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# Senescence induction in renal carcinoma cells by Nutlin-3: a potential therapeutic strategy based on MDM2 antagonism

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ABSTRACT

Although the role of p53 as a tumour suppressor in renal cell carcinoma (RCC) is unclear, our recent analysis suggests that increased wild-type p53 protein expression is associated with poor outcome. A growing body of evidence also suggests that p53 expression and increased co-expression of MDM2 are linked with poor prognosis in RCC. We have therefore examined whether an MDM2 antagonist; Nutlin-3, might rescue/increase p53 expression and induce growth inhibition or apoptosis in RCC cells that retain wild-type p53. We show that inhibition of p53 suppression by MDM2 in RCC cells promotes growth arrest and p53-dependent senescence – phenotypes known to mediate p53 tumour suppression *in vivo*. We propose that future investigations of therapeutic strategies for RCC should incorporate MDM2 antagonism as part of strategies aimed at rescuing/augmenting p53 tumour suppressor function.



## Introduction

The incidence of cancer of the kidney is increasing in the developed world. For example the age-adjusted incidence has risen from 5 to approximately 11/100,000 in the last 30 years in the UK [1]. Despite advances in understanding of the molecular biology of RCC and related drug developments, targeting consequences of VHL mutations (increased VEGF) or mTOR dependence, patients with metastatic disease have an extremely short life expectancy [2]. Accordingly, identifying other pathways that define novel therapeutic targets remains a high priority. In the present study we have focused on the role of the p53 pathway in renal cancers (reviewed in [3]). Although the literature on p53 in RCC contains seemingly conflicting data [3], a recent review of the evidence suggests that a re-interpretation of the existing information on the status of the p53/MDM2 pathway provides a way to resolve this discrepancy [3]. Specifically, whilst mutations in the p53 gene (*TP53*) were not predictive of outcome, up-regulation of p53 expression was and moreover, p53 expression is strongly linked with expression of MDM2 [4,5], a conclusion further supported by our own recent study [6]. In a cohort of 90 renal cell carcinoma patients, we found that p53 expression was linked with MDM2 ($P = 0.000013$) and that co-expression was linked with reduced disease specific survival in both univariate ($P = 0.036$) and Cox multivariate ($P = 0.027$) analyses [6,7]. These results are similar to those obtained in the only two other studies to have previously examined p53 and MDM2 expression in RCC, demonstrating associated co-expression [4,5] and in the one case where outcomes were analysed, co-expression was also linked with poorer outcome [4]. It seemed likely that the mechanism linking p53 and MDM2 expression was due to p53-dependent transcription of MDM2 in these cells [8]. Of course, this explanation demands that the p53 up-regulated in these cells is wild-type, and not surprisingly, our study found that indeed, in 86% of cases, up-regulated p53 was wild-type, thus providing support for this explanation for co-expression [3].

This then begs the question of how up-regulation of wild-type p53 can be linked with poor prognosis in cancer. The most probable explanation may be that the associated MDM2 expression can promote phenotypes associated with more aggressive disease. For example, MDM2 can promote increased cell motility and invasiveness [9] and thus it may be that the contribution of wild-type p53 is indirect and is mediated via increased MDM2 expression.

Critically, the observed association between retention of up-regulated wild-type p53 in RCC patients and co-expression of MDM2 also provides support for *in vitro* data from previous studies by ourselves and others showing that p53 in RCC cells is at least partially functional [10–12].

If wild-type p53 is retained in RCCs and promotes co-expression of MDM2 as a consequence of wild-type p53-mediated

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transcription of the *MDM2* gene, then this suggests that although p53 is partially transcriptionally active in these cells, it critically fails to promote tumour-suppressive activities in RCC. We have therefore investigated whether we could rescue p53 in RCC cells by targeting its negative regulator MDM2, to promote phenotypes that contribute to tumour suppression: apoptosis and/or senescence. A key question arising from this model (since expression of MDM2 is up-regulated in response to p53 up-regulation, but apparently fails to promote efficient degradation of p53) is whether targeting of MDM2 in these cells will prove an effective strategy to activate a tumour suppressing phenotype in RCC cells. As proof of principle we show here that Nutlin-3, the small molecule inhibitor of MDM2-p53 interaction, effectively induces growth arrest and senescence in RCC cells and that this senescence is p53-dependent. Thus due to the unusual nature of the p53 pathway in RCC there exists a unique opportunity to rescue wild-type p53 function in a sub-set of poor prognosis and biomarker-defined patients (i.e. wild-type p53+ve/MDM2+ve) which clearly warrants further investigation.

## Materials and methods

### *Antibodies*

Antibodies for β-actin (C-2), p21 (F-5) and BAX (N-20) were obtained from Santa Cruz Biotechnology. Antibodies for p53 (DO-1) and MDM2 (IF2) were from Calbiochem and anti-BrdU (B44) for flow cytometry was from Becton Dickinson. Anti-mouse and anti-rabbit HRP were from GE Healthcare.

### *Plasmids, siRNAs and transfection*

pRDI-22 was a kind gift of Richard Iggo. siRNAs (Dharmacon) and their use have been described previously [12–14]. ACHN cells expressing shRNA for either p53 or a scrambled control sequence based on previously described siRNAs [12] were generated as follows: oligonucleotide duplexes were cloned into pSUPER-puro vector (Oligoengine) according to the manufacturer's protocol. ACHN cells were transfected with shRNA vectors using GeneJuice (Novagen) essentially as described previously [13], and selected for four days in media supplemented with 1.5 μg/ml puromycin. Subsequently cells were maintained under selection of 0.75 μg/ml puromycin. Colonies were identified, picked and expanded before being analysed by western blotting.

### *Cell culture and western blot analysis*

Cell lines ACHN, A498 and Caki-2 were obtained directly from the ATCC. Cell lines 111, 115, 117 and 154 were generated by Prof. W.M. Linehan and were kindly provided directly to us. In all cases, aliquots of low passage cells were frozen and then as required these were recovered for use in these studies. No cell line was in culture continuously for more than 6 months prior to analysis. Cells were maintained, treated with Nutlin-3 (Sigma), harvested and processed for western blotting as described previously [12–15].

### *Quantitative RT-PCR analysis of MDM2 mRNA*

Q-RT-PCR analysis of transcripts initiated in the P1 and P2 promoters of the human *MDM2* gene was performed as we have previously described [16]. Ct values were converted to relative mRNA transcript abundance using the standard curve method and then normalised to the abundance of *GAPDH* mRNA in the sample.

### *Proliferation and senescence assays*

These were performed as described previously [15]. MTT (Sigma) was added to cells for three hours and absorbance measured using a Thermo-Multiskan plate reader.

### *Flow cytometry*

Flow cytometry was performed as described previously [15], except for BrdU incorporation which was performed as follows: cell nuclei were extracted as described [17], incubated sequentially in anti-BrdU and anti-mouse FITC secondary antibody solutions in PBS with 0.5% Tween-20 and 1% BSA, re-suspended in 1 μg/ml propidium iodide in PBS and analysed using a BD FACScalibur flow cytometer.

## Results

p53 promotes transcription of the *MDM2* gene from the P2 promoter, normally as part of a stress response [8]. We therefore measured the relative levels of P1 and P2 MDM2 transcripts in a panel of RCC cell lines and for comparison also in a wild-type p53 non-RCC cell line (U2Os – osteosarcoma) as Fig. 1 shows (note that none of these lines harbour amplifications of the MDM2 gene – as supplementary data Fig. 1 illustrates). With one exception; 117 cells which harbour wild-type p53 (confirmed functionally (see panel 1C) and by sequence analysis, not shown) all of the p53 lines can be correctly identified as harbouring wild-type or mutant p53 using an empirically determined threshold value of 0.85. Some studies have suggested that the MDM2 P2 promoter is not exclusively up-regulated by p53 [18–20], and in mice, low/basal levels of MDM2 P2 transcripts can be detected in both wild-type and p53-/- animals [21]. However only in p53 wild-type animals is P2 activated by stress and even in studies that have found evidence of other regulators of P2 transcription, p53 status strongly predicted P2 activity [20]. These studies form part of an overwhelming body of evidence which clearly demonstrates that p53-dependent activation of P2 is a primary determinant of MDM2 up-regulation in response to stress. Therefore, since only p53 has been demonstrated to induce MDM2 expression from P2 in response to stress, and since stresses that activate this include oncogenic stress, we conclude that the most likely explanation for the association we observe in RCC cells between p53 status and P2 expression is that the transcription from P2 that we have observed is a consequence of p53 activity.

To resolve the question of p53 function and P2 transcription in the 117 cell line, the only exception to the expected pattern in our study, we have analysed two additional clonal derivative lines which stably express either a dominant negative mutant of p53 (R175H) or wild-type p53 (described previously [9]). Whilst the level of P2 transcript in these cells expressing exogenous wild-type p53 from an integrated plasmid does not reach the threshold level determined for other cell lines, it does increase significantly. Thus it appears that in this cell line p53 activity is in some way suppressed for reasons that are unclear. We therefore examined the p53-depedance of MDM2 expression in these cells by RNAi and found that p53 activity does regulate MDM2 levels in 117 cells as Fig. 1C shows (in accordance with our previously published studies [9,12]). Thus in 9/9 p53 wild-type RCC cell lines analysed (and in 0/5 p53 mutant RCC lines), p53 activity promotes expression of MDM2. This strongly suggests that the association identified previously [4,5], which we have subsequently confirmed in patient samples [6] between p53 expression and MDM2 expression in RCC, is likely the result of p53-mediated promotion of MDM2 expression from the P2 promoter of MDM2 [22].

In p53 wild-type RCC lines we have always seen that RNAi-mediated reduction in MDM2 leads to an increase in p53 expression levels [9,12], an observation confirmed by others [10,11]). Thus the question arises whether inhibiting binding of p53 to MDM2 in RCC cells might rescue p53 from the negative regulatory effects of MDM2 and what the consequences of this would be.

We therefore used Nutlin-3, a small molecule inhibitor of p53-MDM2 interaction (which competitively binds to the p53 binding site on MDM2) [23] to examine this question. Figure 2 demonstrates that Nutlin-3 inhibits proliferation in all (5/5) of the p53 wild-type RCC cell lines tested in a dose-dependent manner. We have found that all of these RCC lines express MDMX (MDM4, not shown), another critical negative regulator of p53, and therefore the efficacy of Nutlin-3 suggests that there is insufficient MDMX to inhibit p53 when MDM2-p53 interaction is inhibited by Nutlin-3 in these lines at concentrations where Nutlin-3 would be expected to have little effect on MDMX-p53 interaction. We note that there is no effect of Nutlin-3, even at relatively high concentrations, on the p53 mutant