Activation of Lung p53 by Nutlin-3a Prevents and Reverses Experimental Pulmonary Hypertension

Running title: Mouraret et al.; Role of Cell Senescence in Pulmonary Hypertension

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Abstract:

Background—Induction of cellular senescence through activation of the p53 tumor suppressor protein is a new option for treating proliferative disorders. Nutlins prevent the ubiquitin ligase MDM2 (murine double minute 2), a negative p53 regulator, from interacting with p53. We hypothesized that cell senescence induced by Nutlin-3a exerted therapeutic effects in pulmonary hypertension (PH) by limiting the proliferation of pulmonary-artery smooth muscle cells (PA-SMCs).

Methods and Results—Nutlin-3a treatment of cultured human PA-SMCs resulted in cell growth arrest with the induction of senescence but not apoptosis; increased phosphorylated p53 protein levels; and expression of p53 target genes including p21, Bax, BTG2, and MDM2. Daily intraperitoneal Nutlin-3a treatment for 3 weeks dose-dependently reduced PH, right ventricular hypertrophy, and distal pulmonary artery muscularization in mice exposed to chronic hypoxia or SU5416/hypoxia. Nutlin-3a treatment also partially reversed PH in chronically hypoxic or transgenic mice overexpressing the serotonin-transporter in SMCs (SM22-5HTT+ mice). In these mouse models of PH, Nutlin-3a markedly increased senescent p21-stained PA-SMCs; lung p53, p21, and MDM2 protein levels; and p21, Bax, PUMA, BTG2, and MDM2 mRNA levels; but induced only minor changes in control mice without PH. Marked MDM2 immunostaining was seen in both mouse and human remodeled pulmonary vessels, supporting the use of Nutlins as a PH-targeted therapy. PH prevention or reversal by Nutlin-3a required lung p53 stabilization and increased p21 expression, as indicated by the absence of Nutlin-3a effects in hypoxia-exposed p53+/− and p21−/− mice.

Conclusions—Nutlin-3a may hold promise as a prosenescence treatment targeting PA-SMCs in PH.

Key words: hypertension, pulmonary; cell senescence; muscle; remodeling
Introduction

Pulmonary artery hypertension (PH) occurring as an idiopathic condition or associated with an underlying disease is an unexplained disorder whose severe forms in adults and neonates are fatal and for which no satisfactory treatment is available. Hyperplasia of pulmonary-artery smooth muscle cells (PA-SMCs) is the primary determinant of the pulmonary vessel remodeling process that underlies PH\(^1\). Similarities exist between cancer and PH. Thus, cultured PA-SMCs and pulmonary-artery endothelial cells (PA-ECs) from patients with PH show growth dysregulation\(^2\)-\(^5\), PA-SMCs from patients with PH have decreased susceptibility to apoptosis\(^6\), remodeled pulmonary vessels express cancer biomarkers\(^7\), and drugs designed to treat malignant proliferations exert beneficial effects in animal models of PH\(^8\).

One common abnormality in cancer is an inactivating mutation in the gene encoding the powerful growth-suppressive and proapoptotic transcription factor p53\(^9\). Also common is posttranslational p53 inactivation via interaction of the p53 protein with its negative regulator MDM2 (murine double minute 2), a specific p53 ubiquitin ligase and transcriptional inhibitor\(^10,11\). MDM2 is overexpressed in many human tumors, suggesting that p53-MDM2 interactions may hold promise as a target for cancer therapy\(^12\). Nutlins are cis-imidazoline analogs recently developed as anticancer agents\(^13\). These selective small-molecule p53-MDM2 binding inhibitors stabilize p53, thereby increasing the expression of genes targeted by p53 including the antiproliferative gene BTG2, the prosenescent gene p21, and the proapoptotic genes Bax and PUMA\(^14\). The most potent Nutlin, Nutlin-3a, exhibits antitumor activity via p53 activation in various cancer cells and induces tumor regression when administered chronically to mice\(^13\). Nutlin-3a is being tested for the treatment of lymphoblastic leukemia in humans\(^15\).

Treatments that activate p53 in PA-SMCs might be capable of limiting or reversing the
pulmonary vascular remodeling process that characterizes PH. The ability of Nutlin-3a to stabilize and activate p53 in target cells depends on the expression and/or activity of MDM2 in these cells. MDM2 dysregulation has been reported in SMCs at sites of vascular injury\textsuperscript{16}, as well as in human atherosclerotic tissues and in vascular SMCs from patients with primary aldosteronism\textsuperscript{17,18}. Moreover, MDM2 expression can be induced by hypoxia or by p53\textsuperscript{11,19}. Whether MDM2 expression or activity is altered during progression of hypoxic or nonhypoxic PH remains unexplored. The potential effects of p53 activation in target cells may also vary according to cell status or environmental conditions, leading to cell quiescence, senescence, or apoptosis\textsuperscript{20-23}. Both senescence and apoptosis of PA-SMCs may provide therapeutic benefits in PH, although the accumulation of senescent cells within the vessel wall may potentially affect the remodeling process\textsuperscript{24}. Until now, prosenescence therapy for PH has not been evaluated.

In the present study, we tested the hypothesis that Nutlin-3a hold therapeutic potential in PH via prosenescence effects. To this end, we first examined the effects of Nutlin-3a on cultured human PA-SMCs. Second, we investigated the effects of chronic Nutlin-3a treatment in normoxic mice free of PH; mice with hypoxia-induced PH; chronically hypoxic mice simultaneously treated with SU5416 (SU5416/hypoxia)\textsuperscript{25}; and transgenic mice overexpressing the serotonin transporter in SMCs (SM22-5HTT\textsuperscript{+} mice), which develop spontaneous PH in normoxia\textsuperscript{26}. Finally, we examined the mechanisms of action of Nutlin-3a by investigating p53 and p21 knockout mice exposed to chronic hypoxia.

Materials and Methods

Mice

Adult male mice (C57Bl/6j) were used according to institutional guidelines that complied with
national and international regulations. All animal experiments were approved by the Institutional Animal Care and Use Committee of the French National Institute of Health and Medical Research (INSERM)-Unit 955, Créteil, France. Transgenic mice overexpressing 5-HTT in smooth muscle cells (SMCs) under the control of the SM22 promoter (SM22-5HTT+) were produced and bred as previously described\textsuperscript{26}. These SM22-5HTT+ mice are fertile and have a normal life span and normal growth\textsuperscript{5}. Mice with deletion of the p53 (p53\textsuperscript{−/−}) or p21 (p21\textsuperscript{−/−}) gene were obtained from Jackson Laboratory (Bar Harbor, ME). Only male mice were used for the experiments. Nutlin-3a was administered by intraperitoneal injection in a dosage of 6, 12, or 25 mg/Kg/day. At treatment completion, the lungs were removed and prepared for histological or Western-blot analyses.

**Exposure to chronic hypoxia**

Male mice aged 15-20 weeks were exposed to chronic hypoxia (9% O\textsubscript{2}) in a ventilated chamber (Biospherix, New York, NY)\textsuperscript{27}. To establish the hypoxic environment, the chamber was flushed with a mixture of room air and nitrogen. The chamber was opened every other day for 1 hour to clean the cages, administer drugs, and replenish food and water supplies. Normoxic mice were kept in the same room, with the same light-dark cycle. Mice subjected to SU5416/hypoxia received an intraperitoneal injection of SU5416 20 mg/Kg once a week during a 3-week period of hypoxia exposure\textsuperscript{25}.

**Assessment of pulmonary hypertension**

We anesthetized mice previously exposed to hypoxia, room air, or SU5416/hypoxia; as well as SM22-5HTT+ mice. After incision of the abdomen, a 26-gauge needle connected to a pressure transducer was inserted into the right ventricle through the diaphragm, and right ventricular systolic pressure (RVSP) was recorded immediately. Then, the thorax was opened and the lungs
and heart were removed. The right ventricle (RV) was dissected from the left ventricle plus septum (LV+S), and these dissected samples were weighed for determination of Fulton’s index (RV/LV+S). The lungs were fixed by intratracheal infusion of 4% aqueous buffered formalin. A midsagittal slice of the right lung was processed for paraffin embedding. Sections 5 μm in thickness were cut and stained with hematoxylin-phloxine-saffron for examination by light microscopy. In each mouse, a total of 20 to 30 intraacinar vessels accompanying either alveolar ducts or alveoli were examined by an observer who was blinded to the treatment or genotype. Each vessel was categorized as nonmuscular (no evidence of vessel wall muscularization) or muscular, i.e., partially muscular (SMCs identifiable in less than three-fourths of the vessel circumference) or fully muscular (SMCs in more than three-fourths of the vessel circumference). The percentage of muscularized pulmonary vessels was determined by dividing the number of partially or fully muscular vessels by the total number of vessels in the relevant group of animals. In addition, medial wall thickness of fully muscularized intraacinar arteries was calculated in SU5416/hypoxia mice and expressed as follows: index (%)=(external diameter - internal diameter)/external diameter·100. Cell proliferation (Ki67-stained cells), apoptosis (TUNEL-positive cells), and senescence (p21-stained cells) were assessed in the walls of distal pulmonary vessels and expressed as the number of stained nuclei over the total number of nuclei counted in the media of at least 20 muscularized vessels per mouse.

Echocardiography

Closed-chest transthoracic echocardiography was performed in nonsedated mice as described in the expanded methods section available in the Online Supplement. Images were acquired using a 13-MHz linear-array transducer with a digital ultrasound system (Vivid 7, GE Medical Systems). All measures were averaged on 5 cardiac cycles, and the reader was blinded to treatment group.
Studies on cultured human pulmonary artery smooth muscle cells (PA-SMCs)

Cultured PA-SMCs were collected from pulmonary arteries of patients undergoing lung surgery for localized lung tumors. To assess the effects of Nutlin-3a on PA-SMC proliferation, we exposed PA-SMCs to Nutlin-3a (2.5-10 μM) or vehicle in serum-free medium then added platelet-derived growth factor-BB (PDGF-BB, 50 ng/mL). After 48 hours, tetrazolium salt (MTT, Sigma, Lyon, France) was added to each well (0.2 mg/mL). After 4 hours’ incubation at 37°C, the culture medium was removed and formazan crystals were solubilized by adding 500 μL of DMSO. Tetrazolium salt reduction to formazan within the cells was quantified by spectrophotometry at 520 nm and taken as an indicator of the number of cells.

To assess the effects of Nutlin-3a on PA-SMC apoptosis, cells were trypsinized and resuspended in binding buffer 1X then incubated with annexin V-FITC–conjugated antibody and stained with propidium iodide according to the manufacturer’s instructions (Sigma-Aldrich, St Louis, MO). Annexin V staining and propidium iodide staining were detected by FACS (Becton Dickinson, Franklin Lakes, NJ). Apoptotic cells were propidium iodide-positive cells and annexin V/propidium iodide-positive cells.

To assess the effects of Nutlin-3a on cell senescence, we determined the percentage of beta-galactosidase (β-gal)-positive cells after 48 hours’ incubation with Nutlin-3a with or without vehicle or platelet-derived growth factor (PDGF).

In addition, cell transfection studies were performed using a p53 Cignal Reporter Assay (Qiagen, ZA Courtaboeuf, France) for assessing p53 signaling in response to Nutlin, as described in the online supplement.

Biological measurements in mouse tissues and cultured human pulmonary-artery smooth muscle cells (PA-SMCs)
Western blotting was used to detect and quantitate p53, p21 and MDM2 proteins in mouse tissues and/or human cells as described in the expanded methods section in the Online Supplement. Complementary experiments were performed in mouse tissue to assess phosphorylated p53 levels by using a co-immunoprecipitation procedure or to determine the nuclear and cytoplasmic fractions of total p53 protein. Levels of p21, Bax and PUMA mRNAs in lung tissue and cells were determined using RT-qPCR. Total mRNA was extracted from PA-SMCs using the RNeasy Mini Kit (Qiagen, ZA Courtaboeuf, France). First-strand cDNA was synthesized in reversed transcribed samples, as follows: 1 μg total RNA isolated from cells or lung tissues, 200 U/μL SuperScript II reverse transcriptase, 100 ng Random primers, and 10 mM mixed dNTP (Invitrogen, Life Technologies, Cergy-Pontoise, France). Quantitative PCR was performed in a 7900HT Real-Time PCR system (Applied Biosystems, ZA Courtaboeuf, France), using SYBR green Mix from Invitrogen as described in the expanded methods section in the Online Supplement.

**Immunohistochemistry**

Paraffin-embedded sections were incubated with antibodies against Ki67 or p21 for immunostaining identification of vascular proliferative or senescent cells, respectively. Apoptotic nuclei were labeled with a TUNEL immunostaining assay (Roche, Meylan, France), as described in the expanded Methods section available in the Online Supplement. For immunofluorescence, slides were incubated with anti-MDM2 SMP-14 mouse antibody (1:50, Santa Cruz, Santa Cruz, CA) and anti-αSMA rabbit antibody (1:200, Abcam, Cambridge, UK) then exposed to anti-mouse Alexa Fluor (1:1000, Cell Signaling Technology, Boston, MA) and anti-rabbit Alexa Fluor (1:1000, Invitrogen, Cergy-Pontoise, France) antibodies. Nuclei were stained with Hoechst 33342 (1 μg/mL, Cell Signaling Technology). Chemicals and drugs
Nutlin-3a was purchased from Bertin Pharma (Montigny-le-Bretonneux, France) and diluted in vehicle (0.9% NaCl and 30% DMSO) to obtain concentrations of 6 mg/Kg, 12 mg/Kg, and 25 mg/Kg. Mice received daily intraperitoneal injections of Nutlin-3a for 21 days. SU5416 was purchased from Sigma (Saint Quentin Fallavier, France).

**Statistical analysis**

The data are described as mean±SEM. Parametric tests were used after verification that the variables in each group were normally distributed. One-way analysis of variance (ANOVA) was performed to compare treatment effects obtained with Nutlin-3a in various doses or at various post-dose times. One-way ANOVA was used to compare hypoxia-exposed vehicle-treated mice to normoxia-exposed control mice, as well as to compare transgenic mice to wild-type mice during normoxia or hypoxia. P values lower than 0.05 were considered significant for the initial ANOVA, and Bonferroni’s correction was then used for multiple comparisons (P<0.017).

**Results**

**Effects of Nutlin-3a treatment on cultured human PA-SMCs**

Nutlin-3a treatment of cultured human PA-SMCs was followed by a marked rise in phosphorylated p53 protein levels within 2 hours after treatment, with a peak after 4 hours and persistent elevation until 24 hours (**Figure 1A**); total p53 protein and p53 mRNA levels remained unchanged (**Figure 1A**). Nutlin-3a treatment also induced marked but delayed increases in p21 mRNA and protein levels, which peaked at 24 hours (**Figure 1B**) and were accompanied with increased expression of other p53-target genes including Bax, BTG2 and MDM2 (data not shown). After 24 hours of Nutlin-3a treatment, β-galactosidase-stained cells increased in percentage from 20% to 90% and exhibited a typical senescent phenotype.
characterized by a flat shape and increased size, in the absence or presence of PDGF (Figures 1C and 1D). The number of annexin-V-positive cells was not affected by Nutlin-3a (Figure 1C). Nutlin-3a treatment dose-dependently increased the phosphorylated p53 protein and p21 protein levels without altering the caspase-3 protein level (Figure 1E), indicating that Nutlin-3a treatment induced senescence but not apoptosis of PA-SMCs. In addition, Nutlin-3a applied to cells transfected with the luciferase p53 reporter, led to a marked increase in luciferase activity compared with vehicle-treated control cells (Figure 1F).

**Effects of treatment with Nutlin-3a in mice exposed to chronic hypoxia**

Intraperitoneal treatment of chronically hypoxic mice with 6 to 25 mg/Kg/day of Nutlin-3a attenuated the development of PH, as judged based on RVSP, RV hypertrophy, and distal pulmonary artery muscularization (Figure 2A). This protective effect of Nutlin-3a was accompanied with simultaneous decreases in PA-SMC proliferation and apoptosis, as assessed by the percentage of Ki67- and TUNEL-positive cells, respectively, contrasting with an increased number of senescent p21-positive PA-SMCs (Figures 2A and 2B). Cardiac output and left ventricular ejection fraction remained unchanged in mice treated with 25 mg/Kg/day of Nutlin-3a, whereas pulmonary acceleration time (PAT) and the PAT/ejection time ratio, two alternative indices of pulmonary artery pressure, improved in chronically hypoxic mice (Online Table 1). Nutlin-3a, 12 mg/Kg/d, from day 15 to day 30 partially reversed PH in chronically hypoxic mice (Figure 2C).

Nutlin-3a treatment markedly increased lung levels of p53, p21, and MDM2 protein and of p21, Bax, Bcl2, and BTG2 mRNAs in chronically hypoxic mice but not in normoxic animals (Figures 3A and 3D). Phosphorylated p53 protein levels measured in whole lung proteins and total p53 protein levels measured in the nuclear and cytoplasmic fractions of lung proteins
increased after Nutlin-3a treatment in hypoxic but not normoxic mice (Figure 3B, 3C). Because lung samples were collected exactly 3 hours after the last Nutlin-3a injection in both normoxic and hypoxic mice, the dissimilar response to Nutlin-3a in hypoxic and normoxic mice was interpreted as reflecting increased MDM2 activity during hypoxia. This increased MDM2 activity was measured, although total lung MDM2 protein levels did not change from normoxia to hypoxia (Figure 3A). In contrast to the dose-dependent increase in lung p21, Bax, Bcl2, and BTG2 mRNA levels induced by Nutlin-3a, lung expression of the proapoptotic gene PUMA, which increased from normoxia to hypoxia, was reduced by Nutlin-3a treatment (Figure 3D). The increased p53 protein levels induced by Nutlin-3a also occurred in the kidneys, heart, spleen, and liver from normoxic and hypoxic mice, although at different levels (online Figure 1). A marked (3.5-fold) increase in p53 protein was observed only in the lungs and kidneys of chronically hypoxic mice.

**Effects of Nutlin-3a treatment in SM22-5HTT+ mice and in SU5416/hypoxia mice**

SM22-5-HTT+ mice were studied to investigate whether Nutlin-3a reversed established PH and altered MDM2 activity during normoxia. SM22-5HTT+ mice, which spontaneously develop PH, were treated for 3 weeks with 12 mg/Kg/day Nutlin-3a. Nutlin-3a partially reversed PH in SM22-5-HTT+ mice, decreasing RVSP, RV hypertrophy, pulmonary vessel muscularization, and Ki67-stained cell counts (Figure 4); and substantially increased p21-stained cell counts; without increasing TUNEL-positive cell counts. Similarly to the findings in chronically hypoxic mice, Nutlin-3a treatment increased lung p53, p21, and MDM2 protein levels; as well as mRNA levels of the p53-target genes p21, Bax, Bcl2, and BTG2 (Figure 5 and online Figure 2). Nutlin-3a also increased lung PUMA mRNA levels in SM22-5-HTT+ mice (online Figure 2). No such alterations were observed in wild-type mice, indicating increased lung MDM2 activity in...
SM225-HTT+ mice, similar to chronically hypoxic mice. In contrast to the results in chronically hypoxic mice, total lung p53, p21, and MDM2 protein levels were slightly higher in SM22-5-HTT+ mice than in wild-type mice and increased further in response to Nutlin-3a (Figure 5).

To assess the effects of Nutlin-3a in a third model of PH characterized by more intense pulmonary vascular remodeling, we investigated mice subjected to SU5416/hypoxia\textsuperscript{25}. Treatment with 12 mg/Kg/d during 3 weeks partially prevented PH in SU5416/hypoxia mice, inducing a substantial increase in p21-stained cell counts and marked attenuation of the increased wall thickness of pulmonary vessels (online Figure 3A and 3B). MDM2 protein levels, which were higher in SU5416/hypoxia mice than in wild-type mice, increased further in response to Nutlin-3a (Online Figure 3C).

**Immunolocalization of MDM2 in lung tissues from mice and patients with chronic lung disease**

In both normoxic and chronically hypoxic mice, immunofluorescence staining for MDM2 protein was predominantly found in SMCs from the media of pulmonary vessels, as shown by double-immunofluorescence staining for MDM2 and αSMA of paraffin-embedded lung sections (Figure 6A). Only a few MDM2-stained cells were observed in the bronchi and alveoli. Similar findings were obtained in SM22-5-HTT+ mice. In lung tissues from lung-surgery patients with or without pulmonary vessel remodeling, MDM2 also predominated in SMCs of pulmonary vessels (Figure 6B).

**Effects of treatment with Nutlin-3a in p53\textsuperscript{\textminus/\textminus} mice**

To determine whether Nutlin-3a effects required p53 stabilization, we investigated p53\textsuperscript{\textminus/\textminus} mice and their littermate controls during exposure to chronic hypoxia. Nutlin-3a in a dose of 12 mg/Kg/day did not prevent hypoxic PH in p53\textsuperscript{\textminus/\textminus} mice, in contrast to its protective effect in
control nonmutant mice (Figure 7A). Nutlin-3a treatment also failed to alter p53 downstream signaling in these mice, indicating clearly that p53 was required to mediate Nutlin-3a effects (Figures 7B and online Figure 4). Interestingly, PH was slightly but significantly more severe in p53−/− mice than in control wild-type mice, in accordance with previously published studies.

**Effects of treatment with Nutlin-3a in p21−/− mice**

To determine whether p21 was required to prevent PH in chronically hypoxic mice, we investigated the effects of Nutlin-3a treatment in p21−/− mice. As shown in Figure 8, Nutlin-3a treatment failed to protect hypoxia-exposed p21−/− mice against PH. Interestingly, the ability of Nutlin-3a treatment to enhance Bax and BTG2 expression was reduced but not abrogated in p21−/− mice, whereas Nutlin-3a-induced changes in Bcl2 and PUMA expression were abolished (online Figure 5). In p21−/− mice, PH was slightly but significantly more severe than in wild-type controls, suggesting that p21-induced cell senescence not only was critical in mediating Nutlin-3a effects, but also played a role in the development of hypoxic PH.

**Discussion**

The main finding of the present study is that Nutlin-3a, a potent and selective antagonist of the p53-MDM2 interaction, induces PA-SMC senescence in vitro and attenuates or reverses PH in three distinct experimental mouse models, decreasing the number of proliferating PA-SMCs and increasing the number of p21-stained senescent cells. Nutlin-3a led to marked increases in lung p53 protein and p53 downstream gene expression in mice with PH, whereas only minor effects were noted in control mice without PH. These results are consistent with potentiation of Nutlin-3a effects in PH due to increased MDM2 activity. The ability of Nutlin-3a to prevent or reverse PH required lung p53 stabilization and increased p21 expression, as indicated by the inability of
Nutlin-3a to prevent chronic hypoxia-induced PH in p53−/− and p21−/− mice. Thus, Nutlin-3a holds promise as a prosenescence treatment targeting PA-SMCs during PH progression.

The induction of cell senescence is a novel therapeutic approach to malignant and nonmalignant proliferative disorders29. Inducing PA-SMC senescence is expected to be more efficient than inducing cell quiescence in PH, as cell growth arrest in senescence is stable and largely irreversible. Here, we found that Nutlin-3a treatment of human PA-SMCs led to a large increase in phosphorylated p53 protein levels, with a subsequent increase in p21 expression and acquisition of a typical senescence phenotype characterized by increased size, a flat shape, and β-gal staining. This senescence-induced growth arrest appeared irreversible, since Nutlin-3a removal did not restore PA-SMC proliferation. However, PA-SMCs treated with Nutlin-3a did not become apoptotic, a finding consistent with results obtained using other cell types, including fibroblasts from mice and humans20, 21.

Chronic Nutlin-3a treatment of mice during hypoxia exposure considerably attenuated PH severity by decreasing distal pulmonary artery muscularization. This effect was dose-dependent, and no toxic effects were detected even at the highest dose tested. Nutlin-3a treatment for 3 weeks also attenuated PH in mice subjected to SU5416/hypoxia and partially reversed established PH in SM22-5HTT+ mice and in chronically hypoxic mice, producing similar decreases in pulmonary vessel muscularization. These effects of Nutlin-3a were associated with a marked reduction in Ki67-positive dividing PA-SMCs, together with an increased number of p21-stained PA-SMCs, but with no increase in apoptotic TUNEL-positive cells. Taken together with our in vitro data, these results indicate that prevention or reversal of pulmonary vessel remodeling in our mouse models of PH resulted from the induction of PA-SMC senescence by Nutlin-3a.
We found that the number of p21-stained senescent cells following Nutlin-3a treatment increased to a much greater extent in lungs from chronically hypoxic and SM22-5HTT+ mice than from their respective controls without PH, suggesting potentiation of Nutlin-3a effects during PH. One potential advantage of using Nutlin to treat cancer is that cancer cells overexpress MDM2 and should therefore prove more susceptible than normal cells to growth arrest in response to Nutlin. The results of the present study also suggest greater susceptibility to Nutlin-3a of proliferating PA-SMCs in PH, compared to PA-SMCs from control animals. Nutlin-3a administration was followed by considerably larger increases in lung p53 protein levels in chronically hypoxic mice and SM22-5HTT+ normoxic mice than in their respective controls. These effects were related to increased MDM2 activity in chronically hypoxic mice and to increased MDM2 activity and expression in SM22-5HTT+ mice. Potentiation of Nutlin-3a effects during PH was also supported by the Nutlin-3a-induced expression of p53 downstream genes including p21, Bax, Bcl2, BTG2, and MDM2, which was far greater in hypoxic and SM22-5HTT+ mice than in control mice without PH. Moreover, MDM2 was strongly expressed by PA-SMCs in remodeled vessels from chronically hypoxic mice, from SM22-5HTT+ mice, and from mice subjected to SUGEN/hypoxia compared to those from mice without PH. PA-SMCs from remodeled pulmonary vessels of patients with chronic lung disease also exhibited increased MDM2 immunostaining compared to those from nonremodeled vessels of the same patients. Taken together, these results indicate that prevention or reversal of pulmonary vessel remodeling in these mouse models of PH was due to the induction of PA-SMC senescence and that the effects of Nutlin-3a on PA-SMCs were potentiated during PH, probably because of activation and/or overexpression of the Nutlin-3a target MDM2 protein. Thus, Nutlin-3a holds promise as a targeted treatment for PH.
Of note, we found that Nutlin-3a treatment increased both phosphorylated and nonphosphorylated p53 protein in lungs from hypoxic mice but increased only phosphorylated p53 protein in cultured human PA-SMCs. Neither phosphorylated nor total p53 protein was altered by Nutlin-3a in lungs from normoxic mice. These results are consistent with data suggesting that Nutlin-3a efficacy may vary according to basal MDM2 activity and conditions of Nutlin-3a treatment\textsuperscript{29,30}. Moreover they emphasize the complexity of the interactions between MDM2 and p53. Indeed, the MDM2-p53 complex is known to form primarily in the nucleus and to shuttle subsequently from the nucleus to the cytoplasm, where MDM2 targets p53 for degradation by acting as an ubiquitin-ligase\textsuperscript{30}. The dissimilar effects of Nutlin-3a in hypoxic versus normoxic mice are also consistent with previous studies showing that systemic Nutlin treatment is toxic to tumor tissue but not to normal tissue\textsuperscript{31,32}. Because previous studies reported that p53 activation could lead to heart failure\textsuperscript{33}, we measured p53 protein levels in various organs from normoxic and hypoxic mice treated with Nutlin-3a. We observed only minimal changes in p53 protein levels in the heart, spleen, and liver; and found that cardiac function remained unaltered. Major increases in p53 protein were seen only in the lungs and in the kidneys. Further studies are therefore needed to assess the renal effects of Nutlins in conditions associated with PH, although Nutlins have been reported to improve kidney function in other conditions\textsuperscript{34}.

Although p53 regulation appears to be the central focus of MDM2 activity, the p53-binding domain of MDM2 binds to other proteins that are potentially involved in cell growth and survival\textsuperscript{35}. To investigate whether Nutlin-3a effects were mediated solely by stabilization and overexpression of p53, we investigated p53\textsuperscript{−/−} mice. Nutlin-3a did not prevent PH in p53\textsuperscript{−/−} mice simultaneously exposed to chronic hypoxia. Neither did Nutlin-3a alter p53 downstream
signaling in these mice, clearly indicating that p53 was required to mediate the effects of Nutlin-3a. Another point of interest was whether p53 played a pivotal role in PH development in our mouse models. As previously reported, we found that PH was more severe in p53−/− mice than in control wild-type mice28. However, in contrast to these previous studies showing marked increases in lung p53 protein levels from normoxia to hypoxia28, we observed only minimal alterations in baseline p53 levels in hypoxic vs. normoxic mice and in SM22-5HTT+ mice vs. control wild-type mice. The reason for this difference is unclear but probably involves differences in hypoxia exposure conditions. The relative stability of p53 protein levels from normoxia to hypoxia in our study is probably ascribable to increased p53 protein degradation due to increased MDM2 ubiquitin ligase activity. Suppression of this adaptive response by Nutlin-3a led to major increases in p53 in PA-SMCs, thereby strongly inhibiting the pulmonary vascular remodeling process.

Among the transcriptional targets of p53, p21 has been shown to play a major role as a regulator of cell-cycle progression and to be involved in cell senescence36. We found that the protective action of Nutlin-3a against PH required p21, as indicated by the inability of Nutlin-3a to prevent chronic hypoxia-induced PH in p21−/− mice, despite significant increases in mRNA levels of the antiproliferative protein BTG2 and of the proapoptotic protein Bax. These results indicate a prominent role for p21 in our experimental PH model and support cell-senescence induction as the mechanism by which Nutlin-3a protects against PH. Moreover, the slight increase in PH severity noted in p21−/− mice compared to their hypoxic controls suggests involvement of p21 in the control of hypoxic PH progression.

The present results supporting a beneficial effect of cell-senescence induction in PH may appear to contradict our previous studies supporting a role for senescent cells in mediating
pulmonary vascular remodeling. In these previous studies, we showed that the accumulation of senescent cells within pulmonary vessels contributed to neointima formation in patients with chronic lung diseases\textsuperscript{24}. Indeed, senescent cells are still metabolically active and release various factors including cytokines, growth factors, and matrix components; which promote the proliferation of neighboring cells\textsuperscript{24, 36, 37}. Similar observations have been made for cancer cells, which can be stimulated by neighboring senescent cells\textsuperscript{36}. Thus, an important issue regarding PH is whether senescent cells can accumulate for long periods in pulmonary vessels. In theory, such accumulation is prevented via senescent-cell clearance by the immune system\textsuperscript{38}. However, in age-related diseases such as chronic obstructive lung disease, combined mechanisms, including increased susceptibility to cell senescence and an inability of the immune system to clear senescent cells, probably promote the accumulation of senescent cells within the vascular wall. Whether this may occur in response to Nutlin treatment may depend on various factors such as age, immune system performance, and environmental conditions\textsuperscript{38}. Thus, inducing cell senescence might hold promise as a treatment against pulmonary vascular remodeling, provided the senescent cells are simultaneously cleared by the immune system.

Further studies are therefore needed to identify the mechanisms responsible for senescent-cell clearance in PH. Whether prosenescence strategies such as Nutlin therapy hold promise for the treatment of PH will need to be evaluated.

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**Conflict of Interest Disclosures:** None.
References:


Figure Legends:

Figure 1. Effects of Nutlin-3a treatment on pulmonary-artery smooth-muscle-cells (PA-SMCs). A, time-dependent changes in phosphorylated p53 protein (P-p53), total p53 protein, and p53 mRNA following PA-SMC treatment with 5 μM of Nutlin-3a. B, changes in p21 protein and p21 mRNA following treatment with 5 μM of Nutlin-3a. C, percentage of beta-galactosidase-positive cells (bar graphs) and of apoptotic cells (diamonds) 24 hours after increasing doses of Nutlin-3a with or without 50 ng/mL PDGF-B. D, Representative photographs of cells stained for senescence-associated beta-galactosidase activity. E, Phosphorylated p53 (P-p53), total p53, p21, and caspase-3 protein measured by Western blot in PA-SMCs treated with increasing Nutlin-3a concentrations. Data are mean±SEM of 10 values from four independent experiments. F, Effects of Nutlin on firefly/renilla luciferase activity ratio in PA-SMCs co-transfected with the p53-responsive firefly luciferase construct and with a construct constitutively expressing renilla luciferase. Data are mean±SEM of 6 values. *P<0.01; and †P<0.001 compared to treatment with vehicle instead of Nutlin-3a.
**Figure 2. A**, right ventricular systolic pressure (RVSP); right ventricular hypertrophy index (RV/[LV+S] weight ratio); pulmonary vessel muscularization (percentages of muscularized pulmonary vessels); and percentages of Ki67-positive dividing cells, p21-stained cells, and TUNEL-positive cells in mice studied on day 21 after exposure to hypoxia or to normoxia and treated with daily i.p. injections of vehicle or various Nutlin-3a doses. **B**, representative micrographs of pulmonary vessels stained for Ki67, TUNEL, or p21. No immunoreactivity was detected in sections incubated with secondary anti-rabbit and anti-mouse antibody but no primary antibody. Data are mean±SEM of 6 to 10 animals. *P<0.02 compared with values in vehicle-treated mice after exposure to hypoxia or normoxia. †P<0.05 for comparison between hypoxic and normoxic mice treated with vehicle. **C**, RVSP, RV/[LV+S] weight ratio, and pulmonary vessel muscularization in normoxic and chronically hypoxic mice studied on day 15 and 30 after hypoxia exposure. Nutlin-3a 12 mg/Kg/d was given from day 15 to day 30. *P<0.05 versus day 15 of hypoxia and †P<0.05 versus day 30 of hypoxia in vehicle-treated mice.

**Figure 3. A**, Lung levels of total p53 protein, p21, and MDM2 protein measured by Western blot in mice studied on day 21 after exposure to hypoxia or normoxia and treated with daily i.p. injections of vehicle or various Nutlin-3a doses. Mice were sacrificed exactly 3 hours after the last Nutlin-3a dose. **B**, p53 protein levels measured in the cytoplasmic and nuclear fractions of lung proteins and quantified relative to alpha-tubulin and lamin A/C, respectively. **C**, lung phosphorylated p53 (P-p53) levels determined using a co-immunoprecipitation procedure. **D**, lung mRNA levels of p21, bax, Bcl2, p53, PUMA and BTG2 measured by RT-PCR *P<0.02 compared with values in vehicle-treated mice after exposure to hypoxia or normoxia. Data are mean±SEM of 6 to 10 animals. †P<0.05 compared with values in vehicle-treated mice after
exposure to hypoxia.

Figure 4. **A**, right ventricular systolic pressure (RVSP); right ventricular hypertrophy index (RV/[LV+S] weight ratio); muscularization of pulmonary vessels (percentages of partially and fully muscularized pulmonary vessels); and percentages of Ki67-positive dividing cells, p21-stained cells and TUNEL-positive cells in SM22-5HTT+ mice and wild-type mice studied after 21 days of treatment with vehicle or 12 mg/Kg of Nutlin-3a. **B**, representative micrographs of pulmonary vessels stained for Ki67, TUNEL, or p21. Data are mean±SEM of 6 to 10 animals.*P*<0.05 compared with values in vehicle-treated mice. †*P*<0.05 for comparison between SM22-5HTT+ and control mice treated with vehicle.

Figure 5. Lung protein levels of p53, p21, and MDM2 measured by Western blot in SM22-5HTT+ mice and control mice studied after 21 days of treatment with vehicle or 12 mg/Kg of Nutlin-3a. Mice were sacrificed exactly 3 hours after the last Nutlin-3a dose. Data are mean±SEM of 6 to 10 animals.*P*<0.05 compared with values in vehicle-treated mice. †*P*<0.05 for SM22-5HTT+ versus vehicle-treated control mice.

Figure 6. Immunolocalization of MDM2 in mouse and human lung tissues. **A**, representative photographs of immunofluorescence staining for MDM2 in wild-type normoxic and hypoxic mice and in SM22-5HTT+ mice treated with Nutlin-3a or vehicle for 21 days. MDM2 was predominantly expressed in pulmonary vessels and co-localized with α-SMA in smooth muscle cells (SMCs). **B**, representative photographs of immunofluorescence staining for MDM2 in nonremodeled and remodeled vessels from lung-surgery patients. No immunoreactivity was
detected in sections incubated with secondary anti-rabbit and anti-mouse antibody but no primary antibody.

**Figure 7.** A, right ventricular systolic pressure (RVSP), right ventricular hypertrophy index (RV/[LV+S] weight ratio), and pulmonary vessel muscularization (percentages of partially and fully muscularized pulmonary vessels); B, lung protein levels of p21 and MDM2 in wild-type and p53 knockout (KO) mice studied on day 21 after exposure to hypoxia or normoxia and treated with daily i.p. injections of 12 mg/Kg Nutlin-3a. Data are mean±SEM of 10 animals.*P<0.05 compared with values in vehicle-treated mice. ‡P<0.05 compared with values in vehicle-treated control mice.

**Figure 8.** Right ventricular systolic pressure (RVSP), right ventricular hypertrophy index (RV/[LV+S] weight ratio), pulmonary vessel muscularization (percentages of partially and fully muscularized pulmonary vessels) in wild-type and p21 knockout (KO) mice studied on day 21 after exposure to hypoxia or normoxia and treated with daily i.p. injections of 12 mg/Kg Nutlin-3a. Data are mean±SEM of 6 to 10 animals.*P<0.05 compared with values in vehicle-treated mice. ‡P<0.05 compared with values in vehicle-treated control mice.
Figure 1

**A**

Graph showing the fold increase in protein levels for P-p53/β-Actin, p53 Total/β-Actin, and p53 mRNA/SF3A1 with Nutlin-3a at different concentrations.

**B**

Bar graph showing the percentage of cells (viable, apoptotic, and necrotic) after treatment with FCS and PDGF.

**C**

Images showing apoptotic cells under different treatment conditions.

**D**

Western blot analysis showing protein expression levels of P-p53, Total p53, p21, Caspase 3, and β-Actin with different concentrations of Nutlin-3a.

**E**

Graph showing the fold increase in induction with different concentrations of Nutlin-3a.
Figure 2

Panel A: Graphs showing the effects of normoxia and hypoxia on RVSP, RV/LV+IS, and muscularized pulmonary vessels.

Panel B: Images showing the effects of Nutlin-3a treatment on Ki67, TUNEL, and p21 positive cells in normoxia and hypoxia conditions.
Figure 2, cont'd
Figure 3
Figure 4

**A**

- **Muscularized pulmonary vessels (%)**
- **WT SM22-5HTT +**
- **Ki67 positive cells (%)**
- **WT SM22-5HTT +**
- **p21 positive cells (%)**
- **WT SM22-5HTT +**
- **RV/LV+IS (%)**
- **WT SM22-5HTT +**
- **RVSP (mmHg)**
- **WT SM22-5HTT +**

**B**

- **Ki67**
- **TUNEL**
- **p21**

---

**Figure 4**

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Figure 5
Figure 6
Figure 6

- **B**

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Figure 7
Figure 7, cont’d
Figure 8
Activation of Lung p53 by Nutlin-3a Prevents and Reverses Experimental Pulmonary Hypertension

Nathalie Mouraret, Elisabeth Marcos, Shariq Abid, Guillaume Gary-Bobo, Mirna Saker, Amal Houssaini, Jean-Luc Dubois-Rande, Laurent Boyer, Jorge Boczkowski, Geneviève Derumeaux, Valérie Amsellem and Serge Adnot

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Activation of Lung p53 by Nutlin-3a Prevents and Reverses Experimental Pulmonary Hypertension

Mouraret; Role of Cell Senescence in Pulmonary Hypertension

Mouraret N MSC, Marcos E MSC, Abid S MSC, Gary-Bobo G PhD, Saker M MSC, Houssaini A MSC, Dubois-Rande JL MD, Boyer L MD, Boczkowski J MD-PhD, Derumeaux G MD, Amsellem V PhD, Adnot S MD-PhD
Supplemental methods

Echocardiography

Closed-chest transthoracic echocardiography was performed in nonsedated mice as previously described. Images were acquired using a 13-MHz linear-array transducer with a digital ultrasound system (Vivid 7, GE Medical Systems). Conventional measurements [LV diameters, wall thickness and thickening] were obtained serially from M-mode tracings at the level of the papillary muscles at each time point. LV end-systolic and end-diastolic volumes (LVESV and LVEDV, respectively) were calculated from the M-mode image by the “D3” respectively as LVESD3 and LVEDD3. Stroke volume (SV) was derived from the difference between LVEDV and LVESV. Cardiac output (CO) was calculated as the product of SV and heart rate. The pulsed-wave Doppler recording of pulmonary flow was obtained from the parasternal short-axis view at the level of the aortic valve. The pulsed-wave Doppler sample was positioned within the pulmonary infundibulum at the tip of the pulmonary leaflets. The following variables were measured: systolic time-velocity integral of pulmonary flow (VTI, cm), pulmonary acceleration time (PAT, ms: time from pulmonary flow onset to peak velocity), ejection time (ET, ms: time from onset to end of systolic flow velocity), and the PAT/ET ratio (%). All measures were averaged over five cardiac cycles and the reader was blinded to the treatment of the mice.

Real-time reverse-transcription quantitative PCR (RT-qPCR)

The levels of p53 downstream genes were determined by real-time reverse-transcription (RT)-qPCR using SYBR Green (Invitrogen, Cergy-Pontoise, France). To normalize for cDNA input load, mouse 18s was used as an endogenous standard. Specific primers were as follows: p21 forward, 5’-CAGATCCACAGCGATATCCA-3’ and p21 reverse, 5’-
ACGGGACCGAAGAGACAAC-3’; Bax forward, 5’-AGGATGCAGTCACCAAGAAGCT-3’ and Bax reverse, 5’-TCCGTGTCACGTCAGCAATCA-3’; Bcl2 forward, 5’-GGGGATTGTGCGCCTTCTT-3’ and Bcl2 reverse, 5’-CATCCCAGCCTCCGTATCCT3’; Puma forward, 5’-ACGGCTCCACCTGCCCAC-3’ and Puma reverse, 5’-ACGGGCGACTCTAAGTGTCAC-3’; BTG2 forward, 5’-GAGCGAGCAGAGAGATTGTGGCCTTCTT-3’ and BTG2 reverse, 5’-CGATAGCCAGAACCTTTGGATGG-3’. After addition of cDNA to SYBR Green Master Mix PCR (300 nM of each specific primer, PCR was performed in a total volume of 25 µL in a 7900HT real-time PCR cycler (Applied Biosystems, Courtaboeuf, France). All cDNA samples were tested in duplicate and analyzed using ABI Prism Sequence Detection Software v.1.7 (PE Applied Biosystems). Samples were compared using the relative Ct method. The Ct value, which is inversely proportional to the initial template copy number, is the calculated number of cycles with a fluorescence signal significantly above background levels. Fold induction or repression was measured relative to controls and calculated after adjusting for 18s using $2^{-\Delta \Delta Ct}$, where $\Delta Ct = Ct$ tested gene $– Ct$ 18s and $\Delta \Delta Ct = \Delta Ct$ control $– \Delta Ct$ treatment.

Western Blotting
Tissues were sonicated in lysis buffer (Cell Signaling Technology, Beverly, MA). The lysate was centrifuged for 10 minutes at 2000 rpm at 4°C and the supernatant was kept frozen. The protein concentration of each sample was determined using the Bradford method. Samples were subjected to electrophoresis in 10% or 15% polyacrylamide gels under reducing conditions. After electrophoresis, proteins from the gel were electroblotted onto polyvinylidene difluoride membranes (Millipore, Molsheim, France) for 2 h. After transfer, the membranes were saturated with phosphate buffer saline (PBS)/5% milk then probed
overnight at 4°C with antibodies. The antibodies used were anti-P-p53 Ser15 (Cell Signaling Technology, MA, USA) at a final dilution of 1:1000; monoclonal anti p53 (Cell Signaling Technology) at a final dilution of 1:1000; monoclonal anti-p21Waf1/Cip1 antibody (Cell Signaling Technology) at a final dilution of 1:1000, or polyclonal anti-MDM2 (Abcam, Cambridge, UK) at a final dilution of 1:1000. After three 5-minute washes in TBS-T, membranes were incubated for 1 hour at room temperature with horseradish peroxidase-conjugated secondary antibodies at a final dilution of 1:1000 and washed twice for 5 minutes in TBS-T. Proteins were visualized by electrochemiluminescence (Amersham, Little Chalfont, UK) according to the manufacturer's instructions. Membranes were re-probed with anti-β-actin or anti-GAPDH antibody (Sigma, Saint-Quentin-Fallavier, France) at a final dilution of 1:1000 for normalization. Complementary experiments were performed in mouse tissue to assess phosphorylated-p53 levels by using a co-immunoprecipitation procedure or to determine the nuclear and cytoplasmic fractions of total p53 protein.

**Immunoprecipitation**

Immunoprecipitation was carried out at 4°C. Lung tissue was sonicated in lysis buffer (Cell Signaling Technology, MA, USA). The lysate was centrifuged for 10 minutes at 2000 rpm at 4°C and the supernatant was kept frozen. After assessment of the protein concentration, 1 mg of protein from each sample was incubated with the p53 (1C12) antibody (Cell Signaling Technology) overnight then with protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 3 h. The protein–antibody complexes recovered on beads were subjected to Western blot analysis after separation by SDS–PAGE. The membrane was probed with phosphorylated-p53 (Ser15) primary antibody (Cell Signaling Technology) and with a horseradish-peroxidase-conjugated secondary antibody. Blots were visualized by electrochemiluminescence.
Cytosolic and nuclear fraction isolation

To obtain the nuclear fraction, lung tissue was incubated in hypotonic buffer (20 mM Tris HCl, pH 7.4; 10 mM NaCl; 3 mM MgCl2) for 15 min at 4°C. Triton (0.3%) was added to the lysate, which was then centrifuged at 14,000 g for 10 min at 4°C and separated into soluble and insoluble fractions. The insoluble fraction containing the cytosolic protein was kept at -80°C, and the pellet containing the nuclei was incubated in lysis buffer (Invitrogen, Carlsbad, CA, USA) for 30 min. After extraction, the sample was centrifuged at 14,000 g for 10 min at 4°C, and the supernatant constituted the nuclear fraction. Cytosolic and nuclear protein samples were analyzed by Western blotting. The membranes were incubated with the appropriate primary antibody, p53 (1C12) antibody (Cell Signaling Technology), lamin A/C antibody (Santa Cruz), or alpha-tubulin (Cell Signaling Technology).

Transient tranfection of pulmonary-artery smooth muscle cells

Cells were seeded in 24-well plates and transiently transfected the following day with a p53 reporter construct (p53 Cignal Reporter Assay, Qiagen, Courtaboeuf, France), as well as with positive and negative controls, using Lipofectamin 2000 (Invitrogen) according to the manufacturer’s instructions. After 18 h of transfection, the medium was changed and replaced by complete growth medium, and the cells were treated with Nutlin-3a 2.5 µM, 5 µM, 10 µM, or ethanol alone. A dual-luciferase assay (Promega, Charbonnières les Bains, France) was performed 24 h after treatment. Cells were lysed with lysis buffer, and luciferase activity was quantified using a Tristar luminometer (Berthold, Thoiry, France). Values are expressed as arbitrary units using a construct constitutively expressing Renilla luciferase for internal normalization. Firefly/Renilla activity ratios were generated from experimental and control transfections. Experiments were done in triplicate.
Immunohistochemistry

Paraffin-embedded sections were deparaffinized using xylene and a graded series of ethanol dilutions then incubated in citrate buffer (0.01 M, pH 6) at 90°C for 20 minutes. Tissue was permeabilized with 0.1% Triton X-100 in PBS for 10 minutes. Endogenous peroxidase activity was blocked with 3% H₂O₂ and 10% methanol in PBS for 10 minutes. Slides were incubated for 60 minutes in 1% bovine serum albumin and 5% goat serum in PBS then incubated overnight with anti-p21 mouse antibody (1:200, Santa Cruz Biotechnology) and anti-Ki67 rabbit antibody (1:300, Abcam). We used the ABC Vectastain kit (Vector Labs, Burlingame, CA, USA) to mark the primary antibodies according to the user’s guide. The staining substrate was diaminobenzidine (FastDAB, Sigma-Aldrich, St Louis, MO, USA) and the sections were counterstained with methyl green.

For terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL), epitopes were retrieved by treatment with DNase1 digestion for 10 min at room temperature. After endogenous peroxidase blockade with H₂O₂, tissues were incubated in equilibration buffer and treated with terminal deoxynucleotidyl transferase (TdT) to detect TUNEL-positive nuclei as suggested by the manufacturer (Roche, Meylan, France). Tissues were then incubated with peroxidase-conjugated anti-digoxigenin antibodies and color was developed with DAB. After counterstaining with methyl green, sections were protected with coverslips secured with mounting medium. For immunofluorescence, slides were incubated overnight at 4°C with anti-MDM2 SMP-14 mouse antibody (1:50, Santa Cruz Biotechnology) and anti-αSMA rabbit antibody (1:200, Abcam) then exposed to anti-mouse Alexa Fluor (1:1000, Cell Signaling technology) and anti-rabbit Alexa Fluor (1:1000, Invitrogen) antibodies. Nuclei were stained with Hoechst.
Online Table I

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Echocardiographic parameters in nonsedated mice. Data are mean±SEM. *P<0.05 for comparison between hypoxic mice treated with Nutlin-3a and control untreated hypoxic mice. † p<0.05 for comparison between vehicle-treated hypoxic and normoxic mice. HR, heart rate; LVEF, left ventricular ejection fraction; CO, cardiac output; TVI, systolic time-velocity integral of pulmonary flow; PAT, pulmonary acceleration time; ET, ejection time.
Online Figure 1

Heart

Liver

Spleen

Kidney

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Vehicle  Nutlin-3a, 12mg/kg
Online Figure 2

- p21/18s mRNA
  - WT
  - SM22-5HTT+

- Bax/18s mRNA
  - WT
  - SM22-5HTT+

- BTG2/18s mRNA
  - WT
  - SM22-5HTT+

- Bcl2/18s mRNA
  - WT
  - SM22-5HTT+

- Bax/18s mRNA
  - WT
  - SM22-5HTT+

- p21/18s mRNA
  - WT
  - SM22-5HTT+

- BTG2/18s mRNA
  - WT
  - SM22-5HTT+

- Vehicle
- Nutlin-3a, 12mg/kg
Online Figure 3

A

- RVSP (mmHg)
- RV/LV+IS (%)
- Muscularized pulmonary vessels (%)
- Medial thickness (%)

B

- Ki67 positive cells (%)
- p21 positive cells (%)
- TUNEL positive cells (%)

Comparison of Vehicle and Nutlin-3a, 12mg/kg treatments.

Ki67
TUNEL
p21
Negative Control
Vehicle
Nutlin-3a
Online Figure 3

C

- Bar graph showing the ratio of MDM2/β-actin under Normoxia and Hypoxia conditions with and without SU5416 treatment. The bars represent Vehicle and Nutlin-3a, 12mg/kg conditions.

D

- Images showing immunofluorescence staining for MDM2, α-SMA, Hoechst, and Merge under different conditions: Vehicle, SU5416, and Nutlin-3a.
Online Figure 4

Graphs showing the expression levels of various genes under normoxic and hypoxic conditions for WT and p53 KO mice treated with Vehicle or Nutlin-3a, 12mg/kg. The graphs illustrate the mRNA expression levels of PUMA, Bcl2, Bax, and p21, with Vehicle and Nutlin-3a treatment indicating significant changes in gene expression levels.

- **PUMA**: A significant increase in expression is observed in hypoxic conditions for WT mice treated with Nutlin-3a.
- **Bcl2**: A decrease in expression is observed in hypoxic conditions for both WT and p53 KO mice treated with Nutlin-3a.
- **Bax**: A significant increase in expression is observed in hypoxic conditions for WT and p53 KO mice treated with Nutlin-3a.
- **p21**: No significant change is observed between normoxic and hypoxic conditions for any treatment group.

Key:
- Vehicle
- Nutlin-3a, 12mg/kg
Online Figure 5

- **Bax/18s mRNA**
  - WT p21 KO
  - WT p21 KO
  - Normoxia
  - Hypoxia
  - Significance markers: *
  - Significance markers: †

- **Bcl2/18s mRNA**
  - WT p21 KO
  - WT p21 KO
  - Normoxia
  - Hypoxia
  - Significance markers: *

- **PUMA/18s mRNA**
  - WT p21 KO
  - WT p21 KO
  - Normoxia
  - Hypoxia

- **BTG2/18s mRNA**
  - WT p21 KO
  - WT p21 KO
  - Normoxia
  - Hypoxia
  - Significance markers: *

- **Vehicle**
- **Nutlin-3a, 12mg/kg**
Online figure legends

Online Figure 1
Protein p53 levels measured by Western blot in the heart, liver, spleen, and kidneys in mice studied on day 21 after exposure to hypoxia or normoxia and treated with daily i.p. injections of 25 mg/Kg Nutlin-3a. Mice were sacrificed exactly 3 hours after the last Nutlin-3a dose. *P<0.05 compared with values in vehicle-treated mice after exposure to hypoxia or normoxia. Data are mean±SEM of 6 to 10 animals. †P<0.05 compared with values in vehicle-treated mice after exposure to hypoxia.

Online Figure 2
Lung mRNA levels of p21, bax, Bcl2, BTG2, and PUMA measured by RT-PCR in SM22-5HTT+ mice and control mice studied after 21 days of treatment with vehicle or 12 mg/Kg of Nutlin-3a. Mice were sacrificed exactly 3 hours after the last Nutlin-3a dose. Data are mean±SEM of 6 to 10 animals. *P<0.05 compared with values in vehicle-treated mice. †P<0.05 for SM22-5HTT+ versus vehicle-treated control mice.

Online Figure 3
A, right ventricular systolic pressure (RVSP); right ventricular hypertrophy index (RV/[LV+S] weight ratio); pulmonary vessel muscularization (percentage of partially and fully muscularized pulmonary vessels); medial wall thickness of fully muscularized intraacinar arteries; and percentages of Ki67-positive dividing cells, p21-stained cells, and TUNEL-positive cells in mice studied on day 21 after exposure to hypoxia and simultaneously given a weekly intraperitoneal injection of 20 mg/Kg SU5416, and treated daily by i.p. injections of vehicle or Nutlin-3a. B, representative micrographs of pulmonary
vessels stained for Ki67, TUNEL, or p21. No immunoreactivity was detected in sections incubated with secondary anti-rabbit and anti-mouse antibody but no primary antibody (Negative controls). C, Lung protein levels of MDM2 measured by Western blot. D, representative photographs of immunofluorescence staining for MDM2 in mice subjected to SUGEN/hypoxia and treated with Nutlin-3a or vehicle for 21 days. MDM2 was predominantly expressed in pulmonary vessels and co-localized with α-SMA in smooth muscle cells (SMCs). Data are mean±SEM of 6 to 10 animals.*P<0.05 compared with values in vehicle-treated mice.

Online Figure 4
Lung mRNA levels of p21, Bax, Bcl2, PUMA, and BTG2 in wild-type and p53 knockout (KO) mice studied on day 21 after exposure to hypoxia or normoxia and treated with daily i.p. injections of 12 mg/Kg Nutlin-3a. Data are mean±SEM of 10 animals. *P<0.05 compared with values in vehicle-treated mice. ‡P<0.05 compared with values in vehicle-treated control mice.

Online Figure 5
Lung mRNA levels of Bax, Bcl2, PUMA, and BTG2 in wild-type and p21 knockout (KO) mice studied on day 21 after exposure to hypoxia or normoxia and treated with daily i.p. injections of 12 mg/Kg Nutlin-3a. Data are mean±SEM of 6 to 10 animals. *P<0.05 compared with values in vehicle-treated mice. ‡P<0.05 compared with values in vehicle-treated control mice.
References
