Bisoprolol Delays Progression Towards Right Heart Failure

in Experimental Pulmonary Hypertension

de Man et al: Bisoprolol in Experimental Pulmonary Hypertension

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Abstract

**Background**—In pulmonary arterial hypertension (PH) sympathetic adrenergic activity is highly elevated. Sympathetic over-activity is a compensatory mechanism at first, but might be detrimental for cardiac function at the long run. We therefore investigated whether chronic low-dose treatment with bisoprolol (a cardioselective β-blocker) has beneficial effects on cardiac function in experimental PH.

**Methods and Results**—PH was induced in rats by a single injection of monocrotaline (60 mg/kg). Pressure-telemetry in PH-rats revealed that 10 mg/kg bisoprolol was the lowest dose that blunted heart rate response during daily activity. Ten days after monocrotaline-injection, echocardiography was performed and PH-rats were randomized for bisoprolol-treatment (oral gavage) or vehicle (n=7/group). At end-of-study (body mass loss >5%), echocardiography was repeated, with additional pressure-volume measurements and histomolecular analyses. Compared to control, right ventricular (RV) systolic pressure and arterial elastance (measure of vascular resistance) more than tripled in PH. Bisoprolol delayed time to right heart failure (p<0.05). RV afterload was unaffected, however bisoprolol-treatment increased RV contractility and filling (both p<0.01), and partially restored right ventriculo-arterial coupling and cardiac output (both p<0.05). Bisoprolol restored RV β-adrenergic receptor signaling. Histology revealed significantly less RV fibrosis and myocardial inflammation in bisoprolol-treated PH-rats.

**Conclusions**—In experimental PH, treatment with bisoprolol delays progression towards right heart failure, and partially preserve RV systolic and diastolic function. These promising results suggest a therapeutic role for β-blockers in PH that warrants further clinical investigation.

**Key Words:** pulmonary hypertension; right ventricular dysfunction; β-adrenergic receptor blocker, pressure-volume relationship; Wistar rats
Pulmonary arterial hypertension (PH) is a fatal disease, characterized by progressive vascular remodeling and increased right ventricular (RV) afterload, which eventually leads to manifest right heart failure (RHF) and premature death. Current available medical treatments aim to reduce RV afterload, thereby secondarily improving RV function. No treatment is currently available that improves RV function directly, partially because it was not considered a therapeutic target in PH.

Recently, several reports have shown that sympathetic activity is increased in patients with PH. Similar to left heart failure (LHF), “ventricle-specific” down-regulation of $\beta_1$-adrenergic receptors was observed in RV samples of PH-patients. In addition, we recently demonstrated that exercise training was detrimental in experimental and progressive PH. The deleterious effects could be related to bouts of exercise-induced sympathetic stimulation.

Although increased adrenergic activity is a compensatory mechanism to maintain cardiac function by increasing contractility and heart rate, it became apparent that chronic adrenergic over-activity has – in the long run – detrimental effects on cardiac function. This supports the use of $\beta$-adrenergic blockade in LHF-management, which has been demonstrated to significantly reduce mortality and left ventricular (LV) remodeling.

Nevertheless, and notwithstanding the substantial evidence of their beneficial effects in LHF, the use of $\beta$-blockers is currently not recommended for patients with PH. PH-patients are unable to increase stroke volume during exercise, and as a consequence they are presumed to be highly heart rate dependent to raise cardiac output. Furthermore, in an acute model of PH, it was demonstrated that right ventriculo-arterial uncoupling occurs after intravenous $\beta$-blocker administration.

However, the $\beta$-blockers used in these studies were first generation unselective $\beta$-blockers, with more bronchial and vascular side-effects. In addition, the dosages used in
these studies were relatively high, whereas a low dose could have sufficed and been better tolerated. Furthermore, no data is available on the long-term effects of β-adrenergic receptor blockade in PH-patients. This aspect is important, as the typical time-course of improvement by β-blockers in LHF is preceded by initial functional decline, with significant clinical improvement not to be expected before three months after start of therapy.\textsuperscript{15}

Recently, Bogaard and co-workers provided some hemodynamic and molecular insights in effects of carvedilol (an unselective β-blocker) in PH-rats.\textsuperscript{16} However, in the absence of load-independent measurements of RV function, it is unclear whether the beneficial effects are RV-specific or related to the α\textsubscript{1}-associated pulmonary vascular effects of carvedilol.\textsuperscript{17} In addition, it remains uncertain whether β-blockers can restore β-adrenergic receptor (βAR) signaling and delay progression of right heart failure. In the present study, we therefore 1) assessed the chronic effect of bisoprolol (a cardio-selective β-blocker) on disease progression by sequential echocardiographic measurements, 2) evaluated RV function using load-independent parameters derived from pressure-volume analyses, and 3) studied the βAR- signaling assessing its direct downstream targets.

**Methods**

All experiments were approved by the Institutional Animal Care and Use Committee of the VU University, Amsterdam, The Netherlands. Male Wistar rats were used (150-175g; 30 in total), experimental PH was induced by monocrotaline (60 mg/kg).\textsuperscript{9,18}

**Part I – Dose-finding by pressure-telemetry**

The *minimal* effective dose of bisoprolol that could blunt heart rate response during daily activity (>10%) was determined by telemetry (TA11PA-C40, Data Science International (DSI), St. Paul MN).\textsuperscript{19} Ten days after PH-induction, bisoprolol was given once daily for 3
consecutive days by oral gavage, at start of their active phase (i.e. night: 18:00 – 06:00h): 4 PH-rats received 5 mg/kg bisoprolol once daily and the other four received 10 mg/kg. The effect of bisoprolol on heart rate, systemic blood pressure and physical activity were evaluated (for details, see online Supplement).

Part II – “Clinical” effects of bisoprolol-treatment in experimental PH

In the second part of the study, 22 rats were included (no telemetry): 8 control rats and 14 rats treated with monocrotaline. Ten days after PH-induction, PH-rats were randomized for bisoprolol-treatment (PH+biso; 10 mg/kg) or vehicle/water (PH) by oral gavage (n=7/group). Rats were treated for maximally 3 weeks (day 10 until day 31). Rats that showed clinical signs of manifest RHF (defined as >5% loss in body mass and/or respiratory distress, cyanosis, lethargy) were euthanized earlier, in keeping with the protocol approved by the Institutional Animal Care and Use Committee. Manifest RHF was a survival endpoint and recorded as an event in the survival analyses.9

Hemodynamic evaluation

Rats were evaluated by echocardiography 10 days after (monocrotaline-)injection and at end-of-study (when manifest RHF developed, or 31 days after injection).9,19 At end-of-study, open-chest RV catheterization (Millar Instruments, Houston TX) was performed under general anaesthesia in all rats (isoflurane induction: 4.0% in 1:1 O2/air mix; maintenance: 2.0% in 1:1 O2/air mix; see Supplement).9 Non-invasive estimations of disease progression and RV wall stress are described in the Supplement.

Using custom-made algorithms (programmed in MATLAB 2007b, The MathWorks, Natick MA) RV (peak-)systolic pressures and RV end-diastolic pressures were automatically determined from steady-state measurements, as well as arterial elastance (Ea), a measure for RV afterload.13,20 From occlusion-data, end-systolic elastance (Ees; contractility) and end-
diastolic elastance (Eed; filling) were determined. These parameters represent the slope of the end-systolic and end-diastolic pressure-volume relationships, respectively, and are considered load-independent measures for cardiac contractility (Ees) and filling (Eed). In addition, we calculated the preload recruitable stroke work and the dP/dtmax-end-diastolic volume relation to assess RV contractile performance. The ratio Ees/Ea was calculated as an estimate for ventriculo-arterial coupling (cardiac adaptation in relation to its load).

**Histomorphology of heart and lungs**

After the final hemodynamic assessment, all 22 rats were euthanized (by exsanguination under isoflurane), and heart, lungs and other major organs were harvested. Cardiomyocyte cross-sectional area, cardiac fibrosis, relative wall thickness of pulmonary arterioles, myocardial capillary density (using CD31-antibodies) and myocardial inflammation (using CD45-antibodies) were determined (see also Supplement).

**Protein analyses β-adrenergic signaling**

Phosphorylation of cardiac myosin binding protein C (cMyBPC) and cardiac troponine I (cTnI) was determined as described before. All RV samples were treated with trichloroacetic acid, to preserve phosphorylation of cMyBPC and cTnI. Samples were separated on a gradient gel (Criterion Tric-HCL 4% to 15% gel, Bio-Rad Laboratories, Berkeley CA) and proteins were stained for one hour with ProQ Diamond Phosphoprotein Stain (Molecular Probes, Eugene OR). Fixation, washing, and destaining were performed according to manufacturers guidelines. Subsequently, gels were stained with SYPRO Ruby staining (Molecular Probes) for determination of total protein levels of cMyBPC and cTnI. The phosphorylation status of cMyBPC and cTnI was expressed relative to total protein levels to correct for differences in sample loading.
Statistical analysis

All analyses were performed in a blinded fashion. All data were verified for normal distribution. Data are presented as mean ±SEM. A p-value <0.05 was considered statistically significant.

Comparison of telemetric-registrations of PH-rats before/after bisoprolol-treatment was performed by two-way ANOVA for repeated measurements, the interaction between bisoprolol-treatment and time was tested and reported. One-way ANOVA was used for the analyses of disease progression, pressure-volume relation, autopsy data and protein analyses, with Bonferroni post-hoc comparison between PH-rats with/without bisoprolol-treatment.

Survival estimates were performed by Kaplan-Meier analysis, with post-hoc comparison performed by log-rank (Mantel-Cox) test between PH-rats with/without bisoprolol-treatment (SPSS 16.0 for Windows, SPSS, Chicago IL).

Histological data were analysed using multilevel analysis to correct for non-independence of successive measurements per animal (MLwiN 2.02.03, Center for Multilevel Modelling, Bristol, UK).9,24

Results

Part I – Minimal effective dose of bisoprolol in PH-rats

Echocardiography confirmed the PH-status of all 8 rats at day of bisoprolol-administration (reduced PAAT/cl, increased RV wall thickness). Only 10 mg/kg was able to completely blunt heart rate response during daily activity completely (Figure 1A). At this dose, systemic blood pressure and physical activity were minimally affected, which indicates that this dosage was well-tolerated by PH-rats (Figure 1B,C).
Part II – Effects of 10 mg/kg bisoprolol in established PH

*Bisoprolol delayed the progression towards right heart failure*

In a separate group of rats, we determined PH-status ten days after (monocrotaline-) injection, by echocardiography, right heart catheterization and histomorphology. Monocrotaline-treated rats (n=5) revealed lower PAAT/cl, indicating higher RV systolic pressure, and higher RV wall thickness, indicating (moderate) RV hypertrophy (Supplement: Table S1). In addition, increased RV systolic pressures, pulmonary vascular remodeling and RV hypertrophy at day 10 was confirmed by RV catheterization and histomorphometric analyses (Tables S1,S2). The PH-state before start of bisoprolol-treatment was thereby confirmed in all monocrotaline-treated rats. Compared to vehicle-treated PH-rats, bisoprolol delayed the time to manifest RHF, as defined in the Methods section (Figure 2). This finding was confirmed by serial echocardiography demonstrating that bisoprolol significantly delayed the progression of RV dilatation and reduced the decline in cardiac function, whether measured by TAPSE or cardiac output (Figure 3, Table S3; p<0.05). Preservation of cardiac output was mainly the result of an increase in stroke volume rather than increased heart rate.

At end-of-study, cardiac function was partially maintained by bisoprolol-treatment (TAPSE, control: 4.0±0.1; PH: 1.4±0.1; PH+biso 2.4±0.2 mm, PH vs. PH+biso: p<0.001; cardiac output, control: 88±2.1; PH 17±1.6; PH+biso: 34±1.9 ml/min, PH vs. PH+biso: p<0.001; also Figure 3D,E). No differences were observed in RV wall thickness and RV dilatation between bisoprolol- and vehicle-treated PH-rats (Figure 3B,C). In addition, end-systolic wall stress was similar in bisoprolol- and vehicle-treated PH-rats (RV end-systolic wall stress, control: 80±5; PH: 336±26; PH+biso: 308±14 mmHg; PH vs. PH+biso: p=0.26).
Bisoprolol improved cardiac function, without effecting RV afterload

RV pressure-volume measurements at end-of-study (Figure 3A-C) revealed that RV systolic pressures were significantly elevated in PH-rats compared to control, but no difference was found between bisoprolol- and vehicle-treated PH-rats (Figure 4D), which is in line with previous echo-findings (Figure 3A,B). Ea (measure of vascular resistance) was also elevated in PH, but again, no difference was observed between bisoprolol- and vehicle-treated PH-rats (Figure 4E). This indicates that bisoprolol-treatment did not affect RV afterload. We also found an equal rise in (wet) lung mass observed at autopsy, and comparable remodeling of the pulmonary arteries during histological examination (Table S4,S5).

On the other hand, bisoprolol-treatment increased Ees (measure of contractility; Figure 4F), resulting in partial normalization of the ventriculo-arterial coupling (Ees/Ea; Figure 4G). Preload recruitable stroke work (PRSW, control: 211±35, PH: 1362±156, PH+biso: 2161±312 mmHg; PH vs. PH+biso: p<0.001) and dP/dtmax-end-diastolic volume relation showed similar results (dP/dtmax-EDV, control: 3.7±0.6, PH: 11.1±2.7, PH+biso: 23.3±5.7 mmHg/ms/ml; PH vs. PH+biso: p<0.001). Of note, after normalization of Ees for RV mass,26 no significant difference was observed anymore between vehicle-treated PH-rats and controls, whereas the difference in contractility between bisoprolol- and vehicle-treated PH-rats remained statistically significant (Ees/RVmass, control: 40.3±6.3, PH: 43.6±12.0, PH+biso: 99.0±10.9 mmHg/ml/g; p=0.02 PH+biso vs. PH). After normalization of Ees for RV volume, contractility was significantly reduced in vehicle-treated PH-rats and improved towards normal value in the bisoprolol-treated PH-rats (Ees/RVvolume, control: 2.14±0.13; PH: 1.32±0.08; PH+biso: 2.28±0.14 mmHg/ml²·10³; PH vs. PH+biso: p<0.001). Furthermore, bisoprolol-treatment reduced RV end-diastolic pressures and Eed (measure of filling; Figure 4H,I).
**Bisoprolol reduced RV fibrosis and RV myocardial inflammation**

In line with previous echo-findings, the right ventricles of PH-rats at end-of-study were hypertrophied, compared to controls (Figure 3B). No differences were observed between bisoprolol- and vehicle-treated PH-rats, whether expressed as RV mass (irrespective of normalization), RV / (LV+S) ratio, or RV cardiomyocyte cross-sectional area (Table S4,S5).

At start-of-treatment, no difference was observed between control- and PH-rats in RV capillary density or fibrosis, and there were no signs of cardiac inflammation (Table S2). At end-of-study, the findings for RV capillary density were similar; compared to control, capillary density was reduced in PH, without a significant difference between the two PH-groups (Figure 5A,D,G). More RV interstitial fibrosis was observed between PH-rats and controls; Interestingly, bisoprolol-treatment significantly reduced RV fibrosis (Figure 5B,E,H). Furthermore, the presence of (CD45+) inflammatory cells in RV myocardium of bisoprolol-treated PH-rats was significantly less, compared to vehicle-treated PH-rats (Figure 5C,F,I). Leukocyte infiltration in the left ventricle was increased in bisoprolol- and vehicle-treated PH-rats, however these values were low and comparable to control-values of the right ventricle (Table S5). Autopsy and assessment of LV histology revealed no effect of bisoprolol (Tables S4,S5), compatible with a RV-specific effect of bisoprolol.

**Bisoprolol restored RV beta-adrenergic receptor signaling pathway**

Phosphorylation of both cMyBPC and cTnI (protein kinase A-mediated downstream targets of the βAR) were significantly higher in bisoprolol-treated PH-rats in comparison to vehicle-treated PH-rats (Figure 6).
Discussion

This study investigated the effects of bisoprolol-treatment in experimental PH, focusing on RV function and remodeling. Using a comprehensive set of physiologic and pathologic endpoints, we have demonstrated that:

1) Chronic low-dosed bisoprolol-treatment was well-tolerated, and delayed time to manifest RHF;

2) Bisoprolol-treatment improved cardiac function, by improving RV contractility (Ees), filling (Eed), and ventriculo-arterial coupling (Ees/Ea);

3) The cardiac-selective effects of bisoprolol can be attributed to restoration of RV β-adrenergic receptor signaling, the reduction of RV (interstitial) fibrosis and RV myocardial inflammation.

These results suggest a potential role for β-blocker in PH that warrants further clinical investigation.

Bisoprolol-treatment was well-tolerated

Beta-blockers are currently not recommended, because PH-patients are believed not to tolerate the acute (but transient) negative inotropic and chronotropic effects.\textsuperscript{1,13,14} To address this legitimate argument, we used an approach that was inspired by successful β-blocker use in LHF.

\textit{Low vs. high dose}. By definition, patients with LHF are hemodynamically compromised, and like in PH, their adrenergic system is over-activated as well.\textsuperscript{27} To some extent, these two patient groups are therefore comparable.\textsuperscript{3} Interestingly, most LHF patients (approximately 85\%) enrolled in clinical trials with β-blockers, were able to tolerate short- and long-term treatments with this drug and reached the maximum planned target dose, when β-blockers are introduced at a very low dose followed by gradually dosage-increase (“start low, go slow”).\textsuperscript{11} In addition, whereas the adverse effects of β-blockers are dose-dependent, the beneficial
effects are associated with heart rate reduction, which can be achieved by lower dosages. In this study, we used the minimum dose that effectively blunted heart rate response. This was accompanied by only minimal side-effects, and was therefore well-tolerated by the PH-rats (minor effect on blood pressure, no effect on activity). Compared to other rat-studies that used bisoprolol (typically 60 mg/kg), the dose used in this study can be considered low.

Hemodynamic data at day 10 suggests limited functional hemodynamic compromise of the PH-rats at start-of-treatment. Unfortunately, the progressiveness of the model did not permit to start treatment at a later time-point. However, like in LHF, β-blocker use will most likely be limited to PH-patients with some cardiac reserve, in able to cope with its acute negative inotropic effects.

**Selective vs. unselective beta-blocker.** Of all β-blockers, only bisoprolol, carvedilol and metoprolol have been proven to reduce mortality in LHF. Of these three, bisoprolol is the most β1-cardioselective. We chose bisoprolol to avoid potential harmful effects of β2-mediated blockade. The β2-subtype is the predominant βAR present in the pulmonary vasculature. Blockade of the β2-receptors may lead to smooth muscle contraction, which could result in a further increase in pulmonary vascular resistance and RV pressures. Nevertheless, the clinical relevance of β1-selectivity in PH is unknown.

**Beneficial effects of bisoprolol**

To ease the clinical interpretation of our findings, we used robust and clinical relevant outcome measures to investigate the effects of bisoprolol. We explicitly evaluated pressure-volume relations, because it is considered the gold standard to describe cardiac function, and more specifically, to address the potential risk of ventriculo-arterial uncoupling after β-blocker use in PH, as raised by others. In contrast to what was feared, we observed partial normalization of the ventriculo-arterial coupling, which may be explained by chronic opposed to acute drug administration.
Measurements of RV wall thickness and diastolic diameter at end-of-study protocol failed to detect changes that could explain the beneficial effects of bisoprolol. However, it should be noted that these measurements were obtained at a stage of terminal right heart failure, which was reached at a later time-point in the bisoprolol-treated PH group, than the PH controle group: e.g. the progression of RV dilatation was significantly less in the bisoprolol-treated rats (Figure 3C: RVEDD; Table S3). Hence, the beneficial effect of bisoprolol between both groups are reflected more by the time elapsed to reach right heart failure, than the hemodynamic findings at right heart failure. In addition, we observed less RV inflammation and fibrosis, together with an increase in RV contractility normalized for hypertrophy (Ees/RVmass), in the bisoprolol-treated PH-group compared to the control PH group. This implies that bisoprolol-treatment results in improved intrinsic properties of RV cardiomyocytes that cannot be detected by relative crude measures as RV wall thickness and diastolic diameters.

Only a few studies have evaluated the (chronic) effects of \( \beta \)-blockers in the context of PH.\(^{14}\) A recent paper by Bogaard et al. provides some hemodynamic and molecular insights in the effects of carvedilol (a non-specific \( \alpha_1/\beta_1/\beta_2 \)-adrenergic receptor blocker) on echocardiographic parameters and molecular analyses in PH-rats.\(^{16}\) For the first time, we demonstrate that the positive effects of \( \beta \)-blocker treatment are RV-specific and could delay disease progression, improve RV function, and (partially) restored ventriculo-arterial coupling and \( \beta \)AR-signaling. Bogaard also observed less RV fibrosis after \( \beta \)-blocker treatment. They found an increase in RV capillary density, which we could not confirm. We, on the other hand, observed a reduction in RV inflammation, which was not studied in detail by Bogaard. Differential effects of the \( \beta \)-blockers used might explain these subtle differences. An alternative explanation is related to differences in disease severity of the experimental models used: in our MCT-model, all rats developed right heart failure within 4 weeks, whereas all
hypoxia/Sugen rats survived 8 weeks after induction of PH. As a consequence, treatment
duration in our monocrotaline-model was relative short (median treatment was 2.5 weeks)
compared to Bogaard’s Sugen/hypoxia model (4 weeks). It is possible that bisoprolol-
treatment reduced RV inflammation and RV fibrosis (early effects), but that its treatment
duration was insufficient to enhance RV capillary density (late effect). Usui et al. also
investigated the effect of carvedilol in monocrotaline-treated rats. They too observed
survival benefit with β-blocker, but unfortunately they did not report any measures on cardiac
function, and focussed mainly on LV rather than RV remodeling. Also, no information was
provided on possible side-effects and tolerability. In addition, Ishikawa et al. reported
beneficial effects of arotinolol (a non-specific β-blocker) using the same PH-rat model.
However the clinical implications of this study are limited: arotinolol was studied to prevent
rather than to treat PH-associated RHF, and, unlike bisoprolol, arotinolol is not clinically used
or FDA-approved.

Potential mechanisms

In this proof-of-concept study, we did not perform in-depth analysis on cellular and
molecular effects of bisoprolol. Nonetheless, our histological and protein analysis may
provide some mechanistic insights, based on the experiences with β-blockers in LHF.

Although the β-adrenergic system in LHF is incompletely understood, it is generally
believed to exert their beneficial effects by blunting of the sympathetic over-activity, resulting
in reduced desensitization of the βAR and restoration of βAR-signaling. Protein kinase A
(PKA) is a central player in the βAR-signaling. Therefore, we investigated the effect of
bisoprolol on phosphorylation of contractile proteins cMyBPC and cTnI, two main targets of
PKA. Interestingly, we observed that bisoprolol restored phosphorylation of both cMyBPC
and cTnI, compared to vehicle-treatment. This suggests that bisoprolol restore RV
cardiomyocyte βAR-signaling.
An alternative explanation for the observed beneficial effects of bisoprolol therapy might be that a reduction in heart rate by β-blockers could have prevented sustained high levels of RV wall stress. We previously observed that exercise increased myocardial inflammation in experimental PH, and related this to increased RV wall stress during episodes of activity, comparable to what has been described in detail by Sun et al. This is in agreement with the observation of the present study that bisoprolol prevents rather than reverses RV inflammation, as no inflammation was observed at start-of-treatment, which also argue against direct cardiac inflammatory effects of monocrotaline.

**Clinical relevance**

We demonstrated that β-blocker therapy is beneficial in experimental PH. However, the monocrotaline model exhibits alterations in the β-adrenergic system that resemble those in human PH; others have previously demonstrated that in monocrotaline-treated rats with RHF, heart rate variability is reduced, plasma norepinephrine levels are increased and β1-adrenergic receptor density of the right ventricle is decreased, similar to clinical PH.

Despite bisoprolol-treatment all PH-rats eventually developed right heart failure. However, we want to emphasize that the effects of bisoprolol were achieved in the absence of “traditional” vasodilating therapies (e.g. bosentan) that modulate the progression of pulmonary vascular remodeling. The findings of this study therefore provide a rationale to investigate the role of (cardioselective) β-blockers as an add-on therapy in the management of clinical PH.

**Conclusions**

In our PH-rat model bisoprolol-treatment was well-tolerated and beneficial. It delayed the progression towards RHF, which was attributed to improved RV contractility and compliance,
and accompanied by restored β-adrenergic receptor signaling and reduced RV fibrosis and inflammation. Future studies are necessary to address the clinical implications of our findings.
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Disclosures

None.

References


Figure Legends

**Figure 1. Averaged 24-hr registration of PH-rats before and after treatment of 10 mg/kg bisoprolol.**

This dosage was able to completely blunt heart rate response during the whole active phase (between 18 - 6 hr) of the rats (A: grey area). In addition, only a moderate effect on systemic blood pressure was observed (B), without an (adverse) effect on daily physical activity (C). Data presented as mean±SEM, n=4 (PH/PH+biso). P-values represent interactive term (time*treatment). PH (solid/red line), vehicle-treated PH-rats; PH+biso (dotted/blu line), bisoprolol-treated PH-rats.

**Figure 2. Bisoprolol-treatment in PH significantly delayed time to manifest right heart failure.**

Log rank test: $X^2 = 4.54; p<0.05$. Control: n=8; PH/PH+biso: n=7. *: p<0.05 PH+biso vs. PH.

**Figure 3. Disease progression during treatment-period**

Echocardiography could confirm the PH-status at start of treatment (A,B: first time-point, lower PAAT/cl and higher RV wall thickness). Bisoprolol-treatment (PH+biso: dotted/blue) delayed RV dilatation (C: arrow) and reduced the decline in cardiac function (D,E: arrows), compared to vehicle-treated PH-rats (PH: solid/red), numeric data are found in Table S1. In addition, at end-of-study cardiac function was better maintained in bisoprolol-treated PH-rats. Data presented as mean ±SEM, control: n=8; PH / PH+biso: n=7. ###: p<0.001 PH / PH+biso vs. control; *: p<0.05, ***: p<0.001 PH+biso vs. PH. PAAT/cl, pulmonary artery acceleration.
time normalized for cardiac cycle length (inversely correlated with RV systolic pressure); RVEDD, RV end-diastolic diameter; TAPSE, tricuspid annular plane systolic excursion.

**Figure 4. Pressure-volume analyses.**

Typical examples of the pressure-volume relation are shown for control, PH and PH+biso (A-C: line indicates end-systolic pressure-volume relationship). RV systolic pressures (RVSP) and arterial elastance (Ea; measure of RV afterload) were equally increased in both PH-groups (D,E). However, RV contractility (end-systolic elastance; Ees) was significantly increased by bisoprolol-treatment (F), resulting in partial normalization of ventriculo-arterial coupling (Ees/Ea; G). Bisoprolol-treatment also partially restored RV diastolic function, measured by RV end-diastolic pressure (RVEDP) and Eed (H,I). Control: n=8, PH/PH+biso, n=7. *: p<0.05, **: p<0.01, ***: p<0.001 PH+biso vs. PH.

**Figure 5. Bisoprolol-treatment reduced RV fibrosis and RV myocardial inflammation.**

Histomorphometric analyses revealed significant and selective reduction of RV interstitial fibrosis and RV myocardial inflammation in bisoprolol-treated PH-rats (B,C). No difference was observed for RV capillary density (A).

Typical examples are shown of histological sections of the right ventricle of vehicle- (PH: D-F) and bisoprolol-treated PH-rats (PH+biso: G-I), stained for RV capillarization (D,G: endothelium marker CD31 is stained green, cell membranes red; capillaries appear as small yellow/orange dots), fibrosis (E,H: picrosirius red staining, dark grey), and infiltrating inflammatory cells (F,I: lymphocyte-marker CD45 is stained green, cell membranes red, nuclei blue). Control: n=8; PH/PH+biso: n=7. *: p<0.05 PH+biso vs. PH.
Figure 6. Bisoprolol-treatment restored β-adrenergic receptor signaling.

A typical example of a pro-Q/SYPRO gel is shown for control, vehicle-treated and bisoprolol-treated PH-rats. Analyses revealed increased phosphorylation of downstream βAR-targets myosin binding protein C (cMyBPC) and troponin I (cTnI) after bisoprolol-treatment. Control: n=8; PH/PH+biso: n=7. *:p<0.05 PH+biso vs. PH.
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SUPPLEMENTAL MATERIAL

Bisoprolol Delays Progression Towards Right Heart Failure
in Experimental Pulmonary Hypertension

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Nico Westerhof1,2, Walter J. Paulus2, Anton Vonk-Noordegraaf3

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Expanded Methods

Part I – Dose-finding by pressure-telemetry

A group of 8 pulmonary arterial hypertensive (PH)-rats was studied to determine the minimal effective dose of bisoprolol that could blunt heart rate response during daily activity. This strategy was motivated by our previous observations that episodes of increased heart rate during exercise had deleterious effects in progressive PH.1 Furthermore, a recent meta-analysis demonstrated that the beneficial effects of β-blockers are related to the degree of heart rate reduction and not to the dosage administered, whereas the adverse effects of β-blockers are dose-dependent.2

For this purpose, rats were equipped with an implantable telemetric pressure-transmitter (TA11PA-C40, Data Science International (DSI), St. Paul MN) fitted with a 10-cm long catheter that was placed in the abdominal aorta.3,4 Telemetry does not only allow continuous recordings of heart rate and systemic blood pressure, free of artefacts like stress or anaesthesia, but also informs on (relative) physical activity of the rats, based on changes in signal strength while the rat is moving through its cage. Telemetry-recordings were analyzed off-line, using Dataquest A.R.T. Analysis software (version 4.2, DSI). Rats were given a post-operative 10-day resting-period, ensuring full recovery, indicated by normalization of body mass, heart rate and blood pressure, and return of their normal circadian rhythm.3,4

After full recovery, PH was induced by monocrotaline-injection, and two weeks later their PH-status was confirmed by echocardiography (see below). Three days later, bisoprolol was given once daily for 3 consecutive days by oral gavage, at start of their active phase (i.e. night: 18:00 – 06:00h): 4 PH-rats received 5 mg/kg bisoprolol once daily and the other four received 10 mg/kg. These dosages were based on results from similar pilot-experiments in control rats. The effect of bisoprolol on heart rate, systemic blood pressure and physical activity were evaluated. After these experiments, all rats were euthanized and their organs examined. No additional measurements were performed.

Hemodynamic evaluation

Echocardiography

Rats were evaluated by echocardiography 10 days after (monocrotaline-)injection and at end-of-study (when manifest right heart failure developed, or 31 days after injection).
Transthoracic echocardiographic measurements (ProSound SSD-4000 system equipped with a 13-MHz linear transducer (UST-5542), Aloka, Tokyo, Japan) were performed on anesthetized but spontaneously breathing rats (isoflurane 2.0% in 1:1 O₂/air mix; Pharmachemie, Haarlem, The Netherlands), which allows for serial assessment of cardiac dimensions and hemodynamics in rats with equal diagnostic accuracy as cardiac MRI, due to its high temporal resolution. Analyses were performed off-line (Image-Arena 2.9.1, TomTec Imaging Systems, Unterschleissheim / Munich, Germany). Measured parameters for right ventricular (RV) function were: cardiac output (Doppler-derived stroke volume, heart rate), and tricuspid annular plane systolic excursion (TAPSE). Parameters for RV remodelling were: RV end-diastolic diameter (RVEDD) and RV wall thickness. Pulmonary artery acceleration time normalized for cardiac cycle length (PAAT/cl) was used to a non-invasive estimate for RV systolic pressure (PAAT/cl and RVSP are inversely correlated). Disease progression of PH during treatment-period was expressed as percentage changes in hemodynamics over time, e.g. change in cardiac output: \[ \text{\Delta cardiac output} = \frac{[\text{cardiac output}_{\text{end}} - \text{cardiac output}_{\text{start}}]}{\text{cardiac output}_{\text{start}}} \times 100\% \]. Other parameters for disease progression were calculated similarly.

**RV catheterization**

The rats were sedated by inhalation of isoflurane (induction: 4.0% in 1:1 O₂/air mix; maintenance: 2.0% in 1:1 O₂/air mix), intubated (16 G Teflon tube) and attached to a mechanical ventilator (Micro-Ventilator, UNO, Zevenaar, The Netherlands; ventilator settings: breathing frequency 75/min, pressures 9/0 cmH₂O, inspiratory/expiratory ratio 1:1). The rats were placed on a warming pad to maintain body temperature.

After opening of the thorax, a temporal ligature was placed around the inferior vena cava. Following an apical stab (23G), a combined pressure-volume catheter (SPR-869, Millar Instruments, Houston TX) was inserted into the right ventricle and positioned along its long axis. The signals (processed by MPVS-400, Millar Instruments), obtained at steady state (at least 10s) and during transient vena cava occlusion were digitally recorded (2.0 kHz sampling rate; Chart 5.5.6, ADInstruments, Sydney, Australia) and analyzed off-line, using PVAN 3.6 (Millar Instruments) and custom-made algorithms (programmed in MATLAB R2007b, The MathWorks, Natick MA). Stroke volume (in RVU) derived from the conductance signal was calibrated, using stroke volume (in ml) derived from echo-Doppler as external reference.
RV wall stress was estimated using LaPlace’s law: $RV\ wall\ stress = \frac{RVSP \times RVEDD}{(4 \times RV\ wall\ thickness)}$.

**Histomorphometric analyses of heart and lungs**

After the final hemodynamic assessment, all 22 rats were euthanized (by exsanguination under isoflurane), and heart, lungs and other major organs were harvested. Lungs were weighed and the left lobe was subsequently filled by 1:1 mix of saline and cryofixative (Tissue-Tek O.C.T. compound, Sakura, Fintek, Europe, Zoeterwolde, The Netherlands), and snap-frozen in liquid nitrogen. The right lobe was used to measure wet/dry lung mass ratio. The heart was perfused, weighted, dissected and snap-frozen in liquid nitrogen.

**Bright-field microscopy**

Images were collected by the use of a Leica DMRB microscope (Wetzlar, Germany), a Sony XC-77CE camera (Towada, Japan) and a LG-3 frame grabber (Scion, Frederick MD). ImageJ for Windows 1.42 software (National Institutes of Health, Bethesda MD) was used for image analysis, taking the pixel-to-aspect ratio into account.

*Cardiomyocyte cross-sectional area.* Haematoxylin & eosin (HE)-stained cardiac cryosections (5 μm) were used to determine left ventricular (LV) and RV cardiomyocyte cross-sectional area (CSA). Cardiomyocyte size for each ventricle was expressed as the average CSA of minimally twenty transversally cut cardiomyocytes at the level of the nucleus, randomly distributed over the ventricles.

*Cardiac fibrosis.* The combination of picrosirius red staining (5 μm) and polarized light was used for analysis of cardiac fibrosis. LV and RV fibrosis were expressed as the percentage tissue area positive for collagen, measured over minimally three randomly chosen areas per ventricle.

*Relative wall thickness of pulmonary arterioles.* Pulmonary sections (5 μm) were stained with Elastica von Giesson for morphometric analysis of vascular dimensions. Minimally fifty transversally cut pulmonary arterioles, with an outer diameter between 25 and 100 μm, randomly distributed over the lungs, were measured. Relative wall thickness of pulmonary arterioles (PA) was calculated as:

$$PA\ wall\ thickness = 2 \times \text{medial wall thickness} / \text{outer diameter} \times 100\%.$$

**Immunofluorescence microscopy**

For the analyses of cardiac capillarisation and cardiac inflammation, cardiac cryosections (5μm) were incubated for 60 min with primary CD31- (1:35; sc-1506-R, Santa Cruz Biotechnology, Santa Cruz CA) and CD45-antibodies (1:25; sc-53045, Santa Cruz) for capillary density and leukocyte infiltrations, respectively, followed by appropriate secondary antibody staining as well as WGA (glycocalyx) and DAPI (nuclei) counterstaining. Image acquisition was performed on a Marianas digital imaging microscopy workstation (Intelligent Imaging Innovations (3i), Denver CO). SlideBook imaging analysis software (SlideBook 4.2, 3i) was used to semi-automatically quantify the images.

**Myocardial capillary density.** Capillary density was expressed as the number of capillaries per section area, measured in at least three randomly chosen areas per ventricle, where cardiomyocytes were transversally sectioned.

**Myocardial leukocyte infiltration.** Leukocyte infiltration was expressed as the number of positive CD45-nuclei per section area, measured over minimally three randomly chosen areas per ventricle.

**Additional measurements at start-of-treatment**

Ten days after sham- or monocrotaline-injection, pressure-volume measurements and histomorphological analyses were performed in a separate group of 10 rats (5 control; 5 MCT-treated), to evaluate disease severity and cardiac inflammation in more detail.
Supplemental References


# Table S1: Hemodynamic assessment at start-of-treatment (day 10)

<table>
<thead>
<tr>
<th></th>
<th>Control (n=5)</th>
<th>PH (n=5)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Echocardiography</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV wall thickness (mm)</td>
<td>0.84 ±0.02</td>
<td>0.94 ±0.02</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>TAPSE (mm)</td>
<td>3.6 ±0.1</td>
<td>3.5 ±0.1</td>
<td>0.50</td>
</tr>
<tr>
<td>RV end-diastolic diameter (mm)</td>
<td>3.6 ±0.1</td>
<td>3.6 ±0.1</td>
<td>0.68</td>
</tr>
<tr>
<td>PAAT/CL (%)</td>
<td>22.2 ±1.0</td>
<td>12.6 ±1.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Stroke volume (ml)</td>
<td>0.25 ±0.01</td>
<td>0.23 ±0.01</td>
<td>0.20</td>
</tr>
<tr>
<td><strong>Pressure-volume analyses</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV systolic pressure (mmHg)</td>
<td>26.9 ±0.9</td>
<td>37.0 ±0.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RV end-diastolic pressure (mmHg)</td>
<td>2.2 ±0.2</td>
<td>1.9 ±0.1</td>
<td>0.22</td>
</tr>
<tr>
<td>Ees (mmHg/ml)</td>
<td>135 ±43</td>
<td>143 ±35</td>
<td>0.89</td>
</tr>
<tr>
<td>Ea (mmHg/ml)</td>
<td>130 ±16</td>
<td>212 ±27</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Ees/Ea</td>
<td>0.95 ±0.20</td>
<td>0.74 ±0.12</td>
<td>0.39</td>
</tr>
<tr>
<td>Eed (mmHg/ml)</td>
<td>6.8 ±0.7</td>
<td>7.6 ±2.3</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Data presented as mean ±SEM. TAPSE, tricuspid annular plane systolic excursion; PAAT/CL, pulmonary artery acceleration time / cyclus length; Ees, end-systolic elastance; Ea, arterial elastance; Ees/Ea, ventriculo-arterial coupling factor; Eed, end-diastolic elastance.
Table S2: Histomorphology at start-of-treatment (day 10)

<table>
<thead>
<tr>
<th></th>
<th>Control (n=5)</th>
<th>PH (n=5)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV cross-sectional area (µm²)</td>
<td>286 ±15</td>
<td>360 ±28</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LV cross-sectional area (µm²)</td>
<td>475 ±33</td>
<td>421 ±32</td>
<td>0.24</td>
</tr>
<tr>
<td>PA wall thickness (%)</td>
<td>6.5 ±0.4</td>
<td>20.0 ±1.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RV capillary density (/mm² *1000)</td>
<td>2.99 ±0.23</td>
<td>3.01 ±0.26</td>
<td>0.96</td>
</tr>
<tr>
<td>LV capillary density (/mm² *1000)</td>
<td>2.73 ±0.13</td>
<td>2.78 ±0.06</td>
<td>0.74</td>
</tr>
<tr>
<td>RV fibrosis (area%)</td>
<td>9.3 ±1.5</td>
<td>10.4 ±1.8</td>
<td>0.47</td>
</tr>
<tr>
<td>LV fibrosis (area%)</td>
<td>7.3 ±0.7</td>
<td>8.4 ±1.8</td>
<td>0.57</td>
</tr>
<tr>
<td>RV inflammation (CD45⁺-nuclei /mm²)</td>
<td>22.8 ±7.7</td>
<td>18.6 ±8.5</td>
<td>0.72</td>
</tr>
<tr>
<td>LV inflammation (CD45⁺-nuclei /mm²)</td>
<td>13.4 ±4.0</td>
<td>16.2 ±5.1</td>
<td>0.68</td>
</tr>
</tbody>
</table>

PA wall thickness, relative wall thickness of pulmonary arteries.
**Table S3: Disease progression, assessed by serial echocardiography**

<table>
<thead>
<tr>
<th></th>
<th>Control (n=8)</th>
<th>PH (n=7)</th>
<th>PH+biso (n=7)</th>
<th>p-value</th>
<th>Control vs. PH</th>
<th>PH vs. PH+biso</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔPAAT/cl (%/day)</td>
<td>0.0 ±0.2</td>
<td>-4.0 ±0.7</td>
<td>-2.9 ±0.5</td>
<td>&lt;0.001</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>ΔRV wall thickness (%/day)</td>
<td>0.2 ±0.1</td>
<td>2.8 ±0.3</td>
<td>2.6 ±0.5</td>
<td>&lt;0.001</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>ΔRV end-diastolic diameter (%/day)</td>
<td>0.2 ±0.1</td>
<td>10.0 ±1.9</td>
<td>6.0 ±0.5</td>
<td>&lt;0.001</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>ΔTAPSE (%/day)</td>
<td>0.2 ±0.1</td>
<td>-6.2 ±0.8</td>
<td>-2.1 ±0.6</td>
<td>&lt;0.001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>ΔCardiac output (%/day)</td>
<td>0.2 ±0.1</td>
<td>-8.0 ±0.8</td>
<td>-3.9 ±0.6</td>
<td>&lt;0.001</td>
<td>0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ΔHeart rate</td>
<td>-0.1 ±0.0</td>
<td>-3.2 ±0.5</td>
<td>-1.4 ±0.4</td>
<td>&lt;0.001</td>
<td></td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ΔStroke volume</td>
<td>0.3 ±0.2</td>
<td>-6.8 ±0.6</td>
<td>-3.2 ±0.4</td>
<td>&lt;0.001</td>
<td></td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

ΔPAAT/cl, daily percentage change in pulmonary artery acceleration time normalized for cardiac cycle length; ΔRV wall thickness, daily percentage change in right ventricular wall thickness; ΔRV end-diastolic diameter, daily percentage change in right ventricular end-diastolic diameter; ΔTAPSE, daily percentage change in tricuspid annular plane systolic excursion; Δcardiac output, daily percentage change in cardiac output; ΔHeart rate, daily percentage change in heart rate; ΔStroke volume, daily percentage change in stroke volume; PH, PH-rats (treated with vehicle); PH+biso: PH-rats treated with 10 mg/kg bisoprolol once daily from day 10.
Table S4: Autopsy data

<table>
<thead>
<tr>
<th></th>
<th>Control (n=8)</th>
<th>PH (n=7)</th>
<th>PH+biso (n=7)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass (g)</td>
<td>337 ±6</td>
<td>234 ±4</td>
<td>245 ±5</td>
<td>&lt;0.001 0.13</td>
</tr>
<tr>
<td>BMchange (%/2d)</td>
<td>1.4 ±0.2</td>
<td>-6.1 ±0.9</td>
<td>-6.5 ±0.7</td>
<td>&lt;0.001 0.63</td>
</tr>
<tr>
<td>Tibia length (mm)</td>
<td>36.5 ±0.4</td>
<td>32.1 ±0.4</td>
<td>32.7 ±0.5</td>
<td>&lt;0.001 0.35</td>
</tr>
<tr>
<td>Lungs/tl (g/mm*1000)</td>
<td>33.6 ±1.1</td>
<td>66.8 ±3.0</td>
<td>68.3 ±5.4</td>
<td>&lt;0.001 0.76</td>
</tr>
<tr>
<td>Lung wet/dry ratio</td>
<td>4.7 ±0.1</td>
<td>4.6 ±0.1</td>
<td>4.4 ±0.1</td>
<td>0.84 0.23</td>
</tr>
<tr>
<td>Heart/tl (g/mm*1000)</td>
<td>34.6 ±0.8</td>
<td>39.0 ±1.4</td>
<td>37.7 ±1.6</td>
<td>&lt;0.05 0.47</td>
</tr>
<tr>
<td>RV mass/tl (g/mm*1000)</td>
<td>5.2 ±0.3</td>
<td>8.8 ±0.3</td>
<td>8.9 ±0.4</td>
<td>&lt;0.001 0.80</td>
</tr>
<tr>
<td>LV mass/tl (g/mm*1000)</td>
<td>21.9 ±0.7</td>
<td>16.9 ±0.6</td>
<td>16.0 ±0.6</td>
<td>&lt;0.001 0.39</td>
</tr>
<tr>
<td>RV / (LV+S)</td>
<td>0.24 ±0.02</td>
<td>0.53 ±0.03</td>
<td>0.56 ±0.02</td>
<td>&lt;0.001 0.39</td>
</tr>
<tr>
<td>Liver/tl (g/mm*1000)</td>
<td>367 ±8</td>
<td>249 ±10</td>
<td>271±11</td>
<td>&lt;0.001 0.13</td>
</tr>
<tr>
<td>Spleen/tl (g/mm*1000)</td>
<td>17.5 ±0.5</td>
<td>17.4 ±1.7</td>
<td>16.5 ±1.4</td>
<td>0.93 0.63</td>
</tr>
<tr>
<td>Kidneys/tl (g/mm*1000)</td>
<td>60.7 ±1.6</td>
<td>50.0 ±2.0</td>
<td>52.4 ±1.7</td>
<td>&lt;0.001 0.36</td>
</tr>
</tbody>
</table>

BMchange, percentage change in body mass of the last 2 days; …/tl, organ mass normalized for tibia length; RV / (LV+S), RV-to-LV (including septum) mass ratio.
<table>
<thead>
<tr>
<th></th>
<th>Control (n=8)</th>
<th>PH (n=7)</th>
<th>PH+biso (n=7)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA wall thickness (%)</td>
<td>7.5 ±0.4</td>
<td>40.2 ±2.1</td>
<td>38.5 ±1.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RV cross-sectional area (μm²)</td>
<td>322 ±16</td>
<td>540 ±23</td>
<td>553 ±41</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LV cross-sectional area (μm²)</td>
<td>488 ±16</td>
<td>377 ±18</td>
<td>393 ±18</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LV capillary density (/mm² *1000)</td>
<td>2.71 ±0.09</td>
<td>3.49 ±0.24</td>
<td>3.12 ±0.32</td>
<td>0.11</td>
</tr>
<tr>
<td>LV inflammation (CD45⁺-nuclei/mm²)</td>
<td>28.7 ±3.0</td>
<td>59.5 ±6.7</td>
<td>53.6 ±5.7</td>
<td>0.01</td>
</tr>
<tr>
<td>LV fibrosis (area %)</td>
<td>8.4 ±0.6</td>
<td>11.3 ±0.1</td>
<td>10.8 ±0.8</td>
<td>0.01</td>
</tr>
</tbody>
</table>

PA wall thickness, relative wall thickness of pulmonary arterioles.