 1H, J = 2.1 Hz, J = 7.9 Hz), 7.33 (d, 1H, J = 8.1 Hz), 7.35 (d, 2H, J = 5.9 Hz), 8.48 (brs, 1H), 8.53 (d, 2H, J = 6.1 Hz), 8.74 (s, 1H); MS *m/z*: 431.1 ($M^+ + 3$), 430.1 ($M^+ + 2$), 429.1 ($M^+ + 1$).

*3-(4-Chloro-3-methoxyphenyl)-N-(2-morpholinoethyl)-4-(pyridin-4-yl)-1H-pyrazole-1-carboxamide **Ic***

It was purified by flash column chromatography (silica gel, ethyl acetate then switching to ethyl acetate/methanol 4:1 v/v). Yield 80%; mp: 140–141°C; IR (KBr) [cm^{-1}]: 3350, 3129, 2965, 1734, 1605, 1582, 1512, 1464, 1411, 1339, 1279, 1145, 1119, 1057; ^{1}H-NMR (DMSO-d_6) δ 2.43–2.53 (m, 6H), 3.43 (t, 2H, J = 6.1 Hz), 3.56 (t, 4H, J = 4.0 Hz), 3.77 (s, 3H), 7.03 (d, 1H, J = 2.1 Hz), 7.23 (dd, 1H, J = 2.0 Hz, J = 7.9 Hz), 7.34 (d, 2H, J = 5.9 Hz), 7.50 (d, 1H, J = 6.1 Hz), 8.53 (d, 2H, J = 6.0 Hz), 8.57 (brs, 1H), 8.79 (s, 1H); MS *m/z*: 445.0 ($M^+ + 3$), 444.0 ($M^+ + 2$), 443.0 ($M^+ + 1$).

3-(4-Chloro-3-methoxyphenyl)-N-(2-(2,6-dimethylmorpholino)ethyl)-4-(pyridin-4-yl)-1H-pyrazole-1-carboxamide ***Id***

It was purified by flash column chromatography (silica gel, ethyl acetate). Yield 79%; mp: 87–89°C; IR (KBr) [cm^{-1}]: 3208, 3098, 2975, 1733, 1600, 1583, 1524, 1465, 1408, 1338, 1252, 1148, 1092, 1074; ^{1}H-NMR ($CDCl_3$) δ 1.18 (d, 6H, J = 6.3 Hz), 1.85 (t, 2H, J = 10.1 Hz), 2.62 (t, 2H, J = 6.1 Hz), 2.78 (d, 2H, J = 11.4 Hz), 3.58 (q, 2H, J = 5.8 Hz), 3.68 (t, 2H, J = 5.9 Hz), 3.78 (s, 3H), 7.00 (d, 1H, J = 1.8 Hz), 7.06 (brs, 1H), 7.22 (dd, 1H, J = 1.9 Hz, J = 8.0 Hz), 7.37 (d, 2H, J = 4.5 Hz), 7.68 (d, 1H, J = 7.9 Hz), 8.44 (s, 1H), 8.57 (d, 2H, J = 4.4 Hz); ^{13}C-NMR ($CDCl_3$) δ 158.7, 153.8, 152.9, 143.4, 134.9, 134.0, 132.5, 127.1, 126.7, 125.0, 124.5, 115.8, 75.4, 62.8, 60.0, 59.6, 55.4, 40.6, 22.7; MS *m/z*: 473.0 (M^+ + 3), 472.0 (M^+ + 2), 471.0 (M^+ + 1).

3-(4-Chloro-3-methoxyphenyl)-N-(2-(4-methylpiperazin-1-yl)ethyl)-4-(pyridin-4-yl)-1H-pyrazole-1-carboxamide ***Ie***

It was purified by flash column chromatography (silica gel, ethyl acetate). Yield 44%; mp: 247–249°C; ^{1}H-NMR (DMSO-d_6) δ 2.28 (s, 3H), 2.36–2.51 (m, 8H), 2.63 (t, 2H, J = 5.9 Hz), 3.17 (t, 2H, J = 6.0 Hz), 3.77 (s, 3H), 6.97 (d, 1H, J = 2.0 Hz), 7.17 (dd, 1H, J = 1.9 Hz, J = 7.9 Hz), 7.29 (d, 1H, J = 8.0 Hz), 7.46 (d, 2H, J = 5.4 Hz), 7.95 (brs, 1H), 8.25 (s, 1H), 8.49 (d, 2H, J = 5.2 Hz); MS *m/z*: 457.9 (M^+ + 3), 456.9 (M^+ + 2), 455.9 (M^+ + 1).

N-(2-(4-Acetylpiperazin-1-yl)ethyl)-3-(4-chloro-3-methoxyphenyl)-4-(pyridin-4-yl)-1H-pyrazole-1-carboxamide ***If***

It was purified by flash column chromatography (silica gel, ethyl acetate then switching to ethyl acetate/methanol 4:1 v/v). Yield 66%; mp: 247–248°C; IR (KBr) [cm^{-1}]: 3454, 3094, 3000, 1722, 1703, 1603, 1543, 1522, 1497, 1410, 1262, 1245, 1140, 1099, 1059, 1035; ^{1}H-NMR (CD_3OD) δ 2.10 (s, 3H), 2.48–2.60 (m, 6H), 3.32 (t, 2H, J = 5.0 Hz), 3.52 (t, 4H, J = 4.1 Hz), 3.77 (s, 3H), 7.08 (d, 1H, J = 2.0 Hz), 7.24 (dd, 1H, J = 2.0 Hz, J = 7.9 Hz), 7.36 (d, 1H, J = 8.0 Hz), 7.55 (d, 2H, J = 6.0 Hz), 8.54 (d, 2H, J = 6.1 Hz), 8.60 (s, 1H); MS *m/z*: 486.0 (M^+ + 3), 485.0 (M^+ + 2), 484.0 (M^+ + 1).

3-(4-Chloro-3-methoxyphenyl)-N-(2-(piperidin-1-yl)ethyl)-4-(pyridin-4-yl)-1H-pyrazole-1-carboxamide ***Ig***

It was purified by flash column chromatography (silica gel, ethyl acetate). Yield 80%; mp: 150–152°C; IR (KBr) [cm^{-1}]: 3326, 2937, 1722, 1603, 1522, 1497, 1458, 1245, 1140, 1098, 1058, 1034; ^{1}H-NMR (DMSO-d_6) δ 1.35–1.46 (m, 6H), 2.29 (t, 4H, J = 6.9 Hz), 2.64 (t, 2H, J = 6.0 Hz), 3.08 (t, 2H, J = 5.8 Hz), 3.76 (s, 3H), 6.96 (d, 1H, J = 2.1 Hz), 7.11 (dd, 1H, J = 2.0 Hz, J = 8.1 Hz), 7.28 (d, 1H, J = 8.0 Hz), 7.36 (d, 2H, J = 4.9 Hz), 8.22 (s, 1H), 8.48 (d, 2H, J = 4.6 Hz); MS *m/z*: 443.0 (M^+ + 3), 442.0 (M^+ + 2), 441.0 (M^+ + 1).

3-(4-Chloro-3-methoxyphenyl)-N-(2-(2-methylpiperidin-1-yl)ethyl)-4-(pyridin-4-yl)-1H-pyrazole-1-carboxamide ***Ih***

It was purified by flash column chromatography (silica gel, ethyl acetate). Yield 68%; mp: 219–221°C; ^{1}H-NMR ($CDCl_3$) δ 1.07 (d, 3H, J = 6.2 Hz), 1.54–1.63 (m, 6H), 2.33–2.61 (m, 5H), 3.30 (t, 2H, J = 5.1 Hz), 3.79 (s, 3H), 6.98 (d, 1H, J = 2.1 Hz), 7.10 (dd, 1H, J = 2.0 Hz, J = 8.0 Hz), 7.23 (d, 1H, J = 7.9 Hz), 7.38 (d, 2H, J = 6.0 Hz), 8.23 (s, 1H), 8.54 (d, 2H, J = 5.8 Hz); MS *m/z*: 457.01 (M^+ + 3), 456.08 (M^+ + 2), 455.07 (M^+ + 1).

3-(4-Chloro-3-methoxyphenyl)-4-(pyridin-4-yl)-N-(2-(pyrrolidin-1-yl)ethyl)-1H-pyrazole-1-carboxamide ***Ii***

It was purified by flash column chromatography (silica gel, ethyl acetate). Yield 67%; mp: 244–247°C; IR (KBr) [cm^{-1}]: 3439, 3093, 1729, 1603, 1543, 1522, 1497, 1459, 1245, 1140, 1059, 1035; ^{1}H-NMR ($CDCl_3$) δ 1.84 (t, 4H, J = 5.4 Hz), 2.62 (t, 4H, J = 5.3 Hz), 2.78 (t, 2H, J = 6.1 Hz), 3.31 (t, 2H, J = 6.0 Hz), 3.74 (s, 3H), 7.03 (d, 1H, J = 1.9 Hz), 7.11 (dd, 1H, J = 1.9 Hz, J = 8.1 Hz), 7.24 (d, 1H, J = 8.0 Hz), 7.37 (d, 2H, J = 5.9 Hz), 7.89 (brs, 1H), 8.44 (s, 1H), 8.57 (d, 2H, J = 6.0 Hz); MS *m/z*: 428.9 (M^+ + 3), 427.9 (M^+ + 2), 426.9 (M^+ + 1).

General procedure for preparation of compounds ***IIa–i***

To a solution of compound **Ia–i** (0.1 mmol) in methylene chloride (3 mL), $BF_3 \cdot Me_2S$ (0.13 g, 1 mmol) was added dropwise at room temperature under N_2 and the reaction mixture was stirred at the same temperature for 48 h. The mixture was quenched with saturated aqueous $NaHCO_3$. Ethyl acetate (3 mL) was added and the organic layer was separated. The aqueous layer was extracted with ethyl acetate (3 × 2 mL). The combined organic layer extracts were washed with brine and dried over anhydrous Na_2SO_4. The organic solvent was evaporated under reduced pressure and the residue was obtained.

3-(4-Chloro-3-hydroxyphenyl)-N-(2-(dimethylamino)ethyl)-4-(pyridin-4-yl)-1H-pyrazole-1-carboxamide ***IIa***

It was purified by flash column chromatography (silica gel, ethyl acetate then switching to ethyl acetate/methanol 4:1 v/v). Yield 43%; m.p.: 276–277°C; IR (KBr) [cm^{-1}]: 3392, 3128, 2970, 2925, 1730, 1612, 1593, 1514, 1418, 1346, 1293, 1207, 1155, 1004; ^{1}H-NMR (DMSO-d_6) δ 2.37 (s, 6H), 2.68 (t, 2H, J = 6.9 Hz), 3.60 (t, 2H, J = 6.7 Hz), 6.88 (d, 1H, J = 2.0 Hz), 6.91 (dd, 1H, J = 8.1 Hz, J = 2.1 Hz), 7.08 (d, 1H, J = 8.0 Hz), 7.36 (d, 2H, J = 5.9 Hz), 8.04 (brs, 1H), 8.42 (d, 2H, J = 6.1 Hz), 8.61 (s, 1H); ^{13}C-NMR (DMSO-d_6) δ 153.1, 149.8, 147.7, 141.6, 140.8, 138.3, 134.6, 134.2, 122.1, 120.3, 119.8, 116.2, 113.4, 56.5, 46.3, 37.1; MS *m/z*: 388.97 (M^+ + 3), 387.96 (M^+ + 2), 386.96 (M^+ + 1).

3-(4-Chloro-3-hydroxyphenyl)-N-(2-(diethylamino)ethyl)-4-(pyridin-4-yl)-1H-pyrazole-1-carboxamide ***IIb***

It was purified by flash column chromatography (silica gel, ethyl acetate then switching to ethyl acetate/methanol 6:1 v/v). Yield 55%; mp: 220–222°C; IR (KBr) [cm^{-1}]: 3393, 3126, 2970, 1730, 1610, 1593, 1513, 1417, 1344, 1293, 1206, 1155, 1003; ^{1}H-NMR (CD_3OD) δ 1.14 (t, 6H, J = 7.0 Hz), 2.76 (q, 4H, J = 7.1 Hz), 2.84 (t, 2H, J = 6.6 Hz), 3.45 (t, 2H, J = 6.5 Hz), 6.87 (d, 1H, J = 1.9 Hz), 6.90 (dd, 1H, J = 8.0 Hz, J = 1.9 Hz), 7.05 (d, 1H, J = 8.1 Hz), 7.38 (d, 2H, J = 5.8 Hz), 8.49 (d, 2H, J = 5.9 Hz), 8.64 (s, 1H); MS *m/z*: 416.97 (M^+ + 3), 415.97 (M^+ + 2), 414.97 (M^+ + 1).

3-(4-Chloro-3-hydroxyphenyl)-N-(2-morpholinoethyl)-4-(pyridin-4-yl)-1H-pyrazole-1-carboxamide ***IIc***

It was purified by flash column chromatography (silica gel, ethyl acetate then switching to ethyl acetate/methanol 6:1 v/v). Yield 42%; mp: 220–222°C; IR (KBr) [cm^{-1}]: 3382, 3129, 2920, 1732, 1611, 1562, 1497, 1437, 1352, 1295, 1193, 1148, 1116; ^{1}H-NMR (DMSO-d_6) δ 2.42–2.51 (m, 6H), 3.44 (t, 2H, J = 6.2 Hz), 3.56 (t, 4H, J = 4.1 Hz), 7.00 (d, 1H, J = 2.0 Hz), 7.31-7.38 (m, 3H), 7.48 (d, 1H, J = 8.0 Hz), 8.52 (d, 2H, J = 6.0 Hz), 8.75 (s, 1H); MS *m/z*: 430.91 (M^+ + 3), 429.91 (M^+ + 2), 428.91 (M^+ + 1).

3-(4-Chloro-3-hydroxyphenyl)-N-(2-(2,6-dimethylmorpholino)ethyl)-4-(pyridin-4-yl)-1H-pyrazole-1-carboxamide ***IId***

It was purified by flash column chromatography (silica gel, ethyl acetate then switching to ethyl acetate/methanol 10:1 v/v). Yield 56%; mp: 188–189°C; IR (KBr) [cm^{-1}]: 3398, 3128, 2925, 1728, 1611, 1563, 1495, 1434, 1326, 1295, 1144, 1087, 1053, ^{1}H-NMR (CD_3OD) δ 1.15 (d, 6H, $J = 8.3$ Hz), 2.59–2.94 (m, 6H), 3.36 (t, 2H, $J = 4.9$ Hz), 3.58–3.69 (m, 2H), 6.91 (d, 1H, $J = 2.0$ Hz), 6.99 (dd, 1H, $J = 7.9$ Hz, $J = 2.1$ Hz), 7.25 (d, 1H, $J = 8.0$ Hz), 7.38 (d, 2H, $J = 6.0$ Hz), 8.49 (d, 2H, $J = 6.0$ Hz), 8.62 (s, 1H); MS *m/z*: 458.74 ($M^+ + 3$), 457.87 ($M^+ + 2$), 456.83 ($M^+ + 1$).

3-(4-Chloro-3-hydroxyphenyl)-N-(2-(4-methylpiperazin-1-yl)ethyl)-4-(pyridin-4-yl)-1H-pyrazole-1-carboxamide ***IIe***

It was purified by flash column chromatography (silica gel, ethyl acetate). Yield 49%; mp: 257–259°C; IR (KBr) [cm^{-1}]: 3400, 3126, 2973, 1733, 1610, 1592, 1512, 1404, 1357, 1293, 1155, 1094, 1003; ^{1}H-NMR (DMSO-d_6) δ 2.26 (s, 3H), 2.46–2.58 (m, 8H), 2.64 (t, 2H, $J = 6.1$ Hz), 3.12 (t, 2H, $J = 5.9$ Hz), 6.98 (d, 1H, $J = 1.9$ Hz), 7.14 (dd, 1H, $J = 2.0$ Hz, $J = 8.1$ Hz), 7.27 (d, 1H, $J = 7.9$ Hz), 7.52 (d, 2H, $J = 6.0$ Hz), 8.12 (brs, 1H), 8.44 (d, 2H, $J = 6.1$ Hz), 8.53 (s, 1H); MS *m/z*: 443.95 ($M^+ + 3$), 442.95 ($M^+ + 2$), 441.95 ($M^+ + 1$).

N-(2-(4-Acetylpiperazin-1-yl)ethyl)-3-(4-chloro-3-hydroxyphenyl)-4-(pyridin-4-yl)-1H-pyrazole-1-carboxamide ***IIf***

It was purified by flash column chromatography (silica gel, ethyl acetate then switching to ethyl acetate/methanol 4:1 v/v). Yield 61%; mp: 259–260°C; IR (KBr) [cm^{-1}]: 3347, 3126, 2932, 1731, 1611, 1510, 1433, 1346, 1155, 1003; ^{1}H-NMR (CD_3OD) δ 2.08 (s, 3H), 2.47–2.59 (m, 6H), 2.95 (t, 2H, $J = 5.0$ Hz), 3.46 (t, 4H, $J = 5.1$ Hz), 6.99 (d, 1H, $J = 1.9$ Hz), 7.16 (dd, 1H, $J = 1.9$ Hz, $J = 8.0$ Hz), 7.30 (d, 1H, $J = 7.7$ Hz), 7.48 (d, 2H, $J = 5.9$ Hz), 8.12 (s, 1H), 8.56 (d, 2H, $J = 5.7$ Hz); MS *m/z*: 472.0 ($M^+ + 3$), 471.0 ($M^+ + 2$), 470.0 ($M^+ + 1$).

3-(4-Chloro-3-hydroxyphenyl)-N-(2-(piperidin-1-yl)ethyl)-4-(pyridin-4-yl)-1H-pyrazole-1-carboxamide ***IIg***

It was purified by flash column chromatography (silica gel, ethyl acetate). Yield 44%; mp: 273–276°C; IR (KBr) [cm^{-1}]: 3416, 2932, 1730, 1612, 1510, 1433, 1347, 1154, 1002; ^{1}H-NMR (CD_3OD) δ 1.38–1.56 (m, 6H), 2.24 (t, 4H, $J = 7.1$ Hz), 2.66 (t, 2H, $J = 6.2$ Hz), 3.22 (t, 2H, $J = 6.0$ Hz), 6.97 (d, 1H, $J = 2.0$ Hz), 7.09 (dd, 1H, $J = 1.9$ Hz, $J = 8.1$ Hz), 7.26 (d, 1H, $J = 7.8$ Hz), 7.39 (d, 2H, $J = 5.7$ Hz), 8.04 (s, 1H), 8.49 (d, 2H, $J = 5.6$ Hz); MS *m/z*: 428.94 ($M^+ + 3$), 427.94 ($M^+ + 2$), 426.95 ($M^+ + 1$).

3-(4-Chloro-3-hydroxyphenyl)-N-(2-(2-methylpiperidin-1-yl)ethyl)-4-(pyridin-4-yl)-1H-pyrazole-1-carboxamide ***IIh***

It was purified by flash column chromatography (silica gel, ethyl acetate then switching to ethyl acetate/methanol 10:1 v/v). Yield 50%; mp: 273–276°C; IR (KBr) [cm^{-1}]: 3408, 2932, 1728, 1611, 1509, 1435, 1345, 1156, 1002; ^{1}H-NMR (CD_3OD) δ 1.06 (d, 3H, $J = 5.9$ Hz), 1.36–1.48 (m, 6H), 2.30–2.59 (m, 5H), 3.08 (t, 2H, $J = 5.4$ Hz), 6.92 (d, 1H, $J = 1.8$ Hz), 7.07 (dd, 1H, $J = 1.9$ Hz, $J = 7.9$ Hz), 7.21 (d, 1H, $J = 7.7$ Hz), 7.37 (d, 2H, $J = 5.8$ Hz), 8.50 (d, 2H, $J = 5.8$ Hz), 8.59 (s, 1H); MS *m/z*: 443.0 ($M^+ + 3$), 442.0 ($M^+ + 2$), 441.0 ($M^+ + 1$).

3-(4-Chloro-3-hydroxyphenyl)-4-(pyridin-4-yl)-N-(2-(pyrrolidin-1-yl)ethyl)-1H-pyrazole-1-carboxamide ***IIi***

It was purified by flash column chromatography (silica gel, ethyl acetate). Yield 40%; mp: >300°C; IR (KBr) [cm^{-1}]: 3376, 2974, 2802, 1733, 1610, 1500, 1424, 1368, 1153, 1089, 1003; ^{1}H-NMR (CD_3OD) δ 1.78 (t, 4H, $J = 5.2$ Hz), 2.31 (t, 4H, $J = 5.3$ Hz), 2.62 (t, 2H, $J = 6.3$ Hz), 3.01 (t, 2H, $J = 6.1$ Hz), 7.01 (d, 1H, $J = 2.0$ Hz), 7.09 (dd, 1H, $J = 1.8$ Hz, $J = 7.8$ Hz), 7.22 (d, 1H, $J = 8.0$ Hz), 7.36 (d, 2H, $J = 5.8$ Hz), 8.53 (d, 2H, $J = 6.0$ Hz), 8.62 (s, 1H); MS *m/z*: 414.98 ($M^+ + 3$), 413.96 ($M^+ + 2$), 412.97 ($M^+ + 1$).

Evaluation of the biological activity

A375P cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in Dulbecco's modified eagle medium (DMEM, Welgene, Daegu, Korea) supplemented with 10% foetal bovine serum (FBS, Welgene, Daegu, Korea) and 1% penicillin/streptomycin (Welgene, Daegu, Korea) 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 with various concentrations (3-fold serial dilution, 12 points) of test compounds. The cells were incubated for 48 h after treatment with the 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, USA). The IC_{50} was calculated using GraphPad Prism 4.0 software.

Molecular docking methodology

Docking studies were performed using MOE 2008.10. With this purpose, the crystal structure of V600E-b-Raf Kinase oncogenic mutant was obtained from Protein Data Bank [29] (PDB ID: 3OG7) in order to prepare the protein for docking study. Docking procedure was followed using the standard protocol implemented in MOE 2008.10 and the geometry of resulting complex was studied using the MOE's pose viewer utility.

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