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Pharmacologic p53 Activation Blocks Cell Cycle Progression but Fails to Induce Senescence in Epithelial Cancer Cells

Baoying Huang, Dayanand Deo, Mingxuan Xia, and Lyubomir T. Vassilev

Discovery Oncology, Roche Research Center, Hoffmann-La Roche, Inc., Nutley, New Jersey

Abstract

Cellular senescence is a stress-induced state of irreversible growth arrest thought to act as a barrier to cancer development. The p53 tumor suppressor is a critical mediator of senescence and recent *in vivo* studies have suggested that p53-induced senescence may contribute to tumor clearance by the immune system. Recently developed MDM2 antagonists, the nutlins, are effective p53 activators and potent antitumor agents in cells with functional apoptotic pathways. However, they only block cell cycle progression in cancer cells with compromised p53 apoptotic signaling. We use nutlin-3a as a selective probe to study the role of p53 activation in senescence using a panel of eight epithelial cancer cell lines and primary epithelial cells. Our results reveal that the MDM2 antagonist can induce a senescence-like state in all tested cell lines, but it is reversible and cells resume proliferation upon drug removal and normalization of p53 control. Retinoblastoma family members (pRb, p107, and p130) previously implicated in gene silencing during fibroblasts senescence were found down-regulated in cells with nutlin-induced senescence-like phenotype, suggesting a mechanism for its reversibility. Therefore, selective p53 pathway activation is insufficient for induction of true senescence in epithelial cells *in vitro*. However, elevated expression of several inflammatory cytokines in cancer cells with nutlin-induced senescence-like phenotype suggests a possible *in vivo* benefit of p53-activating therapies. (Mol Cancer Res 2009;7(9):1497–509)

Introduction

Replicative senescence is an irreversible state of proliferative arrest in which mammalian cells enter after a defined number of divisions *in vitro* (1). Senescent cells remain metabolically active but acquire distinct changes in morphology and physiology including enlarged size, increased adhesion to substratum, and expression of senescence-associated β-Galactosidase (SA-β-Gal; ref. 2). In addition to replicative senescence, frequently associated with shortening of chromosome telomeres, another form of senescence, termed premature senescence, has been described as a consequence of treatment with genotoxic chemotherapeutics or other cellular stresses (3, 4). Senescence can be induced by multiple forms of stress including telomere erosion, oncogene activation, DNA damage, oxidative stress, etc., frequently acting in concert (5, 6).

It has been postulated that senescence plays an important tumor suppressor role *in vivo* by limiting the proliferative potential of normal cells (7, 8). Not surprisingly, the main tumor suppressor pathway, p53 pathway, has been found to play a critical role in induction of cellular senescence including premature senescence (5). It is thought that premature senescence could contribute to antitumor efficacy of currently used chemotheraputics (3). Recent studies have shown that restoration of p53 expression to mouse liver tumors *in vivo* induces senescence phenotype and tumor regression likely resulting from clearance of senescent cells by the immune system (9). These findings suggest that pharmacologic p53 activation may have beneficial effect even in cancer cells that have lost their ability to undergo p53-dependent apoptosis but have retained sensitivity to p53-dependent senescence. Therefore, elucidating the mechanisms of p53-induced senescence is of high importance to designing new therapeutic strategies. Despite the fact that the role of p53 in cellular senescence remains undisputed, molecular events leading to the execution of p53-dependent senescence are still poorly understood (6).

The master tumor suppressor p53 is a central node in a complex signal transduction pathway responding to multiple oncogenic stresses by cell cycle arrest and apoptosis (10). As such, p53 also serves as a converging point for multiple forms of senescence-inducing stress and cellular senescence has been thought to be one of the functions of activated p53 (11). It has been well documented that p53-null fibroblasts remain immortal when propagated *in vitro* and p53 is up-regulated in both replicative and premature senescence (5, 12). p53 activation seems to play a critical role not only in the induction but also in the maintenance of cellular senescence because p53 knockdown reverses the senescence phenotype of cultured mouse and human fibroblasts (13, 14). It has been shown that p53 activation is an essential step in the induction of senescence following DNA damage or other forms of stress (15). However, these stresses activate not only p53 but also other cellular pathways, thus complicating data interpretation and the role of nongenotoxic p53 activation in senescence has not been addressed at a molecular level.

Recently developed small-molecule p53-MDM2 binding inhibitors, the nutlins, offer a selective tool for activation of the p53 pathway without DNA damage or other stress-induced

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Note: B. Huang and D. Deo contributed equally to this work.
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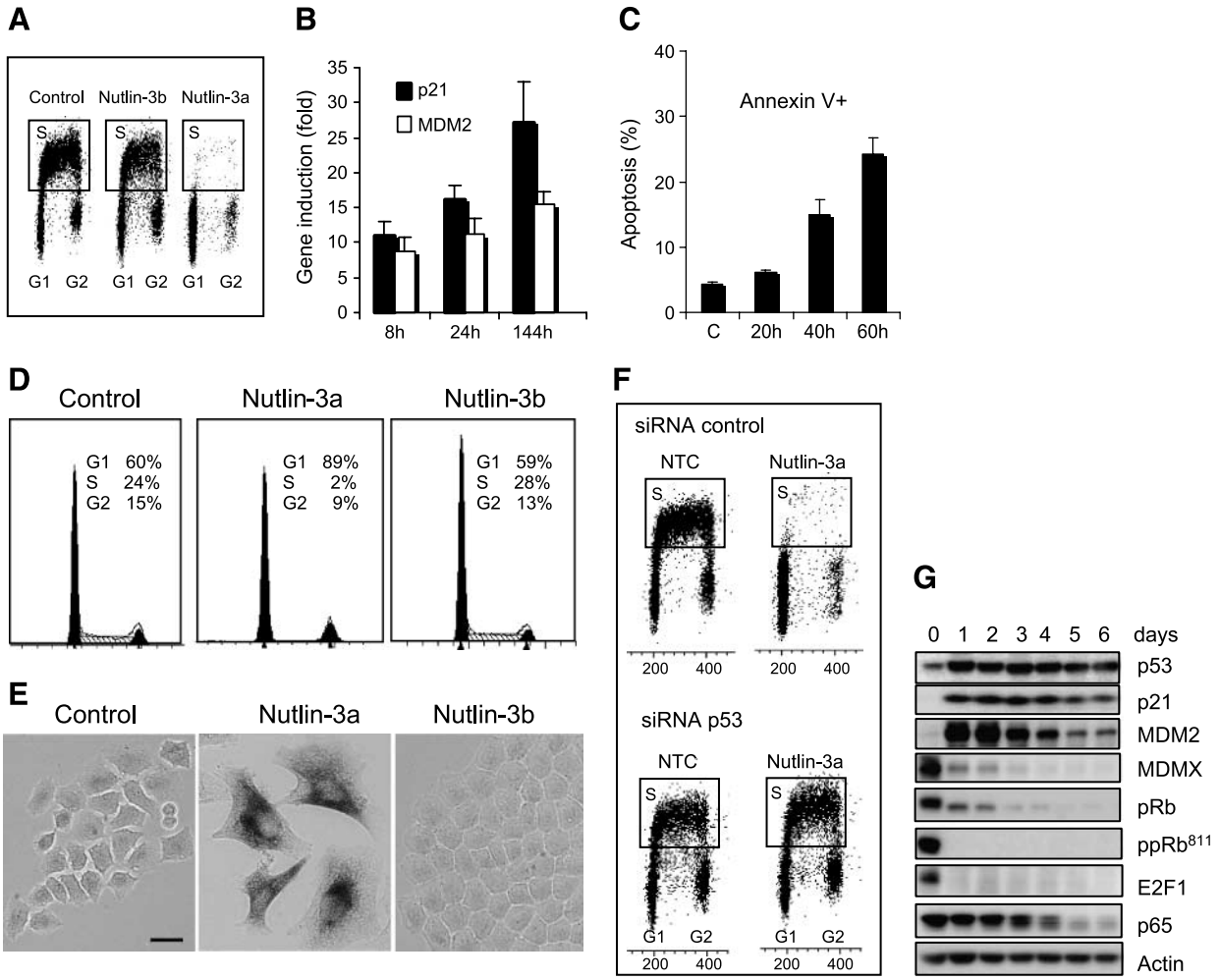

FIGURE 1. Nutlin-3a activates p53 signaling and induces senescence phenotype in apoptosis-resistant H460 cells. **A.** Nutlin-3a arrests H460 cell proliferation. Exponentially growing H460 cells were treated with 10 μmol/L nutlin-3a or nutlin-3b for 24 h. They were labeled with BrdUrd during the last 1 h and cell cycle distribution was analyzed. **B.** Prolonged nutlin treatment causes continuous p53 pathway activation. Messenger RNA levels of p53 target genes, p21 and MDM2, were determined by quantitative PCR and expressed as fold increase ± SD. **C.** Portion of the H460 cell population undergoes p53-dependent apoptosis. Apoptotic cell fractions were determined by measuring the percentage of Annexin V–positive cells following incubation with 10 μmol/L nutlin-3a for indicated time period. **D.** Nutlin-3a maintains the cell cycle block of apoptosis-resistant H460 cells. Cells were incubated with 10 μmol/L nutlin-3a or nutlin-3b for 6 d, and cell cycle profile was analyzed by flow cytometry. Control was treated with DMSO vehicle. **E.** Nutlin-arrested H460 cells acquire senescence phenotype and express SA-β-Gal. H460 cells were treated as in **D** and stained with SA-β-Gal (*scale bar,* 20 μm). **F.** Nutlin-induced cell cycle arrest is p53-dependent. H460 cells were transfected with control siRNA or p53-specific siRNA. Nutlin was added 24 h after transfection. BrdUrd analysis was done 24 h after nutlin treatment. **G.** Western blot analysis of cell lysates from H460 cells exposed to 10 μmol/L nutlin-3a from 1 to 6 d.

signaling events (16, 17). MDM2 is a master regulator of p53 activity and stability directly inhibiting its transcriptional activity and facilitating p53 ubiquitination and proteasomal degradation (18). By blocking p53-MDM2 interaction, nutlins release p53 from negative control, stabilize the tumor suppressor, and activate p53 downstream signaling. Thus, nutlins offer a specific tool to study the role of nongenotoxic p53 activation in senescence in diverse cellular context (19). Recent studies have shown that p53 activation by nutlin treatment can trigger senescence in fibroblasts of mouse and human origin *in vitro* (20, 21). However, fibroblasts are resistant to p53-dependent apoptosis and senescence is the most frequently observed outcome of p53 activation.

Here, we use the MDM2 antagonist nutlin-3a to investigate the role of selective p53 activation in senescence using multiple cancer cell lines of epithelial origin. We find that nutlin treatment leads to activation of p53 signaling and induction of senescence-like phenotype in all studied cell lines. However, this phenotype is readily reversible upon drug withdrawal and normalization of p53 levels, suggesting that p53 activation alone is insufficient for induction of true senescence in epithelial cells *in vitro*.

Results

Nutlin-3a Activates p53 Signaling and Induces Senescence-Like Phenotype in Cancer Cells

Nutlin-3 is a potent small-molecule inhibitor of the p53-MDM2 interaction with a well documented selectivity (22). It has been successfully used in multiple cell lines and showed

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invariable dependence on the presence of wild-type p53 for its activity, indicating that its effect is exerted through p53 stabilization and activation of the p53 pathway (16, 19). Incubation of the lung cancer cell line H460 with 10 μmol/L nutlin-3a, the active enantiomer of nutlin-3, for 24 hours led to an effective cell cycle arrest primarily in G_1 phase (Fig. 1A). The inactive enantiomer, nutlin-3b, a nearly 200-fold less potent MDM2 binder (16), did not show significant changes in cell cycle distribution compared with no treatment controls, indicating that p53 activation results from interference with MDM2 binding (Fig. 1A). Nutlin-3a induced the expression of p53 targets, $p21^{Waf1/Cip1}$ and MDM2 (Fig. 1B), confirming that cell cycle arrest is due to activation of the p53 pathway. As previously shown (19), during the first 20 hours of incubation, nutlin treatment caused an insignificant increase in the Annexin V–positive cell population compared with untreated controls (Fig. 1C). The apoptotic fraction slightly increased over the next 40 hours, reaching 24% after 60 hours of incubation. However, majority of the cells in the population were resistant to nutlin-induced apoptosis (Fig. 1C). Apoptosis-resistant adherent cells maintained elevated expression of p21 and MDM2 (Fig. 1B) and their cell cycle arrest (Fig. 1D) but markedly increased in size, acquired flat morphology, and showed intense SA-β-Gal staining typical for senescent cells (Fig. 1E). Cells exposed to the inactive enantiomer nutlin-3b did not show any difference from controls, indicating that the acquired changes are p53-dependent and result from the mechanism of action of MDM2 antagonists. Knockdown of p53 by specific siRNA that reduced p53 protein levels >85% compared with siRNA controls (data not shown) completely abolished nutlin-induced cell cycle arrest (Fig. 1F), confirming that it is driven by p53 activation. These results point out that continuous activation of the p53 signaling by nutlin induces a senescence-like phenotype in H460 cells that are resistant to p53dependent apoptosis.

Low-density quantitative PCR array analysis of mRNA from H460 cells exposed to nutlin-3a for 6 days detected 22 genes induced >3-fold compared with controls (Table 1). Majority of these genes were previously shown to be affected by activation of the p53 pathway (19). The list included genes implicated in p53-dependent cell cycle arrest (e.g., *CDKN1A*/p21, *GADD45A,* and *BTG2*), apoptosis (e.g., *BBC3, FAS*), or senescence (e.g., Serpine1/*PAI-1*). We also analyzed the changes in several key proteins over a 6-day period of exposure to 10 μmol/L nutlin-3a (Fig. 1G). Western blot analysis showed accumulation of p53 protein, induction of its transcriptional targets, p21 and MDM2 (19), and reduction of MDMX levels through MDM2-mediated degradation as previously reported (23). Retinoblastoma (Rb) protein (pRb) levels decreased gradually, whereas the levels of phospho-pRb^{811}, a preferred CDK2 phosphorylation site, were reduced even more dramatically due to the combined effect of total pRb decrease and inhibition of CDK2 activity by the up-regulated pan-CDK inhibitor, p21 (24). Levels of E2F1 also dropped within 24 hours. These protein changes are consistent with the effective cell cycle arrest imposed by activated p53. Western blotting did not detect expression of protein markers previously associated with senescence: p15 (25), p16 (26), and DcR2 (8) of the H460 cell lysates (data not shown). Despite the intense SA-β-Gal staining (Fig. 1E), we were unable to detect senescence-associated heterochromatin foci in nutlintreated H460 cells (data not shown).

The immediate downstream p53 target, p21, is one of the first genes up-regulated upon p53 activation and has been thought to be the main mediator of p53-dependent cell cycle arrest (11, 27). Therefore, we investigated the role of p21 in the induction and maintenance of the senescence-like state. H460 cells were incubated in the presence of 10 μmol/L nutlin-3a for 4 days until they acquired clear senescence morphology and stained intensely for SA-β-Gal (data not shown). Cells were then transfected with p21-specific and control siRNA and their cell cycle distribution and protein content were analyzed (Fig. 2). Transfections were done in the presence or absence of nutlin to control for a possible effect of lipofectin reagent on intracellular level of nutlin. Protein levels of p21 were substantially reduced compared with the siRNA control, 24 hours after siRNA transfection (Fig. 2A). This led to a partial restoration of cell proliferation in the presence of nutlin, suggesting that p21 up-regulation by nutlin is necessary for maintenance of the senescence-like proliferation arrest (Fig. 2B). There was no difference between transfection in the presence and absence of nutlin during transfection (data not shown).

Activation of the p53 Pathway by Nutlin Induces Senescence-Like State in Multiple Epithelial Cancer Cell Lines

To extend H460 observations to a wider range of tumor cells, we tested the effect of nutlin on a panel of 11 randomly selected solid tumor cell lines of epithelial origin expressing wild-type p53. In addition to H460, these included a second lung cancer line (A459), two colon cancer lines (HCT116 and RKO), two prostate (LNCaP and 22Rv1), two osteosarcoma cell lines with amplified *mdm2* gene (SJSA1 and MHM), one breast (MCF7), one melanoma (LOX), and one renal cancer line (A498). Six days of continuous exposure of

Table 1. Genes Up-Regulated More than 2-Fold after 6 d of Incubation with 10 μmol/L Nutlin-3a Were Determined by TaqMan Low-Density Arrays

Gene	Fold Induction
ACTA2	10.1
BBC3	3.7
BTG2	47.3
p21	54.3
FAS	5.8
FDXR	16.9
FOSB	4.3
GADD45A	8.2
GDF15	39.2
GPR51	3.1
IGFBP3	19.2
LRDD	3.1
MDM2	24.7
NINJ1	3.3
PCNXL2	3.2
PPM1D	3.1
SERPINE1	18.2
TNFRSF10B	6.4
TP53I3	25.0
TP53INP1	22.6
TRAF1	3.1
WIG1	19.2

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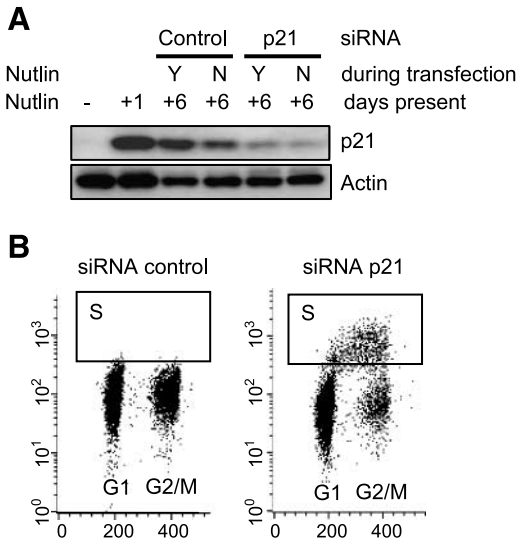

FIGURE 2. p21 up-regulation by p53 is required for induction and maintaining of cell cycle arrest and senescence phenotype. H460 cells were treated with 10 μmol/L nutlin-3a for 4 d, then transfected with p21-specific or nontargeted control siRNA in the presence or absence of nutlin-3a. Nutlin was readded to the cells 24 h after transfection and subjected to Western blotting (**A**) or BrdUrd cell cycle analysis (**B**).

exponentially growing cells to nutlin-3a resulted in variable degrees of apoptosis as measured by the Annexin V assay (Fig. 3). The two osteosarcoma cell lines overexpressing MDM2 (SJSA1 and MHM) and the androgen-dependent prostate cancer line LNCaP were most sensitive to p53-dependent apoptosis and practically all cells lost their viability after 6 days of nutlin treatment (data not shown). The remaining eight cell lines showed either moderate or low apoptosis and substantial fractions of cell populations were viable and adhered to the plate. These lines were selected for investigation of nutlin-induced p53-dependent senescence.

Cells were exposed to 10 μmol/L nutlin-3a for 6 days and analyzed for cell cycle distribution and senescence phenotype (Fig. 4). After 6 days of nutlin treatment, apoptosis-resistant cells from all lines maintained effective cell cycle arrest in G_1 and G_2 phase (Fig. 4A). Although G_1 peak was predominant, an increase of G_2 phase cells was seen in all cell lines, most prominent in RKO, HCT116, and A498 cells. The S-phase fraction in all cell lines was below 1%, indicating effective cell cycle block. As shown previously, nutlin-induced cell cycle arrest is p53-dependent and does not vary among cell lines in contrast to p53-dependent apoptosis, suggesting that the mechanism of cell cycle arrest is preserved in cancer cells with wild-type p53 (19). Nutlin-arrested cancer cells showed a clear senescence-like phenotype: enlarged size, flat morphology, and SA-β-Gal staining (Fig. 4B). Cells treated with the inactive nutlin enantiomer 3b under identical conditions did not show proliferation arrest or any notable difference from the exponentially growing controls (data not shown).

We then investigated the time and concentration dependence of the appearance of this senescence-like phenotype. Three representative cell lines, H460, A549, and LOX, in exponential growth were exposed to 10 μmol/L nutlin-3a for 6 days and SA-β-Gal–stained cells was recorded every day (Fig. 5A). During the first 2 days, fewer than 10% of the cell population showed blue staining with fairly low intensity. However, the number of stained cells increased dramatically to 70% to 80% on day 3 coinciding with notable changes in morphology, indicating that the appearance of senescence-like phenotype requires at least 48 hours of cell cycle arrest. Cell fractions with senescent-like phenotype increased further during the next 2 days reaching a plateau at day 5 when over 97% of the cells in all three lines stained blue. Nutlin concentrations as low as 2.5 μmol/L induced blue staining in over 80% of the cells after 6 days of treatment and the effect of 5 μmol/L was equivalent to 10 μmol/L (Fig. 5B). Despite the fact that lower nutlin concentrations may be equally effective, we chose 10 μmol/L nutlin-3a as a standard dose in all further studies to minimize possible cell-to-cell variability.

Nutlin-Induced Senescence-Like Cell Cycle Arrest Is Reversible

One of the hallmarks of senescence is the inability of cells to re-enter the cell cycle despite presence of all necessary growth factors and nutrients in their media. Therefore, we investigated the reversibility of nutlin-induced senescence-like phenotype. The panel of eight epithelial cancer cell lines was exposed to 10 μmol/L nutlin-3a for 6 days and their ability to proliferate was assessed after several days of growth in complete nutlin-free media and compared with controls. Two different experimental approaches used were colony formation and total cell counting. Colony formation assay can estimate more accurately the fate of each cell in the population but the results can be influenced by their clonogenic growth potential and adherence to the plate. Six days after nutlin removal, six of the cell lines in our panel partially recovered their ability to form colonies but to a variable degree. This variability is likely due to the fact that

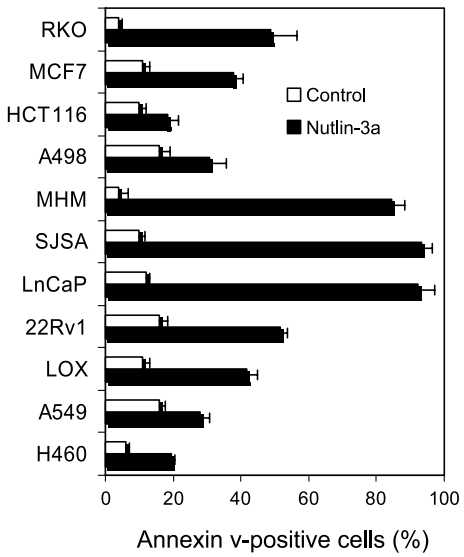

FIGURE 3. Cancer cells vary in sensitivity to p53-dependent apoptosis. A panel of 11 randomly selected cancer cell lines expressing wild-type p53 were treated with 10 μmol/L Nutlin-3a for 6 d, and the percentage of Annexin V–positive cells including 7ADD-positive and 7ADD-negative cells (±SD) was determined using the Guava Nexin kit.

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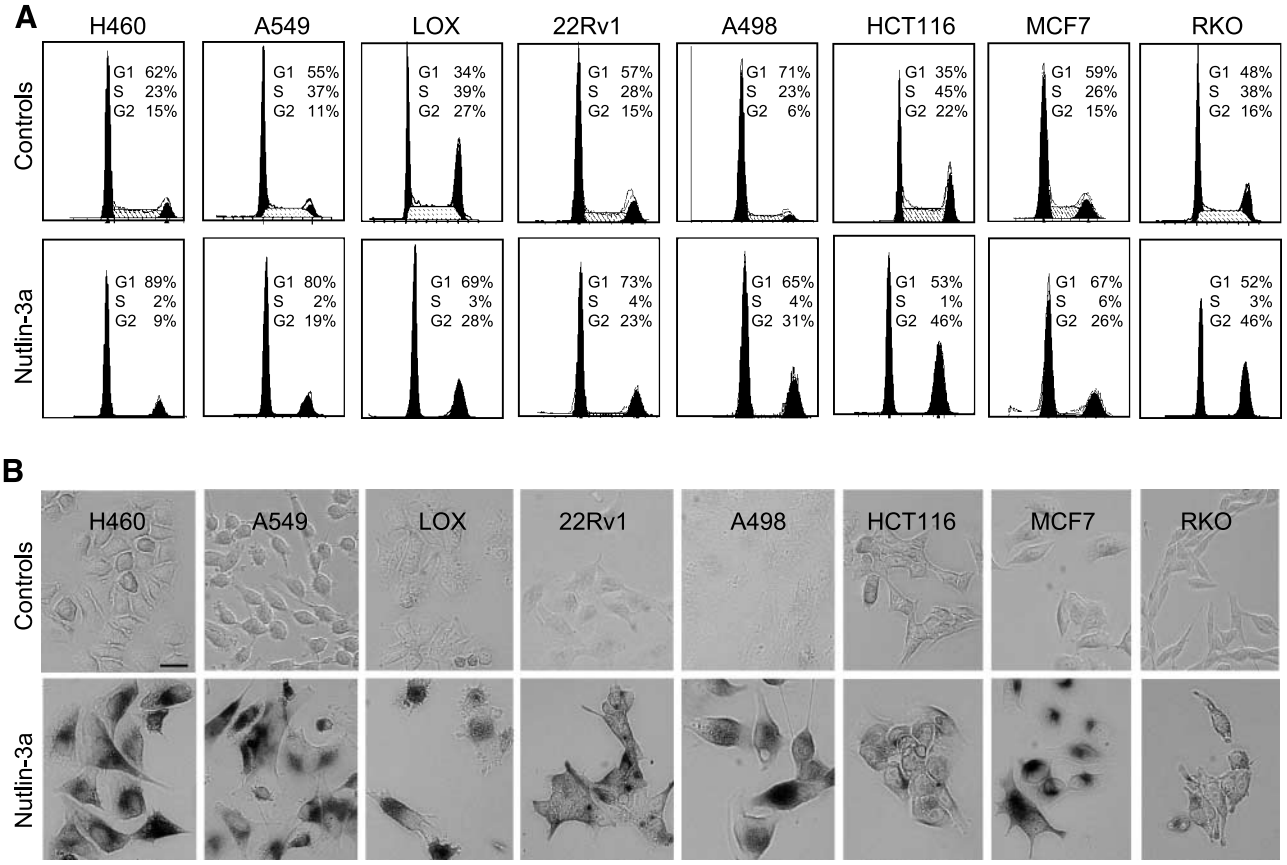

FIGURE 4. Cancer cells resistant to nutlin-induced apoptosis acquire senescence phenotype upon continuous exposure to nutlin-3a. Eight apoptosis-resistant cancer cell lines (Fig. 3) were incubated with 10 μmol/L nutlin-3a for 6 d and the surviving adherent cells were analyzed for cell cycle distribution (**A**) and SA-β-Gal staining (**B**). Cells treated with DMSO vehicle were used as controls (*scale bar,* 20 μm).

a variable fraction of the colony-forming cells were killed by nutlin-induced apoptosis (see Fig. 2) and possible cell loss during drug wash. Low colony recovery in LOX and 22Rv1 is a probable consequence of their relatively low adherence to the plate as individual colony forming cells. The remaining two cell lines, RKO and MCF7, showed no visible colonies suggesting an irreversible block of proliferation (Fig. 6A and B). When proliferative potential was measured by total cell count, 3 days after nutlin removal, the six cell lines that partially recovered their clonogenic growth (H460, A549, LOX, 22Rv1, A498, and HCT116) showed cell number increase comparable with exponentially growing control cells for the same period of time. Again, RKO and MCF7 cells did not re-enter proliferation (Fig. 6C). Bromodeoxyuridine (BrdUrd) labeling of H460, A549, and LOX cells 96 hours after nutlin removal confirmed that majority of the cells in the population have entered S phase (Fig. 6D). Further examination of individual H460 cells with nutlin-induced senescence-like phenotype (10 μmol/L nutlin-3a for 6 days) by time-lapse photography for 6 days after nutlin removal confirmed that practically all cells in the population re-enter the cell cycle (data not shown). These experiments indicated that some cancer cell lines can reverse their senescence-like phenotype and resume proliferation while others maintain continuous cell cycle arrest in the absence of nutlin.

To examine the underlying mechanism of these differences, we analyzed p53 protein levels in RKO and MCF7 cells, the two cell lines that retain senescence-like phenotype, and H460 cells showing a quick recovery after nutlin removal. Western analysis revealed that in H460 cells p53 dropped to the levels seen in untreated controls within 24 hours of nutlin removal. In MCF7 cells, p53 remained as high as in the presence of nutlin up to 3 days after its removal (Fig. 7A). Similarly, high p53 levels were seen in RKO cells after nutlin removal (data not shown). Elevated p53 protein levels that can continuously activate the cell cycle arrest function of the tumor suppressor explain why RKO and MCF7 cells do not resume proliferation within the 3-day recovery period. However, when the recovery period was extended to 10 days, both cell lines restored their proliferative capacity by day 10 (Fig. 7B). Western analysis showed a drop in p53 protein levels in RKO cells between day 4 and 7 (Fig. 7C). On day 7, p53, p21, and MDM2 proteins were at pretreatment levels, indicating that the p53 pathway has returned to its normal uninduced state. Similar results were obtained with MCF7 cells (data not shown).

One cannot exclude the possibility that prolonged exposure to nutlin may lead to selection of resistant cell clones that constitute the bulk of the population with recovered proliferation capacity. To address this possibility, we retreated RKO cells after 6 days of nutlin treatment and 7 days of recovery in the

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absence of nutlin. The second cycle of nutlin treatment and recovery showed that the cells have not lost their ability to activate p53 signaling and they acquired senescence-like phenotype undistinguishable from the first cycle (data not shown; Fig. 7D). Currently, we do not know the mechanism of delayed p53 degradation and cell cycle recovery in RKO and MCF7 cells. However, these experiments clearly show that activation of the p53 pathway by nutlin leads to acquisition of senescence-like phenotype but not irreversible senescence in all eight tested cancer cell lines.

We then extended the period of nutlin treatment from 6 to 14 days and reexamined the reversibility of senescence-like phenotype in H460 and HCT116 cell. Nutlin-3a (10 μmol/L) maintained complete cell cycle arrest over the 2-week treatment period and induced a senescence-like state in both cell lines but as with the 6-day nutlin treatment cells reentered the cycle shortly after nutlin removal (data not shown). These experiments suggested that the reversibility of nutlin-induced senescence-like phenotype is not due to the length of continuous p53 activation. Taken together, our results indicate that selective p53 pathway activation is insufficient for induction of true senescence in epithelial cancer cells.

Is Rb Down-Regulation by Nutlin Responsible for Reversibility of the Senescence-Like Cell Cycle Arrest?

Studies with human fibroblasts have suggested that in addition to the p53 pathway, Rb pathway activation may be essential for induction of the irreversible changes that accompany replicative senescence (28). In fact, the pRb has been shown to play a direct role in silencing of E2F target genes required for permanent exit from the cell cycle (29). Our results showed that activation of the p53 pathway by nutlin-3a leads to a dramatic down-regulation of pRb (data not shown; Fig. 1G). This drop of pRb follows the accumulation of p53 target p21, recently shown to facilitate proteasomal degradation of pRb (30).[1] p21 up-regulation also changes the phosphorylation status of pRb by inhibiting CDK2/CDK4 activity (24). If pRb is essential for gene silencing accompanying the transition from temporary cell cycle arrest to permanent exit from cycling, then its down-regulation may be responsible for reversibility of nutlin-induced senescence-like phenotype.

To test this hypothesis, we examined pRb status during induction of premature senescence by the genotoxic drug doxorubicin. Premature senescence has been defined as irreversible senescence triggered by chemotherapeutic agents (3). Although its mechanism is complex and poorly understood, it is believed that activation of p53 signaling is a critical contributor (4). It has been reported recently that MCF7 cells can be induced to premature senescence by a relatively short period of exposure to high doses of the genotoxic drug doxorubicin (31). We used this model system to examine the importance of pRb in drug-induced senescence. Exponentially growing MCF7 cells were incubated with 1 μmol/L doxorubicin for 2 hours and their cell cycle distribution and pRb status were followed. As previously reported (31), this treatment led to cell cycle arrest, acquisition of typical senescence morphology, and SA-β-Gal staining that was maintained over the 1 week observation period (data not shown). BrdUrd labeling indicated an effective cell cycle arrest (Fig. 8A) and Western analysis showed that doxorubicin treatment caused p53 accumulation and up-regulation of p53 target genes (e.g., p21; Fig. 8B). Similar to nutlin treatment in H460 cells (Fig. 1G), pRb levels were gradually reduced by doxorubicin treatment in MCF7 cells. Rb family member, p107, increased initially but then dropped to its original level, whereas another pRb analogue, p130, gradually increased its cellular levels (Fig. 8B). These results are consistent with results from earlier studies on the role of Rb family members in doxorubicin-induced senescence phenotype (31). It has been suggested that p130 up-regulation may substitute for pRb in induction of senescence, although the mechanism responsible for these changes remains unclear (31).

Then, we examined cellular levels of pRb, p107, and p130 in two of the cancer cell lines from our panel, H460 and MCF7, during acquisition of nutlin-induced senescence-like phenotype (Fig. 8C and D). In both cell lines, nutlin treatment led to a gradual decrease in total pRb. Due to inhibition of CDK activity by elevated p21, the levels of phosphopRb (Ser^{795} and Ser^{811}) preferentially phosphorylated by CDK4 and CDK2, respectively, dropped even faster. However, in contrast to doxorubicin treatment, both p107 and p130 decreased their cellular levels dramatically by day 4. Thus, all three Rb family members were down-regulated in nutlin-treated cells. If protein levels of Rb family members are important for gene silencing

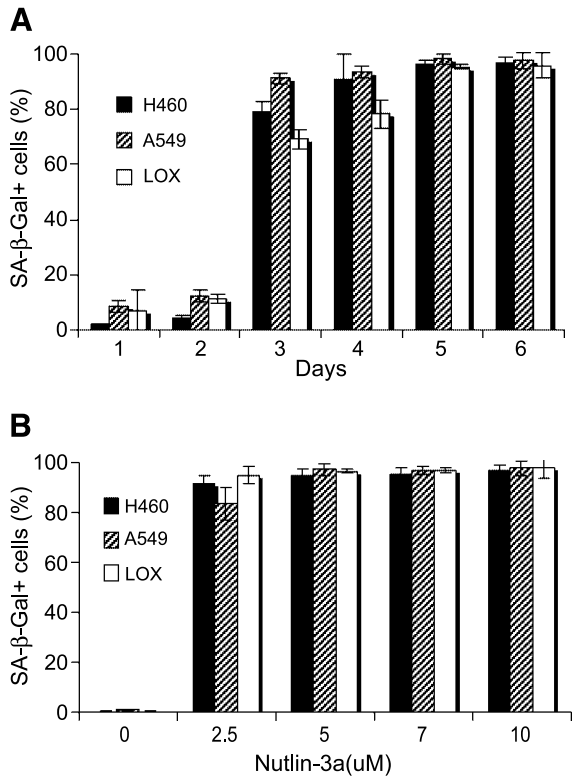

FIGURE 5. Time and concentration dependence of nutlin-induced senescence phenotype. H460, A549, and LOX cells were treated with 10 μmol/L Nutlin-3a for indicated times (**A**) or different nutlin concentration for 6 d (**B**). Percentage of SA-β-Gal stained cells (±SD) was determined by cell counting under the microscope.

[1] Our unpublished results.

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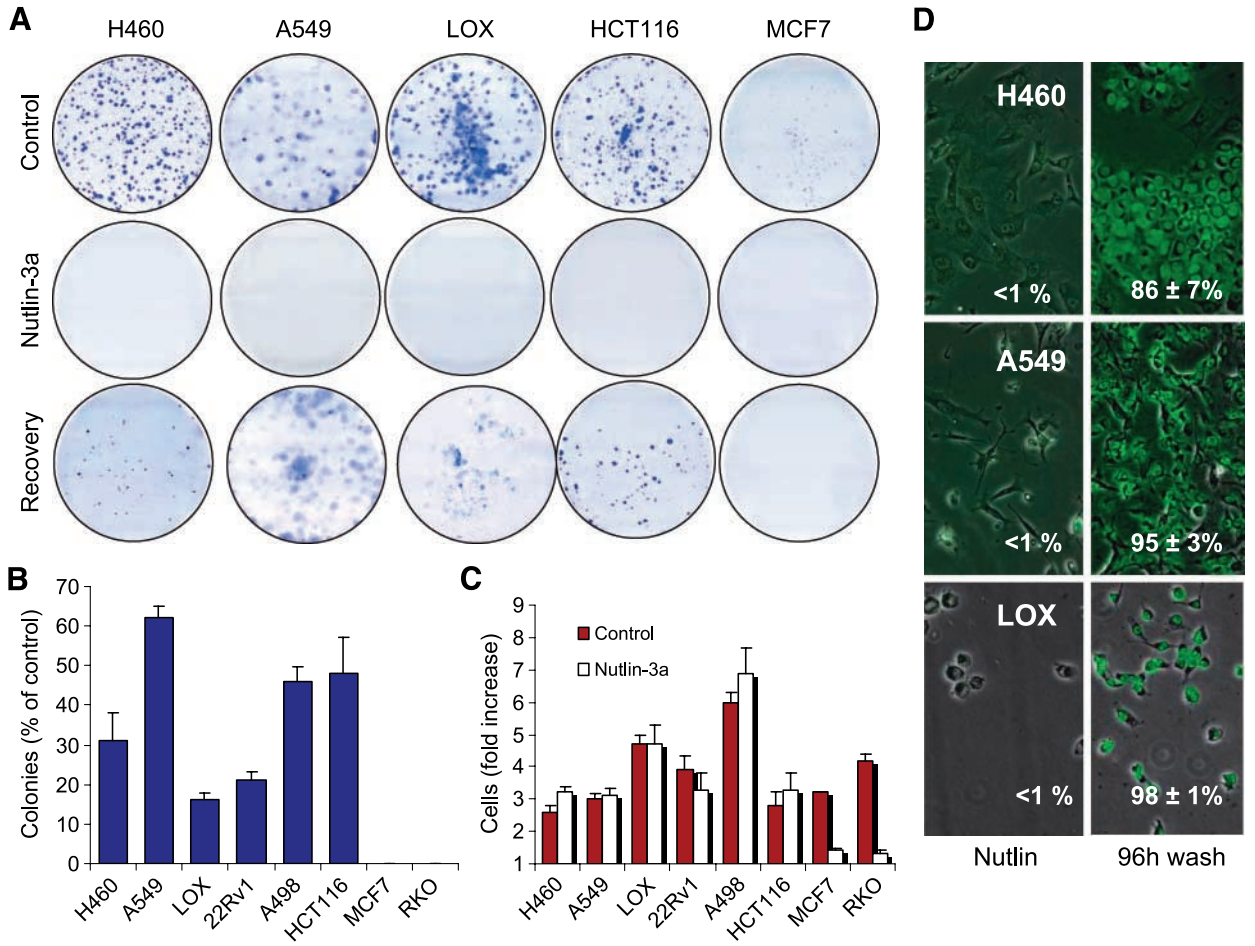

FIGURE 6. Nutlin-induced senescence phenotype is reversible. **A.** Colonogenic growth of cancer cells is effectively suppressed by nutlin-3a but cells can restore their proliferation in the absence of the drug. Cells were seeded and allowed to form colonies during 6 d in the absence (*top*) or presence of 10 μmol/L nutlin-3a (*bottom*). For assessment of recovery, cells were incubated with fresh media for 6 d after nutlin-3a treatment, fixed, and stained with crystal violet (*bottom*). **B.** colonies formed 6 d after release from nutlin-3a block (**A**, *bottom*) were counted and calculated as percentage of untreated control (*top*). **C.** Cancer cells resume proliferation after release from nutlin. Cells were incubated with 10 μmol/L nutlin-3a for 6 d, washed, and incubated in fresh media for another 3 d before counting. Their growth rate was compared with the growth of control untreated cells. **D.** BrdUrd incorporation in cells released from nutlin block. Cells were treated with 10 μmol/L nutlin-3a for 6 d (*left*), washed, and incubated in drug-free media for another 96 h. BrdUrd (20 μmol/L) was added during the last 24 h and its incorporation was detected by FITC-conjugated anti-BrdUrd antibody. Percentage (±SD) of cells incorporated BrdUrd cells was counted and recorded.

during premature senescence in epithelial cells, their reduction during p53 activation by nutlin might explain the inability of cancer cells to become senescent.

Is p53-Dependent Senescence Pathway Defective in Cancer Cells?

Our previous studies on the functionality of the p53 pathway suggested that although p53-dependent cell cycle arrest is preserved in all cancer cells, p53-dependent apoptosis mechanisms may be defective in many cancer cell lines of epithelial origin (19). This leads to a high variability in sensitivity of these cells to nutlin-induced apoptosis. One can assume that p53-dependent senescence mechanisms may also be altered during cancer development in cells expressing wild-type p53. If this is correct, then normal epithelial cells should have intact p53-dependent senescence mechanisms. To test this hypothesis, we investigated the ability of nutlin-3a to induce senescence and its reversibility in primary human epithelial cells.

Two types of primary cells isolated from human prostate epithelium (PrEC) and mammary gland epithelium (HMEC) in log phase were treated with nutlin-3a for 6 days and their phenotype and cell cycle distribution were analyzed (Fig. 9). In agreement with the results with cancer cells (Fig. 1) and previously published data (19), nutlin induced effective cell cycle arrest in G_1 and G_2 phase. Cells acquired senescent-like phenotype and stained for SA-β-Gal (Fig. 9A). Western analysis confirmed the activation of p53 signaling and reduction of pRb, $ppRb^{811}$, and E2F1 (Fig. 9B) similar to its effect in nutlin-treated cancer cells (Fig. 1G). The p53 pathway continued to be activated 4 days after nutlin removal in PrEC and 2 days in HMEC cells. Cell numbers in senescent-like cell populations increased slowly after nutlin removal. However, analysis of their growth curves was consistent with a model in which the bulk of the cell population resumed proliferation with longer population doubling times rather than emergence of a small cell population with normal population doubling

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(Fig. 9C). These results suggest that nutlin-induced senescence-like phenotype and growth arrest in PrEC and HMEC cells are also reversible. Therefore, it is unlikely that reversibility of senescence-like phenotype in cancer cells is due to defective mechanisms of p53-dependent senescence.

Nutlin Induces Inflammatory Cytokines in Cancer Cell With Senescence-Like Phenotype

It has been shown recently that restoration of p53 activity in p53-defficient mouse liver tumors leads to tumor regression *in vivo* (9). Experimental data have suggested that tumor shrinkage in this mouse model is not due to apoptotic response to p53 reactivation but rather to induction of cellular senescence program accompanied by up-regulation of inflammatory cytokines. These observations have led to the hypothesis that innate immune response may play a major role in the clearance of senescent tumor cells (9). We investigated if selective nongenotoxic p53 activation by nutlin can induce inflammatory cytokine expression in the cells with senescence-like phenotype. To this aim, three cancer cell lines (HCT116, RKO, and MCF7) and two primary epithelial cultures (PrEC and HMEC) were incubated with nutlin for 6 days and mRNA level of five cytokines previously found to be affected by p53 activation (CSF1, CCL2, CXCL1, ICAM1, and IL15; ref. 9) were measured by quantitative PCR. Nutlin treatment led to an increased transcription of CSF1, CXCL1, ICAM1, and IL15 in RKO and MCF7 cells and CCL2 in HCT116 cells (Fig. 10). Primary epithelial cells showed only marginal increase in CSF1 and ICAM1 mRNA levels and decreased expression of CCL2, CXCL1, and IL15. Overall, as a result of p53 activation, senescent-like cancer cells appeared to have increased expression of the tested cytokines compared with the primary cells where the effect was a marginal induction (CSF1 and ICAM1) or inhibition (CCL2, CXCL1, and IL15). The relevance of these observations to *in vivo* cytokine expression and secretion and its effect on tumor growth needs to be determined in appropriate animal models. However, by analogy with the published results (9), one can speculate that elevated expression of cytokines may aid in the antitumor activity of p53-activating drugs despite their inability to induce irreversible senescence in cancer cells.

Discussion

The critical role of p53 pathway in induction of cellular senescence has been well documented (5). However, the molecular events leading from p53 activation to senescence phenotype and true senescence are still poorly understood (6). Overwhelming majority of senescence studies have been done with cultured mouse or human fibroblasts as a model system. These experiments have suggested that p53 activity is necessary not only for establishing but also for maintenance of senescence (6). Much less is known about the mechanism of p53-dependent senescence in cancer cells of epithelial origin. Studies with epithelial cells have used cancer chemotherapeutics known to induce senescence-like state termed premature senescence (5). It has been well established that premature senescence involves activation of the p53 pathway primarily via the genotoxic stress mechanism (3). However, DNA damage is known to activate not only p53 signaling but also other cellular pathways making data interpretation challenging. Here, we investigate the role of selective nongenotoxic p53 activation by the MDM2 antagonist nutlin-3a in cellular senescence using a panel of solid tumor–derived cancer cells.

We and others have shown that nutlin-3a, the active enantiomer of nutlin-3, is highly selective for its target, MDM2, in the cellular context. Nutlin treatment disrupts the p53-MDM2 interaction, leading to p53 stabilization and accumulation only in cells expressing wild-type p53 protein (16). Despite the lack of phosphorylation on key NH_2-terminal serine residues, nutlin effectively activates p53-dependent cell cycle arrest and apoptosis (32). Therefore, nutlin represents a unique tool for studying p53 function and its aberrations in cancer. Previous studies have showed that nutlin can induce senescence in transformed mouse fibroblasts (20). The aim of this study was to investigate the ability of selective p53 activation to induce senescence in

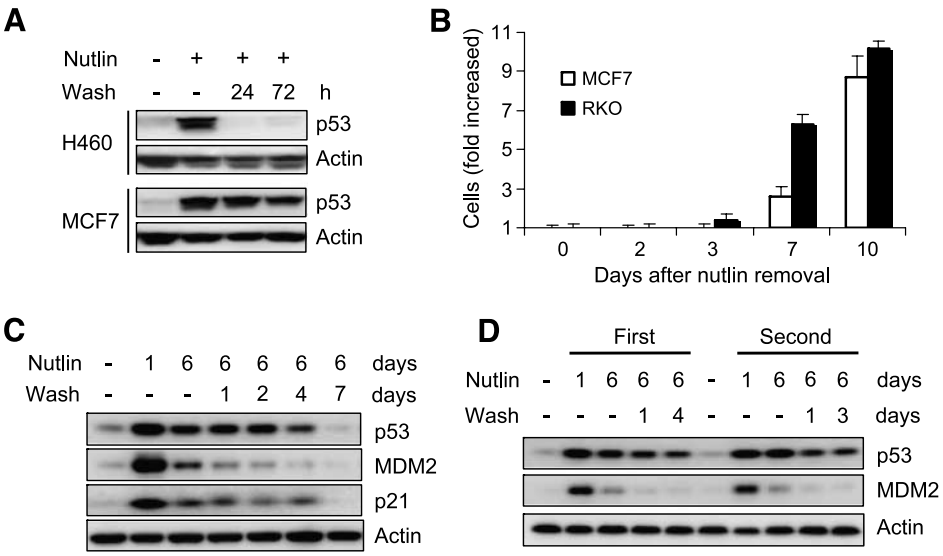

FIGURE 7. Delayed recovery of MCF-7 and RKO cells from nutlin-induced proliferative block. **A.** Changes in p53 cellular levels after release from nutlin block. H460 and MCF7 cells were treated as in Fig. 6C and collected at indicated times after drug washout for Western analysis. **B.** Delayed recovery of MCF-7 and RKO cells. Cells were treated as in **A** and counted at indicated time after removal of nutlin-3a. **C.** Western blot of RKO cell lysates after 6 d of incubation with 10 μmol/L nutlin-3a. **D.** RKO cells do not acquire resistance to nutlin. Cells were treated with 10 μmol/L nutlin-3a for 6 d, and grew in drug-free media for 15 d before a second round of 6-d nutlin treatment. Cell lysates before and after treatment were collected for Western blot.

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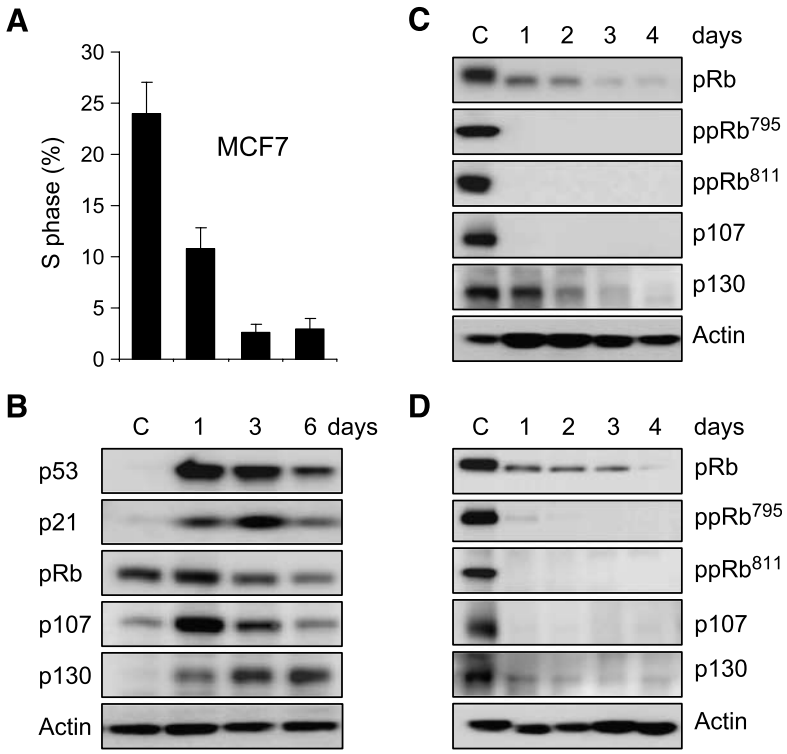

FIGURE 8. Changes in the cellular levels of Rb family members during premature senescence. MCF-7 cells were treated with 1 μmol/L doxorubicin for 2 h followed by drug wash and incubation in drug-free media (**A**) and (**B**). **A.** Cells were labeled with BrdUrd at indicated times and the percentage of BrdUrd-positive (S phase) cells was determined by cell cycle analysis. **B.** Western blotting of key proteins from **A**. H460 (**C**) and MCF7 (**D**) cells were incubated with 10 μmol/L nutlin-3a for indicated times and pRb and related proteins were analyzed by Western blotting.

cancer cells of epithelial origin. To this end, we used a randomly selected panel of 11 human cancer cell lines representing diverse tumor types. Of these, eight lines showed resistance to nutlin-induced apoptosis and majority of the cells in the population retained viability in the presence of high nutlin concentrations. Therefore, these cell lines were used as a model to study the effect of p53 activation on senescence.

Nutlin treatment activates p53 signaling as revealed by the up-regulation of its transcriptional targets p21 and MDM2 (Fig. 1B) and other genes in the p53 pathway including PAI-1(SERPINE1) recently reported as a mediator of p53-induced fibroblast senescence (Table 1; ref. 33). Activated p53-induced cell cycle arrest and apoptosis in cancer cells in which p53-depenedent apoptotic mechanisms are preserved (Figs. 1C and 3; ref. 19). Apoptosis-resistant cell populations were arrested in G_1 and G_2 phase and, within 3 days, gradually acquired a typical senescence phenotype manifested by enlarged size, flat appearance, and SA-β-Gal staining (Figs. 1E and 4B). This senescence-like phenotype was induced in all eight apoptosis-resistant cell lines (Fig. 4), suggesting that they have undergone p53-dependent senescence. However, although this phenotype has been associated with senescence, neither the large cell size or the SA-β-Gal staining are considered true markers of irreversible senescence but rather indicators of prolonged cell cycle arrest (34). Indeed, our experiments indicated that the senescence-like phenotype can be completely reversed as cells restore their proliferation in the absence of nutlin. All cancer cell lines exited the cell cycle and showed visual signs of senescence after 6 days of exposure to nutlin but they restored their proliferative capacity after removal of the drug (Fig. 6). This was accompanied by a drop in cellular p53 to its pretreatment levels (Fig. 7C and D) and a gradual decrease in cell size and SA-β-Gal staining (data not shown).

Due to complete reversibility of nutlin binding, MDM2 restores its p53 binding shortly after nutlin removal, leading to degradation of p53 and deactivation of the p53 pathway (16). In fact, p53 "normalization" was seen within 3 to 8 hours after nutlin removal depending on the cell type.[2] For unknown reasons, this normalization of p53 signaling occurred after a 2- to 4-day delay in RKO and MCF7 cells but eventually both cancer cell lines restored their proliferative capacity. These results suggest that p53 activity is required not only for induction but also maintenance of the senescent-like state in human cancer cells. Therefore, selective p53 activation is insufficient for driving the irreversible changes in gene expression associated with permanent loss of proliferative capacity in epithelial cancer cells. Similar reversibility of senescence has been observed previously upon knockdown of p53 in senescent human fibroblast (13, 14). Only fibroblasts with elevated expression of p16 can maintain their senescent state in absence of continuous p53 activation, suggesting involvement of the Rb pathway (13).

Activation of the Rb pathway has been implicated in senescence and there is experimental evidence that pRb may play a direct role in gene silencing during induction of senescence (29, 35). Because pRb levels are dramatically reduced in nutlin-treated cells (Fig. 1G), one can speculate that despite p53 activation, gene silencing has been hampered and no permanent changes have taken place during the prolonged drug exposure. Similar reduction of pRb has been observed in MCF7 cells

[2] Unpublished data.

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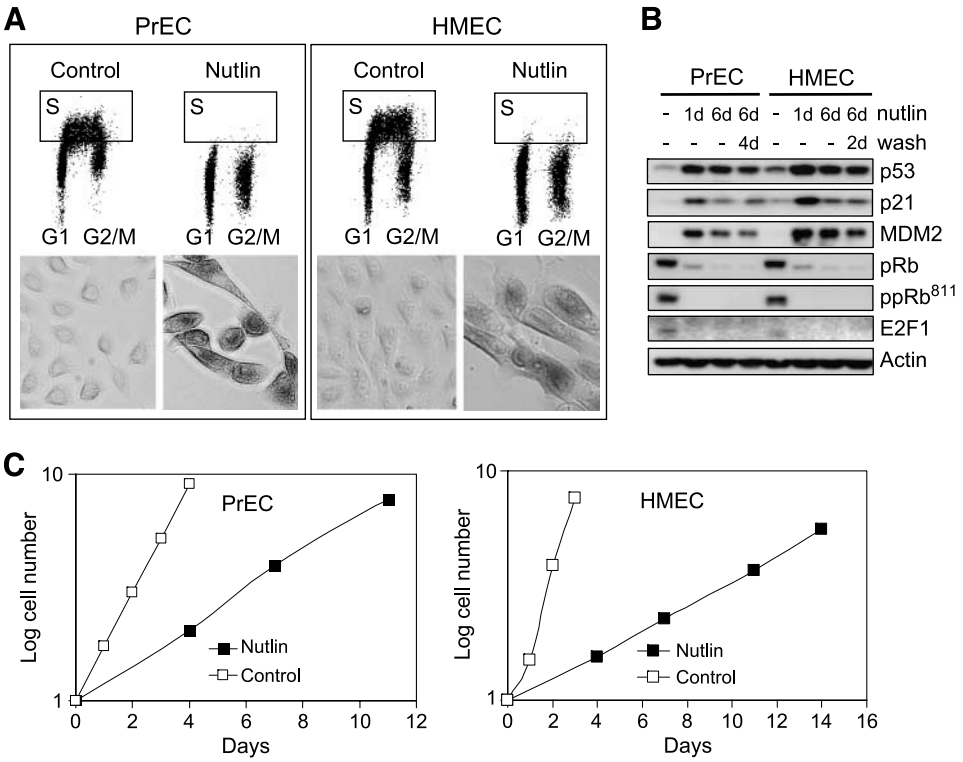

FIGURE 9. Nutlin-3a–induced senescence phenotype in primary epithelial cells is reversible. **A.** PrEC and HMEC cells were treated with 10 μmol/L nutlin-3a for 6 d and analyzed for BrdUrd incorporation and SA-β-Gal staining. **B.** Nutlin-3a activates p53 signaling in normal epithelial cells as revealed by Western analysis. **C.** Cells resume growth after nutlin removal. Early-passage PrEC and HMEC cells were incubated with 10 μmol/L nutlin-3a for 6 d and their growth rate was determined by cell counting at indicated times after drug removal and compared with untreated control cells.

forced to undergo premature senescence by treatment with high doses of doxorubicin (31). However, the Rb family member p130 was found up-regulated in MCF7 cells in response to doxorubicin treatment (Fig. 8B; ref. 31). Furthermore, p130 has been found recruited to the promoters of cyclin A, cyclin B, CDK1, and proliferating cell nuclear antigen in doxorubicin-induced senescent MCF7 cells, suggesting that it plays a role in the changes accompanying genotoxin-induced premature senescence (31). In contrast, in MCF7 and H460 cells in which nutlin treatment induced a clear senescence-like phenotype, all Rb family members were found down-regulated (Fig. 8C and D). Although there is no published evidence that Rb and related proteins are responsible for silencing S-phase genes in epithelial cells, it is reasonable to speculate that Rb down-regulation may contribute to reversibility of senescence-like phenotype induced by nongenotoxic p53 activation.

Defective p53-dependent senescence function in wild-type p53-expressing cancer cells is another possible cause of senescence reversibility. If this is the case, then normal cells should have intact p53-dependent senescence mechanisms. To address this possibility, we tested nutlin effect on two primary cell cultures derived from normal human epithelium, PrEC and HMEC. Similar to the cancer cell panel, primary cells arrested their cell cycle and acquired senescence-like phenotype that was reversed following nutlin removal (Fig. 9). Although cell growth restoration was slower in the primary cell cultures than most of cancer cell lines in the panel, its kinetics suggested recovery of majority of the cells in the population albeit with substantial lengthening of their cycle time. The reasons for extended population doubling time are not clear but factors such as higher sensitivity to stress and elevated concentration of free nutlin may have contributed to this effect. PrEC and HMEC cells grow in media supplemented with 1% serum compared with 10% serum in cancer cell media that effectively increases free concentration of nutlin-3a that is >95% protein bound.[3]

A study published during the course of this work has examined the ability of nutlin-3 to induce senescence in normal and transformed human fibroblasts (21). Similar to previously published study using mouse fibroblasts (20), authors find that p53 activation by nutlin-3 is an effective means of inducing senescence. These results differ from our observations in epithelial cells in which p53 activation by the same concentration of nutlin-3a is insufficient to induce true senescence. Although nutlin induces a clear senescence-like phenotype and intense SA-β-Gal staining in both fibroblast and epithelial cells, the senescence-like state is easily reversible in epithelial cells.

Another recent study has reported senescence-like phenotype in human epithelial cancer cells treated with nutlin-3 (36). However, these observations have been limited to description of cell morphology and SA-β-Gal staining and do not address the most critical issue, reversibility, to distinguish between senescence-like phenotype and true senescence. Our data derived from experiments with multiple cancer cell lines clearly indicate that epithelial cancer cells are unable to undergo irreversible senescence in response to continuous p53 activation for up to 2 weeks. These results suggest that selective pharmacologic p53 activation may not be an effective means for induction of senescence in cancer cells retaining wild-type

[3] Unpublished results.

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p53. However, on the positive side, the inability of normal epithelial cells to undergo senescence upon nongenotoxic p53 activation by MDM2 antagonists may reduce the toxic consequences of irreversible proliferation block *in vivo*.

Cellular senescence is thought to serve as an important barrier to cancer development by blocking cell proliferation. Recent studies using mouse cancer models have derived experimental evidence that p53-dependent senescence may play a tumor suppressor role *in vivo* (29). Restoration of p53 activity in mouse tumors led to emergence of senescence phenotype and tumor shrinkage, suggesting that p53 activating drugs may have utility even in cases where p53-dependent apoptotic signaling is compromised. MDM2 antagonists can activate p53 and have shown great promise as potential cancer therapeutics (17). However, their activity is limited to tumors expressing wild-type p53 that retain relatively intact p53-dependent apoptotic signaling (19). If apoptosis-resistant tumors have preserved their ability to undergo p53-dependent senescence, then MDM2 antagonists or any other p53 activating drugs will have a second chance.

Our results show that selective p53 activation by MDM2 antagonists is insufficient to induce irreversible senescence in epithelial cancer cells. This is unlikely due to defective p53-dependent senescence but rather reflects the need for additional signaling events. Nevertheless, cancer cells are effectively arrested and acquire senescence-like phenotype as long as nutlin is present. It has been reported recently that *in vivo* p53 activation in mouse liver tumors leads to up-regulation of several inflammatory cytokines (e.g., CSF1, CCL2, CXCL1, ICAM1, and IL15) that may facilitate tumor clearance by the immune system (9). If this is true for nutlin-induced senescence-like state, then one can envision possible extra benefit from p53 activating therapy. In our experiments, several of these cytokines were up-regulated in cancer cells with nutlin-induced senescence-like phenotype (HCT116, RKO, and MCF7) but activated to a lesser extent or down-regulated in primary epithelial cells (Fig. 10). Therefore, it is reasonable to speculate that p53-activating drugs may induce innate immune response against tumor tissues. Further exploration of this phenomenon *in vivo* is needed to address this assumption.

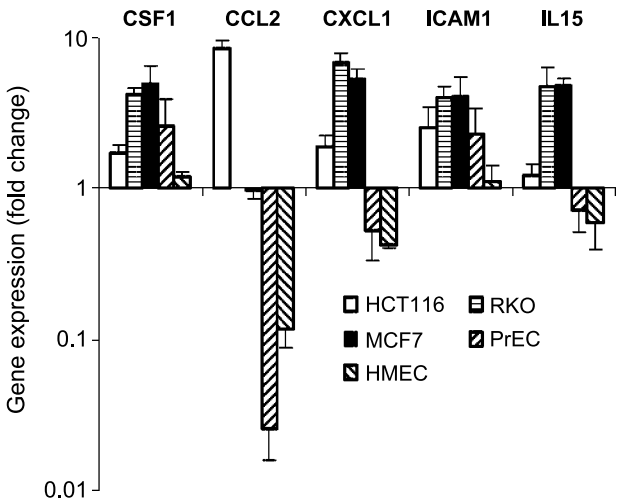

FIGURE 10. Cancer cells with nutlin-induced senescence phenotype express elevated levels of inflammatory cytokines. Cells were incubated with 10 μmol/L nutlin-3a or DMSO vehicle for 6 d and mRNA levels of indicated genes were determined by quantitative PCR. The changes in expression level are shown in log scale.

In conclusion, the experimental results described here show that activation of the p53 pathway by MDM2 antagonists causes prolonged cell cycle arrest and senescence-like phenotype in cultured cancer and normal epithelial cells, which is reversible after drug removal and normalization of p53 control. Therefore, p53 activation is necessary but insufficient for induction of true senescence in epithelial cells.

Materials and Methods

Cells and Drug Treatment

MHM osteosarcoma cells were provided by the Norwegian Radium Hospital. LOX IMVI melanoma cells were provided by the Biological Testing Branch, National Cancer Institute. All other cell lines were purchased from the American Type Culture Collection and grown in the recommended medium in a humidified environment with 5% CO_2. Drugs were dissolved in DMSO and kept at −20°C as 10 mmol/L stock solutions.

Cell Cycle Analysis

For analysis of cell cycle distribution, 10^6 cells were seeded in 75-cm^2 flasks for 24 h before drug treatment. After 6 d of treatment, the adherent cells were washed twice with PBS and collected by trypsinization. They were then fixed in 70% ethanol and stored at −20°C. After thawing, cells were washed twice in cold PBS and resuspended in 0.5 mL PI/RNase staining buffer (BD-Pharmingen), incubated for 15 min at 37°C, and analyzed using FACScalibur flowcytometer (Becton Dickson) using CellQuest software. For BrdUrd incorporation analysis, 20 μmol/L BrdUrd (Sigma) was added 1 h before collecting cells. Cells were fixed as described above, permeabilized with 2N HCl and 0.5% Triton X100 for 30 min, and neutralized with 0.1 mol/L sodium tetraborate (pH 8.5). Cells were then labeled with anti–BrdUrd FITC-conjugated antibody (Becton Dickinson) for 1 h before cell cycle analysis. For analysis of *in situ* BrdUrd incorporation, cells were seeded in six-well plates and incubated with 20 μmol/L BrdUrd for 24 h before fixing with 70% ethanol. Cells were then treated with 0.35 N NaOH for 30 to 60 s, washed twice with PBS, incubated with anti-BrdUrd antibody (FITC conjugated) overnight at 4°C. Samples were then washed with PBS and examined under microscope.

Quantitative PCR

Total RNA was extracted using RNeasy mini kit (Qiagen). Aliquots containing 5 μg total RNA were converted to cDNA using the TaqMan RT kit (Applied Biosystems). Relative quantity of p21 and MDM2 transcripts was determined by TaqMan using gene-specific primer/probe sets described as previously described (23). Primer/probe sets of CSF1, CCL2, CXCL1, ICAM1, and IL15 were purchased from Applied Biosystems. To determine p53 target gene expression level, cDNA were subjected to qPCR with Taqman low-density array cards containing 96 genes (Applied Biosystems). Detailed information regarding the genes in the array is described by Xia et al. (23).

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Apoptosis, Cell Viability, and Senescence Assays

Cells were seeded in six-well tissue culture plates (1×10^5 cells per well) 24 h before drug treatment. After drug treatment for 48 h or 6 d, culture media containing detached cells were collected, attached cells were trypsinized and combined with the corresponding media. For cell viability, an aliquot was removed and stained for 5 min using Guava ViaCount kit. The remaining cells were collected by centrifugation at 1,500 rpm for 10 min at 4°C and stained with the Guava Nexin kit as recommended by the manufacturer. Viability and apoptotic indices were determined using the Guava Personal Cell Analyzer (Guava Technologies). SA-β-Gal activity was assayed in various cancer cell lines as a marker for senescence. Cells were seeded in six-well tissue culture plates (1×10^5 cells per well) 24 h before drug treatment, washed twice with PBS, fixed, and stained using Senescent Cell Staining kit (Sigma). Stained cells were visualized using the Nikon Eclipse TE 2000-U microscope under bright-field and images were taken by the Nikon Digital Camera DXM 1200F using MetaVue software.

Colony Formation Assay

Clonogenic growth of cancer cell lines was determined by seeding 800 cells per well in a six-well plate 24 h before drug treatment. No treatment controls were established in parallel for each cell line. After 6 d of treatment, cells were washed twice with cold PBS, fixed with cold methanol, and stored at −20°C. Cell colonies were stained with 0.5% Crystal Violet (Sigma). To assess growth recovery, cells treated with nutlin-3a for 6 d were washed with drug-free media, incubated for additional 6 d in drug-free media, and stained with 0.5% Crystal Violet. Images of stained colonies were taken by COHU CCD Camera (Cohu, Inc.) using Sorcerer-3 software.

RNA Interference Experiments

Cells were seeded in six-well plates and incubated with 10 μmol/L nutlin-3a for 4 d before transfection with 100 nmol/L nontargeting control siRNAs or human p21-specific SMARTpool siRNAs (Dharmacon) using Lipofectamine 2000 (Invitrogen). Cells were incubated in Lipofectamine/siRNA mix with or without 10 μmol/L nutlin-3a for 24 h, then in Liopfectamine/siRNA-free medium with 10 μmol/L nutlin-3a for additional 24 h and collected for Western blot and BrdUrd analysis. For p53 knockdown experiment, cells were seeded in six-well plates, transfected with 100 nmol/L nontargeting control siRNAs or human *TP53*-specific siGENOME duplex (Dharmacon D-003329-06). Twnety-four hours after transfection, cells were treated with 10 μmol/L nutlin-3a for another 24 h, and then collected for BrdUrd analysis.

Immunoblotting

Cells were lysed in 0.1 to 0.2 mL radioimmunoprecipitation assay buffer and Western blotting was done as previously described (19). Primary antibodies used are as follows: p53 (sc-263) and MDM2 (sc-965) were from Santa Cruz Biotechnology; p21 (OP64) from Calbiochem, human MDMX (A300-287A-1) from Bethyl Laboratories; β-actin (AC-74) from Sigma; Rb, phospho-Rb, and Mouse Anti-$p15^{INK4b}$ from Cell Signaling; and E2F1, p107, p130, and DcR2 antibody (sc-11638) from Santa Cruz Biotechnology. Mouse Anti-$p16^{INK4a}$ antibody was from Chemicon. Secondary antibodies, anti-mouse IgG–HRP (sc-2302) and anti-rabbit IgG-HRP (sc-2301), were purchased from Santa Cruz Biotechnology. For senescence-associated heterochromatin foci detection, cells were cultured in chamber slides, fixed with 4% formaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 1% bovine serum albumin. Primary antibody anti-HP1γ (1:200) and secondary antibody Anti-Rabbit IgG (H+L) F(ab′)2 Fragment (DyLight 488 Conjugate) are from Cell Signaling Technology. Prolong Gold Antifade Reagent containing 4′,6-diamidino-2-phenylindole (Invitrogen) was applied to counterstain DNA.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Pharmacologic p53 Activation Blocks Cell Cycle Progression but Fails to Induce Senescence in Epithelial Cancer Cells

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