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Reversal effects of low-dose imatinib compared with sunitinib on monocrotaline-induced pulmonary and right ventricular remodeling in rats

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ABSTRACT

High-dose imatinib reverses cardiopulmonary remodeling but adverse effects limit its clinical use. Efficacy of the multi-kinase inhibitor sunitinib remains questionable. We compared anti-remodeling effects of imatinib with sunitinib on monocrotaline-induced right ventricular (RV) hypertrophy and pulmonary arterial remodeling in rats, focusing on a lower dose. Fourteen days after monocrotaline injection, oral gavage of imatinib (5, 15, or 50 mg/kg), sunitinib (0.3, 1, 3, or 10 mg/kg), or water for 14 days was started. RV hypertrophy and b-type natriuretic peptide mRNA levels were significantly and dose-dependently reduced, much greater in imatinib- than sunitinib-treated groups. Imatinib normalized muscularization of 20–50 μm intra-acinar pulmonary arteries more significantly than sunitinib. At transcript levels, sunitinib significantly upregulated pulmonary nestin, and downregulated platelet-derived growth factor receptor beta (PDGFR-β), fibroblast growth factor receptor 1, vascular endothelial growth factor receptor-2 and vascular endothelial growth factor (VEGF)-A, but not Raf-1 proto-oncogene serine/threonine kinase mRNAs. Sunitinib also suppressed VEGF-A, but not phosphorylated extra-cellular-signal-related kinase (ERK)-1/2 protein expression. The sole PDGFR-β antagonism of imatinib resulted in significant Raf-1 mRNA and phosphorylated ERK-1/2 protein downregulation, suggesting that the equivocal reversal effect of sunitinib may be due to its VEGF signaling inhibition in the lung. Imatinib's greater dose-dependent reversal on cardiopulmonary remodeling may make a low dose suitable for PAH treatment.

1. Introduction

Pulmonary arterial hypertension (PAH) entails small pulmonary artery proliferation and remodeling, resulting in a rise in mean pulmonary pressure ≥ 25 mmHg at rest [19] and eventually right-sided heart failure [25,36]. With a poor prognosis of reported survival rates of 58%, 41% and 24% in patients receiving a single therapy after 1, 2 and 3 years, respectively [33], dual or triple combination therapy targeting any of the three main mechanistic pathways (endothelin, nitric oxide, and prostacyclin) for PAH treatment improved the survival outcome [17–20,33,48–49]. However, long-term prognosis of PAH treatment remains poor [13,39].

Role of mitogen-activated protein kinase (MAPK) pathway in pathogenesis of PAH has been documented [31,46], which involves upstream signaling from various receptor tyrosine kinases (TK) such as platelet-derived growth factor receptor beta (PDGFR-β) [4,46,55], fibroblast growth factor receptor (FGFR)-1 [5,53,57], endothelial growth factor receptor (EGFR) [9], and C-Kit receptor [14,40]. Imatinib, a TK inhibitor, reverses pulmonary and myocardial remodeling in the rodent models [8,12,46] and significantly improves right ventricular (RV) functions in the PAH patients [21,47,50] and the Phase III Imatinib in Pulmonary Arterial Hypertension, A Randomized, Efficacy Study (IMPRES) [24]. However, an extension of the IMPRES revealed disappointing outcomes and adverse effects in imatinib-treated PAH patients [16]. In 2013, Novartis withdrew imatinib for PAH treatment.

To develop new therapies for PAH, sparing of the receptor TK pathways from the current treatment algorithm is our concern. Many experimental and clinical PAH studies did not investigate whether low-dose TK inhibitors are effective without producing associated deleterious effects. Our study on PAH dogs showed that a low-dose imatinib therapy for 30 days reduced pulmonary arterial pressure and improved cardiac function and hemodynamics [3]. Similarly, a clinical study using low-dose imatinib in PAH patients showed improved diffusion capacity of the lung for carbon monoxide (DLCO) and varying hemodynamic responses [23]. However, these clinical studies did not investigate anti-remodeling effects of the low-dose therapy on cardiopulmonary remodeling assessed at tissue and molecular levels. Thus, we revised the study to investigate several imatinib and sunitinib doses,

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emphasizing on the lowest anti-remodeling dose possible. We also aimed to compare the effects of imatinib with sunitinib on pulmonary and RV remodeling to determine whether imatinib yields a greater anti-remodeling effect than sunitinib.

2. Methods

2.1. Monocrotaline-induced pulmonary arterial remodeling

Eight-week-old, male, Wistar-Imamichi rats were purchased from the Institute for Animal Reproduction, Ibaraki, Japan, and randomized into control, placebo, and treatment groups. Under isoflurane anesthesia, monocrotaline (MCT, Sigma-Aldrich, China) was subcutaneously injected at 60 mg/kg body weight to the placebo and treatment rats to induce pulmonary arterial remodeling. Physiological saline solution was injected to the control rats. Fourteen days after the injection, oral gavage was started once daily, such that the treatment rats received imatinib mesylate [Glivec, Novartis: 5 (Ima-5), 15 (Ima-15), or 50 (Ima-50) mg/kg per day] or sunitinib malate [SUTENT®, Pfizer: 0.1 (Suni-0.1), 1 (Suni-1), 3 (Suni-3), or 10 (Suni-10) mg/kg per day], whereas the control and placebo rats received water. The oral gavage was continued for 14 days after which the rats were euthanized for tissue sampling. All protocols were approved by the Institutional Animal Care and Use Committee of the Tottori University.

2.2. Assessment of RV hypertrophy

The right ventricular (RV) tissue was separated from the left ventricle and septum (LV + S). RV and (LV + S) wet weights were determined to obtain the RV hypertrophy (RVH) index given by the formula: RV/(LV + S).

2.3. Pulmonary artery histology and pulmonary arterial muscularization assessment

The left lung lobe caudal to the bronchus was excised and fixed in 10% formalin neutral buffer solution. Lung tissues were outsourced to Sapporo General Pathology Laboratory Co. Ltd., Japan, for histology and slide preparation, as well as staining with elastic van Gieson (EVG) and double staining of EVG and alpha-smooth muscle actin (α-SMA) antibody. Under light microscopy (400 × magnification), intra-acinar artery (IA) images were captured by an Olympus Digital Camera DP21. Approximately 300 IAs (20–50 μm in diameter) were identified and counted as fully muscular (FMIA), partially muscular (PMIA), and non-muscular (NMIA) to obtain the proportion of each artery type and calculate muscularization percentage (Fig. 1A). For FMIAs between 20 and 50 μm and 51–100 μm in diameter, external diameter (d) and medial wall thickness (MWT) were measured using Image J software. In addition, medial MWT ratio given by MWT normalized to diameter ($2 \times MWT/d$), lumen diameter ($d - 2 \times MWT$), and lumen area [$3.142 \times (d/2)^2$] of the FMIAs were also determined.

2.4. ELISA measurement of serum N-terminal pro-brain natriuretic peptide (NT-proBNP) levels

Blood was sampled via cardiac puncture into plain blood tubes. Serum was obtained after centrifuging the clotted blood at 3500 rpm for 5 min (Kubota 4000, Japan). Enzyme-linked immunosorbent assay (ELISA) for rat serum NT-proBNP was performed using a commercial kit (Cloud-Clone Corp., Wuhan, China), in accordance to the manufacturer's protocol. Absorbance was read at 450 nm by an iMark™ microplate reader (BIORAD, Japan). A standard curve was constructed to give readings of serum NT-proBNP levels (ng/mL).

2.5. RNA extraction, reverse transcription, and semi-quantitative fast real-time polymerase chain reaction

The RV tissue and the right caudal lung lobe were stored in RNA*later*® solution (Ambion™, Austin, TX, USA). Total RNA was isolated from tissue homogenates using TRIzol® Reagent (Ambion™, USA), in accordance to the manufacturer's specifications. First-strand cDNA was synthesized from 2 μg of the total RNA using the Superscript® III First-Strand Synthesis System (Invitrogen, USA). Primers were designed and ordered from the Japan Food Assessment and Management Center (FASMAC) (Fig. 1B). Relative quantifications of the target mRNAs of PDGFR-β, FGFR-1, VGFR-2, VEGF-A, nestin, Raf-1, b-type natriuretic peptide (BNP), and the house-keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were determined by Applied Biosystems 7500 Fast Real-Time PCR System using SYBR® Fast qPCR Mix (Takara Bio Inc., Shiga, Japan) containing 400 nM of each forward and reverse primers for rats.

2.6. Western blotting assay

Lung tissues were homogenized in T-PER® Tissue Protein Extraction Reagent (Thermo Fisher Scientific, USA) containing 1% protease and phosphatase inhibitors (Thermo Fisher Scientific, USA). Total protein concentrations were determined by Bradford assay (Quick Start™ Bradford Protein Assay Kit, Bio-Rad, USA). Lysates containing 50 μg total soluble protein were resolved on 4–15% SDS-polyacrylamide gels (Mini-PROTEAN® TGX™ Precast Protein Gels, Bio-Rad, USA) and transferred to polyvinylidene difluoride membranes. The membranes were blocked in Tris-buffered saline (EzTBS, Atto, Tokyo, Japan) containing 1% bovine serum albumin and then probed with a specific primary antibody of either anti-phospho-ERK 1/2 (Cell Signaling Technology, Inc., USA), anti-ERK 1/2 (C9) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-VEGF-A (Abcam, Cambridge, UK) or anti-β-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), followed by a secondary antibody conjugated to horseradish peroxidase. Chemiluminescence was visualized using Bio-Rad Universal Hood II and quantified by Image Lab Software 6.0 (Bio-Rad Laboratories).

2.7. Statistical analyses

All data are expressed as mean ± standard error of mean (SEM). Data were analyzed for statistical differences using the StatMate3 analysis software (ATMS, Tokyo, Japan). Inter-group differences for normally distributed data were analyzed using one-way analysis of variance, followed by a least significant difference post-hoc test for multiple comparisons. Differences between two independent groups were analyzed using student t-test for normally distributed data or Mann–Whitney U test for non-normally distributed data. The relationship between treatment effects and dosages was analyzed using simple linear regression and Pearson correlation test. $P < 0.05$ was considered to be significant.

3. Results

3.1. Effect of imatinib and sunitinib on RVH and cardiac remodeling biomarkers

The placebo developed a severe RVH with a significant increase in the RV/[LV + S] ratio (0.54 ± 0.03) compared with the control (0.22 ± 0.01) (Fig. 2A). Treatment with ima-15 and ima-50 significantly reversed the RVH, yielding a stronger dose-dependency (R^2: 0.16; $P < 0.05$) than sunitinib (R^2: 0.09; $P < 0.05$) (Fig. 2B). None of the sunitinib-treated groups significantly reduced the RVH, although a weak reversal tendency was observed.

BNP mRNA was markedly increased in the placebo (0.54 ± 0.03)