Synthesis, biological evaluation and molecular modelling of 2,4-disubstituted-5-(6-alkylpyridin-2-yl)-1H-imidazoles as ALK5 inhibitors

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ABSTRACT
A series of 2,4-disubstituted-5-(6-alkylpyridin-2-yl)-1H-imidazoles, 7a–c, 11a–h, and 16a–h has been synthesised and evaluated for their ALK5 inhibitory activity in an enzyme assay and in a cell-based luciferase reporter assay. Incorporation of a quinoxalin-6-yl moiety and a methylene linker at the 4- and 2-position of the imidazole ring, respectively, and a m-CONH2 substituent in the phenyl ring generated a highly potent and selective ALK5 inhibitor 11e. Docking model of ALK5 in complex with 11e showed that it fitted well in the ATP-binding pocket with favourable interactions.

1. Introduction
Transforming growth factor-β (TGF-β) is one of the most potent immunosuppressive cytokines in the tumour microenvironment. Elevated serum levels of TGF-β commonly observed in patients with advanced colorectal cancer, breast cancer, bladder carcinoma, prostate cancer, malignant melanoma, pancreatic ductal adenocarcinoma, and hepatocellular carcinoma have been strongly associated with tumour progression and poor clinical outcome. Overexpression of TGF-β receptors has been implicated in cancer. In patients with advanced hepatocellular carcinoma treated with the first clinically available ALK5 inhibitor, galunisertib (1)13, an approximately two-fold longer overall survival was observed in patients having a TGF-β1 response compared to patients who did not have a TGF-β1 response. Therefore, TGF-β signalling pathway is an attractive target for development of cancer immunotherapeutic agents.

TGF-β signals through two distinct serine/threonine kinase receptors, the type I (activin receptor-like kinase 5 (ALK5)) and type II receptors. Small-molecule ALK5 inhibitors specifically inhibit the Smad pathway by competing with ATP at the hydrophobic ATP binding pocket of ALK5 kinase domain, which is essential for the phosphorylation of its substrates, Smad2/Smad3 proteins. Galunisertib progressed to phase 2/3 clinical trials against pancreatic carcinoma, glioblastoma, hepatocellular carcinoma, and myelodysplastic syndrome; however, Eli Lilly discontinued further clinical development of galunisertib in 2017. Recently, we developed an ALK5 inhibitor, vactosertib (2)15, and it has progressed to phase 1b/2a clinical trials either alone or in combination with pembrolizumab, durvalumab, or pomalidomide against myelodysplastic syndrome, non-small cell lung cancer, gastric cancer, colon cancer, multiple myeloma, etc.16. Another ALK5 inhibitor, LY3200882 (3) has entered a phase 1 clinical trial (Figure 1). Certain imidazo[2,1-b][1,3,4]thiadiazoles and 2,3,4-substituted 5,5-dimethyl-5,6-dihydro-4H-pyrrolo[1,2,3-b]pyrazoles have been reported to possess ALK5 inhibitory activity17,18.

Vactosertib exhibited subnanomolar ALK5 inhibitory activity in a kinase assay and in a cell-based luciferase reporter assay15, high selectivity against a panel of 320 protein kinases including p38β15, moderate oral bioavailability in rats15, and high efficacy in animal models of cancer19–21 and fibrosis22–26. In this report, we examined whether structural modification of vactosertib could increase its subnanomolar ALK5 inhibitory activity, thus further increasing its selectivity. For this purpose, we replaced a [1,2,4]triazolo[1,5-a]pyridin-6-yl moiety of vactosertib with either a benzimidazoles, a quinoxalin-6-yl moiety and inserted either a methylene, an ethylene, or a propylene linker instead of a methyleneamino linker to optimise the distance between a central imidazole ring and a phenyl ring.

2. Materials and methods

2.1. Chemistry
1H NMR spectra were recorded on a Varian Unity-Inova 400 MHz instrument. The chemical shifts are reported in parts per million (ppm). For 1H NMR spectra, CDCl3 was used as solvent, and it served as the internal standard at δ 7.26. Infra-red spectra were recorded on a FT-infra-red spectrometer (Bio-Rad). Electrospray ionisation mass spectra (ESIMS) were obtained on a Q-ToF mass spectrometer (Micromass). Elemental analyses (C, H, and N) were used to determine the purity of all tested compounds, and the
results were within ±0.4% of the calculated values (Carlo Erba 1106 elemental analyzer). Analytical thin-layer chromatography (TLC) was performed on Merck silica gel 60 F-254 glass plates. Medium-pressure liquid chromatography (MPLC) was performed using Merck silica gel 60 (230–400 mesh) with a YFLC-540 ceramic pump (Yamagen).

2.1. General procedure for the preparation of the 4-(benzo[1,3]-dioxol-5-yl)-5-(6-methylpyridin-2-yl)-1H-imidazoles 6a-c

To a stirred solution of 1-(benzo[1,3]dioxol-5-yl)-3-(oxopropyl)benzonitrile (5a), or 4-(4-oxobutyl)benzonitrile (5b), or 4-(4-oxoethyl)benzonitrile (5c) (0.17 mmol) in AcOH (3 mL) were added NH4OAc (1.11 mmol) and 4-(2-oxyethyl)benzonitrile (4) (0.19 mmol), and the mixture was heated at 120 °C for 3 h. The reaction mixture was cooled to 0 °C and neutralised with 1 N HCl to pH ~8. The mixture was extracted with CH2Cl2 (10 mL). The CH2Cl2 solution was washed with water (5 mL) and brine (5 mL), dried over anhydrous Na2SO4, filtered, and evaporated to dryness under reduced pressure. The residue was purified by MPLC on silica gel with CH2Cl2/MeOH as eluent to afford the titled compounds 6a–c as a solid.

2.1.1. 4-(4-(Benzo[d][1,3]dioxol-5-yl)-5-(6-methylpyridin-2-yl)-1H-imidazol-2-yl)methyl)benzonitrile (6a)

Yield 30%; mp 176 – 179 °C; 1H NMR (CDCl3) δ 11.40 (br s, 1 H, NH), 7.45 (d, 2 H, 2 phenyl), 7.44 (overlapped, 1 H, pyridyl), 7.33 (d, 1 H, pyridyl), 7.25 (d, 2 H, 2 phenyl), 7.10 (d, 1 H, pyridyl), 7.07 (s, 1 H, piperonyl), 6.93 (d, 1 H, piperonyl), 6.84 (d, 1 H, piperonyl), 6.00 (s, 2 H, OCH2O), 4.09 (s, 2 H, OCH2O), 2.34 (s, 3 H, CH3); IR (CHCl3) 3441, 1653 cm⁻¹; MS (EI) m/z 409.13 (MH⁺) 409.12 (MH⁺). Anal. Calcd for C26H24N4O3: C, 70.89; H, 5.49; N, 13.14. Found: C, 70.78; H, 4.95; N, 13.48.

2.1.2. 4-(4-(Benzo[d][1,3]dioxol-5-yl)-5-(6-methylpyridin-2-yl)-1H-imidazol-2-yl)ethyl)benzonitrile (6b)

Yield 49%; mp 189 – 192 °C; 1H NMR (CDCl3) δ 10.85 (br s, 1 H, NH) 7.66 (d, 2 H, 2 phenyl), 7.42 (t, 1 H, pyridyl), 7.30 (d, 1 H, pyridyl), 7.19 (d, 2 H, 2 phenyl), 7.07 (d, overlapped, 1 H, pyridyl), 7.05 (s, 1 H, piperonyl), 6.93 (d, 1 H, piperonyl), 6.82 (d, 1 H, piperonyl), 6.37 (br s, 1 H, CONH), 5.99 (s, 2 H, OCH2O), 5.69 (s, 2 H, OCH2O), 3.04 (m, 2 H, CH2), 2.45 (s, 3 H, CH3); IR (CHCl3) 3441, 1653 cm⁻¹; MS (EI) m/z 427.10 (MH⁺). Anal. Calcd for C25H22N4O3: C, 70.41; H, 5.20; N, 13.14. Found: C, 70.23; H, 5.28; N, 12.98.

2.1.3. 4-(3-(4-(Benzo[d][1,3]dioxol-5-yl)-5-(6-methylpyridin-2-yl)-1H-imidazol-2-yl)propyl)benzonitrile (6c)

Yield 36%; mp 60 – 62 °C; 1H NMR (CDCl3) δ 10.63 (br s, 1 H, NH), 7.51 (d, 2 H, 2 phenyl), 7.42 (dd, 1 H, pyridyl), 7.30 (d, 1 H, pyridyl), 7.24 (d, 2 H, 2 phenyl), 7.10 – 7.04 (m, 2 H, 1 pyridyl and 1 piperonyl), 6.93 (d, 1 H, piperonyl), 6.82 (d, 1 H, piperonyl), 5.98 (s, 2 H, OCH2O), 2.74 (m, 4 H, 2 CH2), 2.47 (s, 3 H, CH3), 2.07 (m, 2 H, CH2); IR (CHCl3) 2228, 1573 cm⁻¹; MS (EI) m/z 423.14 (MH⁺).

2.1.2.1. 4-((4-(Benzo[d][1,3]dioxol-5-yl)-5-(6-methylpyridin-2-yl)-1H-imidazol-2-yl)methyl)benzamide (7a)

A stirred solution of 6a–c (0.17 mmol), 6 N NaOH (0.04 mmol), and 28% H2O2 (0.59 mmol) in 95% EtOH (4 mL) was heated at 55 °C for 3 h. The reaction mixture was cooled to 0 °C and neutralised with 1 N HCl to pH ~8. The mixture was extracted with CH2Cl2 (30 mL), and the organic solution was washed with water (15 mL) and brine (15 mL), dried over anhydrous Na2SO4, filtered, and evaporated to dryness under reduced pressure. The residue was purified by MPLC on silica gel with CHCl3/MeOH or CH2Cl2/MeOH as eluent to afford the titled compounds 7a–c as a solid.

2.1.2.2. 4-(2-(4-(Benzo[d][1,3]dioxol-5-yl)-5-(6-methylpyridin-2-yl)-1H-imidazol-2-yl)ethyl)benzamide (7b)

Yield 49%; mp 189 – 192 °C; 1H NMR (CDCl3) δ 10.85 (br s, 1 H, NH) 7.66 (d, 2 H, 2 phenyl), 7.42 (t, 1 H, pyridyl), 7.30 (d, 1 H, pyridyl), 7.19 (d, 2 H, 2 phenyl), 7.07 (d, overlapped, 1 H, pyridyl), 7.05 (s, 1 H, piperonyl), 6.93 (d, 1 H, piperonyl), 6.82 (d, 1 H, piperonyl), 6.37 (br s, 1 H, CONH), 5.99 (s, 2 H, OCH2O), 5.69 (s, 2 H, OCH2O), 3.04 (m, 2 H, CH2), 2.45 (s, 3 H, CH3); IR (CHCl3) 3441, 1653 cm⁻¹; MS (EI) m/z 427.10 (MH⁺). Anal. Calcd for C25H22N4O3: C, 70.41; H, 5.20; N, 13.14. Found: C, 70.23; H, 5.28; N, 12.98.

2.1.2.3. 4-(3-(4-(Benzo[d][1,3]dioxol-5-yl)-5-(6-methylpyridin-2-yl)-1H-imidazol-2-yl)propyl)benzamide (7c)

Yield 52%; mp 178 – 180 °C; 1H NMR (CDCl3) δ 10.60 (br s, 1 H, NH) 7.68 (d, 2 H, 2 phenyl), 7.41 (d, 1 H, pyridyl), 7.30 (d, 1 H, pyridyl), 7.20 (d, 2 H, 2 phenyl), 7.10 – 7.05 (m, 2 H, 1 pyridyl and 1 piperonyl), 6.92 (d, 1 H, piperonyl), 6.82 (d, 1 H, piperonyl), 6.25 (br s, 1 H, CONH), 5.98 (s, 2 H, OCH2O), 5.75 (br s, 1 H, CONH), 2.73 (m, 4 H, 2 CH2), 2.49 (s, 3 H, CH3), 2.07 (m, 2 H, CH2); IR (CHCl3) 3183, 1660 cm⁻¹; MS (EI) m/z 441.12 (MH⁺). Anal. Calcd for C26H22N4O3: C, 70.89; H, 5.49; N, 12.72. Found: 70.63; H, 5.52; N, 12.66.
2.1.3. General procedure for the preparation of the 5-[(alkylpyridin-2-yl)-1-hydroxy-4-(quinoxalin-6-yl)imidazol-2-yl]methyl]benzonitriles (10a–h)

To a stirred solution of 9a–d (0.23 mmol) in rBuOMe (2.5 mL) were added either 5a or 3-(2-oxoethyl)benzonitrile (5d) (0.69 mmol) and NH4OAc (1.15 mmol) dissolved in MeOH (1.2 mL), and the mixture was stirred at room temperature overnight under argon atmosphere. The pH of the reaction mixture was adjusted to pH 8–8 at 0°C with saturated NaHCO3 solution. The reaction mixture was partitioned between CH2Cl2 (40 mL) and water (40 mL). The aqueous layer was extracted with CH2Cl2 (15 mL × 3). The combined organic solution was dried over anhydrous Na2SO4, filtered, and evaporated to dryness under reduced pressure. The residue was purified by MPLC on silica gel with CH2Cl2/MeOH as eluent to afford the titled compounds 10a–h as a solid.

2.1.3.1. 4-[(1-Hydroxy-5-(6-(methyl)pyridin-2-yl)-4-(quinoxalin-6-yl)-1H-imidazol-2-yl)methyl]benzonitrile (10a).

Yield 40%; mp 200–203°C; 1H NMR (CDCl3) δ 8.86 (m, 2 H, 2 quinoxalinyl), 8.35 (d, 1 H, quinoxalinyl), 8.17 (d, 1 H, quinoxalinyl), 8.06 (dd, 1 H, quinoxalinyl), 7.62 (m, 2 H, 2 phenyl), 7.56 (m, 2 H, 2 phenyl), 7.52 (t, 1 H, pyridyl), 7.36 (d, 1 H, pyridyl), 7.06 (d, 1 H, pyridyl), 4.31 (s, 2 H, CH2), 2.61 (s, 3 H, CH3); IR (CHCl3) 3052, 2231, 1575 cm⁻¹; MS (EIS) m/z 419.23 (MH⁺). Anal. Calcd for C24H17N4O3: C 77.56, H 4.93, N 13.13; found: C 77.65, H 4.81, N 13.02.

2.1.3.2. 4-((5-(6-Ethylpyridin-2-yl)-1-hydroxy-4-(quinoxalin-6-yl)-1H-imidazol-2-yl)methyl]benzonitrile (10b).

Yield 37%; mp 84–85°C; 1H NMR (CDCl3) δ 8.86 (m, 2 H, 2 quinoxalinyl), 8.36 (d, 1 H, quinoxalinyl), 8.17 (d, 1 H, quinoxalinyl), 8.07 (dd, 1 H, quinoxalinyl), 7.62 (m, 2 H, 2 phenyl), 7.57 (m, 2 H, 2 phenyl), 7.54 (t, 1 H, pyridyl), 7.37 (d, 1 H, pyridyl), 7.08 (d, 1 H, pyridyl), 4.32 (s, 2 H, CH2), 2.90 (q, 2 H, CH2), 1.38 (t, 3 H, CH3); IR (CHCl3) 2229, 1598, 1571 cm⁻¹; MS (EIS) m/z 447.22 (MH⁺). Anal. Calcd for C25H21N4O3: C 77.70, H 5.64, N 12.66; found: C 77.58, H 5.48, N 12.52.

2.1.3.3. 4-((1-Hydroxy-5-(6-(isopropyl)pyridin-2-yl)-4-(quinoxalin-6-yl)-1H-imidazol-2-yl)methyl]benzonitrile (10c).

Yield 36%; mp 164–166°C; 1H NMR (CDCl3) δ 8.85 (m, 2 H, 2 quinoxalinyl), 8.35 (d, 1 H, quinoxalinyl), 8.17 (d, 1 H, quinoxalinyl), 8.07 (dd, 1 H, quinoxalinyl), 7.75 (s, 1 H, phenyl), 7.70 (m, 1 H, phenyl), 7.53 (m, 2 H, 2 phenyl), 7.42 (t, 1 H, pyridyl), 7.37 (d, 1 H, pyridyl), 7.05 (d, 1 H, pyridyl), 4.29 (s, 2 H, CH2), 2.85 (t, 2 H, CH2), 1.76 (m, 2 H, CH2), 1.43 (m, 2 H, CH2), 0.98 (t, 3 H, CH3); IR (CHCl3) 2957, 1665, 1572 cm⁻¹; MS (EIS) m/z 461.27 (MH⁺). Anal. Calcd for C26H25N4O3: C 78.69, H 6.77, N 12.54; found: C 78.77, H 6.73, N 12.48.

2.1.4. General procedure for the preparation of the 5-[(alkylpyridin-2-yl)-1-hydroxy-4-(quinoxalin-6-yl)-1H-imidazol-2-yl]methyl]benzonitriles (11a–h)

To a stirred solution of 10a–h (0.63 mmol) in a mixture of EtOH (16 mL) and DMSO (4 mL) at room temperature were added 28% H2O2 (6.62 mmol) and 6 N NaOH (0.47 mmol). The mixture was warmed to 55°C for 3 days, cooled to room temperature, and the reaction mixture was partitioned between CH2Cl2 (30 mL) and H2O (50 mL). The aqueous layer was saturated with NaCl and extracted with CH2Cl2 (30 mL × 3). The combined organic solution was washed with brine (30 mL), dried over anhydrous Na2SO4, filtered, and evaporated to dryness under reduced pressure. The residue was dissolved in anhydrous DMF (20 mL) and treated with triethyl phosphite (2.39 mmol). The mixture was heated at 110°C for 3 days, cooled to room temperature, and then evaporated to dryness under reduced pressure. The reaction mixture was partitioned between CH2Cl2 (30 mL) and water (50 mL), and the aqueous layer was extracted with CH2Cl2 (30 mL × 2). The combined organic solution was washed with saturated NaHCO3 solution (40 mL) and brine (50 mL), dried over anhydrous Na2SO4, filtered, and evaporated to dryness under reduced pressure. The residue was purified by MPLC on silica gel with CH2Cl2/MeOH as eluent to afford the titled compounds 11a–h as a solid.

2.1.4.1. 4-((5-(6-Ethylpyridin-2-yl)-1-hydroxy-4-(quinoxalin-6-yl)-1H-imidazol-2-yl)methyl]benzamide (11a).

Yield 36%; mp 227–229°C; 1H NMR (CDCl3) δ 12.01 (br s, 1 H, NH), 8.83 (m, 2 H, 2 quinoxalinyl), 8.38 (s, 1 H, quinoxalinyl), 8.15 (dd, 2 H, 2 quinoxalinyl), 7.55 (d, 2 H, 2 phenyl), 7.42 (dd, 1 H, pyridyl), 7.33 (d, 1 H, pyridyl), 7.21 (d, 2 H, 2 phenyl), 6.95 (d, 1 H, pyridyl), 6.62 (br s, 1 H, CONH), 5.83 (br s, 1 H, CONH), 4.13 (s, 2 H, CH2), 2.29 (s, 3 H, CH3); IR (CHCl3) 3185, 1665, 1616, 1572 cm⁻¹; MS (EIS) m/z 421.14 (MH⁺). Anal. Calcd for
2.1.4.2. 4-((5-(6-Ethylpyridin-2-yl)-4-(quinoxalin-6-yl)-1H-imidazo-
2-yl)methyl)benzamide (11c). Yield 42%; mp 150–152 °C. 1H NMR
(CDCl3) δ 10.33 (br s, 1 H, CONH), 8.84 (s, 2 H, quinoxalinyl), 8.40 (s, 1 H, quinoxalinyl), 8.16 (m, 2 H, 2 quinoxalinyl), 7.61 (d, 2 H, phenyl), 7.43 (t, 1 H, pyridyl), 7.33 (d, 1 H, pyridyl), 7.28 (d, 2 H, phenyl), 6.97 (d, 1 H, pyridyl), 6.35 (br s, 1 H, CONH), 5.68 (br s, 1 H, CONH), 4.28 (s, 2 H, CH2), 3.01 (heptet, 1 H, CH), 1.27 (s, 3 H, CH3), 1.25 (s, 3 H, CH3); IR (CHCl3) 3184, 1666, 1615, 1571 cm⁻¹; MS (EIS) m/z 449.23 (M⁺). Anal. Calcd for C28H26N6O: C, 72.71; H, 5.67; N, 18.17. Found: C, 72.89; H, 5.51; N, 18.03.

2.1.4.5. 3-((5-(6-Methylpyridin-2-yl)-4-(quinoxalin-6-yl)-1H-imidazo-
2-yl)methyl)benzamide (11e). Yield 23%; mp 181 – 182 °C. 1H NMR
(CDCl3) δ 8.79 (s, 2 H, quinoxalinyl), 8.34 (s, 1 H, quinoxalinyl), 8.06 (s, 2 H, quinoxalinyl), 7.71 (s, 1 H, phenyl), 7.55 (d, 1 H, phenyl), 7.41 (t, 1 H, pyridyl), 7.34 (d, 1 H, pyridyl), 7.29 (d, 1 H, phenyl), 7.19 (t, 1 H, phenyl), 6.96 (d, 1 H, pyridyl), 6.83 (br s, 1 H, CONH), 6.30 (br s, 1 H, CONH), 4.19 (s, 2 H, CH2), 2.64 (t, 2 H, CH2), 1.45 (m, 2 H, CH2), 1.24 (m, 2 H, CH2), 0.81 (t, 3 H, CH3); IR (CHCl3) 3196, 1674, 1654 cm⁻¹; MS (EIS) m/z 421.23 (M⁺). Anal. Calcd for C27H24N6O: C, 72.71; H, 4.79; N, 19.99. Found: C, 72.28; H, 5.56; N, 18.02.

2.1.6. General procedure for the preparation of the 5-(6-alkylypyridi-
2-yl)-4-(quinoxalin-6-yl)-1H-imidazoles 16a–h
To a stirred solution of 15a–d (3.79 mmol) in a mixture of t-
BuOMe (35 mL) and MeOH (25 mL) were added either 14a or 14b
(5.69 mmol) and NH4OAc (18.95 mmol), and the mixture was
stirred at 30 °C overnight under argon atmosphere. The pH of the
reaction mixture was adjusted to pH – 8 at 0 °C with saturated
NaHCO3 solution. After removal of solvent, the reaction mixture
was extracted with CH2Cl2 (25 mL × 3), and the organic solution
was dried over anhydrous Na2SO4 filtered and evaporated to dry-
ness under reduced pressure. The residue was purified by MPLC
on silica gel with CH2Cl2/MeOH as eluent to afford the titled com-
ounds 16a–h as a solid.

2.1.7. 3-((5-Isopropylpyridin-2-yl)-4-(quinoxalin-6-yl)-1H-imida-
zol-2-yl)methyl)benzamide (11g). Yield 29%; mp 114–115 °C. 1H NMR
(CDCl3) δ 8.81 (m, 2 H, 2 quinoxalinyl), 8.37 (s, 1 H, quinoxalinyl), 8.11 (m, 2 H, 2 quinoxalinyl), 7.71 (s, 1 H, phenyl), 7.65 (m, 1 H, phenyl), 7.50 (d, 1 H, phenyl), 7.46 (t, 1 H, pyridyl), 7.36 (t, 1 H, phenyl), 7.35 (overlapped, 1 H, pyridyl), 7.02 (dd, 1 H, pyridyl), 6.37 (br s, 1 H, CONH), 5.70 (br s, 1 H, CONH), 4.22 (s, 2 H, CH2), 2.99 (heptet, 1 H, CH), 1.24 (s, 3 H, CH3), 1.22 (s, 3 H, CH3); IR (CHCl3) 3184, 1665, 1574 cm⁻¹; MS (EIS) m/z 449.25 (M⁺). Anal. Calcd for C27H25N6O: C, 72.30; H, 5.59; N, 18.74. Found: C, 72.03; H, 5.52; N, 18.67.

2.1.8. 3-((5-6-Butylpyridin-2-yl)-4-(quinoxalin-6-yl)-1H-imida-
zol-2-yl)methyl)benzamide (11h). Yield 27%; mp 110–111 °C. 1H NMR
(CDCl3) δ 8.83 (m, 2 H, 2 quinoxalinyl), 8.41 (s, 1 H, quinoxalinyl), 8.13 (s, 2 H, 2 quinoxalinyl), 7.81 (s, 1 H, phenyl), 7.70 (d, 1 H, phenyl), 7.55 (d, 1 H, phenyl), 7.42 (m, 2 H, phenyl and pyridyl), 7.32 (br d, 1 H, pyridyl), 6.98 (d, 1 H, pyridyl), 6.20 (br s, 1 H, CONH), 5.60 (br s, 1 H, CONH), 4.27 (s, 2 H, CH2), 2.77 (t, 2 H, 1.63 (m, 2 H, CH2), 1.38 (m, 2 H, CH3), 0.93 (t, 3 H, CH3); IR (CHCl3) 3183, 1666, 1576 cm⁻¹; MS (EIS) m/z 463.25 (M⁺). Anal. Calcd for C28H26N6O: C, 72.71; H, 5.67; N, 18.17. Found: C, 72.89; H, 5.51; N, 18.03.
2.1.6.1. 4-(2-(5-(6-Methylpyridin-2-yl)-4-(quinoxalin-6-yl)-1H-imidazo-2-yl)ethyl)benzamide (16a). Yield 66%; mp 145–147 ºC. δ 8.82 (s, 2 H, 2 quinoxalinyl), 8.36 (s, 1 H, quinoxalinyl), 8.09 (d, 2 H, 2 quinoxalinyl), 7.64 (d, 2 H, 2 phenyl), 7.43 (t, 1 H, pyridyl), 7.32 (d, 1 H, pyridyl), 7.17 (d, 2 H, 2 phenyl), 6.99 (d, 1 H, pyridyl), 6.58 (br s, 1 H, CONH), 6.09 (br s, 1 H, CONH), 3.06 (s, 4 H, 2 CH2), 2.45 (s, 3 H, CH3); IR (CHCl3) 3446, 1652, 1626 cm⁻¹; MS (EIS) m/z 449.26 (MH⁺). Anal. Calcd for C27H24N6O: C, 72.30; H, 5.39; N, 18.74. Found: C, 72.44; H, 5.25; N, 18.58.

2.1.6.2. 4-(2-(5-(6-Ethylpyridin-2-yl)-4-(quinoxalin-6-yl)-1H-imidazo-2-yl)ethyl)benzamide (16b). Yield 63%; mp 123–125 ºC. δ 8.82 (s, 2 H, 2 quinoxalinyl), 8.37 (s, 1 H, quinoxalinyl), 8.10 (m, 2 H, 2 quinoxalinyl), 7.69 (t, 1 H, phenyl), 7.62 (m, 1 H, phenyl), 7.46 (t, 1 H, pyridyl), 7.34 (t, 2 H, phenyl and pyridyl), 7.31 (t, 1 H, phenyl), 7.02 (dd, 1 H, pyridyl), 6.50 (br s, 1 H, CONH), 6.00 (br s, 1 H, CONH), 3.13 (s, 4 H, 2 CH2), 3.00 (heptet, 1 H, CH), 1.25 (s, 3 H, CH3); IR (CHCl3) 3418, 1666, 1570 cm⁻¹; MS (EIS) m/z 463.26 (MH⁺). Anal. Calcd for C28H26N6O: C, 72.71; H, 5.67; N, 18.17. Found: C, 72.88; H, 6.15; N, 17.55.

2.2. Luciferase reporter assay

To establish HaCaT (3TP-luc) stable cells, cells were seeded on six-well plates. Cells were allowed to adhere overnight and then transfected with the p3TP-luc (neo) expression plasmid using PEI reagent (Sigma Aldrich). Transfected cells were cultured for four weeks in the presence of G418 (500 μg/mL). Several single clones were isolated and measured luciferase activity. The clone showing the highest activity was selected and used for further experiments.

2.3. Cell permeability assay

Caco-2 cells were seeded in Transwell® polycarbonate filter at a density of 8 × 10⁴ cells/filter and cultured for 21 days. Culture medium was removed from both apical (AP) and basolateral (BL) chambers of transwell, and the wells were rinsed three times with PBS. AP buffer (HBSS, pH 6.5) containing 10 mM MES and BL buffer (HBSS, pH 7.4) containing 10 mM HEPEs were loaded in AP (500 μL) and BL (1500 μL) chambers, respectively, followed by incubation for 30 min at 37 ºC. Then, test compound (100 μM) was added to the AP side and incubated for 2 h at 37 ºC. After the incubation, BL buffer was collected and analysed using an UV spectrophotometer at a maximum wavelength (wavelength 225–357 nm).
2.4. Docking study

All computational works were performed on the Sybyl-X 2.1.1 (Tripos Inc., St Louis, MO, USA) molecular modelling package with CentOS Linux 5.4. operating system127.

2.4.1. Preparation of ligands and receptor

The 11e was prepared with sketch module embedded in Sybyl package and saved as mol2 format. After sketching the molecule, Gasteiger-Hückel charges were assigned to all atoms. To optimise the ligand, energy minisation was conducted by the standard tripus force field with convergence to maximum derivatives of 0.001 kcal mol$^{-1}$Å$^{-1}$. The X-ray structure of ALKS complexed with 5,6-dihydro-4H-pyrrrolo[1,2-b]pyrazole inhibitor was used as a receptor for docking (PDB id: 1RWB$^{38}$). Receptor structure was retrieved from PDB (http://www.rcsb.org/) and optimised using structure preparation tool embedded in biopolymer module. Native ligand was extracted and all water molecules except key water molecule for water-mediated hydrogen bond network were deleted from the complex structure.

2.4.2. Molecular docking

To examine the binding poses of 11e, docking study was conducted using Surflex-Dock3 embedded in Tripos Sybyl X 2.1.1 software package. For docking performance, the active site was assigned as a protomol generated by using the native ligand in the X-ray structure. Flexible docking was carried out by using default parameter values (threshold = 0.5 and bloat = 0), producing 200 conformers as maximum number of poses per ligand. Binding affinity of each docking pose of ligand was calculated by Surflex-dock score and consensus scoring function (CScore). The total Surflex-Dock score was expressed as −logKd to represent binding affinities. To build the best docking model, key interactions between candidate compound and active site were investigated by visual inspection.

3. Results and discussion

3.1. Chemistry

To develop a potent, selective, and orally bioavailable ALKS inhibitor, as our starting point, we designed three target molecules, 4-(3-oxopropyl)benzonitrile (7b) and its ethyl and n-propyl analogues 7b and 7c to optimise the distance between the two pharmacophores, a central imidazole ring and a phenyl ring.

The 7a–c were prepared as shown in Scheme 1. The 1-(benzo[1,3]dioxol-5-yl)-2-(6-methylpyridin-2-yl)-1H-imidazol-2-yl)methyl)benzamide (7a) and its ethyl and n-propyl analogues, 7b and 7c to optimise the distance between the two pharmacophores, a central imidazole ring and a phenyl ring.

Scheme 1. Reagents and conditions: (a) NH$_4$OAc, AcOH, 120 °C, 3 h; (b) 28% H$_2$O$_2$, 6 N NaOH, EtOH, 55 °C, 3 h.

The 11a–h were prepared as shown in Scheme 2. The 2-(6-alkylpyrpyridin-2-yl)-1-(quinoxalin-6-yl)ethanones 8a–d$^{33}$ were treated with NaNO$_2$ in 5 N HCl to give the 2-(6-alkylpyrpyridin-2-yl)-2-(hydroxyimino)-1-(quinoxalin-6-yl)ethanones 9a–d in 88–99% yields. Condensation of 9a–d with either 5a or 3-(2-oxoethyl)benzonitrile (5d)$^{34}$ and NH$_4$OAc in a mixture of t-BuOMe and MeOH at room temperature afforded the 5-(6-alkylpyrpyridin-2-yl)-1-hydroxy-4-(quinoxalin-6-yl)ethanones 10a–h in 37–77% yields. Conversion of the carbonyl group of 10a–h to the carboxamide group and subsequent dehydroxylation with triethyl phosphite in anhydrous DMF at 110 °C for 3 days gave the target compounds 11a–h in 23–44% yields.

The requisite aldehydes, 4-(3-oxopropyl)benzamide (14a) and 3-(3-oxopropyl)benzamide (14b) for the synthesis of the target compounds 16a–h were prepared as shown in Scheme 3.

Selective hydrolysis of the carbonyl group of 12a$^{31}$ and 12b$^{35}$ to the carboxamide group followed by acidic hydrolysis of 1,3-dioxolanyl protecting group of 13a and 13b afforded 14a and 14b in almost quantitative yield. The 2-(6-alkylpyrpyridin-2-yl)-1-(quinoxalin-6-yl)ethanes 15a–d$^{35}$ was condensed with either 14a or 14b and NH$_4$OAc in a mixture of t-BuOMe and MeOH to obtain the 4-carboxamide analogues 16a–d in modest yields (57–71%) and the 3-carboxamide analogues 16e–h in lower yields (16–20%) (Scheme 4).

3.2. ALKS inhibitory activity in an enzyme assay and in a cell-based luciferase reporter assay

To evaluate whether these potential inhibitors 7a–c, 11a–h, and 16a–h could inhibit ALKS, a kinase assay was performed using the purified human ALKS kinase domain produced in Sf9 insect cells and casein as a substrate (Table 1). The ALKS inhibitory activity of 7b (IC$_{50} =$ 0.093 μM) was 2.4-fold and 3.6-fold higher than those of 7a (IC$_{50} =$ 0.224 μM) and 7c (IC$_{50} =$ 0.338 μM), respectively, in a kinase assay (Table 1). In a cell-based luciferase reporter assay
using HaCaT (3TP-luc) stable cells, 7c (40% inhibition) was much less inhibitory than 7a (72% inhibition) and 7b (74% inhibition) at a concentration of 0.1 μM.

Replacement of a benzo[1,3]dioxol-5-yl moiety in 7a and 7b with a quinoxalin-6-yl moiety increased ALK5 inhibitory activity, thus, the corresponding analogues, 11a (IC50 = 0.088 μM) and 16a (IC50 = 0.030 μM), were 2.5-fold and 3.1-fold more potent than 7a and 7b, respectively, in a kinase assay (Table 1). Similar to kinase assay, 11a (84% inhibition) and 16a (85% inhibition) were much more inhibiting than 7a and 7b in a luciferase reporter assay. It was previously demonstrated that a Me substituent at the six-position of the pyridine ring in the pyridyl-substituted ALK5 inhibitors significantly increased ALK5 inhibitory activity. Therefore, we examined the effect of bulkier alkyl groups such as Et, i-Pr, and n-Bu in ALK5 inhibition. The 6-ethylpyridyl analogues 11b, 11f, 16b, and 16f were 1.3-, 2.1-, 1.4-, and 1.4-fold less potent than the
Corresponding 6-methylpyridyl analogues 11a, 11e, 16a, and 16e, respectively. However, the 6-i-proplyphridyl analogues (11c, 11g, 16c, and 16g) and 6-n-butylpyridyl analogues (11d, 11h, 16d, and 16h) displayed 13–35-fold and 29–71-fold lower inhibitory activity compared to the respective 6-methylpyridyl analogues, indicating that a bulkier group than Et group cannot be accommodated favourably into the ATP binding pocket of ALKS. Regarding the length of a linker, an ethylene linker was generally more beneficial than a methylene linker in the ALKS inhibition as shown in 7a and 7b, thus, 16a, 16b, 16c, and 16g were 2.9-, 2.7-, 3.4-, and 2.1-fold more inhibiting than the respective 11a, 11b, 11c, and 11g. Compounds 16d-f showed the similar level of potency to that of 11d-f, respectively. The position of a carboxamide group in the phenyl ring also influenced ALKS inhibition. The m-CONH2 analogues 11e-h were 1.5–2.6-fold more inhibiting than the respective p-CONH2 analogues 11a-d in a series of compounds having a methylene linker, whereas the p-CONH2 analogues having an ethylene linker, 16a and 16b, were 1.6-fold more inhibiting than the corresponding m-CONH2 analogues 16e and 16f.

### Table 1. ALKS inhibitory activity and Caco-2 cell permeability of 2,4-disubstituted-5-(6-alkylpyridin-2-yl)-1H-imidazoles 7a-c, 11a-h, and 16a-h.

| Compound | R | CONH₂ | n | IC₅₀ IC₅₀ IC₅₀ IC₅₀ |
|----------|---|-------|---|-------------------|
| Mock     |   |       |   | 2 ± 0*             |
| TGF-β    | 7a|       | 1 | 0.224 100 ± 5.0    |
| 7b       | 2 |       | 0.093 26 ± 7.9 23 ± 3.0 |
| 7c       | 3 |       | 0.338 60 ± 9.5 32 ± 1.1 |
| 11a Me   | p-CONH₂ | 1 | 0.088 16 ± 4.5 25 ± 1.3 |
| 11b Et   | p-CONH₂ | 1 | 0.112 33 ± 2.3 26 ± 3.5 |
| 11c m-Pr | p-CONH₂ | 1 | 2.195 39 ± 11.4 30 ± 9.0 |
| 11d n-Bu | p-CONH₂ | 2 | 2.700 85 ± 32.5 41 ± 5.3 |
| 11e Et   | m-CONH₂ | 1 | 0.036 8 ± 5.8 56 ± 26.8 |
| 11f m-Bu | p-CONH₂ | 1 | 0.075 17 ± 3.7 38 ± 13.8 |
| 11g m-Pr | m-CONH₂ | 1 | 1.250 69 ± 6.7 34 ± 15.6 |
| 11h n-Bu | m-CONH₂ | 1 | 1.038 83 ± 21.1 n.d. |
| 16a Me   | p-CONH₂ | 2 | 0.030 15 ± 8.2 45 ± 5.3 |
| 16b Et   | p-CONH₂ | 2 | 0.041 16 ± 8.4 25 ± 4.2 |
| 16c m-Pr | p-CONH₂ | 2 | 0.653 85 ± 38.9 23 ± 3.1 |
| 16d n-Bu | p-CONH₂ | 2 | 2.383 76 ± 5.7 24 ± 1.9 |
| 16e Me   | p-CONH₂ | 2 | 0.047 7 ± 0.7 21 ± 9.5 |
| 16f Et   | m-CONH₂ | 2 | 0.066 56 ± 14.6 6 ± 4.1 |
| 16g m-Pr | p-CONH₂ | 2 | 0.593 48 ± 14.8 0 |
| 16h n-Bu | m-CONH₂ | 2 | 2.875 92 ± 10.4 0 |

*ALKS was expressed in Sf9 insect cells as human recombinant GST-fusion protein by means of the vacuolar expression system. A Proprietary radioisotopic protein kinase assay (13P) Activity Assay was performed at ProQinase GmbH (Freiburg, Germany). The assay contained 70 mM HEPES-NaOH, pH 7.5, 3 mM MgCl₂, 3 mM MnCl₂, 3 mM Na-orthovanadate, 1.2 mM DTT, 50 µg/ml PEG20000, 1 µM [γ-3P]-ATP (approximately 5 × 10¹⁵ cpm per well). One hundred ng/50 µL of ALKS and 1000 ng/50 µL of GSK3 (7–27) as a substrate were used per well. 

### Table 2. ALKS and p38α inhibitory activity of 11e, 1, and 2.

| Compound | IC₅₀ IC₅₀ IC₅₀ IC₅₀ |
|----------|------------------|
| 11e      | 0.013 0.288 22 0.0196 |
| Galunisertib (1) | 0.086 0.320 4 >0.1 |
| Vactosertib (2) | 0.013 1.775 137 0.0165 |

*IC₅₀ for ALKS (µM) b IC₅₀ for p38α IC₅₀ IC₅₀ |

### 3.3. Caco-2 cell permeability assay

To estimate oral absorption of target compounds, their permeability in Caco-2 cells was selected, and its ALK5 inhibitory activity was compared with those of potential competitors, galunisertib and vactosertib in a kinase assay and in a luciferase reporter assay. In a kinase assay, 11e (IC₅₀ = 0.036 µM), 16a, 16b (IC₅₀ = 0.041 µM), and 16e (IC₅₀ = 0.047 µM) exhibited much higher inhibitory activity in a luciferase reporter assay, also (11e: 92%, 16a: 85%, 16b: 84%, 16e: 93%). Among this series of compounds, 11e possessing the most potent ALKS inhibitory activity and the highest permeability in Caco-2 cells was selected, and its ALK5 inhibitory activity was compared with those of potential competitors, galunisertib and vactosertib. In a kinase assay, 11e (IC₅₀ = 0.013 µM) showed the same level of potency to that of vactosertib (IC₅₀ < 0.03 µM), and 6.6-fold higher potency compared to that of galunisertib (IC₅₀ = 0.086 µM) (Table 2). Luciferase activity of HaCaT (3TP-luc) cells was increased by 65-fold after treatment of TGF-β (2 ng/mL), and 11e and vactosertib displayed the similar level of inhibition on the TGF-β-induced luciferase reporter activity with IC₅₀ values of 0.0196 µM and 0.0165 µM, respectively. Similar to a kinase assay, galunisertib displayed much lower inhibition (IC₅₀ > 0.1 µM) compared to 11e and vactosertib (Table 2).

### 3.4. Kinase profiling assay

Considering the ALK5 inhibitory activity in both a kinase assay and a luciferase reporter assay and the Caco-2 cell permeability, 11e was selected as a candidate for preliminary kinase profiling.
Because ALK5 is a serine/threonine kinase, we chose 19 serine/threonine kinases including ALK5 and an ALK family kinase, ACVR1 (ALK5, ACV-R1, Aurora-A, ARKS, B-Raf, CK2α1, COT, DAPK1, IRAK4, JNK3, MAPKAPK5, MST4, NEK2, NLK, PAK1, PIM1, PRK1, S6K, SGK1) and 9 tyrosine kinases (ABL1, CSK, FGF-R1, FLT3, IGF1-R, PDGFR-α, SRC, VEGF-R1, ZAP70). Selectivity profiling of 11e using a panel of 28 protein kinases showed selectivity indices of >100 against all the kinases tested (ProQinase GmbH (Freiburg, Germany)) (Figure 2).

3.5. Docking model of ALK5 in complex with 11e

To determine the binding pose of 11e in the active site of ALK5, we executed docking modelling with flexible molecular docking programme Surflex-dock. We analysed the result of docking considering Surflex-dock docking score (−logKd) and consensus score (obtained from CScore module), and selected the poses of 11e with high scores (−logKd ≥7 and CScore ≥3). To select the best docked pose among them, we also identified key interactions between amino acid residues in the active site and 11e, in comparison with the X-ray pose of native ligand (3-(4-fluorophenyl)-2-(6-methylpyridin-2-yl)-5,6-dihydro-4H-pyrrolo[1,2-b]pyrazole). As shown in Figure 3(A,B), 11e fits well into cavity of active site, with quinoxaline ring and methyl group of 6-methylpyridine ring occupying the hydrophobic pockets. Hydrogen bond interactions between 11e and ALK5 are exhibited in Figure 3(C). The quinoxaline ring nitrogen acts as a hydrogen bond acceptor and interacts with NH of His283 in the backbone of hinge region of ALK5. The 6-methylpyridine ring nitrogen forms a water-mediated hydrogen bond network with the backbone NH of Asp351, and the side chain of both Tyr249 and Glu245. The imidazole ring NH of 11e is also involved in the water-mediated hydrogen bond network. The NH2 group of carboxamide in the meta position of phenyl ring interacts with the side chain of Asn338 by forming hydrogen bond, which cannot be formed for the compounds, 11a-d having the carboxamide group in the para position. Our docking model for the most active compound 11e well supports the key interactions for ALK5 inhibition which was previously reported.
4. Conclusions

In this report, a series of 2,4-disubstituted-5-[(6-alkyl)pyridin-2-yl]-1H-imidazoles, 7a-c, 11a-h, and 16a-h has been synthesised and evaluated for their ALK5 inhibitory activity in an enzyme assay and in a cell-based luciferase reporter assay. The structure–activity relationships in this series of compounds revealed that an ethylene linker at the two-position of the imidazole ring was the most beneficial in ALK5 inhibitory activity. Replacement of a benzof[1,3]-dioxol-5-yl moiety at the four-position of the imidazole ring with a quinoxalin-6-yl moiety markedly increased ALK5 inhibitory activity. Regarding the alkyl substituent at the six-position of the pyridine quinoxalin-6-yl moiety, the compounds having a methyl or an ethyl substituent displayed much higher inhibitory activity than the compounds having an i-propyl or a n-butyl substituent. The m-CONH₂ analogues were more inhibiting than the p-CONH₂ analogues in compounds having a methylene linker, whereas the p-CONH₂ analogues were more inhibiting than the m-CONH₂ analogues in compounds having an ethylene linker. In a cell permeability assay using Caco-2 monolayer, 11e showed the highest permeability in this series of compounds. The 11e was equipotent to vactosertib, but much more potent than galunisertib in an ALK5 kinase assay and in a cell-based luciferase reporter assay. Although 11e was 5.5-fold more selective against p38α than galunisertib, it was 6.2-fold less selective than vactosertib. Therefore, it can be concluded that combination of replacement of a [1,2,4]triazolo[1,5-a]pyridin-6-yl moiety with a quinoxalin-6-yl moiety, insertion of a methylene linker instead of a methyleneamino linker, and a m-CONH₂ substituent in the phenyl ring in vactosertib maintained its high ALK5 inhibitory activity, but decreased its selectivity against p38α. Selectivity profiling of 11e using a panel of 28 protein kinases showed that it is highly selective for ALK5. Our docking results demonstrate that 11e fits well in the ATP-binding pocket of ALK5 with favourable intermolecular interactions.

Disclosure statement
No potential conflict of interest was reported by the author(s).

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