# Analysis of the MDM2 Antagonist Nutlin-3 in Human Prostate Cancer Cells

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**BACKGROUND:.** Small molecule MDM2 antagonists including nutlin-3 have been shown to be effective against a range of cancer cell types and nutlin-3 can inhibit growth of LNCaP xenografts. We compared the efficacy of nutlin-3 in three prostate cancer cell types and provide an insight into the mechanism of nutlin-3.
**METHODS:.** Nutlin-3 efficacy was measured using proliferation assays, cell cycle analysis, apoptosis assays, quantitative RT-PCR, and immunoblotting. Chromatin immunoprecipitation (ChIP) assays were also performed.
**RESULTS:.** Nutlin-3 can specifically inhibit proliferation of LNCaP cells through cell cycle arrest and apoptosis. This coincides with increased levels of the p53-responsive transcripts p21, PUMA, gadd45, and Mdm2 and recruitment of p53 to chromatin. Nutlin-3 also reduces androgen receptor levels, resulting in altered receptor recruitment to chromatin.
**CONCLUSION:.** Our study demonstrates that small molecule MDM2 antagonists might be useful in the treatment of human prostate cancers that retain functional p53 and androgen receptor signaling. *Prostate 67: 900–906, 2007.* © 2007 Wiley-Liss, Inc.

*KEY WORDS:* **prostate cancer; p53; MDM2; nutlin-3; androgen receptor**

## INTRODUCTION

Carcinoma of the prostate (CaP) is the most prevalent tumor type in American males resulting in the death of over 30,000 men per annum [1]. Although advances in early diagnosis have reportedly decreased the death rate by around 4% per annum since 1994, new therapies for prostate cancer are lacking which means that almost all patients with advanced disease will die as a result of CaP [2]. For localized disease the main treatment option is surgery in the form of prostatectomy and 68% of patients remain disease free 10 years post-surgery. For advanced disease androgen ablation via either surgical of chemical castration is the main form of treatment and has been for more than 25 years. Initially, this treatment results in rapid tumor regression, even in cases of disseminated disease. However, almost all patients relapse resulting in the emergence of androgen independent CaP (AIPC). Apart from docetaxol-based therapy which confers a limited survival advantage for these patients [3,4], only palliative treatment options are available for AIPC.

The transcription factor p53 can control the expression of genes involved in cell cycle arrest, apoptosis, and DNA repair in response to various stresses such as DNA damage. The cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$ is a critical factor in p53-mediated cell cycle arrest [5,6], members of the Bcl-2 family such as Noxa and PUMA are involved in p53-mediated apoptosis [7,8] and gadd45 is involved in DNA repair [9]. More than 50% of all tumors carry inactivating mutations in the TP53 gene that encodes

Grant sponsor: PRIMA (Prostate Cancer Integral Management Approach); Grant sponsor: ProMPT (Prostate cancer Mechanisms of Progression and Treatment).

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Received 23 October 2006; Accepted 16 January 2007
DOI 10.1002/pros.20568
Published online 17 April 2007 in Wiley InterScience (www.interscience.wiley.com).

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the p53 tumor suppressor protein [10]. Furthermore, many p53 mutations occur within the DNA binding domain suggesting transcriptional activities are critical to p53-mediated tumor suppression [11]. Interestingly, the incidence of p53 mutations in clinically localized CaP is just 5%, whilst 62% of metastatic tumors are thought to harbor p53 mutations [12]. Controversy therefore exists over the relative importance of p53 mutations in CaP. However, the PTEN tumor suppressor that is a component of the p53 pathway is known to be mutated in approximately 50% of advanced prostate tumors [13–15]. Amplification of the MDM2 oncogene that encodes a negative regulator of p53 occurs in around 7% of tumors derived from all organs, and some prostate tumors overexpress MDM2 [16–19].

The fact that primary prostate tumors have a relatively low incidence of p53 mutations means that reactivation or hyperactivation of wild-type p53 might be of therapeutic benefit [20]. Recently described small molecules that specifically inhibit MDM2 function leading to non-genotoxic activation of p53 increase the prospect of being able to reactivate p53 in tumors [21,22]. Importantly, the MDM2 antagonist nutlin-3, which is particularly effective in causing p53-dependent apoptosis in MDM2-amplified cultured cells, exhibits antitumor activity on human prostate LNCaP and other xenografts in nude mice [22,23]. This cytotoxicity seems to involve 53BP1 [24].

Nutlin-3 has been shown to cause apoptosis and cell cycle arrest in LNCaP cells, although no molecular data derived from LNCaP cells are available to explain these phenomena. Paradoxically, a recent study using LNCaP, PC3, and DU145 cells has shown that antisense-mediated downregulation of MDM2 results in increased p53 target gene expression, apoptosis, and reduced proliferation of prostate cancer cells independently of p53 functional status [25]. LNCaP cells contain wild-type p53 ($p53^{wt/wt}$), DU145 cells contain non-functional mutated p53 ($p53^{mt/mt}$), and PC3 cells are p53 null ($p53^{null}$) [26]. This would suggest that inhibition of MDM2 functions might be of value in prostate cancer cells lacking functional p53. No study has examined the potential of nutlin-3 in the PC3 and DU145 prostate cancer cell lines that lack functional p53. We therefore tested the effects of nutlin-3 on the DU145, PC3, and LNCaP cell lines. Our data suggest that nutlin-3 has no significant effects on the DU145 ($p53^{mt/mt}$) and PC3 ($p53^{null}$) cell lines but does cause cell cycle arrest and apoptosis in LNCaP cells ($p53^{wt/wt}$) which coincides with upregulation of p53-responsive genes. Additionally, we demonstrate for the first time that nutlin-3 has an impact on androgen signaling, which is critical for prostate growth, maintenance, and carcinogenesis.

## MATERIALS AND METHODS

### Cell Culture, Flow Cytometry, and Proliferation Assays

Cells were maintained in RPMI 1640-based medium as described [27]. All cell culture reagents used were supplied by Sigma. For cell cycle analysis cells were resuspended in PBS containing 2% FCS then permeabilized with 1% Triton-X-100 (Sigma), treated with 100 μg/ml RNase (Sigma), and stained with 500 μg/ml propidium iodide (Sigma) before analysis on a BD FACScan instrument. Active caspase-3 measurements were made using a FITC-conjugated active caspase-3 antibody as recommended by manufacturer (BD Pharmingen). WST-1 assays were used to measure proliferation in 96 well plates as described by manufacturer (Roche). Eight replicates were used per condition.

### Quantitative Real-time PCR and Chromatin Immunoprecipitation (ChIP)

Measurements of PUMA transcript levels were performed using Taqman products (Applied Biosystems). p21, PSA, gadd45, and GAPDH transcripts were measured using SYBR Green I as described [28] with oligonucleotide sequences GTGGTAGAAATCTGTCATGCTGGT and GACTCTCAGGGTCGAAAACGG for p21; CCCAAACTATGGCTGCACACT and CCATGCAGGAAGGAAAACTATG for gadd45, and CGACCACTTTGTCAAGCTCA and GGGTCTTACTCCTTGGAGGC for GAPDH, respectively. PSA oligonucleotides were as described [28]. Dissociation curves confirmed PCR product specificity and transcript quantities were corrected to GAPDH values. ChIP was performed as described [28] with minor modifications. Prior to immunoprecipitation, DNA from soluble chromatin fractions was quantified using a Nanodrop spectrophotometer. Fifty micrograms of corresponding chromatin were used per ChIP with specific antibodies against p53 (Ab-6, Oncogene research products) or androgen receptor (AR) (C-19, Santa Cruz). Post-ChIP material and input samples were subject to quantitative PCR with oligonucleotides GGATCTGTGGTAGGTGAGGGTCAGG and GGAATTAGTCACGGGAGGCAGTGCAG to amplify the gadd45 p53 response element [29], GGAGTTGGAGTTGTCAGGAAAAAGGG and GGTTGTGGTCTTTCAGGCCTCCACACC to amplify a gadd45 nonspecific region 2kb downstream [29], and GTGGCTCTGATTGGCTTTCTG and CTGAAAACAGGCAGCCCAAG to amplify the p53 response element of p21 [5]. Oligonucleotides used to amplify AREIII were as described [28].

## RESULTS

### Nutlin-3 Dramatically Reduces Proliferation of LNCaP But not PC3 or DU145 Cells

We firstly tested the effects of nutlin-3 on the proliferation of three cell lines used in this study. Using colorimetric WST-1 assays we determined that nutlin-3 could inhibit LNCaP proliferation by 50% at a dose of 5 μM whereas no effect was observed on DU145 cells at this dose and only a 15% reduction in proliferation was observed in PC3 cells (Fig. 1A). Immunoblotting confirmed that p53 was expressed in DU145 and LNCaP cells, but not in PC3 cells (Fig. 1B). Surprisingly, we found that the level of wild-type p53 in LNCaP cells was not appreciably altered upon administration of nutlin-3 (Fig. 1B) despite previously published data from non-prostate cell lines showing that wild-type p53 is stabilized in response to nutlin-3. The MDM2 gene has previously been shown to be highly dependent upon the transcriptional activity of p53 [30]. To confirm that nutlin-3 could indeed activate p53 target gene expression in our system, the levels of MDM2 transcript and protein were measured in the three cell lines. Quantitative real-time PCR demonstrated that nutlin-3 produced a 2.2-fold increase in MDM2 transcript levels at 5 μM in LNCaP cells and an 8.6-fold increase at 10 μM (Fig. 1C). Additionally, MDM2 protein levels markedly increased in response to nutlin-3 in LNCaP cells (Fig. 1B). No significant changes in MDM2 expression were observed in either DU145 or PC3 cells (Fig. 1B and C). Levels of the p53-responsive gadd45 gene transcript were also upregulated in response to nutlin-3 in LNCaP cells, but not DU145 or PC3 cells (Fig. 1D).

In order to investigate the effects of nutlin-3 on proliferation of the three cell lines, we firstly examined cell cycle arrest in response to nutlin-3. Addition of increasing concentrations of nutlin-3 resulted in a dose-dependent reduction in S-phase in LNCaP cells after

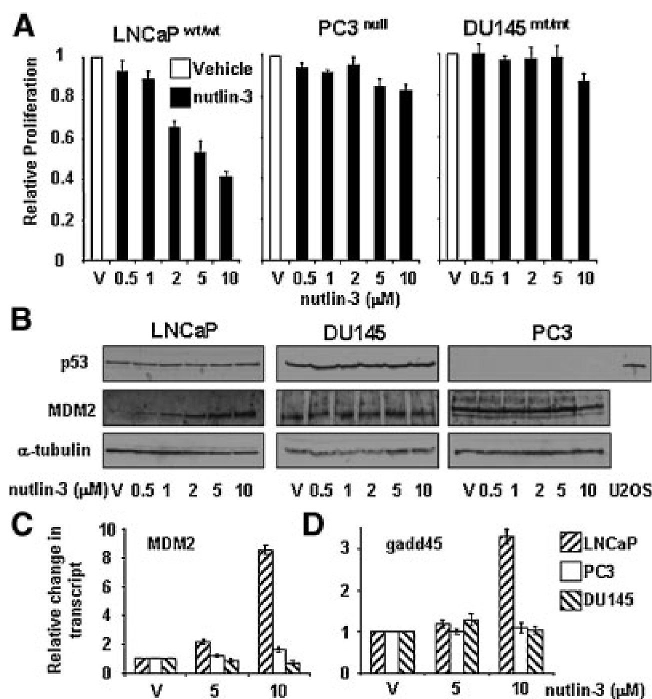
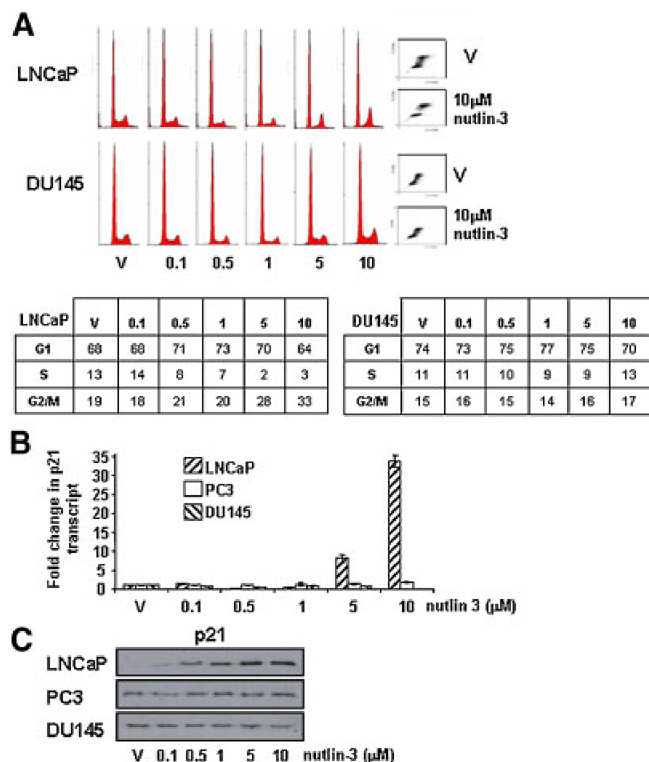



**Fig. 1.** Assessment of nutlin-3 to alter proliferation of prostate cancer cell lines **A**: Cells were seeded in 96 well plates, treated with the indicated doses of nutlin-3 for 48 hr then subject to WST-1 proliferation assays. Experiments were performed three times and data are representative of eight replicates from one experiment. **B**: Cells seeded in 12 well plates were treated with nutlin-3 for 24 hr then equal amounts of whole cell extracts were used for immunoblotting with the indicated antisera. In the case of PC3 cells, U2OS cell extract was used as a positive control for p53 immunoreactivity. **C**,**D**: Cells treated as in B were subject to RNA isolation then cDNA generated was subject to quantitative real-time PCR for MDM2 or gadd45 as indicated. V denotes DMSO vehicle control. All error bars represent standard deviation.




| LNCaP | V | 0.1 | 0.5 | 1 | 5 | 10 |
|---|---|---|---|---|---|---|
| G1 | 68 | 68 | 71 | 73 | 70 | 64 |
| S | 13 | 14 | 8 | 7 | 2 | 3 |
| G2/M | 19 | 18 | 21 | 20 | 28 | 33 |

| DU145 | V | 0.1 | 0.5 | 1 | 5 | 10 |
|---|---|---|---|---|---|---|
| G1 | 74 | 73 | 75 | 77 | 75 | 70 |
| S | 11 | 11 | 10 | 9 | 9 | 13 |
| G2/M | 15 | 16 | 15 | 14 | 16 | 17 |

**Fig. 2.** Effects of nutlin-3 on cell cycle of prostate cancer cell lines **A**: Cells seeded in 12 well plates were treated with the indicated doses of nutlin-3 for 48 hr then stained with propidium iodide and subject to cell cycle analysis by flow cytometry. The table indicates the number of cells in each phase of the cell cycle. V denotes DMSO vehicle control. **B**: Cells were treated as in Figure 1C then cDNA used to quantitate p21 expression by real-time PCR. PCR was performed in triplicate and error bars represent standard deviation. **C**: Whole cell extracts featured in Figure 1B were used for immunoblotting to detect p21 protein. [Color figure can be viewed in the online issue, which is available at www. interscience.wiley.com.]

48 hr exposure (Fig. 2A), with a concordant increase in cell numbers in G2/M phase as previously described [23]. However, no such effects were seen in DU145 (Fig. 2A) or PC3 cells. To explain the cell cycle effects we examined expression of the p53-inducible p21 gene. Although p21 levels have previously been shown to increase in response to nutlin-3 in non-prostate cell lines [22,23], no data from prostate cells have been reported. p21 transcript levels increased by approximately eightfold in LNCaP cells in response to 5 μM nutlin-3 and 34-fold in response to 10 μM nutlin-3 (Fig. 2B) suggesting that LNCaP cells do respond to nutlin-3 in a manner similar to other cell lines that express wild-type p53. No such increases in p21 expression were observed in DU145 or PC3 cell lines (Fig. 2B). Additionally, a strong increase in p21 protein levels was observed by immunoblotting in LNCaP cells, but not in PC3 or DU145 cells (Fig. 2C).

We next examined apoptosis in response to nutlin-3 using caspase-3 cleavage as a flow cytometry based marker of apoptosis. After 48 hr exposure to 5 μM nutlin-3, LNCaP cells underwent 20% apoptosis, which increased to 29% in response to 10 μM nutlin-3 (Fig. 3A). DMSO vehicle-treated cells did not undergo detectable apoptosis (not shown). No increase in apoptotic cell numbers was observed in DU145 or PC3 cells in response to nutlin-3 (Fig. 3A). Expression levels of the proapoptotic p53-responsive gene PUMA increased by approximately fourfold in response to nutlin-3, providing a potential explanation for increased apoptosis (Fig. 3B). No increases in PUMA levels were observed in DU145 or PC3 cells (Fig. 3B).

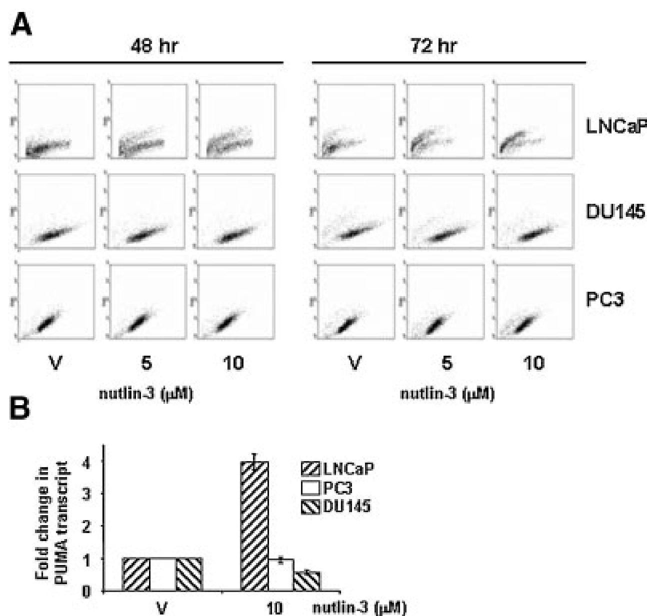



**Fig. 3.** Analysis of apoptosis in response to nutlin-3 **A**: Cells seeded in 12 well plates were treated with either 5 μM or 10 μM nutlin-3 as indicated then harvested after either 48 or 72 hr. They were then stained for active caspase-3 using a FITC-conjugated antibody as recommended (BD Pharmingen) and subject to flow cytometry. V denotes DMSO vehicle control. Apoptotic cells are those which are shifted upwards on the Y axis which is a measure of fluorescence. **B**: Cells were treated as in Figure 1C then cDNA used to quantitate PUMA expression by real-time PCR. Data are represented as fold change from vehicle-treated cells (V). PCR was performed in triplicate and error bars represent standard deviation.

### Nutlin-3 Affects Androgen Signaling in LNCaP Cells

Previous data from our group and others have shown that MDM2 can control expression levels and transcriptional activity of the AR [31,32]. Additional studies have demonstrated that p53 can also interact with the AR leading to repression of AR-mediated transcription [33,34]. As p53 and AR share the same E3 ubiquitin ligase, encoded by the MDM2 oncogene, we wanted to examine the effects of nutlin-3 on the AR. We chose to further focus on the LNCaP cell line as these cells express the AR and respond to androgen treatment. Application of nutlin-3 for 12 hr resulted in a dose-dependent substantial decrease in steady-state AR levels in LNCaP cells (Fig. 4A). Given the reduction in AR levels in response to nutlin-3 we next examined what effects nutlin-3 would have on the transcriptional functions of the AR. Firstly, we examined recruitment of AR to chromatin, using quantitative ChIP. Application of nutlin-3 led to an approximately 70% reduction in AR occupancy at AREIII of the androgen responsive PSA gene promoter (Fig. 4B). p53 has previously been reported to complex with AR and influence AR transcriptional activity, so we also examined p53 recruitment to AREIII. Addition of nutlin-3 led to an approximately fivefold increase in p53 recruitment to AREIII (Fig. 4B). This is the first demonstration that p53 can be recruited to the PSA promoter. Treatment of LNCaP cells with an alternative p53 activating agent, etoposide, also produced the same effects on both AR and p53 recruitment to AREIII (Fig. 4B). We next examined recruitment of both AR and p53 to two p53-responsive genes, p21 and gadd45. Whilst p53 has previously been demonstrated to be recruited to p53 response elements within the promoters of these genes [5,29,35], AR has previously been reported to bind to and regulate only the p21 gene promoter at an ARE-200bp from the transcription start site [36,37]. Addition of nutlin-3 led to an approximately 60-fold increase in p53 recruitment to the p21 promoter and an approximately 22-fold increase in recruitment at the gadd45 p53 response element (Fig. 4B). However, nutlin-3 produced a 50% reduction in AR recruitment to the p21 promoter. Additionally, we found that although AR could be recruited to the gadd45 promoter, treatment with nutlin-3 essentially abolished this recruitment

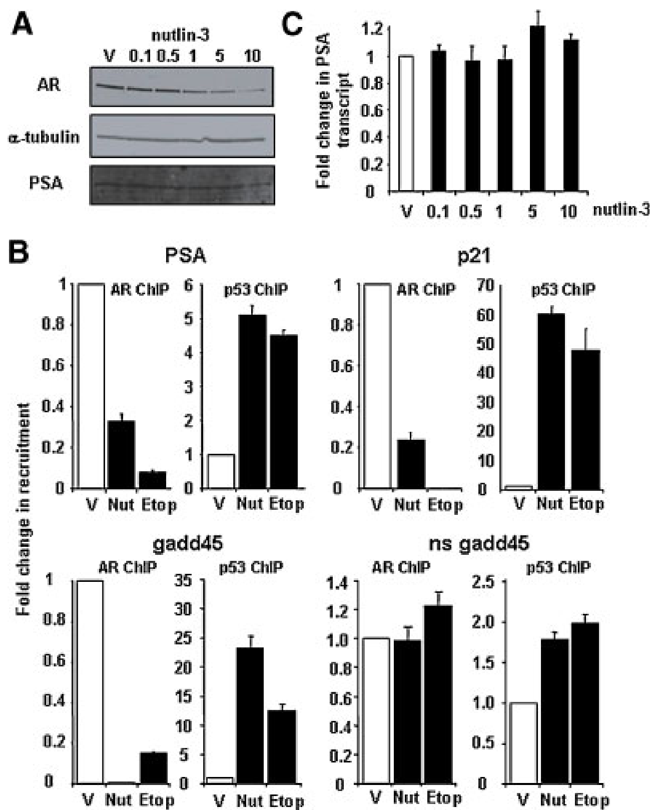



**Fig. 4.** Effect of nutlin-3 on transcriptional regulation **A**: LNCaP cells seeded in 12 well plates were treated with the indicated doses of nutlin-3 or DMSO vehicle (V). After 12 hr cells were harvested and used as in Figure 1B for immunoblotting to detect AR, PSA, or α-tubulin proteins. **B**: LNCaP cells were treated for 12 hr with DMSO vehicle, 5 μM nutlin-3 or 1 μM etoposide then subject to chromatin immunoprecipitation using the indicated antisera. Irrelevant FRS-2 antibody was used as a control (not shown). Recovered material was then subject to quantitative real-time PCR using oligonucleotides corresponding to the PSA, p21, gadd45, or non-specific (ns) region of the gadd45 gene, as indicated. PCR was performed in triplicate and error bars represent standard deviation. **C**: Quantitative real-time PCR was used to measure PSA transcript levels in LNCaP cells treated with nutlin-3 as in Figure 2B. [Color figure can be viewed in the online issue, which is available at www. interscience.wiley.com.]

(Fig. 4B). In order to address the question of specificity in our ChIP assay, we examined recruitment of AR and p53 to a downstream region of the gadd45 gene. We found only small changes in the levels of recruitment to this region of DNA, confirming specificity in our ChIP assay (Fig. 4B). Additionally, ChIP using a non-specific antibody did not recover significant amounts of material (data not shown), further confirming the specificity of our assay.

We next examined expression of the prototypical AR-responsive gene PSA in response to nutlin-3. Surprisingly, PSA transcript levels did not respond to nutlin-3 (Fig. 4C), a result consistent with the lack of effect of nutlin-3 on PSA protein levels (Fig. 4A).

## DISCUSSION

Previously published data have demonstrated that nutlin-3 has antitumor activity in LNCaP cell xenografts in nude mice and that nutlin-3 seems to affect only cells expressing wild-type p53 [22,23]. Here we support this antiproliferative function for nutlin-3 by presenting molecular mechanisms through which nutlin-3 might operate in prostate cancer cells.

We have compared the effects of the MDM2 antagonist nutlin-3 on three different prostate cancer cell lines that have distinct p53 and AR status. LNCaP cells contain wild p53, PC3 cells are p53 null due to a single base deletion in codon 138 that prevents expression, and DU145 cells express mutant p53 that harbors missense mutations in codons 223 and 274 [38]. Additionally, of these three cell lines, only LNCaP cells express the AR which is involved in prostate growth, development, and carcinogenesis [39,40].

We found that the proliferation of LNCaP cells alone was markedly reduced by nutlin-3. Although the proliferation of PC3 cells was affected to a lesser degree by nutlin-3, we could not achieve 50% inhibition of PC3 proliferation using nutlin-3 doses up to 50 μM, compared to DMSO vehicle (data not shown). Additionally, we found that the cell cycle of LNCaP cells was affected by nutlin-3 whereas DU145 and PC3 cells were not. LNCaP cells underwent a G2/M phase cell cycle arrest, with a corresponding reduction in the number of cells in S phase. Consistent with these observations, we show for the first time that p21 levels are increased in LNCaP cells in response to nutlin-3, but not in DU145 or PC3 cells. We also show that nutlin-3 causes apoptosis only in LNCaP cells which appears to involve activation of caspase-3 and upregulation of the p53-responsive gene PUMA.

Because all three of the cell lines express the nutlin-3 target MDM2 but only LNCaP cells express functional p53 one might assume that wild-type p53 is required for nutlin-3 to generate its effects on proliferation of prostate cancer cells. However, we show that nutlin-3 reduces steady-state AR protein levels in LNCaP cells. This is important because androgen signaling is not only required for the proliferation of LNCaP cells but also the development and maintenance of human prostate tumors. Previous studies have demonstrated that AR depletion in LNCaP cells results in inhibition of proliferation [41]. We therefore propose that nutlin-3 might inhibit growth of LNCaP cells through not only p53 signaling but also by downregulation of androgen signaling. Increased levels of the MDM2 protein, which is known to target AR for destruction, could account for the effects of nutlin-3 on AR levels. Although we attempted to examine whether nutlin-3 would affect AR-MDM2 or AR-p53 interaction by

co-immunoprecipitation, the results of these experiments were difficult to interpret due to the fact that application of nutlin-3 produces substantial changes in AR and MDM2 levels (data not shown).

A previous study using antisense targeted to MDM2 demonstrated that MDM2 depletion in DU145, PC3, or LNCaP cells resulted in increased expression of p53-responsive genes, increased apoptosis, and reduced proliferation irrespective of p53 status [25]. At first glance, our data would therefore seem to be contradictory to this study. However, the difference in findings could arise because of the fact that nutlin-3 specifically inhibits the MDM2-p53 interaction [22] whereas antisense treatment would abolish all MDM2 functions within the cell. Nevertheless the intriguing and yet unexplained fact remains that MDM2 does play a role in the proliferation of PC3 and DU145 cells, albeit in a manner that is not affected by nutlin-3. Some studies have shown that nutlin-3 is effective against B-cell chronic lymphocytic leukemia cells [42,43]. Induction of apoptosis by nutlin-3 in these cells seems to involve non-transcriptional mechanisms which might also be worthy of investigation in CaP cells.

Using ChIP, we demonstrated that the changes to AR and p53 levels in LNCaP cells in response to nutlin-3 led to their altered recruitment to chromatin. We show for the first time that application of nutlin-3 leads to increased recruitment of p53 to p53-responsive genes. Whilst this is to be expected, we also found that AR recruitment to the androgen-responsive PSA gene and two p53-responsive genes was altered. In keeping with reduced AR protein levels, we found that AR recruitment to these genes was dramatically reduced. In the case of PSA this surprisingly, did not seem to affect the expression of either PSA transcript or protein levels. This suggests that changes in AR recruitment to the PSA promoter cannot necessarily be used as an indication of altered transcription. Additionally, these findings could indicate that transcriptionally competent AR does not associate with MDM2 and is therefore refractory to MDM2-mediated repression. Interestingly, we show for the first time that AR is recruited to the p53-responsive gadd45 gene promoter. Nutlin-3 essentially abolished this recruitment, and led to a large reduction in AR recruitment to the p21 gene promoter. This would suggest that nutlin-3 induced upregulation of p21 and gadd45 involves co-ordinated recruitment of p53 and loss of AR.

Recent studies have shown that the MDM2 family member MDMX can dictate the outcome to nutlin-3 treatment [44,45]. No published study has examined MDMX expression in CaP, which might prove useful in determining the effectiveness of nutlin-3 or other MDM2 antagonists in CaP.

## CONCLUSIONS

The findings presented here suggest that nutlin-3, or other small molecule MDM2 antagonists should be further evaluated as therapies against prostate cancers that retain functional components of the p53 and AR signaling pathways.

## ACKNOWLEDGMENTS

This work was funded by PRIMA (Prostate Cancer Integral Management Approach) 6th framework programme and ProMPT (Prostate cancer Mechanisms of Progression and Treatment).

## REFERENCES

1. Nelson WG, De Marzo AM, Isaacs WB. Prostate cancer. N Engl J Med 2003;349(4):366–381.
2. Isaacs W, De Marzo A, Nelson WG. Focus on prostate cancer. Cancer Cell 2002;2(2):113–116.
3. Tannock IF, de Wit R, Berry WR, Horti J, Pluzanska A, Chi KN, Oudard S, Theodore C, James ND, Turesson I, Rosenthal MA, Eisenberger MA. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. N Engl J Med 2004;351(15):1502–1512.
4. Petrylak DP, Tangen CM, Hussain MH, Lara PN Jr, Jones JA, Taplin ME, Burch PA, Berry D, Moinpour C, Kohli M, Benson MC, Small EJ, Raghavan D, Crawford ED. Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. N Engl J Med 2004; 351(15): 1513–1520.
5. el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B. WAF1, a potential mediator of p53 tumor suppression. Cell 1993; 75(4): 817–825.
6. Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, Sedivy JM, Kinzler KW, Vogelstein B. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science 1998; 282(5393):1497–1501.
7. Nakano K, Vousden KH. PUMA a novel proapoptotic gene, is induced by p53. Mol Cell 2001;7(3):683–694.
8. Yu J, Zhang L, Hwang PM, Kinzler KW, Vogelstein B. PUMA induces the rapid apoptosis of colorectal cancer cells. Mol Cell 2001;7(3):673–682.
9. Smith ML, Chen IT, Zhan Q, Bae I, Chen CY, Gilmer TM, Kastan MB, O'Connor PM, Fornace AJ Jr. Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. Science 1994;266(5189):1376–1380.
10. Olivier M, Eeles R, Hollstein M, Khan MA, Harris CC, Hainaut P. The IARC TP53 database: New online mutation analysis and recommendations to users. Hum Mutat 2002;19(6):607–614.
11. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. Science 1991;253(5015):49–53.
12. Brooks JD, Bova GS, Ewing CM, Piantadosi S, Carter BS, Robinson JC, Epstein JI, Isaacs WB. An uncertain role for p53 gene alterations in human prostate cancers. Cancer Res 1996; 56(16):3814–3822.
13. Vlietstra RJ, van Alewijk DC, Hermans KG, van Steenbrugge GJ, Trapman J. Frequent inactivation of PTEN in prostate cancer cell lines and xenografts. Cancer Res 1998;58(13):2720–2723.

14. Whang YE, Wu X, Suzuki H, Reiter RE, Tran C, Vessella RL, Said JW, Isaacs WB, Sawyers CL. Inactivation of the tumor suppressor PTEN/MMAC1 in advanced human prostate cancer through loss of expression. Proc Natl Acad Sci USA 1998;95(9):5246–5250.
15. Cairns P, Okami K, Halachmi S, Halachmi N, Esteller M, Herman JG, Jen J, Isaacs WB, Bova GS, Sidransky D. Frequent inactivation of PTEN/MMAC1 in primary prostate cancer. Cancer Res 1997;57(22):4997–5000.
16. Momand J, Jung D, Wilczynski S, Niland J. The MDM2 gene amplification database. Nucleic Acids Res 1998;26(15):3453–3459.
17. Ittmann M, Wieczorek R, Heller P, Dave A, Provet J, Krolewski J. Alterations in the p53 and MDM-2 genes are infrequent in clinically localized, stage B prostate adenocarcinomas. Am J Pathol 1994;145(2):287–293.
18. Leite KR, Franco MF, Srougi M, Nesrallah LJ, Nesrallah A, Bevilacqua RG, Darini E, Carvalho CM, Meirelles MI, Santana I, Camara-Lopes LH. Abnormal expression of MDM2 in prostate carcinoma. Mod Pathol 2001;14(5):428–436.
19. Osman I, Drobnjak M, Fazzari M, Ferrara J, Scher HI, Cordon-Cardo C. Inactivation of the p53 pathway in prostate cancer: Impact on tumor progression. Clin Cancer Res 1999;5(8):2082–2088.
20. Bykov VJ, Wiman KG. Novel cancer therapy by reactivation of the p53 apoptosis pathway. Ann Med 2003;35(7):458–465.
21. Yang Y, Ludwig RL, Jensen JP, Pierre SA, Medaglia MV, Davydov IV, Safiran YJ, Oberoi P, Kenten JH, Phillips AC, Weissman AM, Vousden KH. Small molecule inhibitors of HDM2 ubiquitin ligase activity stabilize and activate p53 in cells. Cancer Cell 2005;7(6):547–559.
22. Vassilev LT, Vu BT, Graves B, Carvajal D, Podlaski F, Filipovic Z, Kong N, Kammlott U, Lukacs C, Klein C, Fotouhi N, Liu EA. In vivo activation of the p53 pathway by small-molecule antagonists of M DM2. Science 2004;303(5659):844–848.
23. Tovar C, Rosinski J, Filipovic Z, Higgins B, Kolinsky K, Hilton H, Zhao X, Vu BT, Qing W, Packman K, Myklebost O, Heimbrook DC, Vassilev LT. Small-molecule MDM2 antagonists reveal aberrant p53 signaling in cancer: implications for therapy. Proc Natl Acad Sci USA 2006;103(6):1888–1893.
24. Brummelkamp TR, Fabius AW, Mullenders J, Madiredjo M, Velds A, Kerkhoven RM, Bernards R, Beijersbergen RL. An shRNA barcode screen provides insight into cancer cell vulnerability to MDM2 inhibitors. Nat Chem Biol 2006;2(4):202–206.
25. Zhang Z, Li M, Wang H, Agrawal S, Zhang R. Antisense therapy targeting MDM2 oncogene in prostate cancer: Effects on proliferation, apoptosis, multiple gene expression, and chemotherapy. Proc Natl Acad Sci USA 2003;100(20):11636–11641.
26. Carroll AG, Voeller HJ, Sugars L, Gelmann EP. p53 oncogene mutations in three human prostate cancer cell lines. Prostate 1993;23(2):123–134.
27. Logan IR, Sapountzi V, Gaughan L, Neal DE, Robson CN. Control of human PIRH2 protein stability: Involvement of TIP60 and the proteosome. J Biol Chem 2004;279(12):11696–11704.
28. Logan IR, Gaughan L, McCracken SR, Sapountzi V, Leung HY, Robson CN. Human PIRH2 enhances androgen receptor signaling through inhibition of Histone Deacetylase 1 and is overexpressed in prostate cancer. Mol Cell Biol 2006;26(17):6502–6510.
29. An W, Kim J, Roeder RG. Ordered cooperative functions of PRMT1, p300, and CARM1 in transcriptional activation by p53. Cell 2004;117(6):735–748.
30. Liang H, Lunec J. Characterisation of a novel p53 down-regulated promoter in intron 3 of the human MDM2 oncogene. Gene 2005;361:112–118.
31. Lin HK, Wang L, Hu YC, Altuwaijri S, Chang C. Phosphorylation-dependent ubiquitylation and degradation of androgen receptor by Akt require Mdm2 E3 ligase. EMBO J 2002;21(15):4037–4048.
32. Gaughan L, Logan IR, Neal DE, Robson CN. Regulation of androgen receptor and histone deacetylase 1 by Mdm2-mediated ubiquitylation. Nucleic Acids Res 2005;33(1):13–26.
33. Sengupta S, Wasylyk B. Physiological and pathological consequences of the interactions of the p53 tumor suppressor with the glucocorticoid, androgen, and estrogen receptors. Ann N Y Acad Sci 2004;1024:54–71.
34. Shenk JL, Fisher CJ, Chen SY, Zhou XF, Tillman K, Shemshedini L. p53 represses androgen-induced transactivation of prostate-specific antigen by disrupting hAR amino- to carboxyl-terminal interaction. J Biol Chem 2001;276(42):38472–38479.
35. Espinosa JM, Verdun RE, Emerson BM. p53 functions through stress- and promoter-specific recruitment of transcription initiation components before and after DNA damage. Mol Cell 2003;12(4):1015–1027.
36. Lu S, Liu M, Epner DE, Tsai SY, Tsai MJ. Androgen regulation of the cyclin-dependent kinase inhibitor p21 gene through an androgen response element in the proximal promoter. Mol Endocrinol 1999;13(3):376–384.
37. Lu S, Jenster G, Epner DE. Androgen induction of cyclin-dependent kinase inhibitor p21 gene: Role of androgen receptor and transcription factor Sp1 complex. Mol Endocrinol 2000;14(5):753–760.
38. Isaacs WB, Carter BS, Ewing CM. Wild-type p53 suppresses growth of human prostate cancer cells containing mutant p53 alleles. Cancer Res 1991;51(17):4716–4720.
39. Culig Z, Bartsch G. Androgen axis in prostate cancer. J Cell Biochem 2006;99(2):373–381.
40. Abate-Shen C, Shen MM. Molecular genetics of prostate cancer. Genes Dev 2000;14(19):2410–2434.
41. Eder IE, Culig Z, Ramoner R, Thurnher M, Putz T, Nessler-Menardi C, Tiefenthaler M, Bartsch G, Klocker H. Inhibition of LncaP prostate cancer cells by means of androgen receptor antisense oligonucleotides. Cancer Gene Ther 2000;7(7):997–1007.
42. Coll-Mulet L, Iglesias-Serret D, Santidrian AF, Cosialls AM, de Frias M, Castano E, Campas C, Barragan M, de Sevilla AF, Domingo A, Vassilev LT, Pons G, Gil J. MDM2 antagonists activate p53 and synergize with genotoxic drugs in B-cell chronic lymphocytic leukemia cells. Blood 2006;107(10):4109–4114.
43. Secchiero P, Barbarotto E, Tiribelli M, Zerbinati C, di Iasio MG, Gonelli A, Cavazzini F, Campioni D, Fanin R, Cuneo A, Zauli G. Functional integrity of the p53-mediated apoptotic pathway induced by the nongenotoxic agent nutlin-3 in B-cell chronic lymphocytic leukemia (B-CLL). Blood 2006;107(10):4122–4129.
44. Patton JT, Mayo LD, Singhi AD, Gudkov AV, Stark GR, Jackson MW. Levels of HdmX expression dictate the sensitivity of normal and transformed cells to Nutlin-3. Cancer Res 2006;66(6):3169–3176.
45. Hu B, Gilkes DM, Farooqi B, Sebti SM, Chen J. MDMX overexpression prevents p53 activation by the MDM2 inhibitor Nutlin. J Biol Chem 2006;281(44):33030–33035.