Supplementary Materials and Methods

Cloning work: The full-length human vasopressin V1b receptor-coding region was obtained by polymerase chain reaction (PCR) using human pituitary gland cDNA as starting material and cloned into pcDNA3.1(V5/His) (Invitrogen). The full-length human vasopressin V1a receptor-coding region was generated by cloning from 3 plasmids obtained from RZPD (pT7T3D-Pac IMAGp998N216850, pT7T3D-Pac IMAGp998F063909, pT7T3D-Pac IMAGp998G015656) containing overlapping partial coding sequences. The full-length coding sequence was amplified from the resulting plasmid and cloned into pcDNA3.1 (V5/His) (Invitrogen). An expression plasmid containing the full-length coding sequence of the human vasopressin V2 receptor was obtained from the Guthrie cDNA Resource Center. The human oxytocin coding sequence is located on two genomic exons. Both exons were amplified separately by PCR from human genomic DNA and the two PCR products assembled by cloning via Hind III into one full-length coding sequence and cloned into pcDNA3 (Invitrogen).

The coding region of the rat vasopressin V1b receptor was amplified by PCR from rat brain cDNA. The PCR product obtained was cloned into the expression vector pcDNA3 (Invitrogen). The coding sequence of the rat vasopressin V1a receptor was amplified using rat total brain cDNA as starting material and cloned into pcDNA3.1(V5/His) (Invitrogen). The rat oxytocin receptor coding sequence is located on two genomic exons. Both exons were amplified separately from rat genomic DNA (Promega G309A) and the two PCR products assembled by cloning via BamHI/Eco47III and EcoRV/XhoI into pcDNA5/FRT/TO digested with BamHI/XhoI to obtain an inducible expression plasmid containing the full-length coding sequence.

Plasmid names, RefSeq IDs and primer sequences are listed in the table below. Nucleotides in the primers corresponding to start and stop codons are written in italics

and highlighted in red. Restriction sites for endonucleases are written in italics and underlined.

species	*receptor*	*plasmid*	*RefSeq*	*Primer 5' -3'*
human				
	V1b	pcDNA3.1(V5 -His) hV1b	NM_000707	GCCACC*ATG*GATTCTGGGCCTCTGTGGGATGCC *CTA*AAAGATGATGGTCTCAGCGGTGCC
	V1a	pcDNA3.1(V5 -His) hV1a	NM_000706	GC*ATG*GACAGCATGCGTCTCTCC CATGAATGCAAGGC*TCA*AGTTGAAACAG
	V2	pcDNA3.1(+) AVPR2	NM_000054	
	Oxytocin	pcDNA3 hOxytocin	NM_000916	EXON3 GGCTCGAAGGCCGGGGCGCACCG GGCCG*AAGCTT*CCTTGGGCGCGTTGGCATCCCAGAC EXON4 CCAAGG*AAGCTT*CGGCCTTCATCATCGTC GCTGGTGGG*TCA*CGCCGTGG
		pcDNA5/FRT/ TO hOxytocin		
rat				
	V1b	?	D45400	?
	V1a	pcDNA3.1(V5 -His) rV1a	NM_053019	CGGACAGC*ATG*AGTTTCCCGC ACGTGGGGCTCAAGTGGAGACA
	Oxytocin	pcDNA5/FRT/ TO rOxytocin	NM_012871	EXON1 CGC*GGATCC**ATG*GAGGGCACGCCAGCAG AAAAAGC*GCTTC*CTTGGGCGCATTGACG EXON2 GGGG*GATATC*TGCCTTCATCATTGCCATGCTC CCCC*CTCGAG**TCA*TGCTGAAGATGGCTGAGAGCAG

Cell culture: Stably or transiently transfected cells (Invitrogen transfection protocol) were grown in DMEM-F12 medium with GlutaMAX I (Invitrogen # 31331), 10% fetal calf serum and geneticin 800 µg/mL (V1b and V2) or 600 µg/mL (V1a and oxytocin) at 37°C in cell factories CF10 (Nunc # 164327) in humidified atmosphere containing 5% CO_2. Cells were washed with phosphate-buffered saline (PBS) and 150 mL trypsin-EDTA (Invitrogen # 25200) was added for 5-10 min at 37°C to detach adherent cells. Thereafter, trypsin was inactivated by adding 300 mL of culture medium and cells were centrifuged at 1,500 x g for 10 min at 4°C.

Membrane Preparation: Recombinant cells expressing human V1b receptors (stable Chinese hamster ovary (CHO) cells, clone 3H2) were harvested into 50 mM Tris-HCl in the presence of protease inhibitors (EDTA-free protease inhibitor complete, Roche # 1836170), homogenized for 2 x 10 s with a Polytron Homogenizer at a setting of 50% followed by a 1 h centrifugation at 40,000 x g. The membrane pellet was homogenized and centrifuged one more time as described and then suspended in 50 mM Tris-HCl, pH 7.4, and vortexed to homogeneity. After determination of protein concentration and preparation of aliquots, the membrane suspension was stored at -170°C in liquid nitrogen.

Recombinant cells expressing rat V1b receptors (stable CHO cells, clone 1) were harvested into lysis buffer in the presence of protease inhibitors (Roche complete Mini # 1836170) leading to cell lysis within 10 min. Aliquots of cell ghosts were stored frozen in liquid nitrogen.

Membranes from recombinant cells expressing human or rat V1a (human: stable CHO cells, clone 5; rat: transient in HEK-293 cells), human V2 (stable CHO cells, clone 23) or human or rat oxytocin receptors (transiently transfected HEK-293 cells) were prepared essentially according to Riordan and Ling (1979). Cells were washed with PBS, resuspended in 10 mM triethanolamine (pH 7.8), 250 mM sucrose, and then homogenized by French Press from Thermo IEC (Thermo Fisher Scientific Inc. Waltham, MA) at 1,500 psi. Membranes were prepared by following differential centrifugation steps: 300 x g for 10 min; supernatant at 10,000 x g for 10 min; resulting supernatant at 100,000 x g for 30 min. After the last spin, the pellet was washed with 50 mM HEPES buffer (pH 7.4) containing protease inhibitors (EDTA-free protease inhibitor complete, Roche # 1836170), and resuspended at 5 mg of protein/mL in the same buffer. Aliquots were stored in liquid nitrogen.

Test Compound Dilutions: Test compound was dissolved at 10 mM in DMSO and diluted to a 50X concentration in DMSO followed by a 1:10 dilution in incubation buffer (50 mM Tris, 10 mM $MgCl_2$, 0.1% bovine serum albumin (BSA), pH 7,4) and a final 1:5 dilution in the final sample volume.

Receptor binding affinity studies: Radioligand competition binding assays for cloned human and rat vasopressin V1b receptors were performed using [3H]-AVP (8-Arg-Vasopressin NET800, PerkinElmer, Boston, USA) as radioligand, and membranes or cell ghosts prepared from cell clones stably expressing the recombinant human and rat receptors. Recombinant cell membranes at a final protein concentration of 50 µg/mL (human V1b receptor) or 500 µg/mL (rat V1b receptor cell ghosts) were incubated with 1.5 nM (human) or 1.0 nM (rat) [3H]-AVP for competition experiments, performed in a 50 mM Tris buffer, pH 7.4, containing 10 mM $MgCl_2$ and 0.1% BSA without or in the presence of A-988315 (0.1 nM – 10 µM). AVP (1 µM) was used for determination of non-specific binding (NSB). After a 1-h incubation period at room temperature (RT), unbound radioligand was removed by vacuum filtration and membranes were washed with 50 mM Tris/HCl, 10 mM $MgCl_2$, pH 7.4, using glass-fibre filter mats grade B (Unifilter 96 GF/B plates, #6005177, PerkinElmer PerkinElmer, Boston, USA) pre-incubated with 0.3% polyethyleneimine solution. Filters were then transferred into scintillation tubes and sample radioactivity was measured by liquid scintillation counting (Tri-Carb®, PerkinElmer, Boston, USA).

Other radioligand assays were performed as described above with specific conditions: Human or rat V1a receptors: 0.04 nM [125I]-AVP (NEX128, PerkinElmer, Boston, USA), or 0.02 nM [125I]-Phenylacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH2 (NEX310, PerkinElmer, Boston, USA) as radioligands and membranes prepared from recombinant cell lines stably expressing human or transiently expressing rat V1a

receptors (NSB: 1 µM AVP). Human V2 receptors: 1 nM [3H]-AVP as radioligand and membranes prepared from a recombinant cell line stably expressing human V2 receptors (NSB: 1 µM AVP). Human or rat oxytocin receptors: 1 nM [3H]-oxytocin as radioligand and membranes prepared from recombinant cells transiently expressing human or rat oxytocin receptors (NSB: 1 µM oxytocin). Ki values from competition binding experiments were determined by non-linear regression analysis of the original data. Fitting was performed according to formulae described by Munson and Rodbard (1980). Reported Ki values were determined by simultaneous fitting of multiple inhibition curves obtained with the same test compound.

Functional antagonism of vasopressin-induced intracellular Ca^{2+} release: For the assessment of antagonistic potency of A-988315, frozen recombinant CHO cells stably expressing the human V1b receptor were quickly thawed and transferred into 50 mL medium at 37ºC. Cells were washed and seeded into FLIPR plates (Falcon 384 plate #353962, Becton Dickinson) at a final concentration of 0.5 to 1 x 10^6 cells/mL and incubated overnight at 37°C and 5% CO_2 in air. The plates were incubated at 37°C for 30 min with FLIPR Calcium No-Wash dyes (Discoverex 90-0080XL or Molecular Devices Calcium 3) in HBSS with 20 mM HEPES and 0.1% BSA, followed by 30-60 min incubation at RT. Stimulation of Ca^{2+} release was performed with 2 nM AVP. A-988315 (at the desired concentrations) was pre-incubated for 10 min before agonist addition. Measurements were performed using a FLIPR®2 reader (Molecular Devices, Sunnyvale, USA).

Pharmacokinetic studies: In order to estimate the pharmacokinetic properties of A-988315 in terms of oral bioavailability, half-life and brain-to-plasma ratio, male Sprague-Dawley rats (n = 3; Janvier, Le Genest-St-Isle, France) were injected intravenously (2

mg/kg) or orally gavaged (10 mg/kg) with A-988315 dissolved in water. Blood was sampled from the tail vein after 30 min, 45 min, 1, 2, 2.5, 3, 4, 6, 8 and 24 h. Blood was collected into K-EDTA vials, centrifuged at 2,000 X g for 10 min at 4°C, and plasma samples were stored at -18°C until analysis. For the brain distribution study, male Sprague-Dawley rats (n = 3 for each time point) were injected intraperitoneally with 10 mg/kg of A-988315. After 30 min, 3 or 8 h, the animals were deeply anesthetized with isoflurane. Blood was drawn via cardiac puncture, the animal sacrificed through cervical dislocation, and the brain removed and stored at -18°C until analysis. Blood was collected into K-EDTA vials, centrifuged at 2,000 X g for 10 min at 4°C, and plasma samples were stored at -18°C until analysis. Experimental procedures were approved by the Animal Welfare Office (Ludwigshafen, Germany) and performed in accordance with the recommendations and policies of the United States National Institutes of Health Principles of Laboratory Animal Care (1996 edition). Animal housing and experiments were conducted in facilities with full Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accreditation. All experiments were performed during the light phase of a 12-h/12-h day/night cycle (lights on: 05:30 AM – 05:30 PM).

An aliquot of plasma (50 µL, sample or spiked standard) or homogenized whole-brain tissue was pipetted into a 96-well polypropylene plate and proteins were precipitated by addition of 400 µL acetonitrile containing an internal standard. Following vortexing, the plate was centrifuged at 2,000 X g for 10 min at 4°C. In an automated manner (Hamilton Starlet), 125 µL of the supernatant was transferred to a clean 96-well plate. Samples were diluted by addition of 125 µL 0.1% formic acid. Samples (10 µL) were injected on an Acquity UPLC BEH C8 1.7 um 2.1 X 100 mm with an acetonitrile gradient (5% - 95%) with 0.1% formic acid (in water) as the second solvent, at a flow rate of 0.3 mL/min. The column temperature was held at RT.

Mass-spectrometric detection was performed on an AB SCIEX Triple Quad™ 5500 with a turbo-ion spray interface by multiple-reaction monitoring. Peak areas were determined using Sciex Analyst™ software. Pharmacokinetic parameters of the compound were estimated using WinNonlin 5.2 (Pharsight, Mountain View, CA), using non-compartmental analysis. Oral bioavailability and brain exposure were determined by comparing the area under the curve (AUC) from zero to the final time point measured (AUC0 –t) of the two compartments.

REFERENCES

Munson PJ, Rodbard D (1980). LIGAND: A versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem* **107**: 220-239.

Riordan JR, Ling V (1979). Purification of P-glycoprotein from plasma membrane vesicles of Chinese hamster ovary cell mutants with reduced colchicine permeability. *J Biol Chem* **254**: 12701-12705.