Senicapoc: a potent candidate for the treatment of a subset of hereditary xerocytosis caused by mutations in the Gardos channel

Hereditary xerocytosis (HX) is a form of stomatocytosis, a red blood cell disease characterized by impaired cation permeability and hemolytic anemia.[1] In HX, red blood cell (RBC) K^+ permeability is largely increased compared to Na^+ permeability thus resulting in a loss of KCl accompanied by water. Up to now, two different genes have been linked to this disease: *PIEZO1*, coding for a non-selective cation channel activated by mechanical forces,[2] and *KCNN4*, a calcium activated K^+ channel also named Gardos channel in RBCs.[3-6] Among the 3 KCNN4 mutations linked to HX, one is in the calmodulin-binding (CaMB) domain (R352H) whereas the two others, V282M and V282E, affect the same residue in the pore domain.[4-6] We have shown that R352H substitution in the CaMB domain changes the Ca^{2+} threshold for activation, increases current density, and delays channel inactivation.[6] RBC shrinking is explained by a greater activity of the mutated channel activated by a small increase in intracellular Ca^{2+} concentration, which would be unable to stimulate the wild-type (WT) channel. The channel hyperactivity results in loss of KCl and water. Whereas V282 residue plays a key role in KCNN4 gating,[7] the way the V282M or V282E substitutions in the pore domain affect channel functioning has not yet been reported. With the finding of mutations in KCNN4 as causative of some HX,[4-6] the possibility to use Senicapoc to treat this disease has emerged. Senicapoc is derived from clotrimazole-1, a KCNN4 inhibitor.[8] It was designed for a long-term sickle cell disease therapy in order to avoid clotrimazole-1 side-effects. Clinical trials did not show significant benefits for patients with sickle cell disease and research was not carried forward.[9] Whereas Senicapoc is a potent inhibitor of WT KCNN4, its efficiency on mutated chan-

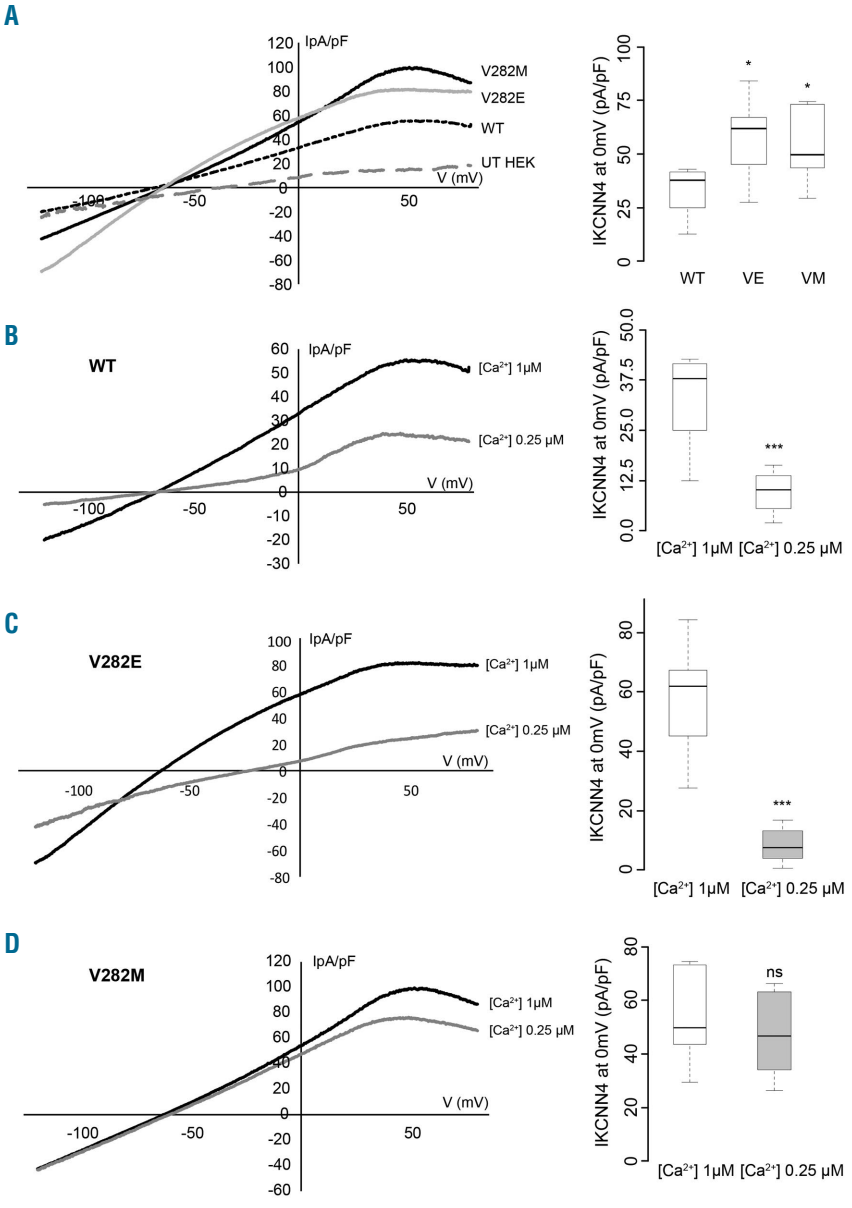

Figure 1. Characterization of KCNN4 mutants in HEK293 cells. Wild-type (WT) KCNN4, V282E KCNN4, or V282M KCNN4, were expressed in HEK293 cells and then subjected to patch clamp experiment in whole cell configuration using a 150ms voltage ramp protocol from -120 to + 80 mV from a holding potential of -60 mV (sampling frequency 10 kHz; filtered 1 kHz). Glass pipettes had a final resistance ranging from 3 to 5 MΩ. The bath solution was in mM: NaCl 145, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, Hepes 10 pH 7.4 adjusted with NaOH (320 mOsm). The intracellular solution was in mM: KCl 145, MgCl2 1, Hepes 10 pH 7.2 adjusted with KOH, CaCl2 0.87, EGTA 1 (corresponding to 1 µM free calcium. Maxchelator was used to calculate free Ca2+ concentration (*http://maxchelator.stanford.edu/CaEGTA-TS.htm*) (305 mOsm). (A) (Left) Mean current/voltage curves from 8-10 experiments. UT: untransfected cells. (Right) Values are represented as Tukey's box plots showing current density measured at 0 mV in cell transfected with WT and mutated KCNN4 (V282E and V282M). Statistical analyses were carried out using Kruskal and Wallis test followed by a Tukey's *post-hoc* test. (n=8-10; **P*<0.05). (B) (Left) Mean current/voltage curves from cells expressing WT KCNN4 recorded with an intracellular solution containing 1 µM free Ca^{2+} (n=8) or containing 0.25 µM free Ca^{2+} (n=4). (Right) Values are represented as a Tukey's plot. Statistical analyses were carried out using Mann-Whitney test (n=4-8; ****P*<0.001). (C) (Left) Mean current/voltage curves from cells expressing V282E KCNN4 recorded with an intracellular solution containing 1 µM free Ca^{2+} (n=10), or containing 0.25 µM free Ca^{2+} (n=8). (Right) Values are represented as a Tukey's plot. Statistical analyses were carried out using Mann-Whitney test (n=8-10; ****P*<0.001). (D) (Left) Mean current/voltage curves from cells expressing V282M KCNN4 recorded with an intracellular solution containing 1 µM free Ca^{2+} (n=8), or containing 0.25 µM free Ca^{2+} (n=8). (Right) Values are represented as a Tukey's plot. Statistical analyses were carried out using Mann-Whitney test (n=8; ns: non significant).

nel was unknown.

The present work intended to assess the effect of mutations V282M/E on channel features and to define the sensitivity of differently mutated KCNN4 to Senicapoc. Our data show that although the three different KCNN4 mutations linked to HX are "gain of function" mutations, they do not share a common mechanism in altering channel characteristics. Moreover, it is shown that Senicapoc sensitivity varies significantly between KCNN4 mutants.

In order to study and compare the different mutations of KCNN4 linked to HX, we used HEK293 cells as a reliable heterologous expression model.

HEK293 cells were transiently transfected with WT KCNN4, V282E or V282M mutants. Currents were then recorded in whole cell configuration. Figure 1A shows that the two substitutions on Val282 increased current density compared to WT. To assess the calcium sensitivity of these currents, the same experiment was performed with two different intracellular Ca^{2+} concentrations: 1

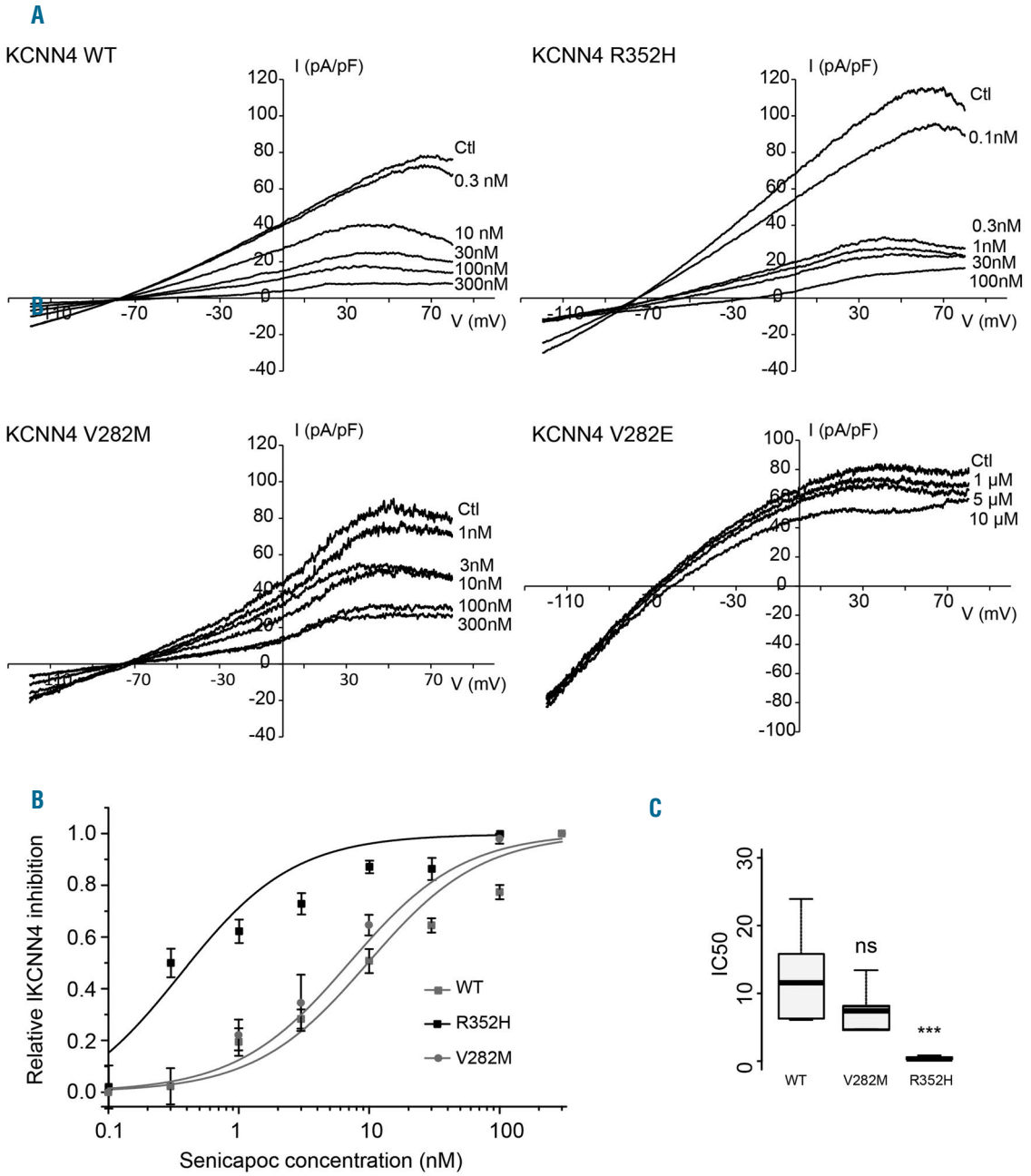

Figure 2. Effect of Senicapoc on KCNN4 mutants in HEK293 cells. (A) Representative traces showing dose-dependent inhibition of wild-type (WT) and mutated KCNN4 (R352H, V282M and V282E) by Senicapoc (n=number of cells recorded in each condition). Currents recorded in a whole cell patch clamp configuration were elicited by 150 ms voltage ramps from -120 to + 80 mV from a holding potential of -60 mV. Each trace corresponds to a different concentration of Senicapoc indicated on the right hand side of the I/V. (B) Normalized K+ currents measured at 0 mV in response to [Senicapoc] was plotted as a function of [Senicapoc] for WT KCNN4 (gray squares), R352H mutated KCNN4 (black squares) and V282M mutated KCNN4 (gray circles). The experimental values (mean ± SEM) were fitted using the Hill equation. (C) Tukey's box plots showing IC50 values for each condition (n=5-11; ***P<0.001). Statistical analysis was performed using a Kruskal and Wallis test followed by a Tukey's *post-hoc* test.

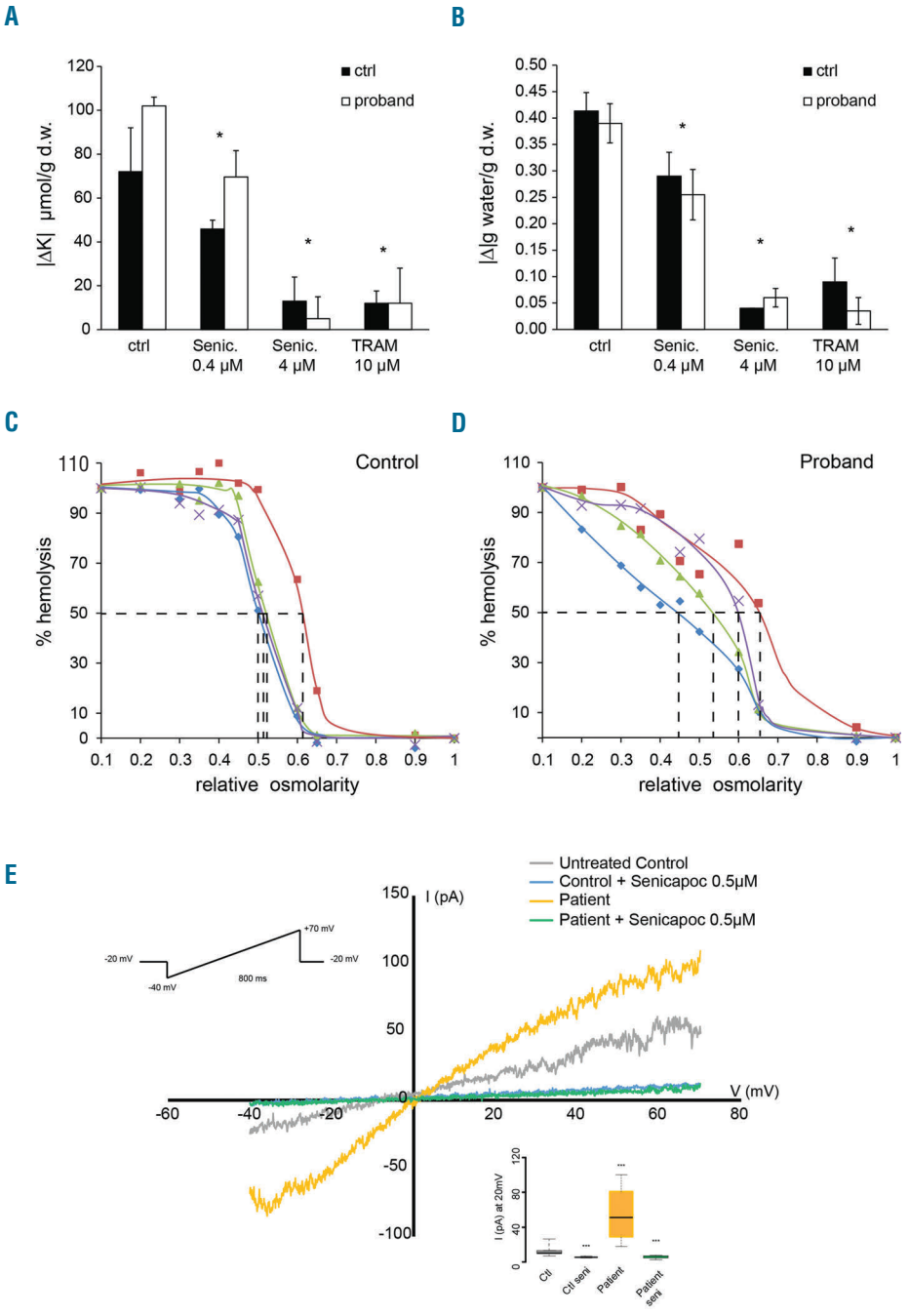

Figure 3. Effect of Senicapoc and TRAM-34 on red blood cells (RBCs) with KCNN4 R352H mutation. Fresh venous blood was obtained by venipuncture from a patient from Family 1 in a previous publication,[6] and healthy volunteers. (A and B) Freshly drawn blood was washed 4 times at room temperature in medium containing in mM: NaCl 147, KCl 5, MgSO4 2, $CaCl_2$ 1, Hepes 10, buffered with NaOH pH7.4 (320 mOsm). 5 mM vanadate was added alone or with 10 µM TRAM-34 or different concentrations of Senicapoc to red blood cell suspension, 30% hematocrit. Samples were treated as previously described.[6] Kinetic of net K+ fluxes and water contents were measured. (A) Absolute value of variation in K^+ content 60 min after vanadate addition was plotted as a function of experimental conditions (*P<0.05). (B) Absolute value of variation in water content 60 min after vanadate addition was plotted as a function of experimental conditions. Data are means±sem of 3 different experiments. Mann-Whitney test was used to compare control *versus* inhibitors for control or patient RBCs (*P<0.05, for clarity purposes only one star was plotted for the two bars compared to their respective control). (C and D) An osmotic fragility test in hypotonic saline solutions, was performed on blood after 25-h incubation at 37 °C in absence (blue) or presence of 10 µM TRAM-34 (red) or Senicapoc at 0.4 (green) or 4 µM (purple). Data are representative of 2-3 different experiments with blood from a single patient and 2 different controls. (E) Endogenous KCNN4 current was recorded using the whole cell patch clamp configuration. Glass pipettes were made to give a final resistance ranging from 17 to 20 MΩ. Currents were elicited by an 800 ms voltage ramp protocol from -40 to + 70 mV from a holding potential of -20 mV (sampling frequency 10 kHz; filtered 1 kHz). The same solution was used for pipette and bath and contained in mM: KCl 150, NaCl 5, $MgCl_2$ 1, Hepes 10, CaCl21, pH 7.4 (320 mOsm). All patch clamp experiments were performed with a PC-controlled EPC 9 patch-clamp amplifier (HEKA, Lambrecht/Pfalz, Germany). Currents were acquired and analyzed with Pulse and Pulsefit softwares (HEKA). Red blood cells were pre-incubated for 5 min with Senicapoc (0.5 µM) and then submitted to patch clamp experiments. Representative current/voltage curves from patients (in yellow, n=4), treated with Senicapoc (in green, n=6) or controls (in gray, n=5), treated with Senicapoc (in blue, n=3), are shown. Inset (lower right) shows quantification of currents recorded from red blood cells. Values are represented as a Tukey's plot (***P<0.001).

μM, corresponding to the EC50 for WT KCNN4, and 0.25 μM, corresponding to the EC50 of R352H mutant.[6] Figure 1B and C shows that both V282E and WT KCNN4 have a similarly increased activity at the higher intracellular calcium concentration whereas this calcium sensitivity is not observed for V282M mutant, which has a similar current density for 0.25 and 1 μM of intracellular Ca^{2+} (Figure 1D). These results show that the two substitutions on V282 are gain of function mutations, as was the R352H mutation. A study of KCNN4 gating by Garneau *et al*. had previously shown that V282 is a key residue in the gating process of the channel[7,10] and that its substitution by Gly makes a constitutively active channel, which conducts ions in the absence of Ca^{2+}. Our data confirm the essential role of V282 in KCNN4 activity. Moreover, it is shown that Ca^{2+}-sensitivity is highly dependent on the substituting residue. Further studies will be necessary to better understand at the molecular level how the Glu and the Met substitutions on V282 alter KCNN4 gating and calcium dependence. In RBCs, the V282M mutant may give a phenotype similar to R352H mutant, i.e. a constitutive channel activity at the resting Ca^{2+} concentration which is around 0.2 μM.[11] Hence, the R352H or V282M mutants should leak K^+ in resting condition and this increased K^+ permeability would explain RBC dehydration. By contrast, the mechanism of K^+ leak in V282E mutants may be different as activation of the channel is still calcium sensitive, like the WT channel. In this case, RBC dehydration would be consecutive to a greater K^+ current only when the channel is activated, whereas activation threshold would not differ from WT.

Senicapoc sensitivity of the three different KCNN4 mutations was assessed on HEK cells transiently transfected with each construct. Figure 2A and *Online Supplementary Figures* show representative current/voltage curves for WT and mutated KCNN4 as a function of different concentrations of Senicapoc. Figure 2B summarizes dose-response curve data of inhibition between WT and the two mutants R352H and V282M. The IC50 around 10 nM is not statistically different between WT and V282M. By contrast, the R352H mutant is approximately 30 times more sensitive than the WT, with an IC50=0.3 nM (Figure 2C). Remarkably, V282E is almost insensitive to Senicapoc, only a slight inhibition being observed for 10 μM Senicapoc, a thousand times IC50 of WT channel.

Differences in Senicapoc efficiency to block KCNN4 mutants on V282 suggest that the size of the side chain in this position plays an important role in inhibitor action. Moreover, the WT channel's IC50 around 10 nM is very similar to the 11±2 nM established in human RBC.[12] HEK293 expression system to assess KCNN4 features is thus relevant to study and compare KCNN4 mutants. As a derivative of clotrimazole, Senicapoc is proposed to bind in the pocket lined by residues from the cytoplasmic ends of the pore, i.e. close to V282.[13] Inhibition may thus be due to obstruction of the ion pathway from the intracellular side of the channel. The low sensitivity of V282E mutant to Senicapoc suggests that the pore is enlarged by Glu substitution preventing efficient blocking of K^+ current. The higher Senicapoc sensitivity of mutant in the CaMB domain (R352H) suggests a role of Ca^{2+} gating in the shape/size of the ion pathway. It confirms the interplay between Ca^{2+} gate at the CaMB domain and the formation of the ion pathway.[7]

Senicapoc was then assessed on fresh RBC samples from a patient carrying the KCNN4 R352H mutation. The efficiency of the inhibitor was evaluated on: 1) RBC K^+ loss following KCNN4 activation; 2) RBC osmotic resistance; and 3) RBC Ca^{2+} activated K^+ current.

1) KCNN4 was activated by incubating RBCs with vanadate a Ca^{2+} pump inhibitor.[14] After 60-min incubation with vanadate a large K^+ efflux was observed in patient and control RBCs (Figure 3A). This K^+ loss was blocked by Senicapoc in a dose-dependent manner. The other KCNN4 inhibitor, TRAM-34, was used for comparison. The K^+ loss was correlated to a decrease in RBC volume (Figure 3B).

2) Freshly drawn blood was stored for 25 h at 37°C in presence of Senicapoc (4 or 0.4 μM) or TRAM-34 (10 μM a KCNN4 reference blocker) and compared to control condition (no blocker). The osmotic resistance of patient RBCs at 37°C was shifted to the left compared to control RBCs, giving 50% hemolysis respectively at 0.45 and 0.5 relative osmolarity (Figure 3C and D, blue curves). The slope of the curve was dramatically reduced for patient compared to control blood. Whereas incubation with Senicapoc did not alter osmotic resistance curve of control blood, there was a dose-dependent effect of Senicapoc only on patient RBCs in which the osmotic resistance was shifted to the right and the steepness of the curves increased in the presence of Senicapoc. TRAM-34 was able to decrease the osmotic resistance in patient but also slightly in control RBCs.

3) Figure 3E illustrates the activity of KCNN4 recorded in patient (R352H mutation) and control RBCs in whole-cell configuration. A significant increase in current was observed for patient RBCs compared to control. Both currents were completely blocked by 0.5 μM Senicapoc.

Thus, Senicapoc is efficient in preventing RBC K^+ loss and dehydration in case of R352H mutation. The similar sensitivity of WT KCNN4 in HEK293 cells and in RBCs[12] suggests that KCNN4 pore structure is not changed by the expression system. As Senicapoc acts through binding in the pore of the channel, it is likely that this drug would also block in RBCs K^+ loss through V282 mutated KCNN4. The discrepancy between mutants in HEK293 cells regarding the IC50 suggests that different dose-response curves might be expected in patient RBCs. In addition, the high hemoglobin concentration could buffer Senicapoc availability, thus, higher doses are expected to be efficient in blood than *in vitro*. The plasma concentration of Senicapoc giving 50% maximal inhibition of KCNN4 was estimated around 166 nM and 70% inhibition was observed with 311 nM Senicapoc.[15]

To conclude, a Senicapoc therapy to treat RBC dehydration due to mutations in KCNN4 is promising even though the different sensitivities of the mutants will require dose adjustments according to patient genotype. A beneficial effect *in vivo* in terms of an improvement in anemia remains to be demonstrated.

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