A novel, highly potent and selective phosphodiesterase-9 inhibitor for the treatment of sickle cell disease

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Supplemental Methods

2.2 K562 and UT-7 erythroid cells

Human erythroleukemic K562 cells (American Type Culture Collection) were cultured in suspension in Iscove's Modified Dulbecco's Medium (ThermoFisher Scientific, France) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine (Thermo Fisher Scientific, France). UT-7 cells were cultured in alpha-MEM (with ribo- and deoxyribonucleosides, ThermoFisher Scientific, France) supplemented with 20% fetal bovine serum and 5 ng/ml human GM-CSF (Peprotech, France). Cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air at raised humidity.

2.3 HbF ELISA assay

K562 cells ($5x10^6$) were centrifuged for 5 minutes at 500×g, and the pellet washed three times in cold PBS and suspended in 1 mL ice-cold PBS. Cells were submitted to freeze (-20°C) and thaw cycles (3 times) and centrifuged at 5,000×g for 10 minutes at 4°C to remove cellular debris. Supernatant was assayed using an ELISA kit for HbF (Cloud Clone Corp, CEA996Hu) according to manufacturer instructions. Protein concentration was measured in a 96-well assay plate by a BCA protein assay (Pierce Thermofisher).

2.4 HbF assessment by flow cytometry

Cells were washed in cold PBS, fixed in cold 0.05% glutaraldehyde at RT for 10 minutes, and then permeabilized in 0.1% Triton X-100 at RT for 10 minutes. Cells were then washed with BD Perm/Wash™ buffer (BD Pharmingen) and stained with PE-mouse anti-human HbF at 1/25 (clone 2D12, BD Pharmingen) or isotype control (PE-mouseIgG1κ, clone MOPC-21, BD Pharmingen) for 30 minutes on ice. The percentage of HbF^+ cells (% HbF) and the HbF levels

(MFI) were determined by flow cytometry (GalliosTM, Beckman) in live cells, using FlowJo software (FlowJo LLC, USA).

2.5 SCD patient cells

Blood was collected in a bag containing acid citrate dextrose solution (ACD) from 5 severe adult SCD patients admitted at the Biotherapy Department of Necker Hospital for an exchange transfusion. All samples used in this study were obtained from patients that signed informed consent forms approved by the ethical committee of Necker Hospital on 11 September 2015 (study IMNIS2015-01). Patient ages ranged from 19 to 33 years with a median age of 32 years.

$CD34^+$ cells were isolated from the low density blood cells from a Ficoll density gradient (GE Healthcare Life Sciences) by positive selection using anti-CD34 antibody magnetic cell sorting on Midi-MACS and LS columns (Miltenyi Biotech). Viable cells were suspended in serum-free medium IMDM (Life Technologies) in a 6-well plate (Corning) at a final concentration of $5x10^5$/ml, in the presence of 15% BIT 9500 (mixture of bovine serum albumin [BSA] + insulin + transferrin from Stem Cell Technologies), 100 U/ml penicillin-streptomycin, 2 mM L-glutamine, 10 ng/mL recombinant human (rh) IL-3 (Peprotech), 100 ng/ml rhIL-6 (Peprotech) and 100 ng/ml rhSCF (Peprotech). Cells were incubated for 7 days at 37°C in 5% CO_2 with media replacement on days 3 and 5 and then were pelleted and resuspended in PBS with 2% fetal bovine serum (FBS). $CD36^+$ cells, selected on Mini-MACS and MS columns following incubation with CD36 antibody (Milteny Biotech) and rat anti-mouse IgG1 antibody coupled magnetic microbeads (Miltenyi Biotech), were cultured in media containing 100 ng/mL rhSCF, 10 ng/mL rhIL-3 and 2 UI/ml erythropoetin (Cilag, France). Erythroid differentiation was evaluated by flow cytometry using the following antibodies: FITC-conjugated CD233/Band3 (Miltenyi Biotech,

Germany), APC-conjugated CD49d/α4-integrin (Biolegend – Ozyme, France), VioBlue-conjugated CD235a/GPA (Miltenyi Biotech, Germany) and LIVE/DEAD® Fixable Aqua Dead Cell Stain (Life Technologies, France). Media was supplemented with DMSO, 30 µM HU or 10 µM IMR-687. Cells were split every 2 days with additions of fresh medium. At the end of the 5 days exposure period, the erythroid cells HbF ($LD^-/GPA^+/Band3^+$) was determined by FACS.

2.6 Animals

2.6.1 Townes model

Experiments were approved by the Animal Care Committee at Paris Descartes University. HbSS-Townes mice[42] on a 129/B6 background (Jackson Laboratory, Bar Harbor, ME, USA, 10-12 weeks old with 7/group) were dosed daily by gavage with vehicle (polyethylene glycol in water 1:3), 50 or 25 mg/kg of HU, or 30 mg/kg of IMR-687. On day 30 mice were anesthetized with isoflurane anesthesia, with blood collected from tail vein by heparinized capillaries into EDTA coated tubes and then mice were euthanized and spleens were collected and weighed. Blood counts were measured on a ProCyte Dx Hematology Analyzer (Idexx, France) and reticulocytes were determined using Retic-COUNT Reagent (BD Biosciences Retic-Count Kit). Blood smears were air dried and stained with May-Grünwald-Giemsa and the percentage of sickled RBC was then determined visually by microscopy. Total bilirubin concentrations in plasma were quantified by a bilirubin assay kit (Sigma Aldrich, France). Plasma LDH activity was measured using a LDH assay kit (Pierce – Thermo Scientific, France). Plasma nitrite was determined using Nitrite/Nitrate assay kit (Sigma Aldrich, France). Fetal hemoglobin was assessed by flow cytometry as described above. Total free hemoglobin levels were determined by measuring

absorbance at 540nm (TECAN infinite 200) on 20 µL of plasma mixed with 180 µL of Drabkin's reagent (Sigma Aldrich).

Myeloperoxidase (MPO) and arginase activities were evaluated in lung tissue homogenates. Protein was isolated from lung in RIPA buffer using tissue-ruptor (Qiagen, France). Total protein concentration was determined the by the bicinchoninic acid protein assay (Pierce – Thermo Scientific, France). MPO and arginase activity were detected by using a colorimetric activity assay kit (Sigma Aldrich).

2.6.2 HbSS-Townes vaso-occlusive crisis model

HbSS-Townes mice[42] on a 129/B6 background (6-17 week old with N=3/group) were treated with vehicle (0.08% w/v methyl cellulose), 100 mg/kg of HU, 10 or 30 mg/kg of IMR-687, or 100 mg/kg HU + 30 mg/kg IMR-687 in their drinking water. Experiments were approved by The University of Minnesota's Institutional Animal Care and Use Committee (IACUC). On day 7 of treatment, the mice were implanted with dorsal skin-fold chambers (DSFCs). Three days later, on day 10 of treatment, mice with DSFCs were anesthetized with a mixture of ketamine (106 mg/kg) and xylazine (7.2 mg/kg), placed on a special intravital microscopy stage, and 20-23 flowing subcutaneous venules in the DSFC window were selected and mapped. Mice were then placed in a hypoxic atmosphere chamber (7% O_2/ 93% N_2) for one hour, after which they were returned to room air. All the selected venules were re-examined after one and four hours of re-oxygenation in room air and the number of static (no flow) venules was counted and expressed as percent stasis.

Following completion of the 4h stasis measurement, mice were euthanized via CO_2 asphyxiation, and heparinized blood collected via cardiac puncture, aliquoted and stored at -80°C. Hematocrit was measured using a micro-capillary reader (IEC) after centrifugation in a

micro-capillary centrifuge (IEC). Reticulocytes were measured from blood smears stained with methylene blue. Reticulocytes and total RBC were counted in 4 separate microscopic fields per mouse. Reticulocytes are expressed as a percentage of total RBC. The total white blood cell (WBC) counts were derived manually using a hemocytometer.

To measure irregular shaped RBC, heparinized whole blood from HbSS- and HbAA (control)-Townes mice was incubated for 15 minutes at $4^{o}C$ with an equal volume of 2% sodium metabisulfite and fixed post-incubation with an equal volume of 10% neutral buffered formalin. Fixed blood smears (10 µL) were prepared on glass slides and 3 representative fields were photographed at 100X magnification. Irregular and normal shaped RBC were counted (mean = 99 ± 15 cells/slide) and the irregular shaped RBC were expressed as a percentage of total RBC. The irregular shaped RBC included teardrop, schistocyte, elliptocyte, bite and sickle cells.[43]

HbF+ cells were stained on blood smears by the Kleihauer-Betke method using a fetal cell stain kit (Simmler).[44] HbF+ cells and total RBC were counted in four separate microscopic fields at 100 X magnification per mouse. Plasma hemoglobin and bilirubin were measured spectrophotometrically by the Fairbanks AII method.[45] Total plasma heme levels were measured colorimetrically at 400 nm using a QuantiChrom™ heme assay kit (BioAssay Systems).

2.7 Fear Conditioning Model

Test articles or vehicle were administered (PO) 30 minutes prior to training and testing.

Training, Day 1, mice were placed into a 7”x7”x12” CFCC enclosure with an electrified grid bottom. After a 120s period of exploration and habituation, they received three tonal cue (30s, 3kHz, 75dB) and mild foot shock (1.5s, 0.65mA) pairings separated by 30 second Inter-Trial-Interval (ITI) rest periods. They were then allowed to remain in the same enclosure for a final

post-shock interval (FPSI, 30s). Animals were then removed from the CCFC enclosure and returned to their home cages.

Contextual testing, Day 2, mice were exposed to the CFCC enclosure for 300 seconds with no tones played and no shocks emitted. Freezing behavior was measured and used as an endpoint for memory over a period of time similar to the original exploration and habituation time. Mice were then returned to their home cage.

Cued testing, Day 2, after contextual session, mice were again placed into the CCFC chamber with a novel context (black insert). After a 300s period of exploration and habituation, they received five CS tonal cues (30 s, 3 kHz, 75 dB) separated by 60s ITIs. Thirty second tones were played at 300s, 390s, 480s, 570s, 660s and the session ended after the final ITI. Mice were then returned to their home cage.

Time spent freezing during the training, contextual, and cued phases of the evaluation were scored automatically via the MedAssociates Software.

2.8 Statistical analyses

Statistical analyses studies were performed using a one-way analysis of variance (ANOVA) test followed by a Dunnett's test (multiple comparison test) or Kruskal-Wallis test (non-parametric) for analysis of treatment effect versus controls. All statistical analyses were derived using GraphPad software (v6.00, San Diego, California, USA). Statistical significance to reject the null hypothesis was identified at the $p<0.05$ level. For illustrative purposes, significance levels of $p<0.01$ and $p<0.001$ were also noted.