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Synthesis, biological evaluation of novel 4,5-dihydro-2*H*-pyrazole 2-hydroxyphenyl derivatives as BRAF inhibitors

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ABSTRACT

A series of novel 4,5-dihydropyrazole derivatives (**3a**–**3t**) containing hydroxyphenyl moiety as potential V600E mutant BRAF kinase ($BRAF^{V600E}$) inhibitors were designed and synthesized. Docking simulation was performed to insert compounds **3d** (*1-(5-(5-chloro-2-hydroxyphenyl)-3-(p-tolyl)-4,5-dihydro-1H-pyrazol-1-yl)ethanone)* and **3m** (*1-(3-(4-chlorophenyl)-5-(3,5-dibromo-2-hydroxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)ethanone)* into the crystal structure of $BRAF^{V600E}$ to determine the probable binding model, respectively. Based on the preliminary results, compound **3d** and **3m** with potent inhibitory activity in tumor growth may be a potential anticancer agent. Results of the bioassays against $BRAF^{V600E}$, MCF-7 human breast cancer cell line and WM266.4 human melanoma cell line all showed several compounds had potent activities IC_{50} value in low micromolar range, among them, compound **3d** and compound **3m** showed strong potent anticancer activity, which were proved by that **3d**: IC_{50} = 1.31 μM for MCF-7 and IC_{50} = 0.45 μM for WM266.5, IC_{50} = 0.22 μM for $BRAF^{V600E}$, **3m**: IC_{50} = 0.97 μM for MCF-7 and IC_{50} = 0.72 μM for WM266.5, IC_{50} = 0.46 μM for $BRAF^{V600E}$, which were comparable with the positive control Erlotinib.

1. Introduction

Cancers arise owing to the accumulation of mutations in critical genes that alter normal programs of cell proliferation, differentiation and death.[1] The RAS/RAF/MEK/ERK/MAP kinase pathway mediates cellular responses to growth signals. RAS is mutated to form about 15% of human cancer. BRAF is a serine/threonine kinase that belongs to this kinase pathway, which is involved in the transduction of mitogenic signals from the cell membrane to the nucleus. It has been reported that this kinase cascade can regulate cell growth, survival and differentiation in a wonderful way.[2] Approximately 90% of activating BRAF mutations in cancer lines are a glutamic acid to valine substitution at position 600 (V600E; formally identified as V599E).[3–7] In cancer cells, $BRAF^{V600E}$, which is ~500-fold more active than the wild-type protein,[8] stimulates constitutive ERK activity and drives proliferation and survival, thereby providing essential tumor growth and remain good functions.[9] $BRAF^{V600E}$ can make contribution to neoangiogenesis by stimulating vascular endothelial growth factor secretion.[10] Overall, these data suggest $BRAF^{V600E}$ as a therapeutic target[11] has offered many valuable and important opportunities for anticancer drug development.

Inhibitors of BRAF have been developed, such as SB-590885,[12] AZ628,[13] sorafenib[14] and PLX4720.[15] Among these inhibitors, SB-590885, one of the hot spots be researched nowadays, is a novel triarylimidazole derivative, and Andrew K. has also evaluated the SAR of a series of imidazole inhibitors based on SB-590885 targeting at BRAF kinase.[16]

Dihydropyrazole, a small bioactive molecule, is a prominent structural motif found in numerous pharmaceutically active compounds.[17] Of late, the dihydropyrazole derivatives used as potent and selective inhibitors have great effect on causing cancer cell death have also been reported.[18–22] Among them, it has been reported many times and have successfully caught many researchers' attention that 4,5-dihydropyrazoles as an important class of heterocyclic small molecules, are important biological agents with a wide range of pharmaceutical (antifungal, antibacteria, antitumor, anti-inflammatory, and antiviral) and agrochemical activities.[23–25] Cox et al. has disclosed a series of 4,5-dihydropyrazole derivative as a potent, selective inhibitor of KSP (Kinesin spindle protein) which is aim to treat human cancer with good potency, pharmacokinetics and water solubility.[26] Some small chemical molecules

Abbreviations: BRAF, V-RAF murine sarcoma viral oncogene homologue B1; $BRAF^{V600E}$, V600E mutant BRAF; IC_{50}, half maximal inhibitory concentration; GI_{50}, the concentration that causes 50% growth inhibition.

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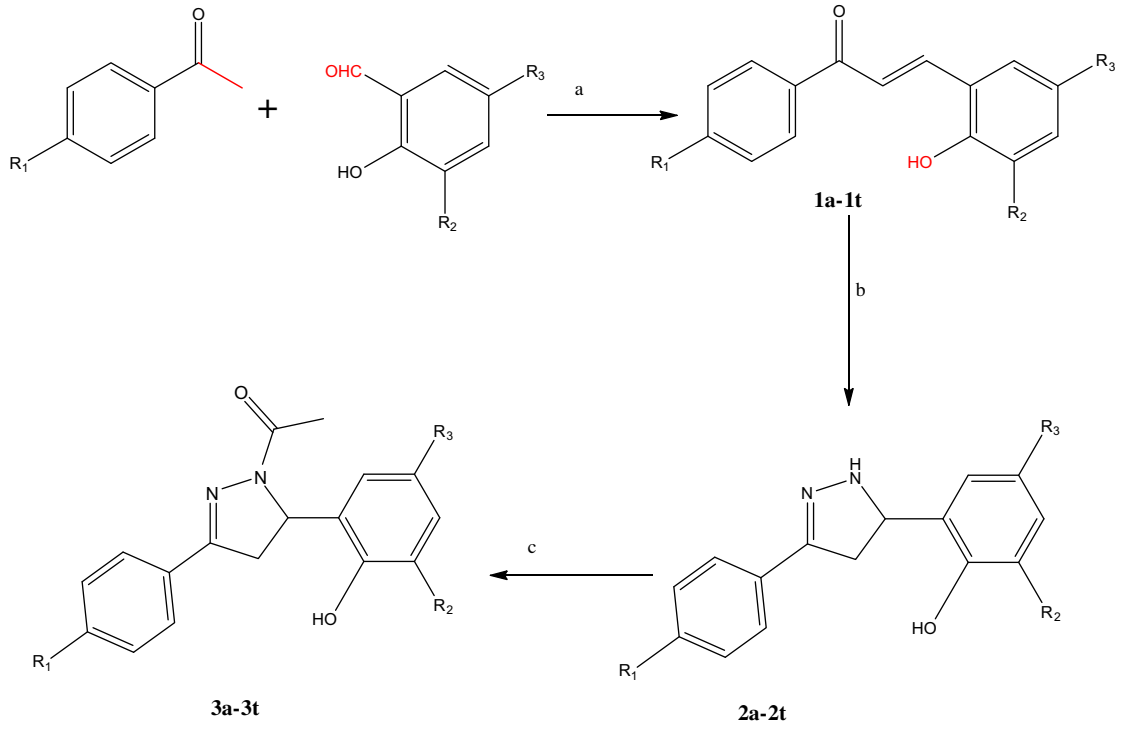
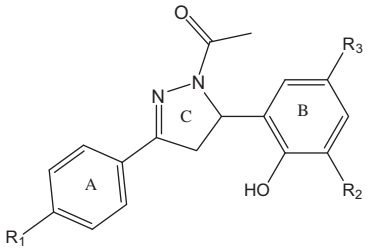


Scheme 1. The synthetic routes of compounds **3a–3t**. Reagents and conditions: (a), ethanol, NaOH, rt, 4–6 h; (b) $NH_2{\cdot}NH_2{\cdot}H_2O$, EtOH, 80 °C; (c) EDC·HCl, CH_2Cl_2, rt.

Table 1
Chemical structures of **3a–3t**



Compound	R_1	R_2	R_3
3a	CH_3	Br	Br
3b	CH_3	Cl	Cl
3c	CH_3	H	Br
3d	CH_3	H	Cl
3e	OCH_3	Br	Br
3f	OCH_3	Cl	Cl
3g	OCH_3	H	Br
3h	OCH_3	H	Cl
3i	F	Br	Br
3j	F	Cl	Cl
3k	F	H	Br
3l	F	H	Cl
3m	Cl	Br	Br
3n	Cl	Cl	Cl
3o	Cl	H	Br
3p	Cl	H	Cl
3q	Br	Br	Br
3r	Br	Cl	Cl
3s	Br	H	Br
3t	Br	H	Cl

containing pyrazole skeleton have been identified as selective inhibitors of $BRAF^{V600E}$ and displayed potent anticancer activities.[27,28] Based on the studies generalized above, a series of novel 4,5-dihydropyrazole derivatives were designed as potential

Table 2
Inhibition (IC_{50}) of MCF-7 and WM266.5 cells proliferation and inhibition of $BRAF^{V600E}$ by compounds **3a–3t**

Compound	IC_{50} ± SD (μM)		
	MCF-7[a]	WM266.5[a]	$BRAF^{V600E}$[b]
3a	3.44 ± 0.06	2.88 ± 0.12	2.68 ± 0.12
3b	2.70 ± 0.01	2.55 ± 0.15	2.87 ± 0.18
3c	2.35 ± 0.02	2.45 ± 0.22	2.41 ± 0.11
3d	1.31 ± 0.01	0.45 ± 0.03	0.22 ± 0.06
3e	3.58 ± 0.05	3.16 ± 0.28	3.02 ± 0.22
3f	3.69 ± 0.04	2.55 ± 0.36	2.56 ± 0.17
3g	3.32 ± 0.11	2.35 ± 0.18	2.08 ± 0.13
3h	3.46 ± 0.03	2.46 ± 0.13	2.65 ± 0.15
3i	23.83 ± 0.06	23.52 ± 0.23	20.83 ± 0.15
3j	28.07 ± 0.34	20.22 ± 1.1	18.31 ± 0.51
3k	19.02 ± 0.25	19.35 ± 1.2	18.62 ± 0.39
3l	19.04 ± 0.22	16.34 ± 0.22	17.31 ± 0.32
3m	0.97 ± 0.02	0.72 ± 0.07	0.46 ± 0.35
3n	4.73 ± 0.17	4.43 ± 0.5	2.62 ± 0.22
3o	4.57 ± 0.17	4.23 ± 0.07	3.34 ± 0.24
3p	3.38 ± 0.15	3.53 ± 0.08	3.22 ± 0.13
3q	7.74 ± 0.13	7.50 ± 0.15	8.14 ± 0.13
3r	8.21 ± 0.19	11.69 ± 0.38	8.68 ± 0.12
3s	5.87 ± 0.13	6.82 ± 0.22	8.01 ± 0.62
3t	6.60 ± 0.09	6.50 ± 0.37	7.86 ± 0.39
Erlotinib	8.97	8.09	0.08

[a] Inhibition of the growth of tumor cell lines.
[b] Inhibition of $BRAF^{V600E}$.

V600E mutant of BRAF kinase ($BRAF^{V600E}$) inhibitors and expected to have a sound cancer therapeutic benefit.

In organic chemistry, phenols, sometimes called phenolics, are a class of chemical compounds consisting of a hydroxyl group (–OH) bonded directly to an aromatic hydrocarbon group. The phenolic hydroxyl group is very important in many chemical reactions and has very good bioactivities. It has been reported that the phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*.[29] Polyphenols, such as

flavones and isoflavones, constitute one of the most represented classes of compounds in higher plants, including medicinal and edible plants. Not only extensive studies but also many in vitro experiments with polyphenols have indicated their broad variety of biological activities of them, including anticancer,[30] anti-inflammatory,[31] antibacterial,[32] cardioprotective,[33] anti-osteoporotic[34] and enzyme-inhibitory[35] activities.

In this study, we want to share our experience about the synthesis, bioassay and the SAR of 4,5-dihydropyrazoles derivatives containing hydroxyphenyl moiety. We have successfully synthesized 20 compounds, and 17 of them are new. Biological evaluation indicated that some of the synthesized compounds are potent inhibitors of $BRAF^{V600E}$. To gain better understanding about the potency of the studied compounds and guide further SAR studies, we proceeded to examine the interaction of compounds with SB-590885 (2FB8.pdb), and docking simulations were performed using the X-ray crystallographic structure of the BRAF in complex with an inhibitor to explore the binding modes of these compounds at the active site.

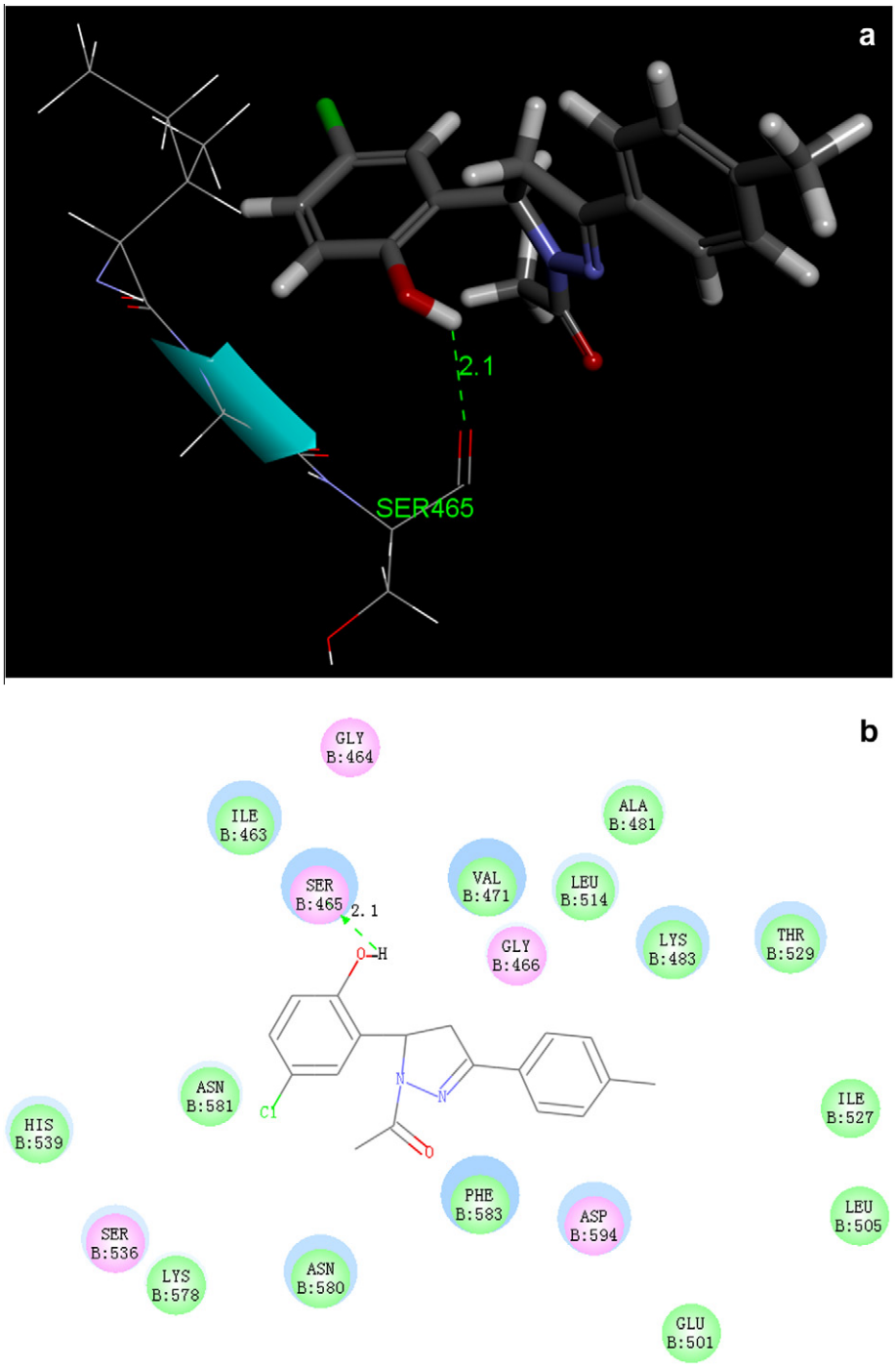


Figure 1. (**a**) Compound **3d** (colored by atom: carbons: gray; nitrogen: blue; oxygen: red; sulfur: yellow) is bond into $BRAF^{V600E}$. The dotted lines show the hydrogen bond and the yellow line show the π–cation interactions. (**b**) 2D Ligand interaction diagram of compound **3d** with $BRAF^{V600E}$ using Discovery Studio program with the essential amino acid residues at the binding site are tagged in circles. The purple circles show the amino acids which participate in hydrogen bonding, electrostatic or polar interactions and the green circles show the amino acids which participate in the Van der Waals interactions.

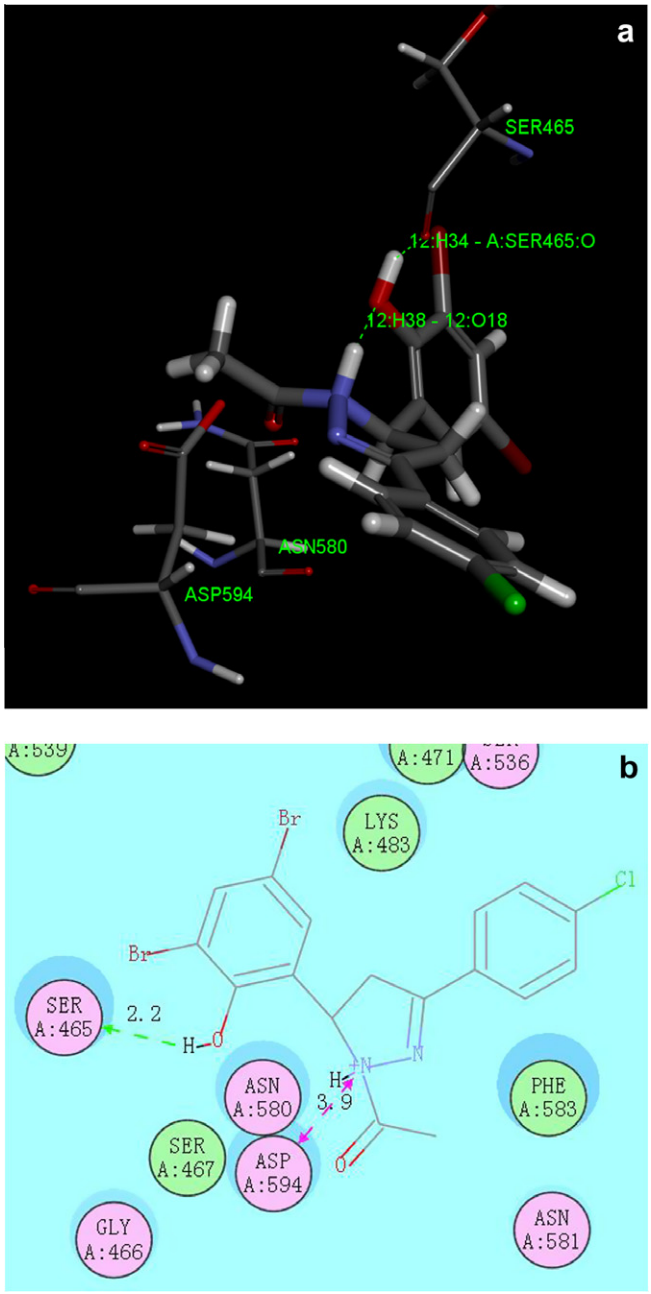


Figure 2. (**a**) Compound **3m** (colored by atom: carbons: gray; nitrogen: blue; oxygen: red; sulfur: yellow) is bond into $BRAF^{V600E}$. The dotted lines show the hydrogen bond and the yellow line show the π–cation interactions. (**b**) 2D Ligand interaction diagram of compound **3m** with $BRAF^{V600E}$ using Discovery Studio program with the essential amino acid residues at the binding site are tagged in circles. The purple circles show the amino acids which participate in hydrogen bonding, electrostatic or polar interactions and the green circles show the amino acids which participate in the Van der Waals interactions.

2. Results and discussion

2.1. Chemistry

The synthesis of compounds (**3a–3t)** is followed the general pathway outlined in Scheme 1 and the structure of these compounds have been shown in Table 1. Compounds (**3a–3t)** are prepared in three steps. Firstly, the chalcones (**1a–1t**) were obtained by the substituted salicylaldehydes and the substituted acetophenone, using 40% potassium hydroxide as catalyst in ethanol.[36] Secondly, treat chalcone derivatives in refluxing isopropanol with hydrazine hydrate for 8 h to obtain the desired compounds (**2a–2t)**. Finally, to a solution of compounds (**2a–2t)** in dichloromethane, Acetic acid derivatives was added, together with EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) and HOBt (hydroxybenzotriazole). The mixture was refluxed under stirring for 8 h to obtain the desired compounds (**3a–3t)**. All the synthetic compounds were characterized by ^1H NMR, elemental analysis and mass spectrum, which were in full accordance with their depicted structures.

2.2. Bioassay

As described above, the $BRAF^{V600E}$ was considered as an important target for the development of small molecule inhibitors in the treatment of human cancers, particularly melanoma. To test the anticancer activities of the synthesized compounds, we evaluated antiproliferative activities of compounds **3a–3t** against MCF-7 and WM266.5 cells. The results were summarized in Table 2. These compounds showed remarkable antiproliferative effects. Among them, compound **3d** and compound **3m** displayed the strong potent inhibitory activity (**3d**: IC_{50} = 1.31 μM for MCF-7 and IC_{50} = 0.45 μM for WM266.5, **3m:** IC_{50} = 0.97 μM for MCF-7 and IC_{50} = 0.72 μM for WM266.5), respectively, comparing with the positive control Erlotinib (IC_{50} = 8.97 μM for MCF-7, IC_{50} = 8.09 μM for WM266.5, respectively).

Structure–activity relationships in these dihydropyrazole derivatives displayed that compounds with *para* electron-donating substituents (**3a–3h**) showed more potent activities than those with electron-withdrawing substituents (**3i–3t**) in the A-ring. A comparison of the *para* substituents on the A-ring demonstrated that an electron-donating group (**3a–3h**) have slightly improved antiproliferative activity and the potency order is $CH_3 > OCH_3$. Comparing with CH_3 (**3a–3d**) and OCH_3 (**3e–3h**), F (**3i–3l**), Cl (**3m–3p**) and Br (**3q–3t**) substituents mostly had minimal effects, which was clearly viewed through Table 2. In the case of constant A ring substituents, change of substituents on B ring could also affect the activities of these compounds. It can be found that compounds with a halogen atom on the 5-position of salicylaldehyde mostly displayed higher anticancer activity than compounds with two halogen atoms on the 3-position and 5-position of salicylaldehyde, however, 1-(5-(3,5-dibromo-2-hydroxyphenyl)-3-(4-fluorophenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone (**3m**) showed better anticancer activity than the compounds with a single halogen atom on the 5-position of salicylaldehyde. Among compounds with a halogen atom on the 5-position of salicylaldehyde and a halogen atom on the 5-position of salicylaldehyde, the strength order is Cl > Br. Thus, the compound **3d** with *para*-Me group in the A ring and Cl in the B ring showed the best activity and compound **3m** also displayed a good anticancer activity. To examine whether the compounds interact with $BRAF^{V600E}$ and inhibit $BRAF^{V600E}$ in vitro, we did the anti-$BRAF^{V600E}$ research and the data had also shown in Table 2, compound **3d** and compound **3m** showed strong potent anti-$BRAF^{V600E}$ activity and 50% $BRAF^{V600E}$ inhibition about **3d**: 0.22 μM, **3m**: 0.46 μM, respectively (the positive control Erlotinib with an IC_{50} of 0.08 μM for $BRAF^{V600E}$). These results indicated the anti-proliferative effect was produced by the direct connection of $BRAF^{V600E}$ and the compounds.

2.3. Docking study

Molecular docking is an application wherein molecular modeling techniques are used to predict how a protein (enzyme) interacts with small molecules (ligands).[37] In the present study, to understand the interactions between compounds and BRAF and

to explore their binding mode, a docking study was performed using the CDOCKER protocol in Discovery Studio 3.1 (Discovery Studio 3.1, Accelrys, Inc., San Diego, CA).

In the current study, we performed docking of 20 4,5-dihydropyrazole derivatives into the active site of the receptor BRAF using CDOCKER in the receptor–ligand Interactions protocol section of Discovery Studio 3.1. The crystal structure of BRAF (PDB Code: 2FB8.pdb)[12] was obtained from the RCSB protein data bank (http://www.pdb.org). After preparing the receptor and ligands, the site sphere was selected based on the ligand binding location of SB-590885.

Binding model between the most potent compound **3d** with BRAF active site was showed in Figure 1. All the amino acid residues which had interactions with $BRAF^{V600E}$ were exhibited in Figure 1b. In the binding model, compound **3d** is nicely bound to the active site of BRAF via hydrogen bond with SER465 (angle O···H–N = 126.66°, distance = 2.1 Å). The receptor surface model was showed in Figure 1a, which revealed that the molecule occupies the ATP-binding pocket and binds to an active conformation of BRAF. The hydrophobic pocket including VAL471, PHE583, ALA481, THR529, LEU514, VAL482 and GLY464 is occupied by acetamide moiety. This binding model confirms the importance of hydrophobic group.

Binding model between the most potent compound **3m** with BRAF active site was showed in Figure 2. All the amino acid residues which had interactions with $BRAF^{V600E}$ were exhibited in Figure 2b. In the binding model, compound **3m** is nicely bound to the active site of BRAF via hydrogen bond with SER465 (angle O···H–N = 104.7°, distance = 2.20 Å) and ASP594(distance = 3.9 Å). The receptor surface model was showed in Figure 2a, which reveals that the molecule occupies the ATP-binding pocket and binds to an active conformation of BRAF. The hydrophobic pocket including LYS483, ASN580, SER467, ASN581 and GLY466 is occupied by acetamide moiety. This binding model also confirms the importance of hydrophobic group as well.

Overall, these results of the molecular docking study showed that the compound **3d** and **3m** could act synergistically to interact with the binding site of $BRAF^{V600E}$, suggested that compound **3d** and **3m** are potential inhibitors of $BRAF^{V600E}$.

3. Conclusion

In summary, a series of novel compounds containing 4,5-dihydropyrazole core with hydroxyphenyl moiety have been synthesized and evaluated for their B-Raf inhibitory and anti-proliferation activities. Results showed these compounds possessed potent antiproliferative activity against BRAF, WM266.4 human melanoma cell line, MCF-7 human breast cancer cell line with IC_{50} inhibitory values in low micromolar range. Among them, compound **3d** and **3m** showed strong potent agent with IC_{50} value (**3d**: IC_{50} = 1.31 μM for MCF-7 and IC_{50} = 0.45 μM for WM266.5, **3m**: IC_{50} = 0.97 μM for MCF-7 and IC_{50} = 0.72 μM for WM266.5, respectively), comparing with the positive control Erlotinib (IC_{50} = 8.97 μM for MCF-7 and IC_{50} = 8.09 μM for WM266.5). And compound **3d** and compound **3m** also showed strong potent anti-$BRAF^{V600E}$ activity and 50% $BRAF^{V600E}$ inhibition about **3d**: 0.22 μM, **3m**: 0.46 μM, respectively (the positive control Erlotinib with an IC_{50} of 0.08 μM for $BRAF^{V600E}$), this result could well show the anti-proliferative effect was produced by direct connection of $BRAF^{V600E}$ and the compounds.

The docking simulation was performed to get binding models and poses, and the result showed compound **3d** and compound **3m** could bind well with the BRAF active site and acted as BRAF inhibitor.

4. Experimental section

4.1. Materials and measurements

All chemicals (reagent grade) used were commercially available. Separation of the compounds by column chromatography was carried out with silica gel 60 (200–300 mesh ASTM, E. Merck, Germany). Developed plates were visualized by a Spectroline ENF 260C/F UV apparatus. 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). Concentration and evaporation of the solvent after reaction or extraction were carried out on a rotary evaporator (Büchi Rotavapor) operating at reduced pressure. Melting points were measured on a Boetius micro melting point apparatus. All the Proton nuclear magnetic resonance (^{1}H NMR) and spectra were recorded on a DPX300 model Spectrometer at 25 °Cwith TMS and solvent signals allotted as internal stands. Chemical shifts were reported in parts per million(d). ESI-MS spectra were recorded on a Mariner System 5304 Mass spectrometer. Elemental analyses were performed on a CHN–O-Rapid instrument and were within 0.4% of the theoretical values.

4.2. General procedure for synthesis of chalcones (1a–1t)

Equimolar portions of the appropriately substituted salicylaldehydes (2 mmol, 1 equiv) and substituted acetophenone (2 mmol, 1 equiv) were dissolved in approximately 10 mL of ethanol. The mixture was allowed to stir for several minutes at 0 °C to let it dissolve. Then, 0.5 mL aliquot of a 40% aqueous sodium hydroxide solution was slowly added dropwise to the reaction flask via a self-equalizing addition funnel. The reaction solution was allowed to stir at room temperature for approximately 4–6 h. The mixture was adjusted to pH 4.0 with dilute hydrochloric acid until the reaction was complete. The reaction was monitored by TLC. Mostly, a precipitate formed and was then collected by suction filtration.

4.3. General method of synthesis of 3,5-diphenyl-4,5-dihydro-1*H*-pyrazole (2a–2t)

To a solution of chalcone derivative (1 mmol) in isopropanol (5 mL) hydrazine hydrate (0.2 mL, 4 mmol) was added. The mixture was refluxed under stirring for 8 h, stored at 4–5 °C for 24 h, and the precipitate formed was filtered off, washed with cool ethanol. The synthesized compound was purified by crystallization from ethanol in refrigerator and allowed to air dry to obtain 4,5-dihydropyrazole derivatives.

4.4. General method of synthesis of compounds (3a–3t)

To a solution of pyrazoline derivatives (1 mmol) in dichloromethane (5 mL) acetic acid (5 ml) was added, together with EDC (1.2–1.5 mmol) and HOBt (1.2–1.5 mmol). The mixture was refluxed under stirring for 8 h. After completion of the reaction, the contents were cooled, and then evaporated to dryness in vacuo. Aqueous hydrochloric acid (0.1 M, 30 mL) was added and the mixture extracted with ethyl acetate (3 × 5 mL). The combined ethyl acetate layers were back-extracted with saturated sodium bicarbonate (3 × 5 mL) and brine (3 × 5 mL), dried over $MgSO_4$, filtered, and evaporated in vacuo. The residue was crystallized from ethanol to obtain target compounds.

4.4.1. 1-(5-(3,5-Dibromo-2-hydroxyphenyl)-3-(*p*-tolyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone(3a)

White powder, yield: 63%. Mp: 246–248 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 2.33 (d, J = 13.3 Hz, 6H), 3.77 (s, 1H), 4.31 (d, J = 4.58 Hz, 2H), 5.65–5.68 (m, 1H), 6.98 (d, J = 2.15 Hz, 1H), 7.27 (d, J = 8.05 Hz, 2H), 7.63–7.67 (m, 3H), MS (ESI): 451 ($C_{18}H_{17}Br_2N_2O_2$, $[M+H]^+$). Anal. $C_{18}H_{16}Br_2N_2O_2$. Calcd for: C, 47.82; H, 3.57; N, 6.20. Found: C, 47.83; H, 3.58; N, 6.15.

4.4.2. 1-(5-(3,5-Dichloro-2-hydroxyphenyl)-3-(*p*-tolyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone(3b)

White powder, yield: 61%. Mp: 237–239 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 2.33 (d, J = 13.3 Hz, 6H), 3.77 (s, 1H), 4.32 (d, J = 4.58 Hz, 2H), 5.66 (s, 1H), 6.95 (d, J = 2.15 Hz, 1H), 7.26 (d, J = 8.05 Hz, 2H), 7.63–7.67 (m, 3H), MS (ESI): 363 ($C_{18}H_{17}Cl_2N_2O_2$, $[M+H]^+$). Anal. $C_{18}H_{16}Cl_2N_2O_2$. Calcd for: C, 59.52; H, 4.44; N, 7.71. Found: C, 59.53; H, 4.48; N, 7.79

4.4.3. 1-(5-(5-Bromo-2-hydroxyphenyl)-3-(*p*-tolyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone (3c)

White powder, yield: 69%. Mp: 215–216 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 2.31 (s, 6H), 4.32 (t, J = 5.03 Hz, 3H), 5.57 (d, J = 7.3 Hz, 1H), 6.78 (s, 1H), 6.84 (d, J = 8.55 Hz, 1H), 6.85 (s, 2H), 6.99–7.01 (m, 1H), 7.71 (d, J = 8.7 Hz, 2H), MS (ESI): 345 ($C_{18}H_{18}BrN_2O_2$, $[M+H]^+$). Anal. $C_{18}H_{17}BrN_2O_2$. Calcd for: C, 57.92; H, 4.59; N, 7.51. Found: C,57.93; H, 4.58; N,7.49.

4.4.4. 1-(5-(5-Chloro-2-hydroxyphenyl)-3-(*p*-tolyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone (3d)

White powder, yield: 78%. Mp: 209–211 °C. ^{1}H NMR (500 MHz, DMSO-d_6)δ: 2.32 (s, 6H), 4.32 (t, J = 5.03 Hz, 3H), 5.57 (d, J = 7.3 Hz, 1H), 6.78 (s, 1H), 6.84 (d, J = 8.7 Hz, 1H), 6.85 (s, 2H), 6.99–7.02 (m, 1H), 7.71 (d, J = 8.55 Hz, 2H), MS (ESI): 329 ($C_{18}H_{18}ClN_2O_2$, $[M+H]^+$). Anal. $C_{18}H_{17}ClN_2O_2$. Calcd for: C, 65.75; H, 5.21; N, 8.52. Found: C, 65.73; H, 5.18; N, 8.49.

4.4.5. 1-(5-(3,5-Dibromo-2-hydroxyphenyl)-3-(4-methoxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone (3e)

White powder, yield: 65%. Mp: 214–215 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 2.41 (d, J = 13.5 Hz, 3H), 4.33 (s, 6H), 5.68 (d, J = 7 Hz, 1H), 7.01 (s, 1H), 7.30 (t, J = 8.85 Hz, 2H), 7.64 (d, J = 2.15 Hz, 1H), 7.83 (t, J = 7.18 Hz, 2H), MS (ESI): 467 ($C_{18}H_{17}Br_2N_2O_3$, $[M+H]^+$). Anal. $C_{18}H_{16}Br_2N_2O_3$. Calcd for: C, 46.18; H, 3.44; N, 5.98. Found: C, 46.13; H, 3.48; N, 5.99.

4.4.6. 1-(5-(3,5-Dichloro-2-hydroxyphenyl)-3-(4-methoxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone (3f)

White powder, yield: 64%. Mp: 206–208 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.41 (d, J = 13.5 Hz, 3H), 3.79–4.32 (m, 6H), 5.66–5.69 (m, 1H), 7.01 (s, 1H), 7.29 (t, J = 5.22 Hz, 2H), 7.63 (s, 1H), 7.83 (t, J = 4.17 Hz, 2H), MS (ESI): 379 ($C_{18}H_{17}Cl_2N_2O_3$, $[M+H]^+$). Anal. $C_{18}H_{16}Cl_2N_2O_3$. Calcd for: C, 57.01; H, 4.25; N, 7.39. Found: C, 56.93; H, 4.28; N, 7.43.

4.4.7. 1-(5-(5-Bromo-2-hydroxyphenyl)-3-(4-methoxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone (3g)

White powder, yield: 67%. Mp: 261–263 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 2.31 (s, 3H), 3.56 (t, J = 8.55 Hz, 3H), 4.22 (t, J = 5.03 Hz, 3H), 5.55 (d, J = 8.7 Hz, 1H), 6.77 (s, 1H), 6.82 (d, J = 8.55 Hz, 1H), 6.85 (s, 2H), 6.99–7.10 (m, 1H), 7.68 (d, J = 8.7 Hz, 2H), MS (ESI): 389 ($C_{18}H_{18}BrN_2O_3$, $[M+H]^+$). Anal. $C_{18}H_{17}BrN_2O_3$. Calcd for: C, 55.54; H, 4.40; N, 7.20. Found: C, 55.58; H, 4.48; N, 7.19.

4.4.8. 1-(5-(5-Chloro-2-hydroxyphenyl)-3-(4-methoxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone (3h)

White powder, yield: 65%. Mp: 262–264 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 2.31 (s, 3H), 3.76 (t, J = 13.88 Hz, 3H), 4.32 (t, J = 5.03 Hz, 3H), 5.57 (d, J = 7.3 Hz, 1H), 6.78 (s, 1H), 6.84 (d, J = 8.55 Hz, 1H), 6.85 (s, 2H), 6.99–7.01 (m, 1H), 7.71 (d, J = 8.7 Hz, 2H), MS (ESI): 345 ($C_{18}H_{18}ClN_2O_3$, $[M+H]^+$). Anal. $C_{18}H_{17}ClN_2O_3$. Calcd for: C, 62.70; H, 4.97; N, 8.12. Found: C, 62.73; H, 4.98; N, 8.19.

4.4.9. 1-(5-(3,5-Dibromo-2-hydroxyphenyl)-3-(4-fluorophenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone (3i)

White powder, yield: 68%. Mp: 215–216 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.33 (s, 3H), 3.61–3.68 (m, 3H), 5.81–5.83 (m, 1H), 6.94 (d, J = 2.1 Hz, 1H), 7.08 (t, J = 8.55 Hz, 2H), 7.51 (d, J = 2.1 Hz, 1H), 7.69–7.74 (m, 2H), MS (ESI): 455 ($C_{17}H_{14}Br_2FN_2O_2$, $[M+H]^+$). Anal. $C_{17}H_{13}Br_2FN_2O_2$. Calcd for: C, 44.77; H, 2.87; N, 6.14. Found: C, 44.73; H, 2.88; N, 6.19.

4.4.10. 1-(5-(3,5-Dichloro-2-hydroxyphenyl)-3-(4-fluorophenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone (3j)

White powder, yield: 61%. Mp: 209–211 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.33 (s, 3H), 3.61–3.66 (m, 3H), 5.81–5.82 (m, 1H), 6.88 (d, J = 2.1 Hz, 1H), 6.99 (t, J = 8.55 Hz, 2H), 7.47 (d, J = 2.1 Hz, 1H), 7.65–7.70 (m, 2H), MS (ESI): 367 ($C_{17}H_{14}Cl_2FN_2O_2$, $[M+H]^+$).Anal$C_{17}H_{13}Cl_2FN_2O_2$. Calcd for: C, 55.60; H, 3.57; N, 7.63. Found: C, 55.53; H, 3.58; N, 7.69.

4.4.11. 1-(5-(5-Bromo-2-hydroxyphenyl)-3-(4-fluorophenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone (3k)

White powder, yield: 71%. Mp: 233–235 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.33 (s, 3H), 3.61–3.65 (m, 3H), 5.79–5.81 (m, 1H), 6.77 (d, J = 1.8 Hz, 2H), 7.07–7.18 (m, 3H), 7.68–7.76 (m, 2H), MS (ESI): 377 ($C_{17}H_{15}BrFN_2O_2$, $[M+H]^+$). Anal. $C_{17}H_{14}BrFN_2O_2$. Calcd for: C, 54.13; H, 3.74; N, 7.43. Found: C, 54.15; H, 3.78; N, 7.39.

4.4.12. 1-(5-(5-Chloro-2-hydroxyphenyl)-3-(4-fluorophenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone (3l)

White powder, yield: 68%. Mp: 212–214 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.32 (s, 3H), 3.61–3.68 (m, 3H), 5.79–5.81 (m, 1H), 6.80 (d, J = 1.8 Hz, 2H), 7.07–7.18 (m, 3H), 7.72–7.76 (m, 2H), MS (ESI): 333 ($C_{17}H_{15}ClFN_2O_2$, $[M+H]^+$). Anal. $C_{17}H_{14}ClFN_2O_2$. Calcd for: C, 61.37; H, 4.24; N, 8.42. Found: C, 61.33; H, 4.28; N, 8.39.

4.4.13. 1-(3-(4-Chlorophenyl)-5-(3,5-dibromo-2-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone (3m)

White powder, yield: 78%. Mp: 208–210 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 2.32 (s, 3H), 3.79–3.81 (m, 1H), 4.31 (t, J = 5.1 Hz, 2H), 5.65–5.68 (m, 1H), 6.88 (d, J = 8.55 Hz, 1H), 7.41 (d, J = 2.4 Hz, 1H), 7.65(s, 2H), 7.69(t, J = 12.88 Hz, 2H), MS (ESI): 471 ($C_{17}H_{14}Br_2ClN_2O_2$, $[M+H]^+$). Anal. $C_{17}H_{13}Br_2ClN_2O_2$. Calcd for: C, 43.21; H, 2.77; N, 5.93. Found: C, 43.23; H, 2.78; N, 5.99.

4.4.14. 1-(3-(4-Chlorophenyl)-5-(3,5-dichloro-2-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone (3n)

White powder, yield: 75%. Mp: 209–210 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 2.33 (s, 3H), 3.79–3.81 (m, 1H), 4.31 (t, J = 8.55 Hz, 2H), 5.65–5.67 (m, 1H), 6.86 (d, J = 8.55 Hz, 1H), 7.39 (d, J = 2.4 Hz, 1H), 7.64 (s, 2H), 7.67 (t, J = 5.1 Hz, 2H), MS (ESI): 385 ($C_{17}H_{14}Cl_3N_2O_2$, $[M+H]^+$). Anal. $C_{17}H_{13}Cl_3N_2O_2$. Calcd for: C, 53.22; H, 3.42; N, 7.30. Found: C, 53.23; H, 3.38; N, 7.29.

4.4.15. 1-(5-(5-Bromo-2-hydroxyphenyl)-3-(4-chlorophenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone (3o)

White powder, yield: 74%. Mp: 270–272 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 2.32 (s, 3H), 3.79–3.81 (m, 1H), 4.31 (t, J = 5.1 Hz, 2H),

5.65–5.68 (m, 1H), 6.88 (d, J = 8.55 Hz, 1H), 7.41 (d, J = 2.4 Hz, 1H), 7.65 (s, 2H), 7.69 (t, J = 12.88 Hz, 2H), MS (ESI): 393 ($C_{17}H_{15}BrClN_2O_2$, $[M+H]^+$). Anal. $C_{17}H_{14}BrClN_2O_2$. Calcd for: C, 51.87; H, 3.58; N,7.12. Found: C,51.93; H, 3.62; N, 7.07.

4.4.16. 1-(5-(5-Chloro-2-hydroxyphenyl)-3-(4-chlorophenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone (3p)

White powder, yield: 71%. Mp: 263–265 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 2.33 (s, 3H), 4.29–4.34 (m, 3H), 5.60 (s, 1H), 6.84 (d, J = 8.55 Hz, 2H), 7.11 (d, J = 8.55 Hz, 1H), 7.52 (d, J = 8.55 Hz, 2H), 7.79 (d, J = 8.55 Hz, 2H), MS (ESI): 349 ($C_{17}H_{15}ClN_2O_2$, $[M+H]^+$). Anal. $C_{17}H_{14}ClN_2O_2$. Calcd for: C, 58.47; H, 4.04; N, 8.02. Found: C, 58.43; H, 3.98; N, 7.97.

4.4.17. 1-(3-(4-Bromophenyl)-5-(3,5-dibromo-2-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone (3q)

White powder, yield: 72%. Mp: 223–225 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.32 (s, 3H), 3.35 (s, 2H), 3.76–3.80 (m, 1H), 5.64–5.66 (m, 1H), 7.01 (d, J = 2.19 Hz, 1H), 7.64–7.78 (m, 5H), MS (ESI): 515 ($C_{17}H_{14}Br_3N_2O_2$, $[M+H]^+$). Anal. $C_{17}H_{13}Br_3N_2O_2$. Calcd for: C, 39.49; H, 2.53; N, 5.42. Found: C, 39.48; H, 2.48; N, 5.43.

4.4.18. 1-(3-(4-Bromophenyl)-5-(3,5-dichloro-2-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl) ethanone (3r)

White powder, yield: 76%. Mp: 237–239 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.32–2.50 (m, 3H), 3.76–3.86 (m, 1H), 4.33 (s, 2H), 5.64–5.67 (m, 1H), 7.01 (d, J = 2.19 Hz, 1H), 7.64–7.73 (m, 5H), MS (ESI): 427 ($C_{17}H_{14}BrCl_2N_2O_2$, $[M+H]^+$). Anal. $C_{17}H_{14}BrCl_2N_2O_2$. Calcd for: C, 47.69; H, 3.06; N, 6.54. Found: C, 47.68; H, 3.05; N, 6.53.

4.4.19. 1-(5-(5-Bromo-2-hydroxyphenyl)-3-(4-bromophenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone (3s)

White powder, yield: 76%. Mp: 297–299 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.32 (s, 3H), 3.75–3.85 (m, 3H), 5.56–5.61 (m, 1H), 6.83 (t, J = 4.38 Hz, 2H), 7.10–7.13 (m, 1H), 7.64–7.73 (m, 4H), MS (ESI): 437 ($C_{17}H_{15}Br_2N_2O_2$, $[M+H]^+$). Anal. C_{17} $H_{14}Br_2N_2O_2$. Calcd for: C, 46.60; H, 3.22; N, 6.39. Found: C, 46.68; H, 3.28; N, 6.33.

4.4.20. 1-(3-(4-Bromophenyl)-5-(5-chloro-2-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone (3t)

White powder, yield: 65%. Mp: 270–272 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.32 (s, 3H), 3.75–3.85 (m, 3H), 5.56–5.61 (m, 1H), 6.63 (t, J = 4.47 Hz, 2H), 7.10–7.13 (m, 1H), 7.64–7.63 (m, 4H), MS (ESI): 393 ($C_{17}H_{15}BrClN_2O_2$, $[M+H]^+$). Anal. $C_{17}H_{14}BrClN_2O_2$. Calcd for: C, 51.87; H, 3.58; N, 7.12. Found: C, 51.78; H, 3.58; N, 7.13.

4.5. Antiproliferative activity

Target tumor cells were cultured in DMEM/10% fetal bovine serum, with 5% CO_2 water saturated atmosphere at 37 °C. After diluting to 2×10^4cells mL^{-1}with the complete medium, 100 μL of the obtained cell suspension was added to each well of 96-well culture plates. The plates were returned to the incubator for 24 h to allow the cells to reattach. These compounds were initially prepared at 20 mM in DMSO. Aliquots (200 μL) were diluted into 20 mL culture medium giving 200 μM, and 10 serial dilutions of 3× prepared. Aliquots (100 μL) of each dilution were added to the wells, giving doses ranging from 0.005 μM to 100 μM. After further incubated at 37 °C for 24 h in a humidified atmosphere with 5% CO_2, the cell viability was assessed by the conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay and carried out strictly according to the manufacturer instructions (Sigma). The absorbance at 590 nm was recorded using LX300 Epson Diagnostic microplate reader. Then IC_{50} was calculated using SPSS 13.0 software.

4.6. Kinase assay

This V600E mutant BRAF kinase assay was performed in triplicate for each tested compound in this study. Briefly, 7.5 ng Mouse Full-Length GST-tagged BRAF^{V600E} (Invitrogen, PV3849) was preincubated at room temperature for 1 h with 1 μL drug and 4 μL assay dilution buffer. The kinase assay was initiated when 5 μL of a solution containing 200 ng recombinant human full length, N-terminal His-tagged MEK1 (Invitrogen), 200 μM ATP, and 30 mM $MgCl_2$ in assay dilution buffer was added. The kinase reaction was allowed to continue at room temperature for 25 min and was then quenched with 5 μL 5× protein denaturing buffer (LDS) solution. Protein was further denatured by heating for 5 min at 70 °C. 10 μL of each reaction was loaded into a 15-well, 4–12% precast NuPage gel (Invitrogen) and run at 200 V, and upon completion, the front, which contained excess hot ATP, was cut from the gel and discarded. The gel was then dried and developed onto a phosphor screen. A reaction that contained no active enzyme was used as a negative control, and a reaction without inhibitor was used as the positive control.

Detection of the effect of compounds on cell based pERK1/2 activity in WM266.4 cells was performed using ELISA kits (Invitrogen) and strictly according to the manufacturer instructions.

4.7. Docking

The pdb file about the crystal structure of the BRAF kinase domain bound to SB-590885(2FB8.pdb) was obtained from the RCSB protein data bank (http://www.pdb.org). The molecular docking procedure was performed by using CDOCKER protocol for receptor–ligand interactions section of Discovery Studio 3.1 (Accelrys Software Inc, San Diego, CA). Initially both the ligands and the receptor were pretreated. For ligand preparation, the 3D structures of all the steroidal compounds were generated with ChemBioOffice 2010 and optimized with MMFF94 method. For enzyme preparation, the hydrogen atoms were added with the pH of the protein in the range of 6.5–8.5. CDOCKER is an implementation of a CHARMm based molecular docking tool using a rigid receptor.[38] It includes the following steps:

(1) A series of ligand conformations are generated using high temperature molecular dynamics with different random seeds.
(2) Random orientations of the conformations are generated by translating the center of the ligand to a specified position within the receptor active site, and making a series of random rotations. A softened energy is calculated and the orientation is kept when it is less than a specified limit. This process repeats until either the desired number of low-energy orientations is obtained, or the test times of bad orientations reached the maximum number.
(3) Each orientation is subjected to simulated annealing molecular dynamics. The temperature is heated up to a high temperature then cooled to the target temperature. A final energy minimization of the ligand in the rigid receptor using non-softened potential is performed.
(4) For each of the final pose, the CHARMm energy (interaction energy plus ligand strain) and the interaction energy alone are figured out. The poses are sorted according to CHARMm energy and the top scoring (most negative, thus favorable to binding) poses are retained. The whole BRAFkinase domain defined as a receptor and the site sphere was selected based on the ligand binding location of SB-590885, then the SB-590885 removed and the ligands prepared by us was placed during the molecular docking procedure. CHARMm was selected as the force field. The molecular docking was

performed with a simulated annealing method. The heating steps were 2000 with 700 of heating target temperature. The cooling steps were 5000 with 300 cooling target temperature. Ten molecular docking poses saved for each ligand were ranked according to their dock score function. The pose with the highest-CDOCKER energy was chosen as the most suitable pose.

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