The dual non-competitive CXCR1/2 inhibitor ladarixin impairs neutrophil extravasation without altering intravascular adhesion

Matteo Napoli,[1] Sebastian Sitaru,[1,2] Agnes Budke,[1] Anna Kammerl,[1] Georgia Querner,[1] Roland Immler,[1] Ina Rohwedder,[1] Sonja Seifert,[1] Bartolo Ferraro,[3] Ludwig Weckbach,[3] Sergio Catz,[4] Andrea Aramini,[5] Pier Giorgio Amendola,[5] Marcello Allegretti,[5] Riccardo Bertini,[6] Monika Pruenster[1#] and Markus Sperandio[1#]

[1]Institute of Cardiovascular Physiology and Pathophysiology, Biomedical Center, Ludwig-Maximilians-University Munich, Munich, Germany; [2]Department of Dermatology and Allergy, School of Medicine, Technical University of Munich, Munich, Germany; [3]Department of Cardiology, Biomedical Center, Ludwig-Maximilians-University Munich, Munich, Germany; [4]Department of Molecular Medicine, The Scripps Research Institute, La Jolla, CA, USA; [5]Research & Early Development, Dompé Farmaceutici S.p.a., L'Aquila, Italy and [6]ATREIUS S.a.s., L'Aquila, Italy

[#]MP and MS contributed equally as senior authors.

Correspondence: M. Sperandio
markus.sperandio@lmu.de

Received: March 20, 2025.
Accepted: August 7, 2025.
Early view: October 2, 2025.

https://doi.org/10.3324/haematol.2025.287877

SUPPLEMENTARY FILE

The dual non-competitive CXCR1/2 inhibitor ladarixin impairs neutrophil extravasation without altering intravascular neutrophil adhesion

Matteo Napoli[1], Sebastian Sitaru[1,2], Agnes Budke[1], Anna Kammerl[1], Georgia Querner[1], Roland Immler[1], Ina Rohwedder[1], Sonja Seifert[1], Bartolo Ferraro[3], Ludwig Weckbach[3], Sergio Catz[4], Andrea Aramini[5], Pier Giorgio Amendola[5], Marcello Allegretti[5], Riccardo Bertini[6], Monika Pruenster[1#], and Markus Sperandio[1#*]

[1]Institute of Cardiovascular Physiology and Pathophysiology, Biomedical Center, Ludwig-Maximilians-University Munich, Germany

[2]Department of Dermatology and Allergy, School of Medicine, Technical University of Munich, Germany,

[3]Department of Cardiology, Biomedical Center, Ludwig-Maximilians-University Munich, Germany,

[4]Department of Molecular Medicine, The Scripps Research Institute, La Jolla, USA,

[5]Research & Early Development, Dompé Farmaceutici S.p.a., L' Aquila, Italy,

[6]ATREIUS S.a.s., L'Aquila, Italy

[#]MP and MS contributed equally as co-last authors

Supplemental Data

Detailed methods

Neutrophil isolation

Human neutrophils were isolated from peripheral blood of healthy human donors. Blood sampling was approved by the ethical committee of LMU München (Az. 611-15). Polymorphprep (Axis Shield) was added and samples prepared according to the manufacturer's instruction. Alternatively, human neutrophils were isolated by EasySep™ Direct Human Neutrophil Isolation kit (STEMCELL TECHNOLOGIES) according to manufacturer's protocols. After isolation, neutrophils were transferred into pH adjusted (pH 7.4) Hanks's balanced salt solution (HBSS containing 10mM HEPES and 0.25%BSA) at a concentration of $1x10^6$ cells/ml until the experiment was started.

Murine Neutrophils were isolated using a Percoll (Sigma-Aldrich) solutions at 1.08 and 1.11g/ml density or by EasySep™ Direct Mouse Neutrophil Isolation kit (STEMCELL TECHNOLOGIES) according to manufacturer's protocols, respectively from bone marrow cells of WT mice. For some assays, murine

neutrophils were matured overnight using WEHI-3 supernatant [1]. Neutrophils were counted and resuspended in HBSS at $1x10^6$ cells/ml.

Leukocyte extravasation in whole mounts of the CXCL1-stimulated cremaster muscle

To investigate the effect of ladarixin on CXCL1-induced leukocyte extravasation, we injected ladarixin or 0.9% NaCl (carrier) into the peritoneal cavity of mice one hour before intrascrotal injection of rmCXCL1 (600ng/mouse). 2h later, mice were anesthetized, cremaster muscles surgically removed, fixed with 4% paraformaldehyde (4% w/v, Applichem) and stained with Giemsa-solution (Merck). Giemsa-stained whole mounts were then used to assess the number of perivascular neutrophils, eosinophils and mononuclear cells. The microscopic analysis of perivascular leukocytes was carried out at the core facility BioImaging of the Biomedical Center using a Leica DM2500 microscope, equipped with a 100x objective (Leica, 1.4NA, oil immersion) and a Leica DMC2900 CMOS camera.

Flow chamber experiments and chemotaxis

Flow chamber experiments were conducted to assess adhesion of human neutrophils as described previously [2]. Briefly, µSlide $VI^{0.1}$ (ibidi GmbH) microflow chambers were coated with rhE-selectin (5 µg/ml, R&D Systems), rhICAM-1 (4 µg/ml, R&D Systems), and rhCXCL8 (10 µg/ml). Isolated human neutrophils were incubated with ladarixin (5µM) or PBS (carrier) for 30min and then perfused through flow chambers at a shear stress level of 1dyne/cm using a high-precision syringe pump (Harvard Apparatus). Number of adherent leukocytes/FOV were assessed over a time period of 20min. Chemotaxis experiments were conducted with bone marrow murine neutrophils, using CXCL1 and CellDirector®2D chemotaxis chambers (Gradientech). Briefly, neutrophils were matured in RPMI 1640 (Sigma-Aldrich) supplemented with 20% WEHI-3B-conditioned medium overnight at 37°C. Chemotaxis chambers were coated with fibrinogen (100 µg/ml, Innovative Research) for 3h at RT and blocked with 5% casein overnight at 4°C. Subsequently, a CXCL1 gradient (0-10nM) was established within the chamber and mouse neutrophils ($1x10^6$ cells/ml) pretreated with PBS (carrier) or ladarixin for 30min at 37°C were transferred into the chamber. All experiments were conducted and recorded at an inverted microscope (Leica DMi8, x10, 0.3 NA, dry objective) at 37 °C. Euclidean distance, crawling directionality and crawling velocity were assessed over 10min to quantify chemotaxis of mouse neutrophils, as described [2].

CXCR2 internalization

Isolated murine neutrophils were incubated with 5µM ladarixin or PBS (carrier) at 37°C for 1h. Then either stimulation with CXCL1 at a concentration of 100ng/ml for 10min at 37°C or NaCl 0,9% treatment followed. Reactions were stopped with 1,5ml BD FACS Lysing Solution, before centrifuging the samples. Cell pellets were stained for Ly6G, CXCR2 (Pacific Blue rat anti-mouse Ly-6G, clone 1A8, 0,8ng/µl and APC rat anti-mouse CXCR2, clone SA044G4, 2ng/µl, both BioLegend) and the

corresponding Isotype controls measured by flow cytometry (CytoFLEX S) and analyzed using Kaluza Flow Analysis (all Beckman Coulter) or FlowJo software. In each experimental group, a ratio of stimulated (CXCL1) against unstimulated (NaCl 0,9%) was calculated.

Neutrophil elastase mobilization assay

Isolated murine or human neutrophils were pretreated with ladarixin or PBS (carrier) 1h. For murine cells, neutrophil elastase (NE) mobilization from intracellular storage pools to the surface was induced by incubating neutrophils in 2%BSA (control) or rmPECAM-1 (2µg/ml), rmICAM-1 (8µg/ml; both R&D Systems) and rmCXCL1 (10µg/ml, PeproTech) coated wells for 30min at 37°C, as described [3]. For human neutrophils we incubated the cells in 2%BSA (control) or rhPECAM-1 (2µg/ml; R&D Systems), rhICAM-1 (8µg/ml; R&D Systems) and rhCXCL8 (10µg/ml) coated wells for 30 min at 37°C. Thereafter, neutrophils were fixed with 2% PFA, blocked, permeabilized and labeled with rabbit anti-mouse NE antibody (polyclonal; Abcam, 5µg/ml) and goat anti-rabbit Alexa Flour 546 (Invitrogen, polyclonal, 5µg/ml). NE surface mobilization (ring formation) was assessed by confocal microscopy (Leica SP8X System, ×40 or x63, 1.3 NA oil immersion objective) at the core facility BioImaging, Biomedical Center, LMU Munich using Fiji software [4]. Briefly, histograms of NE intensity values were obtained for individual cells by drawing a line throughout the middle of the cell. The intensities from 10% of both histogram sides (cell surface) were divided by the intensities measured in the middle 20% of the histogram. Finally, a ratio indicative for NE surface mobilization was calculated. Values >1, indicate more NE signal on the surface while values <1 indicate more signal inside the cell.

Neutrophil elastase activity in vivo

To test perivascular and intravascular neutrophil elastase (NE) activity *in vivo*, we applied an intravital microscopy model in the CXCL1-stimulated mouse cremaster muscle as described earlier [3]. Briefly, we injected NE680FAST (Perkin-Elmer) i.s. (4nmol/mouse) to monitor perivascular NE activity or i.v. to assess intravascular NE activity. NE680FAST injection was applied one hour after i.p. injection of ladarixin or 0.9% NaCl (carrier). One hour later, 0.9%NaCl (control) or CXCL1 (600ng/mouse) was injected i.s. Two hours after CXCL1/NaCl injection, the cremaster muscle was dissected and fixed with 4% paraformaldehyde, permeabilized and blocked with 0.5% Triton X-100/2% BSA in PBS, and finally stained with rat anti-CD31 antibody Alexa Fluor 488 (MEC13.3; BioLegend, 5µg/ml). Cremaster muscles were then embedded on glass slides in Vectashield®plus (Vector Laboratories) and imaged using confocal microscopy (Leica SP8X system, ×40, 1.3 NA oil immersion objective).

Laminin digestion assay in neutrophils in vitro

Laminin (LN) digestion by neutrophil proteolytic activity was assessed

by live cell imaging and using µ-Slide membrane ibiPore flow chambers (Ibidi) containing a 300 nm thick membrane with 5 µm pores and a subjacent rat-tail collagen gel (1.5mg/mL) loaded with rmCXCL1

(1 ng/ml) as chemoattractant. Coating of the upper compartment was performed with laminin (15µg/ml), rmPECAM-1 (2 µg/ml) and rmICAM-1 (8 µg/ml) as described [3]. For microscopic visualization, laminin was stained using an anti-LN antibody conjugated to Alexa Fluor-647 (Novusbio). Isolated murine neutrophils were pretreated with ladarixin or PBS (carrier) for one hour and stained with CellTracker™ Green CMFDA Dye (Thermo Fisher). Thereafter, neutrophils were added to the upper chamber and laminin digestion was observed using time-lapse spinning-disk confocal microscopy (Zeiss Examiner with a x20/1.0 NA water immersion objective with a scanner unit CSU-X1, Yokogawa Electric Corporation and a EMCCD camera Evolve, Photometrics). 3D images were acquired and percentage of neutrophils with digesting capability analyzed using Fiji software [4].

Neutrophil elastase release from human neutrophils

To quantify neutrophil elastase release from human neutrophils, transwell assays were performed as described above. Shortly, filters (5µm pore size, Corning) were coated with rhPECAM-1 (2µg/ml; R&D Systems) and rhICAM-1 (8µg/ml; R&D Systems) for 2h at RT while human neutrophils were isolated and pretreated with 5µM ladarixin or NaCl control for 30min at 37°C. Then, the lower plate compartment was filled with HBSS buffer alone or HBSS buffer containing 1 ng ml-1 CXCL8 and cells were seeded and incubated for 30min at 37°C. Afterwards, the supernatant from the cell suspension in the lower cell compartment was collected, diluted 1:25 and neutrophil elastase concentration was determined via ELISA (Abcam, ab270204).

Supplementary reference List

1. Jakob SM, Pick R, Brechtefeld D, et al. Hematopoietic progenitor kinase 1 (HPK1) is required for LFA-1-mediated neutrophil recruitment during the acute inflammatory response. Blood. 2013;121(20):4184-4194.
2. Immler R, Nadolni W, Bertsch A, et al. The voltage-gated potassium channel KV1.3 regulates neutrophil recruitment during inflammation. Cardiovasc Res. 2021;
3. Rohwedder I, Kurz ARM, Pruenster M, et al. Src family kinase-mediated vesicle trafficking is critical for neutrophil basement membrane penetration. Haematologica. 2020;105(7):1845-1856.
4. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods. 2012;9(7):676-682.