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Syntheses of phenylpyrazolodiazepin-7-ones as conformationally rigid analogs of aminopyrazole amide scaffold and their antiproliferative effects on cancer cells

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ABSTRACT

Recently, we have reported the syntheses and antiproliferative activities of *N*-(5-amino-1-(4-methoxybenzyl)-1*H*-pyrazol-4-yl amide derivatives on melanoma cells. As a continuous work for antiproliferative agents in melanoma, here we report the synthesis of conformationally rigid analogs, phenyl-6,8-dihydropyrazolo[3,4-*b*][1,4]diazepin-7(1*H*)-one derivatives **7a–g, 8a–o** and their antiproliferative activities against A375P melanoma cell line and U937 hematopoietic cell line. Most compounds showed competitive antiproliferative activities to sorafenib, the reference standard. Among them, *N*-(3-(1-benzyl-7-oxo-1,6,7,8-tetrahydropyrazolo[3,4-*b*][1,4]diazepin-5-yl)phenyl)-4-chloro-3-(trifluoro methyl)benzamide-amino-1-(4-methoxybenzyl)-*1H*-pyrazol-4-yl)-5-(3-(4-chloro-3-(trifluoromethyl) phenyl) ureido)-2-methylbenzamide (**7b**) exhibited potent activities (GI_{50} = 0.43 μM and 0.06 μM) on both cell lines. It has been further confirmed to be a potent and selective Raf kinases inhibitor and also mild inhibitor of PI3Kα.

1. Introduction

The irregulation of kinase activity has been considered as a major mechanism of growth and survival of cancer cells. Therefore, there are significant amounts of efforts to develop selective and potent kinase inhibitors and approximately 30 distinct kinase targets are being developed as in Phase I clinical trial to date. Among which, kinases in Ras/Raf/MEK/ERK and phosphatidylinositol 3-kinase (PI3K)/Akt signaling cascades are the most commonly up-regulated in human cancers.[1–4] It is more important that these two signaling cascades cooperate to promote transformed cancer cell survival. Regulation through crosstalk and feedback between the Ras/Raf/MEK/ERK and PI3K/Akt pathways have been demonstrated, indicating the potential of interruption of more than one signaling pathways in cancer therapy.[2,5,6]

The activating mutations in KRAS are found in more than 30% of all human tumors and mutations of B-Raf in 7% of human cancers[7,8] with particularly high frequency in melanoma (50–70%), ovarian (35%), thyroid (30%), and colorectal (10%) cancers. Thus targeting this pathway could have broad therapeutic effects[9] and many small molecule ATP-competitive B-Raf (V600E) kinase inhibitors have been developed and found to have potent antitumor effects on mutant B-Raf (V600E) tumors. Surprisingly, however, they were not potent against RAS mutant tumor models implying that there are more than one targets and inhibitors necessary for this set of disease.[9]

Recently, there are reports that the potent B-Raf (V600E) inhibitor could also trigger the C-Raf activation sequence by independent mechanism,[10] which can lead to enhanced growth of tumors. Therefore, it is proposed that the inhibition of both B-Raf (V600E) and C-Raf should be attained at the same time and we have found several series of compounds fitting this criteria.[11,12]

As shown in Figure 1, we started from imidazolopyrazole bicyclic ring scaffold[11] based on binding mode of sorafenib, which is a potent inhibitor of preactivated C-Raf, V600E B-Raf holding a unique binding mode, namely type II inhibitor.[13] Afterwards, we found imidazolopyrazole bicyclic ring is a novel effective chemotype for both Raf kinases, and to develop more effective hydrogen bonding and hydrophobic interaction, we modified the bicyclic ring into aminopyrazole amide scaffold.[12] We found that they were all potent and selective inhibitors of B-Raf (V600E) and C-Raf as well.

In our docking studies (Fig. 2), the aminopyrazole amide scaffold is supposed to have two hydrogen bonds in the hinge region and one *intramolecular hydrogen bond* between the hydrogen of the 5-amino group and the carbonyl oxygen in the 4-amide group in binding mode. Therefore, we decided to continue this work by introducing a seven-membered ring mimicking *intramolecular hydrogen bond* next to pyrazole ring as a namely 'conformation-restricted analogs'. The scaffold evolution of Raf-inhibitors are shown in Figure 1, and here we report the syntheses of designed

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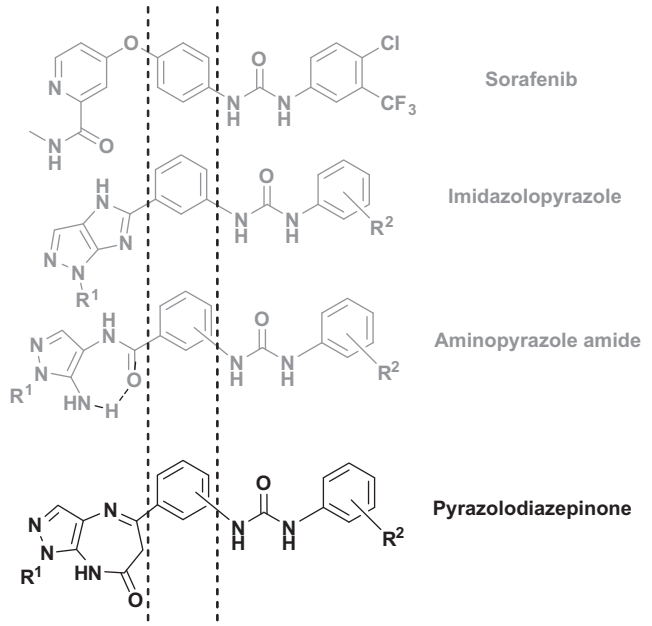


Figure 1. Evolution of Raf inhibitors from Sorafenib to 1-benzyl-5-dihydropyrazolodiazepin-7(1*H*)-one derivatives.

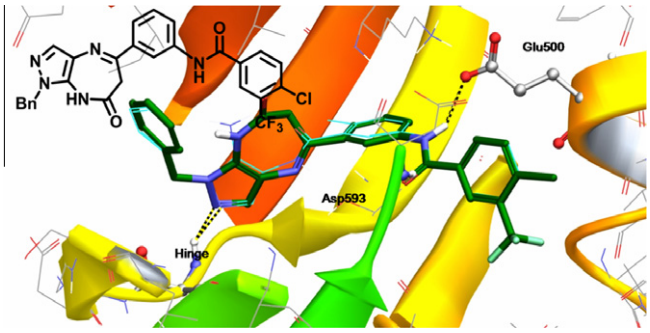


Figure 2. Docking structures of designed pyrazolodiazepinone scaffold (**7b**, bold, green) overlaid with amino-1*H*-pyrazole amide derivatives (thin, cyan)[12] in B-Raf V600E (1uwj).

pyrazolodiazepin-7-one analogs and their antiproliferative effects on two cancer cell lines.

2. Chemistry

The approach taken towards syntheses of the novel 1-benzyl-5-dihydropyrazolodiazepin-7(1*H*)-one analogs is based on formation of a bicyclic key intermediate pyrazolodiazepinone, **5** and is outlined in Scheme 1. The pyrazolodiazepinone was synthesized by two sequential steps; acid-catalyzed imine formation and amide coupling of diamino pyrazole moiety (**4**)[11] and corresponding ethyl β-keto nitrophenylpropano ester (**3**). The ethyl β-keto nitrophenylpropano ester (**3**) was synthesized by thermal ethanolysis of 5-nitrobenzoyl-2, 2-dimethyl-1, 3-dioxane-4, 6-dione (**2**), which was adduct of nitrobenzoyl chloride (**1**) and Meldrum's acid.

1-benzyl (or para-methoxy benzyl) -1*H*-pyrazole-4,5-diamine (**4**) was reacted with ethyl β-keto nitrophenylpropano ester (**3**), under acidic conditions, to smoothly afford ethyl 3-(5-amino-1-benzyl-1*H*-pyrazol-4-ylimino)-3-nitrophenyl propanoate, and this enamine was cyclized using sodium methoxide in dry methanol in situ to furnish the core scaffold, **5**.

Then, the nitro group in **5** was selectively reduced to amino group using iron and ammonium chloride and linked with various aromatic acids under EDCI/HOBt conditions to give amide (**7a**–**7g**) analogs and also directly reacted with aromatic isocynate to give urea (**8a**–**8o**) analogs.

3. Results and discussion

The *N*-[1-benzyl-5-dihydropyrazolodiazepin-7(1*H*)-one]phenyl amide derivatives **7a–g** and *N*-[1-benzyl-5-dihydropyrazolodiazepin-7(1*H*)-one]phenyl urea derivatives **8a–o** were evaluated for their antiproliferative activities against human melanoma cell line, A375P and human hematopoietic cell line, U937. A375P cell line is a well known melanoma cell line, which is an excellent index for V600E B-Raf inhibition, and U937 cell line was selected since it has been known to be a good model to study the Raf/MEK/ERK and PI3K/Akt signaling pathways as well.

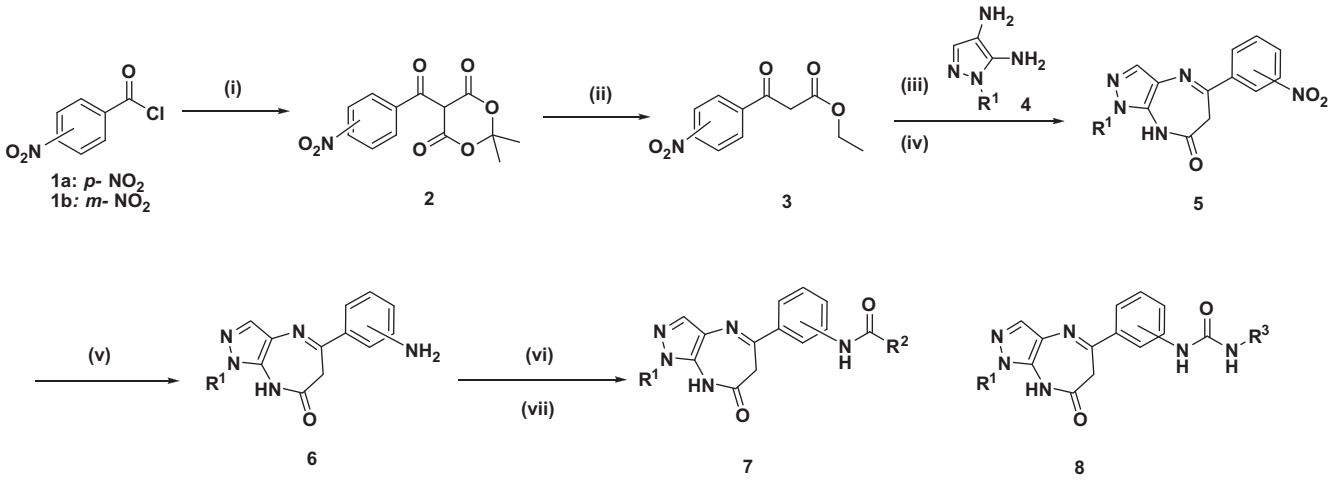
Table 1 shows the antiproliferative activities[14] of *N*-[1-benzyl-5-dihydropyrazolodiazepin-7(1*H*)-one]phenyl amide derivatives linked with various aromatic tail groups by amide bond. Since this scaffold has been designed as conformationally rigid analogs of aminopyrazoleamide series, we focused on the connection in the middle phenyl ring (*m*-/*p*-) with known hydrophobic tails. As shown in Table 1, no growth inhibition was observed for compounds **7a**, **7d**, and **7e** on both cell lines, which indicates whether the connection in the middle phenyl ring is *m*- or *p*-, the preference of substitution pattern of hydrophobic tail benzene is 3-, 4-disubstitution not 3-, 5-disubstitution in amide derivatives. Compound **7b** showed the best activities in this series toward both cell lines. Interestingly, when the middle connection is changed to *p*-(**7c**), it loses its activity on A375P, but it was tolerated in U937 cells. This may indicate that **7c** is not an effective inhibitor of V600E B-Raf in A375P, but potent enough to have an effect on survival signaling in U937 cell, probably impacting on different molecular target. This tendency has also been found the same in case of **7f** and **7g**.

Table 2 shows the antiproliferative activity of *N*-[1-benzyl-5-dihydropyrazolodiazepin-7(1*H*)-one]phenyl urea derivatives **8a–o**. Considering that aminopyrazole amide compounds are more active in their urea derivatives and also the fact that these are conformationally rigid analogs, we expected urea analogs to be more potent. But it was not generally true with the same hydrophobic tails (**7b** > **8a** on both cell lines, **7c** > **8b** on U937). However, we found that compound**8h, 8i, 8n** and **8o** with simpler mono-substitution on benzene tail showed good efficacy and potency on both cell lines. It seems to be better than the reference compound, sorafenib. Notably, replacement of benzyl group in pyrazole moiety (R^1) with PMB (*p*-methoxybenzyl) tolerated in their activity, but removal of this group led to significant decrease or total loss of activity (R^1 = H; **8d**, **8j**, **8m**), which highlight the importance of physicochemical property in cellular potency.

Shown in Table 3, the representative compound **7b** was screened on selected 30 different kinases panel at a single dose concentration of 10 μM[15] and it was revealed that the compound has an excellent selectivity profile. While this compound has effectively inhibited V600E B-Raf, C-Raf, and PI3Kα, the inhibition exerted in most other kinases tested activity was below 20%. Furthermore, we evaluated in vitro enzymatic inhibition of two compounds, **7b** and **8a** toward V600E B-Raf, C-Raf, and PI3Kα. As shown in Table 4, compound **7a** has good to mild potency on three kinases (0.655 μM, 0.96 μM, 21.5 μM, respectively), however, **8a** has only good potency on C-Raf (0.667 μM).

4. Conclusions

A series of *N*-[1-benzyl-5-dihydropyrazolodiazepin-7(1*H*)-one]-phenyl derivatives **7a**–**7g** and **8a**–**8o**, designed as conformationally rigid analogs of aminopyrazole amide has been synthesized and evaluated their antiproliferative activities against A375P human



Scheme 1. Reagents and conditions: (i) Meldrum's acid, DMAP, MC, -15°C, 4 h; (ii) EtOH, reflux, overnight; (iii) **4**, AcOH, MC, rt, 30 min; (iv) NaOMe/MeOH, overnight; (v) Fe, NH_4Cl,THF:H_2O (1:1), 60 °C, 3 h; (vi) R^2-CO_2H, HOBt, EDCI, TEA, DMF, rt; (vii) R^3-NCO, THF, rt.

cell line, together with hematopoietic U937 cell lines. Many compounds in this scaffold showed potent antiproliferative activities, and furthermore, one of the best compound **7b** has been confirmed as a potent and selective Raf kinases and also mild inhibitor of PI3Kα, suggesting that the *N*-[1-benzyl-5-dihydropyrazolodiazepin-7(1*H*)-one]phenyl derivatives could serve as a promising scaffold for new therapeutics of cancer, having absolutely impressive kinase profiling.

5. Materials and methods

5.1. Chemistry general

All chemicals (reagent grade) used were purchased from Aldrich (USA). Separation of the compounds by column chromatography was carried out with silica gel 60 (200–300 mesh ASTM, E. Merck, Germany). The quantity of silica gel used was 50–100 times the weight charged on the column. Thin layer chromatography (TLC) was run on the silica gel coated aluminum sheets (Silica Gel 60 GF254, E. Merck, Germany) and visualized in ultraviolet (UV) light (254 nm). ^{1}H NMR and ^{13}C NMR spectra were recorded on Brucker model digital AVANCE III 400 MHz spectrometer at 25 °C, using tetramethylsilane (TMS) as the internal standard. High-resolution MS (HR/MS) experiments were conducted with a Finnigan LTQ Orbitrap mass spectrometry (Thermo Fisher Scientific Inc., MA, USA) operated in positive-ion electrospray mode.

5.1.1. Synthesis of 2,2-dimethyl-5-(3-nitrobenzoyl)-1,3-dioxane-4,6-dione (2)

To a solution of Meldrum's acid (2.6 g, 17.96 mmol) and *N,N*-dimethylamino pyridine (4.4 g, 35.9 mmol) in methylene chlroride (70 ml) was added nitrobenzoyl chloride (5 g, 26.94 mmol) dropwise at −15 °C. Then the mixture was stirred at the same temperature for 4 h. Methylene chloride wad added (50 ml) to the mixture, and the mixture was washed with brine, dried over Na_2SO_4 and evaporated to dryness. Purification of column chromatography with methylene chlroide/methanol = 10:1 yielded **2** (5 g, 17.05 mmol, 94%) as solid. ^{1}H NMR (400 MHz, $CDCl_3$) 8.50 (1 H, s), 8.44 (1 H, d, *J* = 8.25 Hz), 7.96 (1 H, d, *J* = 7.76 Hz), 7.67 (1 H, t, *J* = 8.03 Hz), 2.63 (1 H, s), 1.86 (6 H, s).

5.1.2. Synthesis of ethyl 3-(3-nitrophenyl)-3-oxopropanoate (3)

2 (5 g, 17.05 mmol) was dissolved in absolute ethanol (40 ml) and heated to reflux (100 °C) for 15 h. After cooling down to ambient temperature, the solvent was removed in vacuo, the pure product 3 (3 g, 12.6 mmol, 74%) was obtained. Further purification was not necessary at this step. (1H NMR (400 MHz, CDCl3) 12.61 (1 H, s), 8.61 (1 H, s), 8.30 (1 H, d, *J* = 8.20 Hz), 8.09 (1 H, d, *J* = 7.82 Hz), 7.61 (1H, t, *J* = 8.0 Hz), 5.76 (1 H, s), 4.29 (2 H, q, *J* = 7.1 Hz), 1.35 (3 H, t, *J* = 7.1 Hz).

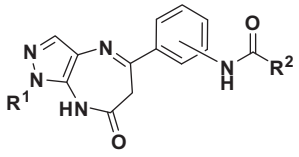
5.1.3. Synthesis of 4, 5-Diamino-1-(4-methoxy-benzyl)pyrazole (4)

To a suspension of 1-(4-methoxybenzyl)-4-nitroso-*1H*-pyrazol-5-amine (100 mg, 0.43 mmol) in EtOH (0.86 ml), was added 35% HCl (0.22 ml, 2.15 mmol), then the mixture was stirred at 80 °C. A solution of $SnCl_2{\cdot}2H_2O$ (213.4 mg, 0.95 mmol) in EtOH (0.25 ml) was added to dissolved mixture over 15 min. Stirring was continued for 30 min and the clear solution was cooled to room temperature, then poured into ice. The pH was made slightly basic (pH 7 ~ 8) by addition of saturated aqueous sodium bicarbonate before being extracted with organic solvent (IPA/$CHCl_3$ = 4:1). The organic phase is thoroughly washed with brine, dried over sodium sulfate. Evaporation of the solvent leaves crude 100 mg of 4,5-diamino-1-(4-methoxy-benzyl) pyrazole **4**, which gives one spot on TLC. (^{1}H NMR (400 MHz, $CDCl_3$) 7.15 (1 H, s), 7.11 (2 H, d, *J* = 8.55 Hz), 6.85 (2 H, d, *J* = 8.64 Hz), 5.11 (2 H, s), 3.76 (3 H, s).

5.1.4. Synthesis of 5-(3-aminophenyl)-1-benzyl-6,8-dihydropyrazolo[3,4-*b*][1,4]diazepin-7(1*H*)-one (6)

To a solution of **3** (252 mg, 1.06 mmol) and 4,5-diamino-1-(4-methoxy-benzyl) pyrazole (300 mg, 1.6 mmol) in methylene chloride (2 ml) was added acetic acid (0.3 mL, 5.3 mmol) dropwise. The mixture was stirred for about 30 min and the solvent was removed in vacuo. To this imine adduct, 25% NaOMe/MeOH solution was added and stirred at 50 °C for 24 h. The reaction mixture was then concentrated under vacuum and the crude product was transferred to the next step without purification. To a suspension of crude **5** (350 mg) in THF/H_2O = 1:1 solution (4 ml), wad added Fe (245 mg, 4.39 mmol) and NH_4Cl (238 mg, 4.45 mmol) was added and heated to 60 °C. Stirring was continued for 4 h, and then the clear solution was cooled room temperature. The pH is made slightly basic (pH 7 ~ 8) by addition of saturated aqueous sodium bicarbonate before being extracted with ethyl acetate. The organic phase was thoroughly washed with brine, dried over sodium sulfate. Evaporation of the solvent leaves oil, and purification of column chromatography with

Table 1
Antiproliferative activity of *N*-[1-benzyl-5-dihydropyrazolodiazepin-7(1*H*)-one]phenyl amide derivatives **7a–g**



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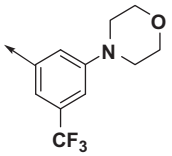
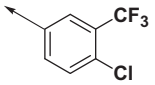
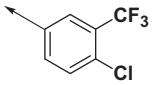
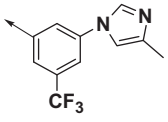
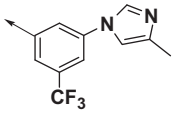
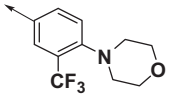
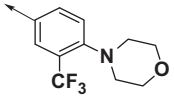
Compd	Substit-ution	R^1	R^2	A375PGI$_{50}$ (μM)	U937 GI$_{50}$ (μM)
7a	*m*-	*Bn*		>30	>30
7b	*m*-	*Bn*		0.43	0.06
7c	*p*-	*Bn*		13.1	0.06
7d	*m*-	*Bn*		>30	>30
7e	*p*-	*Bn*		>30	>30
7f	*m*-	*Bn*		2.91	1.75
7g	*p*-	*Bn*		16.4	0.21
Sorafenib				5.58	2.85

hexane/ethyl acetate = 1:1 to afford compound **6** (250 mg, 69%). ^{1}H NMR (400 MHz, $CDCl_3$) 7.84 (2 H, d, *J* = 8.66 Hz), 7.75 (1 H, bs), 7.72 (1 H, s), 7.33–7.25 (5 H, m), 7.12 (2 H, d, *J* = 6.37 Hz), 6.69 (2 H, d, *J* = 8.63 Hz), 5.32 (2 H, s), 3.95 (2 H, s), 3.53 (2 H, s).

5.1.5. General syntheses of *N*-(3-(1-benzyl-7-oxo-1,6,7,8-tetrahydropyrazolo[3,4-*b*][1,4]diazepin-5-yl)phenyl) amide (7)

A solution of **6** compound (10 mg, 0.028 mmol), substituted benzoic acid (0.028 mmol), HOBt (6.8 mg, 0.05 mmol), EDCI (8.05 mg, 0.04 mmol) and TEA (10 μL, 0.07 mmol) in DMF (0.5 mL) was stirred at room temperature overnight. The reaction mixture was diluted with ethyl acetate and washed with saturated aqueous sodium bicarbonate. The organic layer dried over Na_2SO_4. Purification of column chromatography with Hexane/ethyl acetate = 1:1 to afford compound **7**.

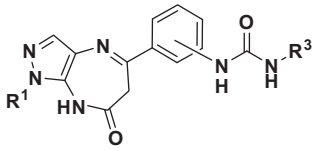
5.1.5.1. *N*-(3-(1-benzyl-7-oxo-1,6,7,8-tetrahydropyrazolo[3,4-*b*]-[1,4]diazepin-5-yl)phenyl)-3-morpholino-5-(trifluoromethyl)-benzamide (7a). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 (2H, d, *J* = 8.68), 7.94–7.97 (2H, m), 7.74–7.77 (3H, m), 7.61 (1H, s), 7.44 (1H, s), 7.30–7.36 (4H, m), 7.14 (2H, d, *J* = 6.24), 5.35 (2H, s), 3.88 (2H, t, *J* = 4.71), 3.58 (2H, s), 3.29 (2H, t, *J* = 4.79); HRMS calcd for $C_{31}H_{28}F_3N_6O_3$ *m/z* 589.2175 Found 589.2166.

5.1.5.2. *N* -(3-(1-benzyl-7-oxo-1,6,7,8-tetrahydropyrazolo[3,4-*b*][1,4]diazepin-5-yl)phenyl)-4-chloro-3-(trifluoromethyl)benz-amide (7b). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.2 (1H, s), 7.99–8.06 (3H, m), 7.96 (1H, s), 7.90 (1H, s), 7.73–7.77 (3H, m), 7.66 (1H, d, *J* = 8.26), 7.32–7.35 (3H, m), 7.15 (2H, d, *J* = 6.7 Hz), 5.35 (2H, s), 3.26 (2H, s); HRMS calcd for $C_{27}H_{20}ClF_3N_5O_2$ *m/z* 538.1258 Found 538.1247.

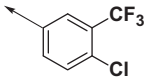
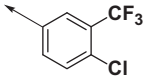
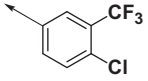
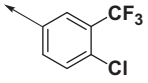
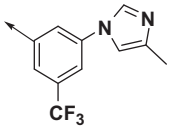
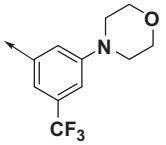
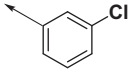
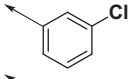
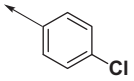
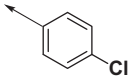
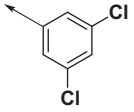
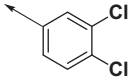
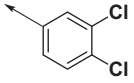
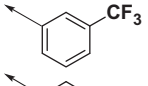
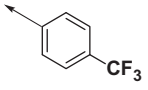
5.1.5.3. *N*-(4-(1-benzyl-7-oxo-1,6,7,8-tetrahydropyrazolo[3,4-*b*]-[1,4]diazepin-5-yl)phenyl)-3-chloro-4-(trifluoromethyl)benz-amide (7c). ^{1}H NMR (400 MHz, DMSO-d_6) 11.40 (1 H, s), 10.73 (1 H, s), 8.41 (1 H, s), 8.28 (1 H, d, *J* = 7.35 Hz), 8.03 (2 H, d, *J* = 8.80 Hz), 7.94 (1 H, d, *J* = 8.34 Hz), 7.90 (1 H, d, *J* = 8.68 Hz), 7.71 (1 H, s), 7.36 (2 H, t), 7.29 (1 H, d, *J* = 7.08 Hz), 7.15 (1 H, d, *J* = 7.88 Hz), 5.75 (2 H, s), 5.37 (2 H, s), 3.48 (2 H, s); HRMS calcd for $C_{27}H_{20}ClF_3N_5O_2$ *m/z* 538.1258 Found 538.1273.

5.1.5.4. *N* -(3-(1-benzyl-7-oxo-1,6,7,8-tetrahydropyrazolo[4,3-*b*][1,4]diazepin-5-yl)phenyl)-3-(4-methyl-1*H*-imidazol-1-yl)-5-(trifluoromethyl)benzamide (7d). ^{1}H NMR (400 MHz, DMSO-

Table 2
Antiproliferative activity of *N*-[1-benzyl-5-dihydropyrazolodiazepin-7(1*H*)-one]phenyl urea derivatives **8a–o**



8

Compd	Substit-ution	R^1	R^2	A375P (GI_{50} (μM)	U937 GI_{50} (μM)
8a	*m*-	*Bn*		4.45	7.31
8b	*p*-	*Bn*		1.19	8.68
8c	*p*-	*PMB*		3.93	7.29
8d	*p*-	*H*		>30	>30
8e	*p*-	*Bn*		>30	>30
8f	*m*-	*PMB*		3.32	>30
8g	*p*-	*Bn*		2.07	0.67
8h	*m*-	*PMB*		0.91	0.63
8i	*m*-	*PMB*		0.64	0.58
8j	*m*-	*H*		>30	>30
8k	*m*-	*PMB*		0.78	6.48
8l	*m*-	*PMB*		3.86	14.9
8m	*m*-	*H*		>30	>30
8n	*m*-	*PMB*		0.25	0.28
8o	*m*-	*PMB*		0.77	0.11
Sorafenib				5.58	2.85

d_6) 10.47 (1 H, s), 9.54 (1 H, s), 8.32 (2 H, m), 8.16 (1 H, d, J = 9.01 Hz), 8.01 (1 H, s), 7.55 (2 H, s), 7.52 (1 H, s), 7.44 (1 H, s), 7.15 (2 H, d, J = 8.22 Hz), 6.88 (2 H, d, J = 8.588 Hz), 5.13 (2 H, s), 5.05 (2 H, s), 3.72 (3 H, s), 2.34 (3 H, s); HRMS calcd for $C_{31}H_{245}F_3N_7O_2$ m/z 584.2022 Found 584.2073.

Table 3
Percentages of enzymatic inhibitions by compound **7b** (10 μM) on selected Protein Kinases

Kinase	Inhibition (%)	Staurosporine IC_{50} (nM)
ABL1	0	105.90
AKT1	0	4.95
ALK	0	3.00
Aurora A	6.5	2.28
B-Raf (V599E)	85.6	4.33[a]
CDK1/cyclin B	7.0	1.30
CDK2/cyclin E	0.9	1.88
c-Kit	3.6	65.19
c-MET	1.3	153.00
c-Src	4.3	3.78
EGFR	1.6	188.30
ERK2/MAPK1	0	3948.00
FAK/PTK2	0	17.15
FGFR2	0	3.36
FGFR3	0	14.71
FLT3	19.3	1.01
FMS	0.5	3.13
GSK3b	17.8	9.23
IGF1R	1.9	85.33
JAK3	0	< 1.0
JNK1	0	1746.00
JNK3	0	6745[b]
KDR/VEGFR2	0	13.73
LCK	5.6	4.49
LYN	4.6	1.30
MEK1	0	8.07
mTOR/FRAP1	0	6796[c]
P38a/MAPK14	0	14.3[d]
PI3Kα	75.2	4.40[e]
PLK1	0	317.90
C-Raf	92.9	4.93[a]
RON/MST1R	4.4	442.50
ROS/ROS1	0	<1.0
SYK	0	<1.0
TRKB	0.2	<1.0

[a] Data of GW5074.[16]
[b] Data of JNKi VIII.[17]
[c] Data of LY294002.[18]
[d] Data of SB202190.[19]
[e] Data of PI103.[20]

Table 4
Enzymatic activities of selected compounds

	IC_{50} (nM)		
	B-Raf V600E	C-Raf	PI3Kα
7b	655.2	960.9	2150
8a	NA	667.8	ND

5.1.5.5. *N* -(4-(1-benzyl-7-oxo-1,6,7,8-tetrahydropyrazolo[4,3-*b*][1,4]diazepin-5-yl)phenyl)-3-(4-methyl-1*H*-imidazol-1-yl)-5-(trifluoromethyl)benzamide (7e). ^{1}H NMR (400 MHz, DMSO-d_6) 11.21 (1 H, s), 9.02 (1 H, s), 8.99 (1 H, s), 8.10 (1 H, s), 7.72 (2 H, d, J = 20.4 Hz), 7.60 (1 H, s), 7.36 (1 H, d, J = 11.22 Hz),, 7.32 (2 H, d, J = 7.72 Hz), 7.27 (1 H, d, J = 7.34 Hz), 7.11 (2 H, d, J = 6.84 Hz), 6.97 (1 H, s), 6.92 (1 H, s), 6.80 (1 H, s), 6.59 (2 H, d, J = 8.61 Hz), 5.87 (1 H, s), 5.73 (2 H, s), 3.47 (2 H, s), 2.15 (3 H, s); HRMS calcd for $C_{31}H_{25}F_3N_7O_2$ *m/z* 584.2022 Found 584.2092.

5.1.5.6. *N* -(3-(1-benzyl-7-oxo-1,6,7,8-tetrahydropyrazolo[3,4-*b*][1,4]diazepin-5-yl)phenyl)-4-morpholino-3-(trifluoromethyl)benzamide (7f). ^{1}H NMR (400 MHz, $CDCl_3$) 8.16 (1 H, d) 8.08 (1H, s), 8.05 (1 H, s), 8.01 (1 H, s), 7.98 (1 H, s), 7.76 (1 H, s), 7.75 (1 H, s), 7.46 (1 H, t, J = 8.0 Hz), 7.39 (2 H, d, J = 8.43 Hz), 7.35–7.31 (3 H, m), 7.14 (2 H, d, J = 7.78 Hz), 5.41 (2 H, s), 3.86 (4 H, m), 3.48 (2 H, s), 3.01 (4 H, m); HRMS calcd for $C_{31}H_{28}F_3N_6O_3$ *m/z* 589.2175 Found 589.2145.

5.1.5.7. *N* -(4-(1-benzyl-7-oxo-1,6,7,8-tetrahydropyrazolo[3,4-*b*][1,4]diazepin-5-yl)phenyl)-4-morpholino-3-(trifluoromethyl)benzamide (7g). ^{1}H NMR (400 MHz, DMSO-d_6) 10.57 (1H, s), 8.27 (1H, s), 8.02 (2H, d, J = 8.85), 7.91 (2H, d, J = 8.91), 7.65–7.71 (3H, m), 7.15 (2H, d, J = 7.16), 6.27–7.36 (4H, m), 5.34 (2H, s), 3.74 (2H, t, J = 4.34), 3.53 (2H, s), 2.97 (2H, t, J = 4.35); HRMS calcd for $C_{31}H_{27}F_3N_6O_3$ *m/z* 589.2175 Found 589.2141.

5.1.6. General syntheses of *N*-(3-(1-benzyl-7-oxo-1,6,7,8-tetrahydropyrazolo[3,4-*b*][1,4]diazepin-5-yl)phenyl) urea (8a–8o)

A mixture of **6** and substituted isocyanate in THF was stirred at room temperature overnight. The reaction mixture was cooled, extracted with CH_2Cl_2. The organic layer was washed with water and brine. After drying over anhydrous $MgSO_4$, and the mixture was evaporated under reduced pressure to give a residue. The residue was purified by silica gel chromatography.

5.1.6.1. 1-(3-(1-benzyl-7-oxo-1,6,7,8-tetrahydropyrazolo[3,4-*b*][1,4]diazepin-5-yl)phenyl)-3-(4-chloro-3-(trifluoromethyl)phenyl)urea (8a). ^{1}H NMR (400 MHz, DMSO-d_6) 11.45 (1H, s), 9.20 (1H, s), 9.13 (1H, s), 8.12 (2H, s), 7.74 (1H, s), 7.59–7.74 (4H, m), 7.26–7.45 (4H, m), 7.15 (2H, d, J = 2.40), 5.38 (2H, s), 3.53 (2H, s); HRMS calcd for $C_{27}H_{21}ClF_3N_6O_2$ *m/z* 553.1367 Found 553.1386.

5.1.6.2. 1-(4-(1-benzyl-7-oxo-1,6,7,8-tetrahydropyrazolo[3,4-*b*]-[1,4]diazepin-5-yl)phenyl)-3-(4-chloro-3-(trifluoromethyl)phenyl)urea (8b). ^{1}H NMR (400 MHz, $CDCl_3$) 8.21 (1H, s), 8.05 (2H, d, J = 8.73), 8.00 (1H, d, J = 8.30), 7.92 (1H, s), 7.78 (1H, s), 7.74 (2H, d, J = 8.78), 7.66 (1H, d, J = 8.33), 7.42–7.32 (4H, m), 7.14 (2H, d, J = 7.86), 5.37 (2H, s), 3.65 (2H, s); HRMS calcd for *m/z* $C_{27}H_{21}ClF_3N_6O_2$ 553.1367 Found 553.1352.

5.1.6.3. 1-(4-chloro-3-(trifluoromethyl)phenyl)-3-(4-(1-(4-methoxybenzyl)-7-oxo-1,6,7,8-tetrahydropyrazolo[3,4-*b*][1,4]diazepin-5-yl)phenyl)urea (8c). ^{1}H NMR (400 MHz, $CDCl_3$) 8.11 (1 H, s), 7.90 (1 H, d, J = 8.44 Hz), 7.83 (1 H, d, J = 8.64 Hz), 7.72 (1 H, d, J = 8.57 Hz), 7.67 (1 H, d, J = 2.13 Hz), 7.55 (1 H, d, J = 9.10 Hz), 7.39–7.31 (2 H, m), 7.10 (2 H, t, J = 8.25 Hz), 6.84 (2 H, d, J = 8.57 Hz), 6.68 (1 H, d, J = 8.66 Hz), 5.26 (2 H, s), 3.76 (3 H, s), 3.48 (2 H, d, J = 5.66 Hz); HRMS calcd for *m/z* $C_{28}H_{23}ClF_3N_6O_3$ 583.1472 Found 583.1418.

5.1.6.4. 1-(4-chloro-3-(trifluoromethyl)phenyl)-3-(4-(7-oxo-1,6,7,8-tetrahydropyrazolo[3,4-*b*][1,4]diazepin-5-yl)phenyl)urea (8d). ^{1}H NMR (400 MHz, DMSO-d_6) 12.86 (1 H, s), 10.62 (1 H, s), 9.44 (1 H, s), 9.33 (1 H, s,), 8.13 (1 H, s), 7.96 (1 H, s), 7.93 (2 H, d, J = 8.74 Hz), 7.67–7.60 (4 H, m), 3.48 (2 H, s); HRMS calcd for $C_{20}H_{15}ClF_3N_6O_2$ *m/z* 463.0897 Found 463.0849.

5.1.6.5. 1-(4-(1-benzyl-7-oxo-1,6,7,8-tetrahydropyrazolo[3,4-*b*]-[1,4]diazepin-5-yl)phenyl)-3-(3-(4-methyl-1*H*-imidazol-1-yl)-5-(trifluoromethyl)phenyl)urea (8e). ^{1}H NMR (400 MHz, DMSO-d_6) 9.11 (1 H, s), 8.99 (1 H, s), 8.01 (1 H, s), 7.73 (1 H, s), 7.72 (2 H, d, J = 16 Hz), 7.36 (1 H, d, J = 11.22 Hz), 7.32 (2 H, d, J = 7.7 Hz), 7.21 (1 H, d, J = 7.6 Hz), 7.11 (2 H, d, J = 6.84 Hz), 6.97 (1 H, s), 6.92 (1 H, s), 6.80 (1 H, s), 6.59 (2 H, d, J = 8.61 Hz), 5.73 (2 H, s), 3.47 (2 H, s), 2.15 (3 H, s); HRMS calcd for *m/z* $C_{31}H_{26}F_3N_8O_2$ 599.2131 Found 599.2132.

5.1.6.6. 1-(3-(1-(4-methoxybenzyl)-7-oxo-1,6,7,8-tetrahydropyrazolo[3,4-*b*][1,4]diazepin-5-yl)phenyl)-3-(3-morpholino-5-(trifluoromethyl)phenyl)urea (8f). ^{1}H NMR (400 MHz, $CDCl_3$) 8.16 (1 H, d) 8.08 (1H, s), 8.05 (1 H, s), 8.01 (1 H, s), 7.98 (1 H, s), 7.76 (1 H, s), 7.75 (1 H, s), 7.46 (1 H, t, J = 8.0 Hz), 7.39 (2 H, d,

J = 8.43 Hz), 7.14 (1 H, d, J = 7.78 Hz), 7.10 (2 H, d, J = 8.25 Hz), 6.84 (2 H, d, J = 8.26 Hz), 5.41 (2 H, s), 3.86 (4 H, m), 4.76 (3 H, s), 3.48 (2 H, s), 3.01 (4 H, m); HRMS calcd for m/z $C_{32}H_{31}F_3N_7O_4$ 634.2390 Found 634.2358.

5.1.6.7. 1-(4-(1-benzyl-7-oxo-1,6,7,8-tetrahydropyrazolo[3,4-*b*][1,4]diazepin-5-yl)phenyl)-3-(3-chlorophenyl)urea (8g). ^{1}H NMR (400 MHz, $CDCl_3$) 7.93 (1 H, d, J = 8.34 Hz), 7.75 (1 H, s), 7.55 (1 H, s), 7.44–7.30 (5 H, m), 7.29–7.21 (2 H, d, J = 7.08 Hz), 7.15 (2 H, d, J = 7.88 Hz), 5.27 (2 H, s), 3.48 (2 H, s); HRMS calcd for $C_{26}H_{22}ClN_6O_2$ m/z 485.1493 Found 485.1494.

5.1.6.8. 1-(3-chlorophenyl)-3-(3-(1-(4-methoxybenzyl)-7-oxo-1,6,7,8-tetrahydropyrazolo[3,4-*b*][1,4]diazepin-5-yl)phenyl)urea (8h). ^{1}H NMR (400 MHz, $CDCl_3$) 8.12 (1 H, s), 8.01 (1 H, s), 7.90 (1 H, d, J = 2.5 Hz), 7.88 (1 H, d, J = 2.5 Hz), 7.76 (1 H, s), 7.71 (2 H, s), 7.46 (1 H, t, J = 8.0 Hz), 7.39 (2 H, d, J = 8.43 Hz), 7.35–7.31 (3 H, m), 7.14 (2 H, d, J = 7.78 Hz), 7.10 (2 H, d, J = 8.26 Hz), 6.84 (2H, d, J = 8.25 Hz), 3.75 (3 H, s), 3.48 (2 H, s); HRMS calcd for m/z $C_{27}H_{24}ClN_6O_3$ 515.1598 Found 515.1575.

5.1.6.9. 1-(4-chlorophenyl)-3-(3-(1-(4-methoxybenzyl)-7-oxo-1,6,7,8-tetrahydropyrazolo[3,4-*b*][1,4]diazepin-5-yl)phenyl)urea (8i). ^{1}H NMR (400 MHz, $CDCl_3$) 8.12 (1 H, s), 8.01 (1 H, s), 7.90 (1 H, d, J = 2.5 Hz), 7.88 (1 H, d, J = 2.5 Hz), 7.76 (1 H, s), 7.75 (2 H, s), 7.46 (1 H, t, J = 8.0 Hz), 7.39 (2 H, d, J = 8.43 Hz), 7.35–7.31 (3 H, m), 7.14 (2 H, d, J = 7.8 Hz), 3.74 (2 H, s), 3.48 (2 H, s); HRMS calcd for $C_{27}H_{24}ClN_6O_3$ m/z 515.1598 Found 515..1559.

5.1.6.10. 1-(4-chlorophenyl)-3-(3-(7-oxo-1,6,7,8-tetrahydropyrazolo[3,4-*b*][1,4]diazepin-5-yl)phenyl)urea (8j). ^{1}H NMR (400 MHz, DMSO-d_6) 13.7 (1 H, s), 10.82 (1 H, s), 9.18 (1 H, s), 9.08 (1 H, s), 8.12 (1 H, s), 8.01 (1 H, s), 7.90 (1 H, d, J = 2.5 Hz), 7.88 (1 H, d, J = 2.5 Hz), 7.76 (1 H, s), 7.75 (2 H, s), 7.46 (1 H, t, J = 8.0 Hz), 7.39 (2 H, d, J = 8.43 Hz), 7.35–7.31 (3 H, m), 7.14 (2 H, d, J = 7.78 Hz), 3.48 (2 H, s); HRMS calcd for m/z $C_{19}H_{16}ClN_6O_2$ 395.1023 Found 395.1094.

5.1.6.11. 1-(3,5-dichlorophenyl)-3-(3-(1-(4-methoxybenzyl)-7-oxo-1,6,7,8-tetrahydropyrazolo[3,4-*b*][1,4]diazepin-5-yl)phenyl)urea (8k). ^{1}H NMR (400 MHz, $CDCl_3$) 8.10 (1 H, s), 7.90 (1 H, d, J = 8.44 Hz), 7.87 (2 H, d, J = 8.2 Hz), 7.78 (1 H, d, J = 8.1 Hz), 7.60 (2 H, s), 7.43 (1 H, s), 7.39–7.31 (2 H, m), 7.15 (1 H, s), 7.10 (2 H, d, J = 8.25 Hz), 6.84 (2 H, d, J = 8.57 Hz), 6.68 (1 H, d, J = 8.66 Hz), 5.26 (2 H, s), 3.76 (3 H, s), 3.48 (2 H, s); HRMS calcd for m/z $C_{27}H_{23}Cl_2N_6O_3$ 549.1209 Found 549.1295.

5.1.6.12. 1-(3,4-dichlorophenyl)-3-(3-(1-(4-methoxybenzyl)-7-oxo-1,6,7,8-tetrahydropyrazolo[3,4-*b*][1,4]diazepin-5-yl)phenyl)urea (8l). ^{1}H NMR (400 MHz, $CDCl_3$) 8.13 (1 H, s), 8.11 (1 H, s), 7.90 (1 H, d, J = 8.44 Hz), 7.83 (1 H, d, J = 8.64 Hz), 7.78 (1 H, d, J = 8.1 Hz), 7.67 (1 H, t, J = 2.13 Hz), 7.60 (2 H, s), 7.51 (1 H, d, J = 5.8 Hz), 7.39–7.31 (4 H, m), 7.15 (1 H, s), 7.10 (2 H, d, J = 8.25 Hz), 6.84 (2 H, d, J = 8.57 Hz), 6.68 (1 H, d, J = 8.66 Hz), 5.26 (2 H, s), 3.76 (3 H, s), 3.48 (2 H, d, J = 5.66 Hz); HRMS calcd for m/z $C_{27}H_{23}Cl_2N_6O_3$ 549.1209 Found 549.1281.

5.1.6.13. 1-(3,4-dichlorophenyl)-3-(3-(7-oxo-1,6,7,8-tetrahydropyrazolo[3,4-*b*][1,4]diazepin-5-yl)phenyl)urea (8m). ^{1}H NMR (400 MHz, DMSO-d_6) 13.7 (1H, s), 10.82 (1 H, s), 9.18 (1 H, s), 9.08 (1 H, s), 8.16 (1 H, s), 8.01 (1 H, s), 7.90 (1 H, d, J = 2.6 Hz), 7.88 (1 H, d, J = 2.6 Hz), 7.76 (1 H, s), 7.54 (1 H, s), 7.46 (1 H, t, J = 8.0 Hz), 7.39 (2 H, d, J = 8.43 Hz), 7.35–7.31 (3 H, m), 7.14 (2 H, d, J = 7.78 Hz), 3.49 (2 H, s); HRMS calcd for $C_{19}H_{15}Cl_2N_6O_2$ m/z 429.0634 Found 429.0603.

5.1.6.14. 1-(3-(1-(4-methoxybenzyl)-7-oxo-1,6,7,8-tetrahydropyrazolo[3,4-*b*][1,4]diazepin-5-yl)phenyl)-3-(3-(trifluoromethyl)phenyl)urea (8n). ^{1}H NMR (400 MHz, $CDCl_3$) 8.13 (1 H, s), 8.11 (1 H, s), 7.90 (1 H, d, J = 8.44 Hz), 7.83 (1 H, d, J = 8.64 Hz), 7.72 (1 H, d, J = 8.57 Hz), 7.67 (1 H, t, J = 2.13 Hz), 7.62 (1 H, s), 7.55 (1 H, d, J = 9.10 Hz), 7.39–7.31 (4 H, m), 7.10 (2 H, t, J = 8.25 Hz), 6.84 (2 H, d, J = 8.57 Hz), 6.68 (1 H, d, J = 8.66 Hz), 5.26 (2 H, s), 3.76 (3 H, s), 3.48 (2 H, d, J = 5.7 Hz; HRMS calcd for C_{28} $H_{24}F_3N_6O_3$ m/z 549.1862 Found 549.1819.

5.1.6.15. 1-(3-(1-(4-methoxybenzyl)-7-oxo-1,6,7,8-tetrahydropyrazolo[3,4-*b*][1,4]diazepin-5-yl)phenyl)-3-(4-(trifluoromethyl)phenyl)urea (8o). ^{1}H NMR (400 MHz, $CDCl_3$) 8.11 (1 H, s), 7.90 (1 H, d, J = 8.44 Hz), 7.83 (1 H, d, J = 8.64 Hz), 7.72 (1 H, d, J = 8.57 Hz), 7.67 (1 H, t, J = 2.13 Hz), 7.62 (2 H, s), 7.61 (2 H, s), 7.55 (1 H, d, J = 9.10 Hz), 7.39–7.31 (2 H, m), 7.10 (2 H, d, J = 8.25 Hz), 6.84 (2 H, d, J = 8.57 Hz), 6.68 (1 H, d, J = 8.66 Hz), 5.26 (2 H, s), 3.76 (3 H, s), 3.48 (2 H, d, J = 5.7 Hz); HRMS calcd for C_{28} $H_{24}F_3N_6O_3$ m/z 549.1862 Found 549.1806.

5.2. Antiproliferative activity

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 concentration (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.

U937 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, US) and maintained in RPMI 1640 medium (Welgene, Daegu, Korea) supplemented with 10% FBS (Welgene), 1% penicillin/streptomycin (Welgene) and 25 mM HEPES (Welgene) in a humidified atmosphere with 5% CO_2 at 37 °C. U937 cells were taken from culture substrate 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 concentration (threefold serial dilution, 12 points) of test compounds. The U937 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 Thiazolyl Blue Tetrazolium Bromide (SIGMA) according to the manufacturer's instructions. The absorbance at 570 nm was recorded using Multiskan EX (Thermo; Waltham, MA, US). The IC50 was calculated using GraphPad Prism 4.0 software.

5.3. Docking simulations

Molecular docking of compound **7b** into 3D X-ray structure of V600E B-Raf (PDB code: 1uwj) was carried out using the Glide (Schrodinger software package Version 9.1).

5.4. Selected kinase profiling

We used Reaction Biology Corp. *Kinase HotSpot*^{SM} service (http://www.reactionbiology.com) for screening of **7b**, and IC_{50} *Profiler*Express 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 [γ-^{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. 10 μL of the reaction is then spotted onto a P30 filtermat and washed three times for 5 min in 75 mM phosphoric acid and once in methanol prior to drying and scintillation counting.

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14. 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% CO2 at 37 °C. A375P cells were taken from culture substrate with 0.05% trypsin-0.02% EDTA and plated at a density of 5 × 103 cells/well in 96 well plates and then incubated at 37 °C for 24 h in a humidified atmosphere with 5% CO2 prior to treatment of various concentration (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.
15. We used Reaction Biology Corp. Kinase HotSpotSM service (http://www.reactionbiology.com) for screening of 7b, 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 [γ-^{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. 10 μL of the reaction is then spotted onto a P30 filtermat and washed three times for 5 min in 75 mM phosphoric acid and once in methanol prior to drying and scintillation counting.
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