Synthesis of ONC-101 and Derivatives:

The imidazo-pyrazine

\[
\begin{align*}
\text{150D07} & \\
\text{ONC92-NX-1}
\end{align*}
\]

\[N,2\text{-diphenylimidazo}\left[1,2\text{-a}\right]\text{pyrazin-3-amine}\]

Synthesis

The synthesis of imidazo[1,2-a]pyrazines is a “simple” three-component-one-pot reaction as shown in Scheme 1:

Scheme 1: The synthesis of imidazo[1,2-a]pyrazines

\[
\begin{align*}
\text{1} & \quad \text{pyrazine} \\
\text{2} & \quad \text{aldehyde} \\
\text{3} & \quad \text{isonitrile} \\
\text{4} & \quad \text{imidazo-pyrazine}
\end{align*}
\]

ONC101-NX-1: \(R_1 = \text{CH}_3\) \(R_2 = \text{H}\) \(R_3 = \text{m-Cl}\)
Multi-component reactions are valuable reactions in combinatorial chemistry, allowing the construction of target compounds with several diversity elements in a single chemical step\(^1\). The synthesis of imidazo-pyrazines, uses three components: a pyrazine, an aldehyde and an isonitrile. The synthesis of any imidazo-pyrazine is limited by the availability of the starting materials and their relative reactivity. Even though aldehyds are commercially available in great variety, this is no longer true for pyrazines with \(R_1 \neq H\) or \(R_1 \neq CH_3\) (both commercially available). Therefore other pyrazines have to be synthesized by a 3-step synthesis\(^4\).

In the following, a general procedure for the preparation of ONC–101 and its precursors is given\(^5\):

**2-Amino-3-methyl-pyrazine (1; \(R_1=CH_3\))**

In an autoclave, 2-chloro-3-methyl-pyrazine (10.0 g, 77.8 mmol) was dissolved in dry methanol (30 ml). Ammonia gas (60 g) was added. The mixture was heated to 150°C for 8 hours. (start pressure: 10 bar, end pressure: 90 bar). After cooling to room temperature, the mixture was evaporated to a brown solid, which was dissolved in 1N hydrochloric acid (100 ml) and washed with dichloromethane. The aqueous layer was slowly poured on cold saturated aqueous ammonia (150 ml), then extracted with dichloromethane (3 x 100 ml). The combined organic layers were dried (Na\(_2\)SO\(_4\)) and evaporated. The product was extracted from the residual solid with hot acetone (200 ml). Evaporation yielded 36% of the product as a yellow solid.
(3-Chloro-phenyl)-(8-methyl-2-phenyl-imidazo[1,2-a]pyrazin-3-yl)-amine (4; ONC101-NX-1)

2-Amino-3-methyl-pyrazine (109 mg, 1.0 mmol), benzaldehyde (106 mg, 1.0 mmol) and 3-chloro-phenylisonitrile (138 mg, 1.0 mmol) were dissolved in a mixture of dry methanol (2.0 ml) and trimethyl orthoformate (2.0 ml) under Argon. The mixture was stirred at 60°C for 3 hours, then cooled to RT. An analytically pure sample was obtained from the crude product using preparative HPLC.

**Supplementary Figure 1:** Schematic summary of findings.

**a,** Ephrin-B2 is essential for VEGF–induced internalization and the full activation (asterisks) of VEGF receptors. Both VEGFR endocytosis and downstream signal transduction are compromised in *Efnb2* knockout endothelial cells.

**b,** Ephrin-B2 and interactions with EphB receptors promote endothelial sprouting and thereby the angiogenic growth of blood vessels. We propose that ephrin-B2 and its downstream (reverse) signal transduction activity integrate VEGF signalling and other Eph/ephrin–regulated cellular processes such as the control of cytoskeletal organisation, motility, invasiveness and adhesion. The exact contribution of EphB receptor tyrosine kinases and their role in VEGFR endocytosis remain to be addressed.
**Supplementary Figure 2:** Ephrin–B2 and EphB4 expression in retinal ECs.

**a,** Whole mount GFP (green) and isolectin B4 (iB4, red) staining in the P5 Efnb2–GFP central retinal vasculature behind the edge of the growing endothelial plexus. Positive cells are found in arteries (A), capillaries and non-vascular cells. Nuclei, TO–PRO3 (blue).

**b,** Whole mount anti–EphB4 immunofluorescence predominantly labels veins (V) but also capillaries in a central region of the P5 wild-type retina.
Supplementary Figure 3: Inducible gene targeting in the vascular system.

a. β-galactosidase–stained E18.5 tissues isolated from Cdh5–CreERT2 transgenics in the ROSA26R Cre reporter background. A, arteries; V, veins; C, capillaries; L, lymphatics.

b. ROSA26R Cre reporter activation in a range of Cdh5–CreERT2 transgenic adult tissues, as indicated.

c. ROSA26–YFP Cre reporter (green) expression in the P5 Cdh5–CreERT2 retinal vasculature. EC were labelled with isolectin B4 (blue).

d. Downregulation of ephrin-B2 protein (green) in the endothelium of dermal blood vessels at E15.5. 5µm thick paraffin sections are shown. Vascular smooth muscle cells are labelled by α-smooth muscle actin (red).
Supplementary Figure 4: Defective endothelial sprouting in the Efnb2\textsuperscript{iΔEC} retina. Shown are representative images of P6 Efnb2\textsuperscript{iΔEC} mutants generated with the Cdh5–CreERT2 transgenic line. Note impaired vascular growth and reduced branching. ECs were labelled with isolectin B4.
Supplementary Figure 5: *efnb2a* knockdown in zebrafish.

**a.** Anti–ephrin–B2a antibody staining of sections labels the dorsal aorta (arrows) and somatic cells in control zebrafish embryos (24hpf). Ephrin–B2a expression in these domains was strongly reduced after injection of two different *efnb2a* morpholinos (MO-1 and MO-2).

**b.** Still images from Supplementary Movies 1 and 2 showing intersegmental sprouts of control and *efnb2a* knockdown *fl*–EGFP zebrafish embryos (27hpf). Control and blunt morphant filopodia (arrows) are marked.

**c.** Network formation by cultured mouse control and *Efnb2* KO ECs. The latter lack cellular protrusions (arrows). Phalloidin (green) labels F-actin.
Supplementary Figure 6: Inducible overexpression of ephrin-B2 in ECs.

a, Immunoprecipitation (IP) and Western blot (WB) of ephrin-B2 (~45kD) and the CFP–ephrin–B2 fusion protein (75kD) from Efnb2^{iGOF} (Tie2-rtTA x tetO-Efnb2) and control tissues. Molecular weights (kD) are indicated.

b, Immunofluorescence on tissue sections of control or Efnb2^{iGOF} mesentery with indicated antibodies. Overexpression of ephrin-B2 in mutant arteries (A), veins (V) and lymphatic vessels (L).

c, Whole mount staining of control and Efnb2^{iGOF} dermal blood vessels at E15.5.
Supplementary Figure 7: Ephrin-B2 and endothelial sprouting.

a and b, PECAM1 (green) and collagen IV (red) staining of whole mount E15.5 (a) and E18.5 (b) control and mutant skin samples as indicated. Arrows mark sprouts, arrowheads empty collagen IV sleeves.

c, Ratio of retracted (PECAM1- collagen IV+) vs. non-retracted (PECAM1+ collagen IV+) sprouts in the E18.5 Ephb2\textsuperscript{ΔEC} dermal vasculature (left). Quantitation of empty collagen IV sleeves (corresponding to abandoned sprouts and lost vascular connections) in Ephb2\textsuperscript{iGOF} mutants.

\( P \) values (c) were calculated using two-tailed Student’s t-test. Error bars, s.e.m. \( n \geq 3 \).
**Supplementary Figure 8:** Morphology of *Efnb2* mutant matrix sleeves and ECs.

**a.** Whole mount anti-collagen IV staining of the E18.5 control and *Efnb2* iGOF dermal vasculature. Note abundance of thin matrix structures in the mutant vasculature (arrowheads).

**b.** Confocal images of collagen IV+ structures in the E18.5 dermal vasculature. Long, sprout-like matrix sleeves (arrows) and thin matrix connections (arrowhead) are characteristic for *Efnb2* ^iGOF^ mutants. Collagen IV sleeves are short and blunt in *Efnb2* loss-of-function mutants.

**c.** Electron micrographs of E18.5 control and *Efnb2* mutant dermal capillaries, as indicated. *Efnb2* ^iGOF^ ECs are thin and highly ruffled whereas *Efnb2* ^iMEC^ capillary ECs are thick and devoid of protrusions.
Supplementary Figure 9:

a. Ephrin-B2 overexpression in individual ECs (asterisk) within a HUVEC monolayer. Shown are still images of long endothelial processes (arrows) from Movie S3.

b. Quantitation of migration speed and distance based on 8 independent microinjection experiments each.
Supplementary Figure 10: Characterisation of Eph kinase inhibitors.

a. Activity and binding profiles (IC_{50} and K_d) of ONC–101.

b. Detection of EphB4 (red) and phosphorylated Eph receptor (green) in microinjected HUVECs overexpressing EphB4. Specific phospho–Eph (p–Eph) signals are absent after treatment with ONC–102 (10μM).

c. Eph tyrosine phosphorylation (green, arrows) in HUVECs surrounding microinjected (dextran-containing) ephrin-B2-overexpressing cells. No p–Eph signal above background level can be seen after treatment with ONC–101 or ONC–102.
Characterization of ONC-101

Physicochemical properties
MW: 334.81 g/mol
Appearance: light brown solid
HPLC: Purity: >99% @ 254 nm
Method: 1% acetonitrile to 100% in 10 min, 0.1% TFA
Flowrate: 1.0 mL/min
ColmMn: HP Hypersil BDS – C18, 125 * 4 mm
Retention: 6.766 min
1H-NMR: consistent
13C-NMR: consistent
MassSpec: consistent (ESI)
Melting Point: N/A
ALOGP: 4.42
Solubility: Kinase buffer (Upstate), 1% DMSO: ~100 μM
Kinase buffer (Upstate), 0.25 % DMSO: ~65 μM

NMR analysis:

Supplementary Figure 11: Characterisation of ONC–101.

Physicochemical properties, the chemical structure of ONC–101 and the NMR analysis confirming the chemical identity of the compound are shown.
Characterization of ONC-101: HPLC and mass spectroscopic analysis

Supplementary Figure 12: Characterisation of ONC–101.
HPLC and mass spectroscopic data confirming the purity of ONC–101 are shown.
Characterization of ONC-102

Physicochemical properties
MW: 364.84 g/mol
Appearance: light brown solid
HPLC: Purity: >99% @ 254 nm
   Method: 1% acetonitrile to 100% in 10 min, 0.1% TFA
   Flowrate: 1.0 mL/min
   Column: HP Hypersil BDS – C18, 125 * 4 mm
   Retention: 6.793 min
1H-NMR: consistent
13C-NMR: consistent
MassSpec: consistent (ESI)
Melting Point: N/A
ALOGP: 4.71
Solubility: Kinase buffer (Upstate), 1% DMSO: ~100 μM
           Kinase buffer (Upstate), 0.25 % DMSO: ~50 μM

NMR analysis:

Supplementary Figure 13: Characterisation of ONC–102.
Physicochemical properties, the chemical structure of ONC–102 and the NMR analysis confirming the chemical identity of the compound are shown.
Supplementary Figure 14: Ephrin-B2 and lymphangiogenesis.

Reduced growth of LYVE1-positive (green) dermal lymphatic vessels in postnatally induced P14 \( Efnb2^{i\Delta EC} \) mutants.
**Supplementary Figure 15:** Loss of ephrin-B affects VEGF-C induced signalling and chemotaxis.

**a.** Detection of surface EGFR in stimulated control and Efnb2 KO ECs by biotinylation.

**b,c.** VEGF–C induced Akt and ERK1/2 activation is compromised in Efnb2 KO ECs.

**d.** Efnb2 KO ECs show compromised VEGF–A or VEGF–C induced migration in Boyden chamber assays.

**e.** Loss of ephrin-B selectively impairs chemotaxis induced by VEGF–C but not angiotensin II (Ang II) or fibroblast growth factor (FGF). Efnb2 KO ECs show enhanced chemotaxis towards epidermal growth factor (EGF).
**Supplementary Figure 16**: VEGFR3 internalisation in *Efnb2* mutants.

**a**, Confocal images of VEGFR3 (green) distribution in P6 control and *Efnb2*EC mesenteric lymphatic vessels after injection of PBS (control for the experiment shown in Figure 4b). Nuclei, DAPI (blue).

**b**, Confocal images of VEGFR3 distribution in P6 control and *Efnb2*EC mesenteric lymphatic vessels after intraperitoneal injection of VEGF–C. Accumulation of VEGFR3 (green) occurs around Prox1-positive (red) control but not mutant LEC nuclei.
**Supplementary Movies:**

**S1,** Fluorescent time-lapse movie showing dynamics of intersegmental vessels in 27 hpf *fli1*-EGFP embryo injected with control morpholino (i.e., *efnb2a*-MO containing 5 point mutations). Numerous filopodia can be seen on angiogenic endothelial sprouts visualized by EGFP very similar to uninjected control embryos (data not shown).

**S2,** Intersegmental vessels in 27 hpf *efnb2a*-MO-injected *fli1*-EGFP embryo showed few filopodia and instead blunt, bleb-like protrusions were seen on the cell surface. Still images from Movies S1 and S2 at time points 0, 30, 60 minutes are shown in Supplementary Figure 4b. Actual duration of both movies is 60 minutes with 61 frames at 1 minute intervals.

**S3,** Ephrin-B2 overexpression in single cells within a confluent monolayer of (uninjected) HUVECs. The injected cells displayed increased motility and dynamic sprout formation. Movie commences 5 minutes after injection and corresponds to 300 minutes (600 frames). Selected still images are shown in Supplementary Figure 8a.