Cyclin-Dependent Kinase Inhibitors Sensitize Tumor Cells to Nutlin-Induced Apoptosis: a Potent Drug Combination

Chit Fang Cheok, Anwesha Dey, and David P. Lane

Institute of Molecular and Cell Biology, Proteos, Singapore

Abstract

Current chemotherapy focuses on the use of genotoxic drugs that may induce general DNA damage in cancer cells but also high levels of toxicity in normal tissues. Nongenotoxic activation of p53 by targeting specific molecular pathways therefore provides an attractive therapeutic strategy in cancers with wild-type p53. Here, we explored the antitumor potential of cyclin-dependent kinase (CDK) inhibitors in combination with a small molecule inhibitor of p53-murine double minute 2 (MDM2) interaction. We show that low doses of CDK inhibitors roscovitine and DRB synergize with the MDM2 antagonist nutlin-3a in the induction of p53 activity and promote p53-dependent apoptosis in a dose- and time-dependent manner. Statistical measurement of the combination effects shows that the drug combination is additive on the reduction of cell viability and synergistic on inducing apoptosis, a critical end point of cytotoxic drugs. The degree of apoptosis observed 24 to 48 h after drug treatment correlated with the accumulation of p53 protein and concomitant induction of proapoptotic proteins Puma and PG3. The antiproliferative and cytotoxic effects of this drug combination are validated in a range of tumor-derived cells including melanoma, colon carcinoma, breast adenocarcinoma, and hepatocarcinoma cells. Furthermore, this drug combination does not induce phosphorylation of Ser15 on p33 and does not induce genotoxic stress in the cell. Given that many cytotoxic drugs rely on their ability to induce apoptosis via DNA damage-mediated activation of p53, the data presented here may provide a new therapeutic approach for the use of CDK inhibitors and MDM2 antagonists in combinatorial drug therapy. (Mol Cancer Res 2007;5(11):1133–45)

Introduction

p53 is a stress-inducible transcription factor that positively and negatively regulates a plethora of genes involved in cell cycle arrest, apoptosis, senescence, and differentiation (1, 2). Recent genome-wide chromatin immunoprecipitation analyses of p53-regulated genes revealed additional unexpected targets, suggesting that the role of p53 in the cell may be more extensive than previously thought (3). Nevertheless, analysis has focused on the growth suppression and proapoptotic activities of p53 and the role it has in protecting normal somatic tissues from damage. The role of p53 in tumor suppression is underscored by the fact that mutational inactivation of p53 signaling occurs frequently in tumors. Approximately 50% of all human tumors harbor mutations in the p53 gene, and among many of the remaining tumors the signaling pathway of p53 is defective (4). One common lesion in cancers with wild-type p53 is the overexpression of murine double minute 2 (MDM2), a negative regulator of p53. MDM2 negatively regulates p53 by binding to its transactivation domain and promoting ubiquitin-dependent degradation (5-7). Amplification and overexpression of MDM2 have been shown to confer tumorigenic potential (8, 9).

Disruption of the p53-MDM2 interaction therefore provides an attractive strategy for activating p53 function in tumors with wild-type p53 and has been the underlying rationale for the ongoing development of small molecule inhibitors of this functional interaction. Recently, a class of potent and selective antagonist of the p53-MDM2 interaction, termed nutlins, were shown to activate p53 in vitro and in vivo (10, 11). Nutlins bind to the p53 binding pocket on the surface of MDM2 in an enantiomer-specific manner, releasing p53 from its negative regulatory loop. In cancer cells, this leads to cell cycle arrest and apoptosis, whereas in normal cells, the response is largely limited to a reversible growth arrest (12, 13). The low toxicity of nutlin in animal models and the shown synergistic effects with traditional chemotherapeutic agents support the use of nutlin as an anticancer drug (11-15). Furthermore, nutlin may offer normal cells partial protection from chemotherapy (16, 17).

Another class of drugs that functions by a distinct mechanism from nutlins to activate p53 are cyclin-dependent kinase (CDK) inhibitors. CDKs regulate two processes essential for cancer cell survival: cell cycle progression and gene transcription. Roscovitine, often referred to as a selective inhibitor of CDKs, inhibits primarily CDK2, CDK5, CDK7, and CDK9 in vitro (18). Accumulating evidence suggests that CDK7 and CKD9 are involved in the activation of RNA polymerase II–dependent transcriptional initiation and elongation via direct phosphorylation of the RNA polymerase II COOH-terminal domain (19). Similar to many other inhibitors of RNA polymerase II...
transcription, roscovitine induces p53-dependent cell cycle arrest and apoptosis. Another mechanism by which roscovitine may function is through direct inhibition of CDK2, resulting in the phosphorylation of p53 on Ser15 (20). However, at lower drug concentrations that do not activate Ser15 phosphorylation, the nongenotoxic events induced by roscovitine effectively activate p53 cellular functions (21). Currently, roscovitine (CYC202; Seliciclib) is in phase II clinical trials in combination with cisplatin/gemcitabine for non–small-cell lung cancer and as a single agent for lung and B-cell malignancies. Another CDK inhibitor known to induce p53 nuclear accumulation in cells is DRB (22). DRB, a classic inhibitor of transcription, principally inhibits CDK9, the kinase component of positive transcription elongation factor b, and blocks global RNA polymerase II–dependent transcription (22, 23). Additionally, DRB also inhibits other protein kinases involved in cellular metabolism such as casein kinase type II (24, 25).

Whereas current chemotherapeutic agents offer potential suppression of tumor growth and regression of tumors, the efficacy of anticancer therapy is limited by acquired drug resistance of tumor clones, collateral DNA damage, and toxicity to normal tissues. These considerations prompted us to explore the effects of drug combinations on p53 activity. Using a p53-dependent reporter gene construct, we show that nutlin-3a, a potent and selective small molecule antagonist of MDM2, in combination with nongenotoxic CDK inhibitors, DRB and roscovitine, synergistically activates p53-dependent transcription. Importantly, this report provides evidence that subthreshold concentrations of drugs that activate p53 poorly on their own can be used in combination to synergistically induce p53 signaling pathways. Furthermore, this combination drug treatment is independent of the phosphorylation of Ser15 on p53 and does not induce genotoxic stress in the cell. Our data support the therapeutic use of this nongenotoxic drug combination to increase drug efficacy without imposing genotoxic burden on normal tissues.

Results
Activation of p53 by CDK Inhibitors and Nutlin-3a, a Small Molecule Antagonist of p53-MDM2 Interaction
To study the activation of cellular p53 by CDK inhibitors and MDM2 antagonist, we used a derivative of A375 melanoma cell line (ARN8) that contains a stably integrated β-galactosidase gene reporter fused to the ribosomal gene clustal (RGC) p53 consensus binding sequence (26). Using this cell line, we evaluated the effects of the combination of CDK inhibitors and an MDM2 antagonist on p53 activation. To select appropriate drug concentrations for the more complex combination studies, we carried out a careful dose titration of each of the drugs used in this study. Treatment of ARN8 cells with a potent MDM2 antagonist, nutlin-3a, induced relatively high levels of p53-dependent gene expression 16 h after drug treatment (Fig. 1). In contrast, treatment with the CDK inhibitors DRB and roscovitine resulted in relatively weak induction of p53 activity. Specifically, low concentrations of DRB (<10 μmol/L) or roscovitine (<10 μmol/L) did not activate p53-dependent gene transcription, whereas a mild increase in the levels of p53-dependent transcription was observed when cells were treated with 20 μmol/L of DRB or roscovitine, and a more evident increase was observed with 40 μmol/L of DRB or roscovitine (Fig. 1A).

Synergistic Activation of p53 by Combined Treatment with Roscovitine, DRB, and Nutlin-3a
Next, we asked if roscovitine would synergize with nutlin-3a in the induction of p53 activity when both drugs are administered at low threshold concentrations. Treatment of ARN8 cells with low doses of nutlin-3a (2.5 μmol/L), complemented with 0 to 10 μmol/L of roscovitine, which does not lead to any appreciable increase in p53 activity on its own, results in an increase in p53-dependent transcription in a roscovitine concentration-dependent manner (Fig. 1B). Here, using a cell-based p53-dependent transcription model system, we show that roscovitine and nutlin cooperate to activate p53-dependent transcription, which may support the observation that roscovitine sensitizes neuroblastoma cells to nutlin-induced p53-dependent cell death (27).

Both roscovitine and DRB have been shown to be potent inhibitors of CDK7 and CDK9, kinases that phosphorylate the COOH-terminal domain of the largest subunit of RNA polymerase II (18, 22, 28). Given that many CDK inhibitors share an overlapping spectrum of targets, it would not be surprising if DRB and roscovitine may share cellular activities by targeting similar kinases. Furthermore, both kinase inhibitors are known to activate p53, resulting in nuclear accumulation of p53 protein and p53-dependent apoptosis (29-33). To this end, we investigated whether DRB could act in a similar manner to roscovitine and found that DRB effectively synergizes with nutlin-3a in a DRB concentration–dependent manner (Fig. 1C). Strikingly, maximal synergistic activation was observed when DRB and nutlin-3a treatment was complemented with roscovitine, resulting in >50-fold increase in the level of reporter activity at 20 μmol/L DRB compared with DMSO-treated controls (Fig. 1C and D). This synergistic effect is dependent on nutlin-3a because roscovitine and DRB do not synergize in the activation of p53-dependent gene transcription (Fig. 1C). Roscovitine and DRB potentiate nutlin-induced p53 activation at all concentrations of nutlin tested (Fig. 1E). Whereas single-drug treatment with low concentrations of roscovitine (5 μmol/L), DRB (20 μmol/L), or nutlin-3a (2.5 μmol/L) gave rise to <10-fold increase in β-galactosidase/p53 activity, treatment with a combination of these three drugs showed >50-fold increase in the activation of p53. Taken together, these data showed that both protein kinase inhibitors, roscovitine and DRB, synergize with nutlin-3a, and this synergistic effect is strictly dependent on nutlin-3a.

To characterize the activation of p53 by the combined drug treatment with DRB, roscovitine, and nutlin-3a, we analyzed the kinetics of p53 activation. Cells were treated with low doses of roscovitine, DRB, or nutlin-3a individually or in combination, and the levels of β-galactosidase enzyme were measured over a 16-h time course. As shown in Fig. 2A, combined drug treatment results in a rapid increase in p53 activity. In contrast, any of the single drug treatments activate p53 poorly. Western blot analysis of the DRB/roscovitine/nutlin-3a–treated cells reveals a direct correlation between the β-galactosidase activity and the rapid accumulation of p53 protein seen as early
as 3 h after drug treatment (Fig. 2B). This is followed by a delayed appearance of p21. Treatment with 2.5 μmol/L nutlin-3a alone also induced p53 protein accumulation with similar kinetics as the triple drug treatment whereas low doses of roscovitine and DRB alone did not alter the levels of p53 protein when compared with the DMSO-treated control (Fig. 2A and B; data not shown). To analyze if the kinetics of p53 accumulation in cells treated with the triple drug combination is similar to that in cells treated with roscovitine or DRB alone, we used higher concentrations of roscovitine and DRB that are known to induce p53 protein accumulation. When cells were treated with 50 μmol/L DRB, p53 can be detected as early as 3 h after drug treatment (Fig. 2D). Roscovitine-treated cells showed a slight delay in the accumulation of p53 (Fig. 2D). Our results show that the kinetics of drug-induced activation of p53 and accumulation of p53 protein were comparable under combined drug treatment with roscovitine, DRB, and nutlin-3a or single-drug exposure to DRB or nutlin-3a.

**Drug Combination Leads to Nuclear Accumulation of p53 and Activation of p53 Signaling Pathways**

To study how the drugs affect p53 protein levels and p53-dependent pathways, we analyzed the cellular localization of p53 by immunostaining and the protein levels of p53 and p53 transcription targets by Western blot. Immunofluorescence studies showed a rapid nuclear accumulation of p53 protein as early as 3 h after combined treatment with DRB, roscovitine, and nutlin-3a alone (Fig. 3A), which persists beyond 16 h of drug treatment (data not shown). However, p53 nuclear staining is not observed in cells treated with 5 μmol/L roscovitine or 20 μmol/L DRB. In agreement with this, Western blot analysis showed that cells treated with 2.5 μmol/L nutlin-3a showed a mild increase in p53 levels whereas cells treated with 5 μmol/L roscovitine or 20 μmol/L DRB showed no change in p53 levels (Fig. 3B). However, the increase became evident when the drugs were combined, indicating a synergistic induction of p53 by roscovitine, DRB, and nutlin-3a (Fig. 3B). The synergistic up-regulation of p53 levels

*FIGURE 1. Enhanced induction of p53 activity through combination of low doses of DRB, roscovitine, and nutlin-3a. A. Differential effects of DRB, roscovitine (ros), and nutlin-3a on p53 transcriptional activity. ARN8 cells, a subclone of A375 melanoma cell line that contains a stably integrated p53 response element reporter construct, were exposed to various concentrations of DRB, roscovitine, and nutlin-3a for 16 h. p53 transcriptional activity was assessed by changes in the level of β-galactosidase as described in Materials and Methods and represented as fold activation over DMSO (0.1%)–treated controls. B. Effects of combined drug treatment with roscovitine and nutlin-3a on p53 transcription activity. ARN8 cells were exposed to 2.5 μmol/L nutlin-3a only or, in addition, increasing concentrations of roscovitine for 16 h. C. Effects of increasing concentrations of DRB on p53 transcriptional activity in the presence of 5 μmol/L roscovitine and/or 2.5 μmol/L nutlin-3a. The levels of β-galactosidase were determined 16 h after drug treatment. D. Summary of the induction of p53-dependent transcription in ARN8 cells with fixed dose combinations of DRB, roscovitine, and nutlin-3a at 20, 5, and 2.5 μmol/L, respectively, or individual drug treatments (data from C). E. Effects of dose titration of nutlin-3a on the induction of p53-dependent transcription in the presence of roscovitine and DRB. Unless otherwise stated, each result is a mean of at least three independent experiments; bars, SD.
is consistent with the synergistic activation of p53-dependent transcription in ARN8 cells.

We next asked if the drug combination imposed any genotoxic stress on the cells. Phosphorylation of Ser\(^{15}\) on p53 has been shown to be ATM dependent and is synonymous with the activation of DNA damage–dependent pathways in response to cellular insults (34-36). We therefore probed for Ser\(^{15}\) phosphorylation on p53. The lack of induction of Ser\(^{15}\) phosphorylation on p53 by DRB, roscovitine, and nutlin treatment despite the accumulation of high levels of p53 is in agreement with previous findings that DRB and nutlin are nongenotoxic drugs (Fig. 3B). Using a phospho-H2AX detection ELISA method to assess the levels of phospho-H2AX, a surrogate marker for DNA double-strand breaks, we verified that this drug combination does not result in detectable DNA damage (Fig. 4; refs. 37, 38). However, the accumulated p53 is phosphorylated on Ser\(^{392}\), a phosphorylation event that has been proposed to regulate the transactivation of p53 and the oncogenicity of mutant p53 (Fig. 3B; ref. 39). In vitro, phosphorylation of Ser\(^{392}\) results in an increase in the tetramerization of p53 and a corresponding increase in the binding of p53 to its consensus DNA sequence (40).

The accumulation of p53 results in activation of selective p53 signaling pathways as evidenced by the increase in mRNA and protein levels of key p53-regulated genes such as p21, Puma, MDM2, PIG3, and Bax (Figs. 3B and 5) but not others (e.g., Noxa; data not shown). The mRNA and protein levels of these targeted genes largely reflected the increased activity of p53 under the various drug treatments as assessed by the reporter assay (Fig. 5), with the exception of the protein levels of the proapoptotic gene Bax. Taken together, our data suggest that p53 is activated in response to the triple drug combination via a DNA damage–independent and phospho-Ser\(^{15}\)–independent pathway. Rather, the observed increase in p53 activity is a direct consequence of the synergistic accumulation of active p53 protein.

To further explore the p53-dependent activities of the nutlin/CDK inhibitor combination on the activation of these genes, we used the colon carcinoma HCT116 p53\(^{+/+}\) cell line and its derivative, HCT116 p53\(^{-/-}\), which has both copies of p53...
FIGURE 3. Synergistic induction of p53 signaling by DRB, roscovitine, and nutlin-3a. A. Nuclear accumulation of p53 protein following 3-h drug exposure. Cells were stained for p53 (green) and visualized by fluorescence microscopy. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (blue). B. ARN8 cells were treated with various drug combinations of DRB (20 μmol/L), roscovitine (5 μmol/L), and nutlin-3a (2.5 μmol/L) as indicated or with DMSO (0.1%) as control for 16 h. In some cases, doxorubicin (1 μmol/L)–treated cells were included as positive control. Western blot showing accumulation of p53, MDM2, p21, PIG3, Puma, and Bax proteins. The graph above each blot shows the quantification of the protein levels by densitometry.
analysis, and (assays: and analyzed the extent of apoptosis by three independent system. We treated cells with the various drug combinations apoptosis assays, we consistently used ARN8 cells as a model to induce apoptosis. To make a comparison between the results DRB tically Enhanced by Combination with Roscovitine and expected, inhibition of p53 pathways by DDp53 leads to the suppressor of wild-type p53 functions (Fig. 6; ref. 41). As DDp53, in ARN8 cells, which functions as a dominant negative when we transiently overexpressed a p53 truncated protein, asyase-1 cleavage and proapoptotic proteins is further confirmed functional p53 in the induction of poly(ADP-ribose) polymer- 

Antitumor Cell Activity of MDM2 Antagonist Is Synergistically Enhanced by Combination with Roscovitine and DRB

Next, we determined whether p53 activation was sufficient to induce apoptosis. To make a comparison between the results of the drug-induced activation of p53 and the cell viability and apoptosis assays, we consistently used ARN8 cells as a model system. We treated cells with the various drug combinations and analyzed the extent of apoptosis by three independent assays: (a) cellular morphologic changes, (b) DNA content analysis, and (c) Annexin V staining at 24 and 48 h after drug exposure. Treatment with DRB, roscovitine, and nutlin-3a resulted in a dramatic increase in the apoptotic fraction, measurable as Annexin V-positive cells and sub-G1 population, when stained with propidium iodide in a time-dependent manner (Fig. 7B and C). In contrast, when cells were exposed to single-drug treatments with nutlin-3a, roscovitine, or DRB, the percentage of sub-G1 population or Annexin V-positive cells remains <5% even after 72 h (data not shown). As shown in Fig. 7B and C, the percentage increase in the apoptotic fraction at 48 h after drug exposure mirrors the extent of activation of p53 seen at 16 h (Fig. 1D), with the combination of roscovitine, DRB, and nutlin-3a exhibiting the most potent cytotoxic effect, followed by the combination of DRB and nutlin-3a. Combined drug treatment with roscovitine and nutlin-3a resulted in apoptosis at later time points (data not shown). To quantify the antiproliferative effect of roscovitine, DRB, and nutlin-3a, we used similar dose regimens and measured cell viability after 24 and 48 h. Results showed a dramatic reduction in the cell viability of ARN8 cells treated with combination of roscovitine, DRB, and nutlin-3a or combination of DRB and nutlin-3a whereas single-drug treatments with DRB or nutlin-3a showed a modest reduction in cell viability, compared with the DMSO-treated controls. These results show that although low dose of DRB or nutlin-3a is sufficient to induce low levels of p53 activity and p53-dependent inhibition of cell proliferation, combined drug treatment with low doses of roscovitine, DRB, and nutlin-3a effectively drives cells toward apoptosis.

To distinguish between synergistic and additive effects of the combination of roscovitine, DRB, and nutlin-3a on apoptosis, we adopted the Chou and Talalay method (42) to give a quantitative assessment of synergism or additive effects of the drug combination. Details of the statistical analysis are described in Materials and Methods. The extent of apoptosis is quantified as the percentage of Annexin V-positive cells and the dose-effect curves for each drug alone or the drug combination were experimentally determined (data not shown). The results indicated a synergistic interaction of roscovitine, DRB, and nutlin-3a in induction of apoptosis and an additive effect of the drug combination on cell proliferation; the average combination index value calculated from ED25 (25% effective dose), ED50, and ED75 is 0.57 for induction of apoptosis and 0.97 for reduction of cell viability (Table 1). Values of less than 1 indicate synergism and values equal to 1 indicate additive effects.

To confirm that the cytotoxicity of the combined drug treatment with roscovitine, DRB, and nutlin-3a is p53 dependent, we carried out an experiment in parallel using colon carcinoma HCT116 p53+/+ cells and its derived isogenic pair, HCT116 p53−/−. Treatment of wild-type p53 cells with the drug combination resulted in an increased sub-G1 fraction and high Annexin V positivity whereas background staining for Annexin V was observed for HCT116 p53−/− cells (Fig. 8). The antiproliferative and cytotoxic effects of this combined drug treatment were also observed in other tumor-derived cell lines containing wild-type p53, including MCF7 breast adenocarcinoma cell line and HepG2 human hepatoma cell line, suggesting a potential use for this drug combination in different clinical settings (Table 2).

Discussion

Many oncotherapeutics harness the ability of cell to mount an apoptotic response in response to DNA damage. However, their therapeutic efficacy is compromised by the nonspecific genotoxic collateral damage, which may lead to genomic instability, selection of drug-resistant tumor clones, and secondary malignancies. Nongenotoxic means to activate p53 would therefore be an attractive strategy for cancers with intact
p53 signaling pathways. The combination of genotoxic drugs with nongenotoxic nutlin has been reported to synergistically activate p53 functions, paving the way for the potential use of nutlin in combinatorial drug therapy (15, 43). Although the use of combination drugs/therapy has been described for decades, optimizing the combinations of various drugs at a wide dosage range has only recently been explored on a high-throughput scale (44, 45). Combinations provide the multiple levers to modify different pathways simultaneously, a welcomed strategy in manipulating biological signaling networks that often contain multiple, redundant, and/or cooperative pathways. The multiple levels of regulation of the p53 response in the presence of many forms of cellular stress underscore the central role of p53 in determining cell fate. p53 thus represents an attractive candidate for pharmacologic intervention in cancer therapy. Here, we report for the first time that the combination of CDK inhibitors, DRB and roscovitine, and a MDM2 antagonist, nutlin-3a, provides a nongenotoxic way to activate the antiproliferative and cytotoxic functions of p53 synergistically.

The high incidence of p53 pathway inactivation in human cancers has led to the development of diverse approaches to restore p53 functions. Restoration of p53 function leads to tumor regression in mice without affecting normal tissues. Recent studies established that loss of p53 function is required for tumor maintenance and that transient reactivation of p53 leads to tumor regression in vivo, showing the therapeutic potential of restoration of p53 against established tumors (46-48). However, these strategies should be carefully implemented given the potential risk of development of resistance to p53 reactivation as was shown in the Eμ-myc lymphoma mouse model containing a reversibly switchable p53 knock-in allele (46). Nevertheless, the demonstration of tumor regression in mice on p53 restoration suggests that restoration of p53 either by gene therapy approaches or pharmacologic interventions has interesting relevance to human tumors. Moreover, the therapeutic potential of the restoration of p53 function may be optimized through combinations of p53-activating signals (46).

It is now clear that the tight control of p53 activity is largely accomplished by its negative regulator MDM2. About half of all human tumors express wild-type p53, and a large proportion of these cancers overexpress MDM2 or lose expression of the MDM2 inhibitor p14ARF. Therefore, modulating MDM2 levels or the MDM2 interaction with p53 will likely have a significant effect on p53 tumor suppressor functions. Indeed, mice expressing a hypomorphic allele of MDM2, when crossed with mice expressing a mutant APC allele (these mice develop spontaneous intestinal adenomas), resulted in a significant reduction in intestinal adenoma formation and a concomitant up-regulation of p53 activity (49). Modest reduction in the levels of MDM2 provides a strong protective effect from
cancer, thereby supporting the notion that pharmacologic disruption of p53 and MDM2 can have therapeutic benefits in cancer patients (49). The advantage of nutlins as an anticancer modality is supported by the finding that transient induction of the p53 response by disruption of the p53-MDM2 interaction in normal somatic cells results in reversible growth arrest but not apoptosis (12, 13). Furthermore, treatment of nude mice with nutlin at therapeutic doses is well tolerated without significant adverse side effects (11). Our results support the use of nutlin as a therapeutic drug in cancers with wild-type p53. In addition, our data show that although nutlin-3a effectively activates p53, stabilization of p53 by nutlin-3a alone is not sufficient to fully activate the p53 response. The use of CDK inhibitors, DRB and roscovitine, strongly potentiates nutlin-3a-dependent induction of p53 activity. Nevertheless, the combination is strictly dependent on nutlin-3a because DRB and roscovitine together only weakly activate p53. Interestingly, we found that the extent of activation induced by the combination of DRB, roscovitine, and nutlin-3a is greater than that achieved by higher concentrations of nutlin-3a alone. This suggests that DRB and roscovitine do not simply enhance the efficacy of nutlin-3a but rather act in cooperative pathways to promote p53 activation. Synergistic activation of p53 results in p53-dependent cell death in ARN8 melanoma cells as shown by the apoptosis and cell viability assays. Similar effects were also observed in other tumor-derived cell lines including MCF7 breast adenocarcinoma cell line, HCT116 colon carcinoma cell line, and HepG2 human hepatoma cell line, suggesting that the mechanism(s) for the cooperative activation of p53 response by these drugs may be similar in these cell lines.

Of note, although roscovitine seems to contribute significantly to cell death and p53 promoter assay outputs when it is used in combination with DRB and nutlin-3a, it has little effect on target gene expression. One possible explanation is that roscovitine inhibits RNA polymerase II-dependent transcription and influences p53 transactivation-independent functions, which contribute to the cytotoxic effects of the drug combination. Transcription inhibition has been linked to p53-dependent cell death. Although the exact underlying mechanisms are unclear, recent studies support a role for p53 transactivation-independent functions at the mitochondria. Blockade of RNA polymerase II–dependent transcription has been shown to result in the translocation of p53 to mitochondria at later time points of drug treatments, an event that precedes changes in mitochondrial membrane potential, release of cytochrome c, and procaspase-3 activation (50). Recent observations also show that p53 can translocate to the mitochondria, directly resulting in p53 transactivation–independent apoptosis (51, 52). Additionally, the inhibition of transcription also leads to an override of the G1-S checkpoint and cells undergo apoptosis on entry into S phase (50). It will be of interest to further investigate the mechanisms of roscovitine when used in combination with DRB and nutlin in the light of these observations.

Relatively high concentrations of DRB and roscovitine have been shown to induce apoptosis by p53-dependent and p53-independent mechanisms and have thus been proposed as potential therapeutic agents. Our studies have identified a potential use for significantly lower concentrations of DRB and roscovitine as “sensitizers” of the p53 response to therapeutic doses of nutlin-3a. One of the therapeutic advantages of this synergistic effect is an effective dilution of any general cytotoxicity that is associated with high concentrations of each of these drugs to achieve similar or higher levels of p53 activation. Given that both roscovitine and nutlin-3a are not sufficiently dose potent on their own, requiring high drug concentrations in intravenous injections and oral administration, combined drug treatment with roscovitine, nutlin-3a, and DRB may provide a new therapeutic approach to increase drug efficacy with reduced toxicity to normal tissues. Although nutlin has been reported to result in p53-independent apoptosis (53), our results in HCT116 cells show that nutlin, in combination with DRB and roscovitine, promotes cell death strictly in a p53-dependent manner. The discrepancy between our observations and the previous studies could be due to the 6-fold difference in the concentrations of nutlin administered to the cells as well as the use of different cell lines. This again emphasizes our rationale for the use of low doses of drugs in combination such that the general cytotoxicity associated with any of the drugs is greatly reduced. In addition, given that many cellular signals may engage p53 through completely different pathways and cancer cell lines may have suffered invariant inactivation of these pathways, targeting multiple cellular pathways using therapeutic doses of drug combinations may provide a fail-safe mechanism to activate p53 functions.

Our data suggest that modification of at least three distinct signaling pathways leads to the synergistic activation of p53: (a) interference of p53-MDM2 regulatory loop, (b) inhibition of roscovitine-sensitive kinases, and (c) DRB-sensitive kinases. Roscovitine and DRB, both potent inhibitors of transcription, activate p53 effectively when used at higher concentrations. In our study, roscovitine, used at a subthreshold concentration that neither inhibits cellular proliferation nor induces cell death, has a significant effect on cell death when in combination with DRB and nutlin-3a. Our data highlight the rationale that roscovitine

![FIGURE 6. Poly(ADP-ribose) polymerase-1 (PARP-1) cleavage and p21, Puma, MDM2, and PI3G3 protein accumulation are dependent on p53 activity. Left, HCT116 p53+/+ and HCT116 p53−/− cells were treated with roscovitine (5 μmol/L), DRB (20 μmol/L), and nutlin-3a (2.5 μmol/L) for 16 h and Western blot analysis was done for the relevant proteins. Right, ARN8 cells transiently transfected with plasmid encoding dominant negative p53 truncated protein, DDp53, or pCMVneo vector only and treated with similar drug dose regimens as HCT116 cells.](image)
and DRB may inhibit different processes that contribute to the drug synergy. This is supported by gene expression analyses showing that DRB and roscovitine inhibit the transcription of overlapping and distinct subsets of genes and by in vitro assays further showing that there are differences in their kinase specificities (18, 54). One drug compound that shows varying inhibitory activity toward multiple CDK family members (CDK1, CDK2, CDK4, CDK6, and CDK7) and inhibits gene expression broadly with a similar profile to DRB is flavopiridol (54, 55). Both DRB and flavopiridol inhibit CDK9 and, hence, block RNA polymerase II–dependent transcription. Moreover, DRB can substitute flavopiridol in drug combinations, suggesting that both drugs share similar mechanisms of action (56). Given that flavopiridol inhibits RNA polymerase II–dependent transcription at much lower drug concentrations (<100 nmol/L) compared with DRB, which inhibits transcription when used in the micromolar concentrations (>10 μmol/L), flavopiridol may be an attractive drug compound to explore for use in combination with roscovitine and nutlin-3a (57, 58). Flavopiridol is currently in phase I and II clinical trials.

Current advances in technology platforms have allowed for the large-scale screening and molecular profiling of drug combinations. Phenotypic screening of drug combinations independent of the knowledge of the mechanistic basis of drug action provides a fast and efficient way to identify novel drug combinations. New drug combinations may provide a way to lower the effective chemotherapy doses of existing compounds or, in some cases, may lead to the discovery of combinations

![FIGURE 7](image_url)

**FIGURE 7.** Synergistic induction of apoptosis in ARN8 melanoma cells. A. Phase-contrast images of ARN8 cells treated with drugs as indicated. B. Cytotoxic effects of combined drug treatment with DRB, roscovitine, and nutlin-3a. Cells were incubated with indicated combinations of DRB (20 μmol/L), roscovitine (5 μmol/L), and nutlin-3a (2.5 μmol/L) or with single drugs for 24 and 48 h, and DNA content was monitored by flow cytometry. DNA content histograms representative of a single experiment are shown. The percentage of sub-G1 population was calculated from the DNA content histograms and represented in the graph on the right. C. Cells were treated as in B and harvested, stained without fixation with Annexin V, and analyzed by fluorescence-activated cell sorting. Annexin V-FITC–positive cells were represented as a percentage of the total cell population. D. Combined drug treatment significantly reduces viability compared with any single-drug exposure. Survival of ARN8 cells after 24 or 48 h of exposure to the same conditions as in C. Control incubations with DMSO (0.1%) were always included, and the relative viability in each assay was calculated as a percentage of live cells relative to the live cell fraction in the control (set as 100%). Unless otherwise stated, data represent means of three independent experiments; bars, SD.
Table 1. Combination Index Values for the Apoptotic and Inhibitory Effects of the Drug Combination

(A) Combination Index Values for Apoptotic Effects of Roscovitine, DRB, and Nutlin-3a

<table>
<thead>
<tr>
<th></th>
<th>ED_{25}</th>
<th>ED_{50}</th>
<th>ED_{75}</th>
<th>Averaged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roscovitine</td>
<td>0.65</td>
<td>0.56</td>
<td>0.51</td>
<td>0.57</td>
</tr>
<tr>
<td>DRB</td>
<td>1.35</td>
<td>0.83</td>
<td>0.71</td>
<td>0.97</td>
</tr>
</tbody>
</table>

NOTE: Values were determined experimentally and calculated using the Chou-Talalay method (42) and CalcuSyn software (Biosoft). Apoptosis and cell death were assessed 48 h following drug treatment.

ED_{25} refers to the effective dose resulting in 25% increase in cell death over the total cell population.

Average combination index value is calculated from ED_{25}, ED_{50}, and ED_{75}.

ED_{25} refers to the effective dose required for 25% reduction in cell viability relative to DMSO-treated controls.

(B) Combination Index Values for Inhibitory Effects of Roscovitine, DRB, and Nutlin-3a on Reduction of Cell Viability

<table>
<thead>
<tr>
<th></th>
<th>ED_{25}</th>
<th>ED_{50}</th>
<th>ED_{75}</th>
<th>Averaged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roscovitine</td>
<td>0.65</td>
<td>0.56</td>
<td>0.51</td>
<td>0.57</td>
</tr>
<tr>
<td>DRB</td>
<td>1.35</td>
<td>0.83</td>
<td>0.71</td>
<td>0.97</td>
</tr>
<tr>
<td>Nutlin-3a</td>
<td>1.35</td>
<td>0.83</td>
<td>0.71</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Drug Treatments and Transient Transfections

Cells were seeded 24 h before incubation with drugs for 16 h unless otherwise stated. Cells treated with 0.1% DMSO were always included as controls. For transient transfections, cells were plated at a low cell density (6 x 10^5 per 10-cm plate) and then, 24 h later, transfected with plasmid using FuGENE 6 (Roche) according to the manufacturer’s instructions.

Materials and Methods

Cells, Antibodies, and Reagents

A375 melanoma cells and its subclone ARN8 (containing a stably transfected p53-dependent β-galactosidase gene reporter construct, RGCΔfos lacZ) have previously been described (26). MCF7 and HepG2 cell lines were obtained from American Type Culture Collection. HCT116 p53\(^{+/+}\) and p53\(^{--/--}\) cells were kindly gifts from Dr. B. Vogelstein (John Hopkins Kimmel Cancer Center, John Hopkins University School of Medicine, Baltimore, MD); ARN8, MCF7, and HepG2 were cultured in DMEM and HCT116 cells in McCoy’s 5A, all supplemented with antibiotics, 10% FCS (Life Technologies, Inc.), and 2 mmol/L glutamine. Plasmid pCMV-neoDDp53 coding for a dominant negative truncated mouse p53 protein (amino acid residues 1-14 and 302-390) under the control of CMV promoter was previously described (41). The following antibodies were used: mouse monoclonal antibodies DO-1 (anti-p53), 118 (anti-p21), 2A9 (anti-MDM2), and Sp3 (anti–phospho-Ser\(^{392}\) p53), kind gifts from Dr. B. Vogelstein (John Hopkins Kimmel Cancer Center, John Hopkins University School of Medicine, Baltimore, MD); anti-Bax (BD Biosciences); anti-β-actin (AC-74; Sigma Chemical); rabbit polyclonal anti-Puma (Ab-1; EMD Biosciences); and rabbit polyclonal anti-PIG3 (PC268; Calbiochem). Secondary antibodies were antirabbit horseradish peroxidase and antiumose horseradish peroxidase (Amersham Biosciences). Roscovitine, DRB, and nutlin-3a (Calbiochem) were kept as 10 mmol/L stock in DMSO (Sigma) at -20°C.

Our study exemplifies the use of drug combinations to increase the potency of drug compounds, which lack dose potency as individual therapeutic agents, through the targeting of the p53 tumor suppressor pathway. Currently, combination therapy in the clinic often involves the use of a chemosensitizer that induces collateral DNA damage leading to secondary malignancies. Herein, we describe a novel combination of CDK inhibitors and nutlin, which retains the lack of genotoxic stress induction characteristic of individual compounds. Given the recent demonstration of the use of nutlin in combination with a genotoxic drug in reducing tumor burden in a preclinical retinoblastoma model, the next key step would be to test the efficacy of this proposed drug combination in vivo (14). In summary, our data highlight the therapeutic potential of nongenotoxic CDK kinase inhibitors as potentiators of MDM2 antagonists in cancers with a wild-type p53 pathway and support the rationale to further validate the therapeutic potential of this drug combination.
Table 2. Effect of the Drug Combination on the Cell Viability of Various Tumor-Derived Cell Lines

(A) Cell Viability of p53-Positive Tumor-Derived Cells Exposed to Combined or Single-Drug Treatments with Roscovitine (5 μmol/L), DRB (20 μmol/L), and Nutlin-3a (2.5 μmol/L)

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Roscovitine + DRB + Nutlin-3a</th>
<th>DRB</th>
<th>Roscovitine</th>
<th>Nutlin-3a</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARN8</td>
<td>20.41 ± 1.26</td>
<td>76.82 ± 4.71</td>
<td>109.6 ± 4.52</td>
<td>81.45 ± 5.82</td>
</tr>
<tr>
<td>MCF7</td>
<td>22.74 ± 1.44</td>
<td>51.97 ± 2.49</td>
<td>91.45 ± 4.01</td>
<td>90.53 ± 4.55</td>
</tr>
<tr>
<td>HCT116</td>
<td>8.51 ± 0.99</td>
<td>45.81 ± 4.60</td>
<td>106.24 ± 5.46</td>
<td>85.21 ± 4.69</td>
</tr>
<tr>
<td>HepG2</td>
<td>19.43 ± 3.97</td>
<td>53.70 ± 1.11</td>
<td>102.30 ± 3.04</td>
<td>87.85 ± 4.94</td>
</tr>
</tbody>
</table>

(B) Induction of Apoptosis in p53-Positive Tumor-Derived Cells Exposed to Combined or Single-Drug Treatments with Roscovitine (5 μmol/L), DRB (20 μmol/L), and Nutlin-3a (2.5 μmol/L)

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Roscovitine + DRB + Nutlin-3a</th>
<th>DRB</th>
<th>Roscovitine</th>
<th>Nutlin-3a</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARN8</td>
<td>58.23 ± 1.34</td>
<td>2.06 ± 0.37</td>
<td>3.01 ± 1.01</td>
<td>5.63 ± 0.55</td>
</tr>
<tr>
<td>MCF7</td>
<td>52.33 ± 2.45</td>
<td>8.02 ± 0.78</td>
<td>3.46 ± 0.89</td>
<td>4.32 ± 0.79</td>
</tr>
<tr>
<td>HCT116</td>
<td>73.26 ± 2.89</td>
<td>19.06 ± 2.82</td>
<td>7.89 ± 0.98</td>
<td>12.32 ± 1.28</td>
</tr>
<tr>
<td>HepG2</td>
<td>50.23 ± 1.57</td>
<td>7.08 ± 0.77</td>
<td>4.05 ± 1.71</td>
<td>6.06 ± 1.11</td>
</tr>
</tbody>
</table>

NOTE: Cell viability in A is assessed using the WST-1 Cell Proliferation Reagent (Roche). Cell viability refers to the percentage of live cells relative to the live cell fraction in DMSO (0.1%)–treated controls after 48 h drug treatment. Apoptosis was scored as a percentage of Annexin V–positive cells over the total cell population.

Immunostaining and Fluorescence Microscopy

Indirect immunofluorescence was carried out as described previously (29). DO-1 (anti-p53) and antimouse Alexa 488–coupled secondary antibody (Molecular Probes) were used. Immunofluorescence was visualized with a AxioImager Z1 (Zeiss).

Western Blotting

Cells were harvested and lysed in NP40 lysis buffer [50 mmol/L Tris- HCl (pH 8.0), 150 mmol/L NaCl, 1% NP40, 5 mmol/L EDTA (pH 8.0)] supplemented with protease and phosphatase inhibitor cocktails (Sigma). Conditions for SDS-PAGE were described previously (59). Densitometry analysis was carried out using QuantifyOne (Bio-Rad). At least three independent experiments were done for each data set.

Quantitative Reverse Transcription-PCR

Total RNA was isolated using the RNeasy kits (Qiagen). The RNA was quantitated by spectrophotometric analysis and used for quantitative real-time PCR. The primers used for each target analyzed are available on request. The RNA Master Power SYBR Green Mix (Roche) was used for quantification of mRNA levels.

β-Galactosidase Enzyme Assay

Cells were cultured in 96-well plates for 24 h before incubating with drugs for 16 h. The levels of β-galactosidase were then quantified with FluoroReporter lacZ Quantification Kit (Invitrogen) according to the manufacturer’s instructions. Fluorescence was measured with a SPECTRAMax (Molecular Devices). At least three independent experiments were done for each data set.

Cell Viability Assay

Cells were seeded in 96-well plates appropriately for their doubling time (3,000 per well, except for ARN8 cells, which were seeded at 2,000 per well) before incubation with drugs. The plates were assayed at the indicated time points after drug treatment using WST-1 Cell Proliferation Reagent (Roche). Absorbance was read at 440 nm (VERSAmax, Molecular Devices) and the cell viability expressed as a percentage of live cells relative to the live cell fraction in DMSO-treated controls.

Cell Cycle and Apoptosis Assays

Cells were collected by trypsinization and washed twice in PBS. For analysis of cell cycle distribution, cells were fixed in 70% ethanol-PBS solution and stained with propidium iodide–containing solution [25 μg/mL propidium iodide supplemented with 1 mg/mL RNase A, in PBS (pH 7.8); Sigma Chemical] at room temperature for 15 min. For apoptosis assay, cells were harvested without fixation. Apoptosis was evaluated with the Annexin V (FITC)-propidium iodide binding assay (Roche). The extent of apoptosis was quantified as a percentage of Annexin V–positive cells over the total cell population. Flow cytometric analysis was done on a FACSCalibur system (BD Biosciences).

H2AX Phosphorylation Assay

Phosphorylation of H2AX on Ser139 was detected by a chemiluminescence-based assay according to the manufacturer’s instructions (Upstate). The level of H2AX phosphorylation in drug-treated samples relative to the DMSO-treated control was determined. At least three independent experiments were done for each data set.

Statistical Analysis

Synergism, additive effects, or antagonism was assessed using the Chou-Talalay method (42, 60) and CalcuSyn software (Biosoft). The extent of apoptosis in drug-treated samples was quantified as a percentage of Annexin V–positive cells over the total cell population; apoptosis in DMSO-treated controls was
negligible. Cell viability was determined with WST-1 cell proliferation reagent as described above. The effect on cellular proliferation is shown as a percentage reduction of cell viability when compared with DMSO-treated controls. The dose-effect curve for each drug or drug combination was determined and the combination index (CI) value for the experimental combination was determined according to the equation:

\[
CI = \frac{D_A/(D_{xa})_A + D_B/(D_{xb})_B}{D_A/(D_{xa})_A + D_B/(D_{xb})_B + D_B/(D_{xb})_B(D_{xa})_A(D_{xb})_A}
\]

where \(D_A\) and \(D_B\) are the doses of drug A and drug B that have \(x\) effect when used in combination, and \((D_{xa})_A\) and \((D_{xb})_B\) are doses of drug A and drug B that have the same \(x\) effect when used individually. The combination index was calculated based on the more stringent statistical assumption that drugs A and B are mutually nonexclusive drugs. The combination index provides a numerical description of the combination effects. Combination index values of <1, 1, or >1 represent synergism, an additive effect, or antagonism, respectively. To determine if DRB and roscovitine may potentiate the effects of nutlin-3a, we tested the combination effects when nutlin-3a treatment is complemented with roscovitine + DRB in a fixed dose ratio of 1:2:8 (nutlin-3a/roscovitine/DRB). Drug A and drug B hence represent nutlin-3a and roscovitine + DRB.

References

Molecular Cancer Research

Cyclin-Dependent Kinase Inhibitors Sensitize Tumor Cells to Nutlin-Induced Apoptosis: a Potent Drug Combination

Chit Fang Cheok, Anwesha Dey and David P. Lane


Updated version  Access the most recent version of this article at:
http://mcr.aacrjournals.org/content/5/11/1133

Cited articles  This article cites 59 articles, 31 of which you can access for free at:
http://mcr.aacrjournals.org/content/5/11/1133.full.html#ref-list-1

Citing articles  This article has been cited by 5 HighWire-hosted articles. Access the articles at:
/content/5/11/1133.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.