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1 **Title: Activating the apelin receptor with LIT01-196, a metabolically stable**
2 **apelin analog, reverses AVP-induced antidiuresis and hyponatremia**

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21

22 **Abstract:** Apelin and arginine-vasopressin (AVP) are conversely regulated by osmotic stimuli.
23 Targeting the apelin receptor (apelin-R) to treat the Syndrome of Inappropriate Antidiuresis
24 (SIAD), in which increased AVP secretion leads to hyponatremia, may be of interest. We
25 previously developed LIT01-196, a metabolically stable apelin-17 analog, which behaves as a
26 potent full agonist for the apelin-R. We show here that LIT01-196 has an *in vivo* half-life of 156
27 minutes in the bloodstream after subcutaneous (s.c.) administration in alert control rats. We
28 demonstrate that LIT01-196 efficiently decreases the cAMP production induced in collecting
29 ducts by dDAVP via AVP type 2 receptors (V2R). We also show in mouse cortical collecting
30 duct cells that LIT01-196 reduces dDAVP-induced apical cell surface expression of
31 phosphorylated aquaporin 2. This results in a LIT01-196-induced increase in aqueous diuresis in
32 control rats following s.c. administration, associated with a slight 11% increase in water intake
33 and no alteration in renal function after 4 days of treatment. In a rat experimental model of AVP-
34 induced hyponatremia, LIT01-196 s.c. administered (900 nmol/kg) for two days, blocked the
35 antidiuretic effect of AVP and the AVP-induced increase in urinary osmolality highly efficiently,
36 and induced a progressive correction of hyponatremia. These results demonstrate a major role of
37 apelin, together with AVP, in the regulation of water balance, and suggest that apelin-R
38 activation constitutes a new approach for hyponatremia treatment.

39

40

41 **Introduction**

42 Hyponatremia, defined in humans as a plasma sodium concentration below 135 mmol/l, is
43 associated with various diseases, including chronic heart failure (CHF), liver cirrhosis, diuretic
44 treatment and the Syndrome of Inappropriate Antidiuresis (SIAD) ¹. Hyponatremia is also
45 associated with high mortality rates ² in patients with liver failure ³, CHF ⁴ and chronic kidney
46 disease ⁵.

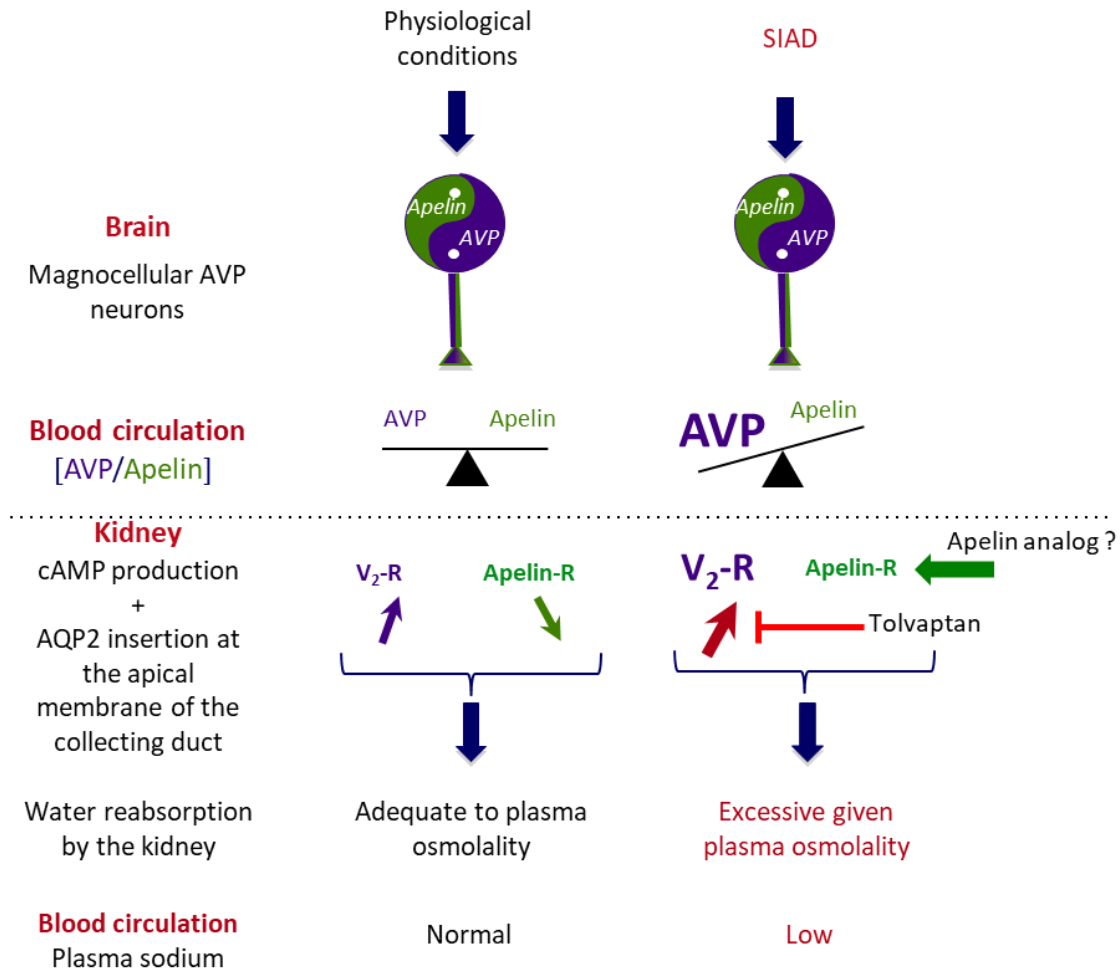
47 Arginine-vasopressin (AVP or antidiuretic hormone, ADH) type 2 receptors (V2-R) are
48 expressed in the kidney collecting ducts (CD). By acting on these receptors, AVP increases
49 cAMP production, leading to the insertion of aquaporin-2 (AQP-2) into the apical membrane of
50 CD, allowing water reabsorption and decreasing urine output (**Fig 1**) ⁶. In SIAD, plasma AVP
51 levels are inappropriately increased with respect to plasma osmolality ¹. V2-R antagonists, such
52 as tolvaptan, block the stimulation of V2-R by AVP, and have been shown to correct
53 hyponatremia efficiently in rodents ⁷ as well as in humans with SIAD ⁸⁻¹⁰. Expected effects of
54 aquaretic agents such as intense thirst, polyuria (24 h diuresis up to 6 L/day) and nocturia were
55 observed ¹¹. Tolvaptan at the dose of 7.5 mg/day was also reported to carry a significant risk of
56 overly rapid sodium correction, in 23 % of hyponatremia patients having baseline serum sodium
57 concentration <125 mmol/l ¹². These patients exhibit the highest risk of developing osmotic
58 demyelination syndrome when overly rapid correction of hyponatremia occurs ^{13,14}. Chronic
59 treatment with V2-R antagonists has also shown efficacy in autosomal dominant polycystic
60 kidney disease (ADPKD). However, aquaresis-related events led 8% of patients with ADPKD
61 receiving tolvaptan to discontinue treatment ¹⁵. Due to this concern and reports of hepatotoxicity
62 associated with the chronic use of high tolvaptan doses¹⁵⁻¹⁷, the development of new therapeutic

63 agents acting on new targets with a different mode of action could be useful for the treatment of
64 disorders linked to excessive AVP secretion such hyponatremia and ADPKD.

65 Apelin is a neuro-vasoactive peptide identified as the endogenous ligand of the human orphan G
66 protein-coupled receptor, APJ^{18,19}. Following the identification of its endogenous ligand, this
67 receptor was renamed the apelin receptor (apelin-R). Three molecular forms of apelin have been
68 identified *in vivo*, apelin-36, apelin-17 (K17F) and the pyroglutamyl form of apelin-13 (pE13F)
69^{20,21}. K17F has an affinity 16 times higher than that of pE13F for apelin-R (Ki, 0.05 nmol/l
70 versus 0.8 nmol/l) but both peptides inhibit similarly forskolin-induced cAMP production in cells
71 expressing the apelin-R. K17F is, however, 20 times more efficient than pE13F for inducing rat
72 apelin-R internalization and β -arrestin mobilization^{22,23}. Apelin and apelin-R^{24,25} are expressed
73 strongly in the supraoptic and paraventricular hypothalamic nuclei, in which double-labeling
74 studies have shown them to colocalize with AVP in magnocellular vasopressinergic neurons
75^{20,24-26}. The central administration of K17F in lactating rats, which display an increased phasic
76 electrical activity of magnocellular vasopressinergic neurons and an increased AVP release from
77 the posterior pituitary into the bloodstream, inhibits the activity of these neurons, leading to a
78 decrease in circulating AVP levels and an increase in aqueous diuresis²⁰. Apelin-R is also
79 expressed in the kidney, both in the glomeruli and in all nephron segments including CD
80 expressing AVP V2-Rs²⁷. The intravenous injection of K17F into lactating rats by decreasing
81 AVP-induced cAMP production, inhibits the AVP-induced AQP-2 insertion into the apical
82 membrane of CD and increases aqueous diuresis²⁸. These data were recently strengthened by the
83 observation that apelin 13 decreases the phosphorylation and the apical membrane expression of
84 AQP-2 in a highly differentiated mouse cortical CD cell line (mpkCCD) expressing the V2R and
85 the ApelinR²⁹. This suggests that the aquaretic effect of apelin is not only due to a central effect

86 involving an inhibition of AVP release into the bloodstream, but also to a direct renal effect in
87 the CD, counteracting the antidiuretic effect of AVP mediated by V2Rs (**Fig. 1**)^{30,31}.
88 Furthermore, plasma AVP and apelin levels are conversely regulated by osmotic stimuli as well
89 in rodents as in humans to maintain body fluid homeostasis^{20,21,32}. In hyponatremia patients with
90 SIAD³³, plasma copeptin levels (a biomarker of AVP release into the bloodstream in humans)
91 are high and inappropriate for plasma sodium levels³³. In addition, sex- and age-adjusted,
92 plasma apelin concentrations were 26% higher in SIAD patients than in healthy subjects.
93 Consequently, the balance between plasma apelin and AVP levels is not reached, as shown by
94 the apelin to copeptin ratio as a function of natremia that was outside the 95% predicted
95 physiological limits for plasma sodium in 86% of SIAD patients³³. This imbalance contributes
96 to the corresponding water metabolism defect in hyponatremia patients with SIAD (**Fig. 1**).
97 We therefore hypothesized that the supplementary activation of the apelin-R by a metabolically
98 stable K17F analog could counteract AVP-induced water reabsorption and correct hyponatremia.
99 Because the half-life of K17F is short (in the minute range *in vivo*), we generated LIT01-196
100 (**Fig. 2A**), a metabolically stable K17F analog, by adding a fluorocarbon chain to the N- terminal
101 part of K17F, to increase its plasma half-life (> 24 h versus 4.6 min for K17F)²². LIT01-196 has
102 a subnanomolar affinity for apelin-R and *in vitro* pharmacological profile similar to that of K17F
103²². In this study, we aimed (1) to determine the *in vivo* half-life of LIT01-196 in the blood
104 circulation and its capacity to enter the brain following systemic administration, (2) to verify the
105 efficiency of LIT01-196, as compared to K17F, to inhibit dDAVP-induced cAMP production in
106 freshly microdissected CD (3) to define if LIT01-196 decreases the apical cell surface expression
107 of phosphorylated aquaporin 2 (AQP-2) in mpkCCD cells (4) to determine the metabolic effects
108 of LIT01-196 administered subcutaneously (s.c.) in alert rats and (4) to assess whether the

109 activation of apelin-R by LIT01-196 reversed AVP-induced antidiuresis and corrected
 110 hyponatremia in a rodent hyponatremic model, using tolvaptan as a reference aquaretic agent.



111
 112 **Fig. 1. Apelin and vasopressin regulation in physiological conditions and in SIAD.** In
 113 physiological conditions, apelin and AVP are released in balanced proportions from the
 114 magnocellular AVP neurons, at levels appropriate for plasma osmolality. In the collecting duct
 115 of the kidney, AVP acts on V₂-R, to increase cAMP production and aquaporin-2 (AQP-2)
 116 insertion, leading to water reabsorption. Conversely, apelin, through its action on apelin-R, has
 117 the opposite effect. In physiological conditions, water reabsorption is adequate and plasma
 118 sodium concentrations are normal. In SIAD, AVP release is excessive relative to plasma
 119 osmolality, leading to excessive water reabsorption by the kidney and hyponatremia. Tolvaptan
 120 blocks the effect of AVP on V₂-R and corrects hyponatremia in SIAD. We hypothesize that the
 121 activation of apelin-R with a metabolically stable K17F analog, LIT01-196, would also correct

122 water homeostasis in this disease. Adapted from De Mota et al²⁰, Hus-Citharel et al²⁸ and
123 Blanchard et al³³.
124

125

126 **Materials and Methods**

127 *Study design*

128 Sample size was determined before the study. For the hyponatremia study, the detection of a
129 mean difference in plasma sodium concentration of 5 mmol/l (expected standard deviation of 3
130 mmol/l) between the treatment group and the control group would require the inclusion of at
131 least 8 animals in each treatment group. AVP-treated animals with an insufficient antidiuretic
132 response (urinary osmolality < 850 mOsm/kg) were excluded. The experimental design is
133 described in **Fig. 4A**. AVP-treated animals were randomly assigned to treatment groups.
134 Blinding was not feasible for treatment administration and sample collection, but the
135 investigators were not aware of the treatment group during sample analysis. Each *in vitro*
136 experiment was performed at least three times in triplicate.

137 *Drugs, antibodies and reagents*

138 K17F was synthesized by PolyPeptide Laboratories (Strasbourg, France). AVP and (Deamino-
139 Cys¹,D-Arg⁸)-vasopressin (dDAVP) were obtained from Bachem (Bubendorf, Switzerland).
140 Tolvaptan was obtained from Activate Scientific (Prien, Germany). LIT01-196 (300 mg) was
141 synthesized by the *Laboratoire d'Innovation Thérapeutique* (CNRS UMR7200, Illkirch, France
142 as previously described ²². [¹²⁵I]-pE13F (monoiodinated on Lys⁸ with Bolton-Hunter reagent)
143 and [¹²⁵I]-(Tyr²Arg⁸)-AVP were purchased from PerkinElmer (Wellesley, MA, USA). A
144 specific AVP-[Arg⁸] Ab was obtained from Peninsula Laboratories International (San Carlo,
145 CA, USA), and rabbit polyclonal antibodies directed against the apelin fragment K17F were
146 produced in the laboratory, as previously described ²⁴. Sodium heparinate, lithium heparinate and
147 EDTA were obtained from SIGMA-ALDRICH (Saint-Quentin Fallavier, France). Isoflurane

148 (Isovet) and meloxicam (Metacam) were obtained from Centravet (Nancy, France). Drugs
149 administered s.c. were diluted in 0.9% NaCl (saline, 1 ml/kg) for injection, except for tolvaptan,
150 which was administered in 100% DMSO (0.2 ml/kg) due to its poor solubility in water.

151 *Animals*

152 Swiss mice (25-30 g) and male Sprague Dawley rats (210-230g) were obtained from Charles
153 River Laboratories (L'Arbresle, France). Whilst housed in our animal facility, they were given
154 free access to normal chow and water, and they were maintained under 12-h light/dark cycles
155 throughout the study. When required for i.v. administration, a right femoral venous catheter was
156 implanted in the rats, by tunneling under the skin, under isoflurane anesthesia (4% for induction,
157 2% for maintenance). Meloxicam (Metacam) was administered postoperatively (1 mg/kg s.c.) for
158 analgesia. All experiments were carried out in accordance with current international guidelines
159 for the care and use of experimental animals, and the experimental protocols were approved by
160 the national animal ethics committee (CEEA, reference numbers 2016-10#3672, 2017-01 #7844
161 and 01966.02) and ethics regional committee for animal experimentation in Strasbourg (APAFIS
162 reference number 1341#2015080309399690).

163 *Cell line sources and culture conditions*

164 CHO-K1 cells were obtained from American Type Culture Collection, Rockville, MD, USA and
165 were maintained in Ham's F12 medium supplemented with 10% fetal calf serum, 0.5 mmol/l
166 glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. HEK-293T cells were obtained
167 from Cancer Research UK, London Research institute and were grown in DMEM medium
168 supplemented with 10% fetal calf serum, 0.5 mmol/l glutamine, 100 units/ml penicillin and 100
169 µg/ml streptomycin. A highly differentiated murine renal cortical collecting duct principal cell
170 (CCD) line, mpkCCDcl4³⁴, which has retained the main characteristics of the parental CCDs

171 from which they were derived³⁵. This cell line was provided by Dr Michel-Robert Popoff from
172 the Pasteur Institute who previously performed studies on these cells³⁶. mpkCCD cells were
173 grown in 1:1 (vol/vol) Ham's F12 : DMEM medium supplemented with 2% fetal calf serum, 20
174 mmol/l D-Glucose, 20 mmol/l Hepes, 1 % penicillin/streptomycin, 5 µg/ml insulin, 50 nmol/l
175 dexamethasone, 60 nmol/l sodium selenate, 1 nmol/l triiodothyronine, 10 ng/ml d'EGF and 5
176 µg/ml transferrin.

177 *Microdissection of rat outer medullary collecting ducts*

178 The left kidney of male rats was prepared for nephron microdissection, as previously described
179 ³⁷. Pieces of outer medullary collecting ducts were isolated under a stereomicroscope.

180 *Membrane preparations and radioligand binding experiments.*

181 Membranes from CHO cells stably expressing rat ApelinR-EGFP were prepared as previously
182 described ²³. We compared the affinities of K17F and the new batch of LIT01-196 by performing
183 classical binding studies with pE13F (Bolton-Hunter radioiodinated on the lysine residue in
184 position 8) as the radioligand. Briefly, membrane preparations (0.5–1 µg total mass of membrane
185 protein/assay) were incubated for 1 h at 20°C with 20 nmol/l [¹²⁵I]pE13F in binding buffer alone
186 (50 mmol/l Hepes, 5 mmol/l MgCl₂, 1% BSA, pH 7.4) or in the presence of pE13F, K17F or
187 LIT01-196 at various concentrations (0.01 pmol/l to 100 µmol/l). The reaction was stopped by
188 adding ice-cold binding buffer and filtering through Whatman GF/C filters. The filters were
189 washed and radioactivity was counted with a Wizard 1470 Wallac gamma counter (Perkin
190 Elmer, Turku, Finland).

191 *cAMP assay.*

192 cAMP assays are described in the Supplementary Materials and Methods.

193 *Measurement of phosphorylated AQP-2 immunoreactivity in mpkCCD cells treated by dDAVP,*
194 *dDAVP +K17F or dDAVP + LIT*

195 mpkCCD cells were seeded in 12 mm Transwell filters of 0.4 μm pore size at a density of 160,
196 000 cells/cm² and were grown for 5 days. Twenty-four hours before the experiment cells were
197 starved with depletion media (1:1 (vol/vol) Ham's F12 : DMEM medium). Then, cells were
198 treated at the basolateral compartment with depletion media (vehicle) or with 10 nmol/l of
199 dDAVP in the presence or in the absence of 10 $\mu\text{mol/l}$ K17F or LIT01-196 for 1h at 37 °C. Cell
200 culture medium was removed and cells were fixed with 4% formaldehyde in PBS1X for 10 min
201 at 4°C, and permeabilized with PBSTriton X-100 0.5% for 5 min at 20 °C. Then, inserts were
202 incubated in 5% PBS-BSA for 60 min at 20°C to block non-specific protein binding. Inserts
203 were subjected to immunostaining with a rabbit anti-AQP-2-pS269 antibody (1/200,
204 Cliniscience, France) and a mouse monoclonal anti-occludin antibody (1/200, Santa Cruz,
205 Cliniscience, France) for 16 hours at 4°C. Inserts were then incubated with goat anti-rabbit
206 Alexa Fluor Plus 647 or goat anti-mouse Alexa Fluor plus 488 secondary antibodies (Invitrogen)
207 for 60 min at 20°C. Inserts were detached and mounted onto microscopy slides with Pro Long
208 Aquafade containing DAPI (Invitrogen). Slides were analyzed in the x-y and x-z axes under a
209 63x/1.4 Oil WD: 0.17 mm objective with a Zeiss Axioobserver Z1 inverted spinning-disk
210 microscope equipped with a camera sCMOS Hamamatsu 2048*2048 / pixel size : 6.45*6.45 μm
211 and the lasers (405 nm; 100mW, 491 nm; 150mW and 642 nm; 100mW). Microscope settings
212 were identical for all the samples and experiments were randomized and blinded.

213 *Apelin and AVP radioimmunoassays*

214 The radioimmunoassays are described in the Supplementary Materials and Methods.

215 *Plasma LIT01-196 measurement by mass spectrometry analysis*

216 The mass spectrometry analysis is described in the Supplementary Materials and Methods.

217 *Plasma protein binding of LIT01-196*

218 Plasma protein binding of LIT01-196 is described in the Supplementary Materials and Methods.

219 *AVP-induced hyponatremia*

220 Hyponatremia was induced in male Sprague Dawley rats with an osmotic minipump (ALZET
221 2001, 200 μ l, flow rate of 1 μ l/h for 7 days), obtained from Durect Corporation (Cupertino, CA,
222 USA) and filled with a solution of saline and AVP (at various concentrations), which was
223 implanted subcutaneously under isoflurane anesthesia. Meloxicam was administered
224 postoperatively for analgesia. Animals were then individually housed in metabolic cages
225 (Techniplast), and given a semi-liquid diet consisting of 25 ml GelDiet Breeding-10 (SAFE,
226 Augy, France) mixed with 25 ml tap water/day, with water available ad libitum. Animal weight,
227 water and food consumption were measured daily. Urine was collected daily, urinary electrolytes
228 (Na^+ , K^+ , Cl^-) were determined with an ISE 3000 (Caretium Medical Instruments, Shenzhen,
229 China) and urinary osmolality was measured with a Cryobasic 1 osmometer (Astori Tecnica,
230 Poncarale, Italy). Blood samples were collected on ice, before implantation of the osmotic
231 minipump, and before drug administration, under isoflurane anesthesia, from the tail vein (4 μ l
232 of lithium heparinate (1600 IU/ml) for 400 μ l of blood). The blood samples were immediately
233 centrifuged (4°C, 2600 x g, 20 min), and plasma electrolyte (Na^+ , K^+ , Cl^-) levels were quantified
234 with an ISE 3000. On the last day of the experiment, blood was also collected on ice by
235 intracardiac puncture. This blood sample was collected into lithium heparinate under isoflurane
236 anesthesia; plasma electrolyte levels were determined as described above and the animals were
237 immediately killed by carbon monoxide inhalation. The experimental protocol is summarized in

238 **Fig. 4A.**

239 *Plasma glucose levels and fractional excretion of water and sodium*

240 Fractional excretion of water (in %) was calculated using the following formula: (plasma
241 creatinine / urine creatinine) x100. Fractional excretion of sodium (in %) was calculated using
242 the following formula: (plasma creatinine x urine sodium / urine creatinine x plasma sodium) x
243 100. Blood and urine creatinine, urea nitrogen and glucose levels were determined using an
244 AU5800 Beckman Coulter chemistry analyzer (Beckman Coulter, Villepinte, France).

245 *Histological analyzes*

246 Histological analyzes methods are described in the Supplementary Materials and Methods.

247 *Blood pressure measurements*

248 Blood pressure measurements are described in the Supplementary Materials and Methods.

249 *Echocardiographic measurements*

250 Left ventricular ejection fraction was determined in mice under isoflurane anesthesia at baseline
251 and 3 hours after s.c. administration of the compound, as previously described³⁸.

252 *Statistical analysis*

253 We used Mann-Whitney U tests or paired Wilcoxon tests for comparisons between two groups,
254 and ANOVA or Kruskal-Wallis tests followed by post-hoc comparisons for comparisons
255 between multiple groups, when appropriate. We used a linear mixed-effects model to compare
256 repeated values recorded over time, with time, treatment group and the measured parameter as
257 fixed effects and the unique animal identification number as a random effect. We assessed the
258 effects of treatment by performing an analysis restricted to measurements taken after treatment
259 administration. The normality of the data was assessed visually and with the Shapiro-Wilk
260 normality test. The data represented are means, with error bars to indicate the standard error of

261 the mean. The number of experiments or animals used is indicated in each figure legend.

262 Statistical analysis was performed with R software version 3.4.3³⁹.

263

264 **Results**

265 *Ability of LIT01-196 to bind and activate ApelinR*

266 We first evaluated the ability of the new batch of LIT01-196 (300 mg), synthesized in large
267 quantities for *in vivo* experiments, to bind to apelin-R and to inhibit forskolin-induced cAMP
268 production in CHO cells stably expressing rat apelin-R-EGFP. As previously reported²², LIT01-
269 196, like K17F, bound to rat apelin-R with an affinity in the subnanomolar range (K_i values for
270 K17F and LIT01-196 of 0.14 ± 0.02 and 0.34 ± 0.05 nmol/l, respectively) (**Fig. S1A**). In CHO
271 cells stably expressing apelin-R-EGFP, LIT01-196 inhibited the cAMP production induced by 5
272 $\mu\text{mol/l}$ forskolin with an IC_{50} of 1.7 ± 0.5 nmol/l, whereas the IC_{50} for K17F was 0.6 ± 0.2 nmol/l
273 (**Fig. S1B**), as previously described²².

274 To ensure the selectivity of action of LIT01-196 on the apelin-R and not on the V2-R, we
275 assessed whether LIT01-196 modified cAMP production induced by V2-R stimulation. In HEK-
276 293 T cells expressing the human V2-R, we observed that both LIT01-196 and K17F at a
277 supramaximal concentration of 1 $\mu\text{mol/l}$ did not modify cAMP production induced by 1 $\mu\text{mol/l}$
278 AVP. Furthermore, K17F or LIT01-196 alone at 1 $\mu\text{mol/l}$ did not modify basal cAMP
279 production in these cells (**Fig. 2B**). We also verified that K17F and LIT01-196 were able to
280 inhibit forskolin-induced cAMP production in a highly differentiated murine renal cortical
281 collecting duct principal cell line (mpkCCD cells). We found in these cells that K17F (1 $\mu\text{mol/l}$)
282 and LIT01-196 (1 $\mu\text{mol/l}$) significantly inhibit cAMP production induced by forskolin (1 $\mu\text{mol/l}$)
283 by 33 % and 32 %, respectively (**Fig. 2C**). Finally, we checked whether K17F and LIT01-196
284 were able to inhibit dDAVP-induced cAMP production in mpkCCD cells. We observed that
285 K17F (10 $\mu\text{mol/l}$) and LIT01-196 (10 $\mu\text{mol/l}$) inhibit dDAVP-induced cAMP production by 75%
286 and 79%, respectively (**Fig. 2D**).

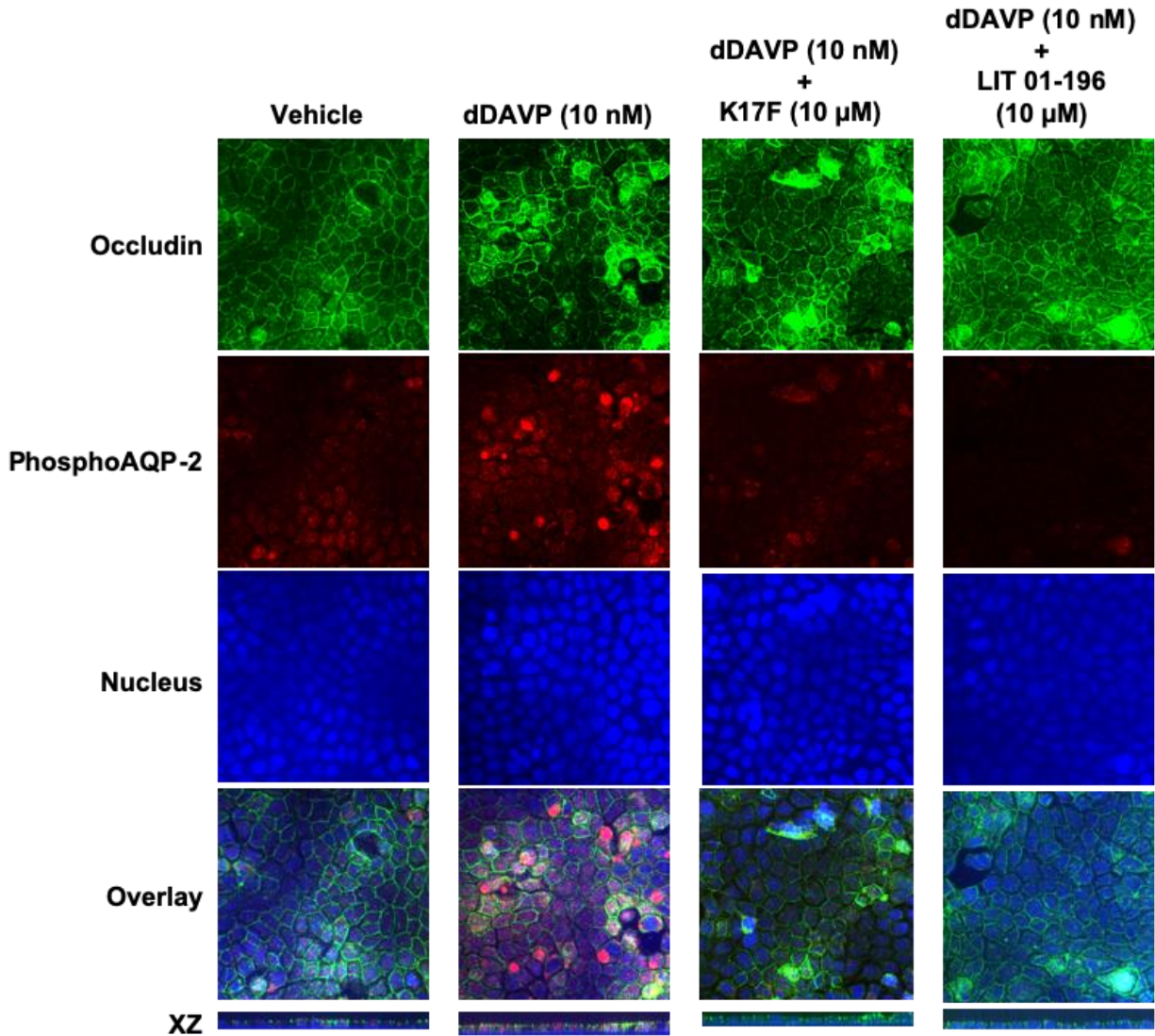
287 *Effects of K17F and LIT01-196 on dDAVP-induced cAMP production in microdissected rat*
288 *outer medullary collecting ducts*

289 We previously showed that dDAVP, a specific and selective V2-R agonist, induced a maximal
290 increase in cAMP production in rat outer medullary collecting ducts (OMCDs) at a concentration
291 of 0.1 nmol/l, and the EC₅₀ was 35 pmol/l²⁸. We show here, in the same experimental
292 conditions, that applying dDAVP (0.1 nmol/l) to microdissected rat OMCDs increased cAMP
293 production (92.6 ± 3.6 vs. 19.3 ± 0.8 fmol cAMP/mm tubular length/10 min for dDAVP and
294 baseline cAMP values, respectively, *P*<0.001, *n*=6). The co-application of dDAVP (0.1 nmol/l)
295 with K17F or LIT01-196 in increasing concentrations (from 50 nmol/l to 50 μmol/l)
296 progressively and significantly inhibited dDAVP-induced cAMP production by 45% and 41%,
297 respectively, in the OMCDs (**Fig. 2E**).

308 cAMP production in mpkCCD cells for 30 min at 37°C. Each data represents the mean of four
309 independent experiments performed in quadruplicate and expressed as the mean \pm S.E.M. **
310 $p < 0.01$ when compared to forskolin alone. **(D)** Effect of K17F and LIT01-196 treatment on
311 dDAVP-induced cAMP production. mpkCCD cells grown on permeable inserts were treated
312 with depletion medium containing 10 nmol/l of dDAVP or in combination of dDAVP and 10
313 μ mol/l K17F or LIT01-196B for 60 min at 37°C. Each data represent the mean of 3 independent
314 experiments performed in quadruplicate and expressed as the mean \pm S.E.M.. *** $p < 0.001$ when
315 compared to dDAVP alone **(E)** Effects of 0.1 nmol/l dDAVP on cAMP production in
316 microdissected rat outer medullary collecting ducts in the presence or absence of different
317 concentrations (50 nmol/l, 500 nmol/l and 50 μ mol/l) of K17F or LIT01-196. Six individual
318 determinations were performed. Each point was compared to dDAVP (10^{-6} mol/l). § denotes a
319 statistical difference ($p < 0.001$) with basal levels (No dDAVP).

320 *Effects of K17F and LIT01-196 on dDAVP-induced phosphorylation of AQP-2 in mpkCCD cells*

321 As expected from the literature^{29,34}, we observed an increase in the phosphorylation of AQP-2 at
322 the apical membrane of the mpkCCD cells treated with 10 nmol/l of dDAVP for 1h. When
323 mpkCCD cells were co-treated with K17F (10 μ mol/l) or LIT01-196 (10 μ mol/l) we observed a
324 decrease in the phosphorylation of AQP-2 as shown by the decrease of the immunolabelling of
325 the phosphoAQP-2 (**Fig. 3**).



326

327 **Figure 3. Effect of K17F and LIT01-196 treatment on dDAVP-induced phosphorylation of**
 328 **aquaporin 2.** mpkCCD cells were grown on transwell filters for 5 days. After 24h of starvation
 329 cells were treated with vehicle or 10 nmol/l of dDAVP with or without 10 μmol/l of K17F or
 330 LIT01-196. Images are shown in XY and XZ plans and represent the immunofluorescence
 331 staining with an anti-occluding (green) and an anti-PhosphoAQP-2 (red) and the staining of the
 332 nuclei with DAPI (blue).

333 *In vivo half-lives of K17F and LIT01-196*

334 Plasma apelin levels were measured by RIA, with the K17F antiserum that we produced and
 335 characterized in a previous study²⁰. The antibody directed against K17F recognized K17F,

336 pE13F and LIT01-196 with a similar affinity (IC_{50} : 0.11 ± 0.01 nmol/l for K17F, 0.26 ± 0.03
337 nmol/l for pE13F, 0.57 ± 0.05 nmol/l for LIT01-196, **Fig. 4A**). It does not recognize K16P²⁰ or
338 Fluoro-K16P ($IC_{50} > 1.0$ μ mol/l for Fluoro-K16P). We determined plasma apelin concentrations
339 at baseline and at different times after the i.v. administration of K17F (250 nmol/kg, i.v.) or
340 LIT01-196 (15 nmol/kg, i.v. or 900 nmol/kg, s.c.) to Sprague-Dawley rats and mice. Plasma
341 apelin concentrations correspond to apelin levels at baseline or to apelin plus LIT01-196-
342 immunoreactive (IR) material after LIT01-196 injection in alert control animals, thus enabling to
343 estimate the *in vivo* half-life of LIT01-196. We estimated the *in vivo* half-life of K17F in the rat
344 blood circulation at 50 seconds (**Fig 4B**), accounting for the very brief (2 minutes) decrease in
345 blood pressure and increase in heart rate observed in rats following the i.v. injection of K17F⁴⁰.
346 In conscious mice, basal plasma apelin levels were 0.98 ± 0.09 pmol/ml. Five seconds after the
347 i.v injection of K17F (250 nmol/kg), plasma apelin levels had risen to 49.2 ± 7.95 pmol/ml, 50
348 times basal levels. Plasma apelin concentration decreased rapidly after 60 seconds, reaching 2.8
349 times the basal level after 300 seconds (**Fig. S2A**). The *in vivo* half-life of K17F in the mouse
350 blood circulation has been estimated at 44 seconds, very similar to that obtained in rats (50
351 seconds) . Both i.v. and s.c. administrations of LIT01-196 to alert rats and mice led to a large
352 increase in plasma apelin-IR levels. We estimated that the *in vivo* half-life of LIT01-196 in the
353 blood circulation in alert rats was 28 minutes after i.v. administration (**Fig. 4C**) and 156 min
354 after a s.c. administration (**Fig. 4D**). Using mass spectrometry, we evaluated the half-life of
355 LIT01-196 after its injection by i.v. route at the dose of 300 nmol/kg in mice (**Fig. 4E**), that was
356 estimated at 25 min, close to that obtained in rats after i.v. administration (28 min) by using the
357 RIA technique. To explain the higher plasma stability of LIT01-196 as compared to apelin, we
358 explored the possibility that the fluorocarbon chain could bind to plasma proteins resulting in the

359 protection of the peptide from enzymatic degradation by steric hindrance. By the classical
360 Diamon equilibrium dialysis chamber analysis⁴¹ we showed that the bound fraction of LIT01-
361 196 to plasma proteins was $69 \pm 7\%$ (value reached at equilibrium).
362 Plasma apelin levels 30 min after s.c. administration of LIT01-196 (900 nmol/kg, **Fig. 5F**) to
363 Sprague Dawley rats were 1.3 ± 0.22 pmol/ml, 3.6 times higher than basal levels (0.36 ± 0.05
364 pmol/ml, $p=0.008$). Conversely, no change was observed in apelin-IR levels measured in the
365 brain of rats treated with LIT01-196 (1.3 ± 0.14 pmol/brain) compared to basal values obtained
366 in rats treated with saline (1.2 ± 0.15 pmol/brain, $p=0.84$, **Fig. 4G**). The increase in plasma
367 apelin-IR was also observed in mice 30 min after s.c. administration of LIT01-196 (900 nmol/kg,
368 **Fig. S2B**). Plasma apelin-IR levels had risen to 12.4 ± 2.5 pmol/ml, 9.7 times basal levels ($1.3 \pm$
369 0.073 pmol/ml, $p=0.01$). At the opposite, no change was observed in apelin-IR levels measured
370 in the brain of mice treated with LIT01-196 (0.440 ± 0.073 pmol/brain) compared to basal values
371 obtained in mice treated with saline (0.391 ± 0.045 pmol/brain, $p=0.8$, **Fig. S2C**).

372 *Effects of K17F and LIT01-196 given by i.v. route on urine output in normonatremic rats*

373 We evaluated the effects on urine output of an intravenous (i.v.) administration of K17F and
374 LIT01-196 in control Sprague-Dawley rats. The i.v. injection of K17F (400 nmol/kg, 855 $\mu\text{g/kg}$)
375 induced no significant change in 24-hour urine output or in urine osmolality in control rats. By
376 contrast, the i.v. administration of LIT01-196 at a dose 27 times lower (15 nmol/kg, 51 $\mu\text{g/kg}$)
377 induced a 93% increase in urine output ($p<0.01$) (**Fig. S2D**) and a 38% decrease in urine
378 osmolality ($p<0.01$) relative to the values obtained for rats receiving saline i.v. (**Fig. S2E**). The
379 i.v. administration of K17F or of LIT01-196 did not induce a significant change in water intake
380 (**Fig S2F**).

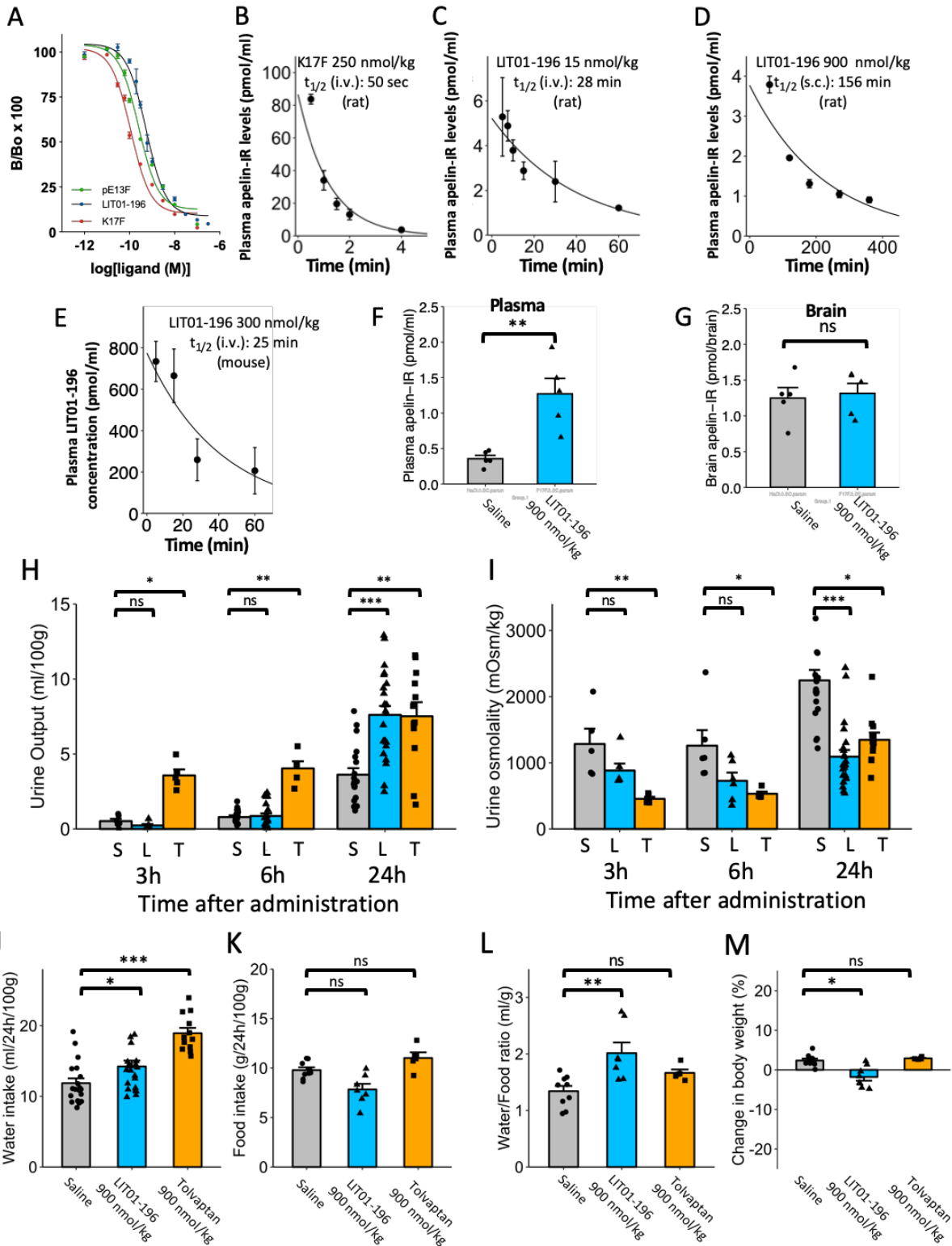
381 *Effects of LIT01-196 and tolvaptan given by s.c. route on metabolic parameters in*
382 *normonatremic rats*

383 We first determined in normonatremic rats the optimal dose of LIT01-196 to induce aqueous
384 diuresis. Subcutaneous LIT01-196 from 300 to 900 nmol/kg induced a progressive increase in
385 diuresis and a decrease in urine osmolality. The maximal effect was observed for doses
386 comprised between 900 and 2400 nmol/kg. The effects of LIT01-196 and tolvaptan on urine
387 output and osmolality were similar at an equimolar dose of 900 nmol/kg. However, increasing
388 the dose of tolvaptan to 6700 nmol/kg was associated with an increased effect on urine output
389 and urine osmolality, while increasing the dose of LIT01-196 to 2400 nmol/kg did not
390 significantly increase its aquaretic effect ~~of LIT01-196~~ (**Fig. S3A and S3B**). We thus used the
391 dose of 900 nmol/kg to study the effects LIT01-196, and compared them to both the equimolar
392 dose of tolvaptan and to a higher dose.

393 In addition, we compared the kinetics of the effects of LIT01-196 and tolvaptan on metabolic
394 parameters in alert normonatremic Sprague-Dawley rats. Relative to saline ($n=19$), LIT01-196
395 (900 nmol/kg, $n=23$) and tolvaptan (900 nmol/kg, $n=12$) significantly increased 24 h-urine
396 output by 108% and 110%, respectively. Urine osmolality decreased similarly in the two groups,
397 by 51% in animals receiving LIT01-196 and 40% in animals receiving tolvaptan. Although the
398 aquaretic effect of tolvaptan was significant at 3, 6 and 24 hours after administration, the
399 aquaretic effect of LIT01-196 was only significant 24 hours after administration, showing an
400 effect occurring later with this compound (**Fig. 4 H-I**). Increasing the dose of LIT01-196 to 2400
401 nmol/kg did not result in an earlier effect on aqueous diuresis (**Fig. S3 C-D**). The increase in
402 urine output was associated with a significant increase in water intake (**Fig. 4J**) in the tolvaptan
403 group (+37%) and a slight increase in the LIT01-196 group (+11%). Food (normal chow) intake

404 **(Fig. 4K)** was similar in the saline and the tolvaptan groups but slightly and non-significantly
405 lower in animals receiving LIT01-196 (20% decrease). Water intake to food intake ratio was
406 significantly increased by 50% in the LIT01-196 group and non-significantly increased by 24%
407 in the tolvaptan group **(Fig. 4L)**. A slight weight loss (-1.8%) **(Fig. 4M)** was observed in the
408 LIT01-196 group.

409 Tolvaptan at a higher dose (6700 nmol/kg, n=13) induced a larger aquaretic effect than LIT01-
410 196 at both 900 and 2400 nmol/kg **(Fig S4 A-B)**, that occurred more rapidly than with LIT01-
411 196 **(Fig S3 C-D)**. LIT01-196 (900 nmol/kg) significantly increased free water excretion fraction
412 by 86%, while tolvaptan (6700 nmol/kg) induced a similar but non-significant increase **(Fig.**
413 **S4C)**. Sodium excretion fraction **(Fig. S4D)** was not significantly modified by the administration
414 of LIT01-196 (900 nmol/kg) or tolvaptan (6700 nmol/kg).



415

416 **Fig 4. In vivo half-life of K17F and LIT01-196 and effects of s.c. saline, tolvaptan, LIT01-**
 417 **196 on water metabolism, food intake, body weight, plasma electrolytes in control rats. (A)**

418 Inhibition of [¹²⁵I]-pE13F binding to anti-K17F serum (1/18000 final dilution) by unlabeled
419 K17F, pE13F or LIT01-196. Data are expressed as a percentage of the maximal binding of [¹²⁵I]-
420 pE13F in the absence of non-radiolabeled ligand and represent three independent experiments
421 performed in triplicate. **(B)** *In vivo* half-life of K17F following i.v. administration in rats. K17F
422 (250 nmol/kg i.v.) was injected in alert rats. Animals (n=3-6 per time point) were killed at
423 various times following injection, trunk blood was collected and plasma apelin-immunoreactive
424 levels were determined in a radioimmunoassay. **(C)** *In vivo* half-life of LIT01-196 following i.v.
425 administration in rats. LIT01-196 (15 nmol/kg i.v.) was administered to alert rats. Animals (n=3-
426 6 per time point) were killed at various times following injection, trunk blood was collected and
427 plasma apelin-immunoreactive levels were determined in a radioimmunoassay. **(D)** *In vivo* half-
428 life of LIT01-196 following s.c. administration (900 nmol/kg) in rats. Animals (n=3-6 per time
429 point) were killed at various times following injection, trunk blood was collected and plasma
430 apelin-immunoreactive levels were determined in a radioimmunoassay. **(E)** *In vivo* half-life of
431 LIT01-196 following i.v. administration in mice (3 animals per time point). LIT01-196 (300
432 nmol/kg i.v.) was administered to alert mice and plasma LIT01-196 concentration was
433 determined by mass spectrometry analysis. **(F)** Plasma and **(G)** brain apelin-immunoreactive
434 levels in rats (n=5) 30 minutes after subcutaneous administration of saline or of LIT01-196 (900
435 nmol/kg). **(H-M)** *In vivo* effects of s.c. saline, tolvaptan, LIT01-196. Evaluation of the effect of
436 saline (1 ml/kg, n=19); LIT01-196 (900 nmol/kg, n=23) and tolvaptan (900 nmol/kg, n=12) in
437 alert male Sprague-Dawley (SD) rats given normal chow on **(H)** Urine output and **(I)** urine
438 osmolality measured 3, 6 and 24 hours after saline (S), LIT01-196 (L) and tolvaptan (T).
439 Metabolic parameters were measured 24 hours after the s.c. administration of saline, LIT01-196
440 or tolvaptan **(J)**, water intake; **(K)**, food intake; **(L)**, water to food intake ratio; **(M)**, body weight
441 change). Multiple comparisons performed by Kruskal-Wallis followed by post-hoc Dunn's tests
442 with Holm's adjustment. Ns: $p>0.05$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

443 *Effects of LIT01-196 and tolvaptan on plasma AVP and apelin levels in control rats*

444 Plasma AVP and apelin (apelin-immunoreactive (IR) material) levels were measured three hours
445 after the s.c. administration of saline (n=9), tolvaptan (6700 nmol/kg s.c., n=6) and LIT01-196
446 (900 nmol/kg s.c., n=8) into alert rats (**Fig. S4 E-G**). Rats receiving tolvaptan had significantly

447 higher plasma AVP levels (134% higher; $P<0.001$) than control animals receiving saline, and no
448 change in plasma AVP levels was observed in rats receiving LIT01-196.

449 Plasma apelin-IR levels in control rats (receiving saline) and in rats receiving tolvaptan were
450 similar. Plasma apelin-IR levels in rats receiving LIT01-196 were significantly higher (140%)
451 than those in control rats ($p<0.001$). Plasma apelin/AVP ratio was not significantly different in
452 rats receiving tolvaptan or saline, but was higher in rats receiving LIT01-196 (91% higher than in
453 control rats; $p=0.003$).

454 *Effects of LIT01-196 on blood pressure, cardiac contractility, kidney function and blood glucose*

455 Following s.c. injection of LIT01-196 (900 nmol/kg) in alert Sprague-Dawley rats, mean arterial
456 blood pressure (MABP) decreased slightly 3 hours after administration, and this change did not
457 reach statistical significance (baseline MABP, 106 ± 2 mmHg, MABP three hours after LIT01-
458 196 administration, 94 ± 2 mmHg, $p=0.063$, $n=5$). MABP measured 24 hours after administration
459 of LIT01-196 did not significantly differ from baseline ($p=0.88$) (**Fig. S5A**). We also evaluated
460 the effects of s.c. LIT01-196 administration on left ventricular ejection fraction (LVEF)
461 estimated by echocardiography in mice ($n=5$) and did not observe any significant effect at the
462 slightly higher dose of 1200 nmol/kg (LVEF 3 hours after administration, 57.1 ± 0.7 % vs $52.6 \pm$
463 3.0 % before administration, $p=0.31$) (**Fig. S5B**). Following the s.c. administration of LIT01-196
464 at the dose of 900 nmol/kg for 4 consecutive days, we performed histological analysis of the
465 kidneys that did not reveal any abnormality (**Fig. S5 C-F**). Blood creatinine, urea nitrogen,
466 sodium and potassium levels (**Fig. S5G-J**) were not changed by LIT01-196 treatment. Repeated
467 administration of LIT01-196 (900 nmol/kg, s.c.) to normonatremic Sprague Dawley rats was not
468 associated with a change in plasma or urine glucose levels (**Fig. S5K and S5L**).

469 *Establishment of a rodent model of AVP-induced hyponatremia*

470 We established a rodent model of AVP-induced hyponatremia by administering AVP
471 subcutaneously and continuously via an osmotic minipump in male Sprague-Dawley rats placed
472 in metabolic cages and fed a liquid diet. The effects of different doses of AVP on urine output,
473 urine osmolality and plasma sodium, water and food intake were evaluated. For a dose of 30 or
474 50 ng AVP/hour, urine output decreased during the four days following insertion of the osmotic
475 minipump (mean decrease of 61% in rats receiving AVP at a dose of 30 ng/h relative to rats
476 receiving saline), urine osmolality increased by 455% and plasma sodium concentration
477 decreased by 26% ($p < 0.001$ for urine output, urine osmolality and plasma sodium). Animals
478 treated with AVP at a dose of 30 ng/h or higher also decreased their food and water intake
479 ($p < 0.001$) (**Fig. S6**). Little or no effect was observed with a lower dose of AVP (20 ng AVP/h).
480 A moderate decrease in body weight was observed during the experiment in both hyponatremic
481 and normonatremic animals, even in the absence of AVP administration, but weight loss was
482 significantly greater in animals receiving 30 ng/h AVP or more (**Fig. S6D**). The experiment was
483 stopped after five days because some of the animals had lost more than 20% of their body
484 weight, the acceptable limit established with the ethics committee. The minimum effective dose
485 of AVP (30 ng/h) was therefore used to study the effects of aquaretic agents on AVP-induced
486 hyponatremia.

487 *Determination of the optimal dose of LIT01-196 in hyponatremic rats*

488 We then determined that the administration of both LIT01-196 and tolvaptan, at the dose of 900
489 nmol/kg, induced a similar increase in diuresis in the 24 hours following s.c. administration in
490 rats receiving 30 ng/h AVP and a liquid diet (increase of 11.7 ± 2.3 and 9.5 ± 2.9 ml/24h/100g,
491 respectively). In contrast, urine output did not change 24 hours after administration of saline. The

492 use of a lower dose of LIT01-196 (180 nmol/kg s.c.) was associated with a smaller increase of
493 urine output (increase of 1.5 ± 2.0 ml/24h/100g), while increasing the dose of LIT01-196 to 2400
494 nmol/kg did not increase the effect of the compound on urine output (increase of 9.2 ± 4.1
495 ml/24h/100g). In contrast, increasing the dose of tolvaptan to 6700 nmol/kg led to an even
496 greater increase in urine output (increase of 27 ± 9.0 ml/24h/100g). Given these results, we used
497 the identical dose of 900 nmol/kg to compare the metabolic effects of LIT01-196 and tolvaptan
498 in a model of AVP-induced hyponatremia.

499 *Evaluation of the effects of LIT01-196 and tolvaptan in a model of AVP-induced hyponatremia*

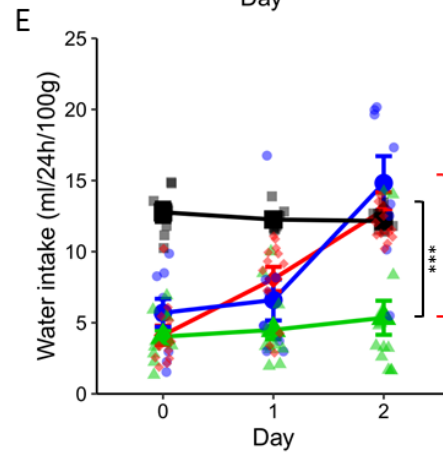
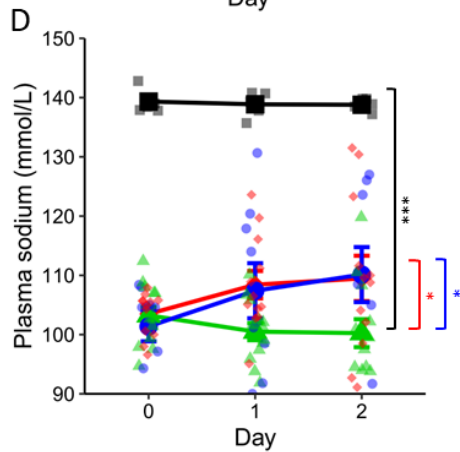
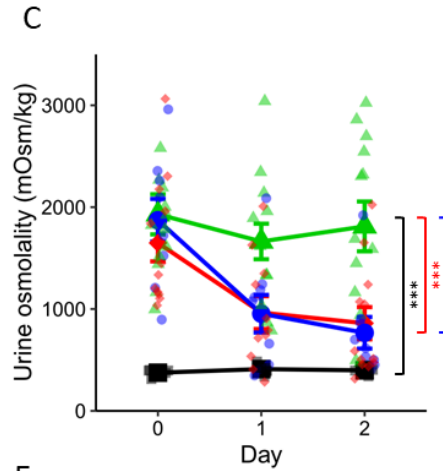
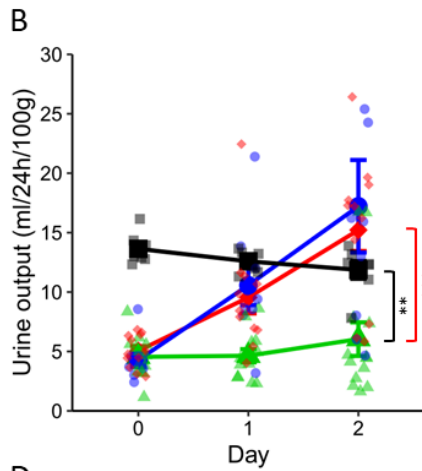
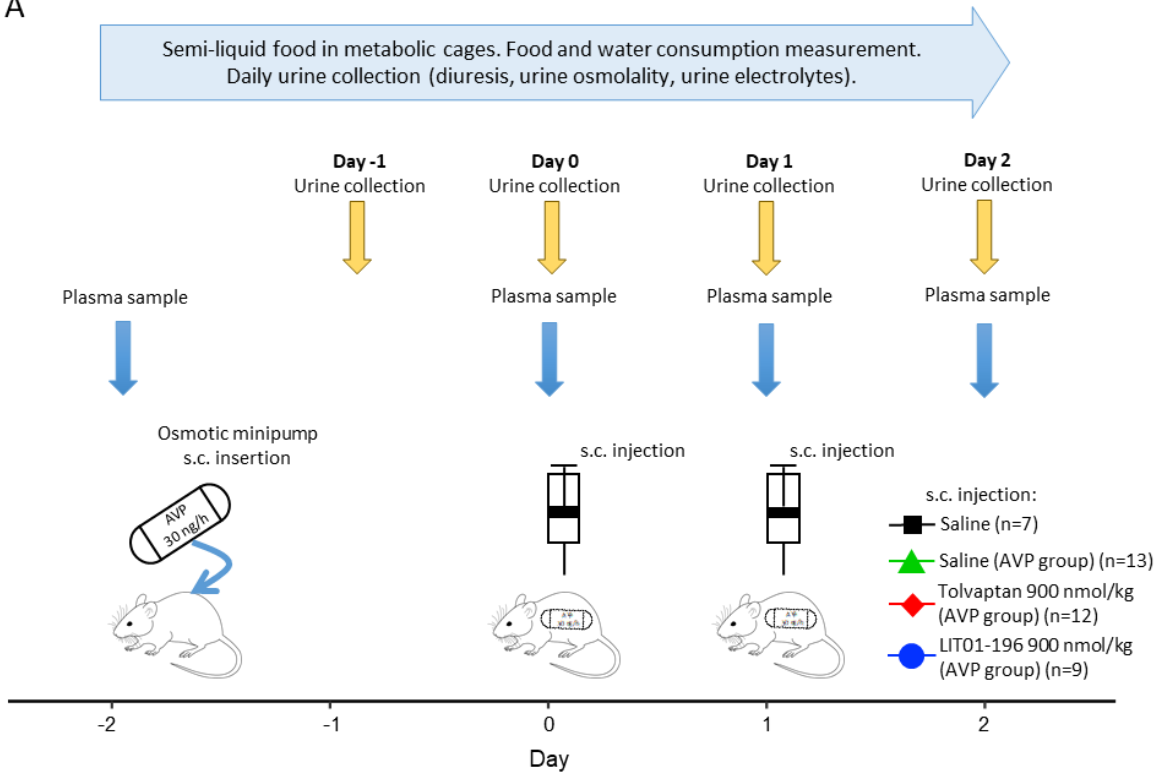
500 As described above, a minipump administering 30 ng AVP/h s.c. was implanted two days before
501 the administration of an aquaretic treatment (tolvaptan or LIT01-196) or saline, and all animals
502 received a semi-liquid diet (50% water, 50% gel-diet, as described in Methods). Water
503 consumption included water mixed with the food as well as water consumed from the bottle.
504 (experimental protocol, **Fig. 5A**). Four experimental groups were studied: the control group
505 (receiving no AVP, $n=7$), the AVP + saline group (continuous s.c. infusion of 30 ng AVP/h +
506 saline 1 ml/kg s.c. on days 0 and 1, $n=13$), the AVP + tolvaptan group (30 ng AVP/h s.c. +
507 tolvaptan 900 nmol/kg s.c. on days 0 and 1, $n=12$) and the AVP + LIT01-196 group (30 ng
508 AVP/h s.c. + LIT01-196 900 nmol/kg s.c. on days 0 and 1, $n=9$). On day 0, before treatment
509 administration, plasma sodium levels were low and similar in all animals receiving AVP ($103 \pm$
510 1 , 103 ± 1 and 101 ± 3 mmol/l, $p=0.6$, for the AVP + saline, AVP + tolvaptan and AVP + LIT01-
511 196 groups, respectively). Animals receiving AVP + saline had significantly lower urine output
512 than controls (49% lower on average between day 1 and day 2, $P=0.002$), higher urine osmolality
513 (355% higher, $P<0.001$), lower water intake (59% lower, $P<0.001$) and lower plasma sodium

514 levels (27% lower, $P<0.001$). These parameters remained stable from day 0 to day 2 (**Fig. 5 B-**
515 **E**).

516 We then evaluated the effects of LIT01-196 in hyponatremic rats. The model was set up as
517 described above, and LIT01-196 (900 nmol/kg s.c.) was administered on days 0 and 1. Relative
518 to AVP-treated animals receiving saline, the LIT01-196-treated animals had significantly higher
519 urine output (185% higher, on average, from day 1 to day 2, relative to the AVP + saline group,
520 $P<0.001$), and lower urine osmolality (58% lower, $P<0.001$). An increase in plasma sodium
521 concentration was observed on day 1, 24 hours after the first administration of LIT01-196,
522 relative to animals receiving saline (109 ± 5 versus 100 ± 2 mmol/l), and on day 2 (110 ± 5
523 versus 100 ± 2 mmol/l), and plasma sodium concentration after LIT01-196 administration were
524 significantly higher than in the AVP + saline group ($P=0.042$). Following treatment with LIT01-
525 196, AVP-treated animals had 177% higher levels of water consumption (including water in
526 diet), on average, from day 1 to day 2 ($P<0.001$, versus the AVP + saline group) (**Fig. 5B-E**).
527 LIT01-196 treatment was associated with a significant increase in food consumption, and
528 thereby a significant increase in sodium intake. Body weight change from baseline was similar in
529 AVP-treated animals receiving LIT01-196 and saline (**Fig. S7**). Subcutaneous administration of
530 tolvaptan at the same dose (900 nmol/kg) on days 0 and 1 was associated with a similar effect on
531 urine output, urine osmolality, plasma sodium and water intake (**Fig. 5B-E**).

532

A



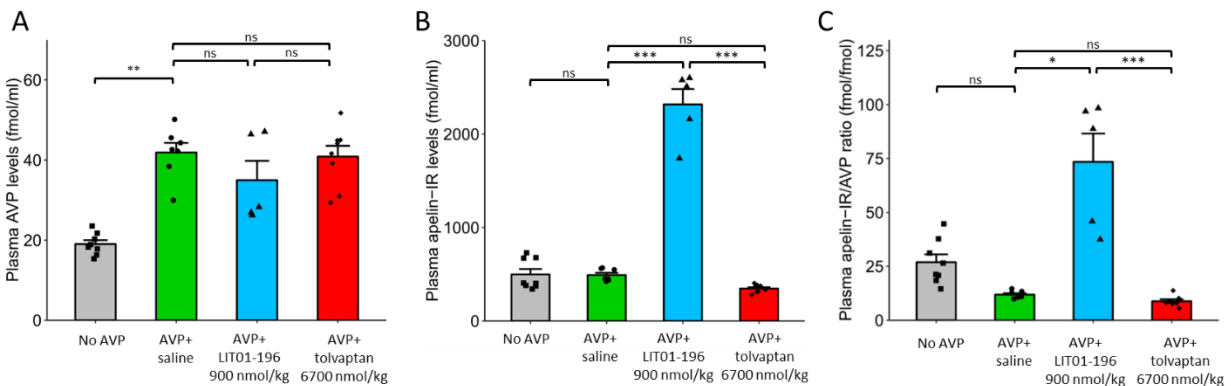
■ Saline ▲ AVP+saline ◆ AVP+tolvaptan 900 nmol/kg s.c. ● AVP+LIT01-196 900 nmol/kg s.c.

534 **Fig. 5. Effects of saline, tolvaptan and LIT01-196 on water metabolism and plasma**
535 **electrolytes in hyponatremic rats. (A)** Experimental protocol for generating hyponatremia. On
536 day -2, a plasma sample was collected from the tail vein of each animal (male SD rats). Except
537 for the control group, which received no AVP, a subcutaneous osmotic minipump delivering
538 AVP at a rate of 30 ng/h was then implanted into each animal. All the animals were given semi-
539 liquid food. On day 0, a plasma sample was collected and each animal treated with AVP received
540 a s.c. injection of saline (green curve, 1 ml/kg, $n=13$), tolvaptan (red curve, 900 nmol/kg, $n=12$)
541 or LIT01-196 (blue curve, 900 nmol/kg, $n=9$). Animals not treated with AVP received a s.c.
542 injection of saline (black curve, $n=7$). On day 1, plasma samples were obtained and each animal
543 received another administration of the same treatment. On day 2, a final plasma sample was
544 collected and the animals were killed. Urine output was measured and analyzed every day from
545 day -1 to day 2, and water and food intake were also measured daily. Change in 24-hour urine
546 output **(B)**, urine osmolality **(C)**, plasma sodium concentration **(D)** and water intake (including
547 water in the bottle and in the food) **(E)** in animals receiving continuous s.c. AVP at a rate of 30
548 ng/h from day -2 to 2, and a s.c. injection of saline (green curve, $n=13$), tolvaptan (red curve, 900
549 nmol/kg, $n=12$) or LIT01-196 (blue curve, 900 nmol/kg, $n=9$) on days 0 and 1. The black curve
550 represents animals receiving no AVP ($n=7$); all animals received a semi-liquid diet. Each group
551 was compared with the AVP + saline group with a linear mixed-effects model, to take repeated
552 measurements over time into account; ns: $P>0.05$, $*P<0.05$, $**P<0.01$, $***P<0.001$.

553 *Plasma AVP and apelin levels following the administration of LIT01-196 or tolvaptan in*
554 *hyponatremic rats*

555 Plasma AVP and apelin-IR levels were measured after the continuous administration of AVP (30
556 ng/h) for 48 hours in male Sprague-Dawley rats receiving a semi-liquid diet, 3 hours after the
557 administration of saline (AVP + saline), LIT01-196 (900 nmol/kg, AVP + LIT01-196) or
558 tolvaptan (6700 nmol/kg, AVP + tolvaptan) and in rats receiving the same diet but no AVP **(Fig.**
559 **6)**. The continuous administration of AVP (30 ng/h) for 48 hours significantly increased plasma
560 AVP levels by 120% in animals receiving AVP + saline **(Fig. 6A)**. No significant difference in
561 AVP levels was observed between AVP-treated rats receiving saline or LIT01-196. Plasma

562 apelin levels were similar in animals receiving no AVP and in those receiving AVP + saline, but
 563 plasma apelin immunoreactive (IR) material levels were 372% higher in rats receiving AVP +
 564 LIT01-196 than in rats receiving AVP + saline, because the antibody against K17F recognizes
 565 K17F and LIT01-196 with similar affinity (**Fig. 6B**). In hyponatremic rats, LIT01-196 treatment
 566 increased plasma apelin-IR/AVP ratio by 518% relative to rats receiving saline (**Fig. 6C**).
 567 Plasma AVP and apelin levels in rats receiving AVP + tolvaptan were similar to those in animals
 568 receiving AVP + saline.



569
 570 **Fig. 6. Plasma AVP and apelin levels in hyponatremic rats receiving saline, LIT01-196 and**
 571 **tolvaptan, relative to normonatremic rats.** Measurements of plasma AVP levels (**A**), plasma
 572 apelin-IR levels (**B**) and plasma apelin-IR/AVP ratios (**C**) in alert male Sprague-Dawley rats
 573 receiving a semi-liquid diet and no AVP (grey bars, $n=8$), a 48-hour s.c. infusion of AVP at a
 574 rate of 30 ng/h together with a s.c. injection of saline (green bars, 1 ml/kg, $n=7$), LIT01-196 (blue
 575 bars, 900 nmol/kg, $n=5$) or tolvaptan (red bars, 6700 nmol/kg, $n=8$) three hours before blood
 576 collection. Multiple comparisons performed by Kruskal-Wallis followed by post-hoc Dunn's
 577 tests with Holm's adjustment. Ns: $p>0.05$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

578
 579

580 **Discussion**

581 We demonstrate in this study that the s.c. administration of LIT01-196, a metabolically stable
582 K17F analog, in a rat experimental model of AVP-induced hyponatremia blocks the AVP-
583 induced decrease in urine output and increase in urinary osmolality, leading to a progressive
584 correction of hyponatremia. These results provide evidence for a major role of apelin, together
585 with AVP, in the regulation of body fluid homeostasis and suggest that the use of apelin-R
586 agonists may represent a new approach for the treatment of hyponatremia.

587 LIT01-196 was previously shown to display, like K17F, full agonist activity for the apelin-R
588 stably expressed in CHO cells, for inhibiting forskolin-induced cAMP production, inducing
589 ERK1/2 phosphorylation, apelinR internalization and β -arrestin recruitment²². In addition,
590 LIT01-196 had a plasma half-life superior to 24 h versus 4.6 min for K17F²². In the present
591 study, we show that LIT01-196, similarly to K17F, dose-dependently inhibits dDAVP-induced
592 cAMP production in CHO cells stably expressing both the Apelin-R and the V2-R. In contrast,
593 LIT01-196, even at a supramaximal concentration of 1 μ mol/l, does not inhibit AVP-induced
594 cAMP production in HEK-293T cells, that overexpress the V2-R but not the ApelinR. This
595 indicates that LIT01-196 does not inhibit dDAVP or AVP-induced cAMP production by binding
596 to the V2-R and acting as a V2-R antagonist. Hence, LIT01-196 by binding to the Apelin-R,
597 activates Gi-protein-coupling, counteracting the increase in cAMP production induced by
598 dDAVP or AVP via Gs-protein coupling of the V2-R. Additional evidence that the inhibitory
599 effect of LIT01-196 on dDAVP-induced cAMP production occurs via a non-V2 mediated
600 mechanism is provided by the fact that LIT01-196 as K17F inhibits forskolin-induced cAMP
601 production in mpkCCD cells, a collecting duct principal cell line expressing both the V2R and
602 the apelin-R^{29,34}. We also show that LIT01-196, similarly to K17F²⁸, inhibits the increase in

603 cAMP production induced by dDAVP in mpkCCD cells and also in freshly microdissected rat
604 OMCD, a more physiological preparation. To investigate if the inhibition by LIT01-196 of the
605 cAMP production induced by dDAVP results in a decrease in AQP-2 translocation to the apical
606 membrane, we show in mpkCCD cells that LIT01-196 greatly reduces dDAVP-induced apical
607 cell surface expression of phosphorylated AQP-2. These data are in agreement with previous
608 studies showing that i.v. administration of K17F into lactating rats inhibited the AVP-induced
609 AQP-2 insertion into the apical membrane of the CD and increased aqueous diuresis by
610 decreasing AVP-induced cAMP production in the CD ²⁸. These data suggested that systemic
611 LIT01-196 administration in rats could induce an increase in aqueous diuresis. Before assessing
612 the *in vivo* metabolic effects of LIT01-196, we first investigated the *in vivo* half-life of LIT01-
613 196 in the blood circulation after i.v. administration in alert control mice and rats, by using two
614 methods, mass spectrometry analysis and radioimmunoassay. Both yielded similar results, 25
615 and 28 min respectively for LIT01-196 versus only 44 and 50 seconds for K17F after i.v.
616 administration in mice and rats, respectively. We then showed in alert control rats that LIT01-
617 196 had an *in vivo* half-life in the bloodstream of 156 min after s.c. administration. The increase
618 of the *in vivo* half-life of LIT01-196 in the blood circulation is probably due to the binding of
619 LIT01-196 to plasma proteins leading to the protection from enzymatic degradation and the
620 reduction of renal clearance, as LIT01-196 has a 69% binding to plasma proteins. This led to
621 evaluate the effects of LIT01-196 on water metabolism.

622 The s.c. administration of LIT01-196 (900 nmol/kg) in control rats increases urine output,
623 decreases urine osmolality, modestly increases water intake, water intake / food intake ratio,
624 increases free water clearance while fractional sodium excretion is unchanged, demonstrating an
625 aquaretic effect. These data are similar to those obtained with tolvaptan, used as a positive

626 control, and administered at an equimolar dose, except for the increase water intake which is
627 higher with tolvaptan (+ 37%) than with LIT01-196 (+ 11 %). The slight increase in water intake
628 induced by LIT01-196 could be related to the fact that i.c.v. administration of apelin-13 in water-
629 deprived rats reduces water intake ²⁴, although it increases water intake in normally hydrated
630 mice ⁴². Moreover, in patients with primary polydipsia, plasma apelin levels are lower than in
631 healthy volunteers ⁴³. Altogether, these results suggest that activation of the apelin receptor may
632 limit water intake associated with aquaresis, without abnormally increasing plasma osmolality.

633 As we previously showed that i.c.v. administration of K17F, by reducing the release of AVP
634 from the posterior pituitary into the bloodstream, increased diuresis in lactating rats ²⁰, it was
635 important to determine whether LIT01-196 given by s.c. route acted through central and/or renal
636 action. Especially, we wanted to determine if LIT01-196 could cross the blood-brain barrier
637 (BBB) after systemic administration. Neuronal apelineric fibers have been visualized in the
638 lamina terminalis of rats, along the anteroventral third ventricle region, including the subfornical
639 organ (SFO), the organum vasculosum and the median preoptic nucleus ²⁵. These structures are
640 all interconnected and project both excitatory and inhibitory signals to the SON and PVN ⁴⁴ to
641 regulate the activity of AVP/apelin magnocellular neurons which also express apelin-R ^{20,24}. We
642 show here in rats and in mice that 30 min after s.c. injection of LIT01-196 (900 nmol/kg), the
643 compound is abundant in the blood circulation but absent in the brain, showing that LIT01-196
644 does not cross the blood-brain barrier (BBB) and does not enter the brain. Furthermore, knowing
645 that among the circumventricular organs, only the SFO expresses apelin-R mRNA ⁴⁵ and that the
646 administration of LIT01-196 by s.c. route at the dose of 900 nmol/kg does not modify AVP
647 release in the blood circulation, our results strongly suggest that LIT01-196 given by s.c. route at
648 this dose does not act on the SFO to regulate magnocellular vasopressineric neuron activity and

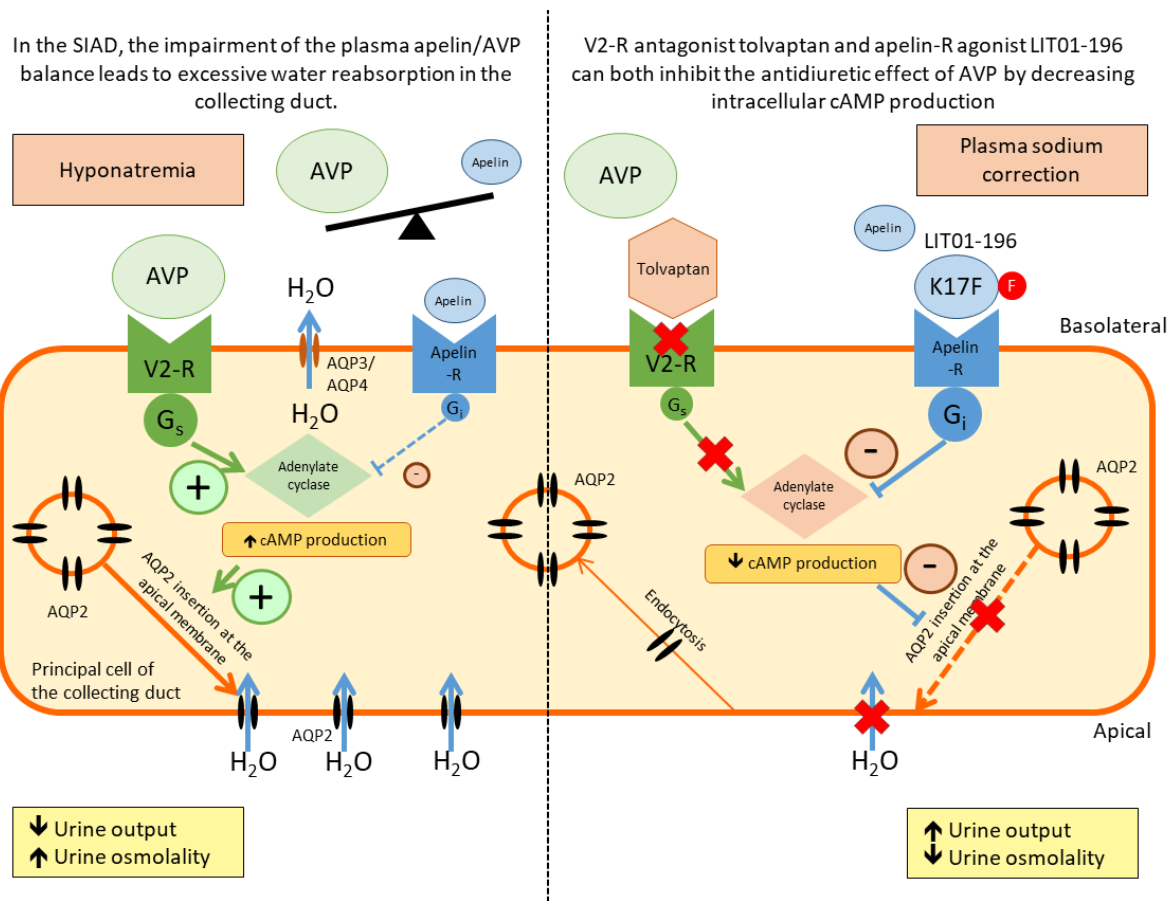
649 systemic AVP release. Therefore, the observed effects of LIT01-196 administered s.c. on water
650 metabolism result from the action of the compound at the kidney level.

651 As the s.c. administration of LIT01-196 increased aqueous diuresis, we investigated whether
652 LIT01-196 could be used to reverse the antidiuretic effect of AVP *in vivo*. We found that a
653 continuous s.c. infusion of AVP (30 ng/h) for four days together with a semi-liquid diet, led to a
654 decrease in urine output and an increase in urine osmolality. We established that, at this dose,
655 plasma AVP levels were 120% higher than those in rats receiving saline, and that plasma sodium
656 levels were decreased and stable at about 100 mmol/l for two to four days after the initiation of
657 AVP infusion. LIT01-196 administered in this rat hyponatremic model at the dose of 900
658 nmol/kg, for two days, via the s.c. route, inhibited the effects of AVP on urine output and urine
659 osmolality highly efficiently, and induced a progressive correction of plasma sodium levels.
660 Considering *ex vivo* and *in vivo* data, this suggests that LIT01-196 by acting on apelin-R present
661 in the CD, inhibits the AVP-induced cAMP production, thereby inhibiting the insertion of AQP-
662 2 at the apical membrane of the CD, resulting in the inhibition of water reabsorption by the
663 kidney and an increase in aqueous diuresis (**Fig. 7**).

664 As expected from previous studies ⁷, tolvaptan also inhibited the antidiuretic effects of AVP.
665 However, while tolvaptan and LIT01-196 at the same dose of 900 nmol/kg resulted similar
666 aquaretic effects in both control and hyponatremic rats, increasing the dose of tolvaptan resulted
667 in an even larger increase in urine output, while increasing the dose of LIT01-196 did not.
668 Activating the apelin-R and thus modifying the apelin/AVP balance in the collecting duct, rather
669 than blocking the effects of AVP, may therefore less often result in severe polyuria and be better
670 tolerated than V2-R antagonists.

671 The apelin-R is widely expressed, as opposed to the V2-R, which could result in unwanted
672 effects following LIT01-196 administration. Apelin has been reported in rodents to induce a
673 transient decrease in blood pressure ⁴⁶, to increase cardiac contractility ⁴⁷ and to improve glucose
674 tolerance ⁴⁸. However, LIT01-196 (900 nmol/kg) given by s.c. route in Sprague Dawley rats did
675 not significantly modify mean arterial blood pressure, blood glucose levels and kidney function.
676 LIT01-196 (1200 nmol/kg s.c.) did not modify left ventricular ejection fraction in mice.
677 Repeated administrations of LIT01-196 were not associated with renal failure or histological
678 alterations of the kidney, and plasma sodium, potassium and glucose levels were not affected by
679 LIT01-196 administration in control rats. The various physiological effects associated with the
680 activation of the apelin-R result from the activation of different signaling pathways. Hence, the
681 development of biased apelin-R agonists that specifically target the protein Gi signaling pathway
682 may improve their specificity of action regarding water metabolism.

683 In conclusion, LIT01-196 is a potent metabolically stable K17F analog that acts as an aquaretic
684 agent. Our model mimics a clinical situation in which the osmotic regulation of circulating AVP
685 levels is impaired, causing SIAD. In this context, LIT01-196 can inhibit the antidiuretic effect of
686 AVP, by increasing urine output, decreasing urine osmolality, enhancing water intake
687 moderately and progressively correcting hyponatremia. Furthermore, our study constitutes a
688 proof of concept that through the activation of apelin-R, metabolically stable apelin analogs,
689 represent promising candidates for the treatment of SIAD. Whether apelin-R agonists could also
690 be beneficial in pathological situations where AVP secretion is in excess and plasma apelin
691 levels are decreased ⁴⁹, such as ADPKD, remain to be determined.



692

693 **Fig. 7. Proposed model of the effects of LIT01-196 on the principal cell of the collecting**
 694 **duct in SIAD.**

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