Design and synthesis of new pyrazolylbenzimidazoles as sphingosine 1-kinase inhibitors

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Abstract

Sphingosine-1 Kinase (SphK-1) is one of the important enzymes of phospholipids and its inhibition is one of the therapeutic strategies for different diseases. SphK1 over expression is observed in different types of cancer that indicating its important role in tumor growth. In search of effective SphK-1 inhibitors, a new series of pyrazolylbenzimidazoles was synthesized and evaluated as sphingosine1-kinase (Sphk-1) inhibitors. In order to evaluate the binding affinities of all the synthesized compounds, all compounds were subjected to docking analysis and fluorescence quenching. The results indicated that there is a consistency between the docking and the fluorescence quenching results which revealed that compounds **47** and **48** exhibited significant decrease in the fluorescence intensity of SphK1 as well as they formed stable protein–ligand complexes. In addition, Enzyme inhibition assay was performed and showed effective inhibitory potential towards SphK-1. Moreover, IC_{50} values was calculated and displayed that compounds **47** and **48** were the most promising compounds. In addition, antiproliferation study for all the synthesized compounds was performed against NCI 60-cell line panel. The target compounds **47** and **48** demonstrated effective antitumor activity inhibitory potential to the SphK-1. Most of these compounds fit well into the ATP-binding site of SphK1 and form significant hydrogen-bonding interactions with catalytically relevant residues as predicted by molecular docking. In this article, insight has been given for the importance of pyrazolylbenzimidazoles as Sphk1 inhibitors and the perspectives that they hold for future research.

Introduction

Sphingolipid is a kind of phospholipid which is a major component of all cell membranes and can form lipid bilayers that maintain the fluidity of membranes [1]. Ceramide (Cer), sphingosine (Sph) and sphingosine1-phosphate (S1P) are metabolites of sphingomyelin which play an important role in different diseases such as cancer [2, 3], fibrosis [4] and Alzheimer's disease [5]. The balance between the sphingolipid metabolites which acting in two opposite ways is crucial in the determination of the cell fate [6, 7]. Ceramide and sphingosine [8, 9] are a pro-apoptotic molecule to mediate the cell cycle and induce apoptosis, while S1P promotes cell proliferation and acts as a 'pro-survival' molecule [10, 11]. Phosphorylation of the proapoptotic D-erythro-sphingosine to the promitogenic sphingosine1-phosphate (S1P) is catalyzed by Sphingosine kinases (SPhK). The two SphK isoforms (SphK1 and SphK2) are known to catalyze this transformation and regulate the sphingolipid metabolism. In particular, SphK1 is more closely linked in many diseases such as cancer, rheumatoid arthritis, diabetes, asthma and pulmonary fibrosis [12–14]. Studies have shown that over expression of SphK1 is observed in many tumor tissues and regulating tumorigenesis, angiogenesis, and chemotherapy resistance which play an important role in cancer progression[15–20]. Hence, inhibition of SphK1 is considered a new therapeutic strategy in the treatment of metastatic cancer and other diseases [21, 22].

SphK inhibitors can be classified into SphK1-selective inhibitors and SphK1/SphK2-dual inhibitors. Many compounds were reported to be a potent selective Sphk1 inhibitors, e.g. *N,N*-Dimethylsphingosine (DMS) **1** [23] and compound (FTY720) **2** [24, 25] (Fig. 1). Moreover, compound (CS0777) **3** [24, 26] and compound (PF-543) **5** [27, 28] are examples of an orally active SphK-1 and (SKI-II-Asp) **4** [24] (Fig. 1). On the other hand, compounds **6** (SKI-III) [29–32] and **7** (Amgen 23) [33] were reported as SphK1/SphK2-dual inhibitors (Fig. 1).

Compounds **8** (SKI-I), **9** (SKI-I-Asp) and **10** (SKI-178), which possess pyrazole ring in their structures, exhibited potent inhibitory effect on Sphk1 (Fig. 2) [24, 34]. Recently, large numbers of 2-substituted benzimidazoles were discovered to have potent inhibitory effect on SphK-1[34–36]. The benzimidazoles **11** and **12** (Fig. 2) were reported as SphK1 [35, 36]. The benzimidazole moiety is well known to interact with kinases by multiple binding modes [37–40].

From the ongoing information, our group has reported different ways for the synthesis of benzimidazoles [41–50] targeting different kinases. In this study our aim is to design and synthesize sphingosine kinase 1 inhibitors by the

combination of both pharmacophoric moieties benzimidazoles and pyrazoles.

The evolution of SphK1 and SphK2 inhibitors has been recently reported [51, 52]. Many SphK inhibitors were designed to have a polar head group and a lipophilic tail region. Studying all structural variations in the sphingosine-based SphKIs and their resulting biological effects in the earlier work, several points will be taken into consideration to improve the potency of the new target compounds possessing both pharmacophoric moieties, benzimidazole and pyrazole: (1) *In the lipophilic region*: increasing lipophilicity by introducing saturated heterocycles to the pyrazole ring as R_2 = morpholinyl, piperidinyl, and pyrrolidinyl in the lipophilic tail as well as the introduction of a phenyl ring will enhance the lipophilicity and the bioavailability by producing a better drug-like profile. (2) *In the polar head*: replacing the hydroxyl group to prevent its phosphorylation by other polar groups seems to be vital in the design of new sphingosine-based SKIs. Modification of the polar head to possess the following groups: R_1 = NO_2 , COOH , was planned so that the nitro group and the carboxylic group will be the polar heads. (Fig. 3)

Results And Discussion

Chemistry

The present study aimed to synthesize new benzimidazole candidates as sphingosine kinase 1 inhibitors (Figure 3) and it was guided by molecular docking study assessing their binding energies taking into consideration that polar substitution of the benzimidazole ring at 5-position was essential for activity. The target pyrazolylbenzimidazoles derivatives were synthesized through two main schemes. The first part of this synthesis was demonstrated in scheme 1 which deals with the preparation of pyrazole derivatives via three steps starting by the synthesis of 3-methyl-1-phenylpyrazol-5-one **13** by the reaction of phenyl hydrazine with ethyl acetoacetate in the presence of glacial acetic acid and ethanol according to the procedure described by Khanal et al [53]. The Vilsmeier-Haack reaction of the previous step afforded 5-chloro-3-methyl-1-phenylpyrazole-4-carboxaldehyde **14** which followed by introduction of nucleophiles i.e secondary amines or phenol derivatives to give compounds **15-21** (Scheme 1) [53-56]. The second part of this synthesis was the coupling of pyrazole-4-carboxaldehydes **15-21** with 1,2-benzendiamine **22**, 3,4-diaminobenzoic acid **23**, 4-methylbenzene-1,2-diamine **24** or 4-nitrobenzene-1,2-diamine **25** to afford different derivatives **26-48** (Scheme 2). Based on the predicted binding affinities and interactions, compounds **47** and **48** were selected as top-scoring compounds and modification of the polar head to possess the NO_2 group, was planned. Unfortunately, the low yield and poor solubility caused no synthesis of other derivatives of nitro-analogues.

Fluorescence binding studies

Fluorescence binding studies were performed for evaluating the binding affinity of all the synthesized compounds **26-48** with SphK1. The gradual loss in the fluorescence intensity upon addition of the selected compounds, **47** and **48**, Figure (4A and 4B) was observed for SphK1, which points towards the formation of a stable protein-ligand complex. The rest of the compounds did not show any quenching and some of them even perturbed the structure of SphK1 since major red shift and increase in the fluorescence intensity was observed when added to protein samples in increasing concentrations (data not shown). The Stern–Volmer plot Figure (4C and 4D) was used to analyze the quenching data to determine the binding affinity (K_a) for each compound. The number of binding sites per SphK1 molecule (n) for these compounds was also determined from the same plot. Compounds **47**, while compounds **48** showed binding in the 10^3 micromolar range (Table 1). Thus, hits obtained from the binding studies showed moderate binding with SphK1 and were further tested for inhibitory activity against SphK1.

Enzyme inhibition assay

Enzyme inhibition potential against compounds **26-48** toward SphK1 were evaluated by malachite green ATPase inhibition assays. During the initial screening, the maximum concentration of all compounds (100 μ M) was used (Table S2), which revealed that most of the studied compounds inhibited SphK1 activity effectively (Table 2). The enzyme inhibitory potential of the synthesized compounds that showed good binding affinity towards the SphK1 was determined by malachite-green based ATPase inhibition assay that revealed IC_{50} values in the micro-molar range (Table 3). The kinase activity of SphK1 is measured in terms of picomolar concentration of phosphate released in the reaction mixture which is represented in Figure 5 (A and B). The absorbance value of the malachite-inorganic phosphate green complex so formed at 620 nm is converted with the help of phosphate standard curve as described [57-64]. The loss in the SphK1 activity followed an inverse relationship between percentage inhibition and an increasing concentration of selected compounds as shown in Figure 5 (C and D) which was used for the calculation of IC_{50} values (Table 3). The compound **47** efficiently inhibited SphK1 kinase activity with lower IC_{50} ($2.48 \pm 0.05 \mu$ M). The IC_{50} value for compound **48** (Figure 6D) inhibited SphK1 activity with higher IC_{50} ($4.02 \pm 0.16 \mu$ M). The enzyme inhibition results overall propose that compounds **47** and **48** act as promising active inhibitors of SphK1.

Anticancer activity against NCI 60-cell line panel:

The synthesized new pyrazolylbenzimidazoles were screened for their in vitro antitumor activity by the Developmental Therapeutics Program (DTP) of the National Cancer Institute (NCI). This involves screening of the compounds at a single dose of 100 μ M [65]. From the obtained results in Table 4, the studied compounds showed a broad spectrum of anti-cancer activity against several NCI cell panels. At 100 μ M concentration, compounds **47** and **48** showed potent inhibition against the leukemia and breast Cancer cell lines. (Table4).

Molecular docking

The molecular docking study of the designed compounds with SphK1 was performed using the AutoDock vina tool [66]. Vina gives the predicted binding poses of the synthesized compounds **26-48** along with the binding affinities in kcal/mol. Based on the predicted binding affinities and interactions, compounds **47** and **48** were selected as top-scoring compounds. The predicted binding affinities of the selected compounds are given in (Table 5). On the basis of non-bonded interactions of compounds with the SphK1, Compounds **47** and **48** showed comparatively better interactions. Figure 6 shows the 2D structure of compound **47** (Figure 6A) along with its interactions with the PF-543 binding site residues of SphK1 (Figure 6B). Compound **47** forms hydrogen bonds with the Thr196 of the PF-543 binding pocket. 2D representation of protein-ligand interactions (Figure 6C) shows compound **47** having π -interactions and van der Waals interaction with the surrounding residues including the Asp178 which is the substrate binding site. These interactions of compound **47** with SphK1 suggest a strong bonding. Surface view of the protein shows compound **47** has strongly occupied the binding cavity of the protein (Figure 6D). A similar pattern is observed for compound **48** where it forms a hydrogen bond with the Thr196 of the PF-543 inhibitor binding site; in addition, Ile174 and Leu302 are showing π -sigma interaction with the ligand (Figure 7C). In addition to that, residues Val177, Leu268, Leu259, Ala274, and Leu319 are showing π -alkyl interactions with the ligand. Besides compounds **46** and **47**, A detailed interaction profile of all the synthesized compounds with SphK1 is given in Table 5. The docking figures for compounds **26-46** are given in the supplementary part (Figures S28-S48 respectively)

Structure activity relationship

Structure-activity relationships were illustrated from the previous results. Concerning the percent of inhibition of the synthesized compounds toward SphK-1, the following points can be explored. Most of the pyrazolylbenzimidazoles exert moderate to potent SphK-1 inhibitory activity. Compounds **47** and **48** with $IC_{50} = 2.48 \pm 0.05$ and 4.02 ± 0.16 , respectively and percentage of inhibition 98.82% and 96.48 % respectively are the most active compounds. It was

observed the importance of substituting the 5-position of benzimidazoles with nitro group. The correlation between the enzyme inhibition results, in vitro cytotoxic activity and docking results was observed. This proves the importance of polar substituents at position-5 of the benzimidazole ring. Unfortunately, the low yield and poor solubility caused no synthesis of other derivatives of nitro-analogues. The unsubstituted benzimidazoles and 5-methylbenzimidazoles were synthesized as was predicted from the docking studies that they might possess good binding affinity but they were found to be the least active compounds. An increase in the inhibitory activity was observed by substitution at position 5 of the pyrazole moiety with N-methyl piperazinyl in compounds **28**, **35** and **42** than pyrrolidinyl derivatives in compounds **29**, **36** and **43** (Table 2). An increase in the inhibitory activities were observed by substituting the phenol ring with two methyl as in compounds **32**, **39** and **46** (Table 2). These data correlated with our rationale, which depends on replacing the hydroxyl group by other polar groups to prevent its phosphorylation, and this was of great importance in the design of novel SphK-1 inhibitors. The effect of the nitro group in compounds **47** and **48** was the most favorable for activity and led to enhancement of both the binding free energy (Table 1) and the hydrogen bonding. In addition, substitution of the pyrazole ring with piperidine in compound **48** instead of morpholine in compound **47** decreased the inhibitory activity and the IC₅₀ (Table 3).

Conclusion

Looking at the major challenges involves in the synthesis of novel inhibitors of SphK-1, the current study is a first step towards the identification of different pyrazolylbenzimidazoles that could be useful in the development of potent SphK-1 inhibitors. A new series of benzimidazole derivatives **26–48** was rationally designed and synthesized. Among the studied compounds, compounds **47** and **48** showed an effective binding affinity to the SphK-1 and significantly inhibited SphK-1. Also, the synthesized molecules were evaluated by the NCI DPT for testing their antiproliferative activity on a panel of 60 cell lines, compounds **47** and **48** exhibited an effective cytotoxic activity against several NCI cell panels. Based on molecular-docking study, most of these synthesized compounds as **47** and **48** occupy the ATP-binding pocket and existing the deep pocket of SphK1 forming several important non-covalent and hydrogen bonding interactions with the active site residues. A considerable correlation was noticed between the docking results and the IC₅₀ values and its worth to mention that, compounds **47** and **48** act as promising inhibitors of SphK1 with low IC₅₀ values in the single-digit.

Material And Methods

Luria broth and Luria agar were purchased from Himedia(Mumbai, India). Plasmid pET28b+, DH5α, and BL21-Gold cells were procured from Invitrogen (USA). Ni-NTA column was purchased from GE Healthcare (GE Healthcare Life Sciences, Uppsala, Sweden). *N*-Lauroyl sarcosine, Tris buffer, DMSO, and other reagents were purchased from Sigma Aldrich (St. Louis, MO, USA). BIOMOL[®] was obtained from Enzo (New York, USA). All the reagents used for buffer and chemical preparation were of analytical grade. Microanalyses and spectral data of the compounds were performed in the Microanalytical center at National Research Centre, and pharmaceutical faculty, Cairo University, Egypt, and Helmholtz Institute for Pharmaceutical Research Saarland (HIPS) – Helmholtz Centre for Infection Research (HZI), Saarbrücken, Germany . The IR spectra (4000-400cm⁻¹) were recorded using KBr pellets in a Jasco FT/IR 300E Fourier transform infrared spectrophotometer on a perkin-Elmer FT-IR 1650 spectrophotometer. The ¹HNMR spectra were recorded using 500 MHz and 400 MHz NMR spectrometer. Chemical shifts are reported in parts per million (ppm) from the tetramethylsilane resonance in the indicated solvent. Coupling constants (*J*) are reported in Hertz (Hz), and integration (where applicable); spectral splitting patterns are designed as follow: singlet (s); doublet (d); triplet (t); quartet (q); multiplet (m) and broad singlet (brs). The samples were referenced to the appropriate internal non-deuterated solvent peak. The data is given as follows: chemical shift (δ) in ppm, multiplicity (where applicable). The mass spectra were recorded using a Finnigan mat SSQ 7000 (Thermo. Inst. Sys. Inc., USA) spectrometer at 70 ev. Chromatography solvents

were HPLC grade and were used without further purification. Thin-layer chromatography (TLC) analysis was performed using Merck silica gel 60 F-254 thin layer plates. Starting materials, reagents, and solvents for reactions were reagent grade and used as purchased. The petroleum ether had a boiling temperature in the 60-80 °C range.

Chemistry

General Procedure for the Synthesis of compounds 26-48.

A suspension of benzene-1,2-diamine derivatives **22-25** (9.2 mmol) and sodium metabisulfite (7 g, 36.8 mmol), dissolved in absolute ethanol (40 mL) was added to a solution of compounds **15-21** [53-55] (9.2 mmol) dissolved in absolute ethanol (30 mL). The mixture was stirred for 6-10 h and monitored by TLC. The reaction mixture was poured onto crushed ice; the resulting precipitate was collected by filtration, dried, and recrystallized from ethanol to give compounds **26-48**.

2-(3-Methyl-5-morpholino-1-phenyl-1*H*-pyrazol-4-yl)-1*H*-benzo[d]imidazole (26).

Yield = 81 %; mp: 258-260 °C; R_f = 0.34 (Petroleum ether / EtOAc = 1:1); IR (KBr): $\nu_{\max}/\text{cm}^{-1}$: 3470 (NH), 3058 (CH aromatic), 2947 (CH aliphatic), 1627 (C=N), 1593 (C=C); $^1\text{H-NMR}$ (DMSO- d_6 , 500 MHz): δ_{H} 2.25 (s, 3H, CH₃), 2.90 (m, 4H, H-3, H-5 morpholine protons), 3.49 (m, 4H, H-2, H-6 morpholine protons), 7.20 (m, 2H, aromatic protons), 7.40 (m, 1H, aromatic proton), 7.52 (m, 3H, aromatic protons), 7.67 (d, J = 7.5 Hz, 1H, aromatic proton), 7.73 (d, J = 7.5 Hz, 2H, aromatic proton), 12.34 (s, 1H, NH); $^{13}\text{C-NMR}$ (DMSO- d_6 , 125 MHz): δ_{C} 13.4 (CH₃), 50.5, 66.5, 104.0, 112.0, 119.1, 122.3, 123.1, 124.7, 128.2, 129.8, 134.7, 139.5, 143.6, 146.1 (C-N), 148.4 (C=N), 149.4 (C-O); MS (EI, 70 Ev): m/z (%) 360 (100) [M+1]⁺; Anal. Calcd for C₂₁H₂₁N₅O (FW: 359): C, 70.17; H, 5.89; N, 19.48. Found: C, 70.35; H, 5.76; N, 19.54.

2-(3-Methyl-1-phenyl-5-(piperidin-1-yl)-1*H*-pyrazol-4-yl)-1*H*-benzo[d]imidazole (27).

Yield = 81 %; mp: 218-220 °C; R_f = 0.47 (Petroleum ether/ EtOAc = 1:1); IR (KBr): $\nu_{\max}/\text{cm}^{-1}$: 3426 (NH), 3061 (CH aromatic), 2927 (CH aliphatic), 1627 (C=N), 1594 (C=C); $^1\text{H-NMR}$ (DMSO- d_6 , 500 MHz): δ_{H} 1.37 (brs, 6H, H3, H4, H5 piperidine protons), 2.21 (s, 3H, CH₃), 2.84 (s, 4H, H-2, H-6 piperidine protons), 7.19 (m, 2H, aromatic protons), 7.36 (m, 1H, aromatic proton), 7.51 (m, 2H, aromatic protons), 7.59 (brs, 2H, aromatic protons), 7.72 (d, J = 8.0 Hz, 2H, aromatic protons), 12.19 (brs, 1H, NH); $^{13}\text{C-NMR}$ (DMSO- d_6 , 125 MHz): δ_{C} 13.1 (CH₃), 23.3, 25.2, 51.0, 103.4, 121.6, 123.7, 127.0, 128.9, 139.5, 146.1, 147.5 (C-N), 149.9 (C=N); MS (EI, 70 Ev): m/z (%) 358.21 (100) [M+1]⁺; Anal. Calcd for C₂₂H₂₃N₅ (FW: 357): C, 73.92; H, 6.49; N, 19.59. Found: C, 73.85; H, 6.61; N, 19.67.

2-(3-Methyl-5-(4-methylpiperazin-1-yl)-1-phenyl-1*H*-pyrazol-4-yl)-1*H*-benzo[d]imidazole (28).

Yield = 50 %; mp: 236-238 °C; R_f = 0.35 (EtOAc/ ethanol = 2: 0.5); IR (KBr): $\nu_{\max}/\text{cm}^{-1}$: 3424 (NH), 3069 (CH aromatic), 2925 (CH aliphatic), 1630 (C=N), 1589 (C=C); $^1\text{H-NMR}$ (DMSO- d_6 , 500 MHz): δ_{H} 2.27 (s, 3H, CH₃), 2.60 (s, 3H, CH₃), 2.85 (s, 4H, piperazine protons), 3.11 (s, 4H, piperazine protons), 7.22 (s, 2H, aromatic proton), 7.43 (d, J = 9.0 Hz, 1H, aromatic proton), 7.54 (m, 2H, aromatic protons), 7.61 (s, 2H, aromatic protons), 7.71 (d, J = 9.5 Hz, 2H, aromatic protons), 12.41 (brs, 1H, NH); $^{13}\text{C-NMR}$ (DMSO- d_6 , 125 MHz): δ_{C} 13.7 (CH₃), 43.1 (CH₃), 47.5 (CH-3 + CH-5 piperazine carbons), 53.5 (CH-3 + CH-5 piperazine carbons), 105.2, 123.7, 124.5, 125.7, 127.9, 129.9, 131.5, 139.4, 145.5; MS (EI, 70 Ev): m/z (%) 372.21 (100) [M]⁺; 373.21 [M+1]⁺ (25%). Anal. Calcd for C₂₂H₂₄N₆ (FW: 372): C, 70.94; H, 6.49; N, 22.56. Found: C, 70.82; H, 6.57; N, 22.43.

2-(3-Methyl-1-phenyl-5-(pyrrolidin-1-yl)-1H-pyrazol-4-yl)-1H-benzo[d]imidazole (29). Yield = 64.5 %; mp: 241-243 °C; R_f = 0.43 (Petroleum ether / EtOAc = 1:1); IR (KBr): $\nu_{\max}/\text{cm}^{-1}$: 3423 (NH), 3060 (CH aromatic), 2958 (CH aliphatic), 1622 (C=N), 1589 (C=C); $^1\text{H-NMR}$ (DMSO- d_6 , 500 MHz): δ_{H} 1.70 (m, 4H, H-3, H-4 pyrrolidine protons), 2.20 (s, 3H, CH₃), 2.95 (m, 4H, H-2, H-5 pyrrolidine protons), 7.17 (m, 2H, aromatic protons), 7.38 (m, 1H, aromatic protons), 7.50 (m, 2H, aromatic protons), 7.57 (m, 4H, aromatic protons), 12.30 (brs, 1H, NH); $^{13}\text{C-NMR}$ (DMSO- d_6 , 125 MHz): δ_{C} 13.2 (CH₃), 25.170 (CH-3, CH-4 of pyrrolidine carbons), 50.4 (CH-2, CH-5 of pyrrolidine carbons), 101.0, 121.5, 124.2, 127.1, 129.0, 140.0, 146.5 (C-N), 147.7 (C-N), 147.8 (C=N); MS (EI, 70 Ev): m/z (%) 344.2 (100) [M+1]⁺, 345.2 (25) [M+2]⁺; Anal. Calcd for C₂₁H₂₁N₅ (FW: 343): C 73.44; H, 6.16; N, 20.39. Found: C, 73.25; H, 6.29; N, 20.45.

2-(3-Methyl-5-phenoxy-1-phenyl-1H-pyrazol-4-yl)-1H-benzo[d]imidazole (30).

Yield = 79%; mp: 97-99 °C; R_f = 0.58 (Petroleum ether / EtOAc = 1:3); IR (KBr): $\nu_{\max}/\text{cm}^{-1}$: 3424 (NH), 3069 (CH aromatic), 2925 (CH aliphatic), 1630 (C=N), 1589 (C=C); $^1\text{H-NMR}$ (DMSO- d_6 , 500 MHz): δ_{H} 2.63 (s, 3H, CH₃), 6.94 (m, 3H, aromatic protons), 7.13 (m, 2H, aromatic protons), 7.21 (m, 2H, aromatic protons), 7.32 (m, 1H, aromatic proton), 7.48 (m, 4H, aromatic protons), 7.66 (d, J = 7.5 Hz, 2H, aromatic protons), 12.14 (brs, 1H, NH); $^{13}\text{C-NMR}$ (DMSO- d_6 , 125 MHz): δ_{C} 14.6 (CH₃), 101.5, 115.4, 121.7, 122.1, 123.7, 127.5, 129.4, 129.9, 137.1, 144.1, 146.1 (C-N), 148.2 (C=N), 155.8 (C-O); MS (EI, 70 Ev): m/z (%) 367.1 (100) [M+1]⁺, 368.2 (25) [M]⁺; Anal. Calcd for C₂₃H₁₈N₄O (FW: 366): C, 75.39; H, 4.95; N, 15.29. Found: C, 75.25; H, 4.80; N, 15.40.

2-(3-Methyl-1-phenyl-5-(pyridin-2-yloxy)-1H-pyrazol-4-yl)-1H-benzo[d]imidazole (31).

Yield = 56%; mp: 114-117 °C; R_f = 0.33 (Petroleum ether / EtOAc = 1:3); IR (KBr): $\nu_{\max}/\text{cm}^{-1}$: 3424 (NH), 3069 (CH aromatic), 2925 (CH aliphatic), 1630 (C=N), 1589 (C=C); $^1\text{H-NMR}$ (DMSO- d_6 , 500 MHz): δ_{H} 2.62 (s, 3H, CH₃), 7.10 (m, 2H, aromatic protons), 7.14 (m, 2H, aromatic protons), 7.20 (m, 2H, aromatic protons), 7.32 (m, 1H, aromatic proton), 7.45 (m, 2H, aromatic protons), 7.52 (m, 2H, aromatic protons), 7.65 (d, J = 8.0 Hz, 2H, aromatic protons), 12.36 (brs, 1H, NH); $^{13}\text{C-NMR}$ (DMSO- d_6 , 125 MHz): δ_{C} 14.6 (CH₃), 101.1, 115.4, 121.9, 122.2, 123.8, 127.5, 129.4, 129.9, 137.1, 143.9, 145.2 (C-N), 148.2 (C=N), 155.8 (C-O); MS (EI, 70 Ev): m/z (%) 367.1 (100) [M]⁺, 368.2 (25) [M+1]⁺; Anal. Calcd for C₂₂H₁₇N₅O (FW: 367): C, 71.92; H, 4.66; N, 19.06. Found: C, 71.76; H, 4.53; N, 19.19.

2-(5-(2,5-Dimethylphenoxy)-3-methyl-1-phenyl-1H-pyrazol-4-yl)-1H-benzo[d]imidazole (32).

Yield = 72%; mp: 102-104 °C; R_f = 0.53 (Petroleum ether/ EtOAc = 1:3); IR (KBr): $\nu_{\max}/\text{cm}^{-1}$: 3441 (NH), 3069 (CH aromatic), 2856 (CH aliphatic), 1625 (C=N), 1594 (C=C); $^1\text{H-NMR}$ (DMSO- d_6 , 500 MHz): δ_{H} 1.97 (s, 3H, CH₃), 2.34 (s, 3H, CH₃), 2.60 (s, 3H, CH₃), 6.28 (s, 1H, aromatic proton), 6.63 (d, J = 7.5 Hz, 1H, aromatic proton), 7.00 (d, J = 7.5 Hz, 1H, aromatic proton), 7.14 (m, 2H, aromatic protons), 7.32 (m, 1H, aromatic proton), 7.44 (m, 2H, aromatic protons), 7.51 (m, 2H, aromatic protons), 7.58 (d, J = 8.0 Hz, 2H, aromatic proton), 12.07 (brs, 1H, NH); $^{13}\text{C-NMR}$ (DMSO- d_6 , 125 MHz): δ_{C} 14.5 (CH₃), 15.6 (CH₃), 20.5 (CH₃), 101.3, 113.4, 121.7, 122.5, 123.2, 124.1, 127.8, 129.2, 131.0, 136.4, 137.2, 144.2, 146.6 (C-N), 148.0 (C=N), 154.0 (C-O); MS (EI, 70 Ev): m/z (%) 395.2 (100) [M+1]⁺, 396.2 (25) [M+2]⁺; Anal. Calcd for C₂₅H₂₂N₄O (FW: 394): C, 76.12; H, 5.62; N, 14.20. Found: C, 76.31; H, 5.73; N, 14.41.

2-(3-Methyl-5-morpholino-1-phenyl-1H-pyrazol-4-yl)-1H-benzo[d]imidazole-5-carboxylic acid (33).

Yield = 73.5%; mp: 192-194 °C; R_f = 0.23 (Petroleum ether/ EtOAc = 1: 3.5); IR (KBr): $\nu_{\max}/\text{cm}^{-1}$: 3423 (NH), 3097 (CH aromatic), 2966 (CH aliphatic), 2855 (OH), 1627 (C=N), 1590 (C=C), 1693 (C=O); $^1\text{H-NMR}$ (DMSO- d_6 , 400 MHz): δ_{H} 2.29 (s, 3H, CH₃), 2.93 (s, 4H, H-3, H-5 morpholine protons), 3.50 (s, 4H, H-2, H-6 morpholine protons), 7.41 (m, 1H, aromatic

proton), 7.54 (m, 2H, aromatic protons), 7.70 (m, 3H, aromatic protons), 7.85 (d, $J = 7.2$ Hz, 1H, aromatic proton), 8.21 (s, 1H, aromatic proton), 12.63 (brs, 1H); ^{13}C -NMR (DMSO- d_6 , 125MHz): δ_{C} 13.3($\underline{\text{C}}\text{H}_3$), 50.0, 65.9, 103.7, 123.4, 124.2, 127.5, 129.0, 139.1, 147.5($\underline{\text{C}}\text{-N}$), 148.9($\underline{\text{C}}\text{=N}$), 167.9($\underline{\text{C}}\text{=O}$); MS (EI,70 Ev): m/z (%) 404.0 (100) $[\text{M}+1]^+$ Anal. Calcd for $\text{C}_{22}\text{H}_{21}\text{N}_5\text{O}_3$ (FW: 403): C, 65.50; H, 5.25; N, 17.36. Found: C, 65.65; H, 5.42; N, 17.47.

2-(3-Methyl-1-phenyl-5-(piperidin-1-yl)-1H-pyrazol-4-yl)-1H-benzo[d]imidazole-5-carboxylic acid (34).

Yield = 80.5 %; mp: 156-158 °C; $R_f = 0.18$ (Petroleum ether/ EtOAc = 1: 4); IR (KBr): $\nu_{\text{max}}/\text{cm}^{-1}$: 3427 (NH), 3088 (CH aromatic), 2924 (CH aliphatic), 1833 (C=O), 1626 (C=N), 1596 (C=C); ^1H -NMR (DMSO- d_6 , 400 MHz): δ_{H} 1.36 (m, 6H, H-3, H-4, H-5 piperidine protons), 2.23 (s, 3H, CH_3), 2.83 (m, 4H, H-2, H-6 piperidine protons), 7.38 (m, 1H, aromatic proton), 7.52 (m, 2H, aromatic protons), 7.67 (m, 3H, aromatic protons), 7.84 (d, $J = 8.0$ Hz, 1H, aromatic protons), 8.21 (s, 1H, aromatic proton), 12.69 (brs, 1H); ^{13}C -NMR (DMSO- d_6 , 125 MHz): δ_{C} 13.2($\underline{\text{C}}\text{H}_3$), 23.3($\underline{\text{C}}\text{H-4}$ of piperidine carbons), 25.2($\underline{\text{C}}\text{H-3}$, $\underline{\text{C}}\text{H-5}$ of piperidine carbons), 51.0($\underline{\text{C}}\text{H-2}$, $\underline{\text{C}}\text{H-6}$ of piperidine carbons), 103.0, 123.9, 127.2, 129.0, 139.4, 147.4($\underline{\text{C}}\text{=N}$), 150.1($\underline{\text{C}}\text{-O}$), 167.9 ($\underline{\text{C}}\text{=O}$); MS (EI,70 Ev): m/z (%) 401.2 (100) $[\text{M}]^+$. Anal. Calcd for $\text{C}_{23}\text{H}_{23}\text{N}_5\text{O}_2$ (FW: 401): C, 68.81; H, 5.77; N, 17.44. Found: C, 68.95; H, 5.76; N, 17.59.

2-(3-Methyl-5-(4-methylpiperazin-1-yl)-1-phenyl-1H-pyrazol-4-yl)-1H-benzo[d]imidazole-5-carboxylic acid (35).

Yield = 42 %; mp: 289-291 °C; $R_f = 0.53$ (EtOAc /methanol = 1:1); IR (KBr): $\nu_{\text{max}}/\text{cm}^{-1}$: 3439 (NH), 3066 (CH aromatic), 2962 (CH aliphatic), 1689 (C=O), 1625 (C=C), 1594 (C=C); ^1H -NMR (DMSO- d_6 , 500 MHz): δ_{H} 2.11 (s, 3H, CH_3), 2.24 (m, 7H, CH_3 + 4H piperazine protons), 2.92 (m, 4H, piperazine protons), 7.39 (m, 1H, aromatic proton), 7.51 (m, 2H, aromatic protons), 7.64 (d, $J = 8.5$ Hz, 1H, aromatic proton), 7.69 (d, $J = 7.5$ Hz, 2H, aromatic proton), 7.84 (d, $J = 8.5$ Hz, 1H, aromatic proton), 8.20 (s, 1H, aromatic proton), 12.63 (brs, 1H); ^{13}C -NMR (DMSO- d_6 , 125 MHz): δ_{C} 13.6 ($\underline{\text{C}}\text{H}_3$), 40.0($\text{N-}\underline{\text{C}}\text{H}_3$), 48.6($\underline{\text{C}}\text{H-3}$ + $\underline{\text{C}}\text{H-5}$ piperazine carbons), 53.9($\underline{\text{C}}\text{H-2}$ + $\underline{\text{C}}\text{H-6}$ piperazine carbons), 103.7, 125.1, 128.9, 130.3, 139.4, 148.0($\underline{\text{C}}\text{-N}$), 149.0($\underline{\text{C}}\text{=N}$), 149.2($\underline{\text{C}}\text{=N}$), 170.9 ($\underline{\text{C}}\text{=O}$); MS (EI,70 Ev): m/z (%) 417.2 (50) $[\text{M}+1]^+$. Anal. Calcd for $\text{C}_{23}\text{H}_{24}\text{N}_6\text{O}_2$ (FW: 416): C, 66.33; H, 5.81; N, 20.18. Found: C, 66.49; H, 5.72; N, 20.27.

2-(3-Methyl-1-phenyl-5-(pyrrolidin-1-yl)-1H-pyrazol-4-yl)-1H-benzo[d]imidazole-5-carboxylic acid (36).

Yield = 51.4 %; mp: 169-171 °C; $R_f = 0.25$ (Petroleum ether / EtOAc/ ethanol = 1: 2: 0.5); IR (KBr): $\nu_{\text{max}}/\text{cm}^{-1}$: 3423 (NH), 3060 (CH aromatic), 2927 (CH aliphatic), 1695 (C=O), 1624 (C=N), 1542 (C=C); ^1H -NMR (DMSO- d_6 , 500 MHz): δ_{H} 1.71 (m, 4H, H-3, H-4 pyrrolidine protons), 2.23 (s, 3H, CH_3), 2.96 (m, 4H, H-2, H-5 pyrrolidine protons), 7.40 (m, 1H, aromatic proton), 7.50 (m, 2H, aromatic protons), 7.56 (d, $J = 7.0$ Hz, 2H, aromatic protons), 7.62 (d, $J = 8.5$ Hz, 1H, aromatic protons), 7.82 (dd, 1H, $J = 8.5$ Hz, $J = 1.5$ Hz, aromatic proton), 8.16 (s, 1H, aromatic proton), 12.60 (brs, 1H); ^{13}C -NMR (DMSO- d_6 , 125 MHz): δ_{C} 13.3 ($\underline{\text{C}}\text{H}_3$), 25.3($\underline{\text{C}}\text{H-3}$, $\underline{\text{C}}\text{H-4}$ of pyrrolidine carbons), 51.1($\underline{\text{C}}\text{H-2}$, $\underline{\text{C}}\text{H-5}$ of pyrrolidine carbons), 100.5, 125.1, 127.4, 129.1, 141.0, 147.5 ($\underline{\text{C}}\text{=N}$), 168.2 ($\underline{\text{C}}\text{=O}$); MS (EI,70 Ev): m/z (%) 388.1 (100) $[\text{M}+1]^+$; Anal. Calcd for $\text{C}_{22}\text{H}_{21}\text{N}_5\text{O}_2$ (FW: 387): C, 68.20; H, 5.46; N, 18.08. Found: C, 68.35; H, 5.65; N, 18.26.

2-(3-Methyl-5-phenoxy-1-phenyl-1H-pyrazol-4-yl)-1H-benzo[d]imidazole-5-carboxylic acid (37).

Yield = 75%; mp: 138-140 °C; $R_f = 0.53$ (Petroleum ether/ EtOAc: ethanol 1: 2: 0.25); IR (KBr): $\nu_{\text{max}}/\text{cm}^{-1}$: 3435 (NH), 3066 (CH aromatic), 2927 (CH aliphatic), 1690 (C=O), 1629 (C=N), 1594 (C=C); ^1H -NMR (DMSO- d_6 , 400 MHz): δ_{H} 2.62 (s, 3H, CH_3), 6.96 (m, 3H, aromatic protons), 7.22 (m, 2H, aromatic protons), 7.35 (m, 1H, aromatic proton), 7.47 (m, 3H, aromatic protons), 7.67 (m, 2H, aromatic protons), 7.75 (s, 1H, aromatic protons), 8.17 (s, 1H, aromatic proton), 12.37

(s, 1H); MS (EI,70 Ev): m/z (%) 411.1 (100) [M+1]⁺, 412.2 (25) [M+2]⁺. Anal. Calcd for C₂₄H₁₈N₄O₃ (FW: 410): C, 70.23; H, 4.42; N, 13.65. Found: C, 70.33; H, 4.35; N, 13.71.

2-(3-Methyl-1-phenyl-5-(pyridin-2-yloxy)-1H-pyrazol-4-yl)-1H-benzo[d]imidazole-5-carboxylic acid (38).

Yield = 55%; mp: 147-150 °C; R_f 0.43 (Petroleum ether/ EtOAc/ethanol = 1: 3: 0.5); IR (KBr): ν_{max}/cm⁻¹ : 3424 (NH), 3069 (CH aromatic), 2925 (CH aliphatic), 1630 (C=N), 1589 (C=C); ¹H-NMR (DMSO-*d*₆, 400 MHz): δ_H 2.65 (s, 3H, CH₃), 6.95 (m, 3H, aromatic protons), 7.21 (m, 2H, aromatic protons), 7.33 (m, 1H, aromatic proton), 7.47 (m, 2H, aromatic protons), 7.66 (d, *J* = 7.6 Hz, 2H, aromatic protons), 7.77 (brs, 1H, aromatic protons), 8.10 (s, 1H, aromatic proton), 12.40 (brs, 1H); ¹³C-NMR (DMSO-*d*₆, 125 MHz): δ_C 15.1(CH₃), 101.3, 111.5, 114.1, 115.5, 118.9, 121.2, 125.1, 128.5, 130.2, 135.1, 137.9, 143.8, 146.4 (C=N), 149.5(C=O), 156.1(C=O), 168.3; MS (EI,70 Ev): m/z (%) 411 (100) [M]⁺, 412 (24) [M+1]⁺; Anal. Calcd for C₂₃H₁₇N₅O₃ (FW: 411): C, 67.15; H, 4.16; N, 17.02. Found: C, 67.08; H, 4.23; N, 17.22.

2-(5-(2,5-Dimethylphenoxy)-3-methyl-1-phenyl-1H-pyrazol-4-yl)-1H-benzo[d]imidazole-5-carboxylic acid (39).

Yield = 37%; mp 153-155 °C; R_f 0.53 (Petroleum ether / EtOAc / methanol = 1:2:0.5); IR (KBr): ν_{max}/cm⁻¹ : 3432 (NH), 3066 (CH aromatic), 2925 (CH aliphatic), 1689 (C=O), 1595 (C=N), 1498 (C=C); ¹H-NMR (DMSO-*d*₆, 500 MHz): δ_H 1.96 (s, 3H, CH₃), 2.34 (s, 3H, CH₃), 2.61 (s, 3H, CH₃), 6.29 (s, 1H, aromatic protons), 6.63 (d, *J* = 8.5 Hz, 1H, aromatic protons), 6.99 (d, *J* = 8.0 Hz, 1H, aromatic protons), 7.32 (m, 2H, aromatic protons), 7.44 (m, 2H, aromatic protons), 7.58 (m, 2H, aromatic protons), 7.78 (d, *J* = 9.5 Hz, 1H, aromatic protons), 8.13 (m, 1H, aromatic protons); ¹³C-NMR (DMSO-*d*₆, 125 MHz): δ_C 13.6(CH₃), 16.7(CH₃), 22.1(CH₃), 102.4, 104.6, 106.7, 110.9, 115.1, 122.5, 123.6, 130.1, 130.1, 132.0, 132.3, 137.3, 148.1(C=N), 154.6(C-O), 168.8(C=O); MS (EI,70 Ev): m/z (%) 439.2 (100) [M+1]⁺, 440.2 (25) [M+2]⁺; Anal. Calcd for C₂₆H₂₂N₄O₃ (FW: 438): C, 71.22; H, 5.06; N, 12.78 Found: C, 71.39; H, 5.21; N, 12.66.

5-Methyl-2-(3-methyl-5-morpholino-1-phenyl-1H-pyrazol-4-yl)-1H-benzo[d]imidazole (40).

Yield = 85%; mp: 129-132 °C; R_f 0.65 (Petroleum ether/ EtOAc = 1:2); IR (KBr): ν_{max}/cm⁻¹ : 3423 (NH), 3068 (CH aromatic), 2960 (CH aliphatic), 1629 (C=N), 1594 (C=C); ¹H-NMR (DMSO-*d*₆, 500 MHz): δ_H 2.24 (s, 3H, CH₃), 2.42 (s, 3H, CH₃), 2.89 (s, 4H, H-3, H-5 morpholine protons), 3.48 (s, 4H, H-2, H-6 morpholine protons), 7.02 (d, *J* = 8.0 Hz, 1H, aromatic proton), 7.39 (m, 2H, aromatic protons), 7.51 (m, 3H, aromatic protons), 7.73 (d, *J* = 7.5 Hz, 2H, aromatic protons), 12.23 (brs, 1H, NH); ¹³C-NMR (DMSO-*d*₆, 125 MHz): δ_C 13.2(CH₃), 21.4 (CH₃), 50.0(CH-3, CH-5 of morpholine carbons), 66.0(CH-2, CH-6 of morpholine carbons), 104.5, 124.0, 125.3, 127.2, 129.0, 139.2, 145.5(C-N), 147.7(C=N), 148.6 (C-O); MS (EI,70 Ev): m/z (%) 374.2 (100) [M+1]⁺, 375.2 (25) [M+2]⁺. Anal. Calcd for C₂₂H₂₃N₅O (FW: 373): C, 70.76; H, 6.21; N, 18.75. Found: C, 70.89; H, 6.35; N, 18.92.

5-Methyl-2-(3-methyl-1-phenyl-5-(piperidin-1-yl)-1H-pyrazol-4-yl)-1H-benzo[d]imidazole (41).

Yield = 80%; mp: 116-118 °C; R_f 0.68 (Petroleum ether / EtOAc = 1:2); IR (KBr): ν_{max}/cm⁻¹ : 3440 (NH), 3068 (CH aromatic), 2933 (CH aliphatic), 1629 (C=N), 1594 (C=C); ¹H-NMR (DMSO-*d*₆, 500 MHz): δ_H 1.36 (brs, 6H, H-3, H-4, H-5 piperidine protons), 2.19 (s, 3H, CH₃), 2.42 (s, 3H, CH₃), 2.82-2.83 (m, 4H, H-2, H-6 piperidine protons), 7.02 (d, *J* = 8.0 Hz, 1H, aromatic proton), 7.37 (m, 2H, aromatic protons), 7.46 (d, *J* = 8.0 Hz, 1H, aromatic protons), 7.37 (m, 2H, aromatic protons), 7.71 (d, *J* = 7.5 Hz, 2H, aromatic protons); ¹³C-NMR (DMSO-*d*₆, 125 MHz): δ_C 13.1(CH₃), 21.3(CH₃), 23.3, (CH-4 of piperidine carbons), 25.2, (CH-3, CH-5 of piperidine carbons), 51.0(CH-2, CH-6 of piperidine carbons), 103.3, 123.1, 123.6, 127.0, 128.9, 130.8, 139.5, 145.6(C-N), 147.4 (C=N), 149.9(C-N); MS (EI,70 Ev): m/z (%) 372.2 (100) [M+1]⁺; Anal. Calcd for C₂₃H₂₅N₅ (FW: 371): C, 74.36; H, 6.78; N, 18.85. Found: C, 74.20; H, 6.63; N, 18.69.

5-Methyl-2-(3-methyl-5-(4-methylpiperazin-1-yl)-1-phenyl-1*H*-pyrazol-4-yl)-1*H*-benzo[d]imidazole (42).

Yield = 40%; mp: 103-104 °C; R_f = 0.58 (Petroleum ether / EtOAc = 1:2); $^1\text{H-NMR}$ (DMSO- d_6 , 400 MHz): δ_{H} 2.28 (s, 3H, CH₃), 2.44 (s, 3H, CH₃), 2.71 (s, 3H, CH₃), 3.05 (brs, 4H, piperazine protons), 3.17 (brs, 4H, piperazine protons), 7.04 (d, J = 7.9 Hz, 1H, aromatic proton), 7.43 (m, 2H, aromatic protons), 7.52 (m, 2H, aromatic protons), 7.71 (d, J = 7.7 Hz, 2H, aromatic protons), 12.19 (brs, 1H, NH); $^{13}\text{C-NMR}$ (DMSO- d_6 , 125 MHz): δ_{C} 13.7 (CH₃), 21.8 (CH₃), 43.1 (N-CH₃), 47.5 (CH-3 + CH-5 piperazine carbons), 53.2 (CH-2 + CH-6 piperazine carbons), 105.2, 123.7, 124.5, 125.7, 127.9, 129.7, 131.4, 139.4, 145.5 (C-N), 147.8 (C=N), 147.9 (C=N); Anal. Calcd for C₂₃H₂₆N₆ (FW: 386): C, 71.48; H, 6.78; N, 21.74. Found: C, 71.59; H, 6.90; N, 21.89.

5-Methyl-2-(3-methyl-1-phenyl-5-(pyrrolidin-1-yl)-1*H*-pyrazol-4-yl)-1*H*-benzo[d]imidazole (43).

Yield = 85%; mp: 133-135 °C; R_f = 0.60 (Petroleum ether 60-80 °C / EtOAc = 1:3); IR (KBr): $\nu_{\text{max}}/\text{cm}^{-1}$: 3424 (NH), 3069 (CH aromatic), 2965 (CH aliphatic), 1630 (C=N), 1593 (C=C); $^1\text{H-NMR}$ (DMSO- d_6 , 500 MHz): δ_{H} 1.68 (brs, 4H, H-3, H-4 pyrrolidine protons), 2.19 (s, 3H, CH₃), 2.41 (s, 3H, CH₃), 2.93 (brs, 4H, H-2, H-5 pyrrolidine protons), 6.99 (d, J = 7.5 Hz, 1H, aromatic proton), 7.36 (m, 2H, aromatic proton), 7.44 (d, J = 8.0 Hz, 1H, aromatic proton), 7.50 (m, 2H, aromatic protons), 7.58 (d, J = 7.0 Hz, 2H, aromatic protons); $^{13}\text{C-NMR}$ (DMSO- d_6 , 125 MHz): δ_{C} 13.2 (CH₃), 21.3 (CH₃), 25.0 (CH-3, CH-4 of pyrrolidine carbons), 50.4, (CH-2, CH-5 of pyrrolidine carbons), 101.1, 122.9, 124.1, 127.1, 129.0, 130.7, 140.0, 145.1 (C-N), 147.7 (C=N), 147.7 (C=N); MS (EI, 70 Ev): m/z (%) 358.2 (100) [M+1]⁺, 359.2 (25) [M+2]⁺; Anal. Calcd for C₂₂H₂₃N₅ (FW: 357): C, 73.92; H, 6.49; N, 19.59. Found C, 73.74; H, 6.32; N, 19.39.

5-Methyl-2-(3-methyl-5-phenoxy-1-phenyl-1*H*-pyrazol-4-yl)-1*H*-benzo[d]imidazole (44).

Yield = 85%; mp: 128-130 °C; R_f = 0.57 (Petroleum ether / EtOAc = 1:3); IR (KBr): $\nu_{\text{max}}/\text{cm}^{-1}$: 3437 (NH), 3066 (CH aromatic), 2924 (CH aliphatic), 1627 (C=N), 1595 (C=C); $^1\text{H-NMR}$ (DMSO- d_6 , 500 MHz): δ_{H} 2.37 (s, 3H, CH₃), 2.61 (s, 3H, CH₃), 6.94 (m, 4H, aromatic protons), 7.20 (m, 2H, aromatic protons), 7.32 (m, 2H, aromatic protons), 7.40 (d, J = 8.0 Hz, 1H, aromatic proton), 7.45 (m, 2H, aromatic protons), 7.66 (d, J = 8.0 Hz, 2H, aromatic protons); $^{13}\text{C-NMR}$ (DMSO- d_6 , 125 MHz): δ_{C} 14.9 (CH₃), 21.8 (CH₃), 101.6, 115.8, 122.9, 124.4, 124.6, 128.5, 130.2, 130.7, 132.4, 137.6, 144.1 (C-N), 147.1 (C=N), 149.1 (C=N), 156.4 (C-O); MS (EI, 70 Ev): m/z (%) 381.2, (100) [M+1]⁺, 382.2 (26.2) [M+2]⁺; Anal. Calcd for C₂₄H₂₀N₄O (FW: 380): C, 75.77; H, 5.30; N, 14.73. Found: C, 75.64; H, 5.41; N, 14.82.

5-Methyl-2-(3-methyl-1-phenyl-5-(pyridin-2-yloxy)-1*H*-pyrazol-4-yl)-1*H*-benzo[d]imidazole (45).

Yield = 66 %; mp: 88-90 °C; R_f = 0.52 (Petroleum ether / EtOAc = 1:3); IR (KBr): $\nu_{\text{max}}/\text{cm}^{-1}$: 3424 (NH), 3069 (CH aromatic), 2925 (CH aliphatic), 1630 (C=N), 1589 (C=C); $^1\text{H-NMR}$ (DMSO- d_6 , 500 MHz): δ_{H} 2.37 (s, 3H, CH₃), 2.60 (s, 3H, CH₃), 6.94 (m, 3H, aromatic protons), 7.21 (m, 2H, aromatic protons), 7.28 (brs, 1H, aromatic protons), 7.32 (m, 1H, aromatic protons), 7.38 (d, 1H, J = 8.0 Hz, aromatic protons), 7.46 (m, 2H, aromatic protons), 7.65 (d, 2H, J = 7.5 Hz, aromatic protons), 12.06 (brs, 1H, NH); $^{13}\text{C-NMR}$ (DMSO- d_6 , 125 MHz): δ_{C} 14.6 (CH₃), 21.3 (CH₃), 101.5, 115.4, 122.1, 123.2, 123.7, 127.4, 129.4, 129.9, 137.1, 143.6 (C-N), 146.0 (C=N), 148.1 (C=N), 155.8 (C-O); (EI, 70 Ev): m/z (%) 381.1 (100) [M]⁺, 382.2 (25) [M+1]⁺; Anal. Calcd for C₂₃H₁₉N₅O (FW: 381): C, 72.42; H, 5.02; N, 18.36. Found: C, 72.58; H, 5.21; N, 18.49.

Dimethylphenoxy)-3-methyl-1-phenyl-1*H*-pyrazol-4-yl)-5-methyl-1*H*-benzo[d]imidazole (46).

Yield = 78%; mp: 123-125 °C; R_f 0.63 (Petroleum ether / EtOAc = 1:2); IR (KBr): $\nu_{\max}/\text{cm}^{-1}$: 3424 (NH), 3069 (CH aromatic), 2925 (CH aliphatic), 1630 (C=N), 1589 (C=C); $^1\text{H-NMR}$ (DMSO- d_6 , 500 MHz): δ_{H} 1.98 (s, 3H, CH₃), 2.34 (s, 3H, CH₃), 2.38 (s, 3H, CH₃), 2.58 (s, 3H, CH₃), 6.27 (s, 1H, aromatic proton), 6.64 (d, J = 9.0 Hz, 1H, aromatic proton), 6.97 (m, 2H, aromatic proton), 7.32 (m, 2H, aromatic protons), 7.42 (m, 4H, aromatic protons), 7.61 (m, 2H, aromatic protons), 11.82 (brs, 1H, NH); $^{13}\text{C-NMR}$ (DMSO- d_6 , 125 MHz): δ_{C} 14.5 (CH₃), 15.6 (CH₃), 20.5 (CH₃), 101.6, 113.3, 122.3, 123.2, 124.0, 127.5, 129.2, 129.2, 131.1, 136.4, 137.2, 146.4 (C=N), 147.9 (C=N), 154.1 (C-O); MS (EI, 70 Ev): m/z (%) 409.2 (100) $[\text{M}+1]^+$, 410.2 (410.5) $[\text{M}+2]^+$; Anal. Calcd for C₂₆H₂₄N₄O (FW: 408): C, 76.45; H, 5.92; N, 13.72. Found: C, 76.59; H, 5.85; N, 13.64.

2-(3-Methyl-5-morpholino-1-phenyl-1H-pyrazol-4-yl)-5-nitro-1H-benzo[d]imidazole (47).

Yield = 55%; mp: 94-96 °C; R_f 0.53 (Petroleum ether / EtOAc = 1:3); IR (KBr): $\nu_{\max}/\text{cm}^{-1}$: 3424 (NH), 3069 (CH aromatic), 2925 (CH aliphatic), 1630 (C=N), 1589 (C=C), 1430, 1544 (NO₂); $^1\text{H-NMR}$ (DMSO- d_6 , 500 MHz): δ_{H} 2.31 (s, 3H, CH₃), 2.95 (m, 4H, H-3, H-5 morpholine protons), 3.51 (m, 4H, H-2, H-6 morpholine protons), 7.42 (m, 1H, aromatic protons), 7.53 (m, 4H, aromatic protons), 7.72 (m, 3H, aromatic protons); MS (EI, 70 Ev): m/z (%) 405.13 (100) $[\text{M}+1]^+$. Anal. Calcd for C₂₁H₂₀N₆O₃ (FW: 404): C, 62.37; H, 4.98; N, 20.78. Found: C, 62.29; H, 5.21; N, 20.87.

2-(3-Methyl-1-phenyl-5-(piperidin-1-yl)-1H-pyrazol-4-yl)-5-nitro-1H-benzo[d]imidazole (48).

Yield = 46%; mp: 103-105 °C; R_f 0.57 (Petroleum ether / EtOAc = 1:3); IR (KBr): $\nu_{\max}/\text{cm}^{-1}$: 3424 (NH), 3069 (CH aromatic), 2925 (CH aliphatic), 1630 (C=N), 1589 (C=C), 1436, 1546 (NO₂); $^1\text{H-NMR}$ (DMSO- d_6 , 500 MHz): δ_{H} 1.39 (brs, 6H, H-3, H-4, H-5 piperidine protons), 2.27 (s, 3H, CH₃), 2.87 (brs, 4H, H-2, H-6 piperidine protons), 7.54 (m, 6H, aromatic protons), 8.12 (s, 1H, aromatic proton), 8.49 (s, 1H, aromatic proton), 12.87 (brs, 1H, NH); $^{13}\text{C-NMR}$ (DMSO- d_6 , 125 MHz): δ_{C} 14.00 (CH₃), 23.6 (CH-4 piperidine carbons), 25.9 (CH-3, CH-5 of piperidine carbons), 51.5, (CH-2, CH-6 of piperidine carbons), 103.1, 108.8, 112.4, 114.7, 118.4, 122.4, 124.1, 124.6, 128.0, 128.9, 140.0, 143.4 (C=N), 148.0 (C=N), 151.3 (C-NO₂); MS (EI, 70 Ev): m/z (%) 403.2 (100) $[\text{M}+1]^+$, 404.2 (25) $[\text{M}+2]^+$. Anal. Calcd for C₂₂H₂₂N₆O₂ (FW: 402): C, 65.66; H, 5.51; N, 20.88. Found: C, 65.50; H, 5.43; N, 20.67.

Biological evaluation

Molecular Docking

The crystal structures of SphK1 in complex with its co-crystallized native ligand: PF-543, as a potent and selective Inhibitor of SphK1, The PDB structure of SphK1 (PDB ID: 4V24) was retrieved from the Protein Data Bank, <http://www.rcsb.org/pdb/home/home.do>. The key amino acids of the active site were identified using data in PDB sum, <http://www.ebi.ac.uk/pdbsum/>.

Molecular-docking studies of the newly synthesized compounds with SphK1 were performed to gain insight into the predicted binding affinity and interaction patterns. The 2D and 3D structures of the synthesized compounds were generated using the Chem Draw Ultra v12.0. AutoDock Tools [66] was used for the preparation of docking files supported by AutoDock Vina [66]. In this study; we have performed site specific molecular docking. The docking was done within 15 Å diameters from the reference PF-543 ligand. The binding site of the crystal structure of SphK1 is composed of the following amino acids: Leu167, Ser168, Ala170, Phe173, Ile174, Val177, Asp178, Phe192, Thr196, Leu259, Leu261, Leu268, Ala274, Phe288, Val290, Leu302, Phe303, Met306, His311, and Ala339. The binding site was defined by including all residues constituting the binding pocket of reference PF-543 ligand.

AutoDock Vina was used for running molecular docking. The docked poses of the newly synthesized compounds with SphK1 were ranked based on the predicted binding affinity and interaction patterns. Intermolecular interactions were studied using PyMol molecular [67]. The 2D plots for protein ligand interaction were created using the Discovery Studio Visualizer. The top-ranked compounds selected from the analysis are listed in Table 2.

Expression and purification of SphK1

The secondary cultures of SphK1 were induced by 1 mM IPTG for 4 h followed by centrifugation at 7000 rpm for 15 minutes to get the cell pellet, which was later resuspended in the lysis buffer and inclusion bodies were prepared as described [57]. Finally, inclusion bodies were solubilized in the solubilization buffer (pH 8.0) comprising 0.5% sarcosine, 50 mM Tris and 150 mM NaCl. SphK1 was purified using Ni-NTA affinity chromatography, followed by dialysis for 24 h to get the refolded native protein. The purified protein was loaded on SDS-PAGE and the concentration was calculated using a molar absorption coefficient of $48275 \text{ M}^{-1}\text{cm}^{-1}$ at 280 nm on the Jasco V-660 UV-visible spectrophotometer.

Fluorescence binding studies

The Jasco Spectrofluorometer at 25 °C was used for the binding studies of all the synthesized compounds. The compounds were first dissolved in DMSO to get the 20 mM stock solution and then diluted to a working concentration of 1 mM in 20 mM Tris and 100 mM NaCl buffer (pH 8.0). The quenching studies were performed with a fixed concentration of SphK1 (5 μM) and the compounds were added gradually in increasing concentration from the 1 mM stocks into the protein solution until the achievement of saturation point. The emission spectra were recorded from 300–400 nm with excitation of SphK1 at 280 nm. The blank titrations (buffer with selected compounds) were subtracted to obtain the final spectra and the quenching data was corrected for the inner filter effect according to the formula, $F = F_{obs} \text{antilog} [(A_{ex} + A_{em})/2]$, where A_{ex} and A_{em} is the absorbance of the selected compound at the excitation and emission wavelength respectively [68]. The quenching spectra obtained for selected compounds were plotted and the inverse correlation between the gradual decrease in the fluorescence intensity with increasing concentration of compounds was used for determining the kinetic parameters (K_a and n) from a modified Stern–Volmer equation (Equation (1)) as described [69] where F_0 denotes fluorescence intensity of SphK1 without the compound and F denotes the fluorescence intensity of SphK1 at a specific concentration of compound at λ_{max} .

$$\log \frac{(F_0-F)}{F} = \log K_a + n \log [\text{Harmaline}] \quad (1)$$

Enzyme inhibition assay

A standard Malachite Green (BIOMOL® GREEN reagent) microtitre-plate assay was performed to evaluate the inhibitory potential of all the synthesized compounds against SphK1. Briefly, compounds were incubated with SphK1 (4 μM) for 1 h at 25 °C and then later freshly prepared ATP (200 μM) and 10 mM MgCl_2 were added to the protein-ligand mixture. The reaction was allowed to proceed for 30 minutes at 25 °C. After the required incubation period, the reaction was ended by adding the double amount of BIOMOL® reagent. Finally, a green-colored complex was formed in 10 minutes and the absorbance readings were recorded on ELISA reader at 620 nm. The reaction with ligands and no protein was also performed to subtract the background reading of inorganic phosphate. A standard phosphate curve was used to determine the loss in activity of SphK1 in terms of amount of phosphate released on treatment with increasing concentrations of selected compounds. The inhibition in SphK1 activity was plotted for selected compounds in terms of percentage as described [70].

Declarations

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Compliance with ethical standards Conflict of interest

The authors declare that they have no conflict of interest.

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Tables

Table 1. The binding affinity constants and number of binding sites as determined from the molecular docking and fluorescence binding experiments.

Compound ID	Predicted Affinity	[¥] Binding	[¥] Number
	ΔG^e (kcal/mol)	Affinity constant (K_a), M ⁻¹	of binding sites (n)
33	- 8.3	2.57 10 ³	0.8
37	- 7.7	1.64 10 ⁴	0.9
39	- 8.0	1.41 10 ³	0.7
41	- 8.7	1.05 10 ⁴	0.9
42	- 8.2	4.51 10 ⁴	1.0
43	- 8.0	2.44 10 ³	0.8
45	- 8.8	2.82 10 ³	0.8

Binding parameters of the synthesized compounds with SphK1 evaluated through molecular docking and fluorescence[¥] binding studies.

Table 2. IC₅₀ values of the selected compounds for SphK1 inhibition calculated from the ATPase inhibition assay.

Compound ID	LogIC ₅₀ (μM)	IC ₅₀ (μM)
33	0.88 ± 0.01	7.60 ± 0.25
37	0.66 ± 0.10	4.61 ± 1.04
39	0.77 ± 0.09	5.90 ± 1.19
41	0.53 ± 0.03	3.39 ± 0.21
42	0.70 ± 0.01	5.05 ± 0.13
43	0.80 ± 0.06	6.34 ± 0.86
45	0.72 ± 0.11	5.31 ± 1.28

Table 3. *In vitro* growth inhibition % (GI %) of some synthesized *benzimidazole derivatives* against a panel of tumor cell lines at 10 mM.

Subpanel	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
Leukemia																									
CCRF-CEM	- ^a	-		-	-	-	-	-	-	-	-	-	-	-	-	11	18	-	32	-	11	41	19	21	40
HL-60(TB)	-	-	-	-	-	-	-	-	-	-	-	-	-	23	21	-	-	-	20	-	12	28	-	-	29
K-562	-	15	-	16	22	28	-	-	-	-	23	-	15	11	19	-	13	10	25	19	19	41	13	18	40
MOLT-4	-	-	-	18	17	27	-	-	-	13	25	-	10	14	26	15	22	13	-	-	-	40	14	18	42
PRMI-8226	-	10	-	18	19	23	-	-	14	-	19	14	-	-	18	15	23	23	12	12	47	49	19	34	55
SR	-	14	26	19	24	38	-	20	-	25	26	-	25	12	21	26	25	16	11	15	12	15	27	17	54
Non-small cell lung Cancer																									
A549/ATTC	-	-	-	12	-	19	-	-	-	-	-	-	-	-	11	-	13	-	29	-	30	29	-	-	32
EKVX	-	-	-	-	19	-	-	-	-	-	-	-	10	-	-	12	-	-	-	-	-	-	-	-	-
HOP-62	14	12	-	-	13	12	10		-	-	10	10	-	-	-	14	-	-	18	-	24	34	15	26	28
HOP-92	10	10	-	-	-	14	13		-	-	10	-	-	-	-	20	-	-	10	-	24	37	-	15	21
NCI-H226	15	-	-	-	-	-	-	-	-	12	-	-	14	-	-	26	-	-	21	-	11	24	-	15	10
NCI-H23	19	18	11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	20	-	-	10	-	32	-
NCI-H322M	-	-	-	-	14	24	-	-	10	-	-	-	23	-	-	-	-	-	-	-	-	10	-	-	-
NCI-H460	-	-	-	-	-	13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	17	18	17	-	18
NCI-H522	-	-	-	-	13	nd ^b	10	-	-	-	-	-	12	19	16	12	-	19	23	15	27	22	-	21	24
Colon Cancer																									
COLO 205	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	21	22	23	-	-
HCC-2998	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	23	24	25	-	-
HCT-116	10	17	-	19	26	33	-	-	-	-	19	-	28	-	15	-	10	-	12	-	29	26	27	22	27
HCT-15	-	-	-	-	11	11	-	-	-	-	-	-	15	-	18	-	-	-	23	-	-	35	20	17	24
HT29	-	-	-	-	-	nd	-	-	-	-	-	-	17	-	-	-	-	-	13	-	-	-	-	20	17
KM12	-	-	-	-	-	14	-	-	-	-	-	-	21	-	12	-	-	-	12	-	27	37	38	34	26
SW-620	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	26	-	-	20	10	50	-
CNS Cancer																									
SF-268	-	-	-	-	11	10	-	-	12	-	-	-	12	10	-	-	-	-	21	-	21	-	13	21	22
SF-295	-	-	-	-	-	-	-	-	-	-	-	-	13	-	-	-	-	-	18	-	15	-	-	10	16
SF-539	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	32	-	23	-	-	16	37
SNB-19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	42	-
SNB-75	20	16	-	14	14	12	15	18	-	13	-	-	31	-	-	15	21	17	13	11	-	-	19	30	50
U251	14	11	-	-	-	10	-	-	-	-	-	-	12	-	-	11	-	-	27	-	15	-	-	-	30
Melanoma																									
LOX IMVI	-	11	-	11	15	11	-	-	12	-	10	-	-	-	18	17	-	-	-	-	13	-	10	12	21
MALME-3M	11	-	19	-	-	13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	14	-	12	11	14
M14	-	-	-	11	-	-	-	10	-	-	-	-	-	-	-	-	-	-	-	-	15	-	23	-	21
MDA-MB-435	-	-	21	-	-	-	-	-	-	-	-	-	19	-	12	-	-	-	12	-	26	-	24	34	27
SK-MEL-2	-	-	-	nd	-	-	-	-	-	-	-	-	-	-	-	nd	-	-	-	-	-	-	13	23	-
SK-MEL-28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	45	-	15	-	12	24	15
SK-MEL-5	-	-	-	-	-	-	-	-	-	-	-	-	11	-	-	-	-	-	-	-	23	-	21	18	25
UACC-257	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	32	-	-
UACC-62	-	28	15	23	23	43	11	10	21	14	30	19	20	16	37	22	26	14	21	-	34	-	12	14	32
Ovarian Cancer																									
IGROV1	13	20	-	31	33	51	-	-	-	-	26	15	11	-	17	18	-	-	-	-	18	-	12	18	11
OVCAR-3	19	10	-	11	13	23	-	-	-	-	-	-	-	-	-	-	-	-	12	-	26	-	10	20	23
OVCAR-4	-	13	-	13	16	21	-	-	-	-	14	-	14	-	18	12	19	-	12	-	19	-	23	16	15
OVCAR-5	-	29	-	-	-	16	-	-	-	-	-	-	-	-	-	-	-	-	13	-	-	-	12	-	-
OVCAR-8	-	-	-	-	11	12	-	-	-	-	-	-	11	-	-	-	-	-	14	-	43	-	14	35	44
NCI/ADR-RES	-	10	-	-	-	10	-	-	-	-	10	-	23	-	10	-	-	-	21	-	19	-	15	38	11
SK-OV-3	-	-	-	-	11	14	-	-	-	-	12	-	10	-	-	-	-	-	11	-	-48	-	17	49	45
Renal Cancer																									
786-0	-	-	-	-	-	12	-	-	-	-	-	-	-	-	-	-	-	-	11	-	15	18	32	18	16
A498	29	44	30	35	45	48	-	21	29	30	39	32	39	23	46	29	21	-	14	33	16	41	15	15	43

ACHN	13	10	-	-	10	14	-	-	-	10	14	-	-	-	11	13	-	11	15	-	38	30	12	35	32
CAKI-1	24	29	21	40	35	58	18	20	23	17	33	14	13	20	27	21	21	16	10	-	16	38	54	45	37
RXF 393	-	-	-	-	-	-	-	-	-	-	-	-	12	-	-	-	-	-	12	-	12	32	23	23	35
SN 12C	-	-	-	-	-	-	-	-	-	-	-	-	23	-	-	-	-	-	32	-	23	36	39	37	16
TK-10	-	-	-	-	-	nd	-	-	-	-	-	-	15	-	-	-	nd	-	25	-	36	34	11	32	12
UO-31	30	29	24	33	38	37	21	13	30	25	24	29	26	18	27	30	15	15	18	21	14	29	31	20	35
Prostate Cancer																									
PC-3	13	17	-	18	17	23	10	-	13	-	19	14	24	-	24	13	22	15	31	-	39	36	35	31	39
DU-145	-	-	-	-	-	-	-	-	-	-	-	-	11	-	-	-	-	-	17	-	38	14	13	15	16

^a Growth inhibition % produced by the compound is below 10%. ^b Not determined.

Table 4: Interaction profile of compounds 33, 37, 39, 41, 42, 43 and 45 with SphK1

Compound ID	Interaction Type	No. of Interactions	Interacting Residues
33	van der Waals	9	Phe173, Thr196, Leu259, Leu261, Met272, Ala274, Phe288, Leu299, His311
	Hydrogen bond	1	Leu299
	π -Cation	2	Asp178, Phe192
	π -Sigma	3	Ile174, Leu302, Phe303
	π -Sulfur	1	Met306
	π -Alkyl	4	Val177, Leu268, Val290, Leu319
37	van der Waals	11	Leu169, Phe173, Leu259, Gly269, Leu261, Ala274, Leu299, His311, Val340, Asp341, Gly342
	Hydrogen bond	3	Thr196, Leu268, His311
	π -Cation/Anion	4	Asp178, Phe192, Met272, Met306
	π -Alkyl	6	Ala170, Ile174, Val177, Val290, Leu319, Ala339
	π -Sigma	1	Leu302
	π - π Stacked	1	Phe303
39	van der Waals	18	Leu167, Ser168, Ala170, Phe173, Val177, Glu189, Leu259, Leu261, Gly269, Ala274, Leu299, Leu302, Phe303, Met306, His311, Ala339, Asp341, Gly342
	Hydrogen bond	6	Asp81, Asp178, Arg185, Thr196, Phe188, Glu343
	π -Anion	1	Asp81
	π -Alkyl	6	Ala115, Ile174, Phe192, Leu268, Met272, Leu319
	π -Sulfur	1	Met272
41	van der Waals	10	Ala115, Ser168, Thr196, Leu261, Leu259, Gly269, Ala274, Leu299, His311, Gly342
	Hydrogen bond	4	Ser168, Asp178, Leu268, Gly342
	π -Cation/Anion	2	Asp178, Phe192
	π -Sigma	3	Ile174, Leu302, Phe303
	π -Alkyl	6	Ala170, Phe173, Val177, Val290, Leu319, Ala339
	π -Sulfur	2	Met272, Met306
42	van der Waals	8	Leu169, Leu261, Gly269, Ala274, His311, Val340, Asp341, Gly342
	Hydrogen bond	4	Ser168, Thr196, Leu268, Ala339
	π -Sigma	1	Leu302
	π -Cation/Anion	3	Asp178(2), Phe192
	π -Alkyl	13	Phe173, Ile174(2), Ala170, Val177, Leu259, Leu268, Val290, Leu299, Phe303, Met306, Leu319, Ala339
	π - π Stacked	2	Phe192, Phe303
	π -Sulfur	1	Met272
43	van der Waals	10	Ala115, Leu169, Leu261, Gly269, Ala274, Leu299, His311, Val340, Asp341, Glu343
	Hydrogen bond	5	Ser168, Thr196, Leu268, Ala339, Gly342

45	π -Cation/Anion	2	Asp178, Phe192
	π -Alkyl	9	Ala170, Phe173, Ile174, Val177, Leu259, Leu268, Val290, Leu319, Ala339
	π -Sigma	2	Ile174, Leu302
	π -Pi Stacked	2	Phe192, Phe303
	π -Sulfur	2	Met272, Met306
	van der Waals	15	Gly26, Gly80, Gly80, Ser112, Gly113, Asn114, Ala170, Arg185, Glu189, Thr196, Leu261, Gly269, Ala339, Asp341, Glu343
	Hydrogen bond	6	Asp81, Ser168, Asp178, Thr196, Leu268, Gly342
	π -Cation/Anion	3	Asp81, Asp178, Arg191,
	π -Alkyl	7	Ala115(2), Ile174, Leu167, Val177, Leu268, Met272,
	π - π Stacked	1	Phe192
	π -Sulfur	1	Met272

Figures

Figure 1

Examples of sphingosine kinase1 inhibitors

Figure 2

Examples of benzimidazoles and pyrazole derivatives as SphK-1 inhibitors.

Figure 3

Rational design of new 5-substituted 2-pyrazolylbenzimidazoles as sphingosine kinase1 inhibitors.

Figure 4

Binding studies of selected synthesized compounds with SphK1. Fluorescence emission spectra representing SphK1 quenching on the addition of an increasing amount of (A) compound 47 (0–31.1 mM), (B) compound 48 (0–41.1 mM), SphK1 was excited at 280 nm and emission spectra were recorded in the range of 300–400 nm. Modified Stern-Volmer plot was used to analyze the quenching data and to estimate the binding constant (K_a) for (C) compound 47, (D) compound 48.

Figure 5

Inhibition of SphK1 ATPase activity by selected compounds. The amount of phosphate released from the hydrolysis of ATP was measured using the standard phosphate curve. Dose-response curve depicting the effect of increasing concentrations of (A) compound 47 (0–20 μM) on the ATPase activity of SphK1. Dose-response curve depicting the effect of increasing concentrations of (B) compound 48 (0–25 μM). . Plots represent percent inhibition in ATPase

activity of SphK1 as a function of increasing concentrations of (C) compound 47 and (D) for compound 48, The IC50 value was calculated by fitting the curve obtained from two independent experiments.

Figure 6

Compound 47 binding to SphK1 A) chemical structure of compound 47 B) Graphical representation of compound 47 interacting with binding site residues of SphK 1 C) 2D scheme of protein-ligand interactions D) Surface view of Sphk1 binding pocket occupied by compound 47.

Figure 7

Compound 48 binding to Sphk 1 A) chemical structure of compound 48 B) Graphical representation of compound 48 interacting with binding site residues of SphK1 C) 2D scheme of protein-ligand interactions D) Surface view of SphK1 binding pocket occupied by compound 48.

Supplementary Files

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