Small-Molecule Activation of p53 Blocks Hypoxia-Inducible Factor 1 α and Vascular Endothelial Growth Factor Expression In Vivo and Leads to Tumor Cell Apoptosis in Normoxia and Hypoxia[∇]

Jun Yang,^{1†} Afshan Ahmed,^{2†} Evon Poon,² Nina Perusinghe,¹ Alexis de Haven Brandon,³ Gary Box,³ Melanie Valenti,³ Suzanne Eccles,³ Kasper Rouschop,⁴ Brad Wouters,⁵ and Margaret Ashcroft^{1,2*}

Cell Growth Regulation and Angiogenesis Team, Cancer Research UK Centre for Cancer Therapeutics, the Institute of Cancer Research, 15 Cotswold Rd., SaHon, Surrey SM2 5NG, United Kingdom¹; Hypoxia Signalling and Angiogenesis Laboratory, Centre for Cell Signalling and Molecular Genetics, Department of Metabolism and Experimental Therapeutics, Division of Medicine, University College London, Rayne Building, 5 University Street, London WC1E 6JJ, United Kingdom²; Tumor Biology and Metastasis Team, Cancer Research UK Centre for Cancer Therapeutics, the Institute of Cancer Research, 15 Cotswold Rd., Sutton, Surrey SM2 5NG, United Kingdom³; Maastricht Radiation Oncology (Maastricht), University of Maastricht, 6200 Maastricht, The Netherlands⁴; and Ontario Cancer Institute, Princess Margaret Hospital, Toronto, Ontario, Canada M5G OA3⁵

Received 17 June 2008/Returned for modification 3 September 2008/Accepted 30 January 2009

The p53 tumor suppressor protein negatively regulates hypoxia-inducible factor 1 α (HIF-1 α). Here, we show that induction of p53 by the small-molecule RITA (reactivation of p53 and induction of tumor cell apoptosis) [2,5-bis(5-hydroxymethyl-2-thienyl) furan] (NSC-652287) inhibits HIF-1 α and vascular endothelial growth factor expression in vivo and induces significant tumor cell apoptosis in normoxia and hypoxia in p53-positive cells. RITA has been proposed to stabilize p53 by inhibiting the p53-HDM2 interaction. However, induction of p53 alone was insufficient to block HIF-1 α induced in hypoxia and has previously been shown to require additional stimuli, such as DNA damage. Here, we identify a new mechanism of action for RITA: RITA activates a DNA damage response, resulting in phosphorylation of p53 and γ H2AX in vivo. Unlike other DNA damage response-inducing agents, RITA treatment of cells induced a p53-dependent increase in phosphorylation of the α subunit of eukaryotic initiation factor 2, requiring PKR-like endoplasmic reticulum kinase activity, and led to the subsequent downregulation of HIF-1 α and p53 target proteins, including HDM2 and p21. Through the identification of a new mechanism of action for RITA, our study uncovers a novel link between the DNA damage response-p53 pathway and the protein translational machinery.

Solid tumors require blood vessels to supply them with oxygen and nutrients in order to grow beyond the macroscopic level or metastasize to other organs. Characteristically, solid tumors contain areas of low oxygen tension (hypoxia). The cellular response to hypoxia is primarily mediated by hypoxia-inducible factors (HIFs) hypoxia-inducible factor 1 (HIF-1) and HIF-2 (12, 42). HIFs are transcription factors that are often deregulated in cancer and play a key role in promoting angiogenesis and tumor progression. Hypoxia usually confers tumor resistance to chemotherapy and radiotherapy (8), and HIF-1 is an important determinant for this resistance (7, 35, 38, 49). Thus, targeting HIF has the potential not only to block tumor angiogenesis but also to improve the efficacy of chemotherapy and radiotherapy within a given cancer setting.

HIF-1 and HIF-2 are heterodimeric complexes consisting of HIF-1 α and HIF-2 α (HIF- α), respectively, that dimerize to a constitutively expressed β subunit (HIF-1 β). HIF transcriptional activity is regulated by the availability of the α subunit which is hydroxylated at conserved prolyl and asparaginyl res-

idues in normoxia. Hydroxylation allows for the binding of von Hippel-Lindau protein (pVHL) E3 ligase that targets HIF- α for ubiquitin-mediated degradation by the proteasome (24, 25). In hypoxia, inhibition of hydroxylation results in stabilization of HIF- α and leads to transcriptional activation of target genes involved in angiogenesis, cell survival, and metabolic adaptation (42).

The p53 tumor suppressor protein is induced and activated in response to a variety of cellular stressors, including DNA damage, oxidative stress, and cellular senescence (3, 48), and is a potent negative regulator of HIF-1 α , mediating both apoptotic (20, 43) and antiangiogenic effects when overexpressed (27, 39). p53 plays an important role in apoptosis in hypoxic tumor cells in that only wild-type p53-expressing cells undergo strong apoptosis (20), while apoptosis is significantly reduced when tumors express mutant p53 (20, 43). HIF-1 α accumulation is blocked by overexpression (39) or activation (27) of p53. In addition, HIF-1-dependent transcription negatively correlates with p53 status (41). p53 is mutated in about 50% of human cancers, and several agents that can reactivate mutant p53 (11, 19) or activate wild-type p53 (23, 45, 47) in tumor cells have been reported. However, many of these emerging p53-targeted agents have not yet been evaluated for their ability to affect the HIF-1 pathway or assessed for their effectiveness at mediating tumor cell death in hypoxia. Recently, a small-molecule activator of p53, RITA (reactivation of p53 and in-

* Corresponding author. Mailing address: Centre for Cell Signalling and Molecular Genetics, Division of Medicine, University College London, Rayne Building, 5 University Street, London WC1E 6JJ, United Kingdom. Phone: 44 (0) 207 679 0799. Fax: 44 (0) 207 679 6211. E-mail: m.ashcroft@ucl.ac.uk.

† These authors contributed equally to this work.

[∇] Published ahead of print on 17 February 2009.

duction of tumor cell apoptosis) [2,5-bis(5-hydroxymethyl-2-thienyl) furan] was described (23). RITA was shown to induce tumor cell apoptosis in a p53-dependent manner and to inhibit tumor growth in vivo (23). RITA was proposed to stabilize and activate p53 by disruption of the p53-HDM2 interaction (23). However, subsequent nuclear magnetic resonance (NMR) studies did not support this proposed mechanism (31).

In this study, we assess the effects of RITA on HIF-1 α accumulation and apoptotic responses in normoxia and hypoxia. We demonstrate that RITA induces and activates p53, mediating significant tumor cell apoptosis in both normoxia and hypoxia. Furthermore, we demonstrate that RITA blocks HIF-1 α and vascular endothelial growth factor (VEGF) expression in vitro and in vivo. We propose that RITA functions by inducing a DNA damage response indicated by increased p53 and γ H2AX phosphorylation in vitro and in vivo. Unlike other DNA-damaging agents, RITA treatment of cells induces phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF-2 α) and subsequently leads to the downregulation of HIF-1 α and p53 target proteins, including HDM2 and p21, in a dose-, time-, and p53-dependent manner. Our study provides new mechanistic insight into p53-dependent antiangiogenic and apoptotic responses mediated by activation of the DNA damage response-p53 pathway.

MATERIALS AND METHODS

Cell culture. All tumor cell lines were maintained in Dulbecco modified Eagle medium. Medium was supplemented with 10% fetal calf serum purchased from Harlan (Oxford, United Kingdom), 100 IU/ml penicillin, 100 μ g/ml streptomycin, and 2 mM glutamine (all purchased from Gibco/Life Technologies, Paisley, United Kingdom). The matched colorectal cell lines p53^{-/-} HCT116 and p53^{+/+} HCT116 have been described previously (10). The tetracycline-responsive (TetON) p53-inducible Saos-2 cell line was a gift from Karen Vousden (Beatson Institute for Cancer Research, Glasgow, United Kingdom) and has been described previously (36). The tetracycline-inducible HCT116-pCDNA5 (control) and HCT116-PERK Δ C (PKR-like endoplasmic reticulum kinase [PERK] dominant-negative) cell lines were a kind gift from Kasper Rouschop (University of Maastricht, Maastricht, The Netherlands) and Brad Wouters (Ontario Cancer Institute, Toronto, Ontario, Canada). The breast carcinoma cell lines MCF-7 and MDA-MB-231 were purchased from the American Type Culture Collection (ATCC).

siRNA duplexes and transient transfection. The small interfering RNA (siRNA) to p53 (human p53, 5'-GCATCTTATCCGAGTGGAA-3') was obtained as a gel-purified annealed duplex from Dharmacon (Lafayette, CO) and used at a final concentration of 5 nM. The siRNA to p21 was described previously (4) and purchased from Dharmacon (Lafayette, CO). The nonsilencing control siRNA duplex (5'-AATTCTCCGAACGTGTACAGT-3') was obtained from Qiagen (Crawley, United Kingdom). Transient transfections with siRNA duplexes were carried out using HiPerfect transfection reagent (Qiagen) according to the manufacturer's instructions.

Antibodies. The HIF-1 α monoclonal antibody was purchased from BD Transduction Laboratories (Oxford, United Kingdom). The p53 monoclonal antibody (DO-1) was purchased from Calbiochem (Merck Biosciences, Nottingham, United Kingdom). The p53 polyclonal antibody, the monoclonal anti-phospho-S15-p53, polyclonal anti-phospho-S51-eIF-2 α , anti-phospho-T172-AMPK α (antibody to AMP-activated protein kinase [AMPK] with T172 phosphorylated), anti-AMPK α , anti-phospho-T37/46-4E-BP1, and anti-phospho-T389-p70S6K were all purchased from Cell Signaling Technologies (Danvers, MA). The anti-phospho-S139- γ H2AX monoclonal antibody was purchased from Upstate (Millipore, United Kingdom).

Inductions and drug treatments. Physiological hypoxia was achieved by incubating cells in 1% O₂, 5% CO₂ and 94% nitrogen in a LEEC dual gas incubator (GA-156). The hypoxic mimetic agent deferoxamine mesylate was used at a final concentration of 500 μ M. RITA [2,5-bis(5-hydroxymethyl-2-thienyl) furan] was obtained from the National Cancer Centre, Drug Therapeutic Program, Frederick, MD (NSC-652287) and dissolved in dimethyl sulfoxide (DMSO). Nutlin-3 (Sigma, Gillingham, United Kingdom) was used at a final concentration of 4 μ M. The

proteasome inhibitor MG132 (Calbiochem-Merck Biosciences, Nottingham, United Kingdom) was used at 10 μ M unless otherwise stated, and the caspase-3 inhibitor Z-DEVD-FMK [Z-Asp(OCH₃)-Glu(OCH₃)-Val-Asp(OCH₃)-FMK] (Calbiochem) was used at the indicated concentrations.

Western blot analysis and immunoprecipitation. After treatment, cells were washed in ice-cold phosphate-buffered saline (PBS) and lysed in 2 $\times$ sample buffer (125 mM Tris [pH 6.8], 4% sodium dodecyl sulfate, 0.01% bromophenol blue, 10% β -mercaptoethanol, 10% glycerol). Alternatively, cells were harvested in NP-40 lysis buffer (100 mM Tris [pH 8.0], 100 mM NaCl₂, 1% NP-40) containing an EDTA-free protease inhibitor cocktail (Boehringer Mannheim-Roche Diagnostics Ltd., Burgess Hill, United Kingdom) to determine total protein concentration using a standard protein assay (Bio-Rad, Hemel Hempstead, United Kingdom). For immunoprecipitation of p53 complexes, cells were lysed in 40 mM HEPES (pH 7.5), 120 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 50 mM NaF, 1.5 mM Na₂VO₄, and 1% Triton X-100 containing EDTA-free protease inhibitor cocktail (Boehringer Mannheim). After centrifugation, 5 μ g of the p53 monoclonal antibody DO-1 (Calbiochem) was added to the supernatant and rotated for 3 h at 4°C. Then, 50 μ l of 50% slurry of protein G-Sepharose (Pierce Biotechnology Inc., Rockford, IL) was added and rotated for another 2 h at 4°C. Immunoprecipitated complexes were washed three times with lysis buffer. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and assessed by Western blotting using standard procedures. Western blots were quantified by densitometric analysis as described recently (12).

VEGF quantification. Following treatment, conditioned medium was removed from cells and analyzed by an enzyme-linked immunosorbent assay for secreted vascular endothelial growth factor (VEGF) (Quantiglo; R&D Systems, Minneapolis, MN). Samples were assessed in duplicate, and a calibration curve was performed for each experiment.

FACS. Cell death was analyzed by fluorescence-activated cell sorting (FACS) using a Beckman Coulter Diagnostics machine (High Wycombe, United Kingdom). Briefly, total populations of cells, including floating and adherent cells, were fixed in 70% ethanol and stained with propidium iodide (50 μ g/ml). RNase was added at 100 μ g/ml. The percentage of cells with a sub-G₁ DNA content was taken as a measurement of apoptosis.

Immunofluorescence. HCT116 (p53^{-/-} and p53^{+/+}), MCF-7, and MDA-MB-231 cells were cultured on sterile glass coverslips in six-well dishes. Following treatment, cells were fixed for 1 h with 4% paraformaldehyde in 1 $\times$ PBS and then blocked with IFF buffer (PBS containing 1% bovine serum albumin and 2% fetal calf serum) for 1 h. Cells were permeabilized with PBS containing 0.5% Triton X-100 for 10 min and washed with PBS. The p53 (DO-1; Calbiochem) and phospho-Ser139- γ H2AX (Upstate-Millipore, United Kingdom) monoclonal antibodies were used at 2 μ g/ml in IFF blocking buffer. The secondary antibody Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin G1 (Molecular Probes, Leiden, Netherlands) was used at 1/200 in IFF buffer. Nuclei were visualized by TO-PRO-3 staining (Molecular Probes).

Real-time quantitative PCR. Total RNA was extracted from cells using the RNeasy minikit (Qiagen, Crawley, United Kingdom). Five micrograms of total RNA was used for first-strand cDNA synthesis using the SuperScript II first-strand synthesis system (Invitrogen, Paisley, United Kingdom) and random hexamers according to the manufacturer's instructions. Real-time PCR was performed using DyNAmo Sybr green quantitative PCR kit (Finnzymes, GRI Ltd., Baintree, United Kingdom) and the DNA Engine Opticon2 system (GRI Ltd., Baintree, United Kingdom) as we have previously described (12). Primer sequences used are as follows: 5'-GCAAGCCCTGAAAGCG-3' (forward) and 5'-GGCTGTCCGACTTTGA-3' (reverse) for HIF-1 α , 5'-GTTCTTGTGGAG CCGGAGC-3' (forward) and 5'-GGTACAAGACAGTGACAGGTC-3' (reverse) for p21, and 5'-ATGGGGAAGGTGAAGGTGC (forward) and 5'-TA AAAGCAGCCCTGGTGACC-3' (reverse) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Where indicated, PCR samples were separated by 3% agarose gel electrophoresis in the presence of ethidium bromide.

In vivo analysis. All procedures involving animals were performed within guidelines set out by The Institute of Cancer Research's Animal Ethics Committee and the United Kingdom Co-ordinating Committee for Cancer Research Committee on the Welfare of Animals in Experimental Neoplasia (46). To determine the distribution of RITA in mice, RITA was prepared at 2.5 mg/ml in PBS containing 10% DMSO and 5% Tween 20 and administered intraperitoneally (25 mg/kg of body weight) to female BALB/c mice. Mice were terminated by exsanguination under anesthesia at 0.08, 0.25, 0.5, 1, 2, 6, and 24 h after administration of RITA, and plasma, liver, spleen, kidney, and lung samples were removed for pharmacokinetic analysis. Tissue samples were homogenized with 3 $\times$ (vol/wt) PBS, and 50 μ l was extracted by the addition of 150 μ l of methanol. Plasma samples (50 μ l) were extracted by protein precipitation with 150 μ l of methanol. Extracts were analyzed by liquid chromatography-mass spectrometry

FIG. 1. RITA induces p53-dependent inhibition of HIF-1 α and VEGF in normoxia and hypoxia. (A to C) Western blot analysis shows HIF-1 α and p53 protein levels in MCF-7 cells (A), MDA-MB-231 (B), and p53^{-/-} HCT116 or p53^{+/+} HCT116 cells (C) treated with RITA over a concentration range for 16 h in normoxia or hypoxia (1% O₂). Actin was used as a load control. (D) The graph shows VEGF (pg/ml) secreted into cell culture medium from p53^{-/-} and p53^{+/+} HCT116 cells treated with RITA (0 to 1 μ M) for 16 h in hypoxia (1% O₂).

using a reverse-phase Synergi Polar-RP (Phenomenix) analytical column (50 by 2.1 mm) and positive-ion mode electrospray ionization and single-ion monitoring. To assess the effects of RITA on p53, HIF-1 α , HDM2, p21, VEGF, and phosphorylated γ H2AX proteins, 2 million p53^{+/+} HCT116 cells were injected subcutaneously into bilateral flanks of female NCr athymic mice at 6 to 8 weeks of age and treated intraperitoneally with either solvent control or 10 mg of RITA per kg. Each group contained eight mice. One tumor per mouse was used for pharmacokinetic analysis (as described above), and samples from the second tumor were bisected. Half the samples were snap-frozen using liquid nitrogen for examination of protein expression by Western blotting, and the remaining samples were fixed in 10% formalin for immunohistochemical analysis. For protein extraction, frozen tumors were ground using a bead grinder homogenizer (Pre-celllys; Stretton Scientific, Stretton, United Kingdom) in freshly prepared lysis buffer. Samples were assessed for total protein concentration using a standard protein assay (Bio-Rad) and subjected to Western blot analysis.

RESULTS

RITA induces p53-dependent downregulation of HIF-1 α and VEGF expression. RITA has been shown to induce p53, mediate apoptosis, and inhibit tumor growth in vivo (23). Given that active p53 downregulates HIF-1 α expression (27, 39), we hypothesized that RITA could potentially mediate antiangiogenic effects by inhibiting HIF activity. To address this, we assessed the effects of RITA on a series of tumor cells that express either wild-type p53 (p53^{+/+} HCT116, MCF-7, U2OS, and RCC4), mutant p53 (MDA-MB-231 and HT29) or are p53 null (p53^{-/-} HCT116 and Saos-2). We found that RITA induced p53 to comparable levels in normoxia and hypoxia and blocked HIF-1 α induction in hypoxia in MCF-7 cells (Fig. 1A). In addition, HIF-1 α induction in response to the hypoxia mimetic agent deferoxamine mesylate was also blocked by RITA in p53^{+/+} HCT116 cells (data not shown). However, RITA did not induce p53 or affect HIF-1 α levels in MDA-MB-231 cells, which express mutant p53 (Fig. 1B). Similar results were obtained for the wild-type p53-expressing U2OS and RCC4 cells, while HIF-1 α levels were not affected by RITA in HT29 cells, which express mutant p53, or in p53-

null Saos-2 cells (data not shown). These data suggest that the ability of RITA to block HIF-1 α was p53 dependent. To assess this further, matched p53^{-/-} HCT116 and p53^{+/+} HCT116 cells (10) were used. We found that RITA blocked HIF-1 α induction only in response to hypoxia in p53^{+/+} HCT116 cells (Fig. 1C) and resulted in a significant inhibition of VEGF expression (Fig. 1D). Our data indicate that RITA blocks HIF-1 α and VEGF induction in response to hypoxia and that this is p53 dependent.

RITA induces p53-dependent cell death in normoxia and hypoxia. Induction of apoptosis by p53 contributes to the antitumor effect of most conventional chemotherapeutic drugs (14, 33). However, hypoxia confers tumor cell resistance to killing by chemotherapy and radiotherapy (1, 8). Since RITA selectively kills tumor cells bearing wild-type p53 (23) and inhibits HIF-1 α expression (Fig. 1A to C), we next determined whether RITA could induce cell death in tumor cells in response to hypoxia. We found that RITA could induce a clear increase in rounding and displacement of cells from monolayers in tissue culture, indicative of apoptosis, to a similar extent in normoxia and hypoxia (Fig. 2A). Using FACS, we also found that there was a marked increase in the sub-G₁ content of cells (28 to 30%) induced by RITA (Fig. 2B) and an increase in poly(ADP-ribose) polymerase (PARP) cleavage (Fig. 2C), indicating that RITA induced apoptosis in cells exposed to normoxia and hypoxia. Consistent with the effects we observed for HIF-1 α (Fig. 1A to C), RITA did not mediate cell death in MDA-MB-231 cells in either normoxia or hypoxia (Fig. 2A) or in p53^{-/-} HCT116 cells (Fig. 2B). To further confirm the role of p53 in RITA-induced cell death, we used siRNA to knock down p53. We found that p53 knockdown reduced PARP cleavage induced by RITA in both normoxia and hypoxia, indicating that p53 is important for RITA-induced apoptosis (Fig. 2C). In addition, we found that inhibition of caspase-3 activation by the inhibitor Z-DEVD-FMK could block PARP

FIG. 2. RITA induces p53-dependent cell death in normoxia and hypoxia. (A) Photographic images of MCF-7 and MDA-MB-231 cells treated with RITA (1 μM) for 24 h in normoxia and hypoxia (1% O₂). Cells treated with RITA have displaced into the culture medium. (B) FACS analysis of p53^{-/-} and p53^{+/+} HCT116 cells treated with RITA (1 μM) for 24 h in normoxia or hypoxia (1% O₂). Graphs show DNA content of cells (events) measured by propidium iodide (PI) stain and FACS analysis. The percentage of sub-G₁ content (the percentage shown under the M1 bar) indicates the apoptotic population of cells. (C) Western blot analysis shows p53 protein and cleaved PARP cleavage as an indicator of apoptosis in p53^{+/+} HCT116 cells transiently transfected with siRNA to p53 or a nonsilencing control (NSC) siRNA duplex. Twenty-four hours after transfection, cells were treated with RITA (+) (1 μM) for 16 h in normoxia or hypoxia (1% O₂). Actin was used as a load control. (D) Western blot analysis shows HIF-1α, cleaved PARP, and p53 proteins in p53^{-/-} and p53^{+/+} HCT116 cells treated with RITA (+) (1 μM) for 16 h in normoxia and hypoxia (1% O₂) in the presence or absence (–) of the caspase-3 inhibitor Z-DEVD-FMK at 25 μM or 50 μM.

cleavage induced by RITA in normoxia and hypoxia in a dose-dependent manner without affecting the ability of RITA to block HIF-1α accumulation in hypoxia (Fig. 2D). These data suggest that the cell death induced by RITA per se is not responsible for the inhibition of HIF-1α, indicating that these are potentially mechanistically separable responses. Taken together, our data show that RITA blocks HIF-1α and VEGF induced in hypoxia and mediates p53-dependent tumor cell apoptosis in normoxia and hypoxia.

RITA induces p53-dependent eIF-2α phosphorylation and blocks HIF-1α protein. HIF-1α expression is usually regulated at the level of protein stability (24, 25) and synthesis (32, 44). To examine the mechanism by which RITA inhibits the expression of HIF-1α, we next assessed the effects of RITA on HIF-1α expression in the presence of the MG132 proteasome inhibitor. We found that MG132 did not affect the ability of RITA to block HIF-1α expression in either normoxia or hypoxia (Fig. 3A). Again, these effects were dependent on p53 (Fig. 3A). In addition, RITA could significantly block HIF-1α protein constitutively stabilized in RCC4 cells in normoxia due

to loss of pVHL function (Fig. 3B). Furthermore, quantitative PCR analysis showed that RITA had no significant effect on HIF-1α mRNA expression either in the presence or absence of p53 (Fig. 3C). Collectively, these data indicate that RITA potentially affects HIF-1α at the level of protein synthesis. Recent studies have shown that activation of p53 by genotoxic stress blocks protein translation via inhibition of mammalian target of rapamycin (mTOR) signaling (9, 15, 17). To address this here, components of the translational machinery were evaluated. We found that phosphorylation of the downstream effector of mTOR, p70S6K was moderately increased in response to RITA in a p53-dependent manner (Fig. 3D), while phosphorylated 4E-BP1 was not significantly altered in RITA-treated cells (data not shown and Fig. 4B), suggesting that the mTOR pathway may not be involved here. In contrast, we found that RITA induced a marked phosphorylation of eIF-2α on Ser51 in a p53-dependent manner (Fig. 3D).

Activation of p53 by genotoxic stress has been shown to induce the phosphorylation of AMP-activated protein kinase (15, 17) and blocks translation via the mTOR pathway (9). To

FIG. 3. RITA induces phosphorylation of eIF-2α and blocks HIF-1α protein. (A) Western blot analysis shows HIF-1α and p53 protein levels in p53^{+/+} HCT116 cells or p53^{-/-} HCT116 cells treated with the indicated concentrations of RITA for 16 h in normoxia or hypoxia (1% O₂) in the presence or absence of the proteasome inhibitor MG132 (50 μM). Actin was used as a load control. (B) Western blot analysis shows HIF-1α protein levels in RCC4 cells treated with RITA (1 μM) for 16 h. Actin was used as a load control. (C) Evaluation of HIF-1α transcripts by quantitative real-time PCR. p53^{+/+} HCT116 cells were treated with the indicated concentrations of RITA for 16 h in hypoxia (1% O₂). Total RNA was prepared, and quantitative real-time PCR was performed. The graph shows the HIF-1α transcript levels relative to the level of the GAPDH control. PCR products were separated by 3% agarose gel electrophoresis and visualized by ethidium bromide. GAPDH was used as a load control. (D) Western blot analysis shows phosphorylated eIF-2α (P-eIF-2α), phosphorylated p70S6K (P-70S6K), and eIF-2α proteins in p53^{-/-} HCT116 and p53^{+/+} HCT116 cells treated with the indicated concentrations of RITA for 16 h in normoxia or hypoxia (1% O₂). Actin was used as a load control. (E) Western blot analysis shows phosphorylated AMPK (P-AMPK) and total AMPK protein in p53^{+/+} HCT116 cells treated with the indicated concentrations of RITA for 16 h in normoxia or hypoxia (1% O₂). Actin was used as a load control.

FIG. 4. RITA induces phosphorylation of eIF-2 α and downregulates p53 targets. (A and B) Western blot analysis shows HIF-1 α , p53, HDM2, p21, phosphorylated eIF-2 α (P-eIF-2 α), total eIF-2 α , cleaved PARP, and phosphorylated 4E-BP1 (P-4E-BP1) in p53^{+/+} HCT116 cells treated with RITA over a concentration range (0 to 500 nM) for 16 h in normoxia (A) and hypoxia (1% O₂). (B) Actin was used as a load control. (C) The graph shows the results of densitometric analysis of the Western blot for hypoxia conditions as in panel B. HIF-1 α , HDM2, and p21 protein levels are represented relative to the actin load control, and phosphorylated eIF-2 α levels are represented relative to total eIF-2 α protein. (D) Total RNA was prepared from p53^{+/+} HCT116 cells treated with RITA and exposed to hypoxia as described above for panel A, and quantitative real-time PCR was performed. The graph shows the p21 transcript levels relative to the level of the GAPDH control and averaged for two independent experiments. Western blot analysis shows p53 and p21 proteins. Actin was used as a load control.

address whether phosphorylation of AMPK was induced in response to RITA, we treated cells with RITA at increasing concentrations curve and assessed phosphorylated AMPK protein. We found that AMPK phosphorylation was not significantly altered in response to RITA treatment of cells, while eIF-2 α phosphorylation increased in a dose-dependent manner (Fig. 3E). Taken together, our data indicate that RITA affects protein translation via eIF-2 α .

RITA induces p53-dependent eIF-2 α phosphorylation and downregulates p53 targets. The α subunit of eukaryotic initiation factor 2 is responsible for binding of Met-tRNA^{Met} to the 40S ribosome for protein translation initiation (2). Phosphorylation of eIF-2 α on Ser51 usually results in general protein synthesis inhibition (13). To evaluate this further, p53^{+/+} HCT116 cells were treated with RITA over a concentration range in normoxia and hypoxia. We found that RITA induced p53 and the p53 targeted p21 and HDM2 in a dose-dependent manner in normoxia and hypoxia (Fig. 4A and B). Surprisingly,

as p53 levels continued to increase in response to increasing concentrations of RITA, the expression levels of not only HIF-1 α but also of HDM2 and p21 proteins were also downregulated (Fig. 4A and B). Concurrently, eIF-2 α phosphorylation increased and cell death was induced by PARP cleavage (Fig. 4B and C). Further analysis indicated that the downregulation of p21 protein induced by RITA treatment of cells was not due to a decrease in p21 mRNA (Fig. 4D). These findings are consistent with our findings for HIF-1 α (Fig. 3C). Interestingly, knockdown of p21 using siRNA could increase basal PARP cleavage in HCT116 cells and enhance the overall cleaved PARP induced in response to RITA (data not shown), suggesting that knocking down p21 can drive the p53-mediated apoptotic response induced by RITA.

PERK activity is important for RITA-mediated induction of eIF-2 α phosphorylation. Recent studies have shown that hypoxia mediates a time-dependent transient induction of eIF-2 α phosphorylation, which results in inhibition of protein synthe-

FIG. 5. PERK activity is important for RITA-mediated induction of eIF-2 α phosphorylation. (A) Western blot analysis shows HIF-1 α , phosphorylated eIF-2 α (P-eIF-2 α), and total eIF-2 α proteins in p53^{+/+} HCT116 cells treated with RITA (1 μ M) and exposed to hypoxia (1% O₂) for the times indicated (in hours). Actin was used as a load control. (B) Western blot analysis shows phosphorylated eIF-2 α and total eIF-2 α proteins in HCT116 cells stably expressing a pCDNA5 control plasmid (control) or a dominant-negative form of PERK (PERK Δ C) under the control of the tetracycline promoter. Cells were treated with RITA (1 μ M) and exposed to hypoxia (1% O₂) for 16 h. Actin was used as a load control.

sis (29, 30). We found that eIF-2 α phosphorylation was induced and maintained in hypoxia by treatment of cells with RITA (Fig. 5A), suggesting that protein synthesis was blocked by RITA. In hypoxia, PKR-like endoplasmic reticulum kinase is activated to control translation via direct phosphorylation of eIF-2 α (5, 50). To assess whether PERK activity is important for the induction of eIF-2 α phosphorylation in response to RITA treatment of cells, we used a dominant-negative form of PERK (PERK Δ C). We found that the expression of PERK Δ C blocked basal eIF-2 α phosphorylation and significantly inhibited the ability of RITA to induce eIF-2 α phosphorylation in cells (Fig. 5B), indicating that PERK activity is important for the p53-mediated eIF-2 α phosphorylation induced in response to RITA.

RITA does not disrupt the p53-HDM2 interaction. Since RITA could induce significant cell death in both normoxia and hypoxia, we next addressed its mechanism of action. RITA was originally proposed to stabilize and activate p53 by disruption of p53-HDM2 interaction by binding to the N-terminal domain of p53 (23). Recent studies using NMR techniques have indicated that RITA does not block the p53-HDM2 interaction in vitro (31). In addition, we found that RITA induced a p53-dependent induction of HDM2 at very low concentrations (<100 nM), but at doses which induced apoptosis, RITA actually downregulated HDM2 expression (Fig. 4A and B). To evaluate this further, p53 complexes were immunoprecipitated from p53^{+/+} HCT116 cell lysates, and associated HDM2 was assessed in the presence of RITA. We found that RITA was not able to disrupt immunoprecipitated p53-HDM2 complexes even at submillimolar concentrations in vitro (data not shown). In contrast, we found that the small-molecule nutlin-3 that has been shown to disrupt the p53-HDM2 interaction by binding to

HDM2 (47) could significantly impair the ability of HDM2 to coimmunoprecipitate with p53 (Fig. 6A), indicating that RITA may activate p53 via mechanisms that do not target the p53-HDM2 interaction. Interestingly, we found that induction and activation of p53 in the absence of eliciting a stress response either by using a tetracycline-inducible system (Fig. 6B) or by treatment of cells with nutlin-3 (Fig. 6C) was not sufficient to inhibit HIF-1 α in response to hypoxia, further supporting the possibility that RITA may induce and activate p53 via mechanisms other than disruption of the p53-HDM2 interaction.

RITA induces a DNA damage response. RITA has previously been reported to cause protein-DNA and DNA-DNA intrastrand cross-links (37, 40), suggesting that it may activate p53 by inducing a DNA damage response. To investigate this, we first assessed the phosphorylation status of p53 in response to RITA, since phosphorylation of p53 within the N terminus is usually induced by genotoxic stress (3, 26). p53^{+/+} HCT116, MCF-7, and U2OS cells were treated with RITA in normoxia or hypoxia and assessed for phosphorylation of p53 at Ser15 (Fig. 7A and D). We found that RITA induced N-terminal phosphorylation of p53, while nutlin-3 was unable to mediate p53 phosphorylation (Fig. 7A). In addition, assessment of γ H2AX, a histone protein usually phosphorylated by DNA damage-induced stress (6, 28), showed that RITA induced the phosphorylation of γ H2AX at Ser139 and that p53 and phosphorylated γ H2AX proteins were induced by RITA and localized to the nucleus in both normoxia and hypoxia (1% O₂) to a similar extent (Fig. 7B and C). Similar data were obtained for other p53-positive cells, such as MCF-7 and U2OS cells, indicating that these responses were not specific to HCT116 cells (data not shown). Previous studies have shown that γ H2AX phosphorylation is induced in extreme hypoxia (<0.02% O₂)

FIG. 6. RITA does not disrupt the p53-HDM2 interaction. (A) Western blot analysis of p53-HDM2 immunoprecipitated (IP) complexes. p53^{+/+} HCT116 cells were treated with RITA (1 μ M) or nutlin-3 (4 μ M) in the presence of MG132 (10 μ M) for 16 h. Cell lysates were immunoprecipitated with a monoclonal antibody to p53 (DO-1), and immunoprecipitated p53 complexes were assessed for associated HDM2 protein by Western blotting. Total immunoprecipitated p53 protein was evaluated using a polyclonal antibody. WCE, whole-cell extracts. (B) Western blot analysis shows HIF-1 α and p53 proteins in TetON p53-inducible Saos-2 cells treated with doxycycline (Dox) (800 ng/ml) (+) for 24 h, then washed with PBS, and treated with RITA (1 μ M) (+) for a further 16 h in normoxia or hypoxia (1% O₂). (C) Western blot analysis shows HIF-1 α , HDM2, and p53 proteins in p53^{+/+} HCT116 cells treated with nutlin-3 (4 μ M) (+) in normoxia (N), hypoxia (H), or deferoxamine mesylate (D) for 16 h. Actin was used as a load control.

and is localized to discrete nuclear foci (21). However, we found that exposure of cells to hypoxia at 1% O₂ did not induce γ H2AX phosphorylation in cells (Fig. 7C), consistent with another study (21). Intriguingly, we found that unlike other agents that activate a DNA damage response, RITA induced eIF-2 α phosphorylation, which correlated with a significant inhibition of HIF-1 α in p53-positive cells (Fig. 7D). Taken together, our study identifies a new mechanism of action for RITA: RITA functions by activating a DNA damage response.

RITA induces p53 and γ H2AX phosphorylation and blocks HIF-1 α and VEGF expression in vivo. RITA has previously been shown to mediate antitumor activity in a subcutaneous HCT116 colon carcinoma xenograft model (23). We next determined whether RITA induced a DNA damage response and downregulation of HIF-1 α and VEGF expression in p53^{+/+} HCT116 cell-derived tumor xenografts. Consistent with a previous study (23), we found that RITA was well-tolerated in mice at 10 to 25 mg/kg daily dosing. In addition, we detected RITA in the plasma and tissues of mice (kidney, liver, lung, and spleen) by liquid chromatography-mass spectroscopy (data not shown), indicating that the compound was distributed in vivo. We found that p53, Ser15 phosphorylation of p53, and Ser139 phosphorylation of γ H2AX were induced at 24 h with a reduction in HIF-1 α and HDM2 in tumors from the RITA-treated group compared with the solvent (DMSO) control group (Fig. 8A). We also observed a marked decrease in VEGF levels in tumor lysates from the RITA-treated group compared to those of the DMSO-treated control group (Fig. 8B). Immunohistochemical analysis indicated that p53 and

phosphorylated γ H2AX staining correlated with increased tumor cell apoptosis (data not shown). Our analyses confirm that RITA induces a DNA damage response and mediates a reduction in HIF-1 α and VEGF expression in vivo.

DISCUSSION

The p53 tumor suppressor protein is a potent negative regulator of HIF-1 α and VEGF in hypoxia (39). In addition, the induction of apoptosis by p53 contributes to the antitumor effects of most conventional chemotherapeutic drugs (14, 33). However, hypoxia and HIF-1 α status in tumor cells are important determinants for resistance to chemotherapeutic agents and radiotherapy (7, 35, 38, 49). Therefore, the possibility that small-molecule activation of wild-type p53 could potentially mediate both antiangiogenic effects via blockade of the HIF pathway and apoptosis in hypoxic tumor cells is of particular interest.

In this study, we have explored the mechanistic properties of the small-molecule activator of p53, RITA. RITA was originally identified in a cell-based screen using the National Cancer Institute (NCI) compound library and has been shown recently to mediate p53-dependent antitumor activity in vivo (23). Here, we show that RITA blocks HIF-1 α and VEGF expression in vivo and mediates significant cell death responses in hypoxic tumor cells in a p53-dependent manner. Inhibition of HIF-1 α was not due to an effect on protein degradation or changes in mRNA levels, indicating an effect on HIF-1 α protein synthesis. We discovered that RITA induced p53-depend-

FIG. 7. RITA induces a DNA damage response. (A) Western blot analysis shows HDM2, p53, and phosphorylated-S15-p53 (P-S15-p53) proteins in p53^{+/+} HCT116 cells treated with RITA (1 μ M) or nutlin-3 (4 μ M) for 16 h. (B and C) Immunofluorescence analysis shows p53 (B) and phosphorylated-S139- γ H2AX proteins (fluorescein isothiocyanate [FITC]) (C) in p53^{+/+} HCT116 cells treated with RITA (1 μ M) in normoxia or hypoxia (1% O₂) for 16 h. Nuclei (blue) were visualized with TO-PRO-3 staining. Images ($\times 40$) were captured using a confocal microscope (Leica). (D) Western blot analysis shows HIF-1 α , p53, phosphorylated S15-p53, HDM2, p21, eIF-2 α , phosphorylated eIF-2 α , and PARP proteins in p53^{-/-} and p53^{+/+} HCT116 cells treated with DMSO, 1 μ M RITA, 1 mM hydroxyurea (HU), doxorubicin (Dox), cisplatin (Cisp), or 25 μ M etoposide (Etop) for 16 h in hypoxia (1% O₂). The status of p53 is shown above the lanes: +, present; -, absent.

dent phosphorylation of eIF-2 α , which correlated with its ability to block HIF-1 α induction in hypoxia. Concurrently, we found that RITA also blocked expression of the p53 target proteins HDM2 and p21, indicating that RITA may affect protein synthesis via phosphorylation of eIF-2 α . Of particular interest is how the p53-dependent response induced by RITA signals to the translational machinery at the level of eIF-2 α , since other known activators of p53 used in this study did not induce phosphorylation of eIF-2 α . Activation of p53 by RITA did not appear to significantly block components of mTOR signaling, ruling out the possibility that downregulation of HIF- α , p21, and HDM2 were due to p53 cross talk to AMPK-mTOR signaling as reported recently (9, 15–17). Interestingly, we discovered that the activity of PERK, an upstream kinase that phosphorylates eIF-2 α in response to hypoxia, was important for the induction of eIF-2 α phosphorylation in response to RITA (5), suggesting that p53 signals to the translational machinery by regulating PERK-eIF-2 α activation. Indeed, a recent report has linked the unfolded protein response pathway

with p53 accumulation via a PERK-dependent mechanism (51).

Further investigation into the mechanism of action of RITA indicated that RITA did not appear to block the p53-HDM2 interaction as originally proposed to be its mechanism of action for inducing p53 (23). In fact, we found that RITA activated a DNA damage response in vitro and in vivo. Previous studies have shown that RITA causes protein-DNA and DNA-DNA intrastrand cross-links (37, 40), supporting the notion that RITA could induce and activate p53 by eliciting a DNA damage response in cells. Indeed, the ability of RITA to mediate both apoptotic and antiangiogenic effects in hypoxia may be due to its ability not only to induce p53 but also to elicit a concurrent DNA damage response. Intriguingly however, unlike other agents that mediate a DNA damage response, RITA-mediated effects are entirely dependent on p53.

Activation of the DNA damage response by RITA was apparent in both normoxia and hypoxia to a similar extent. While transient overexpression of p53 has been reported to block

FIG. 8. RITA induces p53 and γ H2AX phosphorylation and blocks HIF-1 α and VEGF expression in tumor xenografts. (A) Western blot analysis of p53^{+/+} HCT116 tumor xenograft lysates. Mice bearing p53^{+/+} HCT116 cell-derived tumor xenografts were administered with a single intraperitoneal dose of RITA at 10 mg/kg for 24 h. Tumor cell lysates from eight tumors were analyzed per group. Western blots show a representation of two independent tumors (tumor 1 [T1] and T2) from the DMSO- and RITA-treated groups, assessed for HIF-1 α , HDM2, p53, phosphorylated-S15-p53 (P-S15-p53), and phosphorylated-S139- γ H2AX (P-S139-H2AX) proteins. Actin was used as a load control. (B) The graph shows VEGF levels in tumor lysates. Four data points are shown for each condition. Experiments show two independent tumors (T1 and T2) in two independent experiments (diamonds and circles). Abbreviations: IP, intraperitoneally; hr, hours.

HIF-1 α and VEGF induced in hypoxia (39), a previous report has shown that DNA damage is a prerequisite for p53-mediated downregulation of HIF-1 α (27). Interestingly, we found that RITA's ability to inhibit HIF-1 α was not affected by blockade of apoptosis, indicating that the p53-dependent apoptotic and antiangiogenic responses mediated by RITA are mechanistically separable processes. While the p53-dependent apoptotic function induced by RITA did not appear to influence the observed HIF-1 α blockade, conversely we found that in renal carcinoma cells expressing constitutively high basal levels of HIF- α protein due to loss of pVHL function, RITA was at least fivefold less potent in blocking tumor cell growth than in renal carcinoma cells expressing pVHL. In addition, we found that p21 knockdown by siRNA could enhance basal PARP cleavage and increase overall PARP cleaved in response to RITA (data not shown). These observations are consistent with the mTOR inhibitor RAD001 (4). Collectively, these findings indicate that the HIF- α status in cells contributes to RITA's apoptotic outcome and that downregulation of p21 by RITA may help drive the apoptotic response induced by p53.

The ability to both induce apoptosis and suppress tumor angiogenesis by targeting p53 is of significant interest therapeutically. Clinical studies have shown that the combined administration of chemotherapeutic agent 5-fluorouracil and bevacizumab, a monoclonal antibody targeted against VEGF, produced a dramatic increase in survival in colorectal cancer patients (18, 22). However, bevacizumab did not yield long-term survival benefits as a single agent (34). This suggests that combination of drugs that lead to both tumor cell death and suppression of angiogenesis would be more clinically desirable, even when one agent is a classical cytotoxin. Indeed, RITA appears to be able to mediate both processes as a single agent: RITA induces a DNA damage response, resulting in p53-mediated apoptosis, and also blocks HIF-1 α expression, resulting in a significant reduction in VEGF expression.

In conclusion, our study utilizes the small-molecule activator

of p53 RITA to evaluate mechanisms by which p53 can both induce apoptosis and suppress tumor angiogenesis. By identifying a new mechanism of action for RITA, we have uncovered the existence of a DNA damage response that when activated leads to the death of hypoxic tumor cells and blockade of the HIF-1 α /VEGF pathway in vivo. Further evaluation of this response and the identification of other agents or activators of this response will be of particular interest and may lead to improved targeting of HIF/hypoxia signaling in tumor cells.

ACKNOWLEDGMENTS

We thank Galina Selivanova (Microbiology and Tumor Biology Center, Karolinska Institutet, Stockholm, Sweden) for initial discussions and the Developmental Therapeutics Program, National Cancer Institute, Maryland for supplying RITA (NSC-652287). We thank Karen Vousden (Beatson Institute for Cancer Research, Glasgow, United Kingdom) for TetON p53-inducible cells and Bert Vogelstein (Johns Hopkins School of Medicine, Baltimore, MD) for the p53^{-/-} HCT116 and p53^{+/+} HCT116 cells. We thank Nicola Wilsher and Amin Mizra (Cancer Research-United Kingdom Centre for Cancer Therapeutics, The Institute of Cancer Research, United Kingdom) for mass spectroscopy analysis and NMR confirmation of the RITA structure. For input regarding the measurement of DNA damage, we thank Neville Ashcroft, Penny Jeggo, Keith Caldecott (Genome Damage and Stability Centre, University of Sussex, United Kingdom), and Apolinar Maya-Mendoza and Dean Jackson (Faculty of Life Sciences, University of Manchester, MIB, Manchester, United Kingdom). We thank all other members of the Ashcroft lab for their input. Finally, we thank Neville Ashcroft for critical review of the manuscript and Patrick Maxwell (University College, London, United Kingdom) for useful discussions.

This work was funded by University College London, The Institute of Cancer Research, United Kingdom and Cancer Research United Kingdom (CUK) program grant number C309/A2187 (M.A., N.P., A.H.B., G.B., M.V., and S.E.). J.Y. and A.A. were funded by Cancer Research United Kingdom studentships C7358/A4420 and C7358/A8020, respectively. E.P. was funded by Cancer Research United Kingdom project grant C7358/A9958.

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