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Survivin Modulation in Experimental Pulmonary Arterial Hypertension

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Survivin Modulation in Experimental Pulmonary Arterial Hypertension

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Running head: Survivin Modulation in Pulmonary Hypertension.

Abstract

Pharmacological manipulation of survivin seems to be an attractive option in the treatment of several conditions of pathological remodeling. In the present study, we characterized *in vivo* effects of a pharmacological inhibitor of survivin, terameprocol (TMP), in the monocrotaline (MCT) model of PAH. Adult male Wistar rats received a subcutaneous injection of MCT (60 mg/Kg) or equal volume of vehicle. Rats injected with MCT were treated with TMP (166 mg/Kg, ip; MCT-TMP, n=15) or vehicle (MCT-V, n=15) on days 7, 12 and 17 after injection and compared with SHAM animals, treated with either TMP (SHAM-TMP, n=10) or vehicle (SHAM-V, n=10). TMP treatment reduced RV hypertrophy and pulmonary arterial wall thickness, decreased RV peak systolic pressure, dP/dt_{max} and dP/dt_{min} and normalized cardiac output, thereby reversing the pathological phenotype induced by MCT injection. Our findings suggest an important pathological role of survivin in PAH development. Furthermore, terameprocol could be an effective and highly selective therapeutic strategy for PAH by reversing cardiac and pulmonary remodeling and improving hemodynamic features.

Keywords

Pulmonary arterial hypertension; pulmonary heart disease; apoptosis; survivin; terameprocol.

Introduction

The vascular obstruction seen in Pulmonary arterial hypertension (PAH) results from a combination of increased pulmonary vasoconstriction, abnormal vascular remodeling and *in situ* thrombosis of pulmonary vessels, affecting all vessel layers [40]. Obstructive vascular remodeling is characterized by increased cellular proliferation and resistance to apoptosis in both intima and media and is now thought to be the major cause of increased pulmonary vascular resistance in PAH. [3, 40].

Survivin is a multifunctional protein, involved in the control of mitosis, the regulation of apoptosis and the cellular stress response, globally promoting proliferation and apoptosis-resistance [2, 11, 44] . It is highly expressed during embryonic and fetal development and in most liquid and solid tumours [23, 25, 33, 45], but it is almost undetectable in most differentiated tissues in the absence of stress conditions. Moreover, survivin is an unfavourable prognostic marker in several malignancies, correlated with decreased overall survival [33]. Survivin has also been studied in other conditions of pathological remodeling, including in vascular injury after angioplasty or vein bypass graft surgery [13, 17]. In the pulmonary vasculature, survivin was found to be expressed in the PAs of patients with and several animal models of PAH [14, 28, 30, 46, 51]. Additionally, gene therapy targeting survivin reversed established MCT-induced PAH [30]. Pharmacological manipulation of survivin seems an attractive option. Terameprocol (tetra-O-methyl nordihydoguaiaretic acid, M4N or EM-1421) is an inhibitor of surivivin gene expression by binding the transcription factor Sp1 [12, 16, 20]. Its efficacy has been established both *in vitro* and *in vivo*, displaying increased tumor cell apoptosis and growth arrest with decreased tumor growth rates as well as a direct tumoricidal activity [12, 16, 20, 36, 43]. So far, no relevant systemic toxicity has been reported with terameprocol treatment.

In the present study, we aim to characterize survivin role in the pathophysiology of PAH induced by MCT by abrogation of survivin expression with its pharmacological inhibitor, terameprocol.

Material and Methods

Chemical and Drugs

MCT and dimethylsulfoxide (DMSO) were from Sigma (Barcelona, Spain). TMP was from Cayman Chemical (Michigan, USA). TMP was dissolved in DMSO.

Experimental Design

Animal experiments were performed according to the Portuguese law for animal welfare and conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 2011). Adult male Wistar Han rats (Charles River Laboratories, Barcelona, Spain) weighing 180-200 g were housed in groups of 5 animals/cage, in a controlled environment under a 12:12-h light-dark cycle at a room temperature of 22ºC, with free supply of food and water. Rats randomly received a subcutaneous injection of MCT (60 mg/kg) (MCT groups, n=30) or an equal volume of vehicle (1 mL/kg of 0.9% NaCl) (SHAM groups, n=20). At day seven after MCT or vehicle injection, DMSO (1mL/Kg, n=25) or TMP (166 mg/Kg, n=25) were administered intraperitoneally and at every five days until hemodynamic evaluation (21 days after MCT or vehicle injection). The time point for DMSO or TMP administration was based on a preliminary study, thus coinciding with the first evidence of survivin expression in pulmonary hypertensive animals. Four experimental groups were thus created: i) SHAM injected with DMSO (n=10); ii) SHAM injected with TMP (n=10); iii) MCT injected with DMSO (n=15); iv) MCT injected with TMP (n=15).

Hemodynamic Analysis and Tissue Sampling

Animals were anesthetized by inhalation of mixture of sevoflurane (4%) and oxygen, intubated for mechanical ventilation (Dual Mode, Kent Scientific, Connecticut, USA) and placed over a heating pad (body temperature was maintained at 37°C). Under binocular surgical microscopy (Wild M651.MS-D, Leica; Herbrugg, Switzerlad), the right jugular vein was cannulated for fluid administration (prewarmed 0.9% NaCl solution) to compensate for perioperative losses.

The heart was exposed by a median sternotomy and the pericardium was widely opened. Bi-ventricular hemodynamic function was measured with pressure-volume (PV) catheters (PVR-1045 for RV and PVR-1035 for LV, Millar instruments, Houston, USA). Data was continually acquired (MPVS 300, Millar Instruments, Houston, USA) and digitally recorded at 1000Hz (ML880 PowerLab 16/30, Millar TM instruments, Houston, USA). After complete instrumentation, the animal preparation was allowed to stabilize for 15 min. Hemodynamic recording was made under basal conditions and under vena cava, ascending aorta or pulmonary artery occlusion with respiration suspended at end-expiration. Heart rate (HR), RV and LV peak systolic pressure (P_{max}), end-diastolic pressure (EDP), peak rate for pressure rise (dP/dt_{max}), peak rate of pressure decline (dP/dt_{min}), constant time of isovolumetric pressure decline (Tau), ejection fraction (EF), cardiac output (CO) and maximal elastance (Ea) were obtained and analysed using PVAN 3.5 and LabChart 7.0 (Millar Instruments, Houston, USA). After complete hemodynamic assessment, animals were euthanized by exsanguination under anesthesia. The heart (H), lungs (L) and right *gastrocnemius* muscle were excised and weighted. The right tibia was also excised and its length was measured with a millimetric ruler. Under binocular magnification (x3.5, Wild M651.MS-D, Leica; Herbrugg, Switzerlad), the RV free wall was dissected from the left ventricle + septum (S) and weighted separately. Heart, lungs, RV and LV+S weights were normalized to body weight (BW) and *gastrocnemius* weight was normalized to tibial length. Samples from heart and lung were fixed and included in paraffin for light microscopy, or frozen with liquid nitrogen for molecular studies.

Morphometric Analysis

Samples of RV, LV (midway between the apex and base) and lung were fixed in 4% (v/v) buffered paraformaldehyde and included in paraffin blocks. Serial sections (4 μm of thickness) of paraffin blocks were cut and stained for haematoxylin-eosin. Studied samples were observed at light microscopy (Dialux 20, Leitz, Wetzlar, Germany), photographed with a digital camera (XC30, Olympus, California, USA) and measured with a digital image analyzer (cell^B life science basic imaging software, Olympus, California, USA). Five images of random

microscopic fields (magnification of x400) were obtained from each section to compensate for variations within sections. Only round to ovoid muscle fibers with a nuclear profile were counted to measure the cardiomyocytes surface area (CSA) with 50 cardiomyocytes analyzed per animal. On pulmonary specimens, external diameter and medial wall thickness in muscular arteries (20-25 arteries/animal) were analyzed.

Statistical Analysis

Statistical analysis was performed using Graph Pad Prism software (version 5.0, Graph Pad software, California, USA). All data are presented as mean ± SEM and were compared using Two Way ANOVA. When treatments were significantly different, Students-Newman Keuls post-hoc test was selected to perform pairwise multiple comparisons. Results were considered significantly different when $p<0.05$.

Results

Morphometric Analysis

The effects of TMP on the morphometric progression of MCT-induced PAH are summarized in Table 1. MCT-treated animals exhibited lower body weight when compared with the Sham+Vehicle group, that was not altered by treatment with TMP.

In the lung, MCT increased L/BW ratio, which was attenuated by TMP administration (Figure 1B). Pulmonary remodeling was also evident at the histological level, with increased medial hypertrophy of small caliber pulmonary arteries in MCT+Vehicle treated animals (Figures 1A and 1C). TMP targeted the abnormal pulmonary remodeling, reducing medial hypertrophy in the MCT-treated group (Figures 1A and 1C). No significant differences were noted in the Sham groups.

Regarding cardiac remodeling, MCT treatment induced cardiac hypertrophy, as evidenced by increased heart weight (HW) to BW ratio in the MCT+vehicle group. Cardiac hypertrophy was most likely due to RV hypertrophy, since RV/LV+S weight and RV/BW ratios were also found to be increased (Figure 2A). Treatment with TMP abrogated MCT-induced cardiac hypertrophy, resulting in normalization of HW/BW, RV/BW and RV/LV+S weight ratios (Figure 2A).

These results were further confirmed by histological analysis of the RV (Figure 2B). Cardiomyocyte cross-sectional area (CSA) was increased in MCT+vehicle animals, supporting the macroscopic finding of RV hypertrophy. Likewise, treatment with TMP resulted in a significant reduction of cardiomyocyte CSA, with no statistically significant difference between MCT+TMP treated animals and controls.

Hemodynamic evaluation

Table 2. summarizes the results from the bi-ventricular hemodynamic evaluation.

RV function was markedly impaired in MCT+vehicle treated animals. In this group, RV systolic dysfunction in connection with increased RV afterload was suggested by the combination of increased dP/dt_{max}, dP/dt_{min}, Ea and P_{max} and decreased EF and CO (Figures 3A and 3B). TMP administration normalized these parameters, proving to be beneficial in the regularization of RV strain and RV systolic impairment (Figures 3A and 3B). Diastolic function was also compromised, as evidenced by increased EDP and Tau time constant in the MCT+vehicle group, which was also ameliorated by TMP treatment. No significant changes were noted in the hemodynamic evaluation of the LV.

Discussion

In the present study, we have demonstrated that inhibiting survivin throughout the early stages of MCT-induced PAH prevents the complete development of the pulmonary and cardiac remodeling as well as the RV dysfunction that characterizes the disease. Our findings thus indicate that survivin upregulation is an essential maladaptive phenomenon that contributes to the full establishment of PAH pathology.

Traditionally, PAH was considered a disease of excess vasoconstriction [3] but the observation that more than 85% of patients are unresponsive to current available vasodilator therapies at the time of diagnosis has challenged this view [47]. Abnormal vascular remodeling affecting all vessel layers, on the other hand, has emerged as the major contributor to disease initiation and progression [47]. Several mechanisms have been implicated in the switch from a quiescent state to a proliferative, apoptosis-resistant cellular phenotype, including: loss-of-function mutations in bone morphogenetic protein receptor 2 (BMPR2) [21]; upregulation of growth factors, [22, 37, 41, 42] and dysregulation of mediators of apoptosis, such as bcl-2 or survivin [15, 30, 39]. Several studies are currently trying to identify which of these mechanisms are amenable to therapeutic modulation, with encouraging results [7, 18, 24, 35, 41].

Survivin presents unique advantages over these candidate modulators of PAH; as the paradigm of a "nodal protein" survivin is involved in multiple cell signalling circuits, thereby representing a central player in a non-redundant network for the maintenance of disordered proliferation and apoptosis [1]. This central role of survivin is highlighted by its upregulation by several growth factors [14, 17, 28, 49], vasoactive molecules [14, 19, 28, 34] and inflammatory cytokines [14, 46], providing further support for survivin as a downstream effector in the signalling cascades of various neurohormones and other modulators of PA cell proliferation and apoptosis.

These "survivin networks" may also provide a new insight into RV dysfunction in PAH. Bogaard et al. [6] brought into question the commonly held concept that RV failure is due strictly to the increased RV afterload by comparing a RV pressure overload model with an

established model of angioproliferative PAH and consistently demonstrating features of right heart failure in the latter but not the former model. Two important results from a previous unpublished study from our group also support this notion: 1) RV survivin expression preceded the hemodynamic manifestations of PAH, including the increase in PA systolic pressure, by 7 days, coinciding with the onset of cardiomyocyte hypertrophy determined by histological analysis; 2) LV survivin expression was also increased when compared to control animals. We thus hypothesize that the release of neurohormones and paracrine factors from the injured and remodeling PA is the initiating mechanism of RV failure in PAH, by inducing RV cardiomyocyte survivin upregulation and the consequent switch to a hypertrophic, apoptosis-resistant cellular phenotype. The concomitant increase in RV afterload would aggravate RV dysfunction eventually leading to RV failure [5, 10]. In accordance, Levkau et al. [27] observed significant load-dependence of survivin expression in the setting of left heart failure and showed that cardiac-specific deletion of survivin resulted in left heart failure and premature cardiac death. Survivin also appears to be protective after acute myocardial infarction, in doxorubicin-induced cardiomyopathy and in the failing heart of aged spontaneously hypertensive rats, despite being selectively expressed in diseased animals [26, 29, 32] . Thus, it seems that survivin upregulation is an expected and adaptive response to injury, contributing to injury repair. Continued injury and cellular stress as well as its related signalizing molecules lead to maintained survivin overexpression and its deleterious, maladaptive effects, such as the pathological PA and RV remodeling in PAH.

Survivin antagonists might present an unique therapeutic opportunity in PAH: they may function not as single protein inhibitors but, in fact, as broader pathway inhibitors suitable for disabling multiple downstream signalling cascades within PA cells [1] and they may target not only the pathological remodeling of PAs but also act directly on the RV to abrogate the hypertrophic profile of RV cardiomyocytes. In the present study, the pharmacological inhibitor of survivin terameprocol was administered throughout the progression of MCT-induced PAH and we are thus unable to conclude on its therapeutic potential other than as a preventive

strategy. Unfortunately, most patients present with advanced disease, limiting the direct clinical implications of this study. Nevertheless, our current results merit additional investigation into the possible use of terameprocol in established PAH.

Further studies are also warranted to better delineate the survivin axis in PAH, including identification of the neurohumoral and paracrine mediators of survivin expression with various candidate molecules mentioned above. Of special note, mitochondrial-metabolic abnormalities are emerging as an unifying mechanism for the pathological remodeling of PAH, in accordance with previous observations in cancer [4, 9, 50]. In both PAH and cancer, mitochondrial hyperpolarization and disordered mitochondrial metabolism and redox signalling lead to a pseudohypoxic redox state characterized by normoxic decreases in reactive oxygen species, a shift from oxidative to glycolytic metabolism, HIF-1α activation and decreased Kv1.5 K+ channel expression [4, 8, 38]. Therapies targeting these mitochondrial abnormalities have provided good outcomes in a pre-clinical level [29, 32]. Survivin function in mitochondrial biology suggests a likely interaction with this mechanism.

Lastly, it is also important to note that in spite of being a classic and thoroughly studied model of PAH, MCT-induced PAH does not fully mimic human pathology [4, 31]. Limitations of MCT models include lack of neointimal thickening and plexiform lesions and a rapid time course for disease development [48]. Newer models have been developed, e.g. the SU5416 and hypoxia, IL-6 overexpression or the fawn-hooded rat, some with better correlation with human disease. Notwithstanding these advances, no preclinical model completely recapitulates human PAH at present [48].

In conclusion, we have shown that pharmacological suppression of the survivin pathway attenuated the pathological and hemodynamic phenotype of MCT-induced PAH thereby establishing survivin's crucial role in the pathophysiology of PAH. Furthermore, we have unraveled a potential, highly selective therapeutic strategy in the treatment of PAH, capable of

effectively halting progression of the disease and providing an opportunity to alter its still irrevocable natural history.

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References

1. **Altieri DC**. Survivin, cancer networks and pathway-directed drug discovery. *Nat Rev Cancer* 8: 61-70, 2008.
2. **Altieri DC**. Targeting survivin in cancer. *Cancer Lett* 332: 225-8, 2013.
3. **Archer SL, Weir EK,Wilkins MR**. Basic science of pulmonary arterial hypertension for clinicians: new concepts and experimental therapies. *Circulation* 121: 2045-66, 2010.
4. **Archer SL, Gomberg-Maitland M, Maitland ML, Rich S, Garcia JG,Weir EK**. Mitochondrial metabolism, redox signaling, and fusion: a mitochondria-ROS-HIF-1alpha-Kv1.5 O2-sensing pathway at the intersection of pulmonary hypertension and cancer. *Am J Physiol Heart Circ Physiol* 294: H570-8, 2008.
5. **Bogaard HJ, Abe K, Vonk Noordegraaf A,Voelkel NF**. The right ventricle under pressure: cellular and molecular mechanisms of right-heart failure in pulmonary hypertension. *Chest* 135: 794-804, 2009.
6. **Bogaard HJ, Natarajan R, Henderson SC, Long CS, Kraskauskas D, Smithson L, Ockaili R, McCord JM,Voelkel NF**. Chronic pulmonary artery pressure elevation is insufficient to explain right heart failure. *Circulation* 120: 1951-60, 2009.
7. **Bonnet S, Rochefort G, Sutendra G, Archer SL, Haromy A, Webster L, Hashimoto K, Bonnet SN,Michelakis ED**. The nuclear factor of activated T cells in pulmonary arterial hypertension can be therapeutically targeted. *Proc Natl Acad Sci U S A* 104: 11418-23, 2007.
8. **Bonnet S, Michelakis ED, Porter CJ, Andrade-Navarro MA, Thebaud B, Bonnet S, Haromy A, Harry G, Moudgil R, McMurtry MS, Weir EK,Archer SL**. An abnormal mitochondrial-hypoxia inducible factor-1alpha-Kv channel pathway disrupts oxygen sensing and triggers pulmonary arterial hypertension in fawn hooded rats: similarities to human pulmonary arterial hypertension. *Circulation* 113: 2630-41, 2006.

9. **Bonnet S, Archer SL, Allalunis-Turner J, Haromy A, Beaulieu C, Thompson R, Lee CT, Lopaschuk GD, Puttagunta L, Bonnet S, Harry G, Hashimoto K, Porter CJ, Andrade MA, Thebaud B,Michelakis ED**. A mitochondria-K+ channel axis is suppressed in cancer and its normalization promotes apoptosis and inhibits cancer growth. *Cancer Cell* 11: 37-51, 2007.

10. **Bronicki RA,Baden HP**. Pathophysiology of right ventricular failure in pulmonary hypertension. *Pediatr Crit Care Med* 11: S15-22, 2010.

11. **Ceballos-Cancino G, Espinosa M, Maldonado V,Melendez-Zajgla J**. Regulation of mitochondrial Smac/DIABLO-selective release by survivin. *Oncogene* 26: 7569-75, 2007.

12. **Chang CC, Heller JD, Kuo J,Huang RC**. Tetra-O-methyl nordihydroguaiaretic acid induces growth arrest and cellular apoptosis by inhibiting Cdc2 and survivin expression. *Proc Natl Acad Sci U S A* 101: 13239-44, 2004.

13. **Conte MS,Altieri DC**. Survivin regulation of vascular injury. *Trends Cardiovasc Med* 16: 114-7, 2006.

14. **Courboulin A, Tremblay VL, Barrier M, Meloche J, Jacob MH, Chapolard M, Bisserier M, Paulin R, Lambert C, Provencher S,Bonnet S**. Kruppel-like factor 5 contributes to pulmonary artery smooth muscle proliferation and resistance to apoptosis in human pulmonary arterial hypertension. *Respir Res* 12: 128, 2011.

15. **Geraci MW, Moore M, Gesell T, Yeager ME, Alger L, Golpon H, Gao B, Loyd JE, Tuder RM,Voelkel NF**. Gene expression patterns in the lungs of patients with primary pulmonary hypertension: a gene microarray analysis. *Circ Res* 88: 555-62, 2001.

16. **Heller JD, Kuo J, Wu TC, Kast WM,Huang RC**. Tetra-O-methyl nordihydroguaiaretic acid induces G2 arrest in mammalian cells and exhibits tumoricidal activity in vivo. *Cancer Res* 61: 5499-504, 2001.

17. **Hoel AW, Wang GJ, Simosa HF,Conte MS**. Regulation of vascular smooth muscle cell growth by survivin. *Vascular* 15: 344-9, 2007.

18. **Hoeper MM, Barst RJ, Bourge RC, Feldman J, Frost AE, Galie N, Gomez-Sanchez MA, Grimminger F, Grunig E, Hassoun PM, Morrell NW, Peacock AJ, Satoh T, Simonneau G, Tapson VF, Torres F, Lawrence D, Quinn DA,Ghofrani HA**. Imatinib mesylate as add-on therapy for pulmonary arterial hypertension: results of the randomized IMPRES study. *Circulation* 127: 1128-38, 2013.

19. **Horowitz JC, Ajayi IO, Kulasekaran P, Rogers DS, White JB, Townsend SK, White ES, Nho RS, Higgins PD, Huang SK,Sisson TH**. Survivin expression induced by endothelin-1 promotes myofibroblast resistance to apoptosis. *Int J Biochem Cell Biol* 44: 158-69, 2012.

20. **Huang RC, Chang CC,Mold D**. Survivin-dependent and -independent pathways and the induction of cancer cell death by tetra-O-methyl nordihydroguaiaretic acid. *Semin Oncol* 33: 479-85, 2006.

21. **International PPHC, Lane KB, Machado RD, Pauciulo MW, Thomson JR, Phillips JA, 3rd, Loyd JE, Nichols WC,Trembath RC**. Heterozygous germline mutations in BMPR2, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension. *Nat Genet* 26: 81-4, 2000.

22. **Izikki M, Guignabert C, Fadel E, Humbert M, Tu L, Zadigue P, Dartevelle P, Simonneau G, Adnot S, Maitre B, Raffestin B,Eddahibi S**. Endothelial-derived FGF2 contributes to the progression of pulmonary hypertension in humans and rodents. *J Clin Invest* 119: 512-23, 2009.

23. **Jeon C, Kim M, Kwak C, Kim HH,Ku JH**. Prognostic role of survivin in bladder cancer: a systematic review and meta-analysis. *PLoS One* 8: e76719, 2013.

24. **Klein M, Schermuly RT, Ellinghaus P, Milting H, Riedl B, Nikolova S, Pullamsetti SS, Weissmann N, Dony E, Savai R, Ghofrani HA, Grimminger F, Busch AE,Schafer S**. Combined tyrosine and serine/threonine kinase inhibition by sorafenib prevents progression of experimental pulmonary hypertension and myocardial remodeling. *Circulation* 118: 2081-90, 2008.

25. **Krieg A, Werner TA, Verde PE, Stoecklein NH,Knoefel WT**. Prognostic and clinicopathological significance of survivin in colorectal cancer: a meta-analysis. *PLoS One* 8: e65338, 2013.

26. **Lee PJ, Rudenko D, Kuliszewski MA, Liao C, Kabir MG, Connelly KA,Leong-Poi H**. Survivin gene therapy attenuates left ventricular systolic dysfunction in doxorubicin cardiomyopathy by reducing apoptosis and fibrosis. *Cardiovasc Res* 2014.

27. **Levkau B, Schafers M, Wohlschlaeger J, von Wnuck Lipinski K, Keul P, Hermann S, Kawaguchi N, Kirchhof P, Fabritz L, Stypmann J, Stegger L, Flogel U, Schrader J, Fischer JW, Hsieh P, Ou YL, Mehrhof F, Tiemann K, Ghanem A, Matus M, Neumann J, Heusch G, Schmid KW, Conway EM,Baba HA**. Survivin determines cardiac function by controlling total cardiomyocyte number. *Circulation* 117: 1583-93, 2008.

28. **Liu Y, Zhang J, Yi B, Chen M, Qi J, Yin Y, Lu X, Jasmin JF,Sun J**. Nur77 Suppresses Pulmonary Artery Smooth Muscle Cell Proliferation Through Inhibition of the STAT3/Pim-1/NFAT Pathway. *Am J Respir Cell Mol Biol* 2013.

29. **McMurtry MS, Bonnet S, Wu X, Dyck JR, Haromy A, Hashimoto K,Michelakis ED**. Dichloroacetate prevents and reverses pulmonary hypertension by inducing pulmonary artery smooth muscle cell apoptosis. *Circ Res* 95: 830-40, 2004.

30. **McMurtry MS, Archer SL, Altieri DC, Bonnet S, Haromy A, Harry G, Bonnet S, Puttagunta L,Michelakis ED**. Gene therapy targeting survivin selectively induces pulmonary vascular apoptosis and reverses pulmonary arterial hypertension. *J Clin Invest* 115: 1479-91, 2005.

31. **Meyrick B, Gamble W,Reid L**. Development of Crotalaria pulmonary hypertension: hemodynamic and structural study. *Am J Physiol* 239: H692-702, 1980.

32. **Michelakis ED, McMurtry MS, Wu XC, Dyck JR, Moudgil R, Hopkins TA, Lopaschuk GD, Puttagunta L, Waite R,Archer SL**. Dichloroacetate, a metabolic modulator, prevents and reverses chronic hypoxic pulmonary hypertension in rats: role

of increased expression and activity of voltage-gated potassium channels. *Circulation* 105: 244-50, 2002.

33. **Mita AC, Mita MM, Nawrocki ST,Giles FJ**. Survivin: key regulator of mitosis and apoptosis and novel target for cancer therapeutics. *Clin Cancer Res* 14: 5000-5, 2008.

34. **Ohashi H, Takagi H, Oh H, Suzuma K, Suzuma I, Miyamoto N, Uemura A, Watanabe D, Murakami T, Sugaya T, Fukamizu A,Honda Y**. Phosphatidylinositol 3-kinase/Akt regulates angiotensin II-induced inhibition of apoptosis in microvascular endothelial cells by governing survivin expression and suppression of caspase-3 activity. *Circ Res* 94: 785-93, 2004.

35. **Pankey EA, Thammasiboon S, Lasker GF, Baber S, Lasky JA,Kadowitz PJ**. Imatinib attenuates monocrotaline pulmonary hypertension and has potent vasodilator activity in pulmonary and systemic vascular beds in the rat. *Am J Physiol Heart Circ Physiol* 305: H1288-96, 2013.

36. **Park R, Chang CC, Liang YC, Chung Y, Henry RA, Lin E, Mold DE,Huang RC**. Systemic treatment with tetra-O-methyl nordihydroguaiaretic acid suppresses the growth of human xenograft tumors. *Clin Cancer Res* 11: 4601-9, 2005.

37. **Perros F, Montani D, Dorfmuller P, Durand-Gasselin I, Tcherakian C, Le Pavec J, Mazmanian M, Fadel E, Mussot S, Mercier O, Herve P, Emilie D, Eddahibi S, Simonneau G, Souza R,Humbert M**. Platelet-derived growth factor expression and function in idiopathic pulmonary arterial hypertension. *Am J Respir Crit Care Med* 178: 81-8, 2008.

38. **Rehman J,Archer SL**. A proposed mitochondrial-metabolic mechanism for initiation and maintenance of pulmonary arterial hypertension in fawn-hooded rats: the Warburg model of pulmonary arterial hypertension. *Adv Exp Med Biol* 661: 171-85, 2010.

39. **Sakao S, Taraseviciene-Stewart L, Lee JD, Wood K, Cool CD,Voelkel NF**. Initial apoptosis is followed by increased proliferation of apoptosis-resistant endothelial cells. *FASEB J* 19: 1178-80, 2005.

40. **Schermuly RT, Ghofrani HA, Wilkins MR,Grimminger F**. Mechanisms of disease: pulmonary arterial hypertension. *Nat Rev Cardiol* 8: 443-55, 2011.

41. **Schermuly RT, Dony E, Ghofrani HA, Pullamsetti S, Savai R, Roth M, Sydykov A, Lai YJ, Weissmann N, Seeger W,Grimminger F**. Reversal of experimental pulmonary hypertension by PDGF inhibition. *J Clin Invest* 115: 2811-21, 2005.

42. **Schultz K, Fanburg BL,Beasley D**. Hypoxia and hypoxia-inducible factor-1alpha promote growth factor-induced proliferation of human vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol* 290: H2528-34, 2006.

43. **Smolewski P**. Terameprocol, a novel site-specific transcription inhibitor with anticancer activity. *IDrugs* 11: 204-14, 2008.

44. **Song Z, Yao X,Wu M**. Direct interaction between survivin and Smac/DIABLO is essential for the anti-apoptotic activity of survivin during taxol-induced apoptosis. *J Biol Chem* 278: 23130-40, 2003.

45. **Stauber RH, Mann W,Knauer SK**. Nuclear and cytoplasmic survivin: molecular mechanism, prognostic, and therapeutic potential. *Cancer Res* 67: 5999-6002, 2007.

46. **Steiner MK, Syrkina OL, Kolliputi N, Mark EJ, Hales CA,Waxman AB**. Interleukin-6 overexpression induces pulmonary hypertension. *Circ Res* 104: 236-44, 28p following 244, 2009.

47. **Stenmark KR,Rabinovitch M**. Emerging therapies for the treatment of pulmonary hypertension. *Pediatr Crit Care Med* 11: S85-90, 2010.

48. **Stenmark KR, Meyrick B, Galie N, Mooi WJ,McMurtry IF**. Animal models of pulmonary arterial hypertension: the hope for etiological discovery and pharmacological cure. *Am J Physiol Lung Cell Mol Physiol* 297: L1013-32, 2009.

49. **Taeger J, Moser C, Hellerbrand C, Mycielska ME, Glockzin G, Schlitt HJ, Geissler EK, Stoeltzing O,Lang SA**. Targeting FGFR/PDGFR/VEGFR impairs tumor growth, angiogenesis, and metastasis by effects on tumor cells, endothelial cells, and pericytes in pancreatic cancer. *Mol Cancer Ther* 10: 2157-67, 2011.

50. **Voelkel NF, Cool C, Lee SD, Wright L, Geraci MW,Tuder RM**. Primary pulmonary hypertension between inflammation and cancer. *Chest* 114: 225S-230S, 1998.

51. **Yan J, Shen Y, Wang Y,Li BB**. Increased expression of hypoxia-inducible factor-1alpha in proliferating neointimal lesions in a rat model of pulmonary arterial hypertension. *Am J Med Sci* 345: 121-8, 2013.

Figure captions

Figure 1: Effect of terameprocol in pulmonary remodeling. Terameprocol attenuated the characteristic pulmonary remodeling of monocrotaline-treated animals evaluated by the histological appearance of small pulmonary arteries stained with hematoxylin and eosin (A), lung weight/body weight ratio (L/BW) (B) and percentage of medial hypertrophy of pulmonary arteries (C). Sham: Sham group, MCT: Monocrotaline group, TMP: Terameprocol. Data are mean±SEM. $^{\alpha}p < 0.05$ vs. Sham+V, $^{\beta}p < 0.05$ vs. Sham+TMP and $^{\gamma}p < 0.05$ vs. MCT+V.

Figure 2: Effect of terameprocol in right ventricular remodeling. Terameprocol reversed right ventricular hypertrophy in the monocrotaline groups evaluated by right ventricle/ body weight ratio (RV/BW) (A) and cardiomyocyte cross sectional area (CSA) (B). Sham: Sham group, MCT: Monocrotaline group, TMP: Terameprocol. Data are mean±SEM. $^{\alpha}p < 0.05$ vs. Sham+TMP, $^{\beta}p < 0.05$ vs. MCT+V.

Figure 3: Effect of terameprocol in right ventricular hemodynamics. Terameprocol normalized RV maximal systolic pressure (A) and RV cardiac output (B). P_{max}: maximal systolic pressure. CO: cardiac output. Data are mean±SEM. $^{\alpha}p < 0.05$ vs. Sham+V, $^{\beta}p < 0.05$ vs. Sham+TMP and $^{\gamma}p < 0.05$ vs. MCT+V.

Tables

Table 1: Effect of terameprocol in morphometric parameters.

	Sham		MCT	
	Vehicle	**TMP**	**Vehicle**	**TMP**
Body weight (g)	300.1 ± 8.2	268.6 ± 11.4	258.6 ± 5.6$^{\alpha}$	249.7 ± 8.0$^{\alpha}$
HW/BW (g/kg)	3.013 ± 0.103	2.734 ± 0.096	3.634 ± 0.317$^{\alpha,\beta}$	3.068 ± 0.176$^{\gamma}$
RV/(LV+S) (g/g)	0.3043 ± 0.0109	0.3401 ± 0.0299	0.4589 ± 0.0362$^{\alpha,\beta}$	0.3685 ± 0.0189$^{\gamma}$
(LV+S) /BW (g/Kg)	1.856 ± 0.052	1.771 ± 0.063	2.035 ± 0.081	1.837 ± 0.044
G/Tib (g/cm)	0.5200 ± 0.0147	0.4857 ± 0.0092	0.4951 ± 0.0138	0.4290 ± 0.0177

HW/BW: heart weight/body weight; RV/(LV+S): right ventricle/(left ventricle+septum); (LV+S)/BW: (left ventricle+septum)/body weight; G/Tib: gastrocnemius/tibia. Sham: Sham group; MCT: Monocrotaline group; TMP: Terameprocol. Data are mean±SEM. $^{\alpha}p < 0.05$ vs. Sham+Vehicle; $^{\beta}p < 0.05$ vs. Sham+TMP; $^{\gamma}p < 0.05$ vs. MCT+Vehicle.

Table 2: Effect of terameprocol in hemodynamic parameters.

	Sham		MCT	
	Vehicle	**TMP**	**Vehicle**	**TMP**
Heart rate (bpm)	380.5 ± 8.402	376.5 ± 8.425	365.4 ± 5.608	386.1 ± 7.373
RV Function				
dP/dt $_{max}$ (mmHg/sec)	1500 ± 26.55	1627 ± 65.47	3450 ± 468.6$^{\alpha\beta}$	2107 ± 83.22$^{\gamma}$
dP/dt $_{min}$ (mmHg/sec)	-1485 ± 151.4	-1325 ± 18.62	-2903 ± 165.4 $^{\alpha\beta}$	-1763 ± 27.86 β $^{\gamma}$
EF (%)	65.42 ± 5.536	70.04 ± 3.858	29.41 ± 4.383 $^{\alpha\beta}$	70.56 ± 4.902 $^{\gamma}$
EDP (mmHg)	2.951 ± 0.1836	3.561 ± 0.4696	6.424 ± 1.137 $^{\alpha\beta}$	3.165 ± 0.6209 $^{\gamma}$
Ea (mmHg/µL)	0.1528 ± 0.0115	0.1468 ± 0.0079	0.7701 ± 0.1588 $^{\alpha\beta}$	0.2661 ± 0.0372 $^{\gamma}$
Tau (ms)	10.84 ± 0.7640	10.43 ± 0.7636	15.96 ± 0.3210 $^{\alpha\beta}$	9.229 ± 0.7491 $^{\gamma}$
LV Function				
P_{max} (mmHg)	84.89 ± 5.706	94.14 ± 1.525	97.17 ± 4.977	94.66 ± 3.743
dP/dt $_{max}$ (mmHg/sec)	5582 ± 460.5	6517 ± 297.9	5724 ± 217.8	5518 ± 321.0
dP/dt $_{min}$ (mmHg/sec)	-5180 ± 888.4	-6785 ± 430.0	-5242 ± 458.3	-6434 ± 380.8
EF (%)	73.82 ± 3.931	72.89 ± 4.410	71.47 ± 2.945	68.72 ± 3.969
EDP (mmHg)	5.427 ± 1.538	4.376 ± 1.272	4.531 ± 0.7069	6.576 ± 1.885
Ea (mmHg/µL)	0.4007 ± 0.076	0.4911 ± 0.0645	0.7408 ± 0.1039	0.7330 ± 0.1712
Tau (ms)	8.609 ± 0.3356	9.136 ± 0.8681	9.015 ± 0.5702	9.754 ± 0.6173

Sham: Sham group, MCT: Monocrotaline group. TMP: Terameprocol; P_{max}: maximum pressure; dP/dt_{max}: peak rate of pressure rise; dP/dt_{min}: peak rate of pressure fall; EF: Ejection fraction; EDP: end-diastolic pressure; Ea: arterial elastance; Tau: time constant of ventricular decay. Data are mean±SEM. $^{\alpha}p < 0.05$ vs. Sham+Vehicle; $^{\beta}p < 0.05$ vs. Sham+TMP; $^{\gamma}p < 0.05$ vs. MCT+Vehicle.

Figure 1.

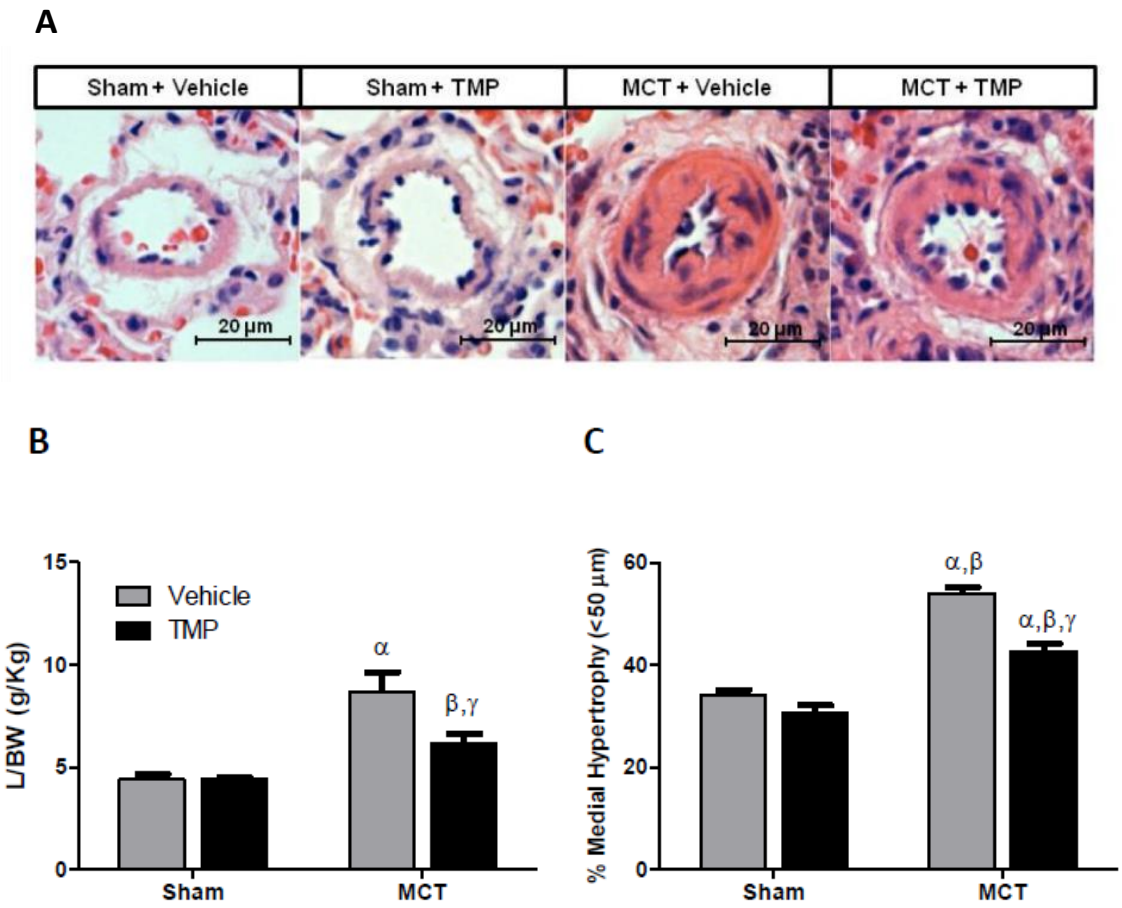
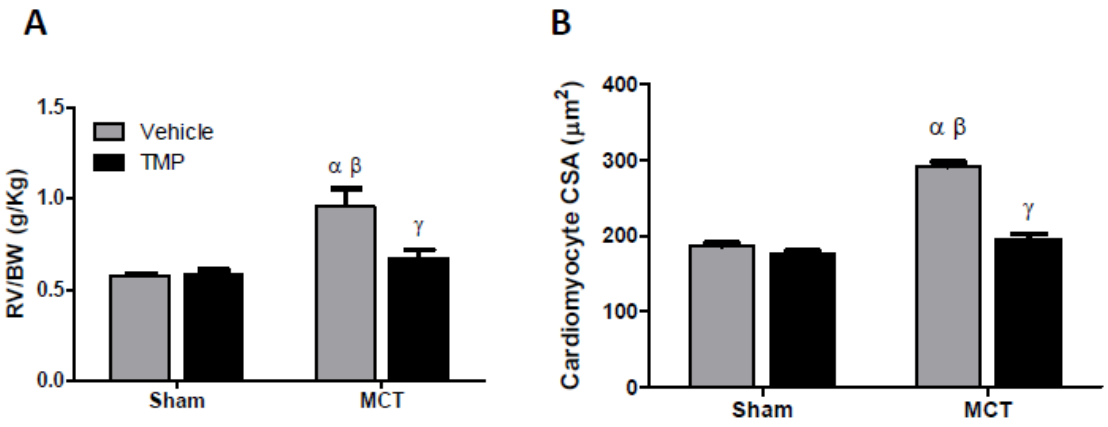

A
Sham + Vehicle
Sham + TMP
MCT + Vehicle
MCT + TMP
20 µm
B
L/BW (g/Kg)
Vehicle
TMP
α
β,γ
Sham
MCT
C
% Medial Hypertrophy (<50 µm)
α,β
α,β,γ
Sham
MCT

Figure 2.


A
1.5
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0.5
0.0
RV/BW (g/Kg)
Vehicle
TMP
α β
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Sham
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400
300
200
100
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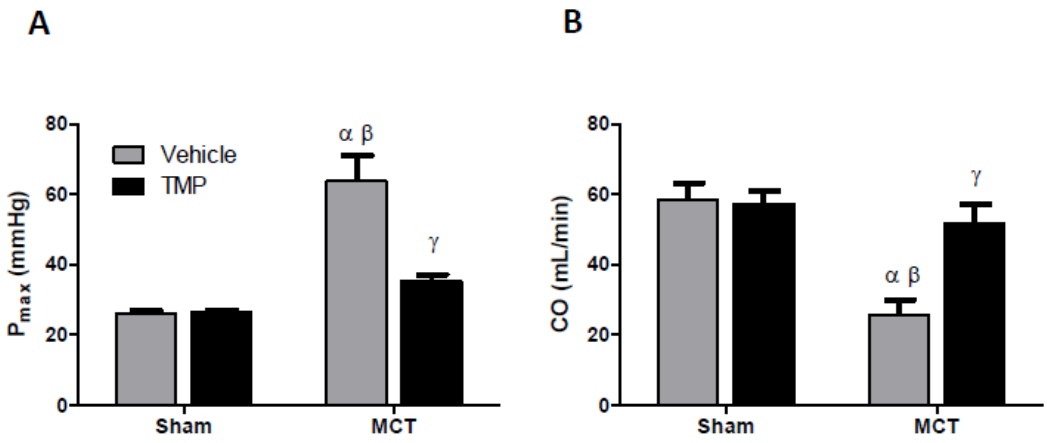
Cardiomyocyte CSA (μm²)
α β
γ
Sham
MCT

Figure 3.



Agradecimentos

A presente dissertação e o trabalho experimental que lhe serviu de base dependeram da colaboração direta de várias pessoas, às quais dirijo as seguintes palavras de agradecimento. Ao meu orientador, Professor Tiago Henriques-Coelho, que ao iniciar-me na investigação científica possibilitou o enriquecimento do meu percurso académico e que, ao longo dos anos, contribuiu para o desenvolvimento da minhas capacidades enquanto investigador e futuro médico. A Manuel Pinto, pelos diversos votos de confiança e pelo contínuo encorajamento. A Ana Filipa Silva, Joana Justino, Rita Ferreira e Joana Brandão, pelo apoio e companheirismo na execução do trabalho laboratorial. Por fim, não poderia deixar de agradecer aos meus pais pelo apoio incondicional e por me fornecerem as condições para cumprir os meus objetivos pessoais e profissionais.

ANEXO

Elaborado segundo as normas da revista: *American Journal of Physiology – Heart and Circulatory Physiology*

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APS has published an editorial on the use of statistics, and authors are encouraged to consult it.

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Acknowledgements

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- Each table should have a brief title; explanatory notes should be in the legend, not in the title.
- Nonsignificant decimal places in tabular data should be omitted.
- Short or abbreviated column heads should be used and explained if necessary in the legend.
- Statistical measures of variations, SD, SE, etc., must be identified. (Example: "Values are means ± SE.")
- Table footnotes should be listed in order of their appearance and identified by standard symbols: *, †, ‡, § for four or fewer; for five or more, consecutive superscript lowercase letters should be used (e.g., a, b, c, etc.).

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Mathematical equations should be simplified as much as possible and carefully checked.

- Use the slant line (/) for simple fractions $(a + b)/(x + y)$ in the text rather than the built-up fraction $a + b$[over]$x + y$, which should only be used if the equation is offset from the text.
- Use subscripts or superscripts wherever feasible and appropriate to simplify the equations.
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- If the model equation(s) require solution, the method of solution should be described in sufficient detail to permit readers to duplicate the solution in their own laboratories. Algorithms from commercial software libraries should be so identified. Details of the solution strategy may be summarized in an **Appendix**.
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important parameters (covering ranges of values relevant to the manuscript) to determine how the model predictions are affected by numerical parameter values.

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