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(54) **Title**: NEW METHOD FOR TREATING CARDIOVASCULAR DISEASES

(57) **Abstract**: The present invention relates to a compound which is selected from the group consisting of AT1-R/α2C-AR heterodimerization inhibitors, AT1-R/α2C-AR antagonists or AT1-R/α2C- AR expression inhibitors for use in the prevention and treatment of cardiovascular diseases in a subject in need thereof.

NEW METHOD FOR TREATING CARDIOVASCULAR DISEASES

FIELD OF THE INVENTION:

The present invention relates to a compound which is selected from the group consisting of AT1-R/α2C-AR heterodimerization inhibitors, AT1-R/α2C-AR antagonists or AT1-R/α2C-AR expression inhibitors for use in the prevention and treatment of cardiovascular diseases in a subject in need thereof.

BACKGROUND OF THE INVENTION:

The pathogenesis of arterial hypertension (HT) and heart failure (HF) is multifactorial, but hyperactivity of both the sympathetic nervous system (SNS) and the renin-angiotensin-aldosterone system (RAAS) is fundamental in their development [Guyenet P.G. et al., 2006 and Paulis J. et al., 2010]. Both systems are functionally interconnected since norepinephrine (NE) secretion through the SNS stimulates angiotensin II (AngII) production and vice versa. Consequently, HT and HF management is currently based on the use of similar pharmacological classes. However, the efficacy of drugs reducing RAAS activity, like AngII AT_1 receptors (AT_1R) antagonists on morbidity and mortality remains uncertain. Moreover, although centrally-acting drugs such as α_2-adrenergic agonists efficiently reduce SNS activity they have never proven to effectively reduce morbi-mortality [Perk J. et al., 2012]. Therefore, a better understanding of the molecular mechanisms regulating NE secretion could help to develop new efficient therapeutics.

NE secretion by SNS endings is controlled by a balance between inhibitory and stimulatory presynaptic G-protein-coupled receptors (GPCRs). The α_2-adrenergic receptors (α_{2A}-AR and α_{2C}-AR), the main NE-inhibitory secretion system, play a prominent role in controlling sympathetic neural output, and their dysfunction constitute a central mechanism for sympathetic hyperactivity in HT/HF [Hein L. et al., 1999 and Lymperopoulos A. et al., 2007]. In contrast, AT1-R facilitate NE secretion [Rump L.C. et al., 1995]. Several ex vivo studies have highlighted the existence of a functional crosstalk between the α_2 and AT_1 receptors in the modulation of NE secretion but led to inconsistent results [Costa M et al., 1988; Cox S.L. et al., 2000 and Talaia C. et al., 2006]. It has been suggested that α_2-AT_1 crosstalk relies on intracellular communication involving $G\beta\gamma$ from Gi/o proteins coupled to α_2-AR [Talaia C. et al., 2006]. However, it is now well-established that GPCRs can exist both as monomeric and

oligomeric entities and that GPCR dimerization, specifically heterodimerization, impacts on different levels of each receptor protomer including traffic, ligand binding and signalling [Smith N.J. et al., 2010]. Recently, GPCR heterodimers were suggested to play a role in physiology and pathophysiology [Gonzales S. et al 2012; Gonzales-Maeso J. et al., 2008; Kern A. et al., 2012; Rivero-Muller A. et al., 2009 and Rozenfeld R. et al, 2011]. Numerous GPCRs have been shown to dimerize and α_2-AR or AT_1-R are no exception [Rozenfeld R. et al, 2011; Abdalla S. et al., 2001; Jordan B.A. et al., 2003; Small K.M. et al., 2006]. However, AT_1-R/α_2-AR heterodimerization is not reported. In this context, the inventors hypothesized that the crosstalk between AT_1-R and α_2-AR for NE release regulation could rely on the existence of AT_1-R/α_2-AR heterodimers. The fact that AT_1-R stimulates NE secretion and that α_{2C}-AR subtype does not influence NE secretion per se but acts as a regulator of α_{2A}-AR-promoted NE secretion prompted the inventors to examine the heterodimerization of AT_1-R with α_{2C}-AR in the context of neurohumoral activation.

SUMMARY OF THE INVENTION:

In this study, the inventors used different biophysical and newly-developed technical approaches, combined with in vitro and in vivo functional assays, to provide an exhaustive characterization of the structure/function of the α_{2C}-AR/AT_1-R heterodimer. They demonstrated that NE/AngII co-stimulation creates a new pharmacological entity of the α_{2C}-AR/AT_1-R heterodimer, signalling through an original Gs/cAMP/PKA signalling pathway and associated with both NE hypersecretion and sympathetic nerve hyperactivity in vivo. These results present the α_{2C}-AR/AT_1-R heterodimer as a promising potential new pharmacological target for the management of HT/HF-associated sympathetic hyperactivity.

Thus, the invention relates to a compound which is selected from the group consisting of AT1-R/α2C-AR heterodimerization inhibitors, AT1-R/α2C-AR antagonists or AT1-R/α2C-AR expression inhibitors for use in the prevention and treatment of cardiovascular diseases in a subject in need thereof.

DETAILED DESCRIPTION OF THE INVENTION:

Therapeutic methods and uses

In a first aspect, the present invention relates to a compound which is selected from the group consisting of AT1-R/α_{2C}-AR heterodimerization inhibitors, AT1-R/α_{2C}-AR antagonists or AT1-R/α_{2C}-AR expression inhibitors for use in the prevention and treatment of cardiovascular diseases in a subject in need thereof.

In one aspect, the invention relates to a compound which is selected from the group consisting of AT1-R/α_{2C}-AR heterodimerization inhibitors, AT1-R/α_{2C}-AR antagonists or AT1-R/α_{2C}-AR expression inhibitors for use in the prevention and treatment of diseases characterized by hyperactivity of the sympathetic nervous system (SNS) in a subject in need thereof. In a particular embodiment, these diseases are cardiovascular diseases.

In one aspect, the invention relates to a compound which is selected from the group consisting of AT1-R/α_{2C}-AR heterodimerization inhibitors, AT1-R/α_{2C}-AR antagonists or AT1-R/α_{2C}-AR expression inhibitors for use in the prevention and treatment of cardiovascular diseases with hyperactivity of the sympathetic nervous system (SNS) and the rennin-angiotensin-alsodsterone system (RAAS) in a subject in need thereof.

As used herein, the term "subject" denotes a mammal. In a preferred embodiment of the invention, a subject according to the invention refers to any subject (preferably human) afflicted with a cardiovascular diseases. In a particular embodiment, the subject is an obese subject.

According to the invention, cardiovascular diseases include but are not limited to heart failure, hypertension, cardiomyopathies and cardiac rhythm disturbances.

As used herein, the term "heart failure" denotes inability of the heart to supply sufficient blood flow to meet the body's needs and this pathology is well-described in medicine practice. This term encompasses chronic heart failure, acute heart failure, myocardial infarction, unstable angina, diastolic dysfunction, systolic dysfunction and diabetic cardiomyopathy.

A patient with a heart failure is classified according to an international gradation namely the New York Heart Association (NYHA) functional classification. Functional classification of heart failure is generally done by the New York Heart Association Functional Classification (Criteria Committee, New York Heart Association. Diseases of the heart and blood vessels). Nomenclature and criteria for diagnosis, 6th ed. Boston: Little, Brown and co, 1964;114). This classification stages the severity of heart failure into 4 classes (I-IV).

As used herein, the term "hypertension" encompasses arterial hypertension, venous hypertension and pulmonary hypertension.

As used herein, the term "cardiomyopathies" encompasses hypertrophic cardiomyopathy, dilated cardiomyopathy, Takotsubo cardiomyopathy.

As used herein, the term "cardiac rhythm disturbances" encompasses any abnormality of heart rhythm related to excessive activity of SNS including but not limited to ventricular tachycardia, ventricular fibrillation.

As used herein, the term "the sympathetic nervous system (SNS)" denotes one of the two main divisions of the autonomic nervous system, the other being the parasympathetic nervous system. The autonomic nervous system functions to regulate the body's unconscious actions. The sympathetic nervous system's primary process is to stimulate the body's fight-or-flight response. It is, however, constantly active at a basic level to maintain homeostasis. The sympathetic nervous system is described as being complementary to the parasympathetic nervous system which stimulates the body to "rest-and-digest" or feed and breed. The sympathetic nervous system is responsible for up- and down-regulating in many homeostatic mechanisms in living organisms. Fibres from the SN innervate tissues in almost every organ system, providing at least some regulatory function to things as diverse as glucidic metabolism, blood pressure, heart rate, pupil diameter, gut motility, sexual function, urinary system output and function.

As used herein, the term "the renin-angiotensin-aldosterone system (RAAS)" denotes a hormone system that regulates blood pressure and water (fluid) balance. Angiotensin II has a variety of effects on the body and for example throughout the body, it is a potent pro-hypertensive factor through a direct action on vascular receptors, increased release of norepinephrine from SNS endings and effects on brainstem centres involved in blood pressure regulation.

As used herein, the term "AT1-R" for "angiotensin II receptor type 1" has its general meaning in the art and refers to an angiotensin receptor. It has vasopressor effects and regulates aldosterone secretion. It is an important effector controlling blood pressure and volume in the cardiovascular system.

As used herein, the term "α_{2C}-AR" for "adrenoceptors subtype α2C" has its general meaning in the art and refers to a G protein-coupled receptor (GPCR) associated with the Gi

heterotrimeric G-protein. Catecholamines like norepinephrine (noradrenaline) and epinephrine (adrenaline) signal through the α2-adrenergic receptor in the central and peripheral nervous systems.

The term "expression" when used in the context of expression of a gene or nucleic acid refers to the conversion of the information, contained in a gene, into a gene product. A gene product can be the direct transcriptional product of a gene (e.g., mRNA, tRNA, rRNA, antisense RNA, ribozyme, structural RNA or any other type of RNA) or a protein produced by translation of a mRNA. Gene products also include messenger RNAs which are modified, by processes such as capping, polyadenylation, methylation, and editing, and proteins (e.g., AT1-R and/or α_{2C}-AR) modified by, for example, methylation, acetylation, phosphorylation, ubiquitination, SUMOylation, ADP-ribosylation, myristilation, and glycosylation.

An "inhibitor of expression" refers to a natural or synthetic compound that has a biological effect to inhibit the expression of a gene.

In one embodiment, the compound of the invention is an AT1-R/α_{2C}-AR heterodimerization inhibitor.

As used herein, the term "AT1-R/α_{2C}-AR heterodimerization inhibitor" refers to a compound that selectively prevents or blocks AT1-R/α_{2C}-AR heterodimerization. The term "AT1-R/α2C-AR heterodimerization inhibitor" refers to a compound that targets AT1-R and/or α_{2C}-AR proteins and blocks AT1-R/α_{2C}-AR heterodimerization. Typically, a AT1-R/α_{2C}-AR heterodimerization inhibitor is a small organic molecule, a peptide, a polypeptide, an aptamer, an intra-antibody or a nanobody.

In one embodiment, the compound of the invention is an AT1-R/α_{2C}-AR antagonist.

The term "AT1-R/α_{2C}-AR antagonist" refers to a compound that selectively blocks or inactivates the AT1-R/α_{2C}-AR. As used herein, the term "selectively blocks or inactivates" refers to a compound that preferentially binds to and blocks or inactivates AT1-R/α_{2C}-AR with a greater affinity and potency, respectively, than its interaction with the other receptor (α_{2C}-AR or AT1-R). Compounds that prefer AT1-R or α_{2C}-AR, but that may also block or inactivate other nuclear receptor sub-types, as partial or full antagonists, are contemplated. Typically, an AT1-R/α_{2C}-AR antagonist is a small organic molecule, a peptide, a polypeptide, an aptamer, an intra-antibody or a nanobody.

In another embodiment, the AT1-R/α_{2C}-AR heterodimerization inhibitor or AT1-R/α_{2C}-AR antagonist of the invention is an aptamer. Aptamers are a class of molecule that represents an alternative to antibodies in term of molecular recognition. Aptamers are oligonucleotide sequences with the capacity to recognize virtually any class of target molecules with high affinity and specificity. Such ligands may be isolated through Systematic Evolution of Ligands by EXponential enrichment (SELEX) of a random sequence library, as described in Tuerk C. and Gold L., 1990. The random sequence library is obtainable by combinatorial chemical synthesis of DNA. In this library, each member is a linear oligomer, eventually chemically modified, of a unique sequence. Possible modifications, uses and advantages of this class of molecules have been reviewed in Jayasena S.D., 1999. Peptide aptamers consists of a conformationally constrained antibody variable region displayed by a platform protein, such as E. coli Thioredoxin A that are selected from combinatorial libraries by two hybrid methods (Colas et al., 1996). Then after raising aptamers directed against AT1-R/α_{2C}-AR of the invention as above described, the skilled man in the art can easily select those inhibiting AT1-R/α_{2C}-AR heterodimerization or inhibiting AT1-R/α_{2C}-AR.

In one embodiment, the compound of the invention is an inhibitor of AT1-R/α_{2C}-AR expression.

Inhibitors of AT1-R/α_{2C}-AR expression for use in the present invention may be based on antisense oligonucleotide constructs. Anti-sense oligonucleotides, including anti-sense RNA molecules and anti-sense DNA molecules, would act to directly block the translation of AT1-R and/or α_{2C}-AR mRNA by binding thereto and thus preventing protein translation or increasing mRNA degradation, thus decreasing the level of AT1-R and/or α_{2C}-AR proteins, and thus the expression of the AT1-R/α_{2C}-AR heterodimer , in a cell. For example, antisense oligonucleotides of at least about 15 bases and complementary to unique regions of the mRNA transcript sequence encoding AT1-R and/or α_{2C}-AR can be synthesized, e.g., by conventional phosphodiester techniques and administered by e.g., intravenous injection or infusion. Methods for using antisense techniques for specifically alleviating gene expression of genes whose sequence is known are well known in the art (e.g. see U.S. Pat. Nos. 6,566,135; 6,566,131; 6,365,354; 6,410,323; 6,107,091; 6,046,321; and 5,981,732).

Small inhibitory RNAs (siRNAs) can also function as inhibitors of AT1-R/α_{2C}-AR expression for use in the present invention. AT1-R/α_{2C}-AR gene expression can be reduced by contacting the subject or cell with a small double stranded RNA (dsRNA), or a vector or construct causing the production of a small double stranded RNA, such that AT1-R and/or α_{2C}-

AR expression is specifically inhibited (i.e. RNA interference or RNAi). Methods for selecting an appropriate dsRNA or dsRNA-encoding vector are well known in the art for genes whose sequence is known (e.g. see Tuschl, T. et al. (1999); Elbashir, S. M. et al. (2001); Hannon, GJ. (2002); McManus, MT. et al. (2002); Brummelkamp, TR. et al. (2002); U.S. Pat. Nos. 6,573,099 and 6,506,559; and International Patent Publication Nos. WO 01/36646, WO 99/32619, and WO 01/68836).

Ribozymes can also function as inhibitors of AT1-R/α_{2C}-AR expression for use in the present invention. Ribozymes are enzymatic RNA molecules capable of catalysing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Engineered hairpin or hammerhead motif ribozyme molecules that specifically and efficiently catalyse endonucleolytic cleavage of AT1-R and/or α_{2C}-AR mRNA sequences are thereby useful within the scope of the present invention. Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites, which typically include the following sequences, GUA, GUU, and GUC. Once identified, short RNA sequences of between about 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site can be evaluated for predicted structural features, such as secondary structure, that can render the oligonucleotide sequence unsuitable. The suitability of candidate targets can also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using, e.g., ribonuclease protection assays.

Both antisense oligonucleotides and ribozymes useful as inhibitors of AT1-R/α_{2C}-AR expression can be prepared by known methods. These include techniques for chemical synthesis such as, e.g., by solid phase phosphoramadite chemical synthesis. Alternatively, anti-sense RNA molecules can be generated by in vitro or in vivo transcription of DNA sequences encoding the RNA molecule. Such DNA sequences can be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Various modifications to the oligonucleotides of the invention can be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5' and/or 3' ends of the molecule, or the use of phosphorothioate or 2'-O-methyl rather than phosphodiesterase linkages within the oligonucleotide backbone.

Antisense oligonucleotides siRNAs and ribozymes of the invention may be delivered in vivo alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable

of facilitating the transfer of the antisense oligonucleotide siRNA or ribozyme nucleic acid to the cells and preferably cells expressing AT1-R and/or α_{2C}-AR. Preferably, the vector transports the nucleic acid to cells with reduced degradation relative to the extent of degradation that would result in the absence of the vector. In general, the vectors useful in the invention include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the antisense oligonucleotide siRNA or ribozyme nucleic acid sequences. Viral vectors are a preferred type of vector and include, but are not limited to nucleic acid sequences from the following viruses: retrovirus, such as moloney murine leukemia virus, harvey murine sarcoma virus, murine mammary tumor virus, and rouse sarcoma virus; adenovirus, adeno-associated virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a retrovirus. One can readily employ other vectors not named but known to the art.

Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses (e.g., lentivirus), the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes in vivo. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in KRIEGLER (A Laboratory Manual," W.H. Freeman C.O., New York, 1990) and in MURRY ("Methods in Molecular Biology," vol.7, Humana Press, Inc., Cliffton, N.J., 1991).

Preferred viruses for certain applications are the adeno-viruses and adeno-associated viruses, which are double-stranded DNA viruses that have already been approved for human use in gene therapy. The adeno-associated virus can be engineered to be replication deficient and is capable of infecting a wide range of cell types and species. It further has advantages such as, heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages, including hematopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular

DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression characteristic of retroviral infection. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

Other vectors include plasmid vectors. Plasmid vectors have been extensively described in the art and are well known to those of skill in the art. See e.g., SANBROOK et al., "Molecular Cloning: A Laboratory Manual," Second Edition, Cold Spring Harbor Laboratory Press, 1989. In the last few years, plasmid vectors have been used as DNA vaccines for delivering antigen-encoding genes to cells in vivo. They are particularly advantageous for this because they do not have the same safety concerns as with many of the viral vectors. These plasmids, however, having a promoter compatible with the host cell, can express a peptide from a gene operatively encoded within the plasmid. Some commonly used plasmids include pBR322, pUC18, pUCl9, pRC/CMV, SV40, and pBlueScript. Other plasmids are well known to those of ordinary skill in the art. Additionally, plasmids may be custom designed using restriction enzymes and ligation reactions to remove and add specific fragments of DNA. Plasmids may be delivered by a variety of parenteral, mucosal and topical routes. For example, the DNA plasmid can be injected by intramuscular, intradermal, subcutaneous, or other routes. It may also be administered by intranasal sprays or drops, rectal suppository and orally. It may also be administered into the epidermis or a mucosal surface using a gene-gun. The plasmids may be given in an aqueous solution, dried onto gold particles or in association with another DNA delivery system including but not limited to liposomes, dendrimers, cochleate and microencapsulation.

In one embodiment, the present invention relates to a method of preventing and treating cardiovascular diseases in a subject in need thereof, comprising the step of administering to said subject a compound which is selected from the group consisting of AT1-R/α_{2C}-AR heterodimerization inhibitors, AT1-R/α_{2C}-AR antagonists or AT1-R/α_{2C}-AR expression inhibitors.

Pharmaceutical composition

The compound of the invention may be used or prepared in a pharmaceutical composition.

In one embodiment, the invention relates to a pharmaceutical composition comprising the compound of the invention and a pharmaceutical acceptable carrier for use in the prevention and treatment of cardiovascular diseases in a subject in need thereof.

Typically, the compound of the invention may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions.

"Pharmaceutically" or "pharmaceutically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

In the pharmaceutical compositions of the present invention for oral, sublingual, subcutaneous, intramuscular, intravenous, transdermal, local or rectal administration, the active principle, alone or in combination with another active principle, can be administered in a unit administration form, as a mixture with conventional pharmaceutical supports, to animals and human beings. Suitable unit administration forms comprise oral-route forms such as tablets, gel capsules, powders, granules and oral suspensions or solutions, sublingual and buccal administration forms, aerosols, implants, subcutaneous, transdermal, topical, intraperitoneal, intramuscular, intravenous, subdermal, transdermal, intrathecal and intranasal administration forms and rectal administration forms.

Preferably, the pharmaceutical compositions contain vehicles which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium) especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions.

In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

Solutions comprising compounds of the invention as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The compound of the invention can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetables oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatine.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with several of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder

of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

In addition to the compounds of the invention formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms include, e.g. tablets or other solids for oral administration; liposomal formulations; time release capsules; and any other form currently used.

Screening method

In a further aspect, the present invention relates to a method of screening a candidate compound for use as a drug for the prevention and treatment of cardiovascular diseases in a subject in need thereof, wherein the method comprises the steps of: i) providing candidate compounds and ii) selecting candidate compounds that blocks the action of AT1-R/α_{2C}-AR heterodimers.

In a further aspect, the present invention relates to a method of screening a candidate compound for use as a drug for the treatment of cardiovascular diseases in a subject in need thereof, wherein the method comprises the steps of:

(i) providing AT1-R/α_{2C}-AR heterodimers., providing a cell, tissue sample or organism expressing the T1-R/α_{2C}-AR heterodimers,

(ii) providing a candidate compound such as small organic molecule, intra-antibodies, peptide or polypeptide,

(iii) measuring the activity of the AT1-R/α_{2C}-AR heterodimers,

(iv) and selecting positively candidate compounds that blocks AT1-R/α_{2C}-AR heterodimerization, blocks the action of AT1-R/α_{2C}-AR or inhibits AT1-R/α_{2C}-AR expression.

Methods for measuring the activity of the AT1-R/α_{2C}-AR heterodimers are well known in the art. For example, measuring the AT1-R/α_{2C}-AR heterodimers activity involves measuring the activity of the Gs/cAMP/PKA and thus a simple test. Indeed, the activity of the dimer could be measured by quantification of global cAMP production in a cell model expressing the AT1-R/α_{2C}-AR heterodimers by sensitive hTRF assay (femto cAMP kit, *Cisbio, Bedford, USA,* based on a competitive immunoassay using Lumi4-Tb^{TM} cryptate-labeled antibodies anti-cAMP and d2-labeled cAMP). In this assay, activity of the heterodimer can be easily measured by the potentiating of cAMP production in the presence of both Norepinephrine and Angiotensin II agonists compared to the cAMP production triggered by Angiotensin II stimulation alone. Candidate compounds that will block the heterodimer activity will be screened based on their ability to block the potentiating effect of NE+AngII costimulation on cAMP production.Activities of the candidate compounds, their ability to bind AT1-R/α_{2C}-AR heterodimers and their ability to inhibit AT1-R/α_{2C}-AR activity may be tested using HEK293T cells Human embryonic kidney 293 cells or isolated sympathetic neurons in primary culture.

Another assay to evaluate the activity of the heterodimer can rely on a BRET-based assay measuring the interaction between a Luciferase-split (L1+L2) AT1-R/α_{2C}-AR heterodimer (AT1-R-L1 + α_{2C}-AR-L2) and Venus-Gαs overexpressed in HEK293T cells Human embryonic kidney 293 cells. This assay can measure the recruitment of the Gs in the presence of both Norepinephrine and Angiotensin II agonists as an increase of the BRET signal. Candidate compounds that will block the heterodimer activity will be screened based on their ability to block the increase in the BRET signal.

In another screen assay, *in vivo* assays may be used to assess the potency and selectivity of the candidate compounds to reduce AT1-R/α_{2C}-AR heterodimers activity. Specifically, compounds will be tested on hypertensive rats model (SHR, Spontaneous Hypertensive Rat) for their ability to reduce basal sympathetic activity.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1. Characterization of the heterodimerization between AT_1-R and α_{2C}-AR in HEK293T cells. BRET was measured in HEK293T cells co-expressing a fixed amount of the indicated Rluc8-tagged receptor and increasing amounts of the indicated GFP2-tagged receptors. Results were analyzed by a non-linear regression assuming a model with one site binding (GraphPad Prism 4.0) on a pooled data set from three independent experiments.

Figure 2. Characterization of individual agonist effects on the AT_1-R/α_{2C}-AR heterodimer. BRET was measured in HEK293T cells co-expressing HA-α_{2C}-Rluc8 and HA-AT_1-R-GFP2 (A, B, D, E) or HA-α_{2C}-AR-Rluc8 and HA-AT_1-R-Venus (C) or HA-α_{2C}-GFP2 and HA-AT_1-R-Rluc8 (F) stimulated or not for 1 min (A, B, C, E, F) with 10 µM or increasing concentrations (A) of norepinephrine (NE), epinephrine (E), angiotensin II (AngII) or [Sar(1),Ile(4),Ile(8)]AngII (SII), as indicated. In (B), similar BRET experiments were performed by first pretreating cells with 10 µM specific antagonist (Yohimbine, Yo or Candesartan, Cande) for 10 min before agonist incubation. Results are expressed as the difference in BRET signals measured in the presence and absence of agonist. Data represent the mean ± s.e.m. of at least four independent experiments. The statistical significance between stimulated and unstimulated cells was assessed using a paired Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) and statistical difference between ligand treatments was assessed using ANOVA followed by a Tukey's test (#, $P < 0.05$; ###, $P < 0.001$). n.s, not statistically significant. (D) BRET kinetics were measured every 200 ms for 12 s before and after 1 µM AngII (black) or NE (gray) injection (arrow). Agonists were injected 2 s after the beginning of the reading. (Inset) kinetics of net agonist-promoted BRET using data from (D). Results were analyzed by non-linear regression assuming a model with one phase exponential association (GraphPad Prism 4.0). The figure is representative of one experiment performed twice in triplicate.

Figure 3. The nature of ligands dictates different β-arrestin2 trafficking pathways activated by the AT_1-R/α_{2C}-AR heterodimer. β-arrestin2 recruitment to AT_1-R/α_{2C}-AR heterodimer was visualized by confocal microscopy on HEK293T cells co-expressing βarr2-mCherry, HA-AT_1-R-V1 and HA-α_{2C}-AR-V2, stimulated or not (basal) for 10, 30 or 120 min with 10 μM NE, 10 μM AngII or 10 μM of both (AngII+NE). Colocalization coefficient of mCherry and Venus (AT_1-R/α_{2C}-AR) fluorescence was quantified on at least 20 cells per condition over 3 independent experiments and results were plotted on a whisker bar graph. The statistical significance was assessed using an unpaired Student's *t*-test (*, $P < 0.05$; **, P<0.001; n.s, not statistically significant).

Figure 4. NE potentiates AngII-induced increase in NE-release in neurons or renal sympathetic nerve activity (RSNA) in mice through a PKA pathway. (**a**) NE-release was assayed in superior cervical ganglion neurons (SCGNs) primary cultures stimulated or not with NE (1 μM), AngII (10 μM) or both and pretreated or not during 10 min with (A) the PKA antagonist Rp-cAMP (100 μM, Rp) or (B) the AT_1-R antagonist candesartan (10 μM, Cande). Ligand modulated NE-release is depicted as the percentage of release in the presence of drugs versus basal (release using vehicle). Data represent the mean ± s.e.m. of 10 (left panel) to 12 (right panel) independent experiments. C) RSNA was measured after randomized intravenous injection of AngII (4 $ng.g^{-1}$), NE (40 $ng.g^{-1}$) or combination of both (AngII+NE) without (vehicle, white bars, n=14) or with H89 pretreatment (5 mg/kg i.p. black bars, n=7) in hexamethonium-treated (30 $\mu g.g^{-1}$, i.v.) mice. Results are depicted as the percentage of the corresponding basal value normalized to 100 %. Data represent the mean ± s.e.m of 7 to 14 independent experiments. The statistical significance was assessed using one-way ANOVA followed by Newman-Keuls test (**a**) or ANOVA for repeated measures followed by Dunett's test (**b**). \$ $P < 0.05$, \$\$ $P < 0.01$, \$\$\$ $P < 0.001$ for the indicated pairs; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs basal value using paired Student's t test; ns: non-significant.

Figure 5. AngII-NE co-stimulation of the AT_1-R/α_{2C}-AR heterodimer generates a new Gs/cAMP/PKA signaling. BRET was measured in HEK293T cells co-expressing HA-AT_1-R-DmrC and Myc-α_{2C}-AR-DmrA, with GFP10-$G\gamma_2$, $G\beta_1$ and $G\alpha_s$-*R*luc8 stimulated or not with 1 μM NE, AngII or both (AngII+NE) for 1 min following 10 min pretreatment with 10 μM Yohimbine (YO), Candesartan (CAN) or both. Attached cells were pre-treated (+ A/C) or not (-A/C) with 400 nM A/C for 1 h to force AT_1-R-α_{2C}-AR before the BRET experiment.

Results are expressed as the difference in the BRET signal measured in the presence and absence of ligands. Data represent the mean ± s.e.m. of 3 to 8 independent experiments. The statistical significance was assessed using a paired Student's *t*-test comparing ligand-stimulated and unstimulated cells (**, $P < 0.01$; ***, $P < 0.001$).

EXAMPLE:

Material & Methods

Live animals. Two month old male or 1- to 3-d-old C57BL/6 mice were used in accordance with the NIH guidelines. Experiments were approved by the local animal ethical committee.

Materials. Angiotensin II (AngII), L-(-)-Norepinephrine (NE), (-)-Epinephrine (E), 3-isobutyl-1-methylxanthine (IBMX), and Yohimbine were purchased from Sigma-Aldrich. Candesartan was kindly provided by JL Hansen. Coelenterazine 400a and *h* were purchased from Biotum. Levo-[7-^{3}H]-Norepinephrine and [^{125}I]-Tyr4-Angiotensine II were purchased from Perkin-Elmer.

cDNA expression vectors. Plasmids encoding rat HA-AT_1-R and HA-AT_1-R-GFP2 were a generous gift from J. L. Hansen. All receptors were fused in frame at their C-terminus to either *R*luc8, GFP2 or *R*luc8 or Venus splits. Human HA-α_{2C}-AR-GFP2 was obtained by inserting receptor coding sequence lacking its stop codon into the humanized pGFP2-N1 vector (Perkin Elmer, Lifescience). HA-α_{2C}-AR-*R*luc8 was obtained from pGFP2-MCS-Rluc8 (customized) vector by replacing the GFP2-MCS sequence with the receptor coding sequence of α_{2C}-AR. HA-AT_1-R-V1, HA-AT_1-R-V2, HA-α_{2C}-AR-V1, HA-α_{2C}-AR-V2 and HA-AT_1-R-L1, HA-AT_1-R-L2, HA-α_{2C}-AR-L1, HA-α_{2C}-AR-L2 were constructed from HA-AT_1-R-GFP2 and HA-α_{2C}-AR-GFP2 by replacing GFP2 with the coding sequence of the different Venus fragments (from CD8-V1 and CD8-V2, kindly provided by J.A Javitch) or *R*luc8 fragments (from D2-L1 and D2-L2, kindly provided by J.A Javitch). Plasmids encoding HA-AT_1-R-Venus, α_{i1}-91*R*luc8, α_q-97*R*luc8, α_s-113*R*luc8, GFP10-$G\gamma_2$, $G\beta_1$, *R*luc-βarr_2, RIα-*R*luc8 and GFP2-Cα were previously described. α_{i1}-Venus and α_s-Venus were a generous gift from J.A

Javitch. α_q-Venus was obtained from α_q-97*R*luc8 by replacing *R*luc8 with the coding sequence of Venus. HA-AT_1-R-DmrC and Myc-α_{2C}-AR-DmrA were generated from HA-AT_1-R-V1 and Myc-α_{2C}-AR-V1 respectively by replacing V1 by the encoding sequence of FRB (DmrC) or FKBP12 (DmrA) respectively (obtained by PCR from pHet-1 and pHet-Nuc1 vectors, iDimerize™ Inducible Heterodimer System, Clontech). All generated constructs were confirmed by DNA sequencing. Rluc-Barr2 and barr2-cherry vectors were kindly provided by S Marullo and were already described. Pm-Epac2-camps was a generous gift from Viacheslav Nikolaev.

Cell culture and transfection. Human embryonic kidney 293 cells (HEK293T) cells were cultured in DMEM Glutamax supplemented with 10% (v/v) FBS and 100 units/ml penicillin/streptomycin at 37°C in a humidified atmosphere at 5% CO_2. Transient transfections were performed 24 h after cell seeding using polyethylenimine (PEI, Polysciences Inc.) or Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. Mouse superior cervical ganglion neurons were cultured as previously described. Briefly, superior cervical ganglions were dissected from 1- to 3-d-old C57BL/6J mice and dissociated for 20 min in 3 mg/ml collagenase/0.5 mg/ml trypsin, followed by washing (10% FBS in DMEM). Cells were plated and incubated with 3% fetal bovine serum in UltraCulture medium containing NGF for 2 h at 37°C to allow fibroblasts to adhere. Supernatant medium (containing SCG cells) was centrifuged, resuspended in medium supplemented with FBS and NGF, and cultured on poly-D-lysine- and collagen-coated glass-bottom plates for 14 days before experiments (treating with 1 μM 5-fluoro-5-deoxyuridine).

Immunofluorescence microscopy. HEK293T cells were seeded and transfected using Lipofectamine 2000 in 6-well plates containing glass coverslips precoated with poly-L-lysine (1 mg/ml; Sigma). Forty-eight hours after transfection, cells were washed twice with PBS, fixed with 4% paraformaldehyde for 15 min, blocked in PBS-0.2 % BSA and incubated for 2 hours with a mouse anti-HA antibody (16B12, Covance) or a mouse anti-Myc tag (9E10, SantaCruz). Immunoreactivity was revealed using an Oregon green- or Texas Red-conjugated secondary goat anti-mouse antibody (Molecular probes). Cell membranes and nuclei were respectively stained with Texas Red-conjugated Wheat Germ Agglutinin (WGA) (Molecular probes) and DAPI (Sigma). Fluorescent images were acquired with an Axio Observer Z.1m inverted microscope associated with ApoTome (Carl Zeiss Jena, Germany) and a x63 objective lens

(Plan-Apochromat, 1,4 Oil DIC). Colocalization was assessed by overlay using Zeiss microscopy software AxioVision Rel. 4.8. Confocal imaging was performed using a LSM 780 microscope, piloted by manufacturer software, with a × 63 Plan-Neofluar objective (Carl Zeiss) and colocalization parameter (correlation coefficient) was calculated for each cell using ZEN software.

FRET imaging. PC12 cells were electroporated with the Epac2-pm cAMP FRET sensor together with HA-AT_1-AR and Myc-α_{2C}-AR according the Neon Transfection kit (InVitroGen) recommendation. Twenty four hours post transfection cells were placed in a differentiation media (RPMI 1 % HS, 70 ng/mL NGF, Pen/Strep) and H9C2 cells were added on the culture 8h after. Cells were imaged 4 to 6 days post differentiation using a confocal LSM 780 microscope piloted by ZEN software, with a × 63 Plan-Neofluar objective (Carl Zeiss). Contact between PC12 and H9C2 was imaged with the following parameters: electronic zoom 2, confocal pinhole 1.5 Airy units no frame averaging, 6 slices z-stack. CFP and EYFP were excited using 452 and 514 nm laser lines (from the 488nm Argon laser), and emitted light collected at 475–510 nm and 520–555 nm (CFP and EYFP or FRET imaging, respectively). A first image with brightfield, CFP and YFP excitation was acquired to choose the cell then to study agonist-induced changes in FRET, we acquired the data in the CFP channel and in the FRET channel only with CFP excitation. 10 images before stimulation and 20 images post stimulation were done with a 10 s interval. At least 25 cells were analyzed per condition over 2 experiments. After background subtraction (bleed through correction), net FRET was calculated as the ratio of the FRET channel over the CFP channel using ImageJ. The difference in net FRET between stimulated and unstimulated for 1-5 ROI (region of interest) was then expressed as a percentage and plotted over time.

Bioluminescence Resonance Energy Transfert (BRET). Receptors, G protein subunits and β-arrestin-encoding vectors were transiently transfected into HEK293T cells as indicated in the figure legends. Forty-eight hours after transfection, cells were washed with PBS, detached in PBS / 5 mM EDTA and resuspended in PBS / 0.1 % (w/v) glucose at room temperature. Cells were then distributed (80 µg of protein per well) into a 96-well microplate (Wallac, PerkinElmer Life and Analytical Sciences) and incubated in the presence of the different ligands for 1 min (or 5 min as indicated), except for the kinetics study (Figure 2D) and PKA activation (data not shown). BRET between native or complemented *R*luc8 and GFP10 or complemented GFP2 was measured after the addition of the *R*luc substrate coelenterazine 400a

(5 µM, Interchim). For BRET^1 experiments, BRET between *R*luc and native or reconstituted Venus was measured following addition of the *R*luc substrate coelenterazine *h* (5 µM, Interchim). Where indicated, BRET signal values were corrected by subtracting the background signal detected when a Rluc8/Rluc-tagged construct was expressed alone from the BRET signal detected in cells co-expressing both Rluc8/Rluc-tagged and GFP2/Venus constructs (Net BRET).

For titration experiments (Figure 1), the expression level of each tagged protein was determined by direct measurement of total fluorescence and luminescence in aliquots of the transfected cells. Total fluorescence was first measured with an excitation filter at 400 nm and an emission filter at 510 nm for GFP2. Then, the same sample was incubated for 8 min with 5 µM coelenterazine *h* and the total luminescence was measured using a modified Infinite F500 (Tecan Group Ltd).

For kinetics analysis of α_{2C}-AR/AT$_1$-R interactions (Figure 2D), coelenterazine 400a was added before injection of the ligand. Readings were then collected at 200 ms intervals for 12 s. Ligands were injected 2 s after the beginning of the reading to allow baseline recording followed by real-time recording of the BRET changes. Net BRET signals were determined for each time point by calculating the ratio of the light emitted by GFP10 over that emitted by *R*Luc8.

BRET readings were collected using a modified Infinite F500 (Tecan Group Ltd). The BRET signal was calculated by the ratio of GFP10/GFP2 (510-540 nm) to *R*luc/*R*luc8 (370-450 nm) for BRET readings or Venus (520-570 nm) to *R*Luc/*R*Luc8 (370-480 nm) for BRET^1 readings.

Anisotropy measurements. Anisotropy measurements were performed on a LSM 710 confocal microscope (Zeiss, Germany) implemented for anisotropy imaging. Two consecutive images were recorded corresponding to the orientation of polarizers in the emission path of the microscope set first to parallel and then to perpendicular respect to the excitation polarization. Images were recorded through a 10x/0.30 objective to prevent mixing of different polarization components. Venus dye was excited with a 488 nm argon laser line and fluorescence emission was recorded from 500 to 600 nm. Anisotropy images were calculated from raw data with ImageJ (NIH) using Equation 1 and a custom-built macro. G-factor was calculated with Equation 2 from the measurement of an isotropic solution of fluorescein.

$$\langle r \rangle = \frac{I_{\parallel} - G \cdot I_{\perp}}{I_{\parallel} + 2 \cdot G \cdot I_{\perp}} \text{ (Eq. 1)} \qquad G = \frac{I_{\parallel}}{I_{\perp}} \text{ (Eq. 2)}$$

Histograms of frequency vs anisotropy values were calculated for each individual anisotropy image and fitted with a Gaussian model to accurately estimate the mean anisotropy value.

Radioligand binding assay. For AT_1-R, whole-cell radioligand binding studies were performed as previously described. Competition studies were done with HEK293T cells transiently transfected with either 1 µg AT_1-R-*R*luc8 or 1µg AT_1-R-*R*luc8 plus 6 µg HA-α_{2C}-AR-GFP2, in the presence of 10^{-10} M [^{125}I]-Tyr4 AngII ([^{125}I]-AngII, Perkin Elmer) and increasing concentrations (from 10^{-12} to 10^{-6} M) of unlabelled AngII, in the presence or in the absence of 10 µM norepinephrine.

For α_{2C}-AR, competition experiments were done as previously described on crude membrane from HEK293T cells transiently transfected with either 10 µg HA-α_{2C}-AR-*R*luc8 or 10µg HA-α_{2C}-AR-*R*luc8 plus 10 µg HA-AT_1-R-GFP2, using 10^{-8} M [^{3}H]-(-)Norepinephrine ([^{3}H]-NE, Perkin Elmer) as a tracer and increasing concentrations of unlabeled norepinephrine (from 10^{-12} to 10^{-6} M) as competitor agent, in the presence or in the absence of 10 µM AngII.

Each experiment was realized in triplicate and competition curves were fitted using GraphPad Prism (version 5.04, GraphPad Software, USA) using a one-site competition model, followed by pKi (-log Ki) calculation.

cAMP quantification. Quantification of cAMP levels was performed using the HTRF assay (Homogeneous Time-Resolved Fluorescence): femto cAMP kit (Cisbio, Bedford, USA), based on a competitive immunoassay using Lumi4-Tb^{TM} cryptate-labeled antibodies anti-cAMP and d2-labeled cAMP. For that purpose, HEK293 cells were transiently cotransfected with HA-AT_1R and Myc-α_{2C}R using X-tremeGENE 9 DNA transfection reagent (Roche). Twenty-four hours after transfection, cells were pretreated over-night with PTX (100ng/mL) or vehicle. The day of the experiment, cells were washed and resuspended in PBS/5mM Glucose/2mM IBMX. Then, 35,000 cells/well (384wells-plate) were plated and stimulated for 1h at room temperature with 10µM NE, AngII or NE+AngII in the presence of 10µM propranolol in a final volume of 10 µL. Cells were then lysed using 5 µL of lysis buffer containing d2-labeled cAMP and 5µL of Lumi4-Tb^{TM} cryptate-labeled anti-cAMP. The signal was measured after 1h room temperature inubtaion using a modified Infinite F500 (Tecan Group Ltd). The RET signal was calculated by the ratio of d2-cAMP/Lumi4-Tb^{TM} (665nm/620nm), the specific signal being inversely proportional to the concentration of cAMP

in the sample. For each experiment, a calibration curve was established with cAMP standards allowing the quantification of cAMP levels by regression.

Culture of mouse superior cervical ganglion neurons. Mouse superior cervical ganglion neurons were cultured as previously described. Briefly, superior cervical ganglions were dissected from 1- to 3-d-old C57BL/6J mice and dissociated for 20 min in 3 mg/ml collagenase/0.5 mg/ml trypsin, followed by washing (10% FBS in DMEM). Cells were plated and incubated with 3% fetal bovine serum in UltraCulture medium containing NGF for 2 h at 37°C to allow fibroblasts to adhere. Supernatant medium (containing SCG cells) was centrifuged, resuspended in medium supplemented with FBS and NGF, and cultured on poly-D-lysine- and collagen-coated glass-bottom plates for 14 days before experiments (treating with 1 μM 5-fluoro-5-deoxyuridine).

Determination of [^{3H}]-noradrenaline release. Neurons cultures were washed, incubated in culture medium with 0.2 uM [^{3}H]-norepinephrine (2 hours), and rinsed with fresh culture medium. Subsequently, cultures were washed (1 hour) in a buffer containing (mM) NaCl (120), KCl (6.0), CaCl2 (2.0), MgCl2 (2.0), glucose (10), HEPES (10), ascorbic acid (0.50) and bovine serum albumin (1%), adjusted to pH 7.4 with NaOH. Cultures were treated with 10 μM propranolol and 10 μM cocaine (10 minutes) in above buffer. Inhibitors (Candesartan or Rp-cAMP) or vehicle were added during this preincubation period. Cultured were treated with drugs in above buffer [10 minutes (37 degrees C) including inhibitors] and tritium content was measured by scintillation counting. Cultures were extracted using 0.1% SDS and remaining radioactivity was evaluated by scintillation counting.

***In vivo* measurement of renal sympathetic nerve activity (RSNA).** Experiments were performed on adult C57BL/6J (12-17 weeks-old 25-35 g) male mice housed in a temperature-controlled room with a 12:12-hour light–dark cycle with free access to standard laboratory chow and tap water. The protocol was approved by the local ethic committee for animal investigation.

Mice were anesthetized with isoflurane gas administration (induction: 3%, maintenance: 1.5%). Body temperature was continuously monitored using rectal probe and maintained within a normal range using a heating pad. The trachea was cannulated with PE-90 polyethylene tubing and until the end of the surgical procedure the mice spontaneously breathed isoflurane gas using a mask connected to the cannula. The right jugular vein and left carotid artery were cannulated

with PE-10 polyethylene catheters for drug administration and measurement of arterial pressure respectively. The arterial pressure catheter was connected to a pressure transducer allowing signal amplification (hydraulic pressure Millar Instruments, Houston, Texas, USA). The left kidney was exposed retroperitoneally through a left flank incision. Under the dissecting microscope, the left renal sympathetic nerve was isolated from the surrounding connective tissue and mounted on a bipolar 36-gauge platinum–iridium electrode (Cooner Wire Co, Chadsworth, CA). Once optimal recording parameters were established, the nerve was fixed to the electrode with silicone gel (Kwik-Sil; World Precision Instruments Inc, Sarasota, FL). The nerve electrode was attached to a high-impedance probe (HIP-511; Grass Instruments Co, Quincy, MA). The signal was amplified 105 times with a Grass P5 AC preamplifier and filtered at both low- (100 Hz) and high-frequency (1000 Hz) cut-off. To prevent baroreflex-induced decrease in RSNA subsequent to the increase in arterial pressure induced by NE or AngII, a ganglionic blocker (hexamethonium, 30 μg.g-1, i.v.) was administered and mice were artificially ventilated (MiniVent ventilator, Hugo Sachs Electronik, Harvard Apparatus, USA). Then, mice received in a randomized order i.v. injections of AngII (4 ng.g-1) or NE (NE, 40 ng.g-1) alone or in combination. Arterial pressure and RSNA were sampled at 2000 Hz. and sent to a PowerLab analog–digital converter (Ad Instruments, Castle Hill, New South Wales, Australia) for recording on a PC computer and off-line data analysis (LabChart 7 Pro, Ad Instruments, Castle Hill, New South Wales, Australia). Heart rate (HR) was extrapolated from phasic arterial pressure. Mean arterial pressure (MAP, mmHg), HR (beats per min, bpm) and RSNA (bursts/s) were averaged over a 1-min period before drug injection (baseline) and over a 5 s period 20-30 s after injections, i.e. at the time of the maximal pressure effect of injected drugs. Mean time interval between each drug injection (5 min) was defined in preliminary experiments as allowing MAP to return to baseline values. RSNA was analyzed as previously described subtracting the integrated voltage after death (background noise) from the total integrated voltage.

Data and statistical analysis. Results are expressed as mean values ± s.e.m. of at least three independent experiments. Statistical analysis was carried out using GraphPad Prism 4 software (GraphPad Software Inc.). Statistical tests used are indicated in the figure legends.

Results

α_{2C}-AR and AT_1-R form constitutive heterodimers

The interaction between α_{2C}-AR and AT_1-R was assessed in HEK293T cells by bioluminescence resonance energy transfer (BRET). Saturating BRET signals between AT_1-R and α_{2C}-AR (Figure 1) highlighted constitutive and specific association between both receptors. The specificity of interaction was confirmed by weak BRET signals not linearly increasing with the amount of interacting probes when measured between AT_1-R-RLuc8 or α_{2C}-AR-RLuc8 and CD8-GFP2, an unrelated transmembrane protein (Figure 1). Subcellular localization of α_{2C}-AR/AT_1-R heterodimers was investigated using bimolecular fluorescence complementation (BiFC). Each receptor was fused to complementary non-fluorescent halves of a Venus fluorophore (V1 and V2) at its C-terminus as previously described, resulting in receptor fusion constructs preserving their cell surface trafficking (data not shown) and their cognate G proteins activation (data not shown). HA-AT_1-R-V1 co-expression with HA-α_{2C}-AR-V2 generated a functional fluorescent Venus (data not shown) co-localizing with cell surface HA-staining, indicating that α_{2C}-AR/AT_1-R heterodimer was expressed at the plasma membrane. In contrast, no fluorescence was detected when co-expressing HA-AT_1-R-V1 or HA-α_{2C}-AR-V2 with CD8-V2 or CD8-V1, while Venus fluorescence was observed at the cell surface and in intracellular compartments following co-expression of CD8-V1 and CD8-V2 (data not shown). The expression of α_{2C}-AR/AT_1-R heterodimers using BiFC was also observed at the cell surface of sympathetic like neurons-differentiated PC12 neurites (data not shown).

Distinct active conformations of the α_{2C}-AR/AT_1-R dimer

Few studies have reported how ligand binding impacted on protomers association in a dimer and led to conflicting interpretations [Dalrymple M.B. et al., 2008 and Lohse M.J. et al., 2012], most likely due to the use of poor resolutive RET techniques. Also, to assess ligand modulation of the α_{2C}-AR/AT_1-R heterodimer, we used BRET with high spectral resolution and sensitivity. The natural α_{2C}-AR agonists, Norepinephrine (NE) and Epinephrine (E), decreased the BRET between HA-α_{2C}-AR-Rluc8 and HA-AT_1-R-GFP_2, while the AT_1-R-specific agonists, AngII and SII, increased it (Figure 2A, middle panel). NE- and AngII-dependent BRET modulation correlated with G protein activation promoted by WT HA-AT_1-R or Myc-α_{2C}-AR when expressed alone (Figure 2A, down panel, NE/EC_{50} = 1.2 ± 0.4 nM; AngII/EC_{50} = 3.4 ± 2.2 nM and data not shown). The specificity of ligand-promoted BRET

changes was confirmed by pre-treatment with 10 μM of specific α_{2C}-AR (Yohimbine, Yo) or AT_1-R (Candesartan, Cande) antagonists which completely blocked the NE-mediated decrease or AngII-mediated increase in BRET (Figure 2B). The agonists-induced changes in BRET could indicate enhanced or decreased physical association/dissociation between α_{2C}-AR and AT_1-R but could also reflect conformational rearrangements within protein complexes since this measure relies on the distance/dipole orientation of the energy donor/acceptor. To distinguish between these two possibilities, we manipulated the distance/dipole orientation by exchanging the GFP_2-energy acceptor fused to AT_1-R for Venus and measuring BRET between HA-α_{2C}-AR-*R*luc8 and HA-AT_1-R-Venus. In this configuration, NE and E still decreased the BRET signal while AngII but not SII promoted an increase in BRET (Figure 2C). Since BRET changes are consistent with conformational changes within the dimer, it appears that α_{2C}-AR and AT_1-R agonists stabilize two different conformations of the HA-α_{2C}-AR/HA-AT_1-R heterodimer. Moreover, AT_1-R agonists, AngII and SII also induce different dimer conformations, in agreement with our previous demonstration that SII stabilizes a different AT_1-R entity to that stabilized by AngII. Further evidence for NE and AngII promoting different conformations of the HA-α_{2C}-AR/HA-AT_1-R heterodimer was provided by real-time BRET kinetics which revealed a faster NE-induced decrease in BRET than the AngII-mediated increase in BRET (Figure 2D; $t_{1/2}$: AngII, 517 ± 6 ms *versus* NE, < 200 ms).

NE and AngII hypersecretion observed in some cardiac diseases results in high concentrations of both agonists at sympathetic nerve endings where they could bind simultaneously on putative α_{2C}-AR/AT_1-R heterodimers. Thus, we tested whether the simultaneous presence of the natural agonists of each receptor protomer would further modify the conformation of the α_{2C}-AR/AT_1-R heterodimer. When measuring BRET between HA-α_{2C}-R-Rluc8 and HA-AT_1-R-GFP_2, while NE or AngII increased or decreased respectively the BRET, co-stimulation with both agonists reduced the BRET to a similar extent to that of NE alone (Figure 2E). When the experiment was repeated following energy donor/acceptor exchange between the two receptors, NE- and AngII-promoted BRET modulations remained unchanged, but the co-stimulation had no effect (Figure 2F). These experiments suggest that ligand-modulated BRET results from conformational changes in the α_{2C}-AR/AT_1-R heterodimer, but also show that AngII, NE and AngII/NE stabilize three different conformational states of the heterodimer. This was further supported by fluorescence anisotropy measurements of complemented Venus fluorophore. In HEK cells co-expressing HA-AT_1-R-V1 and HA-α_{2C}-AR-V2, mean Venus anisotropy was different in AngII-treated

cells compared to NE and NE/AngII treatments (data not shown), indicating that AngII promoted an AT_1-R/-α_{2C}-AR dimer conformation different to that stabilized by NE or AngII/NE. Sequential addition of AngII and NE (data not shown) did not modify BRET modulations between AT_1-R and α_{2C}-AR (Figure 2E and F) for each energy donor/acceptor combination. Thus, contrary to previous reports [Smith, N.J. et al., 2010], these results are consistent with a model in which the ligand occupancy of one protomer not necessarily influences the binding properties of the other. In line with this assumption, AngII or NE binding affinities/Bmax were not modified in the presence of the other agonist in HEK cells co-expressing both α_{2C}-AR and AT_1-R (data not shown), suggesting that the ligand binding pocket was not affected, as previously reported for allosteric communication between protomers.

Distinct heterodimer-effector complex conformations

We next explored the consequences of the different α_{2C}-AR/AT_1-R dimer conformations by analyzing the heterodimer interactions with G proteins and β–arrestin. To specifically isolate ligand effects arising from the heterodimer, we used complemented donor-acceptor resonance energy transfer (CODA-RET) combining BiFC and BRET. Thus, HA-α_{2C}-AR and HA-AT_1-R were fused at their C-termini to an N-terminal (L1) or C-terminal fragment (L2) of Rluc8, and to an N-terminal (V1) or C-terminal fragment (V2) of Venus resulting in fusion receptors with preserved cell surface expression and function (data not shown). Analysis of fluorescence or luciferase complementation for α_{2C}-α_{2C}, AT_1-AT_1 and α_{2C}-AT_1 receptors showed that all combinations demonstrated fluorescence or luminescence reconstitution (data not shown), suggesting that α_{2C}-AR and AT_1-R have similar propensity for forming homo or heterodimers.

When HA-AT_1-R-V1 was co-expressed with HA-AT_1-R-V2 in the presence of *R*luc-β-arrestin2, real time BRET measurements showed β-arrestin2 recruitment to the AT_1-R/AT_1-R homodimer only in the presence of AngII (data not shown). Similarly, with α_{2C}-α_{2C} homodimers, no β-arrestin recruitment was measured in the presence of NE or AngII (data not shown), despite cell surface expression of the homodimer (data not shown). These results agree with previous reports for β-arrestin2 recruitment to AT_1-R[24] but not to α_{2C}-AR[18] (data not shown). Interestingly, when considering the α_{2C}AR-AT_1-R heterodimer, AngII and NE, independently, reproduced the same β-arrestin2 recruitment profile than obtained with their cognate receptor homodimers or monomers (data not shown), suggesting that heterodimerization does not impact the functionality of each receptor protomer on the β-

arrestin2 pathway. However, AngII/NE co-stimulation significantly (P=0.0002) potentiated maximal AngII-induced β-arrestin2 recruitment ($BRET_{max}$ AngII+NE= 121.9 % ± 2.3; $BRET_{max}$ AngII= 100.0 % ± 2.0). When similar experiments were performed but using β-arrestin2 fused to the Rluc probe at its C-terminal (β-arrestin2-Rluc), NE was still unable to recruit β-arrestin2 while AngII or AngII-NE co-stimulation led to significant but similar maximal β-arrestin2 recruitment (data not shown; $BRET_{max}$ AngII+NE= 86.1 % ± 10.9; $BRET_{max}$ AngII= 98.6 % ± 14.4). This suggests that different α_{2C}-AT_1 heterodimer conformations stabilized by AngII, NE or AngII/NE translate into three distinct heterodimer/β-arrestin2 conformational changes rather than modifications of β-arrestin2 levels associated to the receptor.

We applied the same strategy to the G protein activity by measuring the BRET between complemented split luciferase receptor fusions and the Gα-Venus subunit of the Gαβγ heterotrimer, a BRET assay accurately sensing R-G complex conformations. Consistent with the cognate G protein coupling of each receptor, AngII increased the BRET signal between the HA-AT_1-R-L1/HA-AT_1-R-L2 homodimer and $G\alpha_q$-Venus or $G\alpha_{i1}$-Venus but not $G\alpha_s$-Venus (data not shown) while a significant BRET increase was detected in the presence of NE but only between the HA-α_{2C}-AR-L1/ HA-α_{2C}-AR-L2 homodimer and the $G\alpha_{i1}$-Venus (data not shown). When the HA-AT_1-R-L1/HA-α_{2C}-AR-L2 heterodimer was co-expressed with the different Gα-Venus isoforms, the R-G complex exhibited distinct conformations depending on the Gα isoform and the nature of the ligand as shown by the different BRET modulations (data not shown). It is noteworthy that despite NE was unable to promote α_{2C}-AR coupling to $G\alpha_q$, it significantly decreased the BRET between the AT_1-R/α_{2C}-AR heterodimer and $G\alpha_q$. Intriguingly, although AT_1-R and α_{2C}-AR do not usually couple to G_s, and as corroborated by the absence of conformational rearrangements between AT_1-R or α_{2C}-AR homodimer receptors and Gs (data not shown), AngII/NE co-stimulation significantly increased the BRET between the heterodimer and $G\alpha_s$ (data not shown), suggesting an original Gs engagement to the heterodimer. Swapping the luciferase splits between AT_1-R and α_{2C}-AR led to similar results for all G proteins except that heterodimer stimulation with AngII promoted a BRET increase instead of a decrease when looking at the α_{2C}-AT_1/$G\alpha_q$ complex (data not shown). This demonstrates that the AT_1-R/α_{2C}-AR-Gq complex adopts different conformations in the presence of AngII, NE or AngII/NE, agreeing with the notion that specific ligand combinations can stabilize different heterodimer conformations.

α2C-AR/AT1-R co-stimulation activates Gs/cAMP/PKA pathway

We next questioned the functional consequences of the different conformations of complexes between AT_1-R/α_{2C}-AR dimer and β-arrestin2 or Gα subunit and stabilized by the three agonist combinations.

Given the central role of β-arrestin in GPCRs internalization, we examined the internalization profile of AT_1-R/α_{2C}-AR dimer in HEK293T cells co-expressing βarrestin2-mCherry together with HA-AT_1-R-V1 and HA-α_{2C}-AR-V2. In agreement with the BRET assay (data not shown), 120 min NE stimulation did not promote β-arrestin2 recruitment (Figure 3), while internalization of the dimer-Venus could be observed. Contrarily, cell treatments with 10 μM AngII or AngII+NE for 10 min significantly recruited β-arrestin2 to the AT_1-R/α_{2C}-AR dimer. However, after 30 min treatments, while AngII-NE co-stimulation sustained β-arrestin2 co-localization with the heterodimer in internalized vesicles, co-localization dramatically decreased with AngII (Figure 3) and persisted over 120 min stimulation. These results indicate that the three heterodimer conformations stabilized by the three ligand combinations traffic via distinct intracellular routes suggesting diverse signaling outcomes.

We next explored the G protein activation pattern associated with the heterodimer. Indeed, α_{2C}-AR and AT_1-R co-expression in HEK293T cells will lead to the formation of monomer, dimers and higher order oligomers, making impossible to detect only heterodimer activity. To bypass this problem, we designed an assay based on the ligand-forcing FRB(DmrC)-FKBP(DmrA) dimerization previously used to explore protein dimerization. Thus, we engineered a chimeric α_{2C}-AR fused to DmrA at its C-terminus (Myc-α_{2C}-AR-DmrA), while AT_1-R was fused to DmrC (HA-AT_1-R-DmrC). Neither these fusions altered cell surface trafficking, nor the receptor activity (data not shown). We directly evaluate the $G\alpha_{i1}$, $G\alpha_q$ and $G\alpha_s$ activation in living cells using a BRET probe measuring the increased distance between the Gα helical domain (Gα-*R*luc8) and the $G\gamma_2$ N-terminus (GFP10-$G\gamma_2$) during the GDP/GTP exchange and sensed as a decrease in the BRET signal following receptor activation. Myc-α_{2C}-AR-DmrA and HA-AT_1-R-DmrC were co-expressed in HEK293T cells with the different Gα BRET probes in the presence of NE, AngII or both. No G protein activation was detected following 1 min ligand stimulation in the absence of fusion receptors expression (data not shown). In the presence of the $G\alpha_{i1}$ BRET probe, NE strongly decreased the BRET signal in the absence of the A/C dimerizing agent, indicating potent $G\alpha_{i1}$ activation in agreement with an action through the Gi-coupled α_{2C}-AR (data not shown). Further addition of AngII did not

modify the $G\alpha_{i1}$ activation profile while AngII alone activated the $G\alpha_{i1}$ but to a much lower extent (data not shown), in agreement with lower efficiency of AngII on AT_1-R-mediated Gi versus Gq coupling. Interestingly, for similar receptor expression, forced dimerization of α_{2C}-AR/AT_1-R with the A/C dimerizer significantly reduced $G\alpha_{i1}$ activation by NE or NE/AngII stimulation, while AngII response was unchanged (data not shown). This result highly suggests that in the absence of the dimerizer, $G\alpha_{i1}$ activation most probably arises from a mixed population of active monomeric, homodimeric and heterodimeric α_{2C} and AT_1 receptors which is disrupted by the dimerizer to favor the heterodimeric population with lower overall level of $G\alpha_{i1}$ activation. In contrast, all ligand combinations activated $G\alpha_q$ and no difference was observed when forcing α_{2C}-AR/AT_1-R dimerization (data not shown). NE-induced $G\alpha_q$ activation was surprising since α_{2C}-AR is thought to exclusively couple to $G_{i/o}$ proteins and NE is unable to bind to AT_1-R. This could indicate that NE-mediated $G\alpha_q$ activation is triggered by the spontaneous formation of the heterodimer with expression level sufficient for $G\alpha_q$ activation since A/C supplementation did not change the activation profile. This hypothesis agrees with our earlier results showing that NE induced conformational changes of the complex formed between α_{2C}-AR-L1/AT_1-R-L2 and Venus-Gαq (data not shown). Finally, we found that only NE/AngII co-stimulation promoted Gs activation in forced α_{2C}-AR/AT_1-R heterodimer conditions (data not shown). This correlates with conformational rearrangements observed within the α_{2C}-AR/AT_1-R-$G\alpha_s$ complex (data not shown) and demonstrates that rearrangement stabilized by dual AngII/NE occupancy of the heterodimer-Gs complex efficiently translated into Gs activation. The lack of detectable Gs activation in the absence of dimerizing agent could argue for production of a peculiar α_{2C}-AR/AT_1-R heterodimer entity coupled to Gs that did not spontaneously form by itself. However, BRET experiments showed that free mobile α_{2C} and AT_1 receptors can constitutively interact (data not shown) and adopt a specific conformation alone (Figure 2E and F) or complexed with Gs (data not shown) in the presence of both AngII and NE. This more likely supports a model whereby dual AngII/NE binding promotes Gs coupling to the α_{2C}-AR/AT_1-R heterodimer in the absence of the dimerizer but that the Gs BRET probe is not sensitive enough for detection.

Validation of Gs activation by dual AngII/NE occupancy of the α_{2C}-AR/AT_1-R heterodimer was determined at the level of cAMP-dependent Protein Kinase (PKA) effector. HEK293T cells were co-transfected with the BRET-based PKA biosensor measuring the separation between RLuc8-tagged regulatory subunits and GFP2-tagged catalytic subunits of PKA as a reflect of activation. In the absence of fusion receptors expression, NE significantly

activated the PKA biosensor and activation was completely blocked by propranolol (data not shown), suggesting the presence of an endogenous Gs-coupled β-adrenergic receptor. While AngII stimulation alone did not promote any PKA response (data not shown), same results were obtained in the presence of both NE/AngII (data not shown). Endogenous β-adrenergic receptor activating PKA was quite puzzling since no Gs activation could be measured following 1 min NE stimulation in the absence of transient receptor expression (data not shown). However, when activation times were extended to 5 min, NE or NE/AngII induced significant Gs activation, consistent with PKA activation in similar conditions (data not shown). In this context, the ability of the heterodimer to activate PKA was studied in the presence of propranolol when using NE. When the PKA biosensor was co-expressed with Myc-α_{2C}-AR-DmrA and HA-AT_1-R-DmrC, no PKA activation was found downstream of NE or AngII treatments regardless of the dimerizing agent presence (data not shown). Significant PKA activation was measured only in the presence of both NE-AngII with forced α_{2C}-AR/AT_1-R dimerization (data not shown). This supports the idea that Gs is specifically activated downstream of the α_{2C}-AR/AT_1-R heterodimer.

To confirm the atypical Gs activation stimulated by the heterodimer, we directly quantified cAMP production in HEK293T cells co-expressing α_{2C}-AR and AT_1-R. While no cAMP inhibition or production was detected in the absence of receptors expression (data not shown), NE inhibited cAMP production in agreement with activation of Gi-α_{2C}-AR (data not shown). More surprisingly, no cAMP production was detected in the presence of NE+AngII as expected based on the Gs/PKA biosensor activation (data not shown). Since the cAMP concentration could result from the concomitant activation of both activator and inhibitory signals whose detection is highly dependent on the activation level of each pathway, we repeated the experiments in the presence of pertussis toxin (PTX) to inhibit the Gi/cAMP pathway. The NE-mediated cAMP production inhibition was totally prevented by PTX pre-treatment but more interestingly, AngII promoted a significant cAMP production which was significantly potentiated by NE (data not shown). The potentiating effect of AngII-NE co-stimulation on cAMP production was reinforced by FRET-based imaging of cAMP levels in living sympathetic like neurons-differentiated PC12 co-expressing α_{2C}-AR, AT_1-R and a cAMP FRET biosensor and co-cultured with H9C2 cardiomyoblasts (to establish neuro-cardiac-synapses), in which only the AngII-NE co-stimulation promoted a decrease of the FRET signal indicative of cAMP production compared to individual NE or AngII treatments (data not shown).

AngII/NE promotes PKA-dependent SNS hyperactivity

Given the Gs coupling of α_{2C}-AR/AT_1-R heterodimer with dual NE/AngII occupancy and the implication of both receptors in NE secretion, we wondered whether a similar pharmacological unit could impact NE secretion. As expected, NE or AngII stimulation significantly decreased or increased respectively NE release in sympathetic neurons primary culture (Figure 4). However, NE potentiated AngII-induced NE secretion (Figure 4) which was completely prevented by Gs/PKA pathway blockage with Rp-8-cAMPS, a selective PKA inhibitor (Figure 4A). Interestingly, pre-treatment with candesartan, an AT_1-R specific antagonist, selectively inhibited AngII-mediated NE release, but had no effect on the NE release potentiation induced by NE-AngII co-stimulation (Figure 4B). This agrees with results showing that candesartan or yohimbine (α_2-AR antagonist), alone or in combination, were unable to block Gs stimulation promoted by NE-AngII co-treatment on the α_{2C}-AR/AT_1-R dimer (Figure 5), and thus suggests that an endogenous α_{2C}-AR/AT_1-R dimer could be involved in the NE secretion in sympathetic neurons

We next assessed the *in vivo* relevance of NE/AngII co-stimulation on sympathetic nerves firing rate which correlates with NE release. We measured renal sympathetic nerve activity (RSNA) in mice following intravenous injections of AngII, NE or both, in the presence of hexamethonium to prevent baroreflex-induced changes in RSNA subsequent to changes in arterial pressure. Hexamethonium significantly lowered mean arterial pressure (MAP) (60±8 vs 88±6 mmHg in controls, $P<0.001$), did not modify heart rate (553±19 vs 537±19 beats.min^{-1} in untreated controls) and decreased RSNA (14.4±5.0 vs 35.7±7.7 bursts/s in controls, $P<0.001$). RSNA significantly increased following AngII administration (Figure 4C). In contrast, NE administration tended to decrease RSNA (Figure 4C). Strikingly, AngII/NE co-administration dramatically increased RSNA by 125.7±17.8 % vs 65.9±6.3 % with AngII alone) (Figure 4C). This RSNA hyperactivity induced by AngII/NE co-stimulation was not related to differences in drug-induced vasoconstriction (data not shown). Blocking the Gs/PKA pathway using H89 PKA inhibitor completely prevented the RSNA potentiation induced by AngII/NE (Figure 4C), indicating the involvement of a Gs-coupled receptor and correlating with the original Gs coupling of the α_{2C}-AR/AT_1-R dimer we identified *in vitro*.

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Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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CLAIMS:

1. A compound which is selected from the group consisting of AT1-R/α2C-AR heterodimerization inhibitors, AT1-R/α2C-AR antagonists or AT1-R/α2C-AR expression inhibitors for use in the prevention and treatment of cardiovascular diseases in a subject in need thereof.

2. A compound which is selected from the group consisting of AT1-R/α_{2C}-AR heterodimerization inhibitors, AT1-R/α_{2C}-AR antagonists or AT1-R/α_{2C}-AR expression inhibitors for use in the prevention and treatment of diseases characterized by hyperactivity of the sympathetic nervous system (SNS) in a subject in need thereof.

3. A compound which is selected from the group consisting of AT1-R/α_{2C}-AR heterodimerization inhibitors, AT1-R/α_{2C}-AR antagonists or AT1-R/α_{2C}-AR expression inhibitors for use in the prevention and treatment of cardiovascular diseases with hyperactivity of the sympathetic nervous system (SNS) and the rennin-angiotensin-alsodsterone system (RAAS) in a subject in need thereof.

4. A compound for use according to claims 1 to 3 wherein the diseases or the cardiovascular diseases are heart failure, hypertension, cardiomyopathies and cardiac rhythm disturbances.

5. A compound for use according to claims 1 to 4 wherein the compound is AT1-R/α2C-AR antagonist.

6. A pharmaceutical composition comprising the compound according to claims 1 to 3 and a pharmaceutical acceptable carrier for use in the prevention and treatment of cardiovascular diseases in a subject in need thereof.

7. A method of screening a candidate compound for use as a drug for the prevention and treatment of cardiovascular diseases in a subject in need thereof, wherein the method comprises the steps of: i) providing candidate compounds and ii) selecting candidate compounds that blocks the action of AT1-R/α_{2C}-AR heterodimers.

8. A method of treating cardiovascular diseases in a subject in need thereof comprising administering the subject with a therapeutically effective amount of the compound according to claims 1 to 3.

5

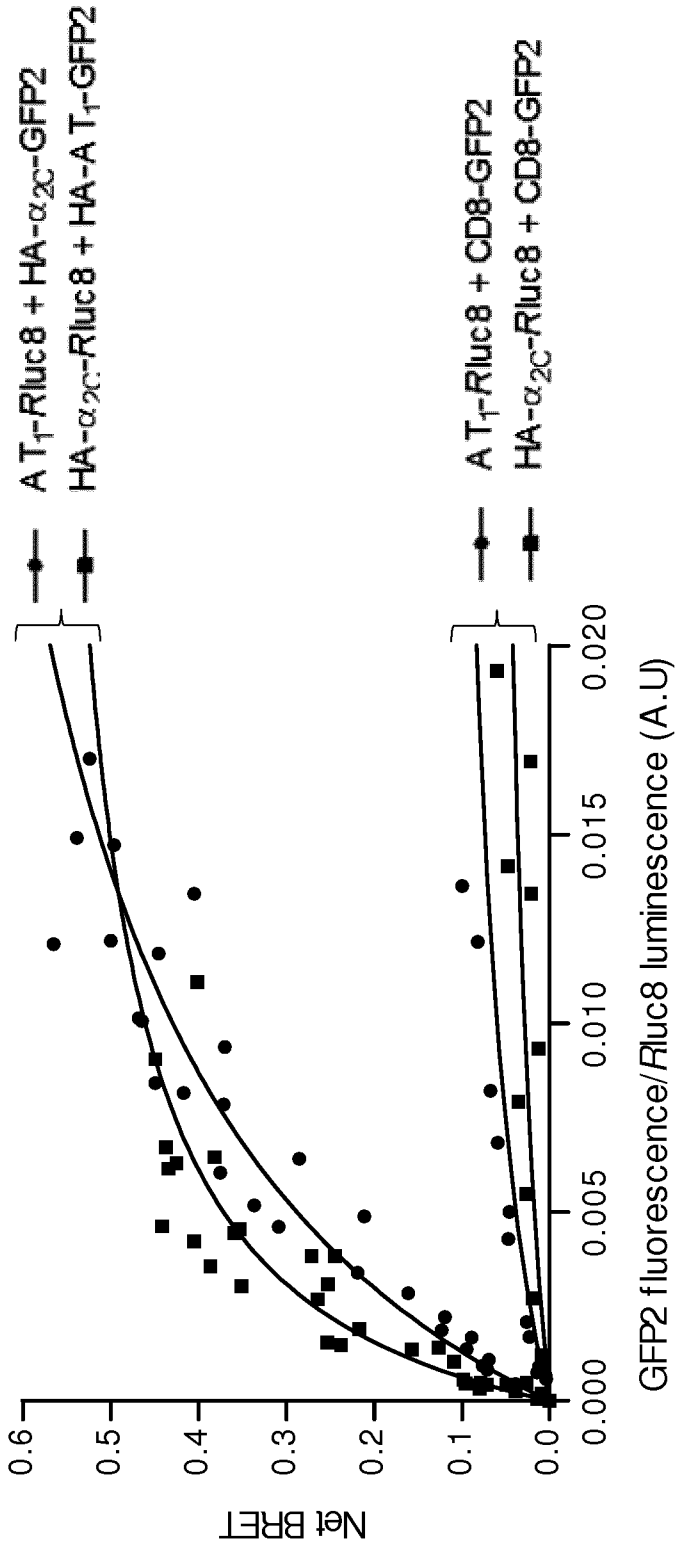
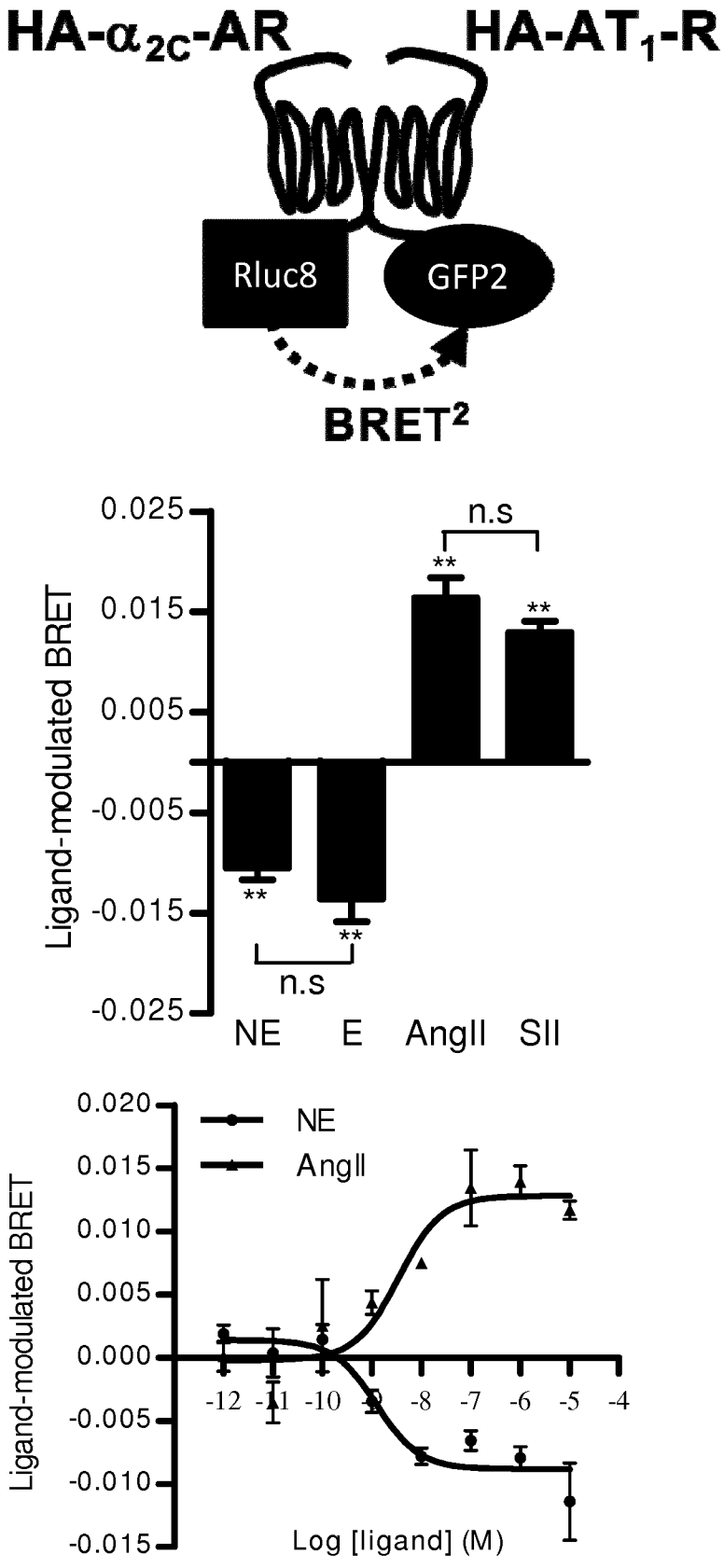

A T1-Rluc8 + HA-α2C-GFP2
HA-α2C-Rluc8 + HA-A T1-GFP2
A T1-Rluc8 + CD8-GFP2
HA-α2C-Rluc8 + CD8-GFP2
Net BRET
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.000
0.005
0.010
0.015
0.020
GFP2 fluorescence/Rluc8 luminescence (A.U)

Figure 1


HA-α2C-AR
HA-AT1-R
Rluc8
GFP2
BRET²
Ligand-modulated BRET
0.025
0.015
0.005
-0.005
-0.015
-0.025
n.s
**
**
**
**
n.s
NE
E
AngII
SII
Ligand-modulated BRET
0.020
0.015
0.010
0.005
0.000
-0.005
-0.010
-0.015
NE
AngII
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-11
-10
-9
-8
-7
-6
-5
-4
Log [ligand] (M)

Figure 2 A

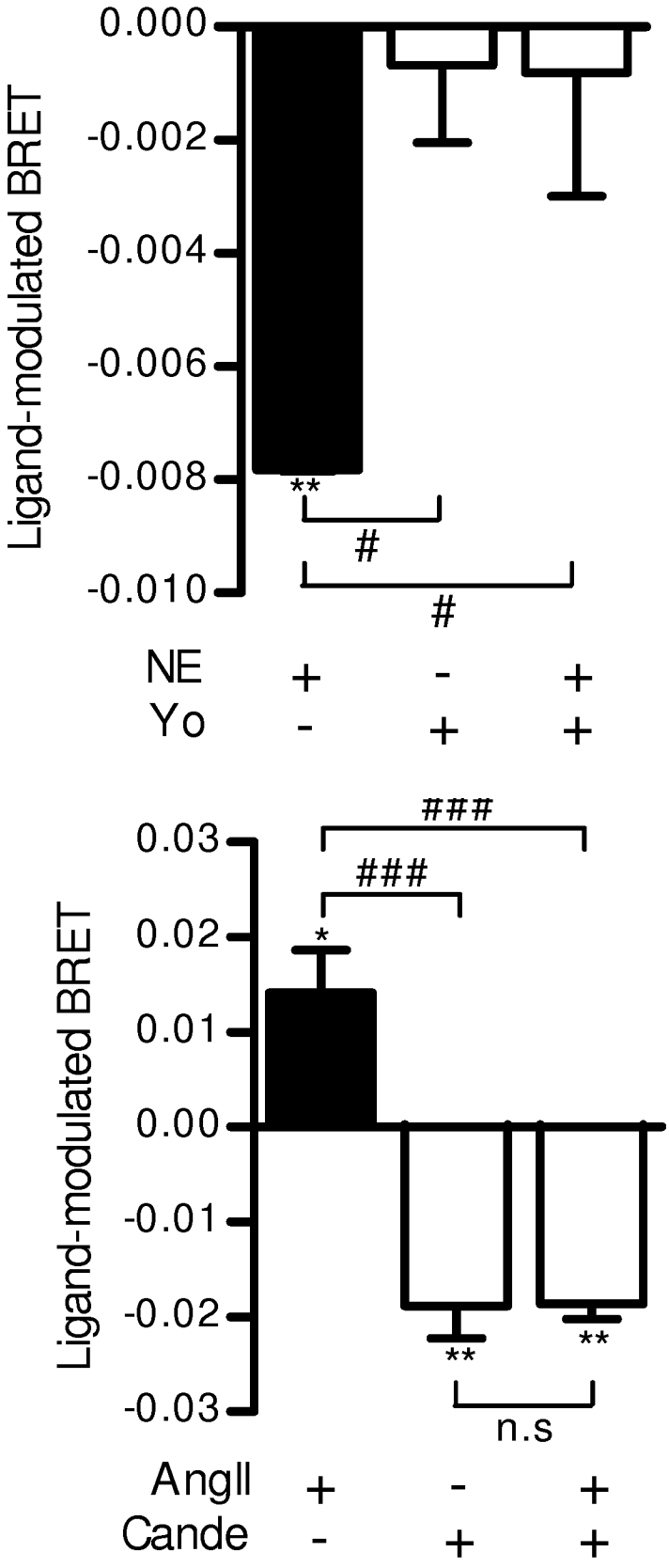

0.000
-0.002
-0.004
-0.006
-0.008
-0.010
Ligand-modulated BRET
**
#
#
NE + - +
Yo - + +
0.03
0.02
0.01
0.00
-0.01
-0.02
-0.03
Ligand-modulated BRET
###
###
*
**
**
n.s
AngII + - +
Cande - + +

Figure 2B

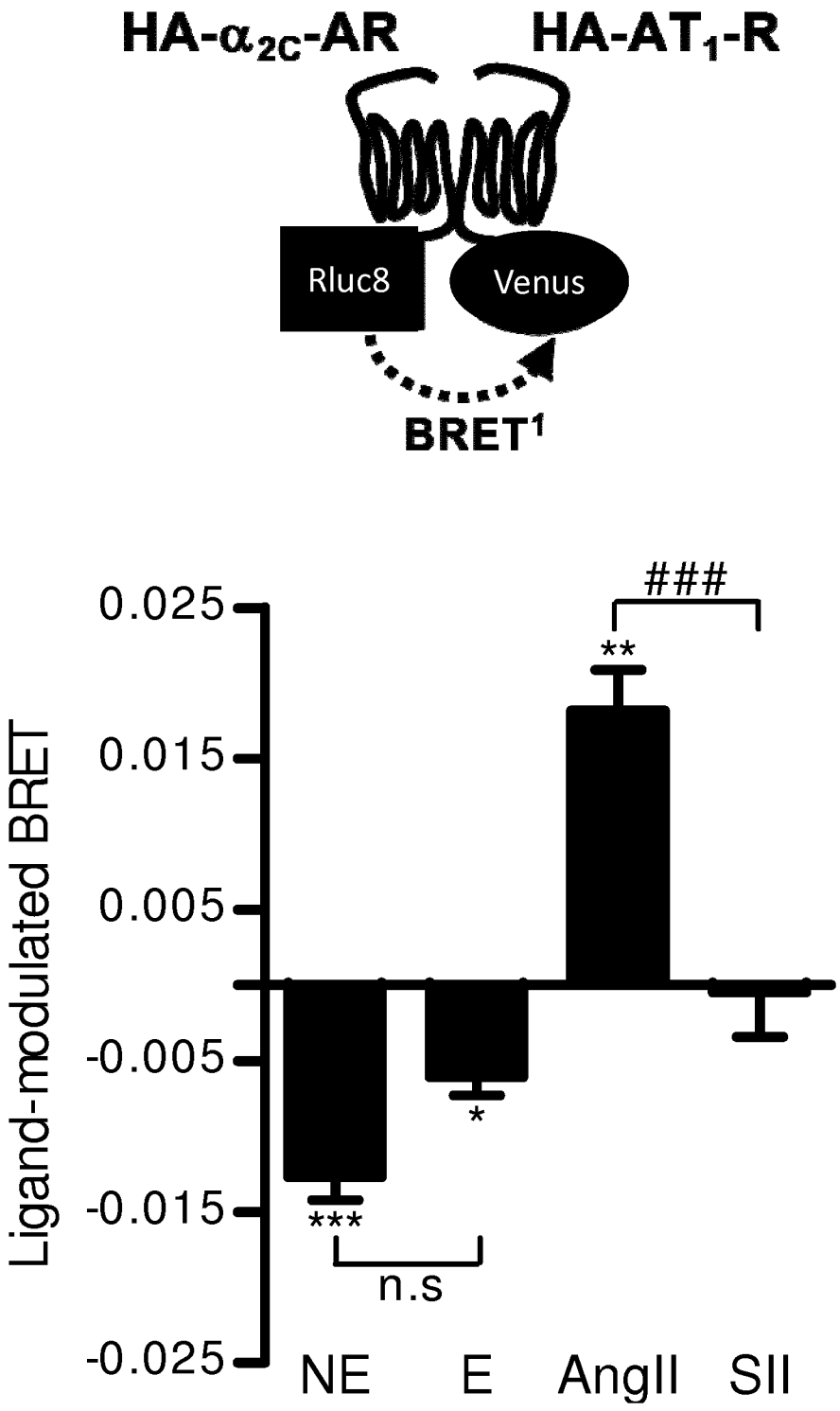

HA-α2C-AR
HA-AT1-R
Rluc8
Venus
BRET1
Ligand-modulated BRET
0.025
0.015
0.005
-0.005
-0.015
-0.025
###
**
*
***
n.s
NE
E
AngII
SII

Figure 2C

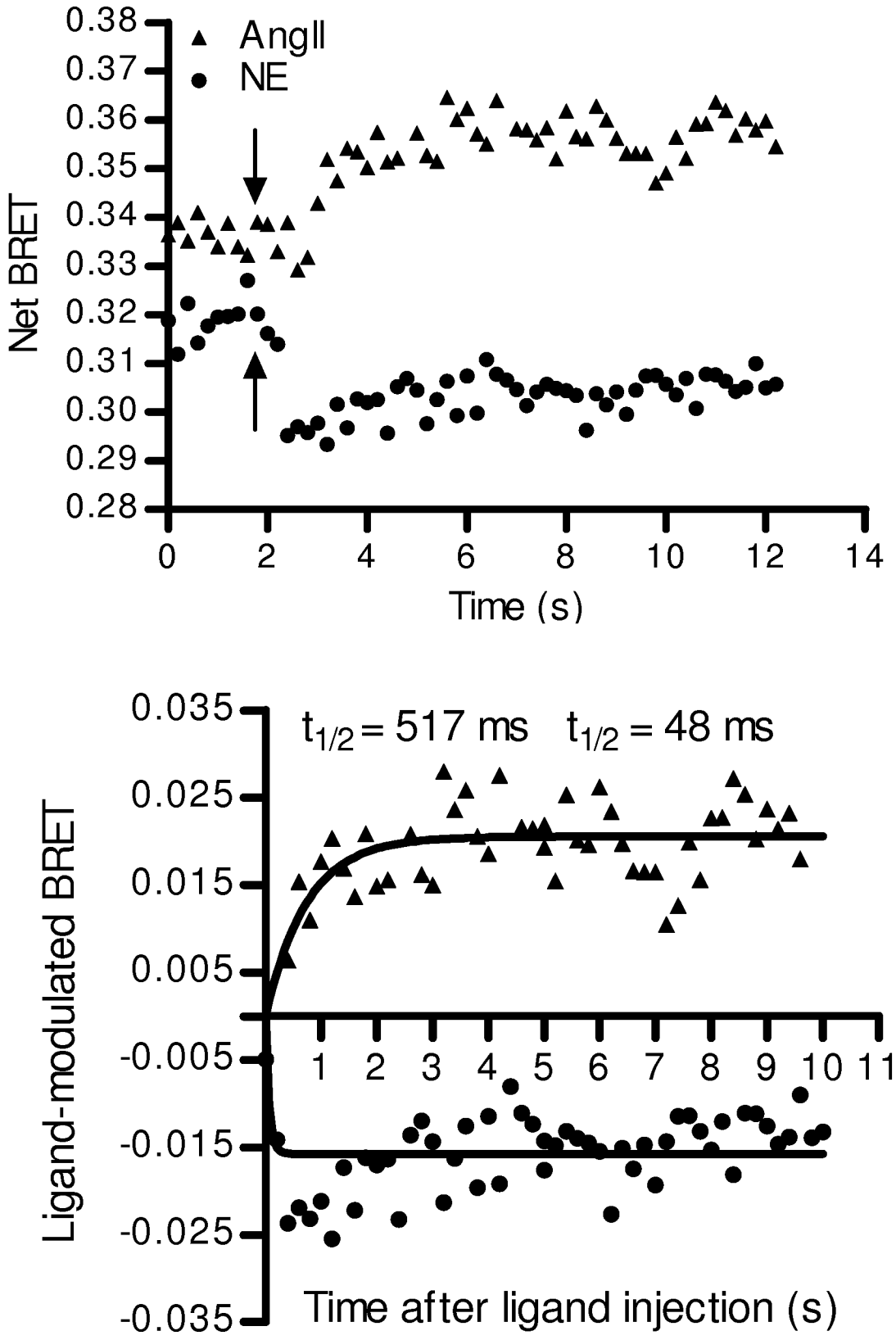
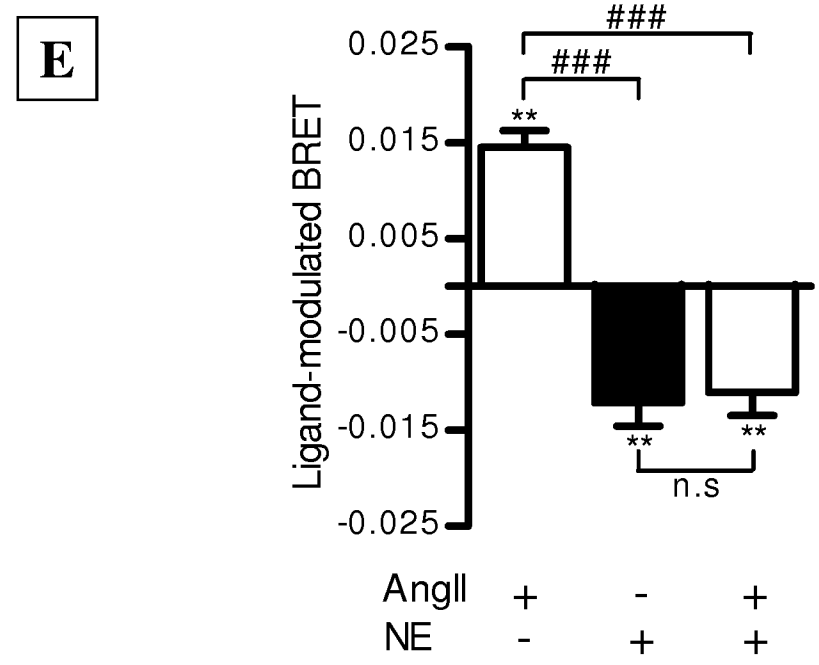

AngII
NE
Net BRET
0.38
0.37
0.36
0.35
0.34
0.33
0.32
0.31
0.30
0.29
0.28
0
2
4
6
8
10
12
14
Time (s)
$t_{1/2}$ = 517 ms
$t_{1/2}$ = 48 ms
Ligand-modulated BRET
0.035
0.025
0.015
0.005
-0.005
-0.015
-0.025
-0.035
1
2
3
4
5
6
7
8
9
10
11
Time after ligand injection (s)

Figure 2D



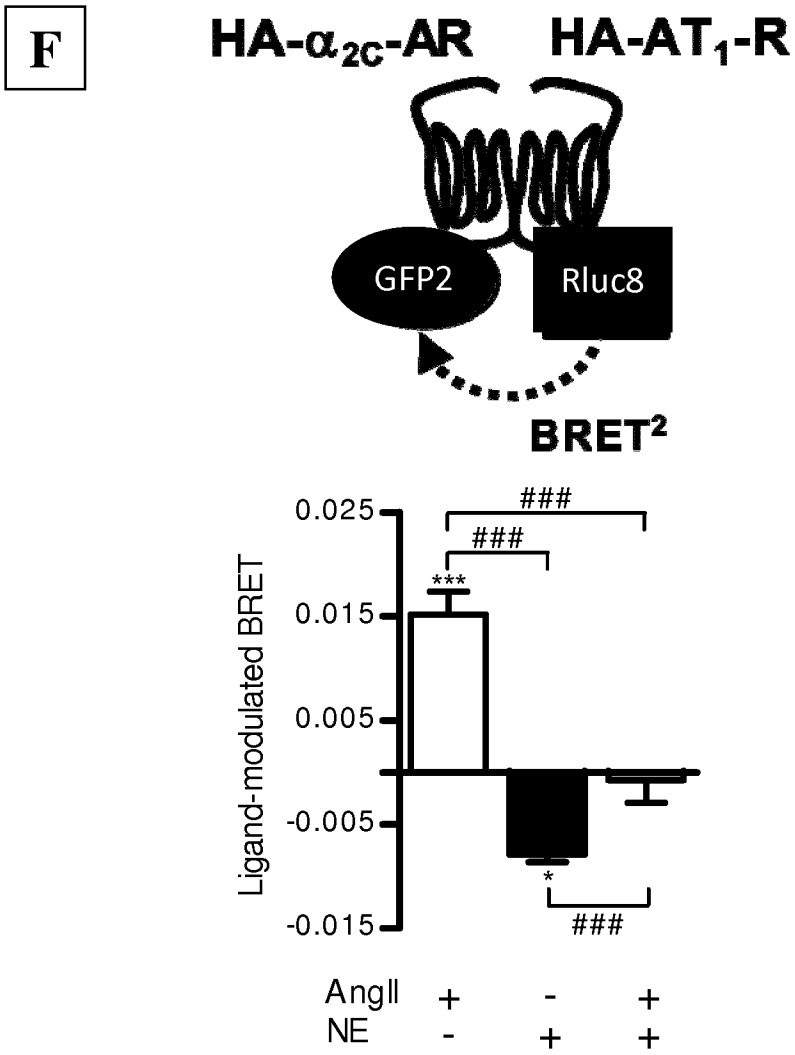


Figure 2E and F

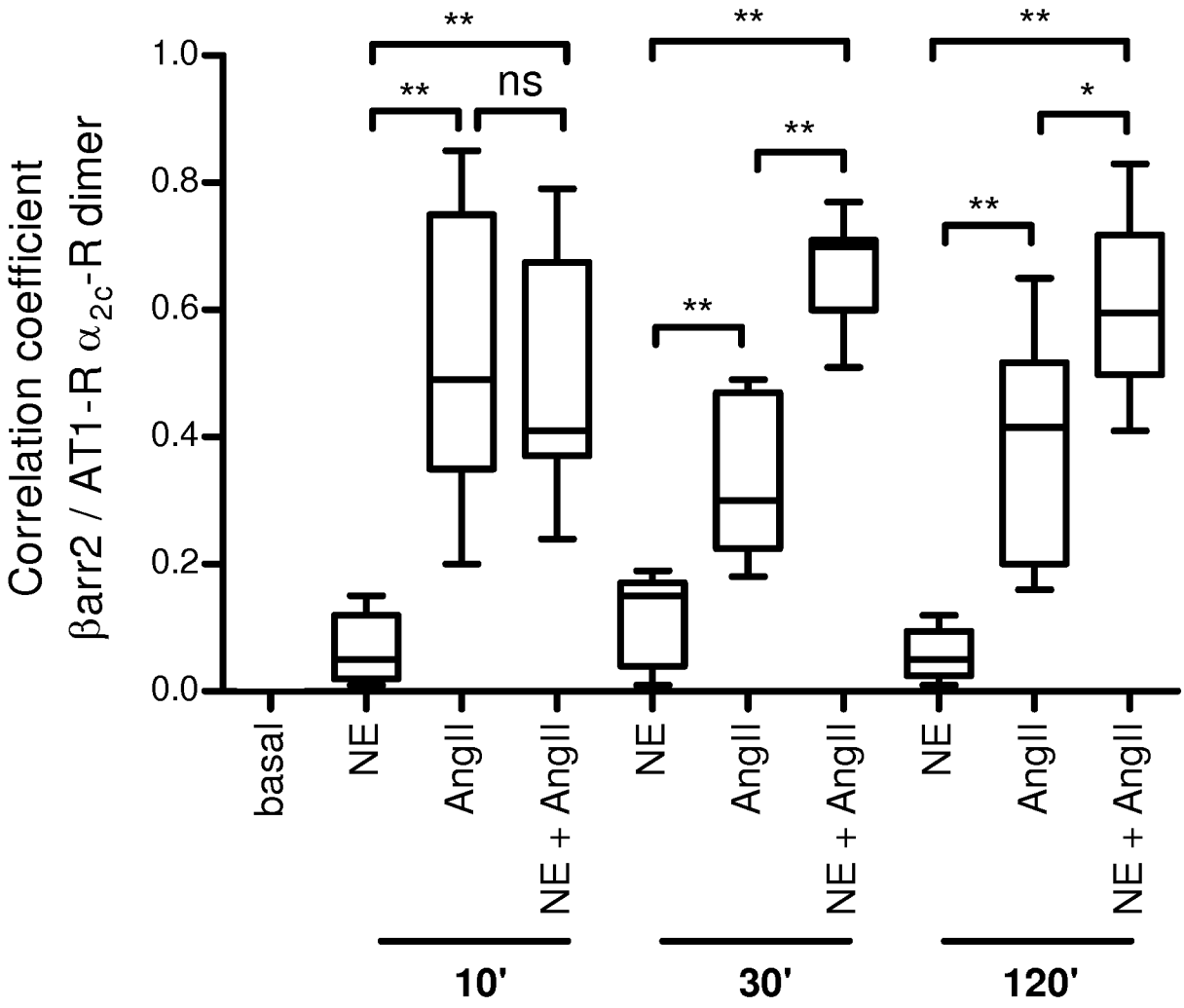

Correlation coefficient
βarr2 / AT1-R α2c-R dimer
1.0
0.8
0.6
0.4
0.2
0.0
**
**
ns
**
**
**
**
*
basal
NE
AngII
NE + AngII
NE
AngII
NE + AngII
NE
AngII
NE + AngII
10'
30'
120'

Figure 3

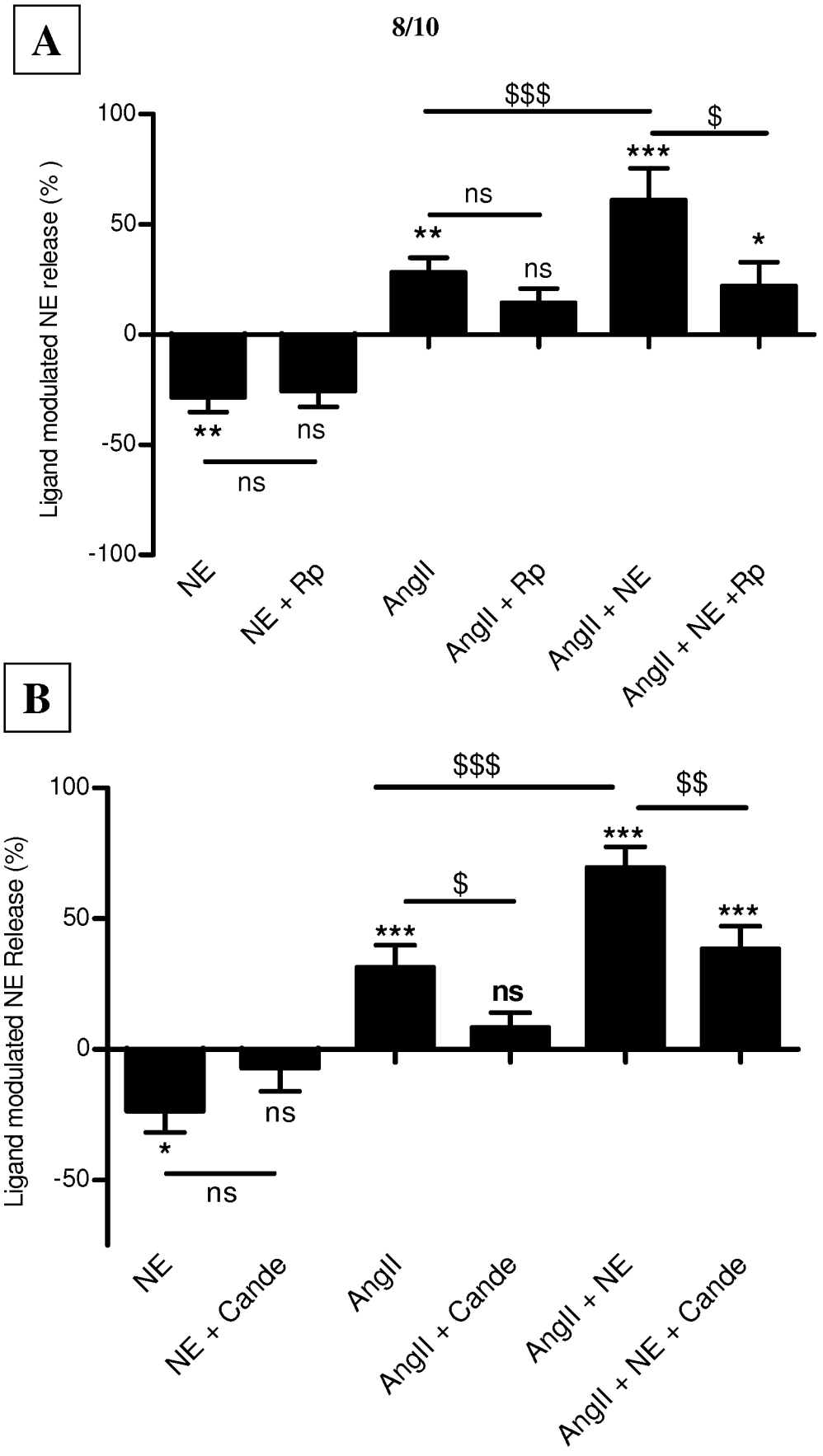


Figure 4 A and B

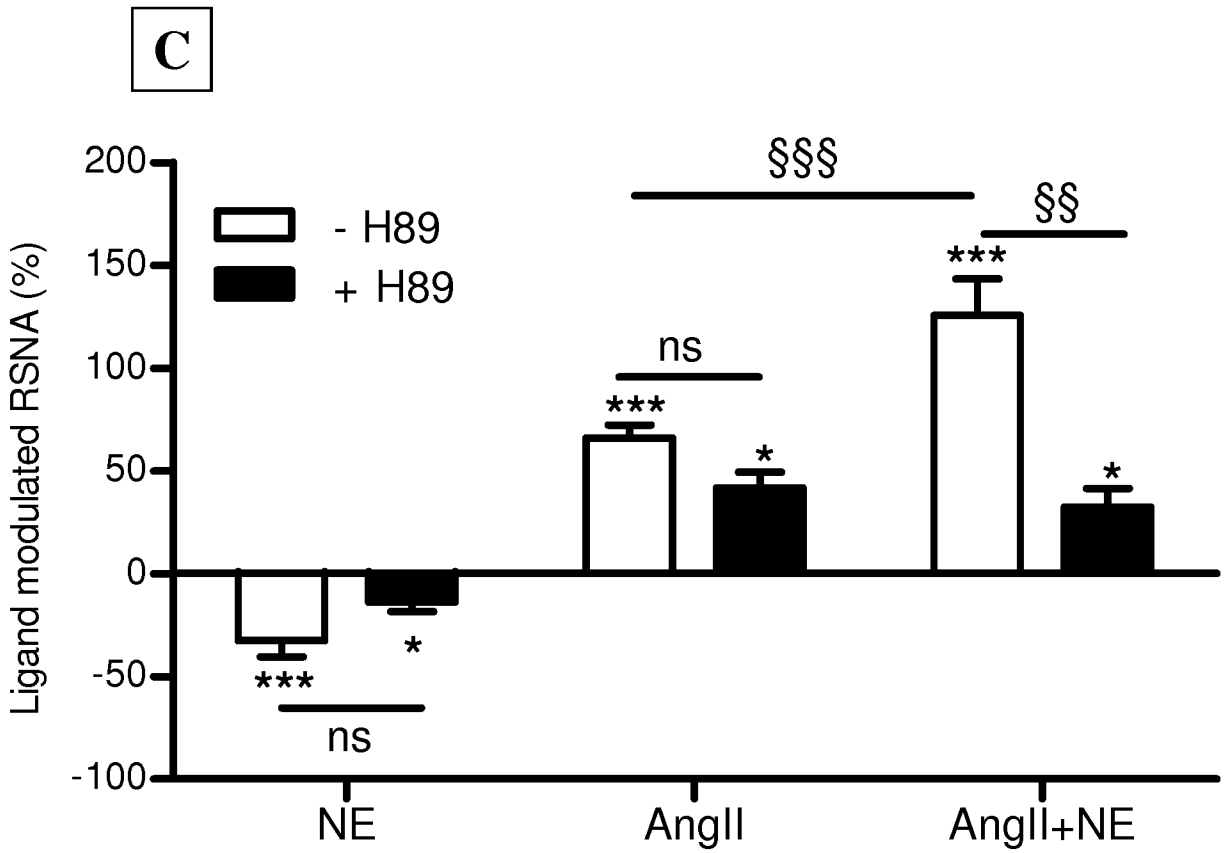

C
200
150
100
50
0
-50
-100
Ligand modulated RSNA (%)
- H89
+ H89
§§§
§§
ns
ns
***
*
***
*
*
***
*
NE
AngII
AngII+NE

Figure 4 C

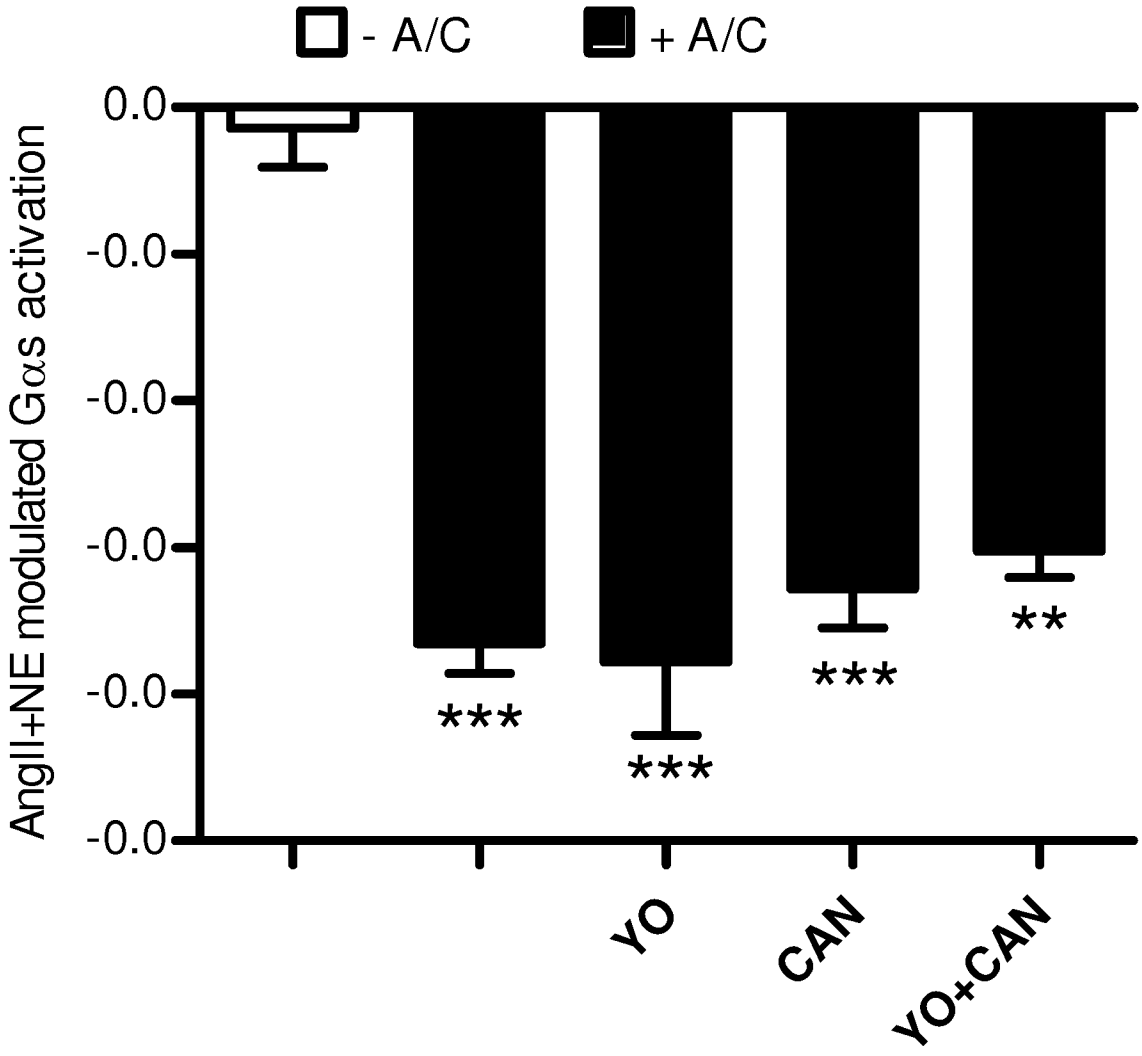

- A/C
+ A/C
AngII+NE modulated Gαs activation
0.0
-0.0
-0.0
-0.0
-0.0
***
***
***
**
YO
CAN
YO+CAN

Figure 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/053521

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/00 G01N33/00 A61P9/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K G01N A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SIYANDA MAKAULA ET AL: "H-89, a Non-Specific Inhibitor of Protein Kinase A, Promotes Post-Ischemic Cardiac Contractile Recovery and Reduces Infarct Size", JOURNAL OF CARDIOVASCULAR PHARMACOLOGY, vol. 45, no. 4, 1 April 2005 (2005-04-01), pages 341-347, XP055200070, NEW YORK, NY ISSN: 0160-2446, DOI: 10.1097/01.fjc.0000156825.80951.14 abstract	1-8

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 April 2016	28/04/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Habedanck, Robert

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/053521

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2009/016514 A2 (ELDRUG S A [GR]; MATSOUKAS JOHN [GR]; GAVRAS CHARALAMBOS [GR]; VLAXAKO) 5 February 2009 (2009-02-05) page 1, lines 3-6; claims 40-44	1-8
A	CHRISTOF BURGDORF ET AL: "Presynaptic Regulation of Norepinephrine Release in a Model of Nonfailing Hypertrophied Myocardium", JOURNAL OF CARDIOVASCULAR PHARMACOLOGY, vol. 41, no. 5, 1 May 2003 (2003-05-01), pages 813-816, XP055200234, ISSN: 0160-2446, DOI: 10.1097/00005344-200305000-00020 abstract	1-8

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/053521

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2009016514 A2	05-02-2009	GR 20070100650 A	15-05-2009
		WO 2009016514 A2	05-02-2009