Structure-activity relationship of pyrazolo pyrimidine derivatives as inhibitors of mitotic kinesin Eg5 and anticancer agents

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ABSTRACT

Human kinesin Eg5 is a potential inhibiting site for cancer chemotherapy. Blocking metaphase by binding foreign inhibitors with Eg5 eventually leads to apoptotic cell death. Here, we report the pyrazolopyrimidine derivatives as potent inhibitors of Eg5 that prevents mitotic kinesin progression. IC50 values were evaluated against the motor domain of Eg5 using steady-state ATPase assay. To better understanding, we have performed molecular docking simulation. It reveals that the interactions of the proposed inhibitors with both the allosteric sites (helices α2, α3 and loopL5, and helices α4 & α6). Out of fifteen pyrazolopyrimidine derivatives, three compounds (12, 25, and 27) have shown significant inhibition of Eg5. The synthesized compounds (12, 25, and 27) were tested for their in-vitro anticancer activity against cervical cancer cell line (HeLa).

1. Introduction

The recent clinical data indicate the extent population affected by cancer directly and indirectly, demands the development new drugs with desired medicinal efficiency that may be synthesized by adopting by simple environmental friendly synthetic methods. Among various mechanisms adopted to control cancer, interfering with the spindle formation has been evolved as a successful mechanism. Spindle formation is one of the important steps in cell multiplication in which spindle formation followed by apoptosis [1–4]. Kinesins a family of molecular motors described as nano-machines convert the chemical energy of ATP into mechanical force used to drive the intracellular cargos towards the plus end of the MT and hence play a critical role in cell cycles [5]. Eg5 known as KSP also a member of kinesin 5 subfamily is an essential factor in mitotic spindle formation hence inhibition of Eg5 will result in monopolar spindles instead of bipolar spindles that may arrest the cell cycle. In recent days several researchers have developed number of Eg5 inhibitors and have established the mechanism of action through SAR studies [6–12]. Various reports reveal that a set of Eg5 inhibitors of biphenyl type such as monastrol, STLC, ispinesib, SB-743921, and ARRY-520 and benzimidazoles bind to the allosteric pocket formed by α2 and α3 helices of L5 at about 10 Å from Eg5 motor domain bound to ATP [5]. It has been observed that these inhibitors established some mutations in various cell culture studies. One of the benzimidazoles, B18 type of KSP inhibitor were reported to bound to an allosteric pocket formed by helices α4 and α6 in addition to α2 and α3 helices of L5. The identification of α4 and α6 allosteric inhibitor-binding pocket (formed by helix α2&α4) provides the opportunity to develop new series of inhibitors that could be used either alone or in combination with existing Eg5 compounds.

Heterocyclic compounds are more common in biological systems as enzyme cofactors, amino acids and proteins that are playing vital role in the metabolism of living systems. The heterocycles involve in number of intermolecular activities such as hydrogen bonding, coordination to metal atoms, and vander Waals hydrophobic interaction. The possible wide range of shapes and sizes of heterocycles favors different structural range of enzyme binding activities. Thus the extremely common occurrence and role in variety of biological activities make the heterocycles as important medicinal compounds [13]. Sarcoma genes (Src) that potentially induces cell malignant transformation plays significant role in gene/kinase. Thus Src inhibitors may be prospective agents in mitigating pancreatic cancer, breast cancer, stomach cancer etc. A series of pyrazole pyrimidine derivatives have been reported to exhibit significant clinical efficiency as Src kinase inhibitors [14]. Zhang et al has reported that phenyl ethyl substituted pyrazole pyrimidine is a potential agent to treat triple negative breast cancer through their in vitro and in vivo studies [15]. They have also reported that this pyrazole pyrimidine can inhibit Src kinase as well as several other kinase
involve in MAPK signal transduction [16]. Pyrazole pyrimidine derivatives exhibited tumor growth suppression efficiency without any toxic effect as a potential drug for EGRF mutant-driven NSCLC [17,18]. Hela et al has reported that pyrazole pyrimidine derivative act as PDE5A inhibitors [19,20]. Pyrazole pyrimidines were also reported to have higher aqueous solubility with enhanced pharmacokinetic properties and hence a better cytotoxic agent against human glioblastoma U87 cell line [21,22]. Zapf et al has reported that acrylamide substituted pyrazole pyrimidines act as non receptor tyrosine kinase (Itk) inhibitor [23]. 4-benzyl-1-(7H-pyrrrolo[2,3-d]pyrimidin-4-yl)peridinin-4-amines was reported to be Akt (PKB) type of inhibitor by preventing deregulation of intracellular signaling pathways [24]. Several pyrazole pyrimidines were reported to have efficient cytotoxic activity even in nanomolar concentrations with promising selectivity against merkinase inhibitor [23].

The maximum yield was obtained in presence of the catalyst Pd(II) acetate (1.5 equiv) in presence of various palladium catalysts such as Pd(OAc)2, Pd(dba)2, PdCl2(PPh3)2 and Pd(PPh3)4 and different bases like NaOEt, KOtBu and K3PO4, Table 1.

The structure of a representative compound from each scheme have been established from the FT-IR, NMR, 2D NMR and LC-MS spectroscopic data given in supporting information. HMB correlation is presented in Fig. 1. 1H NMR spectrum of 12, methylthio protons appear as a singlet at 2.44 ppm (s, 3H) that correlates with C3-H-COSY signals at 13.0 ppm corresponding to the methylthio carbon. The signal at 2.44 ppm also shows HMB correlation contour signals at 154 ppm which is assigned to C-7. The singlet at 3.77 ppm is assigned to the methoxy proton (s, 3H). This signal has a C3-H-COSY contour with the signal at 56.7 ppm. The signal at 3.77 ppm also shows HMB correlation contour signal at 149 ppm and it is assigned to C-3′-

2. Results and discussion

2.1. Chemistry

A set of pyrazolopyrimidine derivatives 8–19 were synthesized by direct reductive amination of scaffold 6 with the corresponding aromatic aldehydes 7a–7l in presence of acetic acid Scheme 1 and another set of derivatives 20–21 obtained from the scaffold 6 and the aldehydes 7m and 7n in presence of triethylamine (TEA) Scheme 2. Similarly, pyrazolopyrimidine derivative 25 was obtained from 23 and 24 through N-alkylation [28–31] (Scheme 3). The morpholine substituted pyrazolopyrimidine 27 was synthesized from 18 and 26 via Suzuki Miyaura cross-coupling reaction (Scheme 4). The synthetic conditions were optimized by using various palladium catalysts such as Pd(OAc)2, Pd(dba)2, PdCl2(PPh3)2 and Pd(PPh3)4 and different bases like NaOEt, KOtBu and K3PO4, Table 1.

All the synthesized compounds were tested against the motor domain of the human mitotic kinesin Eg5 [35]. A steady state ATPase activity assay was carried out with STLC as a standard compound. A set of compounds tested 12, 25 and 27 showed activities in micromolar range concentrations against the Eg5 (Fig. 2).

In order to understand the protein-ligand interactions, we have carried out molecular docking studies using maestro software and analyzed the structures of the best docking poses. The results are presented in the Table 3. It has been reported that the KSP contains two allosteric binding sites where the potential inhibitors could bind. The site-I formed by helix α2, helix α3 & the loop L5 and most of the known compounds bind to this. The site-II which has been identified recently is the helix α4 and helix α6. In order to identify weather, the inhibitor binds to either of the sites or to any one site we have docked the molecules to both positions and analyzed the interactions separately. We have compared the interactions of these molecules with Site 2 to the interactions of two different types of inhibitors Benzimidazole type inhibitor B18 (PDB ID: 3ZCW) and the biphenyl type inhibitor (PDB ID: 3WPW) PVZB1194 that were well established and for the site 1 we have used the tight binding inhibitor STLC (PDB ID: 2WOG) as the reference.

Unlike for site 2 which has only two protein-inhibitor complex structures, plenty of structures of KSP are available with inhibitor present in site 1. The binding energy of the tested compounds significantly higher at site 1 compared to site 2. In site 1, the ligand interactions are primarily hydrophobic and aromatic in nature. In the crystal structure (PDB ID: 2WOG) the reference compound STLC forms hydrophobic interactions with the side chains of Glu215, Glu116, Arg119 and Pro137 and aromatic π–π interactions through Trp127 and Tyr211. Our compounds also form similar type of interactions Figs. 3–5.

Compound 12 forms hydrophobic interaction with residues Arg221, Thr112 and Thr222 through the 1-(2-fluorophenyl)-1H-pyrazolo[3,4-d] pyrimidine and with Gly117, Glu118, Gly217 and Trp127 through the o-cresol group. It also forms weak hydrogen bonds with NH of Arg119 (D⋯A = 2.97 Å). Compound 11 doesn’t form any hydrogen bond but it forms hydrophobic interactions residues Arg221, Thr112 and Thr222 through the 1-(2-fluorophenyl)-1H-pyrazolo[3,4-d]pyrimidine and with Gly117, Glu118, Gly217 and Ala218 through the 3-methoxyphenol group. The compound 27 which doesn’t form any hydrogen bond and surrounded by amino acid residues Thr112, Phe113, Glu118, Arg221,
Ser232, Ser233 and Leu266 within the 4Å region of the site 1. Thenewly identified site 2 defined primarily by helix α4 and α6. For this site 2, two types protein inhibitor complex structures have been reported so far which suggest the interactions are primarily hydrophobic and π-π stacking. The aromatic π interaction with residue Tyr352 has been confirmed by mutational studies. In both reference molecules the Tyr352 residue forms face to face π-π interaction with the pyrazole pyrimidine group of the ligands. All the three active compounds in our study have similar interactions with those residues Fig. 6. The 7H-pyrrolo[3,4-d]pyrimidine group of the compounds 12, 25 forms a hydrogen bond and edge to face π-π interaction with Tyr 352. The amino group attached with the pyrazole pyrimidine of compound 12 and compound 25 forms one hydrogen bond with main chain of oxygen of the Tyr352 (D...A = 2.6 Å). The o-cresol group of compounds 12 and

\[ \text{Scheme 1. Synthesis of pyrazole pyrimidine derivatives 8–19.} \]

\[ \text{Scheme 2. Synthesis of pyrazole pyrimidine derivatives 20–21.} \]

8Reagents and conditions: (a) 1-(2-fluorophenyl)-4-hydrazinyl-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine 6 (1.0 equiv), aromatic aldehyde, 60 °C, DIPEA (2.0 equiv), methanol, 3 h; (b) 1-(2-fluorophenyl)-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine 8 (1.0 equiv), aromatic aldehyde, 60 °C, DIPEA (2.0 equiv), methanol, 3 h; (c) 1-(2-fluorophenyl)-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine 9 (1.0 equiv), aromatic aldehyde, 60 °C, DIPEA (2.0 equiv), methanol, 3 h; (d) 1-(2-fluorophenyl)-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine 10 (1.0 equiv), aromatic aldehyde, 60 °C, DIPEA (2.0 equiv), methanol, 3 h; (e) 1-(2-fluorophenyl)-4-hydrazinyl-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine 11 (1.0 equiv), aromatic aldehyde, 60 °C, DIPEA (2.0 equiv), methanol, 3 h; (f) 1-(2-fluorophenyl)-4-hydrazinyl-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine 12 (1.0 equiv), aromatic aldehyde, 60 °C, DIPEA (2.0 equiv), methanol, 3 h; (g) 1-(2-fluorophenyl)-4-hydrazinyl-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine 13 (1.0 equiv), aromatic aldehyde, 60 °C, DIPEA (2.0 equiv), methanol, 3 h; (h) 1-(2-fluorophenyl)-4-hydrazinyl-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine 14 (1.0 equiv), aromatic aldehyde, 60 °C, DIPEA (2.0 equiv), methanol, 3 h; (i) 1-(2-fluorophenyl)-4-hydrazinyl-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine 15 (1.0 equiv), aromatic aldehyde, 60 °C, DIPEA (2.0 equiv), methanol, 3 h; (j) 1-(2-fluorophenyl)-4-hydrazinyl-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine 16 (1.0 equiv), aromatic aldehyde, 60 °C, DIPEA (2.0 equiv), methanol, 3 h; (k) 1-(2-fluorophenyl)-4-hydrazinyl-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine 17 (1.0 equiv), aromatic aldehyde, 60 °C, DIPEA (2.0 equiv), methanol, 3 h; (l) 1-(2-fluorophenyl)-4-hydrazinyl-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine 18 (1.0 equiv), aromatic aldehyde, 60 °C, DIPEA (2.0 equiv), methanol, 3 h; (m) 1-(2-fluorophenyl)-4-hydrazinyl-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine 19 (1.0 equiv), aromatic aldehyde, 60 °C, DIPEA (2.0 equiv), methanol, 3 h; (n) 1-(2-fluorophenyl)-4-hydrazinyl-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine 20 (1.0 equiv), aromatic aldehyde, 60 °C, DIPEA (2.0 equiv), methanol, 3 h; (o) 1-(2-fluorophenyl)-4-hydrazinyl-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine 21 (1.0 equiv), aromatic aldehyde, 60 °C, DIPEA (2.0 equiv), methanol, 3 h; (p) 1-(2-fluorophenyl)-4-hydrazinyl-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine 22 (1.0 equiv), aromatic aldehyde, 60 °C, DIPEA (2.0 equiv), methanol, 3 h.
27 also forms a hydrogen bond with the main chain oxygen of Tyr352 (D⋯A = 2.9 Å). All these compounds develop hydrophobic interactions with the residues Ile288, Leu293, Glu344, Glu345, Leu347, Ser348 and Glu351. The BI8 forms a hydrogen bond with Thr300 and also some solvent mediated hydrogen bonds while PVZB1194 doesn’t form any hydrogen bond. However, compounds 12 and 25 forms a strong hydrogen bonds with main chain NH2 of Asn271 (D⋯A = 2.6 Å). Compounds 27 form Van der Waals interactions similar to the BI8.

2.3. Cytotoxicity of MTT assay

The best compounds (synthesized pyrazole pyrimidine and thienopyridines) obtained from the steady state ATPase assay were screened for antiproliferative activity in vitro against HeLa cells by performing MTT assay (Fig. 7). All the compounds were screened within a range of 0.25–100 µM concentrations, against HeLa cells for 48 h. Among all the tested analogues, compounds 12, 25 and 27 have shown significantly better inhibition of HeLa cells proliferation in a dose dependent manner. The IC50 values for compound 12, 25 and 27 (4.6 µM, 1.43 µM & 2.28 µM respectively) were obtained from a non-linear regression graph plotted between the percentage of cell viability and Log10 concentration using Graph Pad Prism software. The results suggest that compounds are effective at low concentration against the human cervical cancer cell line.

3. Conclusion

Both the pyrazole pyrimidine and thienopyridines derivatives were tested for activity against human mitotic kinesin eg5. Three compounds (12, 25 and 27) showed to inhibit the eg5 activity at micromolar concentration. Molecular docking studies suggest that they have higher binding affinity to site 1 of eg5 by interacting with the hotspots such as Trp127 and Arg119. These compounds also interact with Tyr352 by forming three hydrogen bonds in the site 2 allosteric binding site of Eg5. The pyrazole pyrimidine derivatives can be used as template to further structure based drug discovery in order to design more inhibitors with improved potency. The present works provides the basis in designing new and more potent leads and understand the activity of the pyrazole pyrimidine derivatives to develop them further as lead compounds for cancer chemotherapy.

4. Experimental

NMR spectra have been taken two different spectrometers n various place at 500, 400 MHz in 1H and 100, 125 MHz 13C respectively using Bruker (Avance) instrument in CDCl3 using tetramethylsilane (TMS) as internal standard. Chemical shifts are reported as δ values (ppm). All one- and two-dimensional NMR spectra were obtained using standard Bruker software throughout. IR spectra were recorded on a JASCO FTIR.

*Reagents and conditions: a) 1-(2-fluorophenyl)-4-hydrazinyl-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine 6 (1.0 equiv), aromatic aldehyde, 60 ° DIPEA (2.0 equiv), methanol, 3 h; b) 4-(2-(6-fluoro-5-methyl-1H-indol-3-yl)methylene)hydrazinyl)-1-(2-fluorophenyl)-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine 23 (1.0 equiv), 1-(bromomethyl)-2,4-dichlorobenzene 24 (1.2 equiv), K₂CO₃, (2.0 equiv), DMF, rt , 10 h;


*Reagents and conditions:a) 18 (1.0 equiv), (4-methoxy-3-(morpholinosulfonyl)phenyl)boronic acid,(1.5 equiv), Pd(dbca)₂ (0.01 equiv), K₂CO₃, (1.5 equiv), 1,4-dioxane:water (4:1), 90 °C in pressure tube 12 h; *isolated yield.

Scheme 4. Synthesis of pyrazole pyrimidine derivatives 27.
Table 1
Screening of catalyst and reaction conditions for pyrazole pyrimidine derivatives. a

<table>
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<th>Base</th>
<th>Yield (%)</th>
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<tr>
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<td>Pd(dba) 3</td>
<td>NaOEt</td>
<td>52</td>
</tr>
<tr>
<td>2</td>
<td>Pd(dba) 3</td>
<td>KOtBu</td>
<td>76</td>
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<tr>
<td>3</td>
<td>Pd(dba) 3</td>
<td>K3PO4</td>
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<td>Pd(OAc) 2</td>
<td>K3PO4</td>
<td>36</td>
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<tr>
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<td>KOtBu</td>
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<td>K3PO4</td>
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<td>KOtBu</td>
<td>31</td>
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<tr>
<td>12</td>
<td>PdCl2(PPh3) 3</td>
<td>K3PO4</td>
<td>26</td>
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a Reagents and conditions: (a) 18 (1.0 equiv), 4-methoxy-3-(morpholinosulfonyl)phenylboronic acid, 26 (1.5 equiv), Pd(dba)<sub>2</sub>, (0.01 equiv), KOtBu, (1.5 equiv), toluene: water (8:2) mL, 90 °C in pressure tube 12 h. b Isolated yield.

IR instrument (KBr pellet). NMR solvent DMSO-<sup>d6</sup> and CDCl<sub>3</sub> were purchased from Sigma Aldrich Company.

4.1. 5-Amino-1-(2-Fluorophenyl)-3-(methylthio)-1H-pyrazole-4-carbonitrile (3)

A mixture of bis(methylsulphanyl)methylenemalononitrile 1 (3.0 g 0.0130 mmol), and 2-fluorophenylhydrazine 2 (3.0 g 0.0130 mmol) in absolute ethanol (40 mL) was refluxed for 6 h. The completion of the reaction was monitored by TLC and the mixture was poured into ice cold water. The solid separated was purified by recrystallisation from absolute ethanol. Isolated as ivory solid: Yield: 85%; mp: 132–135 °C; Calculated Mass: 248.2, Observed LC-MS (M+1): 249.1; Method A: 0.1% Formic acid in water B: Acetonitrile, flow rate: 1.5 mL/min column: Atlantis d C18 (50 × 4.6) mm, 5 µm, +ve mode max chromatogram: RT (min) 3.14: Area. 99.53%; FT-IR (cm<sup>−1</sup>): 3432, 3326, 3224, 3047, 2919, 2857, 2205, 1652, 1594, 1555, 1520, 1450, 1356, 1291, 1019, 978, 924, 764, 699;<sup>1</sup>H NMR (400 MHz, DMSO-<sup>d6</sup>) δ 7.52–7.45 (m, 4H, Ar-H), 4.60 (s, 2H, NH<sub>2</sub>), 2.57 (s, 3H, SMe); 13CNMR (100 MHz, DMSO-<sup>d6</sup>) δ 157.4, 153.3, 149.2, 145.1, 134.7, 123.4, 115.6, 104.5, 12.7.

4.2. 1-(2-Fluorophenyl)-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one (4)

A mixture of 1-(2-fluorophenyl)-3-(methylthio)-1H-pyrazole-4,5-dicarbonitrile 3 (2.5 g, 0.101 mmol) and formic acid (85%, 35 mL) was heated under reflux for 10 h. After completion of the reaction, monitored by TLC, the mixture was filtered to remove excess of 2-fluorophenylhydrazine and the filtrate was poured into ice cold water. The solid formed was filtered and purified by recrystallisation from formic acid. Isolated as ivory white solid: Yield: 76%; mp: 251–253 °C; Calculated Mass: 276.2, Observed LC-MS (M+1): 277.1; Method A: 0.1% Formic acid in water B: Acetonitrile, flow rate: 1.5 mL/min column: Atlantis d C18 (50 × 4.6) mm, 5 µm, +ve mode max chromatogram: RT (min) 3.14: Area. 99.53%; FT-IR (cm<sup>−1</sup>): 3432, 3326, 3224, 3047, 2919, 2857, 2205, 1652, 1594, 1555, 1520, 1450, 1356, 1291, 1019, 978, 924, 764, 699; <sup>1</sup>H NMR (400 MHz, DMSO-<sup>d6</sup>) δ 7.32–7.25 (m, 4H, Ar-H), 4.60 (s, 2H, NH<sub>2</sub>), 2.57 (s, 3H, SMe); 13CNMR (100 MHz, DMSO-<sup>d6</sup>) δ 157.4, 153.3, 149.2, 145.1, 134.7, 123.4, 115.6, 104.5, 12.7.

#Scheme 5. Thiopyridines derivatives 30–32.

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*Reagents and conditions: (a) 3-bromo-7-(5-(morpholinomethyl)thiophene-2-yl)thieno[3,2-c]pyrimi-4-amine 28 (1.0 equiv), 29a–29e Aryl or Heteroaryl boronic acid (1.5 equiv), S-Phos (0.05 equiv), Pd(OAc)<sub>2</sub>, (0.05 equiv), K<sub>2</sub>CO<sub>3</sub> (2.5 equiv), acetonitrile (5 mL), 90 °C in pressure tube 12 h; b Isolated yield.
column: Atlantis d C18 (50 × 4.6) mm, 5 µm, +ve mode max chromatogram: RT (min) 2.53: Area. 99.11%; 1HNMR (400MHz, DMSO-d6) δ 12.47 (s, 1H), 8.17 (s, 1H), 8.04 (dd, J = 9.1, 4.9 Hz, 2H), 7.40 (t, J = 8.8 Hz, 2H), 2.61 (s, 3H); 13CNMR (100MHz, DMSO-d6) δ 161.1, 159.0, 157.0, 153.3, 145.5, 145.2, 123.2, 115.6, 12.7 FT-IR (cm⁻¹): 3329, 3073, 2991, 2926, 2857, 2272, 1662, 1500, 1437, 1383, 1255, 1150, 1088, 864, 657.

**Table 2**
Screening of catalyst and reaction conditions for thienopyridine derivatives.

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*a* Reagents and conditions: (a) 28 (1.0 equiv), 4-methoxyphenyl boronic acid 29a (1.5 equiv), S-Phos (0.05 equiv), Pd(OAc)₂ (0.05 equiv), K₂CO₃ (2.5 equiv), acetonitrile (5 mL), 90 °C in pressure tube 12 h.

*b* Isolated yield.

**Table 3**
Docking scores and Assay results for compounds 5, 11 and 17.

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A suspension of the 1-(2-fluorophenyl)-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine (5)

4.3. 4-Chloro-1-(2-fluorophenyl)-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine (5)

Fig. 1. HMB correlation of 12 and various characteristic ¹H NMR and ¹³C NMR peaks.

Fig. 2. Inhibition of the basal ATPase activity against Eg5, Compounds 12, 25 and 27. Concentration–response plots for the inhibition of the basal Eg5 ATPase activities [IC₅₀ 12 = 15.39 ± 1.61 µM; IC₅₀ 25 = 16.42 ± 3.84 µM and IC₅₀ 27 = 9.98 ± 0.76 µM].

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4.4. 4-Chloro-1-(2-fluorophenyl)-4-hydrazinyl-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine (6)

A mixture of 4-chloro-1-(2-fluorophenyl)-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine 5 (1.8 g, 0.0061 mol) and hydrazine hydrate (99%, 3 mL, 0.0183 mmol) in absolute ethanol (40 mL) was heated under reflux for 3 h. The reaction was cooled, and the separated solid was filtered, dried and crystallized from methanol. Isolated as white solid; Yield: 92%; mp: 165–168 °C; Calculated Mass: 290.3, Observed LC-MS: M+1, 291; Method A: 0.1% Formic acid in water B: Acetonitrile, flow rate: 1.5 mL/min column: Atlantis d C18 (50 × 4.6) mm, 5 µm, +ve mode max chromatogram: RT (min) 2.03: Area. 96.54%; ¹H NMR (500 MHz, DMSO-dma) δ 11.20 (s, 1H, NH), 8.29 (s, 1H), 8.21–8.19 (m, 2H), 8.18–8.13 (m, 2H), 5.09 (s, 2H, NH₂), 2.50 (s, 3H, SMe); ¹³C NMR (100 MHz, DMSO-dma) δ 162.1, 161.0, 148.9, 133.7, 133.5, 126.9, 126.6, 116.2, 113.8, 39.7, 39.4, 39.1, 38.9, 14.4; FT-IR (cm⁻¹): 3328, 2925, 2855, 1501, 1437, 1383, 1255, 1088, 864, 739, 657.

4.5. 4-(2-(4-Chlorobenzylidene)hydrazinyl-1-(2-fluorophenyl)-3(methylthio)-1H-pyrazolo[3,4-d]pyrimidine (8)

A mixture of 4-chloro-1-(2-fluorophenyl)-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine 6 (0.025 g, 0.086 mmol) and the 4-chlorobenzaldehyde 7a (0.014 g, 0.1033 mmol) in methanol (4 mL) and glacial acetic acid (0.1 mL) was heated under reflux for 3 h. The mixture was subsequently diluted with CH₂Cl₂, and the organic layer was sequentially washed with saturated aqueous NaHCO₃ and finally dried over Na₂SO₄. The crude product was purified by flash column chromatography. (CH₂Cl₂/MeOH) yielded 8 (20 mg, 85%) as a white solid; Calculated Mass: 412.06, Observed LC-MS (M+1): 413.1; Method A: 0.1% Formic acid in water B: Acetonitrile, flow rate: 1.5 mL/min column: Atlantis d C18 (50 × 4.6) mm, 5 µm, +ve mode max chromatogram: RT (min) 3.15: Area. 98.40%; ¹H NMR (400 MHz, DMSO-d₆) δ 12.16 (s, 1H), 8.64 (d, J = 7.7 Hz, 1H), 8.57–8.50 (m, 1H), 8.47 (s, 1H), 8.26–8.15 (m, 1H), 8.02 (d, J = 8.6 Hz, 2H), 7.54 (d, J = 10.9 Hz, 8.19 (m, 1H))

Fig. 3. Ligplot image showing the interactions of the inhibitors with the enzyme KSP at site 1.

Fig. 4. Structural analysis of first allosteric binding pocket. (a) Eg5 in complex with Mg²⁺ ADP (orange) and compound12 (yellow) bound in the allosteric inhibitor-binding pocket formed by helices α2 and α3 and loops L7 as well as L9. (b) Close view of site-I with the compound 12 (magenta). (c) The magnification of the inhibitor-binding pocket showing residues involved in compound 12 binding.
4.6. 4-(2-(2-Chloro-4-fluorobenzylidene)hydrazinyl-1-(2-fluorophenyl)-3(methylthio)-1H-pyrazolo[3,4-d]pyrimidine (9)

A mixture of 6 (0.100 g, 0.344 mmol) and the 2-chloro-4-fluorobenzaldehyde (0.054 g, 0.344 mmol) in methanol (6 mL) and glacial acetic acid (0.4 mL) was heated under reflux for 3 h. The mixture was subsequently diluted with CH₂Cl₂ and the organic layer was sequentially washed with saturated aqueous NaHCO₃ and finally dried over Na₂SO₄. The crude product was purified by flash chromatography.

![Fig. 5. Structural analysis of second allosteric binding pocket.](image)

(a) Eg5 in complex with Mg2+ ADP (orange) and compound 12 (yellow) bound in the allosteric inhibitor-binding pocket formed by helices α4 and α6. (b) Close view of site-II with the compound 12 (magenta). (c) The magnification of the inhibitor-binding pocket showing residues involved in compound 12 binding.

![Fig. 6. Ligplot image showing the interactions of the inhibitors with the enzyme KSP at site 2.](image)

![Fig. 7. A non-linear regression graph representing the compound concentration Vs the percentage of cell viability.](image)

1H), 7.44–7.34 (m, 3H), 2.52 (s, 3H); 13CNMR (100MHz, DMSO-d₆) δ 152.4, 149.8, 148.4, 147.4, 144.5, 134.1, 131.7, 129.7, 123.2, 116.0, 101.3, 13.05; FT-IR (cm⁻¹): 3525, 3072, 2926, 2857, 1663, 1500, 1437, 1383, 1254, 1151, 1088, 864, 657.
A mixture of 6 (0.075 g, 0.258 mmol) and the 3-bromo-5-chloro-2-hydroxybenzaldehyde (0.060 g, 0.344 mmol) in methanol (6 mL) and glacial acetic acid (0.3 mL) was heated under reflux for 3 h. The mixture was subsequently diluted with CH₂Cl₂ and the organic layer was washed with saturated aqueous NaHCO₃ for several times and finally dried over Na₂SO₄. The crude product was purified by flash chromatography. CH₂Cl₂/MeOH yielded 7 (50 × 4.6) mm, 5 µm, +ve mode max chromatogram: RT (min) 5.47 Area. 87.90%; 1HNMR (500 MHz, DMSO-d₆) δ 11.98 (s, 1H, NH), 8.38 (s, 1H, ArCH₂), 8.22 (dd, J = 10.4, 6.5 Hz, 2H), 8.05 (dd, J = 8.8, 4.9 Hz, 1H, N = CH), 7.96–7.88 (m, 6H), 7.47 (d, J = 7.2 Hz, 2H), 7.39 (dt, J = 18.1, 8.0 Hz, 6H), 7.13 (d, J = 4.1 Hz, 2H), 5.16 (s, 2H, OCH₂), 3.87 (s, 3H, OMe), 2.60 (s, 3H, CH₃); 13C NMR (125 MHz, DMSO-d₆) δ 161.7, 161.2, 154.2, 150, 149, 146, 144, 137, 128, 128.36, 123, 116, 102, 70, 56, 13.5; FT-IR (cm⁻¹): 3330, 2929, 2857, 1661, 1500, 1437, 1384, 1254, 1150, 1088, 864, 657.

4.10. 4-(2-(4-Benzyloxy)-3-methoxybenzylidene)hydrazinyl-1-(2-fluorophenyl)-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine (13)

A mixture of 6 (0.075 g, 0.26 mmol) and 4-benzyloxy-3-methoxybenzaldehyde (0.062 g, 0.26 mmol) in methanol (6 mL) and glacial acetic acid (0.4 mL) was heated under reflux for 4.5 h. The mixture was subsequently diluted with CH₂Cl₂ and the organic layer was sequentially washed with saturated aqueous NaHCO₃ and finally dried over Na₂SO₄. The crude product was purified by flash chromatography. CH₂Cl₂/MeOH yielded 14 (65 mg, 86%) as a white solid; Calculated Mass: 514.5, Observed LC-MS (M+1): 515; Method A: 0.1% Formic acid in water B: Acetonitrile, flow rate: 1.5 mL/min column: Atlantis d C18 (50 × 4.6) mm, 5 µm, +ve mode max chromatogram: RT (min) 3.14 Area. 94.30%; 1H NMR (500 MHz, DMSO-d₆) δ 11.89 (s, 1H, NH), 8.38 (s, 1H, ArCH₂), 8.22 (dd, J = 10.4, 6.5 Hz, 2H), 8.05 (dd, J = 8.8, 4.9 Hz, 1H, N = CH), 7.96–7.88 (m, 6H), 7.47 (d, J = 7.2 Hz, 2H), 7.39 (dt, J = 18.1, 8.0 Hz, 6H), 7.13 (d, J = 4.1 Hz, 2H), 5.16 (2H, OCH₂), 3.87 (3H, OMe), 2.60 (3H, CH₃); 13C NMR (125 MHz, DMSO-d₆) δ 161.7, 161.2, 154.2, 150, 149, 146, 144, 137, 128, 128.36, 123, 116, 102, 70, 56, 13.5; FT-IR (cm⁻¹): 3330, 2929, 2857, 1661, 1500, 1437, 1384, 1254, 1150, 1088, 864, 657.

4.11. 4-(2-(2,5-Dichlorobenzylidene)hydrazinyl-1-(2-fluorophenyl)-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine (14)

A mixture of 6 (0.100 g, 0.258 mmol) and 2,5-dichlorobenzaldehyde (0.045 g, 0.26 mmol) in methanol (6 mL) and glacial acetic acid (0.4 mL) was heated under reflux for 4 h. The mixture was subsequently diluted with CH₂Cl₂ and the organic layer was sequentially washed with saturated aqueous NaHCO₃ and finally dried over Na₂SO₄. Purification by flash chromatography (CH₂Cl₂/MeOH) yielded 14 (60 mg, 82%) as a white solid; Calculated Mass: 446.3, Observed LC-MS (M+1): 447.7; Method A: 0.1% Formic acid in water B: Acetonitrile, flow rate: 1.5 mL/min column: Atlantis d C18 (50 × 4.6) mm, 5 µm, +ve mode max chromatogram: RT (min) 3.39 Area. 80.07%; 1H NMR (400 MHz, DMSO-d₆) δ 12.17 (s, 1H), 8.65 (d, J = 6.7 Hz, 5H), 8.58 (m, 1H), 8.48 (m, 1H), 8.27 (m, 2H), 8.04 (m, 3H), 7.55 (d, J = 6.6 Hz, 2H), 7.46 (m, 5H), 2.53 (s, 3H); 13C NMR (100 MHz, DMSO-d₆) δ 160.3, 153.7, 150.7–150.5, 148.5–148.3, 146.3, 144.5, 136.6–134.4, 130.0, 128.9, 128.0, 128.3, 123.2, 123.0, 102.5, 69.7, 15.3, 13.8; FT-IR (cm⁻¹): 3543, 3330, 3073, 2927, 2857, 1660, 1500, 1437, 1383, 1255, 1150, 1088, 1063, 864, 657.

4.12. 1-(2-(Fluorophenyl)-3-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine hydraydrazinyl)-1H-pyrazolo[3,4-d]pyrimidine (15)

A mixture of 6 (0.075 g, 0.258 mmol) and 4-fluorophenylbenzaldehyde (0.055 g, 0.26 mmol) in methanol (6 mL) and glacial acetic acid (0.4 mL) was heated under reflux for 3 h. The mixture was subsequently diluted with CH₂Cl₂ and the organic layer was sequentially washed with saturated aqueous NaHCO₃ and finally dried over Na₂SO₄. Purification by flash chromatography (CH₂Cl₂/MeOH) yielded 15 (66 mg, 90%) as a white solid; Calculated Mass: 484.1, Observed LC-MS (M+1): 485.2, Method A: 0.1% Formic acid in water B: Acetonitrile, flow rate: 1.5 mL/min column: Atlantis d C18 (50 × 4.6) mm, 5 µm, +ve mode max chromatogram: RT (min) 3.20 Area. 77.10%; 1H NMR (500 MHz, CDCl₃) δ 11.5 (s, 1H, NH), 8.55 (d, J = 16.2 Hz, 1H), 8.16 (s, 1H), 8.00 (s, 1H, N = CH), 7.83 (d, J = 8.3 Hz, 1H), 7.73 (s, 1H, J = 7.7 Hz, 1H), 7.37 (t, J = 8.1 Hz, 2H), 7.32 (d, J = 7.8 Hz, 1H), 6.96 (d, J = 7.7 Hz, 1H), 6.79 (t, J = 7.8 Hz, 1H), 3.77 (s, 3H, OCH₃), 2.44 (s, 3H, CH₃); 13C NMR (125 MHz, DMSO-d₆) δ 164.8, 161.7, 159.7, 154.3, 149.2, 148.3, 147.2, 146, 144, 135, 123, 126, 121, 119.3, 116.3, 114.1, 111.6, 114.2, 112.1, 143.7, 1384, 1254, 1151, 1088, 1063, 864, 657.
A mixture of 6 (0.075 g, 0.258 mmol) and 4-allyloxybenzaldehyde (0.049 g, 0.26 mmol) in methanol (6 mL) and glacial acetic acid (0.4 mL) was heated under reflux for 5 h. The mixture was subsequently diluted with CH2Cl2, and the organic layer was sequentially washed with saturated aqueous NaHCO3 and finally dried over Na2SO4. Purification by flash chromatography (CH2Cl2/MeOH) yielded 18 (65 mg, 85%) as a white solid; Calculated Mass: 456.1, Observed LC-MS (M+1): 457.0; Method A: 0.1% Formic acid in water B: Acetonitrile, flow rate: 1.5 mL/min column: Atlantis d C18 (50 × 4.6) mm, 5µm, +ve mode max chromatogram: RT (min) 3.22 Area: 90.794%; 1H NMR (400 MHz, CDCl3) δ 2.47 (s, 3H), 2.35 (s, 3H). 13C NMR (100MHz, DMSO‑d6) δ 163.49, 144.19, 142.20, 132.08, 129.26, 128.16, 125.78, 124.15, 119.91, 111.25, 107.72, 102.19, 78.99, 68.90, 57.65, 32.54. FT-IR (cm−1): 3550, 3329, 3073, 2928, 2858, 1661, 1503, 1438, 1384, 1254, 1151, 1088, 864, 657.
4.19. 4-[(2-(6-fluoro-5-methyl-1H-indol-3-yl)methylenedi)hydrazinyl]-1-(2-fluorophenyl)-3-(methyl thio)-1H-pyrazolo[3,4-d]pyrimidine (23)

A mixture of 6 (0.075 g, 0.258 mmol) and 5-fluoro-6-methyl-1H-indole-3-carbaldehyde (0.098 g, 0.26 mmol) in methanol (6 mL) and NaOEt (2.5 equiv) was heated under reflux for 3 h. The mixture was subsequently diluted with CH₂Cl₂, and the organic layer was sequentially washed with water and finally dried over Na₂SO₄. Purification by flash chromatography (CH₂Cl₂/MeOH) yielded 23 (72 mg, 88%) as a white solid.

4.20. 4-[(2-(2,4-dichlorobenzyl)-6-fluoro-5-methyl-1H-indol-3-yl)methylene]hydrazinyl-1-(2-fluorophenyl)-3(methylthio)-1H-pyrazolo[3,4-d]pyrimidine (25)

A mixture of 23 (0.075 g, 0.150 mmol) and 1-bromomethyl-2,4-dichlorobenzene 24 (1.0 equiv), KOTBu (1.5 equiv) was stirred at room temperature for 10 h. The mixture was concentrated under vacuum and diluted with CH₂Cl₂, and the organic layer was sequentially washed with water and finally dried over Na₂SO₄. Purification by flash chromatography (CH₂Cl₂/MeOH) yielded 25 (72 mg, 88%) as a white solid.

4.21. 4-[(4′-(2-(1H-fluoren-3-yl)-3-quinolin-2-yl)thieno[3,2-c]pyridin-4-amine (31)

The reaction of 3-bromo-7-(5-(methylformimidoyl)thiophen-2-yl)thieno[3,2-c]pyridin-4-amine 28 (0.05 g 0.122 mmol) with 4-pyridine boronic acid 29b (0.022 g 0.183 mmol), K₂CO₃ (0.033 g, 0.244 mmol), at 90°C during 12 h in acetonitrile (5 mL) in the presence S-Phos (0.008 g 0.006 mmol), palladium acetate (0.005 g, 0.006 mmol) in pressure tube vessel afforded the corresponding adduct. The reaction mixture was filtered with celite pad and washed with CH₂Cl₂. Then the reaction mixture was evaporated under vacuum. The crude solid was purified by column chromatography using silica gel 60-120 mesh to afford the desired product yielded 31 (26 mg 58%).

The reaction of 3-bromo-7-(5-(methylformimidoyl)thiophen-2-yl)thieno[3,2-c]pyridin-4-amine 28 (0.05 g 0.122 mmol) with 8-quinolinyl boronic acid 29e (0.031 g 0.183 mmol), K₂CO₃ (0.033 g, 0.244 mmol), at 90°C during 12 h in acetonitrile (5 mL) in the presence S-Phos (0.008 g 0.006 mmol), palladium acetate (0.005 g, 0.006 mmol) in pressure tube vessel afforded the corresponding adduct. The reaction mixture was filtered with celite pad and washed with CH₂Cl₂. Then the reaction mixture was evaporated under vacuum. The crude solid was purified by column chromatography using silica gel 60-120 mesh to afford the desired product yielded 32 (32 mg 74%).

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2018.12.014.

References


