Transient Nutlin-3a Treatment Promotes Endoreduplication and the Generation of Therapy-Resistant Tetraploid Cells

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Abstract

p53 activity is controlled in large part by MDM2, an E3 ubiquitin ligase that binds p53 and promotes its degradation. The MDM2 antagonist Nutlin-3a stabilizes p53 by blocking its interaction with MDM2. Several studies have supported the potential use of Nutlin-3a in cancer therapy. Two different p53 wild-type cancer cell lines (U2OS and HCT116) treated with Nutlin-3a for 24 hours accumulated 2N and 4N DNA content, suggestive of G1, and G2 phase cell cycle arrest. This coincided with increased p53 and p21 expression, hypophosphorylation of pRb, and depletion of Cyclin B1, Cyclin A, and CDC2. Upon removal of Nutlin-3a, 4N cells entered S phase and re-replicated their DNA without an intervening mitotic division, a process known as endoreduplication. p53-p21 pathway activation was required for the depletion of Cyclin B1, Cyclin A, and CDC2 in Nutlin-3a–treated cells and for endoreduplication after Nutlin-3a removal. Stable tetraploid clones could be isolated from Nutlin-3a treated cells, and these tetraploid clones were more resistant to ionizing radiation and cisplatin-induced apoptosis than diploid counterparts. These data indicate that transient Nutlin-3a treatment of p53 wild-type cancer cells can promote endoreduplication and the generation of therapy-resistant tetraploid cells. These findings have important implications regarding the use of Nutlin-3a in cancer therapy. [Cancer Res 2008;68(20):8260–8]

Introduction

Wild-type p53 is a tumor suppressor and transcription factor activated by DNA damage and other stresses (1). p53 is normally maintained at low levels through the action of MDM2, an E3 ubiquitin-ligase that binds and ubiquitinates p53 and promotes its proteosomal degradation (2, 3). Stress-induced (DNA damage) phosphorylations, particularly those in the p53 NH2 terminus, inhibit the binding between p53 and MDM2 and thus stabilize p53 and cause its levels to increase (4). The effect of increasing p53 is to stop proliferation, either through G1 and G2 phase cell cycle arrests or apoptosis (1). These effects are mediated by p53-responsive gene products such as p21 (G1-G2 arrest), bax, PUMA, and NOXA (apoptosis).

There is considerable interest in restoring wild-type p53 activity in cancer as a therapeutic strategy. This goal has led to the development of Nutlin-3a (hereafter called Nutlin), a small molecule that binds MDM2 at the pocket used for interaction with p53. Nutlin prevents MDM2 from binding p53 and, consequently, stabilizes and activates p53 (5). At least two strategies have been proposed for Nutlin use in cancer therapy. In the first strategy, Nutlin would be used to treat p53 wild-type cancers due to its ability to trigger p53-dependent growth arrest or apoptosis. Support for this comes from various studies including reports that Nutlin could block the growth of p53 wild-type tumors grown as mouse xenografts, and studies in which Nutlin promoted apoptosis in p53 wild-type leukemia and lymphoma cells (5, 6). In the second strategy, Nutlin would be used to treat tumors that are null or mutant for p53. The notion here is that Nutlin would promote cell cycle arrest in normal tissues and cells that surround a p53-null or mutant tumor, whereas the tumor cells themselves would be unaffected and continue to proliferate. Subsequent treatment with drugs that target proliferating cells would then selectively kill the tumor cells, although having no effect on the arrested normal cells. Support for this comes from studies in which p53 wild-type cells arrested in G1 or G2 phase by Nutlin pretreatment were resistant to killing by the S phase poison gemcitabine or microtubule poison Taxol (7, 8).

In addition to its role in DNA damage and stress responses, p53 also functions in the "tetraploidy checkpoint." Evidence for this comes from studies using microtubule inhibitors (MTI) such as nocodazole and colcemid that block cells in metaphase. Cells arrested in metaphase by prolonged MTI exposure can eventually exit mitosis and enter a pseudo-G1 state with 4N DNA content (tetraploid G1; refs. 9, 10). Endoreduplication refers to the case when these tetraploid cells re-replicate their DNA, giving rise to a polyploid 8N population. Cells lacking p53, p21, or pRb are more sensitive to MTI-induced endoreduplication than wild-type cells, supporting a p53-p21-pRb–dependent "tetraploidy checkpoint" that prevents S phase entry by 4N cells (9–13). Involvement of p21 in endoreduplication has also been revealed in overexpression studies. P21 overexpression arrests cells in G1 and G2 phases. Interestingly, cells released from p21-mediated G2 arrest underwent endoreduplication with an accumulation of polyploid 8N cells (11, 14, 15). It was suggested that endoreduplication and polyploidy resulted from p21-induced depletion at the mRNA level of G2-M regulators and checkpoint proteins, such as Cyclin B1, CDC2, mitotic control proteins MAD2, BubR1, PLK1, and cytokinesis-associated proteins such as PRC1, AIM1, and Citron kinase (15). Another report showed that p21 overexpression via adenovirus promoted endoreduplication but only in cells that lacked p16 function (11). In that report, it was suggested that p21 expression in the absence of p16 may not fully inhibit Cyclin E-CDK2 activity, and that residual Cyclin E-CDK2 activity was likely driving G2-arrested cells into S phase inappropriately. Nutlin-treated p53 wild-type cells express high levels of both p53 and p21. An effect of Nutlin on the tetraploidy checkpoint and endoreduplication has not been described.

There is mounting evidence that aneuploid cancer cells are generated from either asymmetric division or progressive chromosomal
loss from tetraploid precursors (16, 17). For example, the appearance of tetraploid cells in the premalignant condition Barrett’s esophagus correlated with p53 loss and preceded gross aneuploidy and carcinogenesis (18). Tetraploid or near-tetraploid cells have also been described in early-stage cancers, such as cervical cancer (19). Direct evidence for the tumorigenic potential of tetraploid cells was provided by Pellman and colleagues who isolated binucleate, tetraploid mammary epithelial cells from p53-null mice (20). Remarkably, these cells were more susceptible to carcinogen-induced transformation (soft agar growth) than normal diploid cells, and the tetraploid cells formed tumors in nude mice, whereas diploid cells did not (20). In another study, Kroemer and colleagues (16) used prolonged (48 hours) nocodazole treatment to isolate tetraploid cells from two human colon cancer cell lines that express wild-type p53. They found that most tetraploid cells died after attempting to divide, although some survived and gave rise to stable tetraploid clones. Surprisingly, these tetraploid clones were resistant to radiation and certain chemotherapeutic agents compared with normal diploid counterparts (16). These studies and others have sparked sharp interest in how tetraploid cells arise and their susceptibility to conventional therapies. Here, we report that transient exposure of p53 wild-type cells to Nutlin can promote endoreduplication and the generation of therapy-resistant tetraploid clones.

Materials and Methods

Cell lines and culture conditions. U2OS cells were purchased from American Type Culture Collection and grown in DMEM supplemented with 10% fetal bovine serum (FBS). HCT116 cells and its derivatives (p53−/−, p21−/−) were gifts from Dr. Bert Vogelstein (John Hopkins University, Baltimore, MD) and grown in McCoy’s 5A supplemented with 10% FBS. Cells were plated 24 h before being treated with Nutlin-3a (5 μmol/L; Sigma), irradiation (10 Gy), Cisplatin (15 μmol/L; Bedford Laboratory), or Control RNAi (On-target plus siControl nontargeting pool) were purchased from Dharmacon and transfected according to the manufacturer's guidelines using DharmaFECT 1 reagent. Treatments were applied 24 h after transfection.

Results

G2-M (4N) cells arrested by Nutlin treatment re-replicate their DNA after Nutlin removal. Multiple in vitro studies have supported the potential for Nutlin in cancer treatment either as a single agent or in combination with radiation or chemotherapeutic drugs. We examined the combined effects of Nutlin and ionizing radiation (IR) in two commonly used p53 wild-type cancer cell lines, HCT116 and U2OS. Cells were treated with Nutlin alone (5 μmol/L; IR alone (10 Gy), or combination of Nutlin and IR for 24 hours. In some cases, the cells were then rinsed and refed with medium lacking Nutlin (Nutlin removal) and cell cycle profiles determined at multiple time points. Treatment with Nutlin alone in both cell lines caused an accumulation of 2N and 4N cells with little or no S phase, suggestive of G1 and G2 phase arrest (Fig. 1). It should be noted that both cell lines treated with Nutlin plus the mitosis blocker colcemid accumulated almost entirely with 4N DNA (Supplementary Fig. S1), indicating Nutlin-treated cells pass through one division cycle before arresting in G2 phase. In contrast to Nutlin only treatment, cells treated with IR alone or Nutlin plus IR arrested mostly with 4N DNA content, suggesting G2 arrest was the primary response to these treatments. Surprisingly, in cells that had been treated with Nutlin alone or Nutlin plus IR, we observed the emergence of cells with >4N DNA content after Nutlin removal. In both cell lines treated with Nutlin alone, >4N cells became evident 12 hour after Nutlin removal, accumulated in an 8N population at the 18-hour time point, and diminished by the 24-hour time point after Nutlin removal. With Nutlin plus IR treatment, >4N cells became apparent at 18 hour after Nutlin removal and accumulated in an 8N population at the 24-hour time point (Fig. 1).

In HCT116 cells treated with IR alone, we also observed >4N cells at the +18-hour and +24-hour time points, whereas in U2OS cells treated with IR alone, we did not observe a significant accumulation of >4N cells at any time points.

The appearance of cells with >4N DNA content and their accumulation in an 8N population suggested that 4N cells that had accumulated in Nutlin and Nutlin plus IR–treated cells were reinitiating DNA synthesis and replicating their DNA, a process known as endoreduplication. BrdUrd incorporation and propidium iodide staining (DNA content) was used to test whether 4N cells reinitiated DNA synthesis after Nutlin removal. U2OS and HCT116 cells were treated with Nutlin alone, IR alone, or Nutlin plus IR for 24 hours. In some cases, cells were then rinsed and refed with medium lacking Nutlin and allowed to incorporate BrdUrd for the first 12 hours after Nutlin removal (Fig. 2, +12 h). In other cases, cells were rinsed and refed with medium lacking Nutlin and grown for 12 hours, and BrdUrd then added to the medium at this 12-hour
time point. These cells were allowed to incorporate BrdUrd for 2 hours (Fig. 2, +14 h). The results show that U2OS and HCT116 cells treated with Nutlin alone accumulate with 2N and 4N DNA content, and that both 2N and 4N cells reinitiate DNA synthesis (incorporate BrdUrd) within the first 12 hours after Nutlin removal. U2OS cells treated with IR alone accumulated mostly with 4N DNA content, and these 4N cells did not reinitiate DNA synthesis (did not incorporate BrdUrd). In contrast, Nutlin plus IR–treated U2OS cells behaved similarly to cells treated with Nutlin alone, with 4N cells reinitiating DNA synthesis after Nutlin removal. These results suggest Nutlin can drive IR-treated U2OS cells with 4N DNA content into a state from which they can re-replicate their DNA. In HCT116 cells treated with IR alone, we observed 4N cells reinitiate DNA synthesis (incorporate BrdUrd), consistent with the appearance of >4N cells in IR-treated HCT116 in Fig. 1A. In Nutlin plus IR–treated HCT116 cells we also observed 4N cells reinitiating DNA synthesis after Nutlin removal. In total, the results of Figs. 1 and 2 indicate that Nutlin treatment causes U2OS and HCT116 cells to accumulate with 2N and 4N DNA content, and the 4N cells can reinitiate DNA synthesis and replicate their DNA after Nutlin removal, giving rise to polyploid 8N cells.

**Nutlin treatment induces down-regulation of Cyclin B1, Cyclin A, and CDC2.** For endoreduplication to occur, 4N (G2-M) cells must first enter into a G1-like state, called tetraploid G1 (17). This is often associated with decreased expression of Cyclin B, Cyclin A, and/or CDC2, such that a condition of low CDK activity resembling early G1 phase is established in 4N cells (21, 22). Indeed, deletion or conditional inactivation of Cyclin B, Cyclin A, and CDC2 has been shown to establish a tetraploid G1 state and subsequent endoreduplication in different cell systems (23–26). To investigate whether Nutlin promotes a tetraploid G1 arrest, we compared the levels of Cyclin B1, Cyclin A, and CDC2 in cells treated with Nutlin alone, IR alone, or combination Nutlin plus IR 24 hours after treatment and at time points after Nutlin removal (Fig. 3). Cyclin A and Cyclin B1 accumulate during the cell cycle and are high in G2 phase but then decrease rapidly as cells pass through mitosis (27–29). IR arrests cells in G2 phase, in part, through inhibitory phosphorylation of CDC2 at tyrosine-15 (Tyr-15). U2OS and HCT116 cells treated with IR alone expressed increased levels of Cyclin B1, Cyclin A, and Tyr-15–phosphorylated CDC2, indicative of G2 arrest. In contrast, cells treated with Nutlin alone or Nutlin plus IR expresses low/undetectable levels of Cyclin B1, Cyclin A, and Tyr-15 phosphorylated CDC2. Overall CDC2 levels were also modestly decreased in Nutlin and Nutlin plus IR–treated cells. Notably, the effects of Nutlin on Cyclin B1, Cyclin A, and CDC2 were specific because Cyclin E levels were not decreased in Nutlin or Nutlin plus IR–treated cells. To examine whether these cells resembled G1–arrested cells, we monitored expression and phosphorylation of pRB. pRB levels were decreased in both cell lines treated with Nutlin or Nutlin plus IR. In Nutlin or Nutlin plus IR–treated U2OS
cells, pRb was expressed only in the hypophosphorylated form that is typically associated with arrest in G₁ phase, whereas in cells treated with IR alone, pRb remained mostly in a hyperphosphorylated form. HCT116 cells treated with Nutlin alone or Nutlin+IR also expressed hypophosphorylated pRb, whereas HCT116 cells treated with IR alone expressed pRb in both hyperphosphorylated and hypophosphorylated forms. These results are consistent with 4N U2OS and HCT116 cells treated with Nutlin only or Nutlin plus IR being arrested in a tetraploid G₁-like state. p53 levels decreased rapidly (within 2 hours) after Nutlin removal, and p21 levels decreased 8 to 12 hours after Nutlin removal. Decreased levels of p21 coincided with pRb accumulating in a hyperphosphorylated form and the eventual return of Cyclin B1 and Cyclin A levels (Fig. 3).

DNA endoreduplication after transient Nutlin treatment is p53 and p21 dependent. Previous studies support a p53, p21, and...
pRb-dependent checkpoint that blocks endoreduplication. In contrast, transient p21 expression can promote endoreduplication (14, 15). We investigated whether p53, p21, and/or pRb are required for down-regulation of CDC2, Cyclin B1, and Cyclin A in Nutlin-treated cells and endoreduplication after Nutlin removal. First, HCT116 cells transfected with control siRNA or siRNAs targeting p53, p21, or pRb were treated with Nutlin or untreated for 24 hours, and protein lysates examined by immunoblotting (Fig. 4A). Cyclin A and Cyclin B1 expression was markedly decreased in both cell types, and CDC2 expression also decreased after Nutlin treatment in control cells and cells with pRb knocked down but not in cells with either p53 or p21 knocked down. This indicates p53-p21 pathway activation is required for decreased expression Cyclin A, Cyclin B1, and CDC2 in Nutlin-treated cells, whereas pRb is not required. Next, we tested whether p53, p21, and pRb are required for endoreduplication in Nutlin-treated cells. First, U2OS and

Figure 4. DNA endoreduplication after Nutlin removal is p53 and p21 dependent. A, HCT116 and U2OS cells were transfected with siControl (siC), sip53, sip21, sipRb, or no transfection (UT). These cells were treated with Nutlin (5 μmol/L) or untreated for 24 h. Cell lysates were collected and analyzed by Western Blot with indicated antibodies. B, HCT116 and U2OS cells were transfected as in A. These cells were treated with Nutlin (5 μmol/L) followed by Nutlin removal. Cells were harvested at the indicated time points and subjected to flow cytometry for cell cycle analysis. C, HCT116 cells that are wild-type (WT; with intact p53 and p21 genes), p53−/−, and p21−/− were treated with Nutlin (5 μmol/L) followed by Nutlin removal. Cells were harvested at the indicated time points and stained with propidium iodide to measure DNA content. D, the percentage of cells in B with >4n DNA content (>4n) was quantified using Flowjo software; mean ± SE; n = 2. >4n cells were gated as shown in B (UT: +16 h).
HCT116 cells transfected with control siRNA or siRNAs targeting p53, p21, or pRB were treated with Nutlin for 24 hours, followed by Nutlin removal for different periods. FACS analysis was used to monitor the appearance of cells with >4N DNA content after Nutlin removal (Fig. 4B and D). Cells with >4N DNA content were observed in control samples and cells with pRB knocked down at the 16- and 20-hour time points after Nutlin removal. In contrast, cells with >4N DNA content were not observed after Nutlin removal in cells with either p53 or p21 knocked down. This indicates that both p53 and p21 are also required for Nutlin-induced endoreduplication. Second, we compared the extent of endoreduplication in HCT116 cells with intact or deleted p53 and p21 alleles and obtained similar results (Fig. 4C). Cells with an intact p53-p21 signaling pathway treated with Nutlin underwent endoreduplication after Nutlin removal, whereas p53 and p21 null cells did not. Taken together, the results suggest p53-p21 pathway activation in Nutlin-treated cells is required for the cells to undergo a tetraploid G1 arrest (indicated by changes in Cyclin A, Cyclin B1, CDC2, and pRB expression) and subsequent endoreduplication after Nutlin removal.

Stable tetraploid clones isolated after transient Nutlin treatment show resistance to DNA damage–induced apoptosis. U2OS and HCT116 cells with >4N DNA content that emerged after Nutlin removal gave rise to a transient 8N population that eventually diminished (Fig. 1). This suggested these 8N cells might enter mitosis and divide. We considered two possible outcomes: first, the 8N cells could undergo an asymmetric division characterized by abnormal, multipolar mitoses and three or more centrosomes. Quadri-polar and tripolar mitoses were readily apparent in HCT116 cells (observed in 25% of mitotic cells) that had been Nutlin treated for 24 hours followed by Nutlin removal for 16 more hours (Fig. 5A) but not in cells maintained in Nutlin (data not shown). Similarly, cells with 3 or more centrosomes were readily apparent in U2OS cells (observed in 8.5% of cells) that had been Nutlin treated for 24 hours followed by Nutlin removal for 24 more hours (Fig. 5B). Abnormal mitoses and cells with supernumerary centrosomes were not observed in untreated cells (data not shown). These results are consistent with at least some 8N cells entering mitosis and undergoing asymmetric cell division. Notably, asymmetric divisions of this type can lead to whole chromosome aneuploidy in surviving daughter cells. We also considered that 8N cells might divide symmetrically and survive as stable tetraploid clones with twice the normal DNA content. To examine this possibility, U2OS and HCT116 cells were treated with Nutlin for 24 hours, followed by Nutlin removal for an additional 16 hours. The cells were then labeled with the live-cell DNA stain Hoechst 33342. 2N and 8N cells were isolated by flow cytometry and replated at low density for isolation of DNA stain Hoechst 33342. 2N and 8N cells were isolated by flow cytometry and replated at low density for isolation of DNA stain Hoechst 33342. 

We were particularly interested in the stable tetraploid clones that arose after transient Nutlin treatment because of the high frequency with which they appeared. Recent studies have suggested that tetraploid cells may be more resistant than diploid cells to certain anticancer agents but not others (16). We tested whether tetraploid HCT116 clones that arose after Nutlin treatment were more resistant to IR and cisplatin-induced apoptosis than diploid counterparts. The 7 tetraploid and 7 diploid clones isolated from Nutlin-treated cells, and the 10 diploid clones isolated from untreated cells, were treated with IR (10 Gy) or cisplatin (15 μmol/L) doses that can effectively promote HCT116 cell killing (16). Cell death was monitored by sub-G1 DNA content and apoptosis by decreased mitochondrial membrane potential (low ΔΨm) and annexin-V staining 72 hours after treatment. As shown in Fig. 6, the tetraploid clones as a group were significantly more resistant to IR and cisplatin (CP) treatment by each criteria compared with the parental cells and diploid clones isolated from either untreated or Nutlin-treated cells ($P < 0.05$). There was no significant difference between the parental cell line (P) and diploid clones from untreated cells (D) or Nutlin-treated cells (ND). Long-term clonogenic survival after treatment can depend on a combination of senescence, apoptosis, and nonapoptotic death (30). We therefore also examined clonogenic survival after IR in parental HCT116 cells, three diploid and three tetraploid clones (Supplementary Fig. S3). In these studies, the tetraploid clones were slightly, but not significantly, more resistant to IR treatment than diploid clones or parental cells, suggesting clonogenic survival may depend on other pathways in addition to apoptosis. In summary, the data indicate endoreduplication after transient Nutlin treatment can give rise to tetraploid cells resistant to therapy induced apoptosis.

Discussion

Genomic stability is maintained by elaborate checkpoint mechanisms that regulate mitotic entry and exit, as well as entry into S phase. Defects in these checkpoints can result in numerical and structural chromosomal changes that promote tumorigenesis. In normal cells, entry into S phase is dependent on the completion of mitosis and activation of G1 phase Cyclin-CDK complexes. Endoreduplication occurs when G2 or M phase (4N) cells enter a G1-like state (tetraploid G1) and re-replicate their DNA without an intervening mitotic division. There is increasing evidence that aneuploid cancer cells can arise from tetraploid precursors. This has led to sharp interest in how tetraploid cells arise and their susceptibility to conventional therapies. The MDM2 antagonist Nutlin-3a (Nutlin) is being considered as a single or combination agent for cancer treatment. The current report shows that p53 wild-type cancer cells exposed to Nutlin for 24 hours undergo endoreduplication upon Nutlin removal, giving rise to therapy-resistant tetraploid clones.

Two things must happen for endoreduplication to occur: First, 4N cells must exit G2 or M phase prematurely and enter a pseudo-G1 state (tetraploid G1); Second, these tetraploid cells must reinitiate DNA synthesis, a process that requires activation of Cyclin E-CDK2 complexes (17, 31). The p53-p21 pathway inhibits endoreduplication as part of the "tetraploidy checkpoint." Evidence for this comes largely from studies with MTIs, such as nocodazole or colcemid, that block cells in metaphase. Cells that are metaphase arrested in this way for prolonged periods can eventually exit mitosis and enter a tetraploid G1 state from which...
they can re-replicate their DNA (endoreduplication; refs. 9, 10). Cells lacking p53, p21, or pRb are more prone to MTI-induced endoreduplication than normal cells, supporting a p53-p21-pRb checkpoint that inhibits endoreduplication (9–13). However, p21 was also reported to promote endoreduplication in different studies. For example, high p21 expression from an inducible promoter arrested cells in G1 and G2 states (14, 15). Cells released from p21-mediated G2 arrest by promoter shut-off underwent endoreduplication with an accumulation of 8N cells (14, 15). An explanation for these apparently discrepant findings is that high p21 levels drive 4N cells into a tetraploid G1 state, but subsequent DNA synthesis in the tetraploid cells requires p21 levels decrease so that CyclinE-CDK2 complexes can be activated.

Under normal conditions, prereplication complexes (pre-RC) assemble at DNA origins of replication in a process termed origin “licensing” only in late M and early G1 phase when CDK activity is low (32). This involves sequential binding of the origin recognition complex (ORC), CDC6 and Cdt1, and the replicative helicase MCM2-7 complex. Subsequent origin firing/S phase entry occurs upon recruitment of the DNA synthesis machinery and activation of Cyclin E-CDK2. In G2 and early M phase, origin licensing is prevented by Cyclin A/B-CDC2–dependent phosphorylation of factors including ORC, MCM4, and CDC6 that inhibits their origin binding (33–37). Thus, depletion of CDC2, Cyclin B, and/or Cyclin A can establish a “G2-like” state of low CDK activity in G2 or early M phase cells, priming these cells for S phase entry by allowing pre-RC formation. Indeed, depletion of CDC2, Cyclin A and Cyclin B1 has been causally linked with tetraploid G1 arrest and endoreduplication in several studies (22, 24–26). In the current study, IR-treated cells accumulated with 4N DNA content and expressed elevated Cyclin A, Cyclin B1, and Tyr-15 phosphorylated CDC2, consistent with arrest in G2 phase. In contrast, cells treated with Nutlin alone or Nutlin plus IR accumulated with 4N DNA but had either lost or markedly decreased expression of CDC2, Cyclin B1, and Cyclin A. These results support Nutlin and Nutlin plus IR–treated 4N cells being arrested in a tetraploid G1-like state. SiRNA knockdown studies revealed that p53 and p21 are required for Nutlin to cause decreased CDC2, Cyclin B1, and Cyclin A expression. In addition, p53/p21 knockdown or null cells were resistant to endoreduplication after Nutlin removal. In total, these results suggest that Nutlin drives 4N cells into a tetraploid G1 state via p53-p21 pathway activation, and this is required for endoreduplication after Nutlin removal. The tetraploid G1 arrest caused by Nutlin most likely results, at least in part, from down-regulation of CDC2, Cyclin B1, and Cyclin A. We speculated these effects might be through p21-mediated activation of pRb, which could bind E2F complexes and potentially inhibit CDC2, Cyclin B1, and Cyclin A expression as a result. In this regard, it is notable that pRb was not essential for the apparent tetraploid arrest and endoreduplication induced by Nutlin. For example, CDC2, Cyclin B1, and Cyclin A levels still decreased in Nutlin-treated cells in which pRb was knocked down, and pRb knockdown cells underwent endoreduplication after Nutlin removal. Previous studies showed functional overlap...
between pRb-family members (pRb, p107, and p130) in the cell cycle response to p53 (38). Specifically, cells lacking individual pRb family members were only partially resistant to p53-dependent G1 arrest, whereas cells lacking all three members were completely resistant to p53-dependent arrest. It remains to be seen whether p107 and p130 contribute to the p53/p21-dependent effects of Nutlin.

An interesting finding from the current report was that HCT116 cells were susceptible to endoreduplication after IR treatment, whereas U2OS cells were not. This difference correlated with the extent of p21 induction in each cell line. In HCT116 cells, IR (10 Gy) induced p53 and p21 within 6 hours of treatment, whereas in U2OS cells the induction of p21 was delayed (Supplementary Fig. S4). Previous studies found that IR can cause p53 and p21-dependent down-regulation of CDC2, Cyclin A, and Cyclin B1, similar to what we observe for Nutlin (22, 39). This down-regulation was at the mRNA level and, in at least one study, coincided with endoreduplication 1 to 6 days after treatment (22). It was suggested that endoreduplication after IR could also be explained in part by reduced CDC2 levels leading to tetraploid arrest and origin licensing in 4N cells (22, 24). CDC2, Cyclin A, and Cyclin B1 levels were not obviously decreased in IR-treated HCT116 cells in the current study. However, our ongoing studies suggest a fraction of IR-treated HCT116 cells with 4N DNA content lack or express low levels of Cyclin B1 (data not shown). We speculate these cells may undergo endoreduplication after IR treatment, whereas high Cyclin B1 expressing cells may not. Notwithstanding a change in protein levels, it is also likely that inhibition of CDC2 activity after IR treatment contributed to a tetraploid arrest in HCT116 cells that preceded endoreduplication.

Endoreduplicating cells that emerged after Nutlin removal gave rise to a transient 8N population. A common consequence of endoreduplication is supernumerary centrosomes in 8N cells. These supernumerary centrosomes can lead to multipolar mitoses and unequal chromosome segregation in daughter cells, a defect that directly causes whole chromosome aneuploidy. We observed supernumerary centrosomes and tripolar and quadriapolar mitoses in U2OS and HCT116 cells after Nutlin removal. Thus, one apparent fate of 8N cells that arise after Nutlin treatment is to undergo asymmetric division, potentially giving rise to whole chromosome aneuploidy in surviving cells. It has also been observed that tetraploid cells can cluster supernumerary centrosomes in two poles, thereby promoting bipolar mitoses even in the presence of multiple chromosomes (17). Consistent with this, we were able to isolate stable tetraploid clones of HCT116 and U2OS cells that had been treated with Nutlin, and our results indicate that nearly all (97%) of these tetraploid cells divide in a bipolar fashion (data not shown). One possibility is that these clones maintain their tetraploid DNA content through centrosome clustering and continuous bipolar divisions.

The relationship between ploidy and DNA damage (radiation) sensitivity has been examined since the 1950s. Some studies have reported increased radiosensitivity in cells with higher ploidy (40, 41), whereas others reported cells with increased ploidy are equally sensitive or more sensitive than cells with lower ploidy to radiation killing (42, 43). Recently, Castedo and colleagues (16) compared radiation and chemosensitivity of diploid RKO and HCT116 cells with tetraploid clones that arose after prolonged nocodazole treatment. Tetraploid clones were significantly more resistant than diploid clones to certain agents (e.g., IR, cisplatin, and camptothecin), whereas being equally sensitive to other agents (staurosporine and etoposide). However, it is worth noting that it was not tetraploidy per se that afforded radiation and chemoresistance in their study but rather increased expression in certain DNA repair and antiapoptotic factors that were identified by microarray analysis (16). We observed stable tetraploid HCT116 clones that arose after Nutlin treatment were more resistant to IR and cisplatin than diploid clones. It will be interesting to identify genes that are up/down-regulated in these tetraploid clones as potential mediators of this resistance phenotype. Regardless of the mechanism for this resistance, the

Figure 6. Tetraploid clones show resistance to IR and cisplatin-induced (CP) apoptosis. The 7 tetraploid clones (T) and 7 diploid clones isolated from Nutlin treated cells (ND), and the 10 diploid clones isolated from untreated cells (D) were exposed to 15 μmol/L cisplatin or 10 Gy IR for 72 h. The cells were then harvested, stained with indicated fluorophore dyes, and subjected to flow cytometry analysis. A, the percentage of cells with low S/Vm (TMRE staining) of tetraploid clones, diploid clones, and parent cells 72 h posttreatment. B, the percentage of sub-G1, cells (propidium iodide staining) of tetraploid clones, diploid clones, and parent cells 72 h posttreatment. C, the percentage Annexin V–positive cells of tetraploid clones, diploid clones, and parent cells 72 h posttreatment. Columns, mean of three independent experiments; bars, SE. *, a significant difference (P < 0.05) comparing tetraploid clones to diploid clones isolated from untreated cells or Nutlin-treated cells as well as to parental cells (P). No significant difference (P > 0.05) was observed between diploid clones and parental cells. Statistical analysis was done using unpaired Student’s t test (n = 3).
results suggest a potentially adverse side effect of any Nutlin-based therapy is endoreduplication and the generation of therapy-resistant tetraploid cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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