Downloaded from orbit.dtu.dk on: Dec 17, 2017

Technical University of Denmark

DTU

Method for the detection of aquaretic compounds

Jonassen, Thomas; Hadrup, Niels; Petersen, Jørgen Søberg

Publication date:
2003

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
Jonassen, T., Hadrup, N., & Petersen, J. S. (2003). IPC No. A61K38/08. Method for the detection of aquaretic compounds (Patent No. WO2003097857.)

DTU Library
Technical Information Center of Denmark

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 November 2003 (27.11.2003)

PCT

(10) International Publication Number
WO 03/097857 A2

(51) International Patent Classification: C12Q 1/00

(21) International Application Number: PCT/DK03/00328

(22) International Filing Date: 15 May 2003 (15.05.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/381,521 17 May 2002 (17.05.2002) US

(71) Applicant *(for all designated States except US)*: **ZEALAND PHARMA A/S** [DK/DK]; Smedeland 26B, DK-2600 Glostrup (DK).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **JONASSEN, Thomas** [DK/DK]; Svanholmvej 12A, 2. th., DK-1905 Frederiksberg C (DK). **HADRUP, Niels** [DK/DK]; Mariendalsvej 48C, 2. th., DK-2000 Frederiksberg C (DK). **PETERSEN, Jørgen, Søberg** [DK/DK]; Hellebakken 1, DK-3150 Hellebæk (DK).

(74) Common Representative: **ZEALAND PHARMA A/S**; Patent Department, Smedeland 26B, DK-2600 Glostrup (DK).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/097857 A2

(54) **Title:** DETECTION OF AQUARETIC COMPOUNDS

(57) **Abstract:** Disclosed is a method for detecting an aquaretic compound. In one embodiment, the method includes administering to a mammal a candidate compound that modulates a nociceptin receptor. Biological material is isolated from the mammal and expression of aquaporin-2 is measured. Modulation of the aquaporin-2 is taken to be indicative of a candidate compound having aquaretic activity. The invention has a wide spectrum of uses including helping to identify new diuretics that spare unwanted loss of sodium and potassium ions.

DETECTION OF AQUARETIC COMPOUNDS

FIELD OF THE INVENTION

The present invention generally relates to methods for detecting diuretics with known or suspected nociceptin receptor binding activity. The invention has a wide spectrum of applications including providing a highly useful *in vivo* screen for detecting new aquaretic compounds.

BACKGROUND

Diuretics have been described as an important group of therapeutic agents that are used to modulate body fluid volume and composition. Such agents have been used to treat a wide spectrum of medical indications including hypertension, acute and chronic failure of the heart and kidneys, nephrotic syndromes, and liver cirrhosis. Particular diuretics include carbonic anhydrase inhibitors, osmotic diuretics, loop diuretics, thiazide and thiazide-like diuretics, and the potassium (K^+)-sparing diuretics. See generally E. K. Jackson in *The Pharmacological Basis of Therapuetics*, 9th Ed. (Hardman, J.G and L.E. Limbird Eds.) McGraw-Hill, New York Chpt. 29, pp. 685-713 and references cited therein.

A variety of naturally-occurring anti-diuretics have also been reported. For example, arginine vasopressin (AVP) is believed to be an important human anti-diuretic that helps maintain normal body fluid osmolality. It has been disclosed as regulating water permeability in the renal collecting duct by short-term and long-term regulation. There have been reports that water permeability in the kidney collecting duct (CD) increases within a few minutes in response to an acute increase in plasma vasopressin concentration.

More generally, vasopressin is believed to be an ancient modulator of water conservation that facilitated emergence of terrestrial life from primordial seas. See E.K. Jackson, *ibid.* Chpt. 30, pp. 715-731.

There have been efforts to understand mechanisms of vasopressin action. For example, structure and function of the AVP pre-prohormone has been described. Additionally, two vasopressin receptors called V1 and V2 have been reported. With respect to the V2 receptor, a detailed mechanism of V2-receptor-effector coupling has been disclosed. In general, binding between AVP and the V2 receptor is thought to stimulate adenylyl cyclase and boost intracellular cyclic AMP (cAMP). Activation of cAMP-dependent protein kinase A has been reported to be important to vasopressin action in a process that involves protein phosphorylation. See eg., Snyder, H.M et al. (1992) *Am. J. Physiol.* 263: C147-C153.

The structure and function of a variety of naturally-occurring and synthetic vasopressin agonists have been disclosed. See E.K. Jackson, *ibid.* Chpt. 30, pp. 715-731.

Vasopressin-mediated protein phosphorylation is thought to increase the rate of exocytosis of vesicles that include water channels (WCVs). The WCVs have been disclosed as containing fully function water channel proteins. See eg., Knepper, M.A and Nielsen, S. (1993) *Am. J. Physiol.* 265: F214-F224; and E.K. Jackson, *ibid.* Chpt. 30, pp. 715-731.

A vasopressin-regulatable water channel has been isolated and described. The channel is referred to herein as aquaporin-CD or simply as aquaporin-2 (AQP2). The vasopressin effect is thought to involve shuttling of AQP2 from intracellular vesicles into the apical plasma membrane via exocytosis. In the absence of AVP, a large fraction of AQP2 is localized to vesicles in the cytoplasm of CD principal cells. AVP has been reported to stimulate water permeability by inducing the translocation of AQP2 to the apical membrane of the cell. The process is thought to involve phosphorylation of Ser256 in AQP2. For long-term regulation of body water the total amount of AQP2 protein in the principal cells is increased along with increased AQP2 mRNA. This effect is believed to be due in part to increased AQP2 gene transcription. See Fushimi, K et al. (1993) *Nature* 361: 549-552; and You, G. et al. (1993) *Nature* 365: 844-847.

The structure and function of another aquaporin (aquaporin-CHIP) has been disclosed. See Agre, P. et al. (1993) *Am. J. Physiol.* 265: F463-F476.

Prostaglandins such as PGE_2 have been reported to impact renal salt and water
5 excretion eg., by modulating kidney tubules. See Campbell, WB and P.V Halushka in *The Pharmacological Basis of Therapuetics, ibid,* Chpt. 26, pp. 601-616 and references cited therein.

An opioid-like peptide called nociceptin (also referred to as orphanin FQ) has been
10 disclosed. Nociceptin has been reported to bind. a specific receptor named opioid receptor-like one (called the ORL1 or OP4 receptor) with much greater affinity than to the three classical subtypes of opioid receptors. See eg., Dooley et al. *J. Of Pharm. And Exp. Therapeutics*, (1997) 283: 735.

15 There have been efforts to understand the function of the ORL1/OP4 receptor. For instance, low doses of nociceptin increase the renal excretion of water and decrease urinary sodium excretion (i.e., produces a selective water diuresis) which render this compound a candidate for treating hyponatremia. See Daniel R. Kapusta, *Life Science*, 60:15-21, 1997; and US patent No. 5,840,696. The ligand has also been reported to impact blood pressure
20 and heart rate.

A wide spectrum of peptides and peptide conjugates have been disclosed for the treatment of disorders impacted by nociceptin and related opioid-like peptides. See the following PCT application by Larsen, B.D et al. (W0 01/98324 A1 as published on 27
25 December 2001).

There is a need to develop methods for detecting compounds that modulate aquaporin expression. Such screening methods would be useful for identifying new aquaretics ie., diuretics that have a greater impact on water elimination than sodium or potassium excretion.

30

SUMMARY OF THE INVENTION

The present invention relates to methods for detecting compounds that modulate expression of aquaporin-2 (AQP-2). More specifically, the invention relates to highly useful methods that can detect and identify aquaretic compounds. The invention has a wide spectrum of important applications including use as a valuable *in vivo* screening tool for selecting aquaretics from known or suspected nociceptin receptor ligands.

We have discovered that certain ligands of the nociceptin receptor are potent aquaretics. Preferred aquaretic compounds enhance renal water elimination while preferably sparing at least some loss of sodium and/or potassium ions. Our discovery is grounded in the observation that the ligands can eliminate or substantially decrease expression of the AQP-2 water channel protein. Without wishing to be bound to theory, it is believed that compounds detected by the present screening methods by virtue of the AQP-2 expression changes can help eliminate or reduce renal tube water reabsorbtion to produce a potent aquaretic effect.

Figure 1 is a schematic overview of relationship between vasopressin receptor binding and AQP-2 expression in the kidney collecting duct (CD) cells. The present invention substantially adds to this knowledge by showing, for the first time, that AQP-2 expression can be modulated by a nociceptin receptor ligand. More generally, the invention relates potential nociceptin receptor binding to aquaresis. Our finding provides a framework for practicing a wide spectrum of screens intended to test (or confirm) aquaretic activity of a large variety of known (or suspected) nociceptin receptor binding molecules. Examples of such compounds include naturally-occurring, synthetic, and semi-synthetic molecules including nociceptin receptor agonists and antagonists.

Accordingly, and in one aspect, the invention provides *in vivo* methods for detecting diuretic compounds, preferably aquaretics that are or are intended to be nociceptin receptor ligands. Preferred compounds are known (or suspected) to modulate (increase or decrease) activity of the nociceptin receptor, typically but not exclusively, by binding to that receptor as a ligand. In one embodiment, the method for detecting aquaretic compounds includes at least one and preferably all of the following steps.

a) administering to a mammal at least one known or candidate compound that modulates (increases or decreases) activity of the nociceptin receptor in which the administration is for a time sufficient to modulate the receptor in the mammal and/or observe diuresis,

5 b) isolating cells, tissue or an organ from the mammal following the administration,

c) measuring expression of aquaporin-2 (AQP-2) in the cells, tissue or organ; and

d) determining effect of the compound on the AQP-2 expression in which modulation of the AQP-2 is taken to be indicative of the aquaretic compound.

The foregoing invention method provides important advantages.

10 For example, it provides a key *in vivo* screen that can detect aquaretics from a pool or library of known or suspected nociceptin receptor ligands. The method is inherently flexible and can be used eg., as a primary or follow-up screening tool for identifying aquaretics or confirming *in vivo* activity of known or suspected aquaretics. The present methods are fully compatible with a wide spectrum of conventional screening methods including standard *in* 15 *vitro* and *in vivo* assays for detecting ligand binding to the nociceptin receptor. Thus in one invention embodiment, the present methods are combined with one or more of such conventional screens to further detect and/or select compounds with desired aquaretic activity. Examples of such screens have been disclosed in the W0 01/98324 A1 PCT application. Significantly, practice of the invention provides highly useful *in vivo* 20 verification of aquaretic activity.

The *in vivo* detection methods described herein can be readily adapted to suit an intended use.

For example, the invention can be adapted to detect expression of AQP-2 as manifested by presence of phosphorylated AQP-2 (pAQP-2). In this invention, an increase 25 or decrease in pAQP-2 expression is taken to be indicative of aquaretic activity of the candidate compound. Such expression can be detected before, during, after, or as a substitute

for measuring expression of the AQP-2 protein as described above. However in most cases, it will be preferred to measure expression of both AQP-2 and pAQP-2.

In addition, the known or candidate compound can be used in accord with the invention as a sole active agent or in combination with one or more other agents including
5 known diuretics, anti-diuretics and like compounds to be tested. In most instances, the methods (assays) of this invention will be performed with a suitable control assay usually but not exclusively including the same test conditions as described above, but without adding the known or candidate compound to the mammal. For example, saline or other pharmaceutically acceptable solution will often provide a good control. In such instances, a candidate
10 compound can be identified as exhibiting desired aquaretic activity by providing at least about a 10% decrease in AQP-2 and/or pAQP-2 expression relative to a control. Preferably, such a compound will exhibit at least about a 20% decrease in AQP-2 and/or pAQP-2 expression relative to the control, more preferably at least about a 50% decrease in such expression up to about 90%, 100% or 200% decrease relative to the control. It will be
15 apparent to those of skill that once the characteristics of a control have been analyzed, that control need not be repeated each time the invention method is practiced.

The invention is fully compatible with use of a range of mammals such as rodents, non-human primates, and domesticated animals eg., rabbits, dogs, cats, goats, horses, pigs, and the like. In one embodiment, the mammal is manipulated to induce an abnormal water
20 fluid retaining condition such as edema, hypertension, acute or chronic congestion of the heart or kidney, or a nephrotic syndrome. A preferred condition is the fluid imbalance accompanying coronary heart failure (CHF). Thus in one invention embodiment, the mammal used in the method described above is a rodent, non-human primate or domesticated animal that has been surgically (or chemically) manipulated to mimic CHF. Alternatively, the
25 abnormal water fluid retaining condition may be pre-existing in the mammal.

For example, practice of the invention can be achieved using mammals which have been surgically manipulated to induce CHF. In this embodiment, the manipulation includes occluding (partially or essentially completely) at least one coronary artery in the mammal. Occlusion will typically involve ligation eg., with surgical thread although other methods

may also be used. It has been shown that induced congestive heart failure in rats is accompanied by a selective increase in AQP2 expression in the renal collecting duct and enhanced plasma membrane targeting (Nielsen, S. et al., PNAS, Vol. 94, pp. 5450-5455, May 1997) which may render this animal model especially useful as a screen for aquaretic compounds that are nociceptin receptor ligands.

As discussed, the present invention methods involve measuring expression of AQP-2 and preferably also pAQP-2. Such measurements can be accomplished by one or a combination of standard formats including methods based whole or in part on conventional immunological and/or PCR based detection strategies. Such measurements can be conducted at the level of transcription or post-transcriptional level as needed to suit an intended use. For example, in situations in which AQP-2 expression is to be monitored at the level of RNA production, use of Northern blot and/or PCR based detection approaches may be desirable. However, in embodiments in which expression at the protein level is needed (alone or in combination with the Northern and PCT based methods just described), an immunoassay such as those relying on antibody use will be more helpful.

The present methods can be used to measure protein expression of the AQP-2 channel protein in response to administration to the mammal of a known or candidate nociceptin receptor ligand. As mentioned, a significant decrease in such expression (sometimes referred to herein as "downregulation") can be taken to be an indicator of aquaretic activity eg., at least about a 10% decrease in comparison to a control. If desired, phosphorylation of the AQP-2 channel protein can also be measured, either alone or in conjunction with measurement of AQP-2 protein expression. As discussed, it is often preferred to measure both expression of AQP-2 and pAQP-2. In this example of the invention, the method employs an antibody that specifically binds pAQP-2 and does not significantly bind unphosphoryalted (or underphosphorylated) AQP-2. In embodiments in which production of pAQP-2 is examined alone, a substantial decrease in presence of pAQP-2 can be taken as an indicator of aquaretic activity eg., at least about a 10% decrease in phosphorylation of the channel protein when compared to a control, preferably at least about a 50% decrease relative to the control.

Further provided by the present invention are methods for preventing or treating a water retaining condition in a mammal. In a preferred embodiment, the method includes administering a therapeutically effective amount of at least one of the nociceptin ligand compounds disclosed herein to suppress expression of at least one of AQP-2 and pAQP-2 levels to prevent or treat the condition.

The invention also provides methods for modulating (increasing or decreasing) at least one of AQP-2 and pAQP-2 expression in a mammal. Preferably, the method includes administering a therapeutically effective amount of at least one of the compounds disclosed herein to the mammal to modulate the channel protein expression.

The *in vivo* methods of the present invention will help determine (or confirm) use of known or candidate compounds as therapeutic agents with good aquaretic activity. The methods can be performed in a wide variety of cells, tissues and organs eg., kidney, heart, and liver, preferably kidney.

Significantly, use of multiple detection formats (eg., a combination of in vivo and in vitro assays, or multiple in vivo detection strategies) with a single known or candidate compound can readily extend the selectivity and sensitivity of detection as desired. For instance, the present methods are compatible with conventional sodium and/or potassium detection methods to identify aquaretics with significant sodium and/or potassium sparing function. See the PCT application W0 01/98324 A1 (disclosing a variety of suitable *in vitro* and *in vivo* tests for use in conjunction with the present invention).

Additionally, pools or libraries of nociceptin ligands and analogues thereof can be made using standard synthetic methods including combinatorial-type chemistry manipulations and then tested *in vivo* in accord with the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic drawing showing an overview of vasopressin receptor (V2) effector coupling. AVP= arginine vasopressin; G-prot.= stimulatory G protein; AQP-2= aquaporin-2; PGE_2= prostaglandin E_2.

5

Figure 2A is a representation of a Western blot showing suppression of AQP-2 and pAQP-2 expression following administration of Compound 1 to sham operated rats (sham). Figure 2A is a graph showing results of densitometric measurements taken from the blot

10 shown in Figure 2A.

Figure 3A is a representation of a Western blot showing suppression of AQP-2 and pAQP-2 expression following administration of Compound 1 to rats with experimentally induced congestive heart failure (CHF). Figure 3B is a graph showing results of

15 densitometric measurements taken from the blot shown in Figure 3A .

DETAILED DESCRIPTION OF PARTICULAR EMBODIMENTS

As discussed, the present invention provides important *in vivo* methods for screening compounds with aquaretic activity. Preferred compounds are known (or suspected) of

20 increasing or decreasing nociceptin receptor activity, typically but not exclusively by specifically binding the receptor.

The methods described herein employ standard immunoassay such as immunoassays, Northern blots and PCR-based detection strategies. See, e.g., Sambrook et al., *Molecular*

25 *Cloning* (2d ed. 1989); Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1989. A preferred immunoassay involves at least one of: 1) an antigen capture assay, 2) an antibody capture assay, and 3) a multi-antibody sandwich assay. Preferred is a Western blot type assay. See eg., Harlow and Lane in Antibodies: *A Laboratory Manual*, Cold Spring Harbor Laboratory (1988), New York.

30

In invention embodiments in which the mammal to be used in the assay has congestive heart failure (CHF), that condition may be further be characterized by an increase in AQP2 expression. Preferably, the increase in expression will be at least about 1.2-fold eg., between from about 2- to about 5-fold as determined by standard immunological techniques.
5 A preferred technique is a Western immunoblot assay as described herein.

Preferred aquaretic compounds identified by the invention methods exhibit significant suppression of AQP-2 expression as measured by downregulation of AQP-2 and/or pAQP-2. Such aquaretic compounds suitably exhibit at least about a 10% decrease in AQP-2 and/or pAQP-2 expression, preferably at least about a 50% decrease in such activity up to about a
10 100% to 200% decrease when compared to a control assay. In such an assay, at least one known or candidate compound is administered to a mammal such as a rat. After a suitable time post-administration, one or more kidneys are removed from the rat and assayed for AQP-2 protein expression and optionally phosphorylation of that protein as evidenced by pAQP-2 production. A decrease of at least about 10% AQP-2 expression in the assay is
15 taken to be indicative of an aquaretic. Such activity can also be detected by measuring a decrease of at least about 10% of pAQP-2 in the assay.

A generally preferred assay measures AQP-2 expression and optionally pAQP-2 production by:

a) administering to a rat between from about 0.01nmol/min/100g body weight to
20 about 1nmol/min/100g body weight, preferably about 0.1nmol/min/100g body weight of a known or candidate compound in which the administration is for less than about 24 hours, preferably less than about 12 hours, typically for about 2 hours to 10 hours with about 4 hours being generally preferred. A typical administration route is i.v. infusion for about 4 hours.

25 b) following the administration, isolating one or more kidneys from the rat and preparing a kidney lysate in which the lystate is electrophoretically separated and transferred to a solid support sufficient to bind proteins in the lysate,

c) measuring expression of aquaporin-2 (AQP-2) in the kidney lysate by contacting the solid support with at least one antibody that specifically binds AQP-2, preferably one of such antibodies, followed by producing and detecting an antibody-AQP-2 channel protein complex; and

5 d) determining effect of the compound on the AQP-2 expression in which modulation of the AQP-2 expression is taken to be indicative of the aquaretic compound.

Preferably, the method further measures use of at least one antibody to detect the phosphorylation of AQP-2. Such measurements can be conducted before, after or during antibody binding of the AQP-2 protein and generally involve producing and detecting an 10 antibody-pAQP-2 channel protein complex. Examples of such antibodies are described below in Example 2.

A preferred immunological assay for use with the invention is a Western blot using a conventional sandwhich type ELISA assay. Suitable antibodies for binding AQP-2 and pAQP-2 are known and described below. Methods for performing such assays are disclosed 15 below and in Harlow and Lane, *supra*.

Alternatively, the invention can be used to detect modulation of AQP-2 transciption. Preferably, a significant decease in such transcription, mRNA stability (or both) is taken as indicative of the aquaretic. To practice this invention embodiment, the foregoing method steps c) and d) are replaced by one or more of the following steps:

20 ci) measuring production of AQP-2 mRNA by electrophoretically separating the kidney lysate and transferring same to a solid support sufficient to bind nucleic acid and contacting the solid support with hybridizing nucleic acid probe (eg., cDNA or RNA probe). Preferably, the probe binds the immobilized AQP-2 mRNA under high stringency conditions (ie., about 50% formamide, 0.1X SSC at 42°C); and

25 cii) determining effect of the known or candidate compound on production of the AQP-2 mRNA in which a decrease (downregulation) of the AQP-2 mRNA relative to a suitable control is taken to be indicative of the aquaretic compound.

Detailed methods for performing Northern blots have been described in Sambrook et al. Supra. Suitable probes for detecting the AQP-2 mRNA including DNA and cDNA probes have been disclosed. See Fushimi, K et al. (1993) *Nature* 361: 549-552; and You, G. et al. (1993) *Nature* 365: 844-847.

5

See also Deen, P.M et al. *Eur. J. Cell. Biol.* (2000) 79: 523 (reviewing AQP-2 in health and disease). Rutishauser, J. *Eur. J. Endocrinology* (1999) 140: 137 (disclosing AQP-2 channel mutations relating to nephrongeic diabetes). See also the National (U.S) Center for Biotechnology Information, National Institutes of Health (GENBANK) disclosing cDNA
10 sequences for human and rodent AQP-2. See also Agre, P. et al. (1993) *Am. J. Physiol.* 265: F463-F476.

The present invention methods are fully compatible with testing one or a combination of known or candidate compounds. Preferred compounds have demonstrated or suspected
15 capacity to modulate the nociceptin receptor, typically by binding to that receptor as a ligand. A wide variety of such suitable compounds have been disclosed. See U .S Pat. Nos. 5,998,375, 5,840,696 and WO 99/03491 (providing nociceptin and analogues thereof); US. Pat. No. 6,172,067 (2-substituted-1-piperidyl benzimidazoles and derivatives thereof); US. Pat. No. 6,258,825 (2-oxoimidazoles and derivatives thereof); U.S Pat. No. 6,262,066 (high
20 affinity nociceptin receptor ligands); U.S Pat. No. 6,043,366 (1,3,8-triaza Spiro (4,5)decan-7-ones and derivatives thereof); U.S. Pat. No. 6,075,034 (spiro[piperidine-4,1'-pyrrolo[3,4-C] pyrroles and derivatives thereof; WO 01/39775 and EP 1 122 257 A1 (benzimadazole compounds); EP1 072 263 A1 (amide derivatives and nociceptin antagonists) and WO 99/36421 (providing 4-(2-keto-1-benzimidazolinyl)piperidines and derivatives thereof).

25

Preferred candidate compounds for use in the methods described herein are peptide conjugates disclosed in Larsen, B.D et al. (W0 01/98324 A1 as published on 27 December 2001). Examples include, but are not limited to, conjugates having the following general formula I:

30

$$R_1\text{-}Z\text{-}X\text{-}Z'\text{-}R_2 \qquad (I)$$

wherein X represents a hexapeptide of the formula II

A^1-A^2-A^3-A^4-A^5-A^6 (II)

wherein A^1 represents Arg, Lys, His or Asp, A^2 represents Tyr, Trp, or Phe, A^3 represents Tyr, Trp or Phe, A^4 represents Lys, Arg or His, A^5 represents Phe, Tyr, Trp, Leu, Val or Ile, and A^6 represents Arg, Lys or His and wherein each amino acid residue in said hexapeptide may be in the L or D form;

Z represents a charged peptide chain of from 4 to 20 amino acid residues having the D or L configuration or is missing; and Z' represents a charged peptide chain of from 4 to 20 amino acid residues having the D or L configuration or is missing, providing that not both of Z and Z' are missing;

R_1 represents H or an acyl group;

R_2 represents NR_3R_4 where each of R_3 and R_4 independently represents hydrogen, C(1-6)-alkoxy, aryloxy or a lower alkyl as defined herein; or R_2 represents OH; and salts, hydrates and solvates thereof and C-terminally amidated or esterified derivatives thereof with suitable organic or inorganic acids.

As disclosed in the W0 01/98324 A1 PCT application, the formula II is represented by the amino acid sequence (RK)YY(RK)(WI)(RK) wherein alternative amino acid residues at positions 1, 4, 5 and 6 are shown in brackets. Alternatively, the amino acid residues R and K at positions 1, 4 and 6 may each be substituted with Orn, Dab or Dapa. Preferably, Z represents a negatively charged peptide chain of from 4 to 20 amino acid residues, Z' represents a positively charged peptide chain of from 4 to 20 amino acid residues, R_1 represents Ac or Tfa, and R_2 represents NH_2, or R_2 represents NR_3R_4 where each of R_3 and R_4 independently represents hydrogen, methyl or ethyl. Said hexapeptide X is preferably selected form the group consisting of KYYRWR, RYYRWR, KWRYYR, RYYRWK, RYYRWK (all-D), RYYRIK, RYYRIR, RYYKIK, RYYKIR, RYYKWR, and RYYKWK, more preferably the group consisting of RYYRWR, RYYRWK, RYYRIK, RYYKWR, and RYYKWK, more preferably said hexapeptide X is RYYRWK or KYYRWK wherein the amino acid residues are in the L-form unless otherwise specified. Furthermore, the number of amino acid residues in each of Z and Z' is preferably in the range of 4-10 or 5-10.

As provided in the WO 01/98324 A1 PCT application, preferred peptide conjugates have amino acid residues of Z are selected from the group consisting of Q, T, S, P, N, E, and D having the D or L configuration, and the N-terminal amino acid of Z is selected from the group consisting of Q T, N and S having the D or L configuration and the remaining amino acid residues are selected from the group consisting of P, D and E. More specifically, Z is selected from the group consisting of, e.g. $N(E)_7$, $N(E)_6$, $N(E)_5$, $N(E)_3$, $S(E)_7$, $S(E)_6$, $S(E)_5$, $S(E)_3$, $NP(E)_4$, $NP(E)_5$, $N(D)_7$, $N(D)_6$, $N(D)_5$, $N(D)_3$, $Q(E)_7$, $Q(E)_5$, $Q(E)_3$, $QN(D)_7$, $Q(D)_6$, $Q(D)_5$, and $Q(D)_3$, preferably Z is $N(E)_5$.

For use with the present invention, it is often preferred that the amino acid residues of Z' are selected from the group consisting of A, G, K, and R, preferably K, having the D or L configuration, more preferably Z' is selected from the group consisting of $A(K_4)G$, K_5G, AK_5, H_6, K_{10}, K_8, K_6, K_5, and K_4. Examples of conjugates Z-X-Z' are $N(E)_5RYYRWKK_6$, $N(E)_5RYYRWK$, $RYYRWKK_6$, $N(E)_5RYYRWRK_6$, $N(E)_5RYYRWR$, $RYYRWRK_6$, $N(E)_5RYYRIKK_6$, $N(E)_5RYYRIK$, and $RYYRIKK_6$.

More specific peptide conjugates for use with the invention are: Ac-$RYYRWKK_6$-NH_2, Ac-$K_6RYYRWK$-NH_2, $N(E)_5RYYRWKK_6$-NH_2, and salts, preferably pharmaceutically acceptable salts, hydrates, solvates and derivatives thereof including C-terminal derivatives, such as free carboxylic acid.

As disclosed in the W0 01/98324 A1 PCT application, a further peptide conjugate for use with the invention is represented by the general formula III

$$R_1\text{-}X\text{-}Z'\text{-}R_2 \qquad \text{(III)}$$

wherein R_1, X, Z' and R_2 have the same meanings as defined above, and salts, hydrates and solvates thereof and C-terminally amidated or esterified derivatives thereof with suitable organic or inorganic acids. In a preferred peptide conjugate of formula III R_1 represents Ac, X represents a hexapeptide of the formula (RK)YY(RK)(WI)(RK) wherein alternative amino acid residues at positions 1, 4, 5 and 6 are shown in brackets, Z' represents K_n where n is an integer selected from 5, 6 or 7, and R_2 represents NH_2. X is preferably selected from the

group consisting of KYYRWK, RYYRWR, RYYRWK, RYYRWK (all-D), KWRYYR, RYYRIK, RYYKWR, and RYYKWK. The peptide conjugates of formulae I and III are optionally further linked to a transport moiety or an affinity tag, such as H_6, where the linkage between the peptide conjugate and said transport moiety or affinity tag may be by
5 any convenient covalent bond. Said transport moiety is preferably selected from the group consisting of a HIV tat peptide residues 49-57, HIV tat peptide residues 49-56, the tat sequence YGRKKRRQRRR, a polyarginine peptide having from 6 to 20 residues, such as R_6, and transducing peptide sequences, such as the following peptide sequences: YARKARRQARR, YARAAARQARA, YARAARRAARR, YARAARRAARA,
10 ARRRRRRRRR, and YAAARRRRRRR, which are disclosed in WO 99/29721 and in US patent No. 6,221,355 (seq. id. nos. 3-8) the disclosures of which are incorporated by reference.

The peptide sequence of a compound of formulae I and III is optionally in the all-D
15 form, the retro form or the retro all-D form, where the all-D form is more preferred. Optionally, the X sequence of formulae I and III is an all-D form, a retro form, or a retro all-D form of the peptide sequence of formula II or the hexapeptide (RK)YY(RK)(WI)(RK) as defined above, respectively.

20 Specific compounds for use with the present screening methods have been disclosed in the WO 01/98324 A1 PCT application and are shown in Table 1 below:

Compound 1	Ac-RYYRWKKKKKKK-NH_2
Compound 1A	Ac-RYYRWKKKKKKK-NH_2
Compound 1C	Ac-RYYRWKKKKKKK-NH_2
Compound 2	Ac-KKKKKKRYYRWK-NH_2
Compound 3	H-NEEEEERYYRWKKKKKKK-NH_2
Compound 4	Ac-RYNRWKKKKKKK-NH_2
Compound 5	Ac-KKKKKKKWRYYN-NH_2
Compound 6	Ac-KKKKKKKWRYYR-NH_2

Compound 7	Ac-KKKKKKKWRYYR-NH_2 (all D)
Compound 8	Ac-RYYRWKKKKKKK-NH_2 (all D)
Compound 9	Ac-KYYRWKKKKKKK-NH_2
Compound 10	Ac-RYYRIKKKKKKK-NH_2
Compound 11	Ac-RYYRWKAKKKKK-NH_2
Compound 12	Ac-RYYRWKKKKKK-NH_2
Compound 13	Ac-RYYRWKKKKKKKC-NH_2
Compound 14	Tfa-RYYRWKKKKKKK-NH_2

as well as salts, hydrates, solvates and C-terminal derivatives thereof, such as the free carboxylic acid.

5 Other preferred compounds are
Ac-KWRYYNKKKKKK-NH_2
Ac-KWRYYRKKKKKK-NH_2 and
Ac-KWRYYRKKKKKK-NH_2 (all D) and salts, hydrates, solvates and C-terminal derivatives thereof, such as the free carboxylic acid.

Examples of acid addition salts with an organic and an inorganic acid of compounds of the invention are
Compound 1A Ac-RYYRWKKKKKKK-NH_2 x $9CH_3COOH$
and Compound 1C Ac-RYYRWKKKKKKK-NH_2 x 9HCl.

Other preferred compounds are peptides of formula I and III wherein R_1 represents p-
10 anisoyl, p-cyanobenzoyl, p-nitrobenzoyl, p-fluorobenzoyl, p-chlorobenzoyl, p-bromobenzoyl, p-aminobenzoyl, p-thiomethoxybenzoyl, 3-(4-hydroxyphenyl)propionyl, 3-(2-hydroxyphenyl)propionyl, 4-hydroxyphenoxyacetic acid, 2-hydroxyphenoxyacetic acid, 4-(hydroxymethyl)-phenoxyacetic acid, 4-hydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 2-hydroxyphenylacetic acid, 4-azidosalicyloyl radical or 4-azidobenzoyl radical; and
15 wherein X of the compound of formula I and III represents RYYRWK or RYYRIK and wherein the W and I residues are in the D-form; and wherein Z' of the compound of formula

I and III represents a polylysine chain of from 4 to 8 residues, preferably a K_5 or a K_6 peptide. Examples are the compounds p-anisoyl-Arg-Tyr-Tyr-Arg-D-Trp-Lys-Lys-Lys-Lys-Lys-Lys-Lys-OH/NH_2 and p-anisoyl-Arg-Tyr-Tyr-Arg-D-Ile-Lys-Lys-Lys-Lys-Lys-Lys-OH/NH_2.

5

Methods for making and using the peptide conjugates have been fully described in the WO 01/98324 A1 PCT application including references cited therein.

Throughout the description and claims the one letter code for natural amino acids is
10 used as well as the three letter code for natural amino acids and generally accepted three letter codes for other α-amino acids, such as Ornithine (Orn), 2,4-Diaminobutanoic acid (Dab) and 2,3-Diaminopropanoic acid (Dapa). Where the L or D form has not been specified it is to be understood that the amino acid in question has the natural L form, cf. Pure & Appl. Chem. Vol. 56(5) pp595-624 (1984). Where nothing is specified it is to be understood that
15 the C-terminal amino acid of a compound of the invention exists as the free carboxylic acid, this may also be specified as "-OH". Cf. Biochem. J., 1984, 219, 345-373; Eur. J. Biochem., 1984, 138, 9-37; 1985, 152, 1; 1993, 213, 2; Internat. J. Pept. Prot. Res., 1984, 24, following p 84; J. Biol. Chem., 1985, 260,14-42; Pure Appl. Chem., 1984, 56, 595-624; Amino Acids and Peptides, 1985, 16, 387-410; Biochemical Nomenclature and Related Documents, 2nd
20 edition, Portland Press, 1992, pages 39-69; Copyright IUBMB and IUPAC. The term "retro form" of a peptide conjugate of formulae I or III refers to a peptide having the reversed aminoacid sequence of formulae I or III. The term "all-D form" of a peptide conjugate of formulae I or III refers to a peptide wherein all aminoacid units are in the D-form. The term "retro all-D form" of a peptide conjugate of formulae I or III refers to a peptide having the
25 reversed aminoacid sequence of formulae I or III and wherein all aminoacid units are in the D-form (retro-inverse form). Optionally, in a peptide conjugate of the invention the sequence defined by formula II or the formula (RK)YY(RK)(WI)(RK) is in the all-D form, the retro form or the retro all-D form. D-aminoacids are unnatural aminoacids which are stable in a protease rich environment. Thus, a useful way of stabilising the peptide conjugates of the
30 invention against proteolytic degradation is to substitute L-aminoacids with corresponding D-aminoacids. The term " epitopic fraction" refers to a truncated or shortened section of a

peptide sequence having, e.g., 5-8 aminoacid units and is capable of eliciting an antigen response.

The term "alkyl" refers to univalent groups derived from alkanes by removal of a hydrogen atom from any carbon atom: C_nH_{2n+1}-. The groups derived by removal of a hydrogen atom from a terminal carbon atom of unbranched alkanes form a subclass of normal alkyl (*n*-alkyl) groups: $H[CH_2]_n$-. The groups RCH_2-, R_2CH- (R not equal to H), and R_3C- (R not equal to H) are primary, secondary and tertiary alkyl groups respectively. "Alkyl" refers to any alkyl group, and includes C(1-6)alkyl, such as methyl, ethyl, propyl, iso-propyl, butyl, pentyl and hexyl and all possible isomers thereof. By "lower alkyl" is meant C(1-6)alkyl, preferably C(1-4)alkyl, more preferably, methyl and ethyl.

The term "C(1-6)alkoxy" refers to an ester group of the formula R-O- wherein R represents C(1-6)alkyl. The term "aryloxy" refers to an ester group of the formula R-O- wherein R represents phenyl or naphthyl optionally substituted with a lower alkyl group.

The term "acyl " as used herein include acyl radicals which are formally derived from oxoacids $R_kE(=O)_l(OH)_m$ (l not equal to 0) by removal of a hydroxyl cation HO^+, a hydroxyl radical $HO^\cdot$ or a hydroxyl anion HO^-, respectively, and replacement analogues of such intermediates. Acyl radicals can formally be represented by canonical forms having an unpaired electron or a positive charge on the acid-generating element of the oxoacid. Acyl radicals, e.g. $RC^\cdot(=O)$, $RS^\cdot(=O)_2$.

The term "acylated" as used herein indicates the the compound in question carries an acyl group. An acyl group is formed by removing one or more hydroxy groups from an oxoacid, such as a carboxylic acid, that has the general structure $R_kE(=O)_l(OH)_m$ (l not equal to 0), and replacement analogues of such acyl groups. E.g.$CH_3C(=O)$-, $CH_3C(=NR)$-, $CH_3C(=S)$-, $PhS(=O)_2$-, $HP(\equiv N)$-. In organic chemistry an unspecified acyl group is commonly a carboxylic acyl group. Cf. International Union of Pure and Applied Chemistry, Recommendations on Organic & Biochemical Nomenclature, Symbols & Terminology etc.

IUPAC Recommendations 1994. “Ac” indicates an acetyl group and “Tfa” indicates a trifluoroacetyl group.

The term "peptide conjugate" as used herein indicates a fusion between at least two peptide sequences via a peptidic bond or an equivalent bioisosteric bond, such as the peptide bond mimetics described in Table 1 in Tayar et al., Amino Acids (1995) 8:125-139.

The term “transport moiety” as used herein indicates a chemical entity that acts in escorting molecules such as polypeptides across biological membranes. The transport polymers disclosed in WO 98/52612 are all incorporated by reference. Peptide conjugates of the invention are linked via a covalent bond to said transport moiety. The term “HIV tat peptide residues 49-57” refers to a transport moiety having the sequence RKKRRQRRR as disclosed in WO 91/09958.

“Agonist” refers to an endogenous substance or a drug that can interact with a receptor and initiate a physiological or a pharmacological response characteristic of that receptor (contraction, relaxation, secretion, enzyme activation, etc.).

“Antagonist” refers to a drug or a compound that opposes the physiological effects of another. At the receptor level, it is a chemical entity that opposes the receptor-associated responses normally induced by another bioactive agent.

“Partial agonist” refers to an agonist which is unable to induce maximal activation of a receptor population, regardless of the amount of drug applied (See also Intrinsic activity). A "partial agonist" may also be termed "agonist with intermediate intrinsic efficacy" in a given tissue. Moreover, a partial agonist may antagonize the effect of a full agonist that acts on the same receptor.

“Receptor” refers to a molecule or a polymeric structure in or on a cell that specifically recognizes and binds a compound acting as a molecular messenger (neurotransmitter, hormone, lymphokine, lectin, drug, etc.).

"Modulation" of a receptor refers to an increase or decrease in activity of that receptor as determined by one or more of the receptor binding assays disclosed herein and in the PCT application W0 01/98324 A1.

5

The term "salt", as used herein, denotes acidic and/or basic salts, formed with inorganic or organic acids and/or bases, preferably basic salts. While pharmaceutically acceptable salts are preferred, particularly when employing the compounds of the invention as medicaments, other salts find utility, for example, in processing these compounds, or

10 where non-medicament-type uses are contemplated. Salts of these compounds may be prepared by art-recognized techniques. Examples of such pharmaceutically acceptable salts include, but are not limited to, inorganic and organic acid addition salts, such as hydrochloride, sulphates, nitrates or phosphates and acetates, trifluoroacetates, propionates, succinates, benzoates, citrates, tartrates, fumarates, maleates, methane-sulfonates,

15 isothionates, theophylline acetates, salicylates, respectively, or the like. Lower alkyl quaternary ammonium salts and the like are suitable, as well. "Pharmaceutically acceptable anions" as used herein includes the group consisting of CH_3COO^-, CF_3COO^-, Cl^-, SO_3^{2-}, maleate and oleate.

20 The term "peripheral administration" includes all administration forms that exclude delivery of the active substance directly into the central nervous system. "Central administration" as used herein means an administration directly into the central nervous system, such as intracerebroventricular administration (i.c.v. administration).

25 By the phrase "water retaining condition" is meant an ailment that is directly or indirectly impacted by improper fluid homeostasis. Examples of such conditions include but are not limited to edema, hypertension, acute and chronic failure of the heart and/or kidneys, and nephrotic syndromes. Also included are primary Na^+ loss (secondary water gain) associated with, e.g., integumentary loss: sweating, burns; gastrointestinal loss: vomiting,

30 tube drainage, fistula, obstruction, diarrhea; renal loss: diuretics, osmotic diuresis, hypoaldosteronism, salt-wasting nephropathy, postobstructive diuresis, nonoligouric acute

tubular necrosis; Primary water gain (secondary Na^+ loss) associated with, e.g. primary polydipsia; decreased solute intake (e.g., beer potomania); AVP (vasopressin) release due to pain, nausea, drugs; syndrome of inappropriate AVP secretion; glucocorticoid deficiency; hypothyroidism and chronic renal insufficiency; and primary Na^+ gain (exceeded by 5 secondary water gain) associated with, e.g. heart failure, hepatic cirrhosis and nephrotic syndrome.

By the term, "specific binding" or a similar term is meant a molecule disclosed herein which binds another molecule, thereby forming a specific binding pair. However, the 10 molecule does not recognize or bind to other molecules as determined by, e.g., Western blotting ELISA, RIA, mobility shift assay, enzyme-immuno assay, competitive assays, saturation assays or other protein binding assays know in the art. See generally, Ausubel, et al *supra*; Sambrook, et al, *supra*; Harlow and Lane, *supra* and references cited therein for examples of methods for detecting specific binding between molecules.

15

As discussed, the invention provides methods for preventing or treating a water retaining condition in a mammal comprising administering a therapeutically effective amount of at least one of the compounds detected by the present methods. Preferably, the compounds suppresses expression of at least one of AQP-2 and optionally production of 20 pAQP-2. Further provided are methods for modulating at least one of AQP-2 expression and pAQP-2 production in a mammal that includes administering a therapuetically effective amount of at least one of the compounds disclosed herein. Acceptable adminstration regimins have been fully disclosed in the WO 01/98324 A1 PCT application.

25 As will be apparent, the present *in vivo* screening methods can be combined with one or a combination of the *in vitro* and *in vivo* methods for detecting and analyzing nociceptin binding that have been provided in the WO 01/98324 A1 PCT application. Such use of the invention can fine tine aquaretic detection and selection paradigms. Examples of such assays include conventional tests for measuring nociceptin receptor binding and diuresis including 30 sodium and potassium excretion assays. As discussed, preferred compounds identified by such methods have significant sodium and/or potassium sparing activity.

The following examples are illustrative of the present invention.

EXAMPLE 1: Modulation of AQP-2 and pAQP2 expression after treatment with a nociceptin analogue (Compound 1).

An objective of this Example was to evaluate changes in AQP2 and pAQP2 expression in response to a few hours i.v. treatment with Compound 1 in chronically instrumented rats where water balance is maintained, by use of a computer-driven, servo-controlled i.v. volume replacement system. Furthermore, potential differences in Compound 1 induced changes in AQP2 protein and phosphorylation levels between normal rats and rats with congestive heart failure was evaluated.

The following materials and methods were used:

Materials. Barrier-bred and specific pathogen-free male Wistar rats (210-230 g) from Charles River, Hannover, Germany are used. The animals were housed in a temperature (22-24□C) and moisture (40-70%) controlled room with a 12-hour light-dark cycle (light on from 6:00 A.M. to 6:00 P.M.). All animals had free access to tap water and a pelleted rat diet containing approximately 140 mmol/kg of sodium, 275 mmol/kg potassium and 23 % protein (Altromin catalogue no. 1310, Altromin International, Lage, Germany).

Animal preparation: Rats were anaesthetized in an inhalation chamber with 4% isoflurane in O_2. After insertion of an endotracheal tube the animal was artificially ventilated with1.0 % isoflurane in O_2 using af Hugo Basile Rodent ventilator. Tidal volume is set to 8-10 ml/kg b.w. and respiratory rate to 75 min^{-1} which maintain arterial pH between 7.35 and 7.45. During surgery the animal was placed on a heated table that maintains rectal temperature at 37-38°C. Standard ECG (second lead) was measured using a Hugo Sachs ECG Coupler and collected on line at 4,000 Hz in PowerLab. After parasternal thoracotomy and opening of the pericardium the left anterior descending coronary artery (LAD) was localized visually. Rats where the LAD were not visualized are excluded. An atraumatic 6-0 silk is placed around the LAD between the pulmonary trunk and the lower right end of the

left auricle. Then the LAD was occluded. Successful occluding was confirmed by alterations in the ECG (ST-segment elevation and increase in R-wave amplitude). Control rats was sham-operated, i.e. the same surgical procedure without ligation of LCD. To relieve postoperative pain, rats were treated with buprenorfin, 0.2 mg/kg b.w. i.p. twice daily for two
5 days.

Three weeks later, the rats were anaesthetised with fentanyl-fluanisone/midazolam and permanent medical grade Tygon catheters was implanted into the abdominal aorta and into the inferior caval vein via a femoral artery and vein. The catheters were sealed with a 50% glucose solution containing heparin and streptase. A permanent suprapubic bladder catheter
10 was implanted into the urinary bladder and sealed with a silicone-coated stainless steel pin after flushing the bladder with 0.1 % chlorhexidine. Surgical procedures were performed during aseptic conditions. To relieve postoperative pain, rats are treated with buprenorfin, 0.2 mg/kg b.w. i.p. twice daily for two days. After instrumentation, the animals were housed individually.

15 Four weeks after LCD ligation/sham operation the rats were anaesthetised with isoflurane in O_2. The concentration of isoflurane was adjusted to maintain after-load during anaesthesia, i.e mean arterial pressure was stabilised at 95-100 mmHg. A 2F Millar microtip catheter was inserted into the left ventricle via the right carotid artery for measurement of left ventricular end diastolic pressure (LVEDP), and positive and negative dP/dT. Rats with LAD
20 ligation and a LVEDP less than 10 mmHg are excluded. To relieve postoperative pain, rats were treated with buprenorfin, 0.2 mg/kg b.w. i.p. twice daily for two days.

Compound 1 ***treatment.*** Prior to Compound 1 infusion the rats were adapted to the restraining cage used for these experiments by training them for two periods of two hours
25 each. In order to examine the rats at the same level of hydration all experiments was started at 8:00 A.M.

The following protocol provides general guidance on performing the experiments reported in this example:

30

The aquaretic effect of Compound 1 in rats with congestive heart failure

The purpose of this part of the example is to evaluate the aquaretic effect of Compound 1 in rats with congestive heart failure induced by ligation of the left anterior descending coronary artery.

5

Study A: To investigate the aquaretic effect of i.v. treatment with Compound 1 in chronically instrumented rats where water balance is maintained, by use of a computer-driven, servo-controlled i.v. volume replacement system. In order to avoid influence from surgical and mental stress and anaestetics on renal function all experiments will be performed

10 in chronically instrumented, trained and conscious animals.

Study B: The examine the effect of one weeks treatment with Compound 1 on sodium and water balance and renal function in in chronically instrumented rats with congestive heart failure.

15

Methods

Materials. Barrier-bred and specific pathogen-free male Wistar rats (250 g). The animals will be housed in a temperature (22-24°C) and moisture (40-70%) controlled room with a 12-hour light-dark cycle (light on from 6:00 A.M. to 6:00 P.M.). All animals will have

20 free access to tap water and a pelleted rat diet containing approximately 140 mmol/kg of sodium, 275 mmol/kg potassium and 23 % protein (Altromin catalogue no. 1310, Altromin International, Lage, Germany). The number of rats that succesfully will go through the experimental protocol is N=8 in each group.

25 Animal preparation. Rats will be anesthetized in an inhalation chamber with 4% isoflurane in O_2. After insertion of an endotracheal tube the animal will be artificially ventilated with1.0 % isoflurane in O_2 using af Hugo Basile Rodent ventilator. Tidal volume will be set to 8-10 ml/kg b.w. and respiratory rate to 75 min-1 which will maintain arterial pH between 7.35 and 7.45. During surgery the animal will be placed on a heated table that

30 maintains rectal temperature at 37-38°C. Standard ECG (second lead) will be measured using a Hugo Sachs ECG Coupler and collected on line at 4,000 Hz in PowerLab. After parasternal

thoracotomy and opening of the pericardium the left anterior descending coronary artery (LAD) will be localized visually. Rats where the LAD can not be visualized will be excluded. An atraumatic 6□0 silk will be placed around the LAD between the pulmonary trunk and the lower right end of the left auricle. Then the LAD will be occluded. Succesfull occluding will be confirmed by alterations in ECG (ST-segment elevation and increase in R-wave amplitude). It must be expected the the mortality among the LAD ligated rats will be about 40%. Control rats will be sham-operated, e.i. the same surgical procedure without ligation of LAD. To relieve postoperative pain, rats will be treated with buprenorfin, 0.2 mg/kg b.w. i.p. twice daily for two days.

Three weeks later rats with be anesthetized with fentanyl-fluanisone and midazolam and permanent medical grade Tygon catheters will be implanted into the abdominal aorta and into the inferior caval vein via a femoral artery and vein. The catheters will be sealed with a 50% glucose solution containing heparin and streptase. A permanent suprapubic bladder catheter will be implanted into the urinary bladder and sealed with a silicone-coated stainless steel pin after flushing the bladder with 0.1 % Chlorhexidine. Surgical procedures will be performed during aseptic conditions. To relieve postoperative pain, rats will be treated with buprenorfin, 0.2 mg/kg b.w. i.p. twice daily for two days. After instrumentation, the animals will be housed individually (Petersen J.S: et al: *J. Pharmacol. Exp. Ther.* 258: 1-7,1991; Jonassen T.E.N. et al.: Hepatology 31: 1224-1230, 2000).

Four weeks after LAD ligation/sham operation the rats will be anesthetized with isoflurane in O2. The concentration of isoflurane will adjusted to maintain after load during anaesthesia, i.e MAP will be stabilized at 95-100 mmHg. A 2F Millar microtip catheter will be inserted into the left ventricle via the right carotic artery for measurement of left ventricular end diastolic pressure (LVEDP),and positive and negative dP/dT. Rats with LAD ligation that have a LVEDP less than 10 mmHg will be excluded. To relieve postoperative pain, rats will be treated with buprenorfin, 0.2 mg/kg b.w. i.p. twice daily for two days.

Study A:

Renal function will be examined by clearance techniques five weeks after LAD ligation/sham operation. Prior to the renal clearance experiments all rats will be adapted to the restraining cage used for these experiments by training them for two periods of two hours each. Three days before the clearance experiments, the rats' diet will be changed to a similar diet to which Li-citrate (12 mmol Li/kg dry diet) is added. When Li is given by this mode of administration and in this dose, it does not influence renal function. In order to examine the rats at the same level of hydration all experiments will be started at 8:00 A.M. Renal function will be examined by clearance techniques where 14[C]-tetraethylammonium bromide (TEA) clearance is used as a marker for the effective renal plasma flow (Petersen, J.S., and S. Christensen: Renal Physiol. 10: 102-109, 1987), 3[H]-inulin clearance as a marker for glomerular filtration rate and lithium clearance as a marker for the delivery of fluid from the proximal tubule (Thomsen, K., and D.G. Shirley: Nephron 77: 125-138, 1997). Renal clearances (C) and fractional excretions (FE) will be calculated by the standard formula: $C = U \cdot V/P$; $FE = C/GFR$ in which U = concentration in urine, V = urine flow rate, and P = plasma concentration; FE = fractional excretion. During the clearance experiment, mean arterial pressure (MAP) and heart rate (HR) will be measured continuously.

After a 90 minutes equilibration period, urine will be collected during two thirty minutes control periods. Then continuously i.v. infusion of Compound 1 (0.1 nmol/min/100 g b.w.) will start and urine will be sampled in 30 minuts periods for the next 4 hours (study period may be changes when we have the results from study protocol 1 (the tolerance study)). Total body water content will be kept constant during Compound 1 treatment by i.v. replacement of urine losses with 50 mM glucose. Volume replacement will be performed by use of a computer-driven servo-control system in which urine output will be monitored continuously by collecting urine in test tubes placed on an electronic balance that is connected to a computer which in turn controls the infusion rate of an infusion pump (Burgess, W. et al: Clin. Sci. 85: 129-137, 1993; Jonassen T.E.N et al: *Am. J Physiol Renal Physiol* 278: F246F256, 2000).

The rats will receive the following i.v. infusion throughout: 150 mM glucose, 3 mM lithium chloride; 1.0 ml/hour with [3H]-inulin (4.0 μCi/ml) and [14C]-TEA, (2.0 μCi/ml). In

addition to this infusion the rats will receive Vehicle 150 mM glucose at an infusion rate at 0.5 ml/hour during the equilibration and control periods. This infusion will end when the infusion of Compound 1 begins. Compound 1 will be dissolved in 150 mM glucose, infusion rate 0.5 ml/hour. Finally the rats will receive an infusion of 150 glucose with heparin 100
5 U/ml, 0.5 ml/hour, into the arterial catheter throughout.

Arterial blood samples of 300 µl each will be collected into ammonium-heparinized capillary tubes at the end of the equilibration period, at the end of the control periods and every hour during the Compound 1 treatment. In addition to this a 1.0 ml blood sample for
10 measurement of the plasma concentration of vasopressin will be collected during the equilibration period in a prechilled test tube (20 µl 0.5 M EDTA, pH 7.4, and 10 µl 20·106 IE/ml aprotinin) and a 0.3 ml blood sample will be collected for measurement of plasma renin concentration in a prechilled test tube. After centrifugation at 4EC, plasma will be transferred to a prechilled test tube and stored at -20°C for later determination. All blood
15 samples will be replaced immediately with heparinized blood from a normal donor rat.

Analytical procedures.

Urine volume will be determined gravimetrically. Concentrations of sodium, potassium, and lithium in plasma and urine will be determined by atomic absorption
20 spectrophotometry using a Perkin-Elmer (Allerød, Denmark) model 2380 atomic absorption spectrophotometer. Urine and plasma osmolality will be determined by use of a cryomatic osmometer, (Advanced Instruments model 3 CII, Needham Heights, MA). 3[H]-Inulin and 14[C]-tetraethylammonium bromide in plasma and urine will be determined by dual label liquid scintillation using a Packard Tri-Carp liquid scintillation analyser, model 2250CA
25 (Packard Instruments, Greve, Denmark). The plasma concentration of vasopressin will be measured by radioimmunoassay (RIA) using a commercial kit (vasopressin (125I) RIA kit catalog number RK-AR1; Bühlmann Laboratoies, Schwitzerland) and the concentration of PRA will be measured by tænk over om du vil have Ole Skøtt eller KAS Glostrup til at måle renin.

30

Data Analysis:

Renal function will be expressed as the urinary excretions of water, Na and K, by measurement of urine osmolality and by calculation free water clearance (CH_2O) for every 30 minuts urine collection period. Renal clearances of TEA, Inulin and Li and the fractional excretion of Na, K, Li and water will be calculated for each 30 minuts period as well.

5

Data will be presented as mean ± S.E. To evaluate the effects of Compound 1 treatment, the average value during the two 30 minutes control periods will be compared to the average value during the Compound 1 induced steady state diuresis using Student's paired t test. The maximal aquaretic effect of Compound 1 will be expressed as the
10 Compound 1 induced changes in diuresis, CH_2O, and V/CInulin. Differences in Compound 1 induced changes in V, CH_2O and other parameters of interest between the heart failure rats and the sham/operated controls will be performed by student with Student's unpaired t test. Differences will be considered significant at the 0.05 level.

15 Study B:

Renal function (only baseline values) will be examined by clearance techniques five weeks after LAD ligation. The same set up as in study A will be used. After a 90 minutes equilibration period, urine will be collected during six thirty minutes periods. Then an osmotic minipump Alzet model 2ML1 will be placed subcutaneusly in all rats. Half the rat
20 will receive Compound 1, infusion rate 0.1 nmol/min/100 g b.w., the other half will receive vehicle (water). The next morning, the the rats will be transferred into metabolic cages (Techniplast, model 1700, Scandbur A/S, Lellinge, Denmark). The rats will receive demineralized water and granulated standard diet (Altromin catalogue no. 1310, Altromin International, Lage, Germany) which contains approximately 150 mmol Na/kg. Sodium
25 intake will be calculated from the amount of diet ingested per 24 hours and sodium loss will be estimated from the amount of sodium excreted in the urine within the same 24 hours. Twenty four hours urine production will be measured gravimetrically and then the metabolic cage will be rinsed with 40-50 ml of demineralized water in order to optimize the recovery of sodium. The sodium content will be measured in the combined volume of urine and
30 demineralized water and 24 hours sodium balance will then be calculated as sodium intake minus urinary sodium losses. Urine osmolality will be measured as well. After one week in

the metabolic cages the rats will be transferred to the restaining cages an another renal clearance experiment will be performed. Same protocol as the pre-metabolic cage experiment.

5 Plasma samples for vasopressin and renin will be collected in the equilibration period in both the renal clearance studies.

Finally the rats will be anesthetized with halothane-nitrous oxide and the kidneys will be removed and immediately frozen in liquid nitrogen and stored at -80°C prior to analysis.
10 The kidneys will be homogenized and prepared for immunoblotting in order to quintitate the expression of selected renal water and sodium transporters.

Data Analysis:

Renal function will be expressed as the urinary excretions of water, Na and K, by
15 measurement of urine osmolality and by calculation free water clearance (CH_2O) for every 30 minuts urine collection period. Renal clearances of TEA, Inulin and Li and the fractional excretion of Na, K, Li and water will be calculated for each 30 minuts period as well.

Data will be presented as mean ± S.E. To evaluate the effects of Compound 1
20 treatment, the average values during steady state in the pre-treatment clearance study will compared to the average value during steady state in the second pos-ttreatment clearance study using Student's paired t test. Differences in Compound 1 induced changes in sodium and water balance as well as changes in renal function between the Compound 1 treated rats and the vehicle treated rats will be performed by Student's unpaired t test. Differences were
25 considered significant at the 0.05 level.

Time period Study A.

Day 1-8: LAD ligation/sham operations.

Day 22-28: Catheter operations.

30 Day 29-35: LVEDP measument and training of the animals in restraining cages.

Day: 35-42: Clearance studies.

Day: 43-52: Analysing Urine and Plasma samples.

Time period Study B.

Day 1-8: LAD ligation.

5 Day 22-28: Catheter operations.

Day 29-35: LVEDP measument and training of the animals in restraining cages.

Day: 35-42: Clearance studies (pretreatment) and metabolism studies.

Day: 43-52: Clearance studies (postreatment) and analysing Urine and Plasma samples.

10 As controls time-control studies are performed, the only change in the study design is that vehicle is infused throughout.

Tissue preparation for immunoblotting: The rats were anaesthetised and at the end of the experiments the left kidney was removed rapidly. The kidney is then homogenized using 15 a tissue homogenizer in 10 ml of ice-cold membrane-isolation solution, with 250 mM sucrose, 10 mM triethanolamine, 1 µg/ml leupeptin, and 0.1 mg/ml phenylmethylsulfonyl fluoride adjusted to pH 7.6. Protein concentration is measured on the homogenates (Pierce BCA Protein Assay Reagent Kit, Pierce, Rockford, IL). All samples is then diluted with isolation solution to a protein concentration of approximately 2 µg/µl and solubilized at 60°C 20 for 15 min in Laemmli sample buffer. Samples is then stored at -80°C until ready to run on gels.

Electrophoresis and Blotting of Membranes. Initially, 5 µg of protein from each of the samples was loaded on 12% sodium dodecyl sulfate-polyacrylamide gels and electrophoresed.. The proteins are transferred from the gels electrophoretically to PVDF 25 membranes. Blots is blocked with 5% milk in PBS-T for 1 hour, and incubated with the primary antibody. The labeling was visualized with horseradish peroxidase-conjugated secondary antibody (P448; Dako; diluted 1:3,000) using an enhanced chemiluminescence system (ECL+, Amersham). For AQP2 protein blots, we used the polyclonal antibody L126. This antibody was made to the same peptide as previously described L127 and gives a similar 30 labeling pattern. For pAQP2 we used the polyclonal antibody AN-244 who specific

recognize AQP2 phosphorylated at Ser^{256} in the PKA phosphorylation site (Arg-Arg-Gln-Se). Chemiluminescence was detected by use of a multi-imager (Bio-Rad Max2) and band densitometry within the linear range was analysed and quantitated by use of the gel-analysing program Quantity One (Bio-Rad). AQP2 or pAQP2 labelling in samples from the time-
5 control rats rats will be expressed relative to the mean expression in the corresponding material from rats treated with Compound 1 run on the same gel.

Results for the foregoing Western blot experiments are shown in Figures and 2A-B and 3A-B. Figures 2A-B and 3A-B also show that in an experimentally induced model of congestive heart failure (CHF), administration of Compound 1 suppressed expression of
10 phosphorylated AQP-2 (pAQP-2).

Antibodies used in the present example are obtainable from a variety of public sources including the National Institutes of Health (Laboratory of Kidney Research), Bethesda, MD (USA); the University of Aarhus, Denmark (Department of Anatomy); and the University of Copenhagen, Denmark (Department of Pharmacology).

15 Compound 1 is a novel peptide nociceptin analogue, which has been shown to have a marked aquaretic effect in acutely instrumented, conscious rats. The aquaretic effect of continuous i.v. infusion or Compound 1 (1 nmol/kg/min) in conscious rats during conditions where water balance was maintained using a computer-driven, servo-controlled i.v. volume replacement system was examined. The results elicited an aquaretic response of similar
20 magnitude to the response seen with maximal doses of the selective V_2-receptor antagonist OPC-31260. Like treatment with V_2-receptor antagonists the dose of Compound 1 inhibited tubular water reabsorption without concomitant changes in arterial blood pressure, heart rate, renal blood flow, glomerular filtration rate or proximal tubular reabsorption. Furthermore, measurements of plasma vasopressin indicated that the aquaretic effect of Compound 1 was
25 not due to inhibition of vasopressin secretion.

See generally Harlow and Lane, *supra* for a general discussion relating to the immunological methods used in this example.

EXAMPLE 2: Renal Effect of Compound 1 in Rats with Congestive Heart Failure (CHF)

Compound 1 is believed to be a novel ORL-1 receptor agonist that is useful for the treatment of acute decompensated heart failure. The aim of this example was to investigate the renal effect of Compound 1 in rats with CHF and to study the mechanism responsible for the aquaretic actions of Compound 1. Conscious, chronically instrumented rats with congestive heart failure induced by left coronary artery ligation (LCAL) were infused with either Compound 1 (1 nmol/kg/min) or vehicle for 4 hours (n=8/group) while water balance was maintained using a servo-controlled i.v. volume replacement system. Sham operated rats treated with Compound 1 or vehicle, were used as controls (n=8/group). Three weeks after LCAL, LVEDP was significantly elevated in CHF animals (Sham-CHF: 5±1 mmHg; CHF: 13±1 mmHg). Compounds 1 elicited a marked increase in urine flow rate (V) in both CHF and in control rats (ΔV_{CHF}: +43.9±8.2; ΔV_{Sham}: +49.4±5.1 μL/min/100 g b.w; $\Delta(V/GFR)_{CHF}$: +4.5±0.9%; $\Delta(V/GFR)_{Sham}$: +5.8±0.8%) without concomitant changes in systemic and renal hemodynamics or in the circulating levels of vasopressin. At the termination of the renal function study, the rats were anaesthetized and the kidneys were removed and prepared for immunoblotting (see above examples). CHF rats had as previously shown increased aquaporin 2 (AQP2) expression compared to control rats (Control-Vehicle: 100±18 vs. CHF-Vehicle: 171±16 %, $p<0.05$). Compounds 1 treatment significantly decreased the expression of AQP2 in both CHF and control rats (Control-Vehicle: 100±16 vs. Control-Compond 1: 47±7 %, $p<0.05$; CHF-Vehicle: 100±19 vs. CHF-Compound 1: 47±12 %, $p<0.05$). In conclusion, Compound 1 induces a marked aquaretic response in both normal and CHF rats, which is associated with a marked down-regulation of AQP2.

See also Nielsen, S. et al. (1997) *PNAS* 94: 5450 (showing that AQP2 expression is increased in animals with CHF).

All documents mentioned herein are incorporated herein in their entirety.

The invention has been described with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this

disclosure, may make modifications and improvements within the spirit and scope of the invention.

5

THE REST OF THIS PAGE IS LEFT BLANK INTENTIONALLY

What is claimed is:

1. An *in vivo* method for detecting an aquaretic compound comprising:

a) administering to a mammal a known or candidate compound that modulates the nociceptin (ORL1/OP4) receptor, the administration being for a time sufficient to modulate the nociceptin receptor and/or observe diuresis in the mammal,

b) isolating cells, tissue or an organ from the mammal,

c) measuring expression of aquaporin-2 (AQP-2) and/or phosphorylated aquaporin-2 (pAQP-2) in the cells or tissues; and

d) determining effect of the known or candidate compound on the AQP-2 and/or pAQP-2 expression, wherein modulation of the AQP-2 and/or pAQP-2 expression is taken to be indicative of presence of the aquaretic compound.

2. The method of claim 1, wherein the modulation of the AQP-2 or pAQP-2 expression is down regulated in the mammal.

3. The method of claim 1, wherein the known or candidate compound is a peptide conjugate of the following general formula I:

$$R_1\text{-}Z\text{-}X\text{-}Z'\text{-}R_2 \qquad (I)$$

wherein X represents a hexapeptide of the formula II

$$A^1\text{-}A^2\text{-}A^3\text{-}A^4\text{-}A^5\text{-}A^6 \qquad (II)$$

wherein A^1 represents Arg, Lys, or His, A^2 represents Tyr, Trp, or Phe, A^3 represents Tyr, Asn,Trp or Phe, A^4 represents Lys, Arg or His, A^5 represents Phe, Tyr, Trp, Leu, Val or Ile, and A^6 represents Arg, Lys or His and wherein each amino acid residue in said hexapeptide may be in the L or D form;

Z represents a charged peptide chain of from 4 to 20 amino acid residues having the D or L configuration or is missing; and Z' represents a charged peptide chain of from 4 to 20 amino acid residues having the D or L configuration or is missing, providing that not both of Z and

Z' are missing;

R_1 represents H or an acyl group;

R_2 represents NR_3R_4 where each of R_3 and R_4 independently represents hydrogen, C(1-6)alkoxy, aryloxy or a lower alkyl as defined herein; or R_2 represents OH; the peptide
5 conjugates of formula I being optionally further linked to a transport moiety; and salts, hydrates and solvates thereof, and C-terminally amidated or esterified derivatives thereof with suitable organic or inorganic acids.

4. The method of claim 3, wherein the formula II is represented by the amino acid
10 sequence (RK)YY(RK)(WI)(RK) wherein alternative amino acid residues at positions 1, 4, 5 and 6 are shown in brackets.

5. The method of claims 2-4, wherein the number of amino acid residues in Z and Z' is in the range 5 to 10.

15

6. The method of claims 2-5, wherein the amino acid residues in Z and Z' have the L-configuration.

7. The method of claims 2-6, wherein the amino acid residues of Z are selected from the
20 group consisting of Q, T, S, P, N, E, and D.

8. The method of claims 2-7, wherein the N-terminal amino acid of Z is selected from the group consisting of Q, T, N and S and the remaining amino acid residues are selected from the group consisting of P, D, and E.

25

9. The method of claims 2-8, wherein Z is selected from the group consisting of $N(E)_7$, $N(E)_6$, $N(E)_5$, $N(E)_3$, $S(E)_7$, $S(E)_6$, $S(E)_5$, $S(E)_3$, $NP(E)_4$, $NP(E)_5$, $N(D)_7$, $N(D)_6$, $N(D)_5$, $N(D)_3$, $Q(E)_7$, $Q(E)_5$, $Q(E)_3$, $QN(D)_7$, $Q(D)_6$, $Q(D)_5$, and $Q(D)_3$.

10. The method of claim 1, wherein the known or candidate compound is represented by the following general formula III

$$R_1\text{-X-Z'-}R_2 \qquad \text{(III)}$$

wherein R_1, X, Z' and R_2 have the same meanings as defined in the preceding claims; and
5 salts, hydrates and solvates thereof, and C-terminally amidated or esterified derivatives thereof with suitable organic or inorganic acids.

11. The method of claims 1-10, wherein the peptide sequence is in the all-D form, the retro form, or the retro all-D form.

10

12. The method of claims 1-11, wherein the peptide conjugate is optionally further linked to a transport moiety, where said transport moiety is preferably selected from the group consisting of a HIV tat peptide residues 49-57, HIV tat peptide residues 49-56, the tat sequence YGRKKRRQRRR, a polyarginine peptide having from 6 to 20 residues, such as
15 R_6 , and transducing peptide sequences, such as the following peptide sequences: YARKARRQARR, YARAAARQARA, YARAARRAARR, YARAARRAARA, YARRRRRRRRR, and YAAARRRRRRR.

13. The method of claims 1-12, wherein Z' represents a positively charged peptide chain.

20

14. The method of claims 1-13, wherein R_1 represents acetyl or trifluoroacetyl.

15. The method of claims 1-14, wherein R_2 represents NH_2, or R_2 represents NR_3R_4 where each of R_3 and R_4 independently represents hydrogen, methyl or ethyl.

25

16. The method of claims 1-15, wherein the hexapeptide X is selected form the group consisting of KYYRWK, RYYRWR, RYYRWK, RYYRWK (all-D), KWRYYR, RYYRIK, RYYRIR, RYYKIK, RYYKIR, RYYKWR, and RYYKWK.

30 17. The method of claim 16, wherein the hexapeptide X is KYYRWK or RYYRWK.

18. The method of claims 1-17, wherein the amino acid residues of Z' are selected from the group consisting of A, G, K, H and R, preferably K.

5 19. The method of claims 1-18, wherein Z' is selected from the group consisting of $A(K_4)G$, K_5G, AK_5 ,K_{10}, K_9, K_8, K_7, K_6, K_5, K_4.

20. The method of claims 1-19, wherein the peptide conjugate further comprises a terminal cysteinyl residue.

10

21. The method of claims 1-20, wherein the peptide conjugate is selected from the group consisting of:

Compound 1 Ac-RYYRWKKKKKKK-NH_2

Compound 2 Ac-KKKKKKRYYRWK-NH_2

Compound 3 H-NEEEEERYYRWKKKKKKK-NH_2

Compound 4 Ac-RYNRWKKKKKKK-NH_2

Compound 5 Ac-KKKKKKKWRYYN-NH_2

Compound 6 Ac-KKKKKKKWRYYR-NH_2

Compound 7 Ac-KKKKKKKWRYYR-NH_2 (all D)

Compound 8 Ac-RYYRWKKKKKKK-NH_2 (all D)

Compound 9 Ac-KYYRWKKKKKKK-NH_2

Compound 10 Ac-RYYRIKKKKKKK-NH_2

Compound 11 Ac-RYYRWKAKKKKK-NH_2

Compound 12 Ac-RYYRWKKKKKK-NH_2

Compound 13 Ac-RYYRWKKKKKKKC-NH_2

Compound 14 Tfa-RYYRWKKKKKKK-NH_2; and

the C-terminal free acid thereof, esterified derivatives thereof, and pharmaceutically acceptable acid addition salts thereof.

15

22. The method of claims 1-21, wherein the peptide conjugate is one of the following:

Compound 1A Ac-RYYRWKKKKKKK-NH_2 x $9CH_3COOH$
or Compound 1C Ac-RYYRWKKKKKKK-NH_2 x 9HCl.

23. The method of claims 1-22, wherein the peptide conjugate X-Z' is selected from the group consisting of $RYYRWKAK_5$, $KYYRWKK_6$, $RYYRWKK_6$, $RYYRWRK_6$, $RYYRIKK_6$, $RYYRWK_5$ and pharmaceutically acceptable salts and derivatives thereof
5 including N-terminally acetylated and C-terminally amidated or esterified derivatives.

24. The method of claims 1-23, wherein the peptide conjugate is Ac-$RYYRWKK_6$-NH_2 (Compound 1) and salts, hydrates, solvates and N-terminally acylated derivatives or esters thereof.
10

25. A method according to claims 1-10, wherein R_1 of the compound of formula I and III represents p-anisoyl, p-cyanobenzoyl, p-nitrobenzoyl, p-fluorobenzoyl, p-chlorobenzoyl, p-bromobenzoyl, p-aminobenzoyl, p-thiomethoxybenzoyl, 3-(4-hydroxyphenyl)propionyl, 3-(2-hydroxyphenyl)propionyl, 4-hydroxyphenoxyacetic acid, 2-hydroxyphenoxyacetic acid,
15 4-(hydroxymethyl)-phenoxyacetic acid, 4-hydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 2-hydroxyphenylacetic acid, 4-azidosalicyloyl radical or 4-azidobenzoyl radical.

26. A method according to claims 1-11 wherein X of the compound of formula I and III represents RYYRWK or RYYRIK and wherein the W and I residues are in the D-form.
20

27. A method according to claims 1-12 wherein Z' of the compound of formula I and III represents a polylysine chain of from 4 to 8 residues, preferably a K_5 or a K_6 peptide.

28. The method of claim 1, wherein the known compound is a 1,3,8,-triaza spiro (4,5)
25 decan-4-one or a spiro[piperidine-4,1'-pyrrolo[3,4-C] pyrrole]; or a derivative thereof as described in US. Pat. Nos. 6,075,034 and 6,043,366.

29. The method of claim 1, wherein the known compound is a 2-substituted-1-piperidyl benzimidazole or derivative thereof as described in U.S. Pat. No. 6,172,067.

30. The method of claim 1, wherein the known compound is a high affinity ORL-1 ligand as disclosed in U.S Pat. No. 6,262,066.

5 31. The method of claim 1, wherein the known compound is a 2-oxoimidazole or derivative thereof as described in U.S. Pat. No. 6,258,825.

32. The method of claim 1, wherein the known compound is a benzimidazole compound or derivative thereof as described in EP 1 122 257 A1 or W0 01/39775.

10

33. The method of claim 1, wherein the known compound is a 4-(2-keto-1-benzimidazoylinyl)piperidine compound or derivative therof as disclosed in WO 99/36421.

34. The method of claim 1, wherein the known compound is nociceptin or an analogue or

15 derivative thereof as disclosed in the following U.S. Pat. Nos. 5,840,696; 5,998,375; and PCT WO 99/03491.

35. The method of any one of claims 1-34, wherein the tissue isolated from the mammal is associated with the renal system.

20

36. The method of any of one of the claims 1-34, wherein the organ isolated from the mammal is a kidney.

37. The method of any one of claims 1-36, wherein the mammal has been manipulated to

25 mimic at least one of edema, hypertension, acute or chronic congestive heart failure (CHF).

38. The method of claim 37, wherein the mammal is surgically manipulated to induce CHF, the manipulation comprising occluding at least one coronary artery.

30 39. The method of claim 38, wherein the occlusion is achieved by partially or completely ligating the coronary artery.

40. The method of any one of claims 1-39, wherein step c) of the method further comprises measuring expression of at least one of the AQP-2 or pAQP-2.

5 41. The method of claim 40, wherein the expression of the AQP-2 and pAQP-2 proteins is measured immunologically.

42. The method of claim 41, wherein the immunological measurement comprises performing at least one of: 1) an antibody capture assay; 2) an antigen capture assay; and 3)
10 a multi-antibody sandwich assay.

43. The method of claim 42, wherein the immunological measurement comprises performing an ELISA assay.

15 44. The method of claim 43, wherein the ELISA assay comprises use of a second antibody linked to one of biotin, streptavidin, horseradish peroxidase or alkaline phosphatase.

45. The method of claim 1, wherein expression of the AQP-2 is measured by performing a polymerase chain reaction (PCR) assay.

20

46. The method of claims 1-45, wherein the known or candidate compound decreases AQP-2 expression by at least about 10% when compared to a control.

47. The method of claims 1-46, wherein the known or candidate compound decreases
25 transcription of the endogenous AQP-2 gene relative to a control.

48. The method of claims 1-47, wherein the known or candidate compound decreases AQP-2 and pAQP-2 expression by at least about 10% when compared to a control.

30 49. The method of claim 1-48, wherein the known or candidate compound decreases phosphorylation of the AQP-2 channel protein when compared to a control.

50. A method for preventing or treating a water retaining condition in a mammal comprising administering a therapuetically effective amount of at least one of the compounds of claims 1-49 sufficient to suppress expression of at least one of AQP-2 and pAQP-2 and
5 prevent or treat the condition.

51. A method for modulating at least one of AQP-2 and pAQP-2 expression in a mammal comprising administering a therapuetically effective amount of at least one of the compounds of claims 1-49.
10

52. The method of claim 1, wherein the time sufficient to modulate the nociceptin receptor and/or observe diuresis in the mammal is less than about 24 hours.

53. The method of claim 52, wherein the time sufficient to modulate the nociceptin
15 receptor and/or observe diuresis in the mammal is less than about 12 hours.

54. The method of claim 53, wherein sufficient time to modulate the nociceptin receptor and/or observe diuresis in the mammal is about 4 hours.

20 55. The method of claim 37, wherein the mammal has congestive heart failure (CHF), the CHF being characterized by an increase in AQP2 expression compared to a control (sham-operated) mammal.

56. The method of claim 55, wherein the increase in AQP2 expression is at least about a
25 2-fold increase.

57. The method of claim 56, wherein the increase in AQP2 expression is between about 2-fold to about 5-fold increase.