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(54) Title: PEGYLATED BNP

(57) Abstract: A chemically modified human BNP (BNP) prepared by attaching a transient linker which comprises a polyethylene glycol. The chemically modified peptide may have a much longer lasting BNP activity than that of the unmodified BNP, enabling reduced dose and scheduling opportunities and the modified BNP may not cause hypotension. Also includes methods of use for the treatment and/or prevention of diseases or disorders in which use of BNP is beneficial.

PEGylated BNP

FIELD OF THE INVENTION

This invention relates to a pharmaceutical composition comprising suitable pharmaceutical excipients and also comprising a human in vivo clinical effective amount of a transient PEGylated B-type natriuretic peptide (BNP) prodrug, which may not cause hypotension and controls levels of aldosterone.

BACKGROUND ART

B-type natriuretic peptide (also known as Brain Natriuretic Peptide), hereafter referred to as BNP is an endogenous peptide belonging to the group of natriuretic peptides. The natriuretic peptides are a family of peptides, each with a 17 amino acid disulfide ring structure, with distinct renal, endocrine and cardiovascular homeostatic actions. Four peptides have been identified in humans: Atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), C-type natriuretic peptide (CNP) and urodilatin (Uro).

BNP is a 32 amino acid peptide and was originally discovered in extract of porcine brain, leading to the name brain natriuretic peptide. It is present in human brain, but there are significant higher amounts in the cardiac ventricular tissue. BNP is released as response to increased myocardial wall stretch, which is exaggerated in heart failure and is therefore used as a marker for pathology related to high extracellular fluid volumes.

BNP binds to the natriuretic peptide receptor A (NPR-A) where it stimulates release of cGMP, that in turn mediates natriuresis, diuresis, inhibition of renin and aldosterone, as well as other effects such as, vasorelaxant, anti-fibrotic, anti-hypertrophic and lusitropic effects. Hypertension per se is a serious result of an increase in extra-cellular fluid volume and is a major cause of death.

Therapeutic measures related to diseases associated with sodium and water retention are varied and include administration of a variety of diuretic substances. BNP is known to have natriuretic, diuretic, and vasorelaxant properties and may have antagonistic effects on the renin-angiotensin (ANG)- aldosterone (Aldo) system. It is understood that these peptides

and their analogs (such as Atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), C-type natriuretic peptide (CNP) and urodilatin (Uro) are effective in regulating blood pressure by controlling fluid volume and vessel diameter. A number of disease states are characterized by abnormal fluid retention, including congestive heart failure, cirrhosis of the liver and nephrotic syndrome. These diseases are associated with excessive fluid accumulation on the venous side of circulation, and an underperfusion of the kidneys, leading to a fall in glomerular filtration rate (GFR). Since late 1980, BNP was cloned and expressed and a commercial product named Nesiritide has been approved by FDA for clinical indications of management of acute decompressed congestive heart failure (CHF). BNP product and related medical use of BNP regulating blood pressure by controlling fluid volume and vessel diameter are well described in WO8912069 (Scios) and related patent families (EP418308B1, US5114923, US5674710, US65863696).

The natriuretic peptides are cleared from circulation by several mechanisms. Natriuretic peptides, including BNP, bind to Natriuretic Peptide Receptor C, hereafter referred to as NPR-C. BNP binds to NPR-C and is internalized and enzymatically degraded, after which NPR-C returns to the cell surface. Circulating BNP is also degraded by neutral endopeptidases present in the renal tubular cells and vascular cells. BNP is degraded by neutral endopeptidase by initial attack at Met4-Val5. Lastly, a smaller proportion of BNP is excreted through renal filtration.

It is well known in the prior art how to prolong circulations times of peptides which are sensitive to cleavage. The PEGylated BNP may increase plasma residency duration, decrease clearance rate (hereby achieving reduced dosage), improve stability and decrease antigenicity or a combination thereof.

A mechanism for enhancing peptide availability is by conjugation of the peptide with derivatizing compounds, which include, but are not limited to, polyethylene glycol and polypropylene glycol. Some of these benefits recognized include: lowered immunogenicity and antigenicity, increased duration of action, and altered pharmacokinetic properties. [Veronese, F.M. "Enzymes for Human Therapy: Surface Structure Modifications," Chimica Oggi, 7:53-56 (1989)] (Herein reference 5).

There are numerous prior art patent documents available on PEGylation of peptides. US2004020303081 describes modified BNP molecules (substitutions of specific amino acids) as a way to prolong half live. Another way is described in patent application

US2008004206A1, where a fusion protein of BNP and albumin was prepared. In "Heart failure Clin. 2 (2006) 365-373" the so called "AlbuBNP" is a long-acting form of BNP produced by recombinant fusion to human serum albumin. The authors Chen & Burnett (in "Heart failure Clin. 2 (2006) 365-373")) measured that AlbuBNP had approximately the same bioactivity as BNP to activate cGMP in the in vitro assay. This patent application teaches fusions of albumin and BNP hereby obtaining longer circulation times.

Cataliotti et al. (Trends in Cardiovascular Medicine, Vol 17, Issue 1, January 2007, Pages 10-14) describes conjugation of BNP to PEG oligomers. Four distinct conjugation sites are described namely serine-1 (the N-terminal), lysine-3, lysine-14 and ly-sine-27. Cataliotti et al. describe that since BNP binding to NPR-A mostly is believed to occur in the loop region, it is expected that activity will be compromised upon conjugation to lysine-14 and perhaps lysin-27.
It is shown that conjugation to one or two sites in the loop region results in partial or full loss of activity, respectively. Conjugation to lysine-3 retains the biological activity. Cataliotti's interest was to identify positions to where a conjugate could be attached to without losing the biological activity (page 11 of publication "Trends in Cardiovascular Medicine, Vol 17, Issue 1, January 2007, Pages 10-14"). Cataliotti does not describe anything about cleavable linkers and one may objectively say that this article teaches away from using a cleavable linker using a self hydrolysable (autocleavage) transient linker.

WO2006076471 describes orally (transportable through the epithelium) available conjugates of polypeptides, such as BNP. The problem to be solved in WO2006076471 is to provide BNP which is protected against proteolytic enzymes. The solution provided is to conjugate with PEG. The inventors of patent WO2006076471 had no specific interest to inactivate the conjugates and selected actually the most active ones for further evaluation. In this invention, it teaches away from such selection of inactivate conjugates. Also, the main interest in WO2006076471 was to identify conjugates most suitable for transdermal penetration (orally administered).

BNP is approved for acute CFH and patients are obliged to get such treatment in a hospital under intensive care. This is therefore an expensive (hospital time) treatment. The chronic treatment of CFH with BNP is not approved by FDA. There have been reported cases of dose related hypotension and this is also mentioned in the prescribing information of Natrecor (www.natrecor.com) under chapter pharmacody-namic. Hypotension is abnormally low pressure of the blood and called also low blood pressure. One of the adverse effects for

administration of BNP is hypotension. Symptomatic hypotension can be defined as a significant decrease in blood pressure (in excess of what would be intended with an intravenous vasodilator) and can be associated with 1 or more of the following symptoms: lightheadedness, dizziness, feeling faint, or having blurred vision.

The FDA has set a commission to investigate occurrence of dose related hypotension in relation to the administration of BNP. In this study [JAMA . 2002;287:1531–1540], it was noticed that there was no significant difference between nesiritide and nitroglycerine in frequency of hypotension and in renal injury/increased Serum creatine (Cr) levels. Also 4 patients died within 7 days after nesiritide treatment versus 1 in the nitroglycerine group. Recent publications have indicated that clinician tends to omit the bolus dose of nesiritide to avoid risk of hypotension. This may ultimately affect the efficacy of nesiritide treatment.

As discussed above, BNP administration may be beneficial in treating conditions relating to high volumes of extracellular fluid because of a direct natriuretic action, increased cardiac output, and/or decreased aldosterone levels. Aldosterone is important in the pathophysiology of heart failure because of its ability to increase sodium retention and potassium loss. Aldosterone production is activated in failing ventricles of humans. Concentration of aldosterone within the heart is reported to greatly exceed circulating concentrations.

Thus, there are many unmet needs in the management of congestive heart failure.

SUMMARY OF THE INVENTION

The problem to be solved by the present invention is to reduce the administration frequency of recombinant BNP. This problem is solved by use of special types of PEG conjugated to BNP as described herein. The herein described BNP PEG conjugate releases sustainably the correct dosage over time and reduces the risk of inducing hypotension.

According to prescribing information for Natrecor® a bolus injection is necessary. A bolus injection is an injection of a drug (or drugs) in a high quantity (called a bolus) at once. Without being limited to theory, the plasma peak concentration associated with this bolus injection is thought to relate to hypotension.

The current invention does not require a bolus injection in order to reach the correct plasma BNP profile. Further in the present invention is claimed the sustained release of relevant lower dosage of BNP to suppress aldosterone hormone as treatment for chronic congestive heart failure.

As mentioned above, Cataliotti et al. (Trends in Cardiovascular Medicine, Vol 17, Issue 1, January 2007, Pages 10-14) describes conjugation of BNP to PEG oligomers. Cataliotti's interest was to identify positions to where a conjugate could be attached without losing the biological activity. In current invention, it is the purpose to deactivate BNP and to deliver the optimal sustained release in terms of dosage over the time obtaining virtual no hypotension side effects.

WO2006076471 describes orally (transportable through the epithelium) available conjugates of polypeptides, such as BNP. The problem to be solved in WO2006076471 is to provide BNP which is protected against proteolytic enzymes. The solution provided is to conjugate with PEG. It is also discussed so called class 3 modifying moieties which are full hydrolysable. There are no data provided of this class 3 conjugates. Figure 4 shows several conjugates orally dosed, which have longer half-life than did native BNP (tested trypsin digestion rate). The therein described conjugates BN-002, 021, BN-022 and BN-024 have an activity closest to native BNP. The inventors of patent WO2006076471 had thus no specific interest to inactivate the conjugates and selected actually the most active conjugates for further evaluation. In this invention, it teaches away from such selection. Also, the main interest in WO2006076471 was to identify conjugates most suitable for transdermal penetration (orally administered) and the selected candidates are not prodrugs as termed in this invention.

The self hydrolysable transient linker PEGylated BNP conjugated prodrug – as described herein - releases the drug in such a way that native BNP is sustainably released and controls a therapeutic effective concentration in the plasma when administered at appropriate intervals.

Furthermore in vivo linker half life can be engineered to reduce the number of injections needed for optimal BNP coverage as well as reducing the peak-to-trough ratio that would be associated with frequent administration of BNP. This has the advantage that the risk of side effect such as hypotension is significantly reduced and a better drug coverage is achieved.

The term “Peak” herein understood as the maximum drug concentration after administration. As the drug circulates and is cleared, the concentration drops and reaches “Trough” which is the lowest drug level before subsequent administration. The therapeutic window is the acceptable range of concentration of the drug in plasma. The window falls between the maximum acceptable dose (as determined by effect vs. tolerability of side effects) and lowest concentration exhibiting therapeutic effect. The risk of hypotension is observed in the higher dose range. Without being limited to theory, the fluctuation of drug concentration in the plasma it is also believed to contribute to side effects.

The herein relevant properties of the BNP prodrug – as described herein - are:

(1): when PEG is linked to BNP in the prodrug conjugate the prodrug has an BNP activity which is less than 5% of the native BNP; and
(2): PEG is linked to BNP via a self hydrolysable (autocleavage) transient linker, wherein the linker autohydrolysis rate is such that the *in vivo* linker half-life is from 50 hours to 500 hours.

Feature (1) delivers conjugates which are substantially inactive and can be used as useful prodrugs.

Feature (2) ensures release the drug in such a way that native BNP is sustainably released and controls a therapeutic effective concentration in the plasma.

Feature (1) and (2) together delivers the enormous advantage that now the drug can be administered (subcutaneously) for example once weekly. Such administration can be done for example by a nurse visiting the patient or eventually by the patient itself. This saves hospital visits (saving cost), gives the patient comfort and secures that the drug is present in an effective concentration over time without causing risk of hypotension. All this is not possible today with the current way of BNP administration.

Accordingly, a first aspect of the invention relates to a pharmaceutical composition comprising pharmaceutically acceptable excipients and also comprising a PEGylated prodrug capable of releasing an in vivo clinically effective amount of a recombinant human BNP (BNP without PEG), wherein the prodrug is characterized by that:

(1): when PEG is linked to BNP in the conjugate the conjugate has an BNP activity which is

less than 5% of the native BNP without PEG, measured according to the assay to measure BNP PEGylated prodrug and BNP activity of example 1; and

(2): PEG is linked to BNP via a self hydrolysable (autocleavage) transient linker, wherein the linker autohydrolysis rate is such that the in vivo linker half-life is from 50 hours to 500 hours, measured according to the assay to measure autocleavage rate of the transient linker of the prodrug of example 2.

Congestive heart failure (CHF) is reduced ability of the heart to pump blood around the body. The body tries to compensate by retaining water to increase blood volume, but this further weakens the heart. Diuretic drugs reduce water in the body.

BNP is approved for acute CHF and therefore patients suffering of decompensated congestive heart failure (CHF) are obliged to get treatment in the hospital (a typical 4 hour infusion once weekly applied in the hospital intensive care). This is therefore an expensive treatment requiring in some cases prolonged hospital time (staying overnight for extra observations). The present invention overcomes this burden by delivering self hydrolysable transient linker PEGylated BNP conjugated prodrug administered subcutaneously e.g. once weekly. Hereby patients only have to get a once weekly injection in the hospital or outside the hospital (a nurse which passes by the patient for example to give the injection), which of course significantly reduces expensive hospital time.

Figure 2 illustrates a typical BNP plasma profile of a continuous infusion of 15 ng/kg/min for 4 hours. As bolus injections of BNP does not ensure adequate BNP coverage, it is currently necessary to administer BNP as continuous infusion as depicted in Figure 2. As this has to be done in the hospital setting, this is very costly. Furthermore, BNP levels return to baseline within hours of ending the intravenous infusion, which generally lasts 4 hours. For this reason, even with expensive weekly intravenous infusion, the BNP coverage is far from optimal.

Figure 3 shows a plasma profile and is a simulation of a plasma profile of a single subcutaneous injection of a sustained release formulation using transiently conjugated nesiritide (BNP). The dashed line represents BNP bound in the inactive conjugate. Solid line represents free circulating BNP.

Applying the transiently linked BNP prodrug as described in the present invention, it is

possible to administer a therapeutic relevant amount of BNP as a subcutaneous injection on e.g. a weekly basis. Due to the slow autohydrolysis of the linker, native BNP is released in a predetermined fashion ensuring a plasma profile free of significant burst effect and with a peak-to-trough ratio of less than 10. The *in vivo* linker half life can be engineered such that the maximum BNP concentration will be below the concentration inducing symptomatic hypotension. Furthermore, due to the simplicity of subcutaneous administration the need for expensive in hospital intravenous infusion is eliminated.

Accordingly, a second aspect of the invention relates to a clinically effective amount of the pharmaceutical composition comprising BNP PEGylated prodrug of the first aspect for use in a method for the treatment of a BNP related disease in a human, wherein the treatment comprises administering the pharmaceutical composition as one administration during a treatment period and wherein the treatment is characterized by that after the one administration of the pharmaceutical composition is the ratio during the treatment period, between the highest concentration of active BNP drug (BNP without PEG) in plasma during the treatment period and the concentration of active BNP drug in the plasma at the end of the treatment period, less than 10, wherein the treatment period is a period of at least three days.

This second aspect may alternatively be formulated as a method of treating a BNP related disease in a human which comprises administering the pharmaceutical composition comprising the BNP PEGylated prodrug of the first aspect as one administration during a treatment period characterized by that after one administration of the pharmaceutical composition during the treatment period is the ratio, between the highest concentration of active BNP drug (BNP without PEG) in plasma during the treatment period and the concentration of active BNP drug in the plasma at the end of the treatment period, less than 10, wherein the treatment period is a period of at least three days.

As discussed above, Aldosterone is important in the patho-physiology of heart failure because of its ability to increase sodium retention and potassium loss. Aldosterone production is activated in failing ventricles of humans. Concentration of aldosterone within the heart is reported to greatly exceed circulating concentrations. Self hydrolysable transient linker PEGylated BNP conjugated prodrug, releases the drugs in a controlled way and it is an advantage that a specific low concentration of drug is released which can suppress the hormone aldosterone. The suppression of aldoste-rone by sustained release of low concentration of BNP has a therapeutic advantage.

DEFINITIONS

Prior to a discussion of the detailed embodiments of the invention is provided a definition of specific terms related to the main aspects of the invention.

In general, all specific technical terms used herein shall be understood as the skilled person would understand them in the present technical context.

The term “activity” herein is understood as the ability of BNP or a conjugate thereof, to evoke a clinically relevant biological response when administered to a mammal, e.g. in an in vivo model, or to produce a measureable response in an in vitro model as described in examples.

The term “autocleavage” herein is understood as spontaneous cleavage of the bond between the transient linker and the drug molecule BNP under physiological conditions, the latter being defined as a pH in the range 7.0 – 7.8 and a temperature of between 35°C to 40°C.

The term “BNP” herein is understood as B-type natriuretic peptide.

The term “conjugate” herein is understood as a PEG molecule covalently bound to the drug herein being BNP.

The term “*in vivo* linker half life” is understood as the time interval in which 50% of the initial proportion of BNP is released from the PEGylated BNP conjugate after administration to a mammal and corrected for systemic clearance of the PEGylated BNP conjugate and can be measured as described in Example 2.

The term “hypotension” refers to an abnormally low blood pressure. This is best understood as a physiologic state, rather than a disease. Hypotension is the opposite of hypertension, which is high blood pressure. Hypotension can be life-threatening. Hypotension can also lead to renal damage due to lack of blood circulating through the kidneys.

The term “Peak” herein understood as the maximum drug concentration after administration. As the drug circulates and is cleared, the concentration drops and reaches “Trough” which is the lowest drug level before subsequent administration. The therapeutic

window is the acceptable range of concentration of the drug in plasma. The window falls between the maximum acceptable dose (as determined by effect vs. tolerability of side effects) and lowest concentration exhibiting therapeutic effect. The risk of hypotension is observed in the higher dose range. Without being limited to theory, the fluctuation of drug concentration in the plasma it is also believed to contribute to side effects.

The term a "pharmaceutical composition comprising "a human *in vivo* clinically effective amount of a recombinant human BNP PEGylated prodrug" is to be understood as an amount that is sufficiently high to obtain a wanted clinical effect in a human after administration of the pharmaceutical composition to the human – e.g. a wanted clinical effect in relation to treatment of a BNP related disease.

In the present context the skilled person routinely is able to adjust the amount of re-combinant human BNP PEGylated prodrug to be administered in order to get a wanted clinical effect.

The term "PEG" is also known as Poly(ethylene glycol).

The term "Prodrug" herein is understood is any compound that undergoes transformation before exhibiting its pharmacological effects. Prodrugs can thus be viewed as drugs containing specialized non-toxic protective groups used in a transient manner to alter or to eliminate undesirable properties in the parent molecule.

The term "transient" linker is a linker in which the conjugation of drug to PEG molecule is reversible. This implies that autocleavage of the linker releases the drug in its native and active form. Furthermore, cleavage of the linker proceeds at a predictable manner releasing the unmodified drug over many hours independent of enzyme activity.

The term "therapeutic window" is herein understood as the window where the active drug has a therapeutic effect and typically has a peak and a trough, where the peak level is the highest allowable concentration in the plasma and is mostly obtained straight after administration and a minimum lower amount of drug (typically after some time circulation in the body) in the plasma still having a therapeutic effect (also termed Trough).

DRAWINGS

Figure 1: Structure and amino acid sequence for BNP.

Figure 2: Plasma profile of a continuous infusion of 15 ng/kg/min for 4 hours. This profile is cited in Scios' Clinical Pharmacology and Bio-pharmaceutics Review: Natrecor (nesiritide) appendix 4.

Figure 3: Simulated plasma profile of a single subcutaneous injection of a sustained release formulation using transiently conjugated nesiritide (BNP) as described herein. Dashed line represents BNP bound in the inactive conjugate. Solid line represents free circulating BNP.

DETAILED DESCRIPTION OF THE INVENTION

Pharmaceutical composition comprising suitable pharmaceutical excipients

As known to the skilled person a pharmaceutical composition comprises pharmaceutical acceptable excipients and/or carriers.

"Pharmaceutically acceptable" is meant to encompass any excipient and/or carrier, which does not interfere with the effectiveness of the biological activity of the active ingredient and that is not toxic to the host to which it is administered.

In a preferred embodiment the pharmaceutical composition is a composition for subcutaneous administration, intramuscular administration or intravenous injection. Most preferred is a subcutaneous administration.

This is examples of preferred administration routes for treatment of a relevant disorder/disease as described herein.

The pharmaceutical composition may comprise other active ingredients than a BNP PEGylated prodrug as described herein.

Activity BNP with PEG

As discussed above, the BNP PEGylated prodrug as described herein shall have a relatively low activity.

A preferred embodiment is the PEG conjugate which has a BNP activity of less than 4% compared to the native BNP without PEG, more preferably less than 3%, even more preferably less than 1% and most preferably is the PEG-BNP conjugate virtually inactive, measured according to the assay to measure BNP PEGylated prodrug and BNP activity of example 1.

BNP PEGylated prodrug - "transient" linker structures

A PEGylated-prodrug, wherein the drug is for example BNP as described in patent application WO2005099768. In this document is generically/broadly described numerous herein relevant suitable so-called transient linker structures to get a relevant release profile of interest. Other "transient" linker structures are generically/broadly described in e.g. other Complex Biosystems GmbH applications such as WO05034909, WO05099768, WO06003014 and WO2006136586.

More "transient" linker structures are generically/broadly described in e.g. WO9930727 (Enzon Inc).

In order to solve the present problems for BNP as discussed herein one may say that the present inventors have selected suitable preferred transient linker structures to get the herein described relevant functional properties of the BNP PEGylated prodrug. Based on the herein detailed description of preferred linkers structures it is within the skilled person knowledge to make other suitable preferred transient linker structures that could give a BNP PEGylated prodrug with the herein described relevant functional properties.

The herein selected linker structures are described in detail below.

Ideally, a conjugate of the invention will possess one or more of the following features and/or advantages over current BNP conjugates or formulations; can easily be synthesized in good yields, have half life's falling within preferred range, can be purified to provide homogeneous conjugate compositions, exhibit activity after autocleavage such as

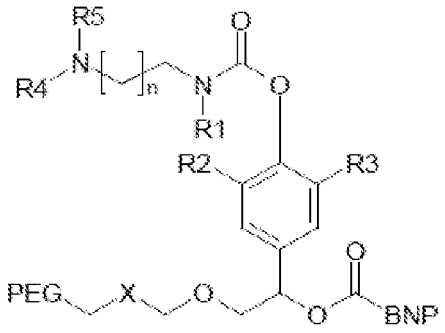
in vitro and in vivo activity and have pharmacodynamic effects superior to unmodified BNP and previously described BNP conjugates and do not cause hypotension.

The herein described structures exhibit release properties as required herein.

Other conjugates exhibit activity after autocleavage such as in vitro and in vivo activity and have pharmacodynamic effects superior to unmodified BNP and previously described BNP conjugates and suppress aldosterone hormone.

A preferred embodiment is a self hydrolysable (autocleavage) transient linker described herein where the linker autohydrolysis rate is such that the *in vivo* linker half life is from 40 hours to 600 hours; more preferred from 72 hours to 300 hours, more preferred from 96 hours to 192 and most preferred 120 hours to 192 hours.

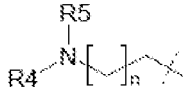
Selected chemical structures are presented below. The preferred self hydrolysable transient linker structure (aromatic) is:



R2, R3, R4, and R5 are selected independently from hydrogen, methyl, ethyl, propyl, isopropyl, butyl, isobutyl

and

R1 is selected from methyl, ethyl, propyl, isopropyl, butyl, isobutyl or



Formula I

wherein, the dashed line indicates the attachment site to the respective nitrogen atom

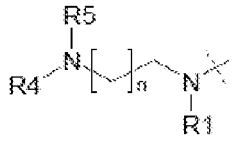
n = 1 or 2 and

X is selected from C1 to C8 alkyl or C1 to C12 heteroalkyl including heterocycloalkyl

and

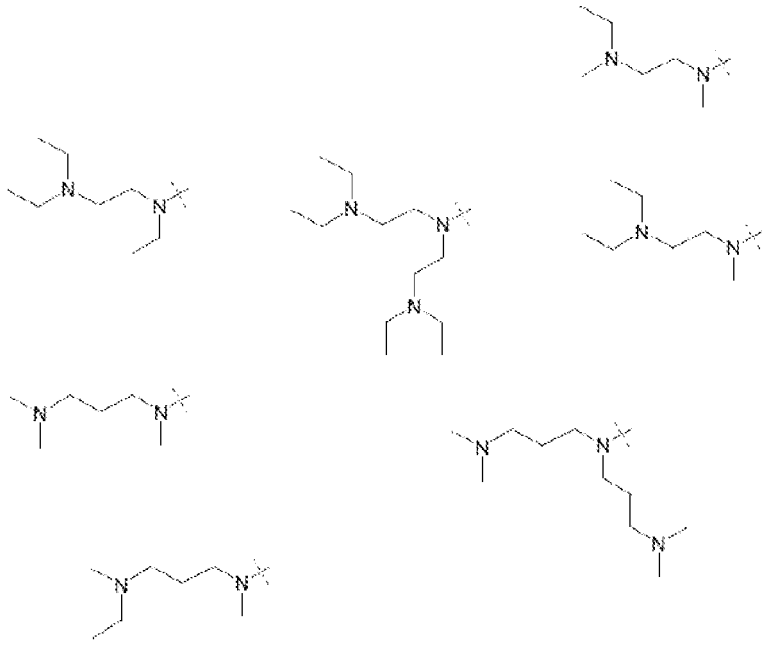
BNP is conjugated to the PEG-linker conjugate via a carbamate bond by one of its amino groups.

Further preferred structures of the formula I, which is a part of the self hydrolysable transient linker structure with the general formula I:



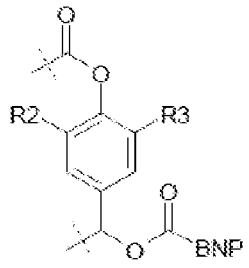
Formula I

and formula I can be selected from the group consisting of:



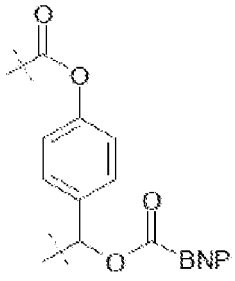
Further preferred structures of a part of the aromatic structure as described above, where

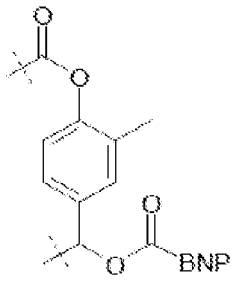
formula II is a part of self hydrolysable transient linker structure and is

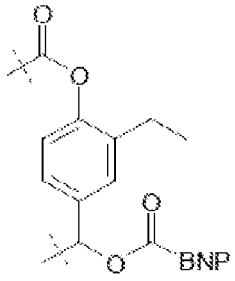


Formula II

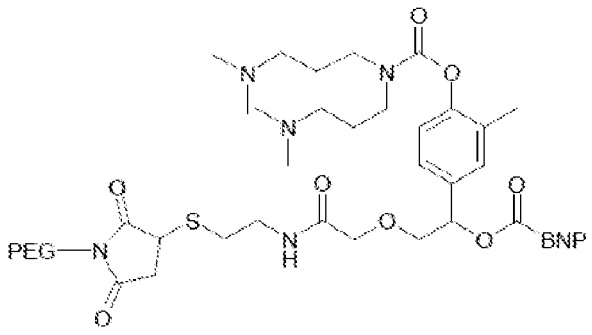
and formula II is selected from the group consisting of:

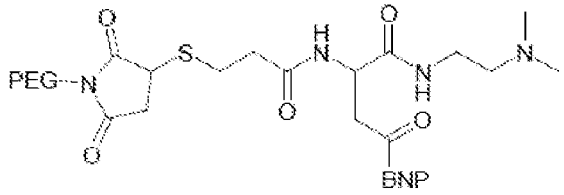




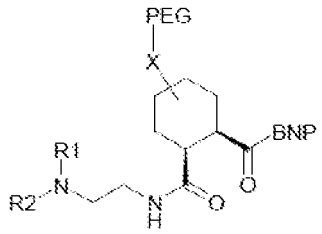


Further a preferred structure of the self hydrolysable transient linker PEGylated BNP prodrug is

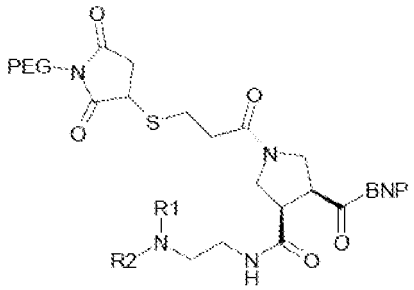




and is



and is



R1 and R2 are selected independently from hydrogen, methyl, ethyl, propyl, isopropyl, butyl, isobutyl, cycloalkyl and

R1 and R2 may be connected to form a C4 to C8 cycloalkyl and X is selected from C1 to C8 alkyl or C1 to C12 heteroalkyl

and

BNP is linked to the PEG-linker moiety (PEG-linker-BNP structure) via an amide bond by one of its amino groups.

PEG structures including linear and branched

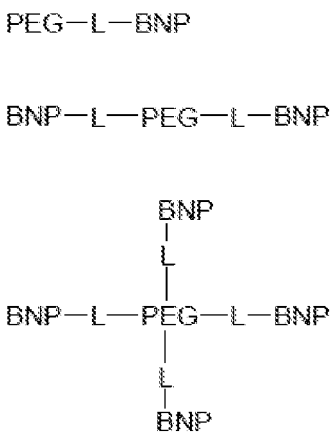
The role of PEG is discussed in the prior art above and preferred PEG structures are preferred embodiments herein.

The PEG structures include linear and branched PEG moieties with molecular weights between 5 kDa and 200 kDa. Preferred PEG structures include linear and branched PEG moieties with molecular weights between 5 kDa and 200 kDa and more preferred PEG molecular weights between 20 and 80 kDa.

Amount of BNP molecules attached to PEG

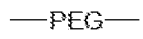
Preferably, one to eight BNP molecules are attached to one PEG molecule. Preferably, one to four BNP molecules are attached to one PEG.

Preferred general structures of PEG-Linker-BNP with one, two or four BNP molecules attached to one PEG molecule include preferred embodiments and can be selected from the group consisting of



where

L is a transient linker and PEG is a polyethylene glycol derivative and are selected from the group consisting of



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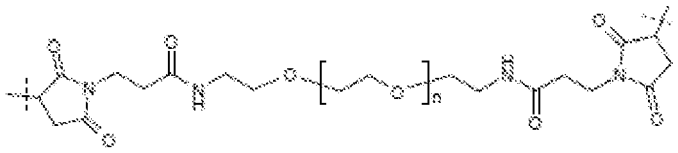
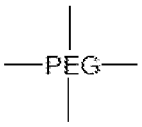


and



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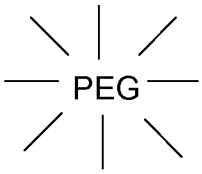
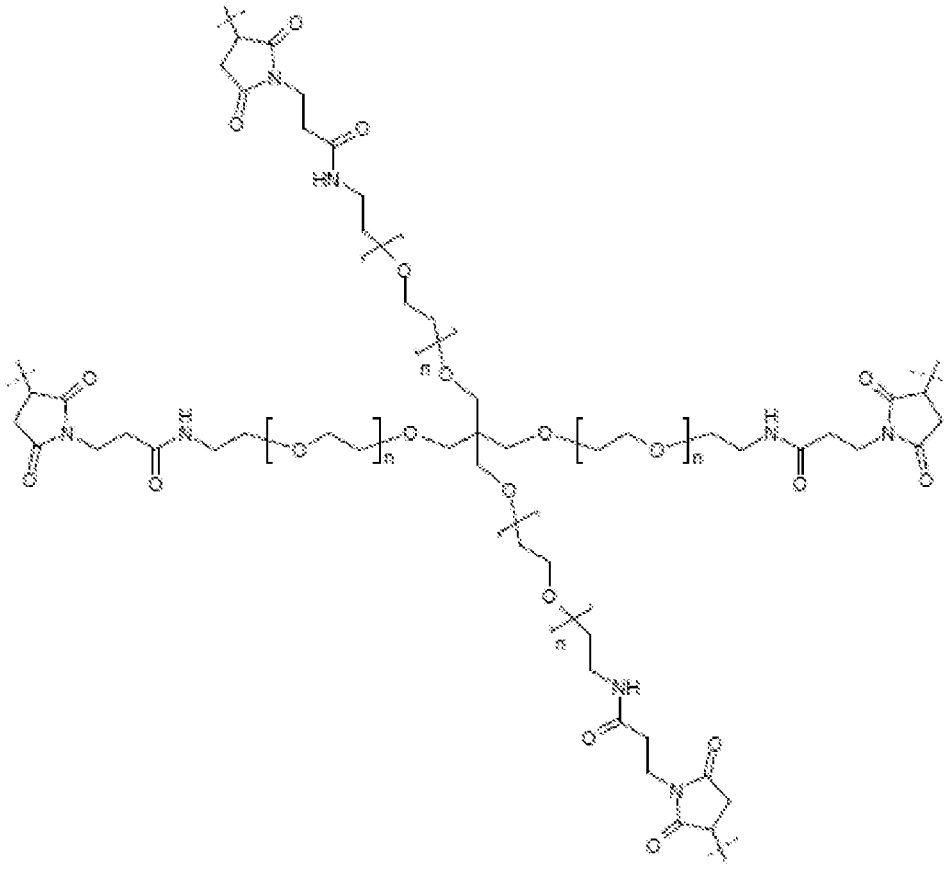


.

In order to reduce the weight to weight ratio of BNP to PEG ratio more than one BNP molecule can be attached to the carrier molecule (PEG). This has the advantage that the amount of BNP, which is a small molecule to be administered, can be optimized in comparison to the bigger PEG molecule.

In a preferred embodiment 4 BNP molecules are attached to one 4 armed PEG as shown below.

A preferred embodiment is therefore:



Hereby, the PEG functions as such especially for a small peptide as BNP, by attaching more BNP molecules per PEG, is maximized.

Recombinant Brain Natriuretic Peptide (BNP)

Beside that the recombinant human BNP is identical in sequence to natural human BNP, the term recombinant Brain Natriuretic Peptide (BNP) relates herein also to so-called generic versions of biological molecules.

The term “biogenerics” herein is understood generic forms of biopharmaceuticals; molecules developed using biological processes, usually through modern biotechnology activity.

Generic chemical pharmaceuticals can be defined as those molecules which, when compared with the originator product have essentially similar activity, are bioequivalent, achieve market authorization through an abbreviated procedure following patent expiry.

As known to the skilled person, it is today routine work to make e.g. minor amino changes of a biologics of interest (herein BNP) and still retain natriuretic peptide activity.

As also known to skilled person in the art fusion peptides of natriuretic peptides can be of interest herein. The fusion peptides are e.g. produced by combining two or more peptide sequences selected from the group of natriuretic peptides.

Other examples of natriuretic peptides that can be objects of the present inventions are peptides with natriuretic activity, such as ANP, CNP, DNP, Urodilatin and albuBNP, as well as the peptides described in the following patents and patent applications, which are incorporated herein in their entirety; WO2008/021872 A1; WO2008/031045 A2; WO2009/015011 A1; WO2009/036448 A2; US 5,583,108; US 6,818,619 B2; US 6,407,211 B1; US2005/0059600 A1; US2009/0069243 A1; WO01/44284 A2; WO94/20534; WO2007/035600 A2; US 7,384,917 A2.

Therapeutic window

The change in BNP levels in the plasma over time with nesiritide treatment is the topic of ongoing investigation. It is expected that levels could rise to above 3 ng/ml during the infusion and then fall rapidly after discontinuation of therapy to levels below baseline (reflecting the lower ventricular filling pressure achieved).

Without being limited to the theory, it is believed that the therapeutic range of BNP in the plasma is in the range of 0.1 ng/ml to 3 ng/ml, more preferred between 0.8 ng/ml and 1.2 ng/ml.

Hypotension effect

In Natrecor prescribing information (page 2 under Cardiovascular), it is described that Natrecor may cause hypotension. In the VMAC trial, in patients given the recommended dose (2 mcg/kg bolus followed by a 0.01 mcg/kg/min infusion) or the adjustable dose, the incidence of symptomatic hypotension in the first 24 hours was similar for Natrecor (4%) and IV nitroglycerin (5%).

The herein described conjugated BNP prodrug compounds, release active BNP and have a preferred incidence of symptomatic hypotension of maximum 5% and most preferred no symptomatic hypotension.

Ratio between the highest and lowest concentration of active BNP drug during treatment period

The highest concentration of BNP in plasma during the treatment period is generally referred to as the peak level. Accordingly, a trough level is generally defined as the lowest plasma concentration and is achieved just prior to re-administration of a drug. Using current modes of administration and if the therapeutic window is narrow, the peak value will be outside the therapeutic range and cause side effects and the trough level will be below the therapeutic window and hence not have therapeutic effect. In the present invention it can now be achieved by engineering the in vivo linker half life in such way, that both the peak and trough levels of released BNP will be within the therapeutic window following a single (one) administration of the pharmaceutical composition.

As figure 2 shows the peak level (this particular graph has a maximum concentration reached after 2 to 4 hours reaching between 1 and 2 ng/ml in the plasma.

After 8 hours, the concentration is below 0.5 ng/ml. The patient in need would now require a new BNP administration for the concentration of BNP in plasma to stay within the therapeutic window.

Peak to trough level can also be more simply described as herein is discussed a ratio between the highest concentration of active BNP in plasma and the lowest concentration of active BNP drug in the plasma during treatment.

In a preferred embodiment the administration of the PEGylated BNP conjugated pro-drug causes BNP to be present at least 3 days in the plasma, more preferred 5 days and most preferred 7 days and the ratio between highest and lowest plasma concentration within the dosing period is less than 10, more preferred less than 5.

As also explained above, the suppression of aldosterone can improve the BNP related disease treatment. It is therefore an advantage which can be delivered by the herein described PEGylated prodrug that a sustained release also helps to suppress aldosterone.

Treatment period

As discussed above the treatment period is a period of at least three days. Preferably, the treatment period is a period of at least four days, more preferably of at least five days, even

more preferably the treatment period is a period of at least six days. Most preferably the treatment period is a period of at least seven days.

The BNP-PEG conjugate as described herein is given as a one time administration during the treatment period. After the treatment period has ended, one may start a new treatment by administrating the BNP-PEG conjugate again.

This new period may e.g. be started by administrating the BNP-PEG conjugate just after the prior (e.g. first) treatment period has ended. For instance, if the treatment period is three days one may administrate the BNP-PEG conjugate at the end of day three and start a new treatment period of e.g. three extra days. In such a case the complete accumulated treatment period will be 6 days.
By such regular administration of BNP-PEG conjugate as described herein one may get a very long total accumulated treatment period, wherein the active BNP concentration is constantly within the therapeutic window during the total accumulated treatment period. For instance, if the treatment period is one week and BNP-PEG conjugate is administered each week for 52 weeks then the patient will have a total accumulated treatment period of 52 weeks, wherein the active BNP concentration is constantly within the therapeutic window.

Accordingly, in a preferred embodiment the treatment of the human is done for at least ten continuous treatment periods – with one administration of the BNP PEGy-lated prodrug in each period – wherein the active BNP concentration is constantly within the therapeutic window during the total accumulated treatment period. The therapeutic window may be understood as the ratio of the second aspect herein.

It is self-evident to the skilled person that one only gets the concentration constantly within the therapeutic window if the BNP-PEG conjugate is administered again relatively close after the prior treatment period. For instance if the treatment period is 7 days and one – after these 7 days – wait further 30 days before a second administration one will not get a concentration constantly within the therapeutic window during the total accumulated treatment period.

For instance for chronic treatment it may be good to a have virtually indefinite (e.g. virtually life-time) total accumulated treatment period. In such a case there may be at least 100 continuous treatment periods or even more e.g. 500 continuous treatment periods.

BNP relates diseases

The term “a BNP related” disease of second aspect simply herein relates to diseases and conditions where a human could benefit from BNP.

This includes, but is not limited to hypertension; salt-sensitive hypertension; congestive heart failure; angina pectoris, peripheral artery disease; diabetic nephropathy; stroke; kidney failure; acute and/or chronic renal failure; acute tubular necrosis; acute renal failure; renal disease; renal glomerular disease; excess fluid in tissues; hypotension; cardiac volume overload; cardiac decompensation; decompensated congestive heart failure; left ventricular dysfunction; dyspnea; treatment for elevated aldosterone levels, which can lead to vasoconstriction, impaired cardiac output and/or hypertension; cardiovascular disease; cardiac failure; myocardial infarction; myocardial re-perfusion injury; left ventricular remodeling; post-myocardial infarction; cardiac surgery; cardiac artery bypass graft; retinopathy, multi organ failure; organ transplant; renal protection; asthma; cancer; vascular regeneration.

Congestive heart failure (CHF) in humans

Congestive heart failure (CHF) occurs when the heart can no longer meet the metabolic demands of the body at normal physiologic venous pressures. Typically, the heart can respond to increased demands by means of one of the following: increasing the heart rate, which is controlled by neural and humoral input, increasing the contractility of the ventricles, secondary to both circulating catecholamines and autonomic input and augmenting the preload, medicated by constriction of the venous capacitance vessels and the renal preservation of intravascular volume.

As the demands on the heart outstrip the normal range of physiologic compensatory mechanisms, signs of CHF occur. These signs include tachycardia; venous congestion; high catecholamine levels; and, ultimately, insufficient cardiac output.

In a preferred embodiment the disease is decompensated congestive heart failure.

EXAMPLES

Example 1: *Assay to measure BNP PEGylated prodrug and BNP activity*

The residual activity of conjugated BNP can be determined using a cell based assay using human aortic smooth muscle cells, for example as described in WO 2006/076471. Human aortic smooth cells are treated with BNP and test materials at varying concentrations. The occurrence of cGMP in the cells were measured and used as indicator for BNP activity. On the basis of cGMP, EC50 values can be calculated and compared to that of native BNP.

Human aortic endothelial cells (HAEC) were obtained from Cambrex (Clonetics). Cells were thawed and placed in a T75 flask prior to use in an experiment. Cells were grown for two days until they reached 70-80 % confluence and were then plated into 12 well plates at 2.5 x 104cells/well. The next day the media was removed and cells were pre-incubated for 10 min at 37 °C with 0.5 mM IBMX to inhibit phosphodieste-rases. Conjugates to be screened were added to the cells for an additional 60 min at 37 °C. Incubation was stopped by lysing cells using Cell Lysis Solution (Molecular Devices). cGMP was then measured using an ELISA-based cGMP kit. (CatchPoint-cyclic GMP Fluorescent Assay Kit, catalog #R8074, Molecular Devices Corp, Sunnyvale, CA). This kit measures cGMP via a competitive immunoassay in 96-well format. Lysates were added to the coated microplate followed by the addition of an anti-cGMP antibody and a horseradish peroxidase (HRP)-cGMP conjugate. Plates were incubated for two hours at room temperature, followed by four washes. As known to the skilled person one may identify a kit with essentially the same characteristics as the kit catalog numbers mentioned above.

A substrate solution was added and the fluorescent intensity of each well was quantitated. The fluorescent signal intensity decreased with increasing levels of cGMP. Native BNP was tested in each experiment as a positive control.

Conclusion:

Based on detailed instructions of this example 1 it is routine work for the skilled per-35 son to measure this residual activity of the prodrug.

Example 2: *Assay to measure autocleavage rate of the transient linker of the BNP prodrug.*

Determination of in vitro autocleavage rate

For determination of in vitro linker cleavage rate of PEG-linker-BNP conjugates, the compounds are dissolved in buffer at pH 7.4 (e.g. 10 mM sodium phosphate, 140 mM NaCl,

3 mM EDTA) and solution is filtered through a 0.22 µm filter and incubated at 37 °C. Samples are taken at time intervals and analyzed by RP-HPLC or size exclusion chromatography at 215 nm. UV-signals correlating to liberated BNP are integrated and plotted against incubation time. Curve fitting software is applied to determine first-order cleavage rates.

Linker cleavage rates in vivo and in vitro/in vivo correlation

Linker cleavage rates in vivo are determined by comparing the pharmacokinetics of permanent PEG-BNP conjugates with the respective transient PEG-linker-BNP conjugate carrying the same PEG moiety after intravenous injection into rat.

Firstly, permanent PEG-BNP is injected intravenously into rats and blood samples are taken at time intervals, plasma prepared, and analyzed for BNP using an ELISA.
Secondly, transient PEG-linker-BNP is injected intravenously in rats, blood samples are taken at time intervals, plasma prepared, and analyzed for BNP using an ELISA. Linker autocleavage half-life is calculated from the ratio of BNP concentration of transient conjugate divided by determined BNP concentration of permanent conjugate at the respective time points and curve fitting. Data are compared to in vitro cleavage rates.

Conclusion

Based on detailed instructions of this example 2 it is routine work for the skilled person to measure the in vivo autocleavage rate of the transient linker of the prodrug and obtaining the relevant bioactive dose.

Example 3: *Assay to measure Hypotension*

Blood pressure in human subjects can be measured with a sphygmomanometer. In animals, blood pressure can be monitored by implantation of a blood pressure transducer, for example as described in WO 2007/115182.
Rats are induced to a surgical plane of anesthesia with isoflurane and maintained on a heating pad. The abdomen is shaved and scrubbed with 70% alcohol and betadine solution. Using aseptic technique, a midline abdominal incision is made in order to expose the descending aorta and vena cava. The contents of the abdomen are retracted gently using wet sterile gauze and retractors. Based on the manufacturer's instructions (described in Data Sciences International's Multiplus TL Series Device Surgical Manual 2000: pp. 3.13.10), the abdominal aorta is carefully dissected from the surrounding fat and connective tissue and the catheter of the blood pressure transducer is inserted. The catheter of the transducer is secured into place using surgical glue and the body of the transducer stabilized by suturing

to the abdominal wall (4-0 silk suture). Care is taken to ensure that hemostasis is maintained during the procedure and that blood flow is not compromised (e.g. aorta will not be occluded for more than 3 minutes at a time). Transducer placement is verified using the telemetry radio signal. After transducer placement, the gauze sponges are removed and the abdominal cavity is flushed with sterile saline. The abdominal incision is then sutured closed with non-absorbable sutures (4-0 silk suture) in a simple interrupted pattern. The skin is closed using absorbable suture (4-0 vicryl). Finally the animal is removed from the isoflurane and placed in a warm environment while being monitored until it is fully awake.

Conclusion

Based on detailed instructions of this example 3 it is routine work for the skilled person to measure the hypotension effect.

Example 4: *Assay to measure aldosterone suppression*

Plasma concentration of aldosterone can be measured using commercial kits. Blood samples are drawn from animal or human subjects after a period of rest, transferred to suitable chilled buffer and centrifuged, for example as described by Yamato et al., Circ J 2003 May; 67(5): 384-90.

Blood samples are drawn by venipuncture after at least 30 min of rest with the patient in the supine position. Samples were placed immediately in a prechilled tube containing sodium EDTA and centrifuged at 3,000 rpm for 10 min. Plasma concentration of aldosterone (SPAC-S Aldosterone Kit; Dainabot Inc, Tokyo, Japan) were measured by radioimmunoassay using the commercial kits.

Conclusion

Based on detailed instructions of this example 4 it is routine work for the skilled person to measure the aldosterone suppression.

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3. Mangano CM, Diamondstone LS., Ramsay JG, Renal dysfunction after myocardial revascularization: risk factors, adverse outcomes, and hospital resource utilization. Ann Intern Med 1998; 128:194-203.

4. Salvador DRK, Rey NR, Ramos GC, Punzalan FER. ["Continuous infusion versus bolus injection of loop diuretics in congestive heart failure"]. Cochrane Database of Systematic Reviews 2004, Issue 1. Art. No.: CD003178. DOI: 10.1002/14651858.CD003178.pub3

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6. WO2006076471

CLAIMS

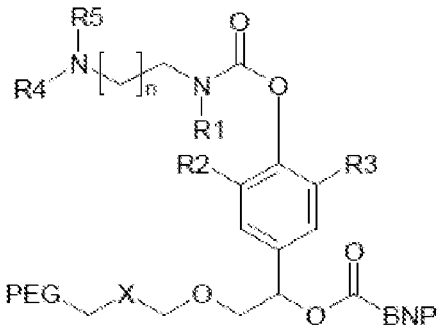
1. A pharmaceutical composition comprising pharmaceutically acceptable excipients and also comprising a PEGylated prodrug capable of releasing an in vivo clinically effective amount of a recombinant human BNP (BNP without PEG), wherein the prodrug is characterized by that:

(1): when PEG is linked to BNP in the conjugate the conjugate has an BNP activity which is less than 5% of the native BNP without PEG, measured according to the assay to measure BNP PEGylated prodrug and BNP activity of example 1; and

(2): PEG is linked to BNP via a self hydrolysable (autocleavage) transient linker, wherein the linker autohydrolysis rate is such that the in vivo linker half-life is from 50 hours to 500 hours, measured according to the assay to measure autocleavage rate of the transient linker of the prodrug of example 2.

2. The pharmaceutical composition of claim 1, wherein the pharmaceutical composition is administered subcutaneously.

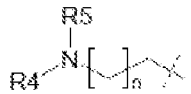
3. The pharmaceutical comprising the self hydrolysable transient linker PEGylated BNP prodrug of claim 1, wherein the self hydrolysable transient linker structure is:



R2, R3, R4, and R5 are selected independently from hydrogen, methyl, ethyl, propyl, isopropyl, butyl, isobutyl

and

R1 is selected from methyl, ethyl, propyl, isopropyl, butyl, isobutyl or



Formula I

wherein, the dashed line indicates the attachment site to the respective nitrogen atom

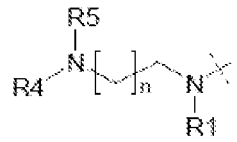
n = 1 or 2 and

X is selected from C1 to C8 alkyl or C1 to C12 heteroalkyl including heterocycloalkyl

and

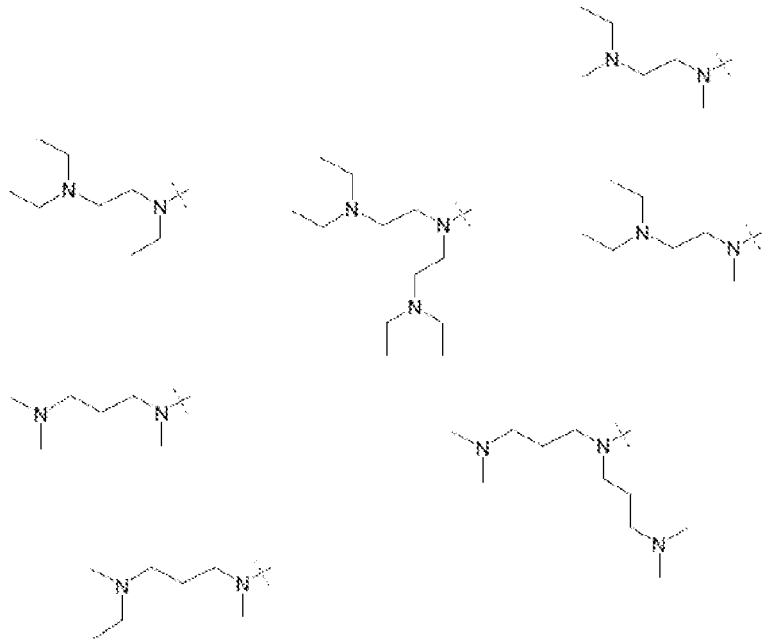
BNP is conjugated to the PEG-linker conjugate via a carbamate bond by one of its amino groups.

4. The pharmaceutical composition of claim 3, wherein the formula I of claim 3 which is a part of the self hydrolysable transient linker structure is

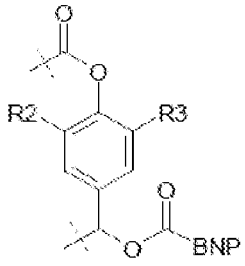


Formula I

and formula I is selected from the group consisting of:

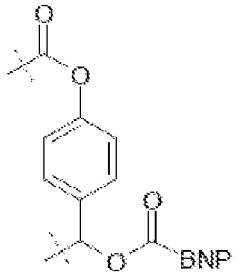


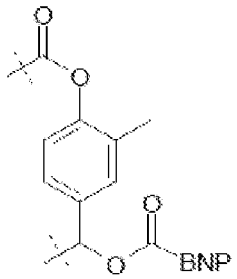
5. The pharmaceutical composition comprising the self hydrolysable transient linker PEGylated BNP prodrug of claim 3, and formula II which is a part of self hydrolysable transient linker structure is

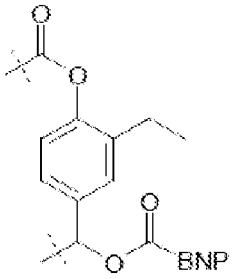


Formula II

and formula II is selected from the group consisting of:

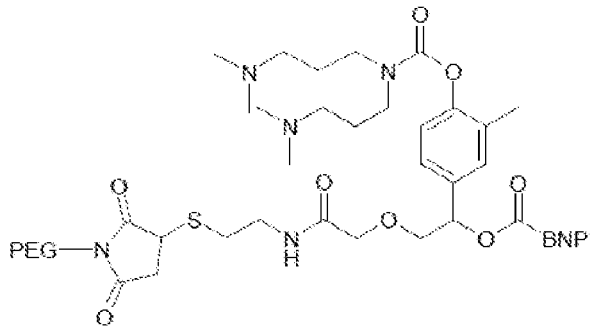




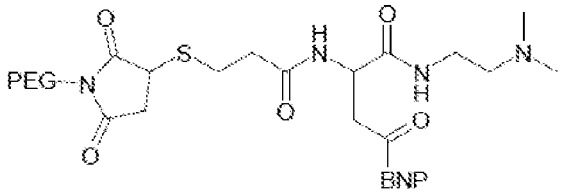


6. The pharmaceutical composition of claim 1, wherein the self hydrolysable transient

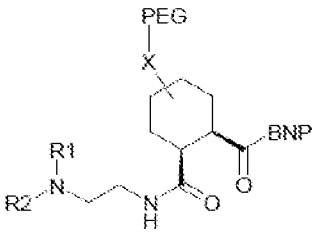
linker PEGylated BNP prodrug is



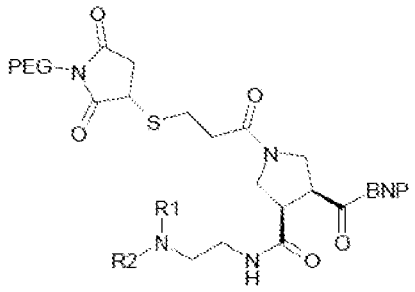
7. The pharmaceutical composition of claim 1, wherein the self hydrolysable transient linker PEGylated BNP prodrug linker structure is a structure selected from the group consisting of:



and



and



R1 and R2 are selected independently from hydrogen, methyl, ethyl, propyl, isopropyl, butyl, isobutyl, cycloalkyl and

R1 and R2 may be connected to form a C4 to C8 cycloalkyl and X is selected from C1 to C8 alkyl or C1 to C12 heteroalkyl

and

BNP is linked to the PEG-linker moiety (PEG-linker-BNP structure) via an amide bond by one of its amino groups.

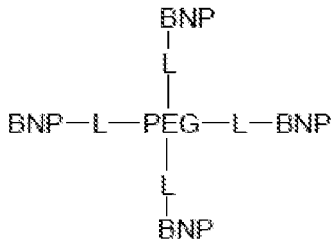
8. The pharmaceutical composition of any of the preceding claims, wherein the PEG structures include linear and branched PEG moieties with molecular weights between 5 kDa and 200 kDa.

9. The pharmaceutical composition of any of the preceding claims, wherein at least 1 BNP molecule is attached to one PEG molecule.

10. The pharmaceutical composition of claim 8 and 9, wherein one, two or four BNP molecules are attached to one PEG molecule and is a structure selected from the group consisting of:

PEG—L—BNP

BNP—L—PEG—L—BNP



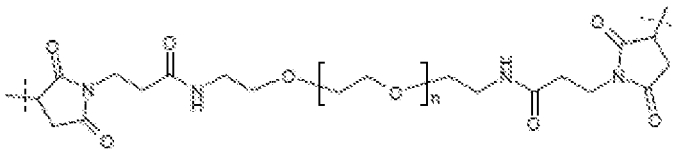
where

L is a transient linker and PEG is a polyethylene glycol derivative.

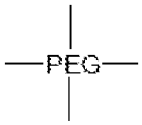
11. The pharmaceutical composition of claim 10, wherein two or four BNP molecules

are attached to one PEG molecule and the PEG derivative is a PEG derivate selected from the group consisting of:

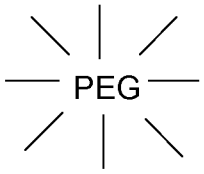
and



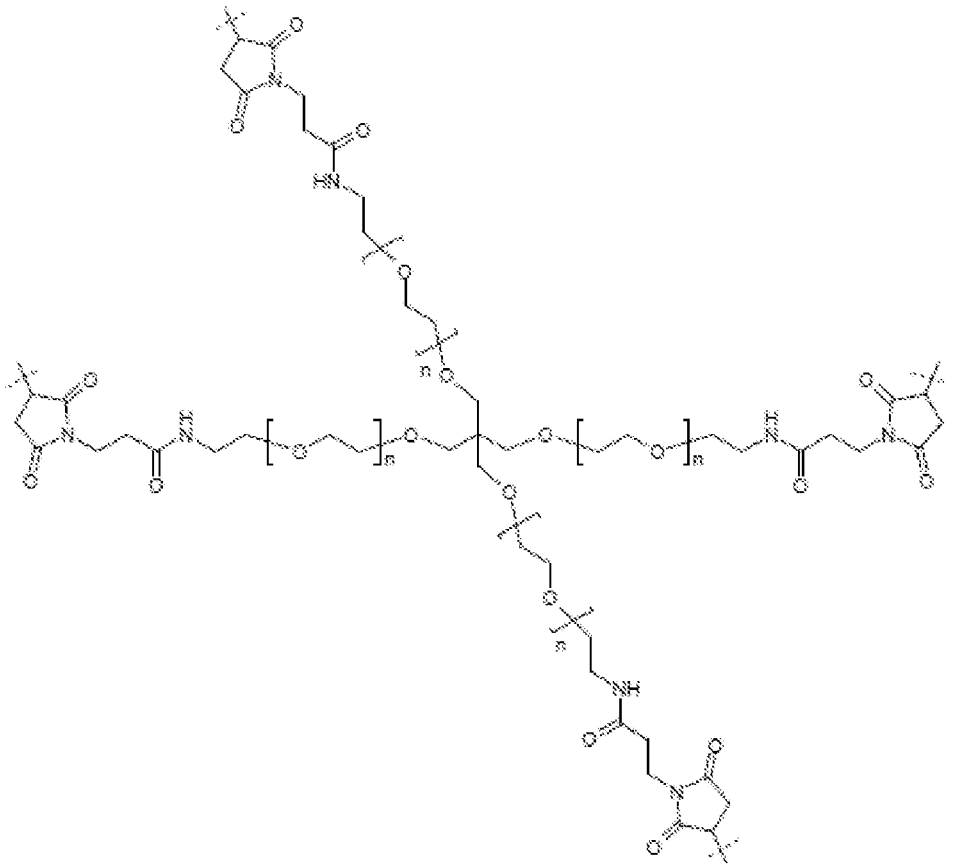
and



and



and



12. The pharmaceutical composition of any of claims 1 to 11, wherein the PEGylated BNP prodrug is a prodrug that in the assay to determine hypotension of example 3 induces symptomatic hypotension in less than 4% of the treated rats.

13. A clinically effective amount of the pharmaceutical composition comprising BNP PEGylated prodrug of any of the preceding claims for use in a method for the treatment of a BNP related disease in a human, wherein the treatment comprises administering the pharmaceutical composition as one administration during a treatment period and wherein the treatment is characterized by that after the one administration of the pharmaceutical composition is the ratio during the treatment period, between the highest concentration of active BNP drug (BNP without PEG) in plasma during the treatment period and the concentration of active BNP drug in the plasma at the end of the treatment period, less than 10, wherein the treatment period is a period of at least three days.

14. The clinically effective amount of the pharmaceutical composition of claim 13, wherein the highest concentration during treatment is less than 3 ng/ml and the lowest concentration is higher than 0.3 ng/ml during treatment in the plasma.

15. The clinically effective amount of the pharmaceutical composition of claim 14, wherein the BNP related disease is congestive heart failure and the treatment of the human is done for at least ten continuous treatment periods – with one administration of the BNP PEGylated prodrug in each period – and wherein the active BNP concentration in the plasma is constantly within the concentration range of claim 14 during the total accumulated treatment period.

Fig 1. Structure and amino acid sequence for BNP.

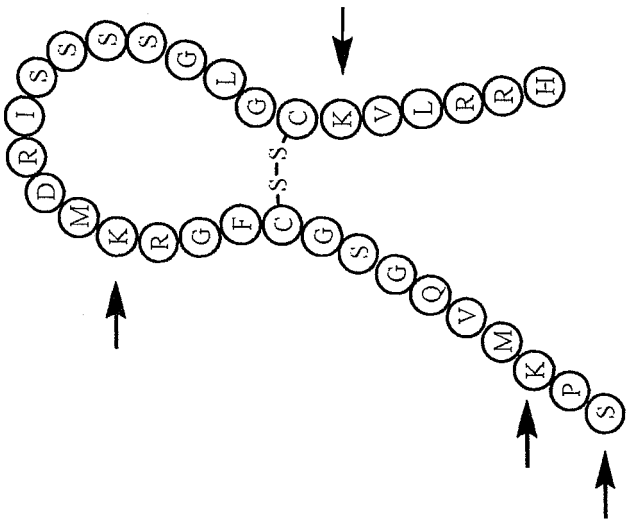


Fig 2. Plasma profile of a continuous infusion of 15 ng/kg/min for 4 hours

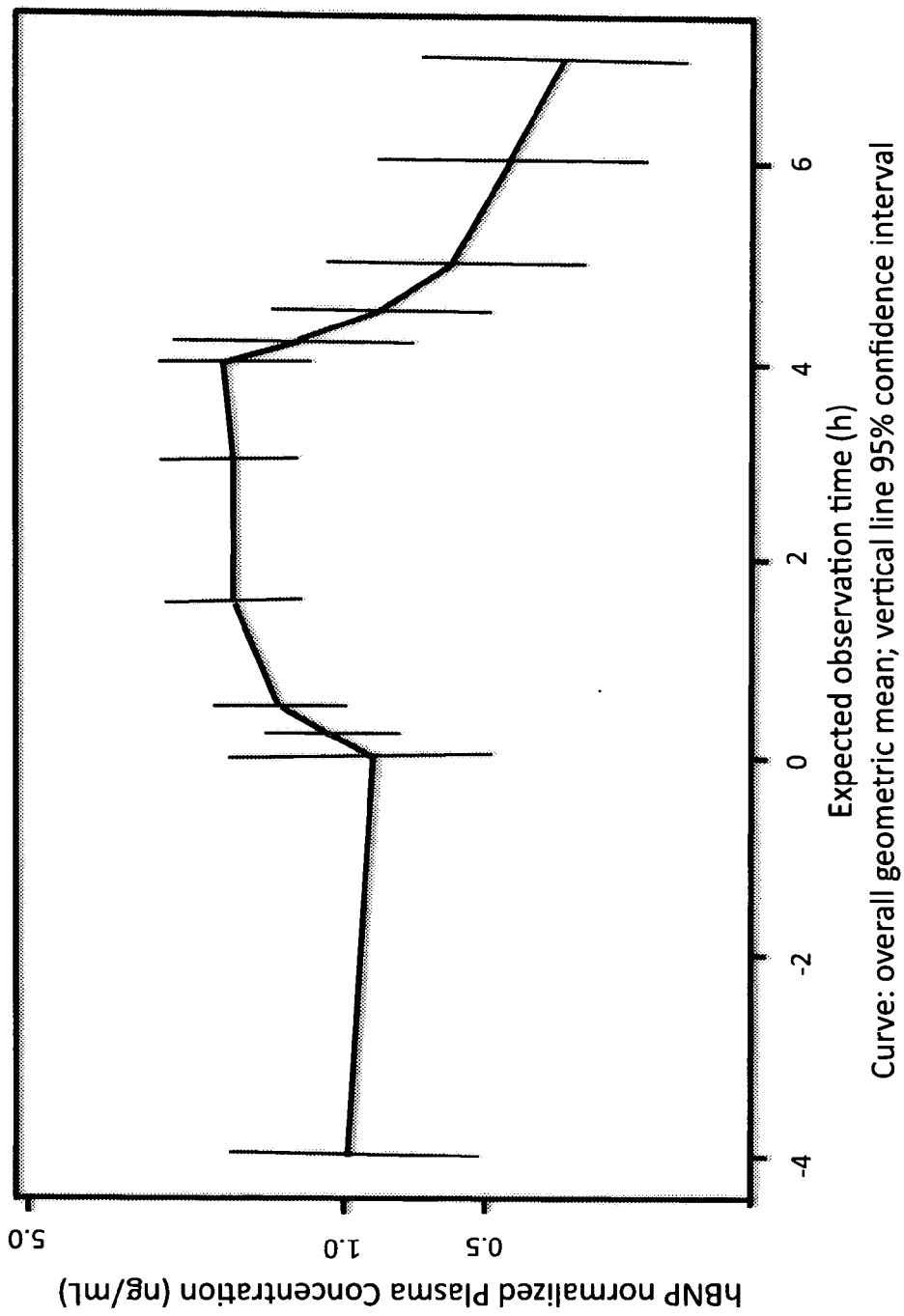
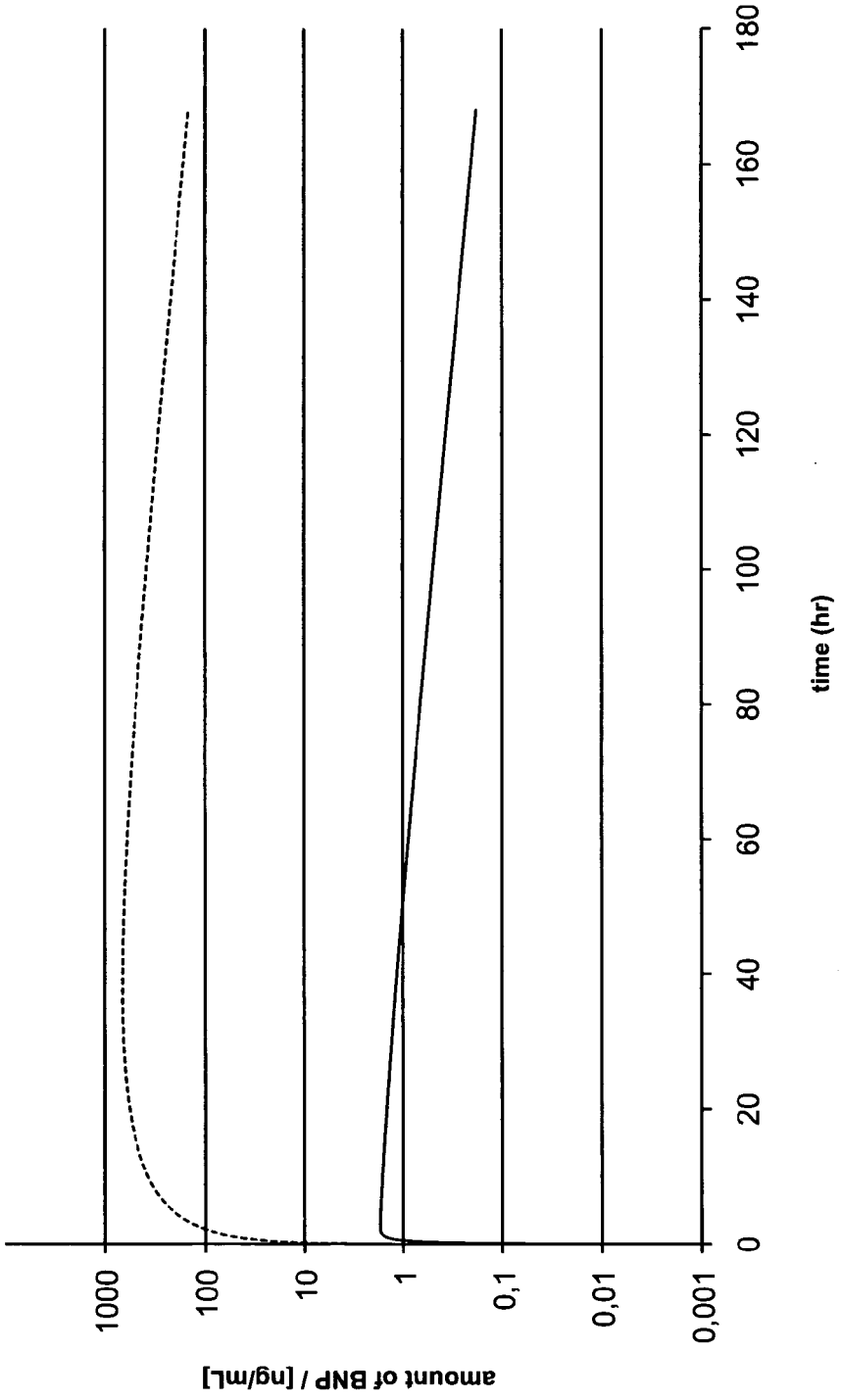


Fig. 3. Simulated plasma profile of a single subcutaneous injection of a sustained release formulation using transiently conjugated nesiritide (BNP)



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/057993

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/48 A61P43/00

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 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 practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2006/076471 A (NOBEX CORP [US]; JAMES KENNETH [US]) 20 July 2006 (2006-07-20) cited in the application pages 33,34, paragraph 153-156 page 79, paragraph 280 page 81, paragraph 286; figures 6a-6d; table 1	1,2, 8-10,12
Y	the whole document	13-15
X	WO 2004/047871 A (NOBEX CORP [US]) 10 June 2004 (2004-06-10) pages 27,28, paragraph 145-148 pages 66-70, paragraphs 224,230-232; table 1	1,2, 8-10,12
Y	the whole document	13-15

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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 document 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
22 July 2009	09/11/2009

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	Orlando, Michele

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2004/203081 A1 (JAMES KENNETH D [US] ET AL) 14 October 2004 (2004-10-14) paragraphs [0162] - [0165], [0326], [0335]; table 1	1,2, 8-10,12
Y	the whole document	13-15
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Y	the whole document page 19, line 1; claim 6 compounds 21(D-F),30A,60	3-6
X	EP 1 625 855 A (COMPLEX BIOSYSTEMS GMBH [DE]) 15 February 2006 (2006-02-15)	1
Y	paragraphs [0038], [0042], [0053]; compounds 20(A,B,D-F), 21(A,B,D-F)	3-6
X	EP 1 579 873 A (COMPLEX BIOSYSTEMS GMBH [DE]) 28 September 2005 (2005-09-28)	1
Y	paragraphs [0036], [0040], [0051]; claims 1,4,24; compounds 9D, 9I	3-6
Y	NESHER MAOZ ET AL: "Reversible pegylation prolongs the hypotensive effect of atrial natriuretic peptide" BIOCONJUGATE CHEMISTRY, vol. 19, no. 1, January 2008 (2008-01), pages 342-348, XP002493772 ISSN: 1043-1802 the whole document page 343, column 1, paragraph 2 page 347, column 1, paragraph 2	1
Y	PELEG-SHULMAN T ET AL: "REVERSIBLE PEGYLATION: A NOVEL TECHNOLOGY TO RELEASE NATIVE INTERFERON ALPHA2 OVER A PROLONGED TIME PERIOD" JOURNAL OF MEDICINAL CHEMISTRY, US AMERICAN CHEMICAL SOCIETY. WASHINGTON, vol. 47, no. 20, 23 September 2004 (2004-09-23), pages 4897-4904, XP008053917 ISSN: 0022-2623 the whole document page 4902, column 2, paragraph 3 - page 4903, column 1, paragraph 1	1

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SHECHTER Y ET AL: "New technologies to prolong life-time of peptide and protein drugs in vivo" INTERNATIONAL JOURNAL OF PEPTIDE RESEARCH AND THERAPEUTICS, vol. 13, no. 1-2, June 2007 (2007-06), pages 105-117, XP002493773 ISSN: 1573-3149 the whole document page 115, column 2, paragraph 3 - page 116, column 1, paragraph 1	1
Y	SHECHTER ET AL: "Reversible PEGylation of peptide YY3-36 prolongs its inhibition of food intake in mice" FEBS LETTERS, ELSEVIER, AMSTERDAM, NL, vol. 579, no. 11, 25 April 2005 (2005-04-25), pages 2439-2444, XP005390209 ISSN: 0014-5793 page 2443	1
Y	CATALIOTTI ET AL: "Oral Brain Natriuretic Peptide: A Novel Strategy for Chronic Protein Therapy for Cardiovascular Disease" TRENDS IN CARDIOVASCULAR MEDICINE, ELSEVIER SCIENCE, NEW YORK, NY, US, vol. 17, no. 1, 18 January 2007 (2007-01-18), pages 10-14, XP005824130 ISSN: 1050-1738 pages 10,11; figure 2	13-15
Y	CATALIOTTI ALESSANDRO ET AL: "Oral human brain natriuretic peptide activates cyclic guanosine 3',5'-monophosphate and decreases mean arterial pressure." CIRCULATION 9 AUG 2005, vol. 112, no. 6, 9 August 2005 (2005-08-09), pages 836-840, XP002493774 ISSN: 1524-4539 page 836, column 1 - page 837, column 1	13-15
A	VERONESE F M: "Peptide and protein PEGylation - a review of problems and solutions" BIOMATERIALS, ELSEVIER SCIENCE PUBLISHERS BV., BARKING, GB, vol. 22, no. 5, 1 March 2001 (2001-03-01), pages 405-417, XP004227886 ISSN: 0142-9612 page 413, paragraph 14	

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ZALIPSKY S ET AL: "Thiolytically cleavable dithiobenzyl urethane-linked polymer-protein conjugates as macromolecular prodrugs: Reversible PEGylation of proteins" BIOCONJUGATE CHEMISTRY 200711 US, vol. 18, no. 6, November 2007 (2007-11), pages 1869-1878, XP002493775 ISSN: 1043-1802	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2009/057993

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers allsearchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search reportcovers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

see annex

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1,2,8-15 (in part); 3-6 (complete)

A pharmaceutical composition according to present claim 1, wherein the self hydrolysable transient linker comprises the chemical structure of present claim 3. Excluding the subject matter of invention 2.

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2. claims: 1,2,8-15 (in part); 7 (complete)

A pharmaceutical composition according to present claim 1, wherein the self hydrolysable transient linker comprises one of the chemical structures of present claim 7. Excluding the subject matter of invention 1.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2009/057993

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2006076471 A	20-07-2006	US 2008207505 A1	28-08-2008
WO 2004047871 A	10-06-2004	AU 2003297583 A1	18-06-2004
		BR 0316560 A	04-10-2005
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		EP 1732607 A2	20-12-2006
		EP 2087910 A2	12-08-2009
		JP 2007530485 T	01-11-2007
		US 2008241102 A1	02-10-2008
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EP 1579873 A	28-09-2005	NONE	