Antiangiogenic Activity of the MDM2 Antagonist Nutlin-3

Paola Secchiero, Federica Corallini, Arianna Gonelli, Raffaella Dell’Eva, Marco Vitale, Silvano Capitani, Adriana Albini, Giorgio Zauli

Abstract—Nutlin-3, a nongenotoxic activator of the p53 pathway, dose-dependently (range 0.1 to 10 µmol/L) inhibited the formation of capillaries in an in vivo matrigel assay, as well as the formation of capillary-like structures in an in vitro coculture system composed of endothelial cells surrounded by fibroblasts. In contrast to the chemotherapeutic agent doxorubicin, nutlin-3 showed no induction of apoptosis in vitro either in the cocultures or in isolated vascular endothelial cells, even when used at the highest concentration (10 µmol/L). However, treatment with pharmacological inhibitors of the nuclear factor κB and phosphatidylinositol 3-kinase/Akt pathways sensitized endothelial cells to nutlin-3–induced apoptosis. Although nutlin-3 and doxorubicin induced a comparable p53 accumulation in endothelial cells, nutlin-3 was significantly more efficient than doxorubicin in upregulating the p53 target genes CDKN1A/p21, MDM2, and GDF-15, as well as in inhibiting cell cycle progression. However, the predominant in vitro effect of nutlin-3 was its strong antimigratory activity observed at concentrations significantly lower (0.1 µmol/L) than those required to inhibit endothelial cell cycle progression. Taken together, our data suggest that the antiangiogenic activity of nutlin-3 observed in vivo was mainly attributable to inhibition of endothelial cell migration, to some extent attributable to cell cycle arrest, and to a lesser extent attributable to induction of apoptosis. (Circ Res. 2007;100:61-69.)

Key Words: angiogenesis ■ endothelial cells ■ cell cycle ■ signaling pathways

Approximately 50% of all human malignancies harbor mutations or deletions in the TP53 gene that disable the tumor suppressor function of the encoded protein.1 This high rate of genetic alterations underscores the important cellular function of the tumor suppressor p53, whose activation can be initiated by diverse stresses, including DNA damage.2 Depending on the subset of activated p53 target genes, different downstream signaling events are initiated, leading to blockage of cell cycle progression, DNA repair or apoptosis, which are the most frequent and important cellular responses to p53 activation.3,4 In fact, the p53 pathway uses the G1/S and G2/M checkpoint mechanisms to arrest cell cycle progression and thus prevent propagation of DNA damage while cells attempt to repair it. However, if the damage is too severe, activation of the p53 pathway results in apoptotic cell death as the ultimate means of preventing possible malignant transformation of the damaged cells. For the purpose of this study, it is noteworthy that p53 also participates in regulating cell migration through the control of Rho signaling and actin cytoskeletal organization5.

The activation of p53 is tightly controlled by murine double minute 2 (MDM2) gene,6,7 whose expression is regulated in part by a p53-responsive promoter. In turn, MDM2, which is an E3 ubiquitin ligase for p53 and, itself, controls p53 half-life via ubiquitin-dependent degradation. In response to cellular stress, the p53–MDM2 interaction is disrupted, p53 undergoes posttranslational modifications on multiple sites and becomes more stable.6,7 Recently, potent and selective small molecule inhibitors of p53–MDM2 interaction, the nutlins, have been developed.8–11 These nongenotoxic compounds bind MDM2 in the p53-binding pocket with high selectivity and can release p53 from negative control, leading to effective stabilization of p53 and activation of the p53 pathway.11 On the other hand, nutlins do not bind to p53 protein and do not interfere with its activities.

Because it has been clearly established that any significant increase in tumor mass must be preceded by an increase in the vascular supply to deliver nutrients and oxygen to the tumor,12,13 much effort has been placed in recent years on translating antiangiogenic molecules into the clinical setting.14 The aim of this study was to investigate the potential biological effect of nutlin-3 on angiogenesis. For this purpose, we studied the ability of nutlin-3 to modulate the formation of capillary networks both in vivo and in vitro, analyzing in particular the effect of different concentrations of nutlin-3 on survival, proliferation, and migration of cultured vascular endothelial cells.
Materials and Methods

Reagents

For the in vivo and in vitro experiments, the following reagents, individually or in combination, were used: nutlin-3, doxorubicin, phytohemagglutinin (PHA) (all from Sigma-Aldrich, St Louis, MO); tumor necrosis factor (TNF)-α (R&D Systems, Minneapolis, Minn); basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF)-A, interleukin (IL)-2 (all from Peprotech, London, UK); LY29400 (Calbiochem, La Jolla, Calif); and parthenolide (Alexis Biochemicals, San Diego, Calif).

In Vivo Angiogenesis

The matrigel sponge model of angiogenesis was used, as described previously.15,16 VTH cocktail (100 ng/mL VEGF-A, 2 ng/mL TNF-α, and heparin) either alone or in combination with nutlin-3 was added to unpolymerized liquid matrigel at 4°C, and the mixture was brought to a final volume of 0.6 mL. (heparin is always added to matrigel samples to avoid cytokine/growth factor trapping by proteoglycans). The matrigel suspension was then slowly injected subcutaneously into the flanks of C57/BL6 male mice (Charles River, Lecco, Italy) with a cold syringe. At in vivo body temperature, the matrigel quickly polymerizes to form a solid gel. Groups of 8 mice were used for each treatment. Control animals received implants of matrigel containing heparin only. Four days after injection, the gels were recovered, minced, and diluted in water to measure the hemoglobin content with a Drabkin reagent kit (Sigma). Some samples were formalin-fixed, paraffin-embedded, sectioned at 2 to 4 μm, and then stained with hematoxylin and eosin for histological analysis or with anti-myeloperoxidase (DAKO, Glostrup, Denmark) antibody (Ab) for immunochemistry.16 Animal care and treatments were conducted in conformity with institutional guidelines in compliance with national and international laws and policies (EEC Council Directive 86/609, OJL 358, December 12, 1987).

In Vitro Tube Formation Assay

In vitro angiogenesis was assessed as formation of capillary-like structures of human umbilical vein endothelial cells (HUVECs) cocultured with matrix-producing fibroblasts (Angiokit assay, TCS Biologicals, Buckingham, UK).17 In selected experiments, monolayers of fibroblasts (obtained from TCS Biologicals) cultured in the absence of HUVECs were used. Briefly, cultures were left untreated or treated with nutlin-3, VEGF-A, or bFGF alone or in combination. Media and treatments were replaced every 3 days, and the status of cell layers was monitored over time by light microscopy and/or after crystal violet staining. The culture supernatants were harvested at different time points and examined for GDF-15 (growth/differentiation factor-15) release. At day 12, the cells were fixed and HUVECs were stained using an anti-CD31 Ab (TCS Biologicals), according to the instructions provided of the manufacturer.

After crystal violet and/or CD31 staining, images were captured and analyzed with the use of a video-based image analysis program (MCID; Imaging Research, St Catharines, Ontario, Canada). In particular, to measure the formation of the capillary network, the number of connections among 3 or more capillary-like structures and the total length of tubes were quantified by image analysis at ×10 magnification. Four to 6 different fields were analyzed per well. In some experiments, cocultures were treated with doxorubicin, and the degree of apoptosis was evaluated by in situ TUNEL technique, as described below.

Cell Cultures

HUVECs were purchased from BioWhittaker (Walkersville, Md) and grown on 0.2% gelatin-coated tissue culture plates in medium 199 endothelial growth medium supplemented with 20% FBS, heparin, and 50 mg/mL endothelial cell growth factor (all from BioWhittaker). In all experiments, cells were used between the third and fifth passage in vitro, as previously described.18

SKW6.4 lymphoblastoid cell line was purchased from American Type Culture Collection (ATCC, Manassas, Va) and cultured in RPMI medium 1640 containing 10% FBS. Peripheral blood was collected in heparin-coated tubes from healthy blood donors following informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density-gradient centrifugation, and enriched T-lymphocyte preparations were seeded in RPMI medium 1640 containing 10% FBS and stimulated with PHA (1 μg/mL; Sigma), IL-2 (20 U/mL; Peprotech) or treated with nutlin-3, VEGF-A, or bFGF alone or in combination. For the in vivo and in vitro experiments, the following reagents, individually or in combination, were used: nutlin-3, doxorubicin, phytohemagglutinin (PHA) (all from Sigma-Aldrich, St Louis, MO); tumor necrosis factor (TNF)-α (R&D Systems, Minneapolis, Minn); basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF)-A, interleukin (IL)-2 (all from Peprotech, London, UK); LY29400 (Calbiochem, La Jolla, Calif); and parthenolide (Alexis Biochemicals, San Diego, Calif).

Viability Assays and Cell Cycle Profile Analysis

For evaluation of cytostatic and/or toxic effects, HUVECs were seeded and grown to subconfluence before treatments with nutlin-3 or doxorubicin. In selected experiments, cells were treated simultaneously with nutlin-3 plus LY29400, a pharmacological inhibitor of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway or parthenolide, an inhibitor of IκB degradation and nuclear factor κB (NF-κB) DNA-binding activity.

The viability of cell cultures was monitored over time by light microscopy and by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Roche, Mannheim, Germany). The detection of apoptosis in adherent HUVECs was performed by using the in situ cell death detection kit (Roche Diagnostics Corp, Indianapolis, Ind), according to the instructions of the manufacturer. The percentage of TUNEL-positive cells was calculated by fluorescent microscopy observation (Axioptoph 100, Zeiss, Germany) after counterstaining with 4',6-diamidino-2-phenylindole (DAPI). Apoptosis was also quantitatively evaluated by double staining with Annexin V and propidium iodide (PI), followed by flow cytometry. For this purpose, cells were stained with fluorescein isothiocyanate–conjugated Annexin-V (Alexis Biochemicals, Lausen, Switzerland) and PI, according to the instruc-
tions of the manufacturer, and analyzed as previously detailed. To analyze the degree of apoptosis in the entire cell population of HUVEC cultures, substrate-attached endothelial cells were harvested by trypsin treatment and pooled with floating cells for the staining.

Flow cytometric analysis was also used to determine the cell cycle profile. Substrate-attached HUVECs, as well as suspensions of activated T lymphocytes and SKW6.4 lymphoblastoid cells, were incubated with 50 μmol/L 5-bromodeoxyuridine (BrdUrd) (Sigma) at 37°C for 1 hour. Anti-BrdUrd Ab was bound to BrdUrd incorporated into neosynthesized DNA, and the complex was detected by fluorescein isothiocyanate–conjugated secondary Ab. Cells were stained with PI (50 μg/mL) and analyzed by flow cytometry. To avoid nonspecific fluorescence from dead cells, live cells were gated tightly using forward and side scatter.

Enzyme-Linked Immunosorbent Assays

Release of GDF-15 was measured in supernatants of Angiokit cocultures, fibroblasts, and HUVECs. Commercially available ELISA kits, purchased from R&D Systems, were used according to the instructions of the manufacturer. The results were read at an optical density of 450 nm using an Anthos 2010 ELISA reader (Anthos Labtec Instruments, Wals Salzburg, Austria). Measurements were performed in duplicate, and results, corrected for the dilution factors, are reported as mean ± SD of at least 3 independent experiments.

Western Blot Analysis

To obtain lysates from HUVECs, cells were lysed with a buffer containing 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and protease inhibitors (protease inhibitor cocktail P8340; Sigma). After protein determination performed with the Bradford assay (Bio-Rad, Richmond, Calif), equal amounts of proteins for each sample were migrated in 10% PAGE, blotted onto nitrocellulose filters, and incubated with the following Abs: anti-p53, anti-MDM2, anti–poly(ADP-ribose) polymerase (anti-PARP) (from Santa Cruz Biotechnology, Santa Cruz, Calif), anti-p21 (Cell Signaling Technology, Beverly, Mass), and anti–β-actin (Sigma) as loading control. Reactions were revealed with the ECL Western blotting detection reagent (Amersham Corp, Arlington Heights, Ill).

Cell Migration Assay

Cell migration assays on HUVECs were performed in Boyden chambers as described previously. Cells (5×10⁴) were extensively washed with PBS, resuspended in serum-free medium, and placed in the upper compartment with or without nutlin-3. The 2 compartments of the Boyden chamber were separated by 12-μm pore-size polycarbonate filters coated with 5 μg/mL collagen IV.

Figure 2. Effect of nutlin-3 on the capillary-like network in the in vitro coculture angiogenesis assays. Formation of capillary-like structures in HUVECs cocultured with matrix-producing cells was analyzed after 12 days of treatment with nutlin-3, used either alone or in combination with angiogenic cytokines (VEGF-A and bFGF) as indicated. A, Representative morphological examples of the endothelial structures, detected after staining with anti–CD31 Ab, in the in vitro angiogenesis assay. Rare capillary structures were observed in the angiogenesis assay left untreated, whereas both VEGF-A and bFGF induced a diffuse network of capillary structures. Original magnification, ×10. B, Cultures, treated as indicated, were observed after 12 days, and results were recorded for quantitative analysis of the total length of tubes per field and the number of capillary connections per field. Data are expressed as means ± SD of results from 5 experiments, each performed in duplicate. *P < 0.05 compared with untreated. C, Supernatants were harvested from the cocultures, and induction of GDF-15 was measured by ELISA. Data are expressed as means ± SD of results from 4 experiments, each performed in duplicate. *P < 0.05 compared with untreated. D, Inverse correlation between the induction of GDF-15, measured at day 3, and the total tube length. R = −0.7; P < 0.05.
bFGF, VEGF-A (both used at 4 ng/mL), or supernatants from NIH3T3 cells were used as chemoattractants in the lower chamber. After 6 hours of incubation at 37°C in 5% CO2, the filters were recovered, the cells on the upper surface were mechanically removed, and those on the lower surface were fixed and stained. The migration was measured by densitometric analysis. The experiments were done in triplicate.

**Statistical Analysis**
Data were analyzed by ANOVA and with the Mann–Whitney rank-sum test. For each set of experiments, values are reported as means±SD. Comparison of group means was performed by Bonferroni method. For EC_{50} calculation, experiments were performed by testing serial dilutions (1:3) of nutlin-3. Correlation coefficients were calculated by the Spearman’s method. Statistical significance was defined as P<0.05.

**Results**

**Nutlin-3 Blocks Capillary Formation Both In Vivo and In Vitro**

The first group of experiments was performed using the in vivo matrigel sponge assay, which represents a rapid and quantitative system for measuring angiogenesis in vivo. A cocktail of VEGF-A, TNF-α, and heparin (VTH) promoted hemorrhagic vascularization of the gels that was readily apparent after 4 days (Figure 1A). Quantification of the extent of angiogenesis by measurement of the hemoglobin content showed that addition of nutlin-3 to the VTH cocktail in the matrigel dose-dependently inhibited the angiogenic response (P<0.05, Figure 1B) with an EC_{50} value of 0.1±0.03 μmol/L.

The effect of nutlin-3 was next analyzed in an in vitro Angiokit assay, a coculture system in which capillaries develop between layers of fibroblasts and morphogenetic processes of tubule formation occur in HUVECs completely surrounded by stromal fibroblasts, as in vivo. As shown in Figure 2A and 2B, morphological observation and morphometric quantification of the vessel-like structures revealed that nutlin-3 dose-dependently suppressed both the total tube length and the number of capillary connections that developed over a 12-day culture time, in cocultures left untreated or stimulated with the proangiogenic cytokines bFGF (25 ng/mL) or VEGF-A (10 ng/mL) (Figure 2A and 2B). The mean EC_{50} value of nutlin-3 inhibition for total tubular length was 0.5±0.02 μmol/L for unstimulated cultures and 0.15±0.01 μmol/L and 0.4±0.02 μmol/L for VEGF- and bFGF-treated cultures, respectively.

To ascertain whether these effects of nutlin-3 were related to the activation of the p53 pathway, we quantitatively evaluated the release in the culture supernatant of GDF-15 (Figure 2C), which represents a sensitive biomarker of p53 activation. In fact, it has been shown that the amount of GDF-15 released in the culture supernatants as well as in the plasma of animal models accurately correlates with the degree of activation of wild-type p53. As shown in Figure 2C, nutlin-3 dose-dependently increased the release of GDF-15, starting from day 3 of culture and peaking at day 6 of culture. Of note, there was a significant inverse correlation between the levels of GDF-15 released in the culture supernatants and the total tube length (Figure 2D).

To exclude the possibility that the reduction in capillary-like network induced by nutlin-3 in the cocultures merely reflected a general cytoxicity of nutlin-3, 2 independent
approaches were used: in situ TUNEL technique (Figure 3A) and crystal violet staining (Figure 3B), analyzed by fluorescence and light microscopy, respectively. In agreement with previous findings, treatment with the chemotherapeutic agent doxorubicin significantly \((P<0.05)\) induced apoptosis in the Angiokit assay (Figure 3A), which resulted in a marked \((P<0.05)\) reduction in the total number of viable cells in the monolayers (Figure 3B). In contrast to doxorubicin, nutlin-3 did not increase the degree of apoptosis over the background levels, even when used at \(10\ \mu\text{mol/L}\) (Figure 3A), whereas it significantly \((P<0.05)\) decreased the total number of cells at light microscopy examination at only the highest concentration used \((10\ \mu\text{mol/L})\) (Figure 3B). These findings are particularly noteworthy because a significant \((P<0.05)\) inhibition in capillary tube formation was observed also at concentrations as low as 1 and \(0.1\ \mu\text{mol/L}\) both in vivo (Figure 1B) and in vitro (Figure 2B).

**Nutlin-3 Induces the Accumulation of p53 Protein and p53 Transcriptional Targets in Endothelial Cells**

Addition of nutlin-3 to HUVECs for 24 hours induced a dose-dependent accumulation of p53 protein, with \(10\ \mu\text{mol/L}\) of nutlin-3 showing a comparable effect to that induced by the genotoxic agent doxorubicin \((0.5\ \mu\text{mol/L})\), used as positive control for its previously documented ability to upregulate the p53 pathway in endothelial cells. The accumulation of p53 was accompanied by the upregulation of the known p53 transcriptional targets \(\text{CDKN1A/p21, MDM2 (Figure 4A) and GDF-15 (Figure 4B). Interestingly, nutlin-3 (10\ \mu\text{mol/L}) was significantly more efficient than doxorubicin in upregulating both p21 and MDM2 (Figure 4A).**

**Nutlin-3 Induces Apoptosis in Endothelial Cells Only in the Presence of Pharmacological Inhibitors of the NF-κB and PI3K/Akt Pathways**

Because activation of the p53 pathway has been reported to induce apoptosis in different cell types, the occurrence of endothelial cell apoptosis was next analyzed after treatment with nutlin-3 or doxorubicin. The degree of apoptosis was evaluated by in situ TUNEL staining of HUVEC monolayers (Figure 5A) or by Annexin V/PI followed by flow cytometry in detached plus floating HUVECs (Figure 5B). Nutlin-3 did not induce a significant increase of apoptosis in HUVECs at any of the concentrations \((0.1\ \text{to}\ 10\ \mu\text{mol/L)}\) used (Figure 5A through 5C), in keeping with the previous findings obtained in the Angiokit assay. On the contrary, doxorubicin induced a significant \((P<0.05)\) increase of endothelial cell apoptosis as evaluated either by TUNEL or by Annexin V/PI staining, and further confirmed by PARP cleavage in Western blot (Figure 5A and 5B). Of note, nutlin-3 \((10\ \mu\text{mol/L})\) significantly \((P<0.05)\) increased the degree of apoptosis when added to the lymphoblastoid SKW6.4 cell line, carrying a wild-type p53 phenotype, but not when added to PHA-activated primary T lymphocytes (Figure 5C), suggesting that the ability of nutlin-3 to promote apoptosis is cell-type specific.

In an additional set of experiments, nutlin-3 induced a significant \((P<0.05)\) increase of endothelial cell apoptosis when added to HUVECs in the presence of pharmacological inhibitors of the NF-κB (parthenolide) and PI3K/Akt (LY29400) pathways. As shown in Figure 5D, parthenolide and LY29400 variably promoted endothelial cell apoptosis, which was augmented by the simultaneous presence of nutlin-3. These data confirm the important roles of NF-κB and PI3K/Akt in mediating survival signaling in HUVECs and that endothelial cells can be sensitized to nutlin-mediated apoptosis by blocking these pathways.

**Nutlin-3 Partially Counteracts Endothelial Cell Cycle Progression**

Besides inducing apoptosis, the p53 pathway has been involved in cell cycle arrest at the G1/S and G2/M borders, predominantly through the transcriptional activation of CDKN1A/p21. Because endothelial cell proliferation represents an essential step of capillary formation, the effect of increasing concentrations \((0.1\ \text{to}\ 10\ \mu\text{mol/L)}\) of nutlin-3 on the cell cycle profile of endothelial cells was next investigated on endothelial cell proliferation, as evaluated by flow cytometric analysis after BrdUrd labeling and PI staining (Figure 6A). Nutlin-3 treatment significantly \((P<0.05)\) decreased the S-phase population in HUVECs at \(1\ \mu\text{mol/L}\) and almost completely depleted the S-phase pool at \(10\ \mu\text{mol/L}\ \ (P<0.01)\) (Figure 6A). On the other hand, at lower concentrations \((0.1\ \mu\text{mol/L})\), nutlin-3 was totally ineffective.

Interestingly, in spite of the comparable ability to induce p53 accumulation (Figure 4A), doxorubicin \((0.5\ \mu\text{mol/L})\) showed modest effects compared with nutlin-3 \(10\ \mu\text{mol/L}\), on the cell cycle profile of endothelial cells (Table), in agreement with its modest ability to upregulate p21 transcription (Figure 4A).
Consistently with the BrdUrd data, addition of nutlin-3 to subconfluent cultures of HUVECs induced a significant (P<0.05, at the concentrations of 1 and 10 μmol/L) decrease in the total number of endothelial cells at both 3 and 6 days of treatment, as evaluated by the MTT assay (Figure 6B). Such inhibitory activity was also observed when increasing concentrations of nutlin-3 were added to HUVECs stimulated with either bFGF (Figure 6C) or VEGF-A (Figure 6D).

To ascertain whether nutlin-3 modulates the cell cycle profile at a similar extent in any p53 wild-type cells, the effect of scalar doses of nutlin-3 were added to HUVECs stimulated with either bFGF (Figure 6C) or VEGF-A (Figure 6D).

Figure 5. Evaluation of apoptosis in vascular endothelial cells, lymphoblastoid SKW6.4 cells, and activated T lymphocytes in response to nutlin-3. A and B, HUVECs were treated with the indicated concentrations of nutlin-3 (Nut.) or doxorubicin (Doxo.). In A, apoptosis was analyzed by in situ TUNEL. Representative areas showing a higher percentage of TUNEL-positive apoptotic cells in HUVECs treated with doxorubicin. After counterstaining with DAPI, the percentage of TUNEL− cells was calculated by counting TUNEL− cells per total nuclei within the cultures. In B, apoptosis was quantitatively evaluated by flow cytometry after Annexin V/PI staining. Data are expressed as means±SD of 3 separate experiments performed in duplicate. Inset, Western blot analysis for PARP cleavage; the proform of PARP (115 kDa) and the cleaved form (80 kDa; arrowhead) are shown. Unt. indicates untreated. C, Representative flow cytometry profiles after Annexin V/PI of lymphoblastoid SKW6.4 cells and activated T lymphocytes for the determination of apoptosis induction in response to nutlin-3. HUVECs are also shown for comparison. Results representative of 3 separate experiments are shown. D, Apoptosis was assessed in HUVECs treated with nutlin-3 (10 μmol/L) in the presence of parthenolide (10 μmol/L) or LY294000 (20 μmol/L) or control vehicle. Data are expressed as means±SD of 3 separate experiments performed in duplicate.

Nutlin-3 Strongly Inhibits Endothelial Cell Migration

Because the p53 pathway has also been involved in actin cytoskeletal organization and regulation of cell migration,5 which represents an essential step in the morphogenetic events accompanying capillary formation,12,13 in the last group of experiments, we have investigated whether nutlin-3 could also affect endothelial cell motility (Figure 7). Nutlin-3 alone did not show any effect of endothelial cell migration in a modified Boyden chamber assay (data not shown), whereas it dose-dependently inhibited (P<0.05; EC50, 0.5±0.2 μmol/L) the direct migration of endothelial cells induced by various chemotactants, such as NIH3T3 fibroblast supernatants, bFGF or VEGF-A (Figure 7).
Angiogenesis is a complex biological process that depends mainly on the proliferation, migration, and tube formation of endothelial cells. Angiogenesis is normally tightly regulated by the balance between proangiogenic and antiangiogenic factors, and, because tumor growth, invasion, and metastasis are angiogenesis-dependent processes, increasing importance has been given to the search for suitable targets that negatively modulate angiogenesis.

In this study, we have demonstrated for the first time that the nongenotoxic activator of the p53 pathway nutlin-3 strongly suppressed the formation of capillary-like structures both in vivo and in vitro. The ability of nutlin-3 to suppress angiogenesis could not be ascribed to a generalized cytotoxic effect. In fact, nutlin-3 did not induce cell death in the in vitro angiogenesis assay, and it induced a marked increase of GDF-15 release in the coculture supernatant. It is also possible that GDF-15, produced by endothelial cells at higher levels compared with the stromal cells (data not shown), may contribute to the antineoplastic activity of nutlin-3 because GDF-15 has been demonstrated to exhibit a cytostatic/cytotoxic activity in several tumor cell types and to mediate the antiangiogenic activity of the natural compound N-(4-hydroxyphenyl) retinamide.

Accumulation of p53 in endothelial cultures was accompanied by the upregulation not only of GDF-15 but also of the p53 target gene p21. This potent cyclin-dependent kinase (CDK) inhibitor is likely responsible for the endothelial cell cycle arrest observed in nutlin-3–treated endothelial cells. These findings obtained on primary normal endothelial cells are particularly noteworthy because there are abundant data regarding the growth suppressive and proapoptotic function of p53 in cancer cells, although much less is known about the consequences of p53 activation in normal proliferating tissues both in vitro and in vivo. In this respect, we have also demonstrated that nutlin-3 interfered with the cell cycle of circulating T cells activated with PHA plus IL-2. However, activated T cells showed a significantly lower sensitivity to the cytostatic effect of nutlin-3 with respect to endothelial cells, clearly indicating that the effect of nutlin-3 was also cell-type specific among primary cells. The relatively low sensitivity of activated T cells to nutlin-3 cytostatic activity is also particularly noteworthy from a therapeutic perspective, taking into account the key role of tumor-specific T cells in mediating the antineoplastic activity of the immune system.

### Discussion

Angiogenesis is a complex biological process that depends mainly on the proliferation, migration, and tube formation of endothelial cells. Angiogenesis is normally tightly regulated by the balance between proangiogenic and antiangiogenic factors, and, because tumor growth, invasion, and metastasis are angiogenesis-dependent processes, increasing importance has been given to the search for suitable targets that negatively modulate angiogenesis.

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Interestingly, in contrast to the genotoxic agent doxorubicin, nutlin-3 did not induce apoptosis of endothelial cells, even at the highest concentration used (10 μmol/L), unless combined with pharmacological inhibitors of the NF-κB and PI3K/Akt pathways. These findings are noteworthy, taking into account that pharmacological inhibitors of these pathways are now in clinical trials for different types of human cancers. On the other hand, the ability of doxorubicin to efficiently induce p53 accumulation and apoptosis in endothelial cells in the context of a poor activation of p53 transcription targets is in line with recent findings indicating that p53 induces apoptosis predominantly via transcriptional-independent mechanisms. However, the inability of nutlin-3 alone to induce apoptosis in primary endothelial cells was not caused by a functional defect of the nutlin-3 stock used in this study, because it efficiently promoted apoptosis in the p53 wild-type SKW6.4 lymphoblastoid cell line.

The last important finding of our study was represented by the ability of nutlin-3 to significantly inhibit the migration of endothelial cells in response to various chemoattractants. This inhibitory effect of nutlin-3 was observed at concentrations of nutlin-3 as low as 0.1 μmol/L, whereas higher concentrations were required to inhibit cell cycle progression. Because a significant inhibition of angiogenesis was noticed in both in vivo and in vitro assays, starting from concentrations as low as 0.1 μmol/L, it is likely that endothelial migration represents the proangiogenic step more sensitive to nutlin-3 inhibition.

From a therapeutic perspective, it is noteworthy that pharmacologically active concentrations of nutlin-3 have been achieved with oral administrations in rodents injected with different types of tumors. Therefore, although it has been clearly demonstrated that tumors harboring mutated or deleted p53, which represent approximately 50% of all human tumors, are not sensitive to the cytotoxic/cytostatic activity of MDM2 antagonists, an important implication of our findings is that a therapeutic regimen including nutlin-3 still offers the possibility to abrogate tumor angiogenesis also in p53 mutated tumors.

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**Disclosures**

None.

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