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Original article

New diarylureas and diarylamides containing 1,3,4-triarylpyrazole scaffold: Synthesis, antiproliferative evaluation against melanoma cell lines, ERK kinase inhibition, and molecular docking studies

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ABSTRACT

Synthesis of a new series of diarylureas and diarylamides possessing 1,3,4-triarylpyrazole scaffold is described. Their *in vitro* antiproliferative activities against 9 human melanoma cell lines were tested. Compounds **12**, **13**, **15**, and **21**–**23** showed the highest potency against A375P melanoma cell line. In addition, compounds **10**–**15** and **19**–**24** showed high potency over the NCI 8 tested melanoma cell-lines panel. The IC_{50} values for compound **23** were 0.36 μM and 0.84 μM over LOX IMVI and M14 cell lines, respectively. Compounds **21** and **23** showed high, dose-dependent inhibition of ERK kinase. Virtual screening was carried out through docking of compound **21** into the domain of V600E-B-RAF and the binding mode was studied.

1. Introduction

The RAS-RAF-MEK-ERK signalling pathway (ERK pathway) plays an important role in tumorigenesis and cancer progression [1]. Sorafenib (Nexavar), a diarylurea derivative, targets ERK pathway. It inhibits basal phosphorylation of ERK (pERK) in numerous cancer cell lines *in vitro*, including melanoma cell lines, independent of their K-RAS and B-RAF mutational status [2]. In addition, sorafenib is a well known inhibitor of B-RAF. Dysregulated signaling through RAF kinase isoforms has been detected in ~30% of human cancers [3]. Constitutive B-RAF activity can be caused by activating oncogenic mutations, such as B-RAF V600E mutation, which is prevalent in melanomas (63%) [4].

Melanoma is the most serious type of skin cancer as a malignant tumor of melanocytes [5]. Early stage melanoma can be cured surgically. However, melanoma metastasizing to major organs (stage IV) is virtually incurable [6]. Patients with advanced melanoma have a median survival time of less than one year, and the estimated 5-year survival rate is less than 15% [7,8]. 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 [9–11].

A number of reports have recently highlighted diarylureas and diarylamides as potential antiproliferative agents against melanoma cell line [12–19]. Sorafenib (Nexavar) is a diarylurea derivative that has been extensively used in clinical trials [20]. Encouraged by the interesting antiproliferative activity of diarylurea and diarylamide derivatives, a new series of diarylureas and diarylamides containing 1,3,4-triarylpyrazole scaffold was synthesized (Fig. 1). Their *in vitro* antiproliferative activities against nine human melanoma cell lines are reported. ERK kinase inhibitory activity of compounds **21** and **23**, in addition to molecular docking of compound **21** into the domain of V600E-B-RAF are also reported.

2. Results and discussion

2.1. Chemistry

The target triarylpyrazole compounds **9**–**24** were synthesized according to the sequence of reactions illustrated in Scheme 1.

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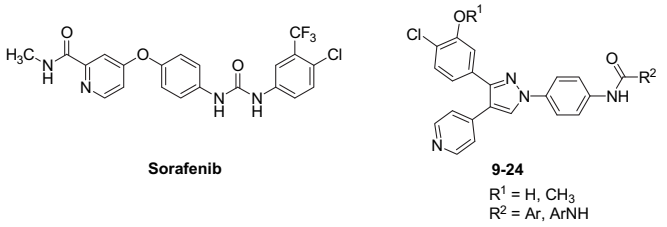


Fig. 1. Structures of Sorafenib and the target compounds **9**–**24**.

Methylation of the phenolic hydroxyl group of 2-chloro-5-methylphenol (**1**) using dimethyl sulfate gave 1-chloro-2-methoxy-4-methylbenzene (**2**) [21]. Oxidation of the methyl group of **2** using $KMnO_4$ produced 4-chloro-3-methoxybenzoic acid (**3**) [22], 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. 1,3,4-Triarylpyrazole derivative **8** with amino group was prepared through *N*-arylation of **6** using 1-iodo-4-nitrobenzene in the presence of anhydrous K_2CO_3, CuI, L-proline, and subsequent reduction of the nitro group of **7** using palladium over carbon in hydrogen atmosphere. Reaction of the amino group of **8** with the appropriate aryl isocyanate derivatives afforded the corresponding urea derivatives **9**–**12**. Synthesis of the methoxy compounds **13**–**16** with amide moiety as a linker was carried out by condensation of the amino compound **8** with the appropriate benzoic acid derivatives in the presence of HOBt, EDCI, and triethylamine. Demethylation of the methoxy group of **9**–**16** using BBr_3 afforded the corresponding hydroxyl derivatives **17**–**24**.

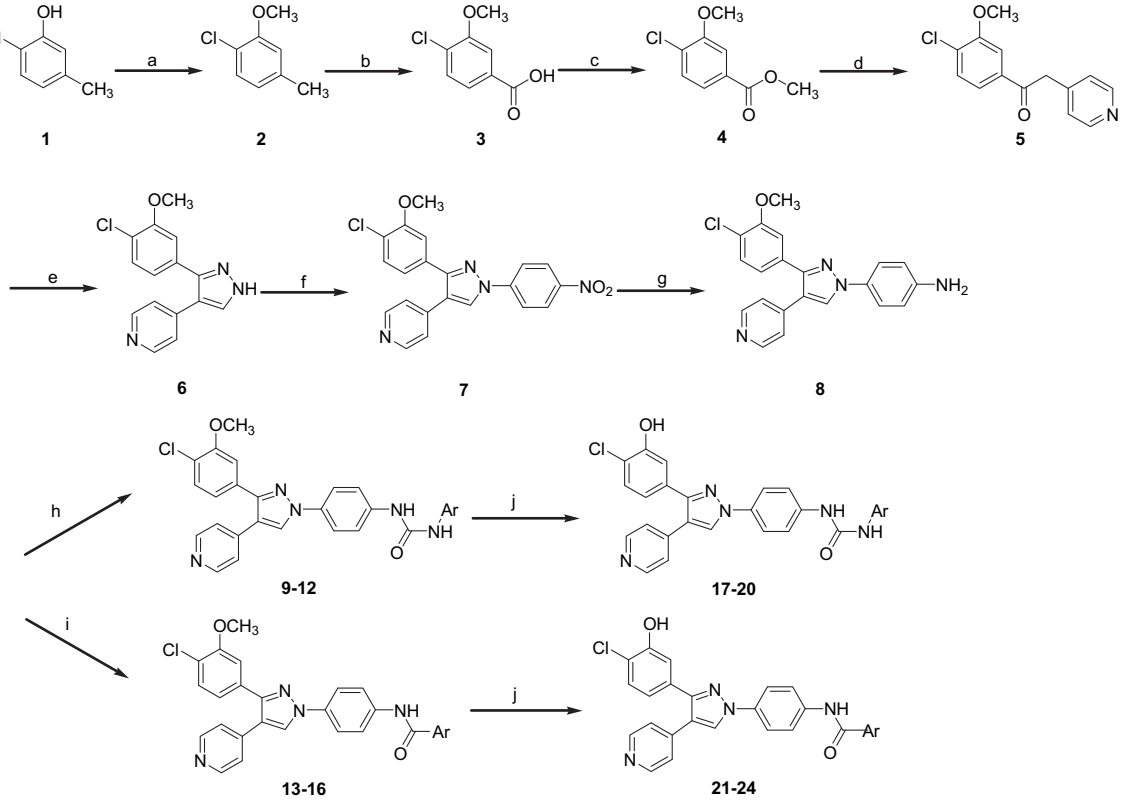
2.2. Biological evaluation

2.2.1. Antiproliferative activity against A375P human melanoma cell line

The antiproliferative activity of the target compounds against A375P human melanoma cell line was tested. Their ability to inhibit the growth of A375P cell line is summarized in Table 1. The results are expressed as IC_{50} values. Sorafenib was selected as a reference standard.

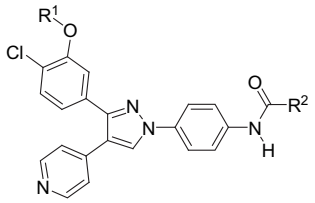
As listed in Table 1, most of the compounds showed moderate activity, while compounds **15** and **21**–**23** having IC_{50} values ranging from 6.7 to 9.8 μM were more potent than Sorafenib (IC_{50} = 11.5 μM). In addition, compounds **12** and **13** exhibited similar potency to that of Sorafenib. Compounds **12**, **13** and **15** possess a methoxy group while compounds **21**–**23** possess a hydroxyl group. In addition, compound **12** possesses a urea linker while compounds **13**, **15**, and **21**–**23** have an amide group as a linker.

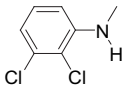
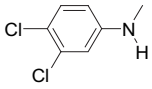
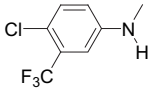
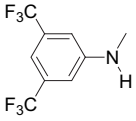
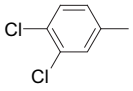
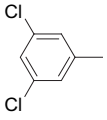
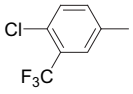
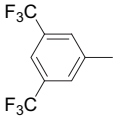
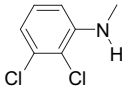
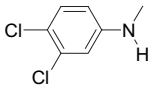
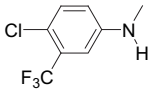
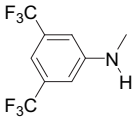
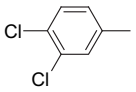
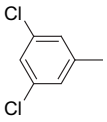
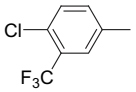
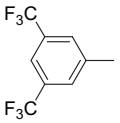
The hydroxyl compounds **17**, **18**, and **21**–**23** were more potent than the corresponding methoxy derivatives **9**, **10**, and **13**–**15**. These results suggest that the hydroxyl group on position 3 of the *p*-chlorophenyl ring at pyrazole ring is optimal for the activity. This may be attributed to hydrogen bond formation at the receptor site. Docking of **21** structure into the domain of V600E-B-Raf kinase crystal structure revealed the formation of one hydrogen bond by the hydroxyl group at the binding site (molecular docking part). Or



Scheme 1. Reagents and conditions: (a) $(CH_3)_2SO_4$, K_2CO_3, acetone, reflux, 1 h; (b) $KMnO_4$, C_5H_5N, H_2O, 50 °C, 24 h, then rt, 13 h; (c) acetyl chloride, CH_3OH, rt, 15 h; (d) 4-picoline, LHMDS, THF, rt, overnight; (e) (i) DMF-DMA, rt, 18 h, (ii) hydrazine monohydrate, C_2H_5OH, rt, overnight; f) 1-iodo-4-nitrobenzene, K_2CO_3, CuI, L-proline, DMSO, 90 °C, 8 h; g) H_2, Pd/C, THF, rt, 2 h; h) appropriate aryl isocyanate, THF, rt, 12 h; i) appropriate benzoic acid derivative, HOBt, EDCI, TEA, DMF, 80 °C, 12 h; j) BBr_3, CH_2Cl_2, -78 °C, 30 min; then rt, 1 h.

Table 1
Antiproliferative activity of the target compounds **9**–**24** against A375P cell line.



Compound No.	R^1	R^2	IC_{50} (μM)
9	CH_3		>20
10	CH_3		>20
11	CH_3		14.0
12	CH_3		11.8
13	CH_3		13.0
14	CH_3		>20
15	CH_3		9.8
16	CH_3		>20
17	H		16.8
18	H		13.5
19	H		14.9
20	H		>20
21	H		6.7
22	H		6.8
23	H		6.8
24	H		>20
Sorafenib			11.5

the *o*-chloro-phenolic moiety may induce additional DNA damage effect in the presence of copper and oxygen.

The effects of the amide and urea moieties as linkers on the activity were also investigated. The amide derivatives **13**, **15**, **21**, and **23** showed higher potency than the corresponding urea derivatives **10**, **11**, **18**, and **19**. In addition, the most potent compounds **15** and **21**–**23** possess an amide linker. They are more potent than the diarylurea reference standard, Sorafenib.

As observed from the data, the best results were obtained with compounds **21**–**23** which possess hydroxyl group on the *p*-chlorophenyl ring and amide linker. So we can conclude that these two moieties are optimum for antiproliferative activity of this series of compounds against A375P human melanoma cell line.

2.2.2. In vitro anticancer screening over 8 melanoma cell lines at the NCI

After initial single-dose screening of the 16 target compounds at the National Cancer Institute (NCI) [23], Bethesda, Maryland, USA, 12 compounds, **10**–**15** and **19**–**24**, with interesting inhibitory activity in single-dose testing were further tested in a five-dose testing mode, in order to determine their potency over 8 melanoma cell lines. For each of these compounds, the IC_{50} (the concentration producing 50% inhibition) values were recorded. The antiproliferative activity of these twelve compounds over 8 melanoma cell lines are summarized in Table 2.

As shown in Table 2, the twelve tested compounds have shown good potency over the 8 tested cell lines. The IC_{50} values of the tested compounds were less than 10 μM over all the cell lines, except for compounds **13** and **14** which exhibited lower potency against few cell lines. Of special interest, the IC_{50} of compound **23** was in sub-micromolar scale against 2 cell lines. It was 0.36 μM and 0.84 μM in case of LOX IMVI and M14 cell lines, respectively. This high potency of compound **23** together with its result against A375P cell line suggest that hydroxyl group, amide linker, and 4-chloro-3-(trifluoromethyl)phenyl terminal moiety are the most optimum for antiproliferative activity of this series of compounds against melanoma cell lines.

2.2.3. ERK kinase inhibition

In order to study the mechanism of action of this series of compounds, the most potent compound against A375P human melanoma cell line **21** and the most potent derivative against the

Table 2
Antiproliferative activity of compounds **10**–**15** and **19**–**24** over 8 melanoma cell lines.

Compound No.	Melanoma cell line (IC_{50}, μM)							
	LOX IMVI	M14	MDA-MB-435	SK-MEL-2	SK-MEL-28	SK-MEL-5	UACC-257	UACC-62
10	1.98	2.56	2.87	3.44	3.94	2.84	3.17	3.35
11	1.43	1.74	1.60	1.63	1.88	1.57	1.64	1.77
12	1.63	1.99	1.88	1.70	1.93	1.53	2.30	1.55
13	2.76	22.40	7.13	5.74	40.30	1.65	>100	6.34
14	3.09	7.39	>100	>100	>100	12.00	>100	4.58
15	1.56	1.92	2.52	3.15	2.80	1.59	5.83	2.18
19	1.44	1.43	1.32	1.70	1.63	1.53	1.51	1.70
20	1.63	1.76	1.77	1.80	2.01	1.67	2.01	2.23
21	1.45	2.35	2.73	4.05	2.94	1.15	4.74	3.22
22	1.19	1.51	3.78	6.91	2.75	1.40	5.70	2.73
23	0.36	0.84	1.20	1.58	1.08	1.07	2.55	2.03
24	2.54	6.34	4.11	6.60	4.88	1.55	8.94	14.4

NCI 8-melanoma cell line panel **23** were screened for ERK kinase inhibitory activity. The ERK-containing A375P cell lysate was treated with three different concentrations of the test compounds (1, 3, and 10 μM) and their inhibitory activities were compared with that of sorafenib. The results showed that **21**, **23**, and sorafenib significantly suppressed phosphorylation of ERK1/2 in a dose-dependent manner. The dichlorophenyl derivative **21** demonstrated higher ERK inhibition than **23** with 4-chloro-3-(trifluoromethyl) phenyl terminal moiety (Fig. 2). These compounds may inhibit melanoma cells proliferation through ERK kinase inhibition.

2.2.4. Molecular docking

V600E-B-RAF kinase is over-expressed in most of melanoma cases [4]. We assumed that the active target compounds might demonstrate antiproliferative activity against melanoma cell lines through inhibition of V600E-B-RAF. In this study, we performed molecular docking of compound **21**, as a representative example of this series of compounds, into the domain of V600E-B-RAF kinase. All calculations were performed using MOE 2008.10 software [24] 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) [25]. The automated docking program of MOE 2008.10 was used for docking of **21** into the domain of V600E-B-RAF kinase. The complex was energy-minimized with a MMFF94x force-field [26] till the gradient convergence 0.01 kcal/mol was reached. The docking study has revealed that the ligand **21** has bound in the active site of one of the protomers in the protein dimer through the formation of two strong hydrogen bonds and one arene–cation interaction between the binding site and the ligand. The hydrogen bonds have been formed between the phenolic hydroxyl hydrogen atom and Val-B590 (1.75 Å); and between the pyrazole *N*2 atom and Lys-B591 (1.61 Å). The arene–cation interaction occurred between the phenolic benzene ring of **21** and Lys-B591 protonated amino group. Figs. 3 and 4 demonstrate the binding model of compound **21** with the binding site of V600E-B-RAF. The results of this molecular docking study can support the postulation that our active compounds may inhibit the growth of melanoma cell lines through inhibition of B-RAF kinase, similar to sorafenib.

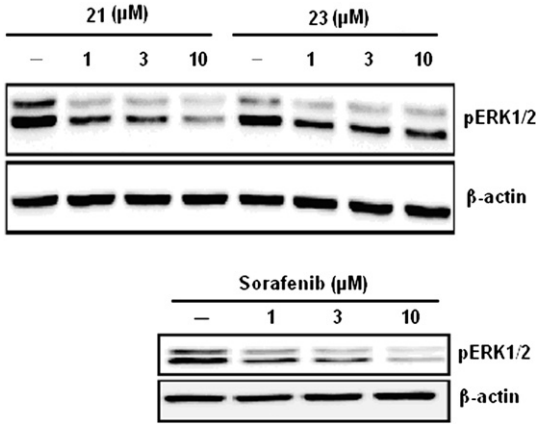


Fig. 2. Inhibition of ERK kinase activity by compounds **21**, **23**, and sorafenib.

3. Conclusions

We have designed and synthesized a new series of diarylureas and diarylamides containing 1,3,4-triarylpyrazole scaffold. A structure-activity relationship (SAR) study has been made to correlate between the compounds structures and antiproliferative activities. Compounds **12**, **13**, **15**, and **21**–**23** showed the highest potency against A375P human melanoma cell line. In addition, all the twelve tested compounds **10**–**15** and **19**–**24** showed good potency over the NCI 8 tested melanoma cell lines panel. Compounds **21** and **23** showed significant and dose-dependent ERK kinase inhibitory activity. In addition, we have studied the binding mode of compound **21** with V600E-B-RAF crystal structure (molecular docking). Based on ERK inhibition data together with the docking results, we may assume that the target compounds may inhibit the growth of melanoma cell lines through inhibition of ERK and/or V600E-B-RAF kinases. Among all the target compounds, compound **23** with hydroxyl group, amide linker, and 4-chloro-3-(trifluoromethyl)phenyl terminal moiety showed the best potency against almost all the tested cell lines. It may be considered as a promising lead for future design of anti-proliferative agents for melanoma.

4. Experimental

4.1. General

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 Detector (Waters, Milford, MA, USA). Nuclear magnetic resonance (NMR) spectroscopy was performed using a Bruker ARX-300, 300 MHz and a Bruker ARX-400, 400 MHz spectrometers (Bruker Bioscience, Billerica, MA, USA) with TMS as an internal standard. Purities of the target compounds **9**–**24** (>95%) were determined by LC-MS analysis using the following system: Waters 2998 photodiode array detector, Waters 3100 mass detector, Waters SFO system fluidics organizer, Waters 2545 binary gradient module, Waters reagent manager, Waters 2767 sample manager, Sunfire™ C18

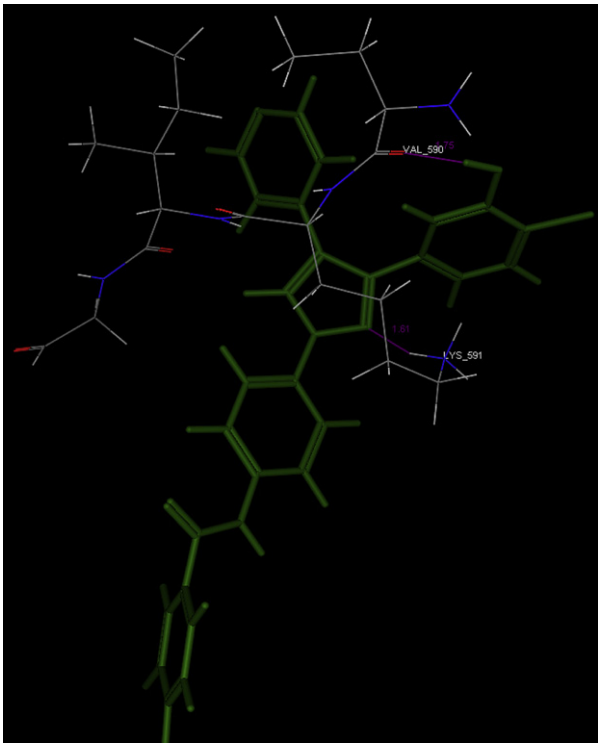


Fig. 3. Interaction between compound **21** (green) and V600E-B-RAF, hydrogen bonds are shown as purple lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

column (4.6 × 50 mm, 5 μm particle size); Solvent gradient = 95% A at 0 min, 1% A at 5 min; solvent A: 0.035% trifluoroacetic acid (TFA) in water; solvent B: 0.035% TFA in CH_3OH; flow rate = 3.0 mL/min; the AUC was calculated using Waters MassLynx 4.1 software. Unless otherwise noted, all solvents and reagents were commercially available and used without further purification.

4.2. 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*: 157.5 $[M + H]^+$.

4.3. 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 x 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%). mp: 215–216 °C (lit. mp: 217–219 °C [22]).

4.4. 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. mp: 49–50 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.62–7.55 (m, 3H), 3.94 (s, 3H), 3.88 (s, 3H).

4.5. 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

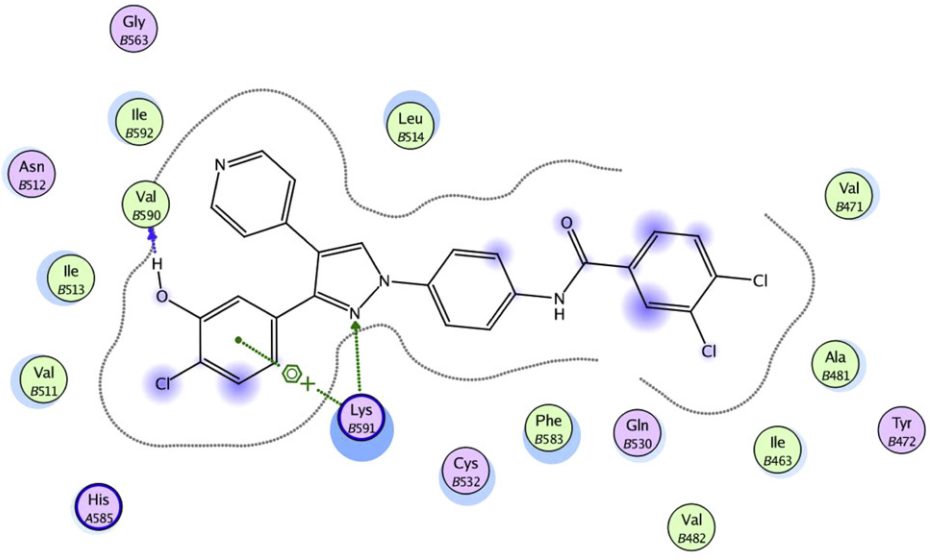


Fig. 4. 2D-presentation for the binding interactions of compound **21** with V600E-B-RAF kinase domain.

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%). mp: 85–88 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.53 (d, 2H, J = 4.5 Hz), 7.69–7.64 (m, 3H), 7.30 (d, 2H, J = 4.1 Hz), 4.53 (s, 2H), 3.96 (s, 3H).

4.6. 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%). mp: 248–251 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.39 (brs, 1H), 8.48 (d, 2H, J = 6.0 Hz), 8.14 (brs, 1H), 7.45 (d, 1H, J = 8.0 Hz), 7.28 (d, 2H, J = 5.9 Hz), 7.18 (s, 1H), 6.97 (dd, 1H, J = 1.5 Hz, J = 1.6 Hz), 3.77 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 154.9, 150.2, 141.3, 130.5, 122.8, 121.6, 117.0, 112.9, 56.4; MS m/z: 287.0 [M^+ + 1].

4.7. 4-[3-(4-chloro-3-methoxyphenyl)-1-(4-nitrophenyl)-1H-pyrazol-4-yl]pyridine (**7**)

A mixture of compound **6** (0.5 g, 1.7 mmol), 1-iodo-4-nitrobenzene (0.9 g, 3.5 mmol), K_2CO_3 (0.7 g, 5.2 mmol), CuI (0.033 g, 0.2 mmol), and L-proline (0.04 g, 0.2 mmol) in DMSO (7 mL) was heated at 90 °C under nitrogen atmosphere for 8 h. The cooled reaction mixture was partitioned between water and ethyl acetate. The organic phase was washed with brine (3 times) 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 1:5 v/v) to yield compound **7** (0.8 g, 86%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.26 (s, 1H), 8.58 (d, 2H, J = 3.3 Hz), 8.43 (d, 2H, J = 6.9 Hz), 8.24 (d, 2H, J = 6.9 Hz), 7.50 (d, 1H, J = 6.1 Hz), 7.37 (d, 2H, J = 3.7 Hz), 7.26 (s, 1H), 7.06 (d, 1H, J = 6.1 Hz), 3.78 (s, 3H).

4.8. 4-[3-(4-chloro-3-methoxyphenyl)-1-(4-aminophenyl)-1H-pyrazol-4-yl]pyridine (**8**)

A mixture of compound **7** (0.5 g, 1.2 mmol) and Pd/C (0.5 g) in THF (5 mL) was stirred at room temperature under hydrogen atmosphere for 2 h. The mixture was filtered through celite and the filtrate was evaporated under reduced pressure to give compound **8** (0.4 g, 86.4%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.55 (d, 2H, J = 4.6 Hz), 7.99 (s, 1H), 7.54 (d, 2H, J = 8.7 Hz), 7.34 (d, 1H, J = 8.1 Hz), 7.24 (s, 1H), 7.15 (s, 1H), 7.06 (d, 1H, J = 8.1 Hz), 6.78 (d, 2H, J = 8.7 Hz), 3.81 (s, 3H).

4.9. 1-{4-[3-(4-chloro-3-methoxyphenyl)-4-(pyridin-4-yl)-1H-pyrazol-1-yl]phenyl}-3-(2,3-dichlorophenyl)urea (**9**)

To a solution of compound **8** (50 mg, 0.1 mmol) in anhydrous THF (1 mL), a solution of 2,3-dichlorophenyl isocyanate (25 mg, 0.1 mmol) in THF (1 mL) was added dropwise at room temperature under N_2. The reaction mixture was stirred at room temperature for 12 h. The mixture was evaporated under reduced pressure, and the residue was purified by column chromatography (silica gel, hexane-ethyl acetate 1:5 v/v) to yield compound **9** (40 mg, 53.3%). mp: 132–135 °C (dec.); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.57 (d, 2H, J = 6.0 Hz), 8.13–8.10 (m, 2H), 7.76 (d, 2H, J = 9.0 Hz), 7.72 (d, 1H, J = 7.5 Hz), 7.64 (d, 2H, J = 9.0 Hz), 7.51 (d, 1H, J = 10.8 Hz), 7.52 (s, 1H), 7.36 (d, 1H, J = 8.0 Hz), 7.15 (s, 1H), 7.07 (d, 1H, J = 8.0 Hz), 3.82 (s, 3H); ESI-MS: 566.0 [M + 1]$^+$.

The synthesis of compounds **10**–**12** was carried out by the same procedure as described for preparation of **9**.

4.9.1. 1-{4-[3-(4-chloro-3-methoxyphenyl)-4-(pyridin-4-yl)-1H-pyrazol-1-yl]phenyl}-3-(3,4-dichlorophenyl)urea (**10**)

Yield 43%; mp: 225–227 °C (dec.); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.58 (d, 2H, J = 5.2 Hz), 8.10 (s, 1H), 7.78 (d, 2H, J = 8.7 Hz), 7.63 (s, 1H), 7.53 (d, 2H, J = 8.0 Hz), 7.40–7.35 (m, 3H), 7.15 (s, 1H), 7.07 (d, 1H, J = 8.5 Hz), 6.53 (s, 1H), 6.48 (s, 1H), 3.82 (s, 3H); ESI-MS: 566.0 [M + 1]$^+$.

4.9.2. 1-{4-[3-(4-chloro-3-methoxyphenyl)-4-(pyridin-4-yl)-1H-pyrazol-1-yl]phenyl}-3-(4-chloro-3-(trifluoromethyl)phenyl)urea (**11**)

Yield 29%; mp: 139–140 °C (dec.); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.58 (d, 2H, J = 6.1 Hz), 8.09 (s, 1H), 7.76 (d, 2H, J = 8.9 Hz), 7.70 (d, 1H, J = 2.4 Hz), 7.61 (d, 1H, J = 8.8 Hz), 7.51 (d, 2H, J = 8.9 Hz), 7.44 (d, 1H, J = 8.6 Hz), 7.36 (d, 1H, J = 8.1 Hz), 7.14 (s, 1H), 7.06 (d, 1H, J = 8.1 Hz), 6.91 (s, 1H), 6.82 (s, 1H), 3.81 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 162.3, 155.0, 152.8, 150.3, 148.5, 140.5, 139.7, 138.5, 134.3, 132.4, 129.8, 129.2, 128.7, 126.3, 123.6, 122.9, 121.6, 119.8, 119.7, 119.4, 117.3, 113.3, 56.6; ESI-MS: 599.1 [M + 1]$^+$.

4.9.3. 1-{4-[3-(4-chloro-3-methoxyphenyl)-4-(pyridin-4-yl)-1H-pyrazol-1-yl]phenyl}-3-(3,5-bis(trifluoromethyl)phenyl)urea (**12**)

Yield 36%; mp: 140–143 °C (dec.); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.59 (d, 2H, J = 6.1 Hz), 8.11 (s, 1H), 7.92 (s, 2H), 7.78 (d, 2H, J = 8.9 Hz), 7.55 (t, 3H, J = 10.2 Hz), 7.36 (d, 2H, J = 12.6 Hz), 7.15 (s, 1H), 7.08 (s, 1H), 6.84 (s, 1H), 3.82 (s, 3H); ESI-MS: 633.0 [M + 1]$^+$.

4.10. 3,4-Dichloro-N-{4-[3-(4-chloro-3-methoxyphenyl)-4-(pyridin-4-yl)-1H-pyrazol-1-yl]phenyl}benzamide (**13**)

A mixture of compound **8** (50 mg, 0.1 mmol), 3,4-dichlorobenzoic acid (38 mg, 0.2 mmol), HOBt (36 mg, 0.3 mmol), and EDCI (38 mg, 0.2 mmol) in DMF (1.0 mL) was cooled to 0 °C under nitrogen atmosphere. Triethylamine (0.03 mL, 0.2 mmol) was added thereto at the same temperature. The mixture was then stirred at 80 °C for 12 h. The reaction mixture was cooled and then partitioned between H_2O and ethyl acetate. The organic layer was separated and the aqueous layer was extracted with ethyl acetate (3 x 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) to yield compound **13** (26 mg, 36.6%). mp: 201–202 °C (dec.); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.58 (d, 2H, J = 4.5 Hz), 8.13 (s, 1H), 8.00 (d, 1H, J = 2.1 Hz), 7.91 (brs, 1H), 7.81 (brs, 4H), 7.73 (d, 1H, J = 8.3 Hz), 7.60 (d, 1H, J = 8.3 Hz), 7.36 (d, 1H, J = 8.1 Hz), 7.28 (brs, 1H), 7.15 (s, 1H), 7.08 (d, 1H, J = 8.1 Hz), 3.82 (s, 3H); ESI-MS: 551.0 [M + 1]$^+$.

The synthesis of compounds **14**–**16** was carried out by the same procedure as described for preparation of **13**.

4.10.1. 3,5-Dichloro-N-{4-[3-(4-chloro-3-methoxyphenyl)-4-(pyridin-4-yl)-1H-pyrazol-1-yl]phenyl}benzamide (**14**)

Yield 36%; mp: 216–218 °C (dec.); ^{1}NMR (300 MHz, $CDCl_3$) δ 8.58 (d, 2H, J = 6.0 Hz), 8.13 (s, 1H), 7.96 (brs, 1H), 7.81 (s, 4H), 7.77

(d, 2H, J = 1.7 Hz), 7.56 (brs, 1H), 7.36 (d, 1H, J = 8.1 Hz), 7.28 (brs, 1H), 7.16 (s, 1H), 7.07 (d, 1H, J = 8.1 Hz), 3.82 (s, 3H); ESI-MS: 551.1 $[M + 1]^+$.

4.10.2. 4-Chloro-3-trifluoromethyl-N-{4-[3-(4-chloro-3-methoxyphenyl)-4-(pyridin-4-yl)-1H-pyrazol-1-yl]phenyl} benzamide (**15**)

Yield 29%; mp: 190–193 °C (dec.); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.58 (d, 2H, J = 4.5 Hz), 8.22 (brs, 1H), 8.13 (s, 1H), 8.02 (d, 1H, J = 8.3 Hz), 7.93 (brs, 1H), 7.82 (brs, 4H), 7.68 (d, 1H, J = 8.2 Hz), 7.36 (d, 1H, J = 8.1 Hz), 7.28 (brs, 1H), 7.16 (s, 1H), 7.08 (d, 1H, J = 8.1 Hz), 3.82 (s, 3H); ESI-MS: 584.1 $[M + 1]^+$.

4.10.3. 3,5-Bis(trifluoromethyl)-N-{4-[3-(4-chloro-3-methoxyphenyl)-4-(pyridin-4-yl)-1H-pyrazol-1-yl]phenyl}benzamide (**16**)

Yield 42%; mp: 206–208 °C (dec.); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.58 (d, 2H, J = 4.5 Hz), 8.36 (brs, 2H), 8.14 (s, 1H), 8.09 (brs, 1H), 8.00 (brs, 1H), 7.84 (s, 4H), 7.37 (d, 1H, J = 8.1 Hz), 7.28 (d, 1H, J = 1.6 Hz), 7.16 (d, 1H, J = 1.8 Hz), 7.08 (d, 1H, J = 8.1 Hz), 3.82 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.9, 160.6, 150.4, 148.5, 140.2, 137.8, 137.3, 135.6, 134.5, 131.1, 130.8, 129.5, 129.0, 125.6, 123.1, 122.2, 121.9, 120.5, 120.0, 119.5, 114.3, 113.4, 56.1; ESI-MS: 618.0 $[M + 1]^+$.

4.11. 1-{4-[3-(4-chloro-3-hydroxyphenyl)-4-(pyridin-4-yl)-1H-pyrazol-1-yl]phenyl}-3-(2,3-dichlorophenyl)urea (**17**)

To a solution of compound **9** (50 mg, 0.1 mmol) in methylene chloride (1 mL), BBr_3 (0.08 mL of a 1M solution in methylene chloride, 1.2 mmol) was added dropwise at −78 °C under N_2 and the reaction mixture was stirred at the same temperature for 30 min. The mixture was allowed to warm to room temperature and stirred for 1 h. The mixture was quenched with saturated aqueous $NaHCO_3$. Ethyl acetate was added and the organic layer was separated. The aqueous layer was extracted with ethyl acetate. The combined organic layer extracts were washed with brine, dried over anhydrous Na_2SO_4. After evaporation of the organic solvent, the residue was purified by short column chromatography (silica gel, hexane-ethyl acetate 1:5 v/v) to yield compound **17** (36 mg, 73.6%). mp: 143–146 °C (dec.). ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.37 (s, 1H), 9.49 (s, 1H), 8.97 (s, 1H), 8.55 (s, 2H), 7.96 (d, 1H, J = 8.0 Hz), 7.91 (d, 1H, J = 8.6 Hz), 7.83 (d, 1H, J = 8.0 Hz), 7.74 (d, 1H, J = 7.9 Hz), 7.55 (d, 2H, J = 7.4 Hz), 7.45 (d, 1H, J = 7.9 Hz), 7.39 (d, 2H, J = 7.4 Hz), 7.37 (s, 2H), 7.15 (s, 1H), 6.92 (d, 1H, J = 8.3 Hz); ESI-MS: 552.2 $[M + 1]^+$.

The synthesis of compounds **18**–**24** was carried out by the same procedure as described for preparation of **17**.

4.11.1. 1-{4-[3-(4-chloro-3-hydroxyphenyl)-4-(pyridin-4-yl)-1H-pyrazol-1-yl]phenyl}-3-(3,4-dichlorophenyl)urea (**18**)

Yield 77%; mp: 191–194 °C (dec.); ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.10 (s, 1H), 9.05 (s, 1H), 8.91 (s, 1H), 8.55 (d, 2H, J = 6.0 Hz), 7.92 (d, 1H, J = 2.4 Hz), 7.85 (d, 2H, J = 9.0 Hz), 7.63 (d, 2H, J = 9.0 Hz), 7.53 (d, 1H, J = 12.7 Hz), 7.40 (s, 1H), 7.34–7.37 (m, 4H), 7.15 (d, 1H, J = 1.9 Hz), 6.92 (d, 1H, J = 8.2 Hz); ESI-MS: 552.0 $[M + 1]^+$.

4.11.2. 1-{4-[3-(4-chloro-3-hydroxyphenyl)-4-(pyridin-4-yl)-1H-pyrazol-1-yl]phenyl}-3-(4-chloro-3-(trifluoromethyl)phenyl)urea (**19**)

Yield 69%; mp: 127–128 °C (dec.); ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.36 (s, 1H), 9.24 (s, 1H), 9.07 (s, 1H), 8.91(s, 1H), 8.54 (d, 2H, J = 5.6 Hz), 8.14 (s, 1H), 7.85 (d, 2H, J = 8.9 Hz), 7.63 (brs, 4H), 7.34–7.39 (m, 3H), 7.14 (s, 1H), 6.91 (d, 1H, J = 8.2 Hz); ^{13}C NMR (75 MHz, DMSO-d_6) δ 153.0, 152.4, 149.8, 148.6, 140.0, 139.2, 138.0, 133.8, 132.4, 132.0, 130.0, 128.8, 126.9, 123.2, 122.5, 119.9, 119.8, 119.3, 119.2, 119.1, 116.9, 116.8, 116.2; ESI-MS: 585.2 $[M + 1]^+$.

4.11.3. 1-{4-[3-(4-chloro-3-hydroxyphenyl)-4-(pyridin-4-yl)-1H-pyrazol-1-yl]phenyl}-3-(3,5-bis(trifluoromethyl)phenyl)urea (**20**)

Yield 42%; mp: 216–217 °C (dec.); ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.34 (s, 1H), 9.49 (s, 1H), 9.23 (s, 1H), 8.92 (s, 1H), 8.55 (d, 2H, J = 6.1 Hz), 8.17 (s, 2 H), 7.87 (d, 2H, J = 9.1 Hz), 7.67 (d, 2H, J = 8.9 Hz), 7.66 (s, 1H), 7.40 (s, 1H), 7.35 (d, 2H, J = 6.1 Hz), 7.15 (s, 1H), 6.92 (d, 1H, J = 8.2 Hz); ^{13}C NMR (75 MHz, DMSO-d_6) δ 153.6, 152.9, 150.4, 149.2, 142.3, 140.4, 138.4, 134.5, 132.9, 131.4, 131.0, 130.5, 129.4, 123.0, 122.0, 120.4, 120.3, 120.1, 119.7, 119.6, 118.6, 116.7; ESI-MS: 619.0 $[M + 1]^+$.

4.11.4. 3,4-Dichloro-N-{4-[3-(4-chloro-3-hydroxyphenyl)-4-(pyridin-4-yl)-1H-pyrazol-1-yl]phenyl}benzamide (**21**)

Yield 47%; mp: 231–233 °C (dec.); ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.57 (s, 1H), 10.32 (s, 1H), 8.95 (s, 1H), 8.55 (d, 2H, J = 4.6 Hz), 8.25 (d, 1H, J = 2.0 Hz), 7.94 (brs, 5H), 7.85 (d, 1H, J = 8.4 Hz), 7.35–7.40 (m, 3H), 7.15 (s, 1H), 6.93 (d, 1H, J = 8.2 Hz); ESI-MS: 537.2 $[M + 1]^+$.

4.11.5. 3,5-Dichloro-N-{4-[3-(4-chloro-3-hydroxyphenyl)-4-(pyridin-4-yl)-1H-pyrazol-1-yl]phenyl}benzamide (**22**)

Yield 87%; mp: 250–252 °C (dec.); ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.59 (s, 1H), 10.31 (s, 1H), 8.95 (s, 1H), 8.55 (d, 2H, J = 4.5 Hz), 8.01 (d, 2H, J = 1.9 Hz), 7.94 (s, 4H), 7.89 (t, 1H, J = 1.9 Hz), 7.40–7.35 (m, 3H), 7.15 (s, 1H), 6.93 (d, 1H, J = 8.2 Hz); ^{13}C NMR (75 MHz, DMSO-d_6) δ 163.2, 153.5, 150.4, 149.3, 140.4, 138.4, 137.8, 135.6, 134.8, 132.8, 131.5, 130.5, 129.5, 127.0, 123.0, 121.7, 120.4, 120.3, 119.8, 119.4, 116.7; ESI-MS: 536.8 $[M + 1]^+$.

4.11.6. 4-Chloro-3-trifluoromethyl-N-{4-[3-(4-chloro-3-hydroxyphenyl)-4-(pyridin-4-yl)-1H-pyrazol-1-yl]phenyl}benzamide (**23**)

Yield 73%; mp: 169–171 °C (dec.); ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.70 (s, 1H), 10.34 (s, 1H), 8.97 (s, 1H), 8.55 (d, 2H, J = 6.0 Hz), 8.42 (brs, 1H), 8.29 (d, 1H, J = 9.3 Hz), 7.95 (brs, 5H), 7.41–7.35 (m, 3H), 7.15 (s, 1H), 6.93 (d, 1H, J = 8.2 Hz); ^{13}C NMR (75 MHz, DMSO-d_6) δ 163.6, 153.6, 150.4, 149.3, 140.4, 137.9, 135.6, 134.4, 133.9, 132.8, 132.5, 130.5, 129.5, 127.6, 123.0, 121.8, 120.4, 120.3, 119.8, 119.4, 116.7; ESI-MS: 570.5 $[M + 1]^+$.

4.11.7. 3,5-Bis(trifluoromethyl)-N-{4-[3-(4-chloro-3-hydroxyphenyl)-4-(pyridin-4-yl)-1H-pyrazol-1-yl]phenyl}benzamide (**24**)

Yield 43%; mp: 289–291 °C (dec.); ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.82 (s, 1H), 10.34 (s, 1H), 8.97 (s, 1H), 8.64 (s, 2H), 8.55 (d, 2H, J = 4.6 Hz), 8.34 (s, 1H), 7.97 (s, 4H), 7.40–7.35 (m, 3H), 7.15 (s, 1H), 6.93 (d, 1H, J = 8.2 Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ 163.1, 153.5, 150.4, 150.0, 149.7, 140.4, 137.8, 137.7, 137.4, 137.1, 135.8, 132.8, 131.1, 130.8, 130.5, 129.5, 129.1, 123.0, 122.0, 120.4, 119.4, 116.7; ESI-MS: 604.16 $[M + 1]^+$.

4.12. Evaluation of the antiproliferative activity against A375P human melanoma cell line

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 A375P 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.

4.13. NCI 8-melanoma cell line screening

8-Melanoma cell line screening was applied at the National Cancer Institute (NCI), Bethesda, Maryland, USA [23], applying the following procedure. The human cell lines are grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. For a typical screening experiment, cells are inoculated into 96-well microtiter plates in 100 μL at plating densities ranging from 5000 to 40,000 cells/well depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates are incubated at 37 °C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of experimental drugs. After 24 h, two plates of each cell line are fixed in situ with TCA, to represent a measurement of the cell population for each cell line at the time of drug addition (Tz). Experimental drugs are solubilized in dimethyl sulfoxide at 400-fold the desired final maximum test concentration and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate is thawed and diluted to twice the desired final maximum test concentration with complete medium containing 50 μg/mL gentamicin. Additional four, 10-fold or 1/2 log serial dilutions are made to provide a total of five drug concentrations plus control. Aliquots of 100 μL of these different drug dilutions are added to the appropriate microtiter wells already containing 100 μL of medium, resulting in the required final drug concentrations. Following drug addition, the plates are incubated for an additional 48 h at 37 °C, 5% CO_2, 95% air, and 100% relative humidity. For adherent cells, the assay is terminated by the addition of cold TCA. Cells are fixed in situ by the gentle addition of 50 μL of cold 50% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4 °C. The supernatant is discarded, and the plates are washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (100 μL) at 0.4% (w/v) in 1% acetic acid is added to each well, and plates are kept for 10 min at room temperature. After staining, unbound dye is removed by washing five times with 1% acetic acid and the plates are air dried. Bound stain is subsequently solubilized with 10 mM trizma base, and the absorbance is read on an automated plate reader at a wavelength of 515 nm. For suspension cells, the methodology is the same except that the assay is terminated by fixing settled cells at the bottom of the wells by gently adding 50 μL of 80% TCA (final concentration, 16% TCA). Using the seven absorbance measurements [time zero, (Tz), control growth, (C), and test growth in the presence of drug at the five concentration levels (Ti)], the percentage growth is calculated at each of the drug concentrations levels. Percentage growth inhibition is calculated as:

- $[(Ti-Tz)/(C-Tz)] \times 100$ for concentrations for which $Ti \geq Tz$
- $[(Ti-Tz)/Tz] \times 100$ for concentrations for which $Ti < Tz$.

Growth inhibition of 50% (IC_{50}) is calculated from $[(Ti-Tz)/(C-Tz)] \times 100 = 50$, which is the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation.

4.14. ERK kinase screening

4.14.1. Protein immunoblotting and immunoprecipitation

For immunoblotting, A375P melanoma cells grown to 70%–80% confluence were harvested in RIPA lysis buffer and disrupted by sonication and centrifuged at 12,000 rpm for 10 min. The quantity of protein was determined with DC protein assay kit (Bio-Rad Lab., Hercules, CA). Protein samples were subjected to SDS-PAGE and immunoblotting.

4.14.2. Suppression of RAF-1/MEK/ERK signaling pathway in A375P cells

To assess the effect of compounds **21** and **23** on the RAF-1/MEK/ERK signaling pathway, A375P cells were treated with **21**, **23**, and sorafenib (1, 3, and 10 μM) for 24 h and immunoblotted with antibodies against phospho-ERK1/2 and β-actin, respectively.

4.15. 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 [25] (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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