

# Nutlin-3 radiosensitizes hypoxic prostate cancer cells independent of p53

Stéphane Supiot, Richard P. Hill,  
and Robert G. Bristow

Princess Margaret Hospital (University Health Network),  
Departments of Medical Biophysics and Radiation Oncology,  
University of Toronto, Toronto, Ontario, Canada

## Abstract

Nutlin-3 is a small-molecule inhibitor that acts to inhibit MDM2 binding to p53 and subsequent p53-dependent DNA damage signaling. Whether Nutlin-3 alters cell toxicity following DNA damage under oxic versus hypoxic conditions has not been studied. The potential radiosensitization (0-10 Gy) properties of Nutlin-3 (dose range, 2-10  $\mu\text{mol/L}$  for up to 24 h) were investigated *in vitro* using three prostate cancer cell lines, 22RV1 [wild-type p53 (WTP53)], DU145 (mutated p53), and PC-3 (p53-null) under oxic (21%  $\text{O}_2$ ), hypoxic (0.2%  $\text{O}_2$ ), and anoxic (0%  $\text{O}_2$ ) conditions. As a single agent, Nutlin-3 (2-10  $\mu\text{mol/L}$ ) stabilized p53 and p21<sup>WAF</sup> levels and was toxic to WTP53-22RV1 cells ( $\text{IC}_{50}$ , 4.3  $\mu\text{mol/L}$ ) but had minimal toxicity toward p53-deficient cells ( $\text{IC}_{50}$ , > 10  $\mu\text{mol/L}$ ). When combined with radiation under oxic conditions, Nutlin-3 decreased clonogenic survival in all three cell lines: 22RV1 [sensitizing enhancement ratio (SER), 1.24], DU145 (SER, 1.27), and PC-3 (SER, 1.12). Anoxia induced p53 protein expression in 22RV1 cells and this was augmented by Nutlin-3 treatment. Furthermore, Nutlin-3 was more effective as a radiosensitizer under hypoxic conditions particularly in WTP53-expressing cells: 22RV1 (SER, 1.78), DU145 (SER, 1.31), and PC-3 (SER, 1.28). The decrease in clonogenic survival with Nutlin-3 was not correlated to altered levels of radiation-induced apoptosis within the three cell lines. Our results indicate that Nutlin-3 can act as a radiosensitizer via p53-independent mechanisms under low  $\text{O}_2$  levels. Nutlin-3 may be a useful adjunct to improve the therapeutic ratio

Received 6/28/07; revised 1/22/08; accepted 2/28/08.

**Grant support:** Terry Fox PMH Hypoxia Program Project Grant (R.G. Bristow and R.P. Hill), National Cancer Institute of Canada Operating Grant (R.G. Bristow), Fondation de France Research Grant (S. Supiot), and PMH-DRO Fellows Program Grant (S. Supiot). R.G. Bristow is a Canadian Cancer Society Research Scientist.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Requests for reprints:** Robert G. Bristow, Princess Margaret Hospital (University Health Network), Departments of Medical Biophysics and Radiation Oncology, University of Toronto, 610 University Avenue, Toronto, Ontario, Canada M5G 2M9. Phone: 416-946-2936; Fax: 416-946-4586. E-mail: Rob.Bristow@rmp.uhn.on.ca

Copyright © 2008 American Association for Cancer Research.

doi:10.1158/1535-7163.MCT-07-0442

using precision radiotherapy targeted to hypoxic cells and warrants further study *in vivo*. [Mol Cancer Ther 2008;7(4):993–9]

## Introduction

Localized prostate cancer is characterized by marked hypoxia and significant heterogeneity in oxygenation, similar to other human tumors (1, 2). Intratumoral hypoxia can be chronic (e.g., diffusion-limited) or acute (e.g., by temporary obstructions or variable blood flow in tumor vessels; ref. 3). The acute and chronic hypoxia found in tumors significantly complicates cancer therapy. Hypoxic cancer cells are considered to be resistant to radiation and chemotherapy and are more prone to develop metastasis (4). Hypoxia can increase radioresistance by up to 3-fold due to a decrease in lethal DNA double-strand breaks (5). Hypoxia can also lead to increased chemoresistance due to limited drug diffusion through hypoxic areas, acquisition of resistance to drug-induced apoptosis, and the decreased production of free radicals required for the cytotoxic action of certain chemotherapy drugs (6). In certain tumors, hypoxia can also induce a metastatic phenotype (4, 7), which decreases cancer-free survival. These effects are thought to occur by hypoxia-induced changes in gene expression and genetic stability including the down-regulation of DNA damage signaling and repair (8).

Hypoxia stabilizes and promotes the activity of hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ). This in turn promotes the transcription of several downstream genes such as vascular endothelial growth factor, carbonic anhydrase IX, and GLUT-1, whose overexpression has been linked to poor prognosis and decreased therapeutic response (8, 9). Additionally, HIF-1 $\alpha$  may increase MDM2, which is an important protein in the ATM-mediated response to DNA damage and activation of DNA repair and cell cycle pathways (10, 11).

MDM2 negatively regulates p53 by inhibiting its transcriptional activity and promoting its degradation by functioning as an E3-ubiquitin ligase in the proteasomal degradation pathway (12). The transcription factor p53 exerts a pivotal role in controlling DNA damage sensing, cell cycle progression, and apoptosis in response to various forms of genotoxic and cellular stress (reviewed in ref. 13). MDM2 also has p53-independent functions that require binding of MDM2 to proteins such as p14<sup>ARF</sup> (14), HIF-1 $\alpha$  (15), NBS1 (16), E2F-1 (17), and ribosomal protein L5 (ref. 18; reviewed in ref. 19). Importantly, MDM2 can be up-regulated by cellular hypoxia and has been shown to increase the metastatic efficiency of a murine fibrosarcoma cell line by increasing resistance to apoptosis (11). Therefore, overexpression or aberrant function of MDM2 can lead to altered DNA damage signaling, hypoxic gene expression, and apoptotic thresholds.

Currently, several molecular signaling inhibitors are being tested both preclinically and clinically for their potential efficiency when combined with ionizing radiation (20). However, few agents are being tested under conditions of hypoxia. This is surprising given that hypoxia is common in tumors and is a major determinant of radioresponse. Nutlins are small molecules that inhibit MDM2 binding to p53 (21). These compounds bind in the p53-binding pocket of MDM2 to displace p53 from the complex and induce p53 stabilization. p53 then activates downstream targets leading to p21<sup>WAF</sup> induction, cell cycle arrest, and apoptosis. In studies of chemosensitization, Nutlin-3a, the active enantiomer of Nutlin-3, increased the sensitivity to cisplatin by inhibiting MDM2 binding to E2F-1 (22). Increased sensitivity was linked to the induction of proapoptotic proteins, such as p73 $\alpha$  and Noxa. Nutlin-3 also prevents the association between MDM2 and HIF-1 $\alpha$  (23), thereby inhibiting vascular endothelial growth factor production and possibly tumor angiogenesis. Finally, a recent study reported that Nutlin-3a can radiosensitize wild-type p53 (WTp53)-expressing lung cancer cells (24). However, whether there are differential radiosensitizing effects of Nutlin-3 on cells that vary in p53 status or levels of oxygenation has not been studied.

We hypothesized that Nutlin-3 inhibition of MDM2-p53 interaction would lead to increased p53 activation under normoxia and hypoxia. Herein, we show novel data indicating that Nutlin-3 radiosensitizes prostate cancer cells under both normoxia and hypoxia independent of p53 status. Nutlin-3 and similar MDM2-p53 inhibitors may therefore be useful adjuncts to radiotherapy to improve clonogenic cell killing in tumor cells that have acquired hypoxia-mediated local or systemic resistance.

## Materials and Methods

### Drug Treatment

Nutlin-3, a racemic mix of Nutlin-3a (active enantiomer) and Nutlin-3b (inactive enantiomer), was purchased from Sigma-Aldrich and dissolved in DMSO at a concentration of 1 mmol/L. Using the stock solution, Nutlin-3 was further diluted in medium for individual experiments. For control experiments, DMSO dissolved in medium at a concentration of 0.5% was used.

### Cell Lines and Hypoxic Treatments

Three human malignant prostate cancer cell lines with varying p53 status were selected for study: DU145 (p53 mutated) and PC-3 (p53 null) were purchased from American Type Culture Collection. The 22RV1 cell line (p53 wild-type) was a kind gift of Dr. J.H. Pinthus (McMaster University). These cell lines were grown in  $\alpha$ -MEM, Ham's F-12K, and RPMI 1640 supplemented with 10% FCS and 1% L-glutamine, respectively. Approximate doubling times for cell cultures under these conditions were as follows: DU145, 18 h; PC-3, 24 h; and 22RV1, 40 h, as described previously (25).

For all experiments,  $1 \times 10^5$  to  $3 \times 10^5$  cells were plated in 10-cm dishes, such that cells were growing exponentially at

all time points tested. The cell lines were passaged in 5% CO<sub>2</sub> and air at 37°C for routine culture. For certain experiments, cells were exposed to either acute hypoxic or chronic anoxic gassing. For acute hypoxia, exponentially growing cells were plated as above, placed in a hypoxic environment (0.2% O<sub>2</sub>) using an *In vivo*<sub>2</sub> 400 chamber (Ruskin Technology) for a minimum of 2 h before irradiation. To induce chronic anoxia, flasks were flushed with a gas concentration of 0% O<sub>2</sub>, 5% CO<sub>2</sub>, and balanced with N<sub>2</sub> for 24 h before irradiation as described previously (26).

### Western Blotting

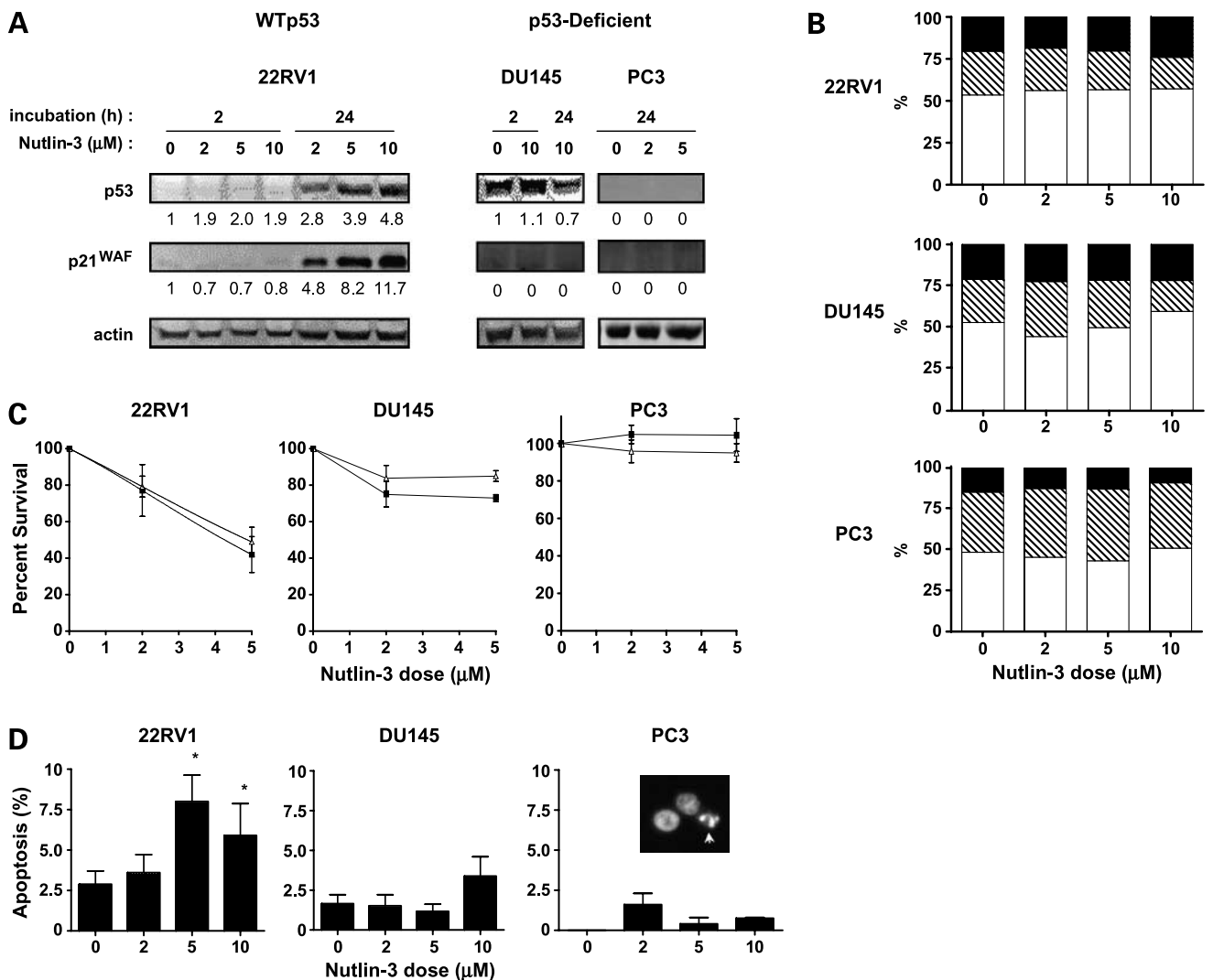
Western blotting was carried out as described previously (26). Primary antibodies were used at the following dilutions: anti-actin rabbit monoclonal antibody (Sigma-Aldrich) 1:50,000, anti-p53 mouse monoclonal antibody clone BP53-12 (Santa Cruz Biotechnology) 1:10,000, anti-p21<sup>WAF</sup> mouse monoclonal antibody (Rockland Immunochemicals) 1:10,000, anti-MDM2 mouse monoclonal antibody (Calbiochem) 1:1,000, and anti-HIF-1 $\alpha$  mouse monoclonal antibody (BD Translab) 1:750. The washed blot was incubated with secondary antibody conjugated to Alexa 680 (Invitrogen) or IRdye-800 (Rockland Immunochemicals) diluted 1:10,000 in LI-COR blocking buffer (diluted 1:1 in PBS) for 30 min at room temperature followed by five 5-min washes with TBS-Tween 20 at room temperature. Western blots were imaged and analyzed using a LI-COR Odyssey infrared imaging system (LI-COR Biosciences) and densitometry values calculated based on actin loading controls.

### Clonogenic Survival, Cell Cycle, and Apoptosis Assays

Clonogenic survival assays were done as described previously (27). Cells were counted and plated at different densities 15 h before Nutlin-3 exposure. Cells were irradiated at room temperature using a <sup>137</sup>Cs  $\gamma$ -ray irradiator (Nordion) at a dose rate of 0.9 Gy/min. Following incubation, drug was removed and fresh medium was added. Colonies were stained and counted under a microscope, with 50 cells as the minimum number to define a surviving colony. The best-fit survival curve was generated according to a linear-quadratic survival model and the mean inactivation dose (area under survival curve) was calculated as described previously (28). The sensitizing enhancement ratio (SER) was defined as SER = mean inactivation dose control irradiation / mean inactivation dose Nutlin-3 + irradiation. A SER value greater than 1.0 is indicative of radiosensitization. Survival curves for combined treatment were corrected for cytotoxicity due to Nutlin-3 alone.

For cell cycle analysis, cells were stained with propidium iodide and counted with a FACScan (Becton Dickinson) as described previously (28).

For apoptosis measurements, cells were collected 48 h after starting treatment with the drug and stained with Hoechst 33342 for microscopic evaluation of the presence of apoptotic bodies as described previously (27). A minimum of 200 cells were counted under a microscope for each experimental point.



**Figure 1.** Nutlin-3 kills cells according to p53 status. **A**, Western blot of Nutlin-3 effects (2–10  $\mu$ mol/L) on protein expression following different doses and incubation time on prostate cancer cell lines, 22RV1 (*p53* WT), DU145 (*p53* Mut), and PC-3 (*p53* Null). Number under the bands, densitometry values that were calculated based on actin loading controls. **B**, effects of 24-h Nutlin-3 treatment (2–10  $\mu$ mol/L) on cell cycle based on qualitative analysis of flow cytometry profiles (open column, G<sub>1</sub>; shaded column, S; dark column, G<sub>2</sub>-M). **C**, cell survival for the three cell lines following 2-h (square) or 24-h (triangle) incubation with 2 and 5  $\mu$ mol/L Nutlin-3. **D**, Nutlin-3 effects (2–10  $\mu$ mol/L) on apoptosis based on Hoechst 33342 staining at 48 h following treatment. Asterisk, statistically significant difference from control. Inset, typical apoptotic cell (arrow).

### Statistical Analysis

The mean  $\pm$  SE (standard error of the mean) was calculated for a minimum of three independent experiments. For survival curves, statistical comparisons were done on mean inactivation dose. For statistical analysis, paired *t*-test was calculated using Instat (Graphpad Software).

### Results

#### Nutlin-3 Alone Increases Clonogenic Cell Death in a p53-Dependent Manner

Nutlin-3 induced p53 and p21<sup>WAF</sup> expression in a dose-dependent manner in 22RV1 cells (WTp53; Fig. 1A). In contrast, p21<sup>WAF</sup> was not induced in the DU145 (mutated

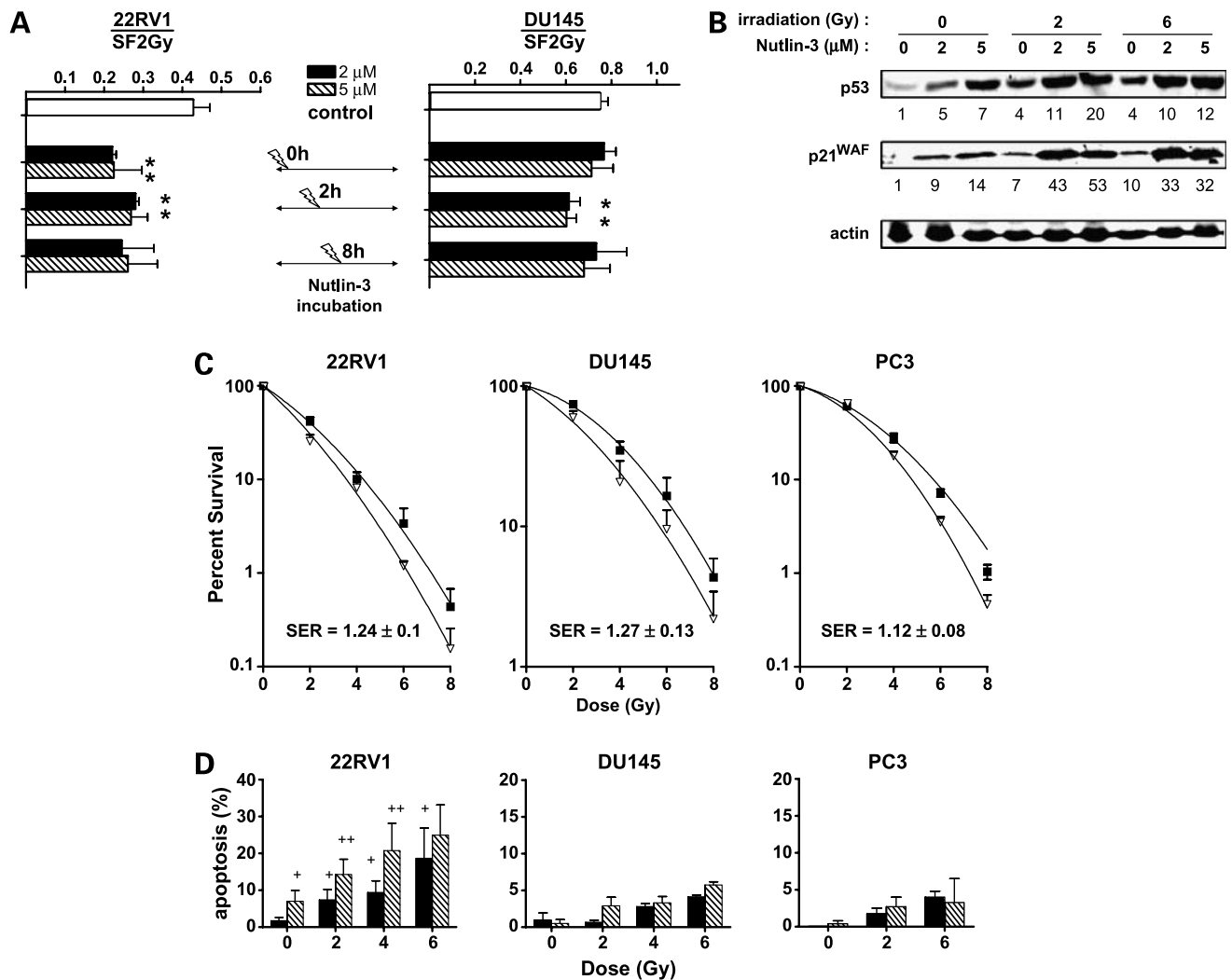
p53) or PC-3 (p53-null) cell lines (data not shown). Short-term cell cycle assays showed that, at a dose of 10  $\mu$ mol/L, Nutlin-3 increased slightly the G<sub>1</sub>-phase fraction and decreased S-phase fraction of all three cell lines (Fig. 1B). Nutlin-3 decreased significantly clonogenic survival of 22RV1 cells (IC<sub>50</sub>, 4.3  $\mu$ mol/L) when compared when DU145 and PC-3 cells (e.g., IC<sub>50</sub>, >10  $\mu$ mol/L; Fig. 1C). Nutlin-3 also induced a significant increase in apoptosis in 22RV1 cells (*P* < 0.05), whereas no significant increase in apoptosis was observed for p53-deficient DU145 or PC-3 cells (Fig. 1D). We conclude that, as a single agent, Nutlin-3 increases apoptosis and decreases clonogenic survival preferentially in WTp53-expressing cells.

### Nutlin-3 Radiosensitizes Prostate Cancer Cell Lines Independently of the p53 Status

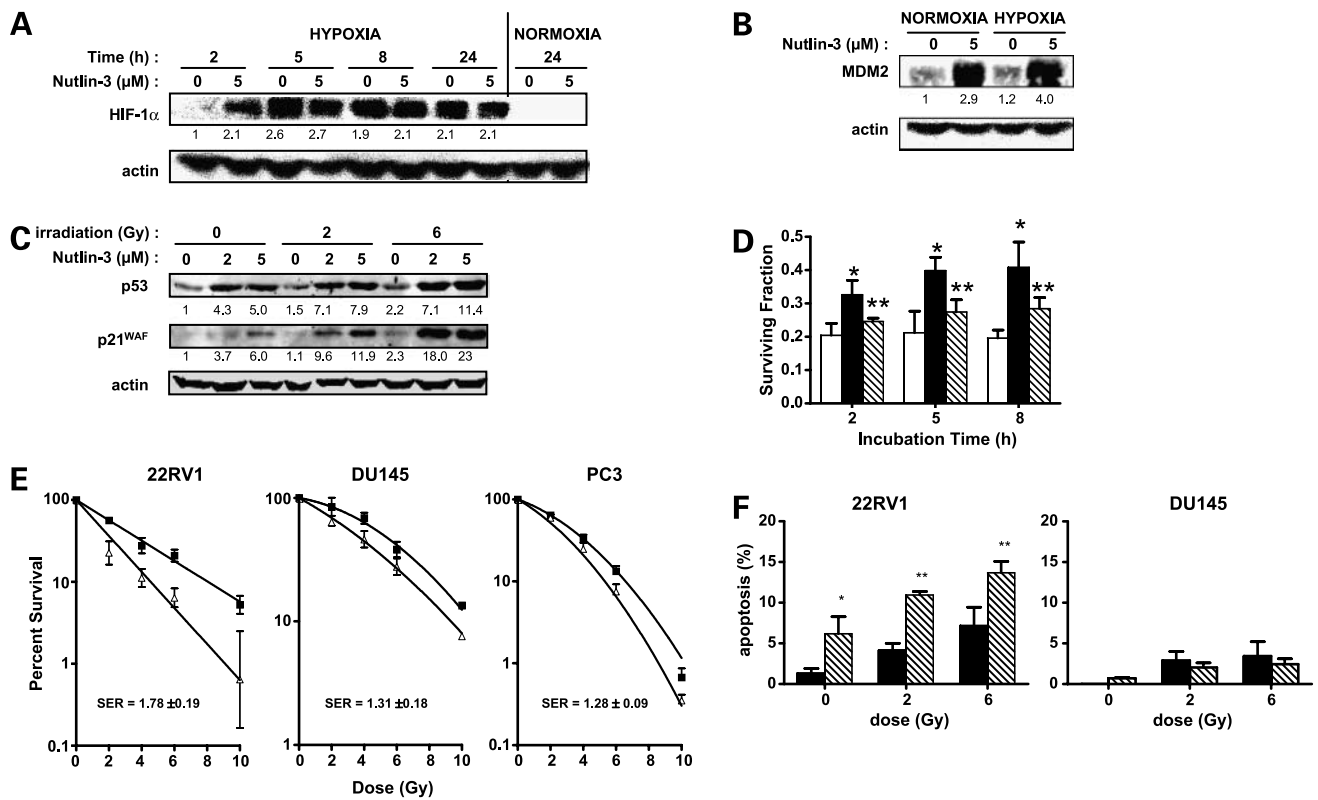
Radiotherapy is usually given as a series of daily fractions of 2 Gy to a total dose of 60 to 80 Gy. To select the optimal combination of Nutlin-3 and irradiation for clonogenic cell killing, initially we incubated cells in Nutlin-3 for 0 to 8 h followed by irradiation at the clinically relevant dose of 2 Gy. In the clonogenic assay, the surviving fraction at 2 Gy (Fig. 2A) decreased significantly when 22RV1 or DU145 cells were treated with Nutlin-3 for 24 h with irradiation at 2 h following the start of incubation. No significant differences were noted between the two Nutlin-3 concentrations tested (e.g., 2 and 5  $\mu\text{mol/L}$ ). Therefore, we selected a 24-h Nutlin-3 incubation (5  $\mu\text{mol/L}$ ) with irradiation at

2 h following the start of this incubation for all further experiments.

We next compared p53 and p21<sup>WAF</sup> expression following DNA damage in the presence of Nutlin-3 among the cell lines. The combination of Nutlin-3 and irradiation induced p53 and p21<sup>WAF</sup> to levels greater than either treatment or radiation alone in the WTp53-expressing 22RV1 cells (Fig. 2B). As expected, no induction was noted in DU145 or PC-3 cells (data not shown). We then tested loss of clonogenic survival and apoptosis following treatment with both Nutlin-3 and irradiation under normoxia. Nutlin-3 significantly increased the radiosensitivity of 22RV1 (SER, 1.24  $\pm$  0.1), DU145 (SER, 1.27  $\pm$  0.13), and PC-3 (SER, 1.12  $\pm$  0.08) cells (Fig. 2C) in the clonogenic assay. Nutlin-3



**Figure 2.** Nutlin-3 radiosensitizes normoxic prostate cancer cells independent of p53 status. **A**, effects of Nutlin-3 (open column, control; dark column, 2  $\mu\text{mol/L}$ ; hatched column, 5  $\mu\text{mol/L}$ ) combined with irradiation on clonogenic survival following 0, 2, or 8 h incubation before irradiation. \*,  $P < 0.05$ , statistically significant difference from control. **B**, Western blot of Nutlin-3 effects (2-5  $\mu\text{mol/L}$ ) on protein expression following irradiation on 22RV1 (p53 WT). Number under the bands, densitometry values that were calculated based on actin loading controls. **C**, effects of Nutlin-3 combined with irradiation on clonogenic survival. Squares, irradiation alone (control); triangles, irradiation and Nutlin-3 (5  $\mu\text{mol/L}$ ). Cells were incubated with Nutlin-3 for 2 h before irradiation and for 22 h following irradiation. **D**, effects of Nutlin-3 (5  $\mu\text{mol/L}$ ) on radiation-induced apoptosis based on Hoechst 33342 staining at 48 h following treatment following irradiation alone (black) or irradiation and Nutlin-3 (hatched). +,  $P < 0.05$ , statistically significant difference from untreated control; ++,  $P < 0.05$ , statistically significant difference from radiation or Nutlin-3 alone.



**Figure 3.** Nutlin-3 radiosensitizes acutely hypoxic prostate cancer cell line to a higher extent than under normoxia. **A**, Western blot of Nutlin-3 effects (5 μmol/L) on HIF-1α expression at various incubation times under acute hypoxia (0.2% O<sub>2</sub>) and normoxia on 22RV1 cell line. *Number under the bands*, densitometry values that were calculated based on actin loading controls. **B**, Western blot of Nutlin-3 effects (5 μmol/L) on MDM2 expression under acute hypoxia (0.2% O<sub>2</sub>) and normoxia on 22RV1 cell line. *Number under the bands*, densitometry values that were calculated based on actin loading controls. **C**, Western blot of Nutlin-3 effects (2-5 μmol/L) combined with irradiation on p53 and p21<sup>WAF</sup> expression at various doses under acute hypoxia (0.2% O<sub>2</sub>) on 22RV1 cell line. *Number under the bands*, densitometry values that were calculated based on actin loading controls. **D**, effects of Nutlin-3 (5 μmol/L) combined with irradiation (5 Gy) on clonogenic survival on DU145 cell line: irradiation under normoxic conditions (*empty columns*), irradiation under acute hypoxia (0.2% O<sub>2</sub>; *plain columns*), and irradiation with Nutlin-3 under acute hypoxia (0.2% O<sub>2</sub>; *hatched columns*). \*, *P* < 0.05, statistically significant difference from normoxia; \*\*, *P* < 0.05, statistically significant difference from irradiated acute hypoxia control without Nutlin-3. **E**, effects of Nutlin-3 combined with irradiation on clonogenic survival. Cells were incubated with Nutlin-3 under hypoxia for 2 h before irradiation and for 22 h following irradiation. *Dark squares*, irradiation alone (hypoxic control); *triangles*, irradiation and Nutlin-3 (5 μmol/L). **F**, effects of irradiation on apoptosis on 22RV1 and DU145 cell lines under hypoxia combined with vehicle (*black*) or Nutlin-3 (5 μmol/L; *hatched*). \*, *P* < 0.05, statistically significant difference from control; \*\*, *P* < 0.05, statistically significant difference from Nutlin-3 or radiation alone.

significantly increased apoptosis when combined with radiation compared with drug or radiation alone only for the WTP53-expressing 22RV1 cells (*P* < 0.05; Fig. 2D).

#### Nutlin-3 Radiosensitizes Prostate Cancer Cells following Acute Hypoxia

Hypoxia can increase MDM2 levels and also induce radioresistance. We therefore tested Nutlin-3 effects under acute hypoxia. Acute hypoxia alone did not lead to clonogenic cell kill. Nutlin-3 clonogenic cell kill was similar under conditions of both normoxia and hypoxia in all three cell lines (data not shown). Hypoxia induced HIF-1α stabilization under these gassing conditions (Fig. 3A). HIF-1α levels increased more rapidly (starting at 2 h) in cells treated with Nutlin-3. As expected, Nutlin-3-induced stabilization of p53 increased MDM2 expression as part of a p53-induced feedback loop. Hypoxia alone did not induce MDM2 expression, but we did observe increased MDM2 levels following treatment with hypoxia plus Nutlin-3 (Fig.

3B). Under acute hypoxia, Nutlin-3 alone, irradiation alone, or both treatments combined induced a dose-dependent activation of p53 and p21<sup>WAF</sup> in 22RV1 cells (Fig. 3C) but not in DU145 or PC-3 cells (data not shown). Hypoxic radioresistance was observed as early as 2 h and reached a plateau at 5 h of gassing (Fig. 3D). Incubation of cells in Nutlin-3 partially reversed this radioresistance. Under acute hypoxia, Nutlin-3 significantly increased killing of 22RV1 (SER, 1.78 ± 0.19), DU145 (SER, 1.31 ± 0.18), and PC-3 (SER, 1.28 ± 0.09) cells (Fig. 3E). These SER values were increased compared with the SER values under normoxia. We conclude that Nutlin-3 increases radiosensitivity under acute hypoxic conditions and that sensitization may be greater in cells that maintain p53 function.

#### Nutlin-3 Radiosensitizes Prostate Cancer Cells under Chronic Anoxia

To ascertain whether Nutlin-3 was a radiosensitizer of anoxic prostate cancer cells, cells were exposed to Nutlin-3

under chronic anoxia for 24 h and then irradiated. Anoxia during 24 h induced HIF-1 $\alpha$  to a similar extent with or without Nutlin-3 (Fig. 4A). Nutlin-3 induced p53 to a greater extent under anoxia compared with normoxia in 22RV1 cells (Fig. 4B). Under anoxia, clonogenic cell killing was more marked when Nutlin-3 was combined with radiation compared with radiation alone (Fig. 4C). Nutlin-3 alone induced more apoptosis under anoxia than normoxia for 22RV1 and DU145 cells (Fig. 4D), but Nutlin-3 increased radiation-induced apoptosis only in 22RV1 cells. No significant differences were noted in this cell line between the apoptotic levels following combined treatment under normoxia or anoxia.

## Discussion

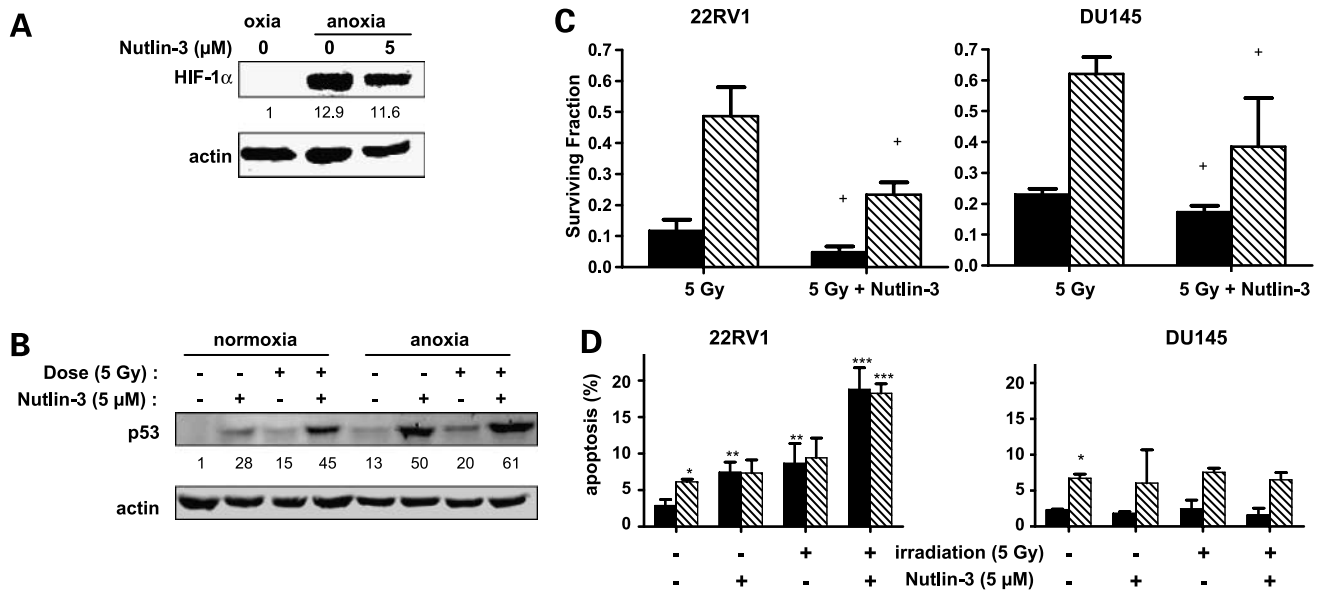
The use of molecular inhibitors of signaling pathways of DNA repair and cell cycle checkpoints is increasing in radiation oncology (20). However, little data are available on whether molecular inhibitors can radiosensitize cancer cells under both oxic and hypoxic/anoxic conditions. This is important as hypoxic and anoxic cells can severely reduce the efficacy of cancer treatment and drive a metastatic phenotype (4).

In our study, we observed that Nutlin-3 is a radiosensitizer under both hypoxic and anoxic conditions, further supporting the use of this agent in solid tumors that contain populations of cells with varying levels of oxygen. The SER were rather small, with the exception of

the effect seen in p53WT cells under hypoxia. Our data support a previous finding (24) that Nutlin-3 radiosensitizes cancer cell lines under oxa. However, in our study, Nutlin-3 was found to radiosensitize cells independent of p53. This supports another report in which MDM2 inhibition using antisense oligonucleotides led to increased radiosensitization through a p53-independent mechanism (29).

Nutlin-3 may radiosensitize WTP53-expressing cells, such as 22RV1, via multiple factors, including p21<sup>WAF</sup> activation and increased apoptosis. However, the mechanism of the radiosensitization of mutated p53-expressing or p53-null cells in our study remains unclear. In treated cells, we did not find a 1:1 correlation between the short-term death endpoint of apoptosis and the longer-term death endpoint of colony formation, suggesting that other forms of cell death (e.g., mitotic catastrophe or terminal growth arrest) may also be factors in Nutlin-associated sensitization.

An important finding of our study is that Nutlin-3 radiosensitized chronically anoxic cells or acutely hypoxic cells to a greater extent than normoxic cells. These radiosensitizing properties of Nutlin-3 were not affected by the oxygen levels (0.2% or 0%) or the duration of hypoxia before radiation (e.g., 2 or 24 h). The molecular mechanisms for the increased radiosensitizing properties of Nutlin-3 under hypoxic or anoxic conditions also remain unclear. Previous studies have shown that Nutlin-3 can alter the binding of MDM2 to both HIF-1 $\alpha$  and p53, which could



**Figure 4.** Nutlin-3 radiosensitizes chronically anoxic prostate cancer cell line independent of p53. **A**, Western blot of Nutlin-3 effects (5  $\mu$ mol/L) on HIF-1 $\alpha$  expression following chronic anoxia and normoxia on 22RV1 cell line. *Number under the bands*, densitometry values that were calculated based on actin loading controls. **B**, Western blot of Nutlin-3 effects (5  $\mu$ mol/L) combined with irradiation (5 Gy) on p53 expression following normoxia or chronic anoxia on 22RV1 cell line. *Number under the bands*, densitometry values that were calculated based on actin loading controls. **C**, effects of Nutlin-3 (5  $\mu$ mol/L) combined with irradiation (5 Gy) on clonogenic survival on 22RV1 and DU145 cell lines under normoxia (O; black) or anoxia (A; hatched). *Plus sign*, statistically significant difference from irradiation without Nutlin-3. **D**, effects of Nutlin-3 (5  $\mu$ mol/L) combined with irradiation on apoptosis on 22RV1 and DU145 cell lines under normoxia (black) or anoxia (hatched). \*,  $P < 0.05$ , statistically significant difference from normoxia; \*\*,  $P < 0.05$ , statistically significant difference from untreated control; \*\*\*,  $P < 0.05$ , statistically significant difference from Nutlin-3 or radiation alone.

modify cell survival. Consistent with these results, our data show that Nutlin-3 increased the level of HIF-1 $\alpha$  at the time of irradiation and that increased apoptosis was observed in WTp53 prostate cells under hypoxic and anoxic gassing conditions.

In conclusion, Nutlin-3 can radiosensitize hypoxic and anoxic prostate cancer cells and should be further evaluated in preclinical studies as a possible radiosensitizer *in vivo* given that hypoxia can occur in 30% to 90% of prostate tumors (8). The SER of Nutlin-3 under hypoxia is similar to the hypoxia-selective nitroacridine drug nitracrine (30) but lower than tirapazamine (SER between 2.55 and 2.70; refs. 31, 32). Further studies need to be completed with tumor cells *in vitro* and *in vivo* that are derived from a range of tumors to determine better the general implications of our data relating to the radiosensitizing properties of Nutlin-3 under hypoxia/anoxia. However, if confirmed for a range of cell lines, using modern radiotherapy techniques to target hypoxic subpopulations in combination with Nutlin-3 could be part of biologically adaptive radiotherapy using boosts (a concept named “dose Painting”; ref. 33). In this manner, the dose to hypoxic/anoxic regions can be selectively increased to enhance radiotherapy clonogenic cell kill. Precise targeting of hypoxic/anoxic subvolumes and the use of drugs that radiosensitize hypoxic cancer cells could improve clonogenic cell kill and maintain the therapeutic ratio. Nutlin-3 could therefore be a useful drug in this instance of combined modality therapy to target radioresistant hypoxic cancer cells within prostate tumors.

## References

- Parker C, Milosevic M, Toi A, et al. Polarographic electrode study of tumor oxygenation in clinically localized prostate cancer. *Int J Radiat Oncol Biol Phys* 2004;58:750–7.
- Carnell DM, Smith RE, Daley FM, Saunders MI, Bentzen SM, Hoskin PJ. An immunohistochemical assessment of hypoxia in prostate carcinoma using pimonidazole: implications for radioresistance. *Int J Radiat Oncol Biol Phys* 2006;65:91–9.
- Brown JM. Tumor microenvironment and the response to anticancer therapy. *Cancer Biol Ther* 2002;1:453–8.
- Cairns RA, Kalliomaki T, Hill RP. Acute (cyclic) hypoxia enhances spontaneous metastasis of KHT murine tumors. *Cancer Res* 2001;61:8903–8.
- Bristow R, Harrington L. Genomic stability and DNA repair. The basic science of oncology. 4th ed. In: Tannock IF, Harrington L and Bristow RG. New York; McGraw-Hill; 2005. p. 77–99.
- Brown JM, Wilson WR. Exploiting tumour hypoxia in cancer treatment. *Nat Rev Cancer* 2004;4:437–47.
- Rofstad EK, Galappathi K, Mathiesen B, Ruud EB. Fluctuating and diffusion-limited hypoxia in hypoxia-induced metastasis. *Clin Cancer Res* 2007;13:1971–8.
- Chan N, Milosevic M, Bristow RG. Tumor hypoxia, DNA repair and prostate cancer progression: new targets and new therapies. *Future Oncol* 2007;3:329–41.
- Bristow RG, Ozcelik H, Jalali F, Chan N, Vesprini D. Homologous recombination and prostate cancer: a model for novel DNA repair targets and therapies. *Radiother Oncol* 2007;83:220–30.
- Bardos JI, Chau NM, Ashcroft M. Growth factor-mediated induction of HDM2 positively regulates hypoxia-inducible factor 1 $\alpha$  expression. *Mol Cell Biol* 2004;24:2905–14.
- Zhang L, Hill RP. Hypoxia enhances metastatic efficiency by up-regulating Mdm2 in KHT cells and increasing resistance to apoptosis. *Cancer Res* 2004;64:4180–9.
- Honda R, Tanaka H, Yasuda H. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett* 1997;420:25–7.
- Appella E, Anderson CW. Post-translational modifications and activation of p53 by genotoxic stresses. *Eur J Biochem* 2001;268:2764–72.
- Honda R, Yasuda H. Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. *EMBO J* 1999;18:22–7.
- Ravi R, Mookerjee B, Bhujwalla ZM, et al. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1 $\alpha$ . *Genes Dev* 2000;14:34–44.
- Alt JR, Bouska A, Fernandez MR, Cerny RL, Xiao H, Eischen CM. Mdm2 binds to Nbs1 at sites of DNA damage and regulates double strand break repair. *J Biol Chem* 2005;280:18771–81.
- Martin K, Trouche D, Hagemer C, Sorensen TS, La Thangue NB, Kouzarides T. Stimulation of E2F1/DP1 transcriptional activity by MDM2 oncoprotein. *Nature* 1995;375:691–4.
- Dai MS, Lu H. Inhibition of MDM2-mediated p53 ubiquitination and degradation by ribosomal protein L5. *J Biol Chem* 2004;279:44475–82.
- Iwakuma T, Lozano G. MDM2, an introduction. *Mol Cancer Res* 2003;1:993–1000.
- Choudhury A, Cuddihy A, Bristow RG. Radiation and new molecular agents. Part I. Targeting ATM-ATR checkpoints, DNA repair, and the proteasome. *Semin Radiat Oncol* 2006;16:51–8.
- Vassilev LT, Vu BT, Graves B, et al. *In vivo* activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* 2004;303:844–8.
- Ambrosini G, Sambol EB, Carvajal D, Vassilev LT, Singer S, Schwartz GK. Mouse double minute antagonist Nutlin-3a enhances chemotherapy-induced apoptosis in cancer cells with mutant p53 by activating E2F1. *Oncogene* 2007;26:3473–81.
- LaRusch GA, Jackson MW, Dunbar JD, Warren RS, Donner DB, Mayo LD. Nutlin3 blocks vascular endothelial growth factor induction by preventing the interaction between hypoxia inducible factor 1 $\alpha$  and Hdm2. *Cancer Res* 2007;67:450–4.
- Cao C, Shinohara ET, Subhawong TK, et al. Radiosensitization of lung cancer by nutlin, an inhibitor of murine double minute 2. *Mol Cancer Ther* 2006;5:411–7.
- Fan R, Kumaravel TS, Jalali F, Marrano P, Squire JA, Bristow RG. Defective DNA strand break repair after DNA damage in prostate cancer cells: implications for genetic instability and prostate cancer progression. *Cancer Res* 2004;64:8526–33.
- Meng AX, Jalali F, Cuddihy A, et al. Hypoxia down-regulates DNA double strand break repair gene expression in prostate cancer cells. *Radiother Oncol* 2005;76:168–76.
- Bromfield GP, Meng A, Warde P, Bristow RG. Cell death in irradiated prostate epithelial cells: role of apoptotic and clonogenic cell kill. *Prostate Cancer Prostatic Dis* 2003;6:73–85.
- Supiot S, Gouard S, Charrier J, et al. Mechanisms of cell sensitization to  $\alpha$  radioimmunotherapy by doxorubicin or paclitaxel in multiple myeloma cell lines. *Clin Cancer Res* 2005;11:7047–52s.
- Zhang Z, Wang H, Prasad G, et al. Radiosensitization by antisense anti-MDM2 mixed-backbone oligonucleotide *in vitro* and *in vivo* human cancer models. *Clin Cancer Res* 2004;10:1263–73.
- Roberts PB, Denny WA, Wakelin LP, Anderson RF, Wilson WR. Radiosensitization of mammalian cells *in vitro* by nitroacridines. *Radiat Res* 1990;123:153–64.
- Herman TS, Teicher BA, Coleman CN. Interaction of SR-4233 with hyperthermia and radiation in the FSaIIc murine fibrosarcoma tumor system *in vitro* and *in vivo*. *Cancer Res* 1990;50:5055–9.
- Brown JM. The hypoxic cell: a target for selective cancer therapy—Eighteenth Bruce F. Cain Memorial Award Lecture. *Cancer Res* 1999;59:5863–70.
- Bentzen SM. Theragnostic imaging for radiation oncology: dose-painting by numbers. *Lancet Oncol* 2005;6:112–7.