derived from inferior vena cava diameter and respiratory change. (NR<35 mmHg);

b. Tricuspid annular plane systolic excursion (TAPSE): measured from M-mode echocardiography from apical four chamber view. TAPSE correlates with right ventricle ejection fraction and its reduction associated with worse prognosis in HF. (NR>16 mm) (*see* Ghio S et al., J Am Coll Cardiol 2001;37(1):183-8); and

c. Right ventricle Sa: measured with tissue Doppler method at right ventricle free wall from apical four chamber view. Sa is a derived parameter of systolic function and correlated with right ventricle ejection fraction. (NR >10 cm/s) (*see* Voelkel NF et al., Circulation 2006;114(17):1883-91; Haddad F et al., Circulation 2008;117(13):1717-31).

6. Other parameters:

a. Mitral regurgitation (MR): evaluated with a visual qualitative assessment ang graded in four categories: none, mild, moderate, and severe (*see* Lancellotti P et al., Eur J Echocardiogr 2010;11(4):307-32); and

b. Inferior vena cava diameter (IVC): measured with M-mode echocardiography from subcostal view at 1-2 cm from the junction with right atrium. This parameter has been used to estimate systolic pulmonary artery pressure. It correlated with right atrium pressure indicating the grade of congestion. Increased IVC diameter is associated with prognosis in patients with HF (NR: ≤1.5 cm) (*see* Pellicori P et al., JACC Cardiovasc Imaging 2013;6(1):16-28; Voelkel NF et al., Circulation 2006;114(17):1883-91).

Table 1A. Cardiac changes at 6 hours of infusion.

Parameter	Ista 0.5 µg/kg/min	Ista 1.0 µg/kg/min	Placebo	p Ista 0.5	p Ista 1.0
LAA (cm^2)	-0.33 ± 1.885	-0.84 ± 2.421	-0.52 ± 1.840	0.663	0.521
LAV (ml)	-1.16 ± 11.265	-4.92 ± 14.390	-3.24 ± 10.047	0.399	0.558
Diastolic function					
E wave (cm/s)	-3.33 ± 14.764	-9.13 ± 17.464	-1.10 ± 10.990	0.451	**0.018**
A wave (cm/s)	1.76 ± 13.572	5.56 ± 17.280	1.55 ± 10.368	0.955	0.348
EDT (ms)	6.83 ± 47.921	20.37 ± 50.849	-0.18 ± 38.939	0.491	0.052

E/A ratio	-0.286 ± 0.866	-0.317 ± 0.898	-0.124 ± 0.866	0.566	0.458
e' (cm/s)	0.61 ± 1.010	0.01 ± 1.154	0.25 ± 1.167	0.146	0.376
E/e' ratio	-3.183 ± 5.628	-2.028 ± 3.652	-0.740 ± 3.994	**0.032**	0.150
Systolic function					
Sa (cm/s) Left V	0.613 ± 1.035	0.908 ± 0.936	0.197 ± 0.919	0.065	**0.001**
S (cm/s) Right V	1.25 ± 2.185	2.00 ± 1.907	0.43 ± 1.441	0.125	**0.003**
Cardiac function					
CO (l/min)	0.385 ± 0.843	0.228 ± 0.760	0.083 ± 0.705	0.094	0.390
CI (l/min/m2)	0.209 ± 0.445	0.140 ± 0.434	0.042 ± 0.400	0.090	0.309
SV (ml/beat)	7.724 ± 11.752	7.269 ± 8.134	2.405 ± 7.244	**0.020**	**0.007**
SVI(ml/beat/m2)	4.198 ± 6.218	4.187 ± 4.641	1.317 ± 4.077	**0.019**	**0.005**

Table 1B. Cardiac changes at 24 hours of infusion.

Parameter	Ista 0.5 µg/kg/min	Ista 1.0 µg/kg/min	Placebo	p Ista 0.5	p Ista 1.0
LAA (cm^2)	-1.70 ± 2.463	-2.56 ± 2.972	-0.31 ± 1.886	**0.008**	**<0.001**
LAV (ml)	-7.94 ± 13.269	-13.81 ± 17.198	-2.95 ± 10.624	0.079	**0.002**
Diastolic function					
E wave (cm/s)	-8.14 ± 17.640	-14.24 ± 24.416	-4.16 ± 12.502	0.267	**0.031**
A wave (cm/s)	5.13 ± 10.990	11.10 ± 16.498	-1.14 ± 7.580	**0.045**	**0.003**
EDT (ms)	12.88 ± 54.954	9.79 ± 52.212	4.58 ± 35.759	0.456	0.628
E/A ratio	-0.647 ± 0.812	-0.722 ± 1.068	-0.164 ± 0.833	**0.005**	**0.004**
e' (cm/s)	0.94 ± 1.089	0.91 ± 1.792	0.26 ± 1.23	**0.013**	0.635
E/e' ratio	-4.548 ± 4.754	-3.191 ± 2.623	-1.285 ± 3.351	**0.001**	**0.011**
Systolic function					
Sa (cm/s) Left V	0.679 ± 0.907	0.803 ± 1.023	0.171 ± 1.029	**0.024**	**0.012**

S (cm/s) Right V	1.10 ± 1.780	1.55 ± 2.012	0.15 ± 1.424	0.052	**0.015**
Cardiac function					
CO (l/min)	0.486 ± 0.696	0.239 ± 0.786	0.195 ± 0.651	0.073	**0.797**
CI (l/min/m2)	0.264 ± 0.369	0.140 ± 0.443	0.116 ± 0.373	0.097	**0.808**
SV (ml/beat)	9.725 ± 12.192	9.339 ± 8.875	4.039 ± 6.259	0.015	**0.005**
SVI(ml/beat/m2)	5.333 ± 6.664	5.318 ± 4.955	2.336 ± 3.516	0.019	**0.005**

Table 1C. Cardiac changes a 48 hours of infusion.

Parameter	Ista 0.5 µg/kg/min	Ista 1.0 µg/kg/min	Placebo	p Ista 0.5	p Ista 1.0
Diastolic function					
E/A ratio	-0.278 ± 1.043	-0.356 ± 0.967	-0.172 ± 0.741	0.729	0.509
E/e' ratio	-2.570 ± 3.654	-2.218 ± 3.193	-1.800 ± 4.013	0.388	0.629
Systolic function					
LVEF (%)	1.28 ± 3.693	2.06 ± 4.975	1.24 ± 3.539	0.968	0.427
Sa (cm/s)	0.224 ± 0.811	0.257 ± 0.771	0.132 ± 0.898	0.640	0.525
Cardiac function					
CO (l/min)	0.350 ± 0.788	0.368 ± 0.735	-0.017 ± 0.691	**0.041**	**0.026**
CI (l/min/m^2)	0.191 ± 0.426	0.205 ± 0.407	-0.007 ± 0.384	**0.044**	**0.027**
SV (ml/beat)	4.815 ± 9.832	5.203 ± 8.078	1.787 ± 7.108	0.139	0.062
SVI (ml/beat/m^2)	2.762 ± 5.603	2.913 ± 4.572	1.039 ± 4.026	0.139	0.071

[74] The quantitative determination of PST 2744 and its metabolite PST 2915 in human plasma was determined by the HPLC-MS/MS method that included a mobile phase of 70:30

[157] The compound PST 3093 was iv administered to STZ injected rats at the dose of 0.22 mg/kg and echocardiographic parameters were measured after 15 and 30 min from the beginning of iv infusion and 10 min after interruption of infusion.

Statistical analysis

[158] Data are reported as mean ± SD, as indicated. Statistical analysis was performed by Student's t-test (pair t test for STZ rats). $P<0.05$ was regarded as statistically significant.

Biological results

In vitro screening

Inhibition of dog renal Na,K-ATPase activity

[159] **Table 2** shows the inhibitory effect of the tested compounds on the enzymatic activity of the purified dog renal Na,K-ATPase. The corresponding IC_{50} are expressed in µM concentration. Istaroxime inhibited the Na,K-ATPase activity with an IC_{50} of 0.14 µM, similar to that of Digoxin, while PST 2915 inhibited the Na,K-ATPase activity with an IC_{50} of 2.1 µM. Further, PST 3093 did not significantly inhibit Na,K-ATPase activity at all (IC_{50} >100 µM).

Table 2 Inhibition of dog renal Na,K-ATPase.

Compound	~~IC_{50}, µM~~
~~DIGOXIN~~	~~0.18~~
~~ISTAROXIME~~	~~0.14~~
~~3093~~	~~>100~~
~~2915~~	~~2.1~~
~~2922~~	~~>100~~

SERCA2a ATPase activity in heart-derived SR microsomes from normal dog

[160] The compounds disclosed herein were tested on SERCA2a ATPase activity prepared from normal and failing dogs in a range of concentrations from 0.1 and 500 nM. The effect has been expressed as % increase of the Vmax activity of a control sample run in the absence of compound. Data are mean ± SD, where n indicates the number of experiments.

[161] In the normal dog SR vesicles, istaroxime, PST 3093 and PST 2915 significantly stimulated SERCA2a activity at concentrations of 0.1 nM and 10 nM (*see* **Table 3**). SERCA2a activation by istaroxime, PST 3093 and PST 2915 was also tested in failing dog preparations. This effect was particularly evident in the failing preparations (data not shown), where SERCA2a activity is known to be depressed compared to a normal heart (Bers DM, Physiology 2006;21:380–387), and therefore implies that the compounds istaroxime and PST 3093 may correct SERCA2a alteration in the failing heart.

[162] In contrast, previous studies showed that Digoxin failed to stimulate SERCA2a activity (Rocchetti M et al., J Pharmacol Exp Ther 2005;313:207-215; Ferrandi M et al., Br J Pharmacol 2013;169:1849-61).

Table 3 SERCA2a ATPase activity in heart-derived SR microsomes from normal dog.

Data are expressed as % increase vs control and are mean ± SD

Compound	Concentration nM (ng/ml)	Vmax (µmol/min/mg prot) mean ± SD	% increase vs control ~~*p<0.05;**p<0.01~~
Istaroxime	~~0~~	1.252 ± 0.083 (n=5)	~~0~~
	0.1 nM (0.039 ng/ml)	1.423 ± 0.123 (n=5)	~~14*~~
	10 nM (3.9ng/ml)	1.505 ± 0.111 (n=5)	~~20**~~
~~3093~~	~~0~~	1.312 ± 0.050 (n=5)	~~0~~
	0.1 nM (0.037 ng/ml)	1.641 ± 0.194 (n=4)	~~25**~~
	10 nM (3.7 ng/ml)	1.556 ± 0.106 (n=4)	~~19**~~

Compound	Concentration nM (ng/ml)	Vmax (µmol/min/mg prot) mean ± SD	% increase vs control *p<0.05;**p<0.01
2915	0	1.312 ± 0.050 (n=5)	0
	0.1 nM (0.041ng/ml)	1.464 ± 0.184 (n=5)	11
	10 nM (4.1 ng/ml)	1.526 ± 0.038 (n=5)	16*
2922	0	1.312 ± 0.050 (n=5)	0
	0.1 nM (0.037 ng/ml)	1.466 ± 0.112 (n=5)	12*
	10 nM (3.7 ng/ml)	1.541 ± 0.170 (n=5)	17*

In vivo studies

Acute toxicity in mouse

[163] The acute toxicity of the tested compound PST 3093 was determined in the mouse (Albino Swiss CD-1, body weight 30 g). Compound PST 3093 was orally administered or intravenously injected at increasing doses to identify the dose causing 50% mortality. Mortality occurred within 30 min after the administration and survival after 24h.

[164] The results for PST 3093 acute toxicity are reported in **Table 4** and indicated that the compound had an LD_{50} >250 and 200 mg/kg after iv or oral administration, respectively. For comparison, the acute toxicity for the reference compound istaroxime has been also included in **Table 4**.

Table 4 Acute toxicity (LD_{50}) of Istaroxime and PS3093 in mouse.

Compound	~~LD_{50}~~ mg/kg
Istaroxime i.v.	~~29-32~~
Istaroxime os	~~200~~
3093 i.v.	~~>250~~
3093 os	~~>200~~

i.v., intravenous

os, oral

Haemodynamics in streptozotocin (STZ) diabetic rats (echocardiography 2M-Doppler-Tissue Doppler)

[165] **Table 5** shows the echocardiographic parameters in STZ diabetic rats before and after 15 and 30 min from iv infusion of PST 3093 at 0.22 mg/kg, and 10 min after interruption of infusion. Data is presented as mean ± SD, and values with an asterisk are statistically significant with at least $p< 0.05$.

[166] The data indicated that in an animal model characterized by a diastolic dysfunction, such as the STZ diabetic rats, PST 3093 administration ameliorated diastolic function. In particular, E wave (which represents the early filling velocity of transmitral inflow during the rapid filling phase and constitutes the energy dependent phase of LV relaxation, mainly mediated by SERCA2a activity) was significantly increased in STZ rats at 15 and 30 min after PST 3093 infusion (**Table 5**). This effect was consistent with a stimulation of SERCA2a activity by the compound, as shown in the *in vitro* assay (**Table 3**), suggesting the ability of PST 3093 to restore SERCA2a function activity, which is depressed in STZ rats as shown by Choi *et al.* (AJP 2002; H1398-H1408).

[167] However, it should be considered that the peak E velocity is influenced by the preload and is directly correlated with heart rate (HR) (Mihm MJ et al., Life Sci. 2001; 22;69(5):527-42; do Carmo JM et al., AJP 2008; 295:H1974-1981). Conversely, the deceleration time of E wave (DT), and the related changes in mitral deceleration index (DT/E) and deceleration slope of E wave (E/DT), are not affected by HR changes and are

stimulatory activity of the compound that, by correcting the depressed cardiac relaxation, increased the amount of blood available for contraction and resulted in an increase of volume of blood pumped from the ventricle (SV).

[172] To evaluate the relevance of the above results to the human condition, the obvious pathophysiological differences between the patients with AHF and the STZ rats should be considered. In the latter, marked changes in the body fluids, sympathetic nervous system and heart rate, may, *per se*, affect echocardiographic parameters, independently from the changes in cellular Ca^{2+} handling and decrease of SERCA2a activity (Mihm MJ et al., Life Sci. 2001; 22;69(5):527-42; do Carmo JM et al., AJP 2008; 295:H1974-1981). Therefore, the similarities between human and rats in DT/E, E/DT and e' changes may be considered as having the same underlying mechanism — a stimulation of the SERCA2a activity by PST 3093.

Table 5 Haemodynamic parameters after 3093 iv infusion in STZ diabetic rats

* P<0.05 compared to basal values

Function	Echo Parameter	STZ before (n=10)	STZ 3093 0.22 mg/kg iv after 15 min (n=10)	STZ 3093 0.22 mg/kg iv after 30 min (n=10)	STZ 3093 after 10 min from STOP infusion (n=8)
	E	0.91±0.197	1.048±0.218*	1.076±0.211*	0.89±0.19
Diastolic function	DT	48.5±12.01	40.2±9.54	36.6±6.09*	41.88±7.86*
	DT/E	56.72±20.46	40.39±15.04*	35.47±10.19*	49.08±13.09*
	E/DT	20.61±9.91	27.51±8.42*	30.36±8.73*	22.36±8.89*
	E/e'	39.55±5.43	38.61±4.71	38.7±4.26	39.45±4.18
	e'	22.94±3.35	27.01±3.4*	27.77±4.27*	22.48±3.35

overall	~~CO~~	~~187.7±43.35~~	~~231.7±64*~~	~~230.6±46.7*~~	~~200.88±51.7~~
	~~HR~~	~~270±54~~	~~271±33~~	~~269±29~~	~~249±28~~
	~~SV~~	~~0.7±0.15~~	~~0.85±0.18*~~	~~0.86±0.14*~~	~~0.81±0.17*~~

E, E wave, early filling velocity of mitral inflow

DT (ms), deceleration time of E wave

DT/E (s^2/m), mitral deceleration index

E/DT (m/s^2), decelaration slope

E/e', index of LV filling pressure

e' (cm/s) TDI, early relaxation velocity

CO (ml/min), cardiac output

HR (beat/min), heart rate

SV (ml/beat), stroke volume

Claims

1. The use of istaroxime or a pharmaceutically acceptable salt, solvate, or hydrate thereof for the manufacture of a medicament formulated for administration by intravenous infusion for the treatment of heart failure in a human subject, wherein the administration comprises an infusion duration longer than 6 hours, whereby diastolic relaxation ~~heart function~~ is improved in the human subject as compared to administration of istaroxime by intravenous infusion for an infusion duration from 3 to 6 hours, and wherein the istaroxime is to be administered at a dose between 0.2 µg/kg/min and 1.5 µg/kg/min.

2. The use according to claim 1, wherein the diastolic relaxation ~~heart function~~ improvement is measured by one or more of:

(a) echocardiographic parameter E/A ratio, A wave, e', E wave deceleration time, E/e' ratio;

(b) diastolic relaxation, increased diastolic blood pressure, decreased heart rate, a decrease in dyspnea, increased stroke volume, increased cardiac index, increased cardiac output, increased pulmonary capillary wedge pressure, or increased stroke volume index; or

(c) sphygmomanometer, an electrocardiogram, cardiac catheterization, a radionuclide ventriculography scan; or

(d) any combination of (a)-(c).

3. The use according to claim 1 or 2, wherein the infusion duration is at least about 24 hours.

4. The use according to claim 1 or 2, wherein the infusion duration is selected from:

at least about 12 hours;

at least about 36 hours; and

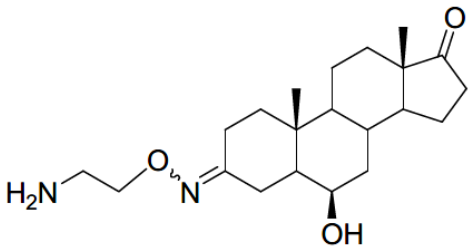
at least about 48 hours.

5. The use according to any one of claims ~~1 to 4~~2-4, wherein the improvement in diastolic relaxation is measured by echocardiography comprising one or more of decreased E wave,

increased E wave deceleration time, increased A wave, decreased E/A ratio, increased e', or decreased E/e' ratio ~~istaroxime is to be administered at a dose between 0.2 µg/kg/min and 1.5 µg/kg/min~~.

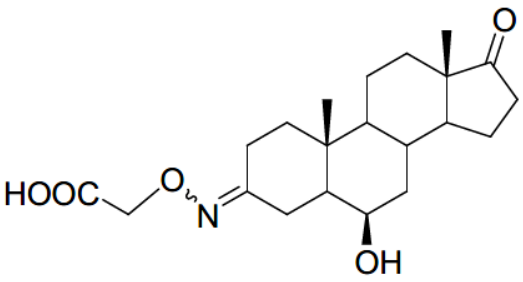
6. The use according to any one of claims 1 to 5, wherein istaroxime is to be administered at a dose between 0.25 µg/kg/min and 1.0 µg/kg/min.

7. The use according to any one of claims 1 to 6, wherein istaroxime is to be administered by infusion for a duration sufficient to produce a plasma level of an istaroxime metabolite, wherein the plasma level is greater than about 5 ng/ml for an accumulation period of at least about 6 hours, and wherein the istaroxime metabolite comprises a formula (II) or a formula (III) compound



PST 2915

Formula (II)



PST 3093

Formula (III)

or a pharmaceutically acceptable salt, an ester a solvate, a hydrate, a polymorph thereof.

8. The use according to any one of claims 1 to 7, wherein the human subject suffers from heart failure with preserved ejection fraction (HFpEF) or mid-range reduction ejection fraction (HFmEF).

9. The use according to any one of claims 1 to 8 wherein the human subject is undergoing a therapeutic treatment for heart failure with one or more further therapeutically active ingredients selected from the group consisting of ACE inhibitors, AIRBs, diuretics, Ca channel blockers, beta-blockers, digitalis, NO donors, vasodilators, SERCA2a stimulators, neprilisin (NEP) inhibitors, myosin filament activators, recombinant relaxin-2 mediators, recombinant NP protein, activators of the soluble Guanylate Cyclase (sGC), and beta-arrestin ligand of Angiotensin II receptor.

10. The use according to claim 9, wherein the diuretic, is selected among furosemide, bumetanide, torasemide, metolazone, an aldosterone antagonist, in particular spironolactone and eplerenone; thiazide diuretics, in particular hydrochlorothiazide, metolazone, chlorthalidone.

11. The use according to claim 9, wherein the ACE inhibitor is selected among Lisinopril and Ramipril.

12. The use according to claim 9, wherein the Angiotensin II receptor blocker is selected among valsartan, candesartan, olmesartan, telmisartan and losartan.

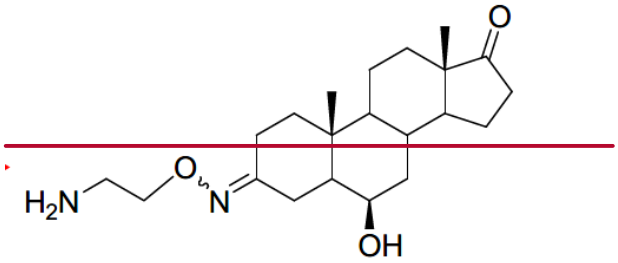
13. The use according to claim 9, wherein the Angiotensin receptor/neprilysin inhibitor is sacubitril.

14. The use according to claim 9, wherein the beta-blocker is selected among carvedilol and metoprolol.

15. The use according to claim 9, wherein the Vasodilator is selected among Hydralazine, optionally combined with isosorbide dinitrate, nitrates, in particular nitroglycerin or isosorbide nitrate, amlodipine, felodipine, non-dihydropyridines, in particular diltiazem or verapamil.

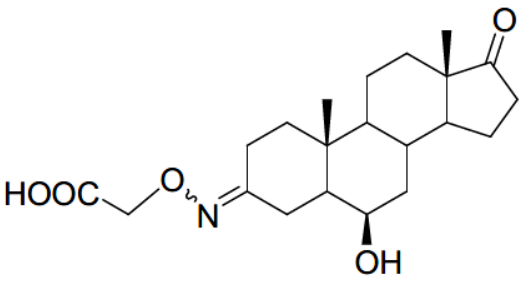
16. The use according to claim 9, wherein the agent for the treatment of heart failure is selected from Digoxin, entresto, omecantiv, serelaxin, ularitide, levosimendan.

17. A synthesized or extracted compound having a ~~formula (II) or~~ formula (III)



PST 2915

~~Formula (II)~~



PST 3093

Formula (III)

or a pharmaceutically acceptable salt, an ester a solvate, a hydrate, a polymorph thereof.

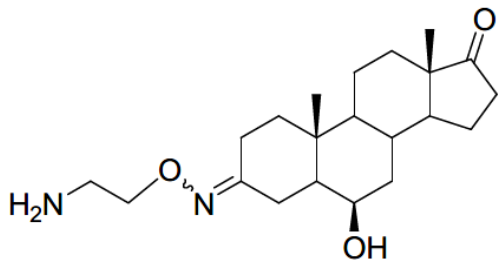
~~18. The compound of claim 17 having the formula (III), or a pharmaceutically acceptable salt, an ester, a solvate, a hydrate, a polymorph thereof.~~

~~19. The compound of claim 17 or claim 18 for use in the treatment of a disease requiring the activation of SERCA2a.~~

~~20~~18. The compound of claim ~~19~~ 17 for use in the treatment of a cardiovascular disease.

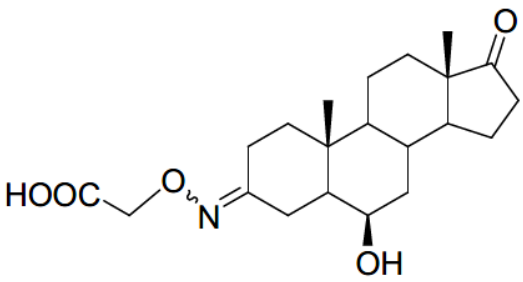
~~21~~19. The compound for use according to claim ~~20~~18, wherein the disease is heart failure.

~~22~~20. A pharmaceutical composition comprising ~~the~~ a compound having formula (II) or formula (III)



PST 2915

Formula (II)



PST 3093

Formula (III)

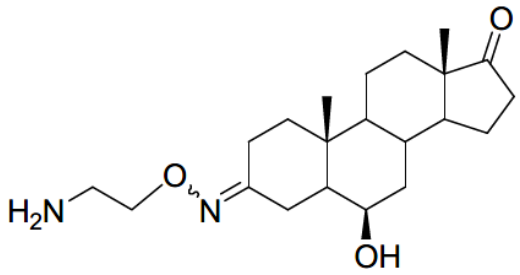
~~of 17 or claim 18,~~ or a pharmaceutically acceptable salt, a solvate, a hydrate, a polymorph thereof in admixture with at least one pharmaceutically acceptable vehicle and/or excipient.

~~23~~21. The pharmaceutical composition according to claim ~~22~~20, further comprising at least one additional therapeutic ingredient.

~~24~~22. The use of istaroxime or a pharmaceutically acceptable salt, solvate, or hydrate thereof for the manufacture of a medicament formulated for administration by intravenous infusion for the treatment of heart failure in ~~an~~ a human individual, wherein the administration is for an infusion duration sufficient to produce in the individual a measured plasma concentration level of an istaroxime metabolite, wherein:

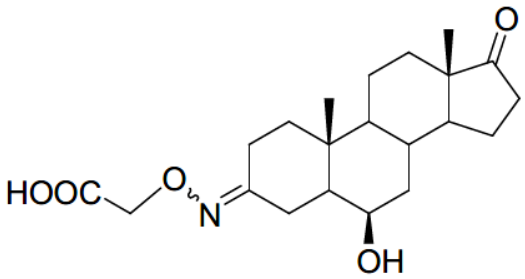
(a) the measured plasma concentration level of the istaroxime metabolite is greater than about 5 ng/ml for an accumulation period of at least about 6 hours; and

(b) the istaroxime metabolite comprises formula (II) or (III):



PST 2915

Formula (II)



PST 3093

Formula (III)

or a pharmaceutically acceptable salt, an ester a solvate, a hydrate, a polymorph thereof;

whereby the diastolic relaxation is improved in the human individual as compared to administration of istaroxime prior to the accumulation period.

~~25~~23. The use according to claim ~~24~~22, wherein the diastolic relaxation improvement is measured by one or more of:

(a) echocardiographic parameter E/A ratio, A wave, e', E wave deceleration time, E/e' ratio;

(b) diastolic relaxation, increased diastolic blood pressure, decreased heart rate, a decrease in dyspnea, increased stroke volume, increased cardiac index, increased cardiac output, increased pulmonary capillary wedge pressure, or increased stroke volume index; or

(c) sphygmomanometer, an electrocardiogram, cardiac catheterization, a radionuclide ventriculography scan; or

(d) any combination of (a)-(c).

24. The use according to claim 22 or claim 23, wherein the improvement in diastolic relaxation is measured by echocardiography comprising one or more of decreased E wave, increased E wave deceleration time, increased A wave, decreased E/A ratio, increased e', or decreased E/e' ratio.

~~26~~25. The use according to any ~~to~~ one of claims 22-24 ~~or 25~~, wherein the infusion duration is sufficient to produce in the human individual a plasma concentration level of an istaroxime metabolite greater than about 10 ng/ml for an accumulation period of at least about 6 hours.

~~27~~26. The use according to claim ~~26~~25, wherein the infusion duration is sufficient to produce in the human individual a plasma concentration level of an istaroxime metabolite greater than about 15 ng/ml for an accumulation period of at least about 6 hours.

~~28~~27. The use according to claim ~~27~~26, wherein the infusion duration is sufficient to produce in the human individual a plasma concentration level of an istaroxime metabolite greater than about 20 ng/ml for an accumulation period of at least about 6 hours.

~~29~~28. The use according to any one of claims 22-27~~24 to 28~~, wherein istaroxime is to be administered at a dose comprised between 0.2 µg/kg/min and 1.5 µg/kg/min.

~~30~~29. The use according to claim ~~29~~28, wherein istaroxime is to be administered at a dose comprised between 0.25 µg/kg/min and 1.0 µg/kg/min.