NEDD8-Targeting Drug MLN4924 Elicits DNA Rereplication by Stabilizing Cdt1 in S Phase, Triggering Checkpoint Activation, Apoptosis, and Senescence in Cancer Cells

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Abstract

MLN4924 is a first-in-class experimental cancer drug that inhibits the NEDD8-activating enzyme, thereby inhibiting cullin-RING E3 ubiquitin ligases and stabilizing many cullin substrates. The mechanism by which MLN4924 inhibits cancer cell proliferation has not been defined, although it is accompanied by DNA rereplication and attendant DNA damage. Here we show that stabilization of the DNA replication factor Cdt1, a substrate of cullins 1 and 4, is critical for MLN4924 to trigger DNA rereplication and inhibit cell proliferation. Even only 1 hour of exposure to MLN4924, which was sufficient to elevate Cdt1 for 4–5 hours, was found to be sufficient to induce DNA rereplication and to activate apoptosis and senescence pathways. Cells in S phase were most susceptible, suggesting that MLN4924 will be most toxic on highly proliferating cancers. Although MLN4924-induced cell senescence seems to be dependent on induction of p53 and its downstream effector p21Waf1, we found that p53−/− and p21−/− cells were even more susceptible than wild-type cells to MLN4924. Our results suggested that apoptosis, not senescence, might be more important for the antiproliferative effect of MLN4924. Furthermore, our findings show that transient exposure to this new investigational drug should be useful for controlling p53-negative cancer cells, which often pose significant clinical challenge.

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Introduction

Duplication of the genetic material is a key event in the cell cycle. In eukaryotes, replication origins are recognized and bound by a 6-subunit complex called ORC (Origin Recognizing Complex; refs. 1–3). Cdc6 and Cdt1 are subsequently recruited independently to those sites in late M or early G1 phase (1, 3, 4), followed by the recruitment of Mcm2–7 complex to initiate DNA replication (5, 6). It is vitally important that the initiation of replication at replication origins is tightly controlled such that it occurs only once during the cell cycle. Mammalian cells have developed different mechanisms to prevent reinitiation and subsequent rereplication of DNA within the same cell cycle. One such mechanism is the inactivation of Cdt1 during S and G2 phases (7, 8). After replication initiation, Cdt1 is either inhibited by a small protein called Geminin (9, 10) or degraded by 2 distinct E3 ligases, cdk-dependent SCFkip2 and Cul4-DDB1dr2 in S or G2/M phase (8, 11). Deregulation of those pathways by depletion of Geminin, Cul4, or Cdt2 activates (or stabilizes) Cdt1 and consequently induces DNA rereplication in different systems (7, 12–14).

Studies have shown that cullin-RING ligases (CRL), a subclass of E3 ligases that includes both SCFkip2 and CRL4dr2, are modified by an ubiquitin-like protein, NEDD8, which subsequently facilitates their ligase activities (15–18). Thus, through the modulation of this activity, the NEDD8 pathway regulates the abundance of CRL substrates. MLN4924, a potential cancer drug currently in phase I clinical trials, is a small molecule inhibitor of NEDD8-activating enzyme (NAE; refs. 19, 20). MLN4924 treatment in HCT116 human colon cancer cell line inhibits NAE, and therefore the NEDD8 conjugation pathway, resulting in an increase in protein abundance of CRL substrates such as Cdt1 (21). This is accompanied by an increase in the percentage of cells containing more than 4N DNA, indicating DNA rereplication was occurring. Cells treated with MLN4924 also undergo significant apoptosis contributing to the drug's antiproliferative activity. Various CRL substrates play critical functions in cellular growth and survival pathways and the question remained as to which substrates are critical for MLN4924-induced rereplication and apoptosis.

In this article, we examine whether Cdt1 is the key factor for the induction of DNA rereplication in HCT116 cells treated with MLN4924. Among the different approaches for stimulating Cdt1 activation, MLN4924 shares a similarity with that of Cdt2 depletion in inactivating the CRL4dr2 E3 ligase, as
opposed to Geminin depletion, which activates Cdt1 by a different pathway. We verified this hypothesis and detected a synergistic effect between MLN4924 treatment and Geminin depletion. Transient exposure of cells to MLN4924 led to DNA rereplication, as well as activation of the apoptosis and senescence pathways. This allowed us to test whether a specific part of the cell cycle was particularly susceptible or resistant to MLN4924. Finally, we compared the sensitivity of wild-type (WT) HCT116 cells and isogenic p53<sup>−/−</sup> or p21<sup>−/−</sup> HCT116 cells to MLN4924 and discovered that WT HCT116 cells were less susceptible to MLN4924-induced cell death. The results indicate that p53-deficient cancer cells may be more sensitive to MLN4924, emphasizing the therapeutic opportunity with this class of investigational drugs.

**Materials and Methods**

**Cell lines and chemicals**

Human colorectal cancer cell lines HCT116 (WT, p53<sup>−/−</sup>, p21<sup>−/−</sup>) were cultured in McCoy’s 5A-modified medium (HyClone) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Isogenic p53<sup>−/−</sup> and p53<sup>−/−</sup> HCT116 cell lines were described earlier (22). Millennium Pharmaceuticals Inc. provided MLN4924, which was then dissolved in DMSO (Sigma). The concentration of Z-Vad-FMK (Calbiochem) used was 50 μmol/L. The concentration of nocodazole (Sigma) used was 40 ng/mL.

**siRNA**

Short interfering (siRNA) oligonucleotides (Invitrogen) were made to the following target sequences (sense): GL2 (control), AACGUACCGGAAUACUUCGA; Cdt1, GCAAU-GUUGGCCAGAUCA; Cdc6, GAUCGAUCUAUCAGGUAU; Mcm7, GAUGUCCUGACGUUUACA; Geminin (Gem), UGC-CAAACCGGAUCAAA; Cdt2, GAAUUAUACUGCUUAUGCUAAGUCA. Transfections were conducted with 20 nmol/L siRNA oligonucleotide duplexes with Lipofectamine RNAiMax (Invitrogen) according to the instructions of manufacturer.

**Antibodies and immunoblotting**

Rabbit anti-Cdt1, rabbit anti-Geminin, and rabbit anti-Cdt2 were raised as described (9, 23). The purchased antibodies were mouse anti-p21 (Lab Vision/Neomarkers); mouse anti-β-actin, mouse anti-Chk1, mouse anti-Chk2 (Sigma); rabbit anti-Chk1-P-S317, rabbit anti-Chk2-P-T68; rabbit anti-PARP, rabbit anti-H3-P-S10 (Upstate). Cells were lysed as described (24), and Western blot analysis was conducted according to standard procedures.

**Flow cytometric analysis (FACS)**

Cells were harvested by trypsinization and fixed with 70% ethanol overnight at −80°C. Cells were then stained and analyzed as described before (12). For FACS analysis with both propidium iodide (PI) and bromodeoxyuridine (BrdU) double staining, cells were labeled with 10 μmol/L BrdU (Sigma) and then harvested as described earlier (12).

**Time-lapse movie analysis**

HCT116 cells were plated at 15,000 cells per well in 6-well culture plates (Becton Dickinson). For continuous treatment, cells were treated with 1 μmol/L MLN4924 for 72 hours. For washout treatment, HCT-116 cells were treated with 1 μmol/L MLN4924 for 8 hours, washed with fresh media to remove compound, and then maintained in fresh compound-free media for 8 days. Time-lapse movie images were taken at times indicated using an automated TE2000U microscope (Nikon Instruments) with Hoffman-modulation optics, 20× objective, with environmental control, and an Orca-ER CCD camera (Hamamatsu) controlled with MetaMorph imaging software (Molecular Devices).

**Measure cell growth and clonogenicity**

The number of viable cells was estimated with a cell proliferation assay (MTT) kit (Promega) according to the manufacturer’s instructions. Cells were seeded into 96-well plates at 500 cells per well, treated with DMSO or MLN4924, and incubated for 7 days before MTT assay. Cell clonogenicity assay was conducted as described (25). Cells were seeded into 6-well plates at 3 × 10<sup>5</sup> cells per well. DMSO or 1 μmol/L MLN4924 was added for 8 hours. Cells were washed twice with PBS and incubated in fresh medium after the washout. Medium was changed every 2–3 days and the colonies were stained with crystal violet to show cell clonogenicity. OD595 was measured to quantify cell colony numbers and normalized to DMSO-treated control sample to obtain cell survival rate.

**SA-β-gal staining assay for senescence**

Senescence β-galactosidase staining assay was conducted in a 6-well plate with staining kit (Cell Signaling Technology; no. 9860). Cells were washed with PBS, fixed, and stained following manufacturer’s instruction. Stained plates were checked under a microscope for development of blue color. For each sample, SA-β-gal-positive and total cell numbers were counted from 5 different microscopic fields (roughly >200 cells per field).

**Results**

**Stabilization of Cdt1 protein is critical for MLN4924-induced rereplication in HCT116 cells**

Consistent with previous results (21), we observed rereplication after 20 hours of treatment of HCT116 cells with MLN4924 (Fig. 1A). To investigate whether the regulation of Cdt1 protein level plays a role in MLN4924-induced rereplication, HCT116 cells were treated with siRNA oligonucleotides targeting Cdt1 for 48 hours prior to the addition of MLN4924. After 20 hours of MLN4924 treatment, Cdt1 protein level increased significantly as expected (Fig. 1B, lanes 1 and 2) and more than 40% of cells were determined to have rereplicated, containing >4N DNA content. However, in the cells depleted of Cdt1 by siRNA, the percentage of rereplicating cells reduced to 15% (Fig. 1A). In these cells, Cdt1 protein expression was effectively repressed (Fig. 1B, lanes 1, 3, and 4), although at a higher exposure, Cdt1 protein level was observed to be modestly induced in.
MLN4924-treated cells (Fig. 1B, lanes 3 and 4), indicating that the drug was still inhibiting its degradation, potentially explaining the 15% of cells with >4N DNA.

Rereplication has been shown to induce both single-strand and double-strand DNA breaks, resulting in activation of checkpoint pathways (24, 26). Indeed, we observed both Chk1 and Chk2 phosphorylation in MLN4924-treated cells (Fig. 1B, lanes 1 and 2). Cdt1 siRNA treatment not only decreased the percentage of cells undergoing rereplication but also decreased the activation of Chk1 and Chk2 (Fig. 1B, lanes 2 and 4), indicating that MLN4924 induces DNA damage primarily through Cdt1-dependent rereplication.

To characterize whether other replication initiators contribute to the rereplication induced by MLN4924, we systematically depleted other components of the prereplicative complex. We treated HCT116 cells hypomorphic for ORC2 (27) with MLN4924 and compared the extent of rereplication with that of WT cells. As shown before, although we could detect ORC2 in 6 μg of extract from WT HCT116 cells, it was hard to detect ORC in even 60 μg of e83 HCT116 cells (Supplementary Fig. S1A). Despite this, there was no significant difference in the amount of rereplication between these cell types (Fig. 1C). Furthermore, we conducted siRNA knockdown of both MCM7 and Cdc6 (Supplementary Fig. S1B and C) and observed no difference in the amount of MLN4924-induced rereplication in both cases (Fig. 1D, 1E). Interestingly, we noticed that MLN4924 treatment could also induce Cdc6 protein expression (Supplementary Fig. S1C), which indicated that Cdc6 could be a potential CRL substrate. Overall, these results show that the ubiquitin-dependent degradation of Cdt1 protein is the rate-limiting step in preventing rereplication and that stabilization of this component of the prereplicative complex by MLN4924 induces rereplication in HCT116 cells. The rereplication leads to DNA damage and activates checkpoint pathways.

**MLN4924 induces rereplication through the inhibition of CRL4<sub>Cdt2</sub>**

Because MLN4924 functions as an NAE inhibitor (21), it is expected to inhibit all cullins, including Cul1 and Cul4.
ubiquitin ligases known to degrade Cdt1 (28–32). Cdt2 depletion, and thus inactivation of CRL4Cdt2, in zebra fish, *Xenopus* egg extracts, and human cancer cells induces DNA rereplication (13, 33). If CRL4Cdt2 inhibition is the primary mechanism by which MLN4924 causes rereplication, one would predict that there should be no synergy between MLN4924 and siCdt2 in induction of rereplication.

To determine whether MLN4924 acts through the same mechanism to induce rereplication as that of Cdt2 depletion, we compared the Cdt1 protein level in cells depleted of Cdt2 by siRNA or treated with MLN4924. As seen in Figure 2A, Cdt2 depletion caused less Cdt1 accumulation than MLN4924 alone (Fig. 2A, lanes 2 and 3) and together the 2 stabilized Cdt1 more (lane 4). However, the extent of rereplication caused by siCdt2 was more than that observed in cells treated with MLN4924 alone, and adding the 2 together did not increase rereplication (Fig. 2B). This suggests that a) pure CRL4Cdt2 inhibition with siCdt2 is more effective at inducing rereplication than inhibiting all cullins by MLN4924, but b) once Cdt1 level has crossed a certain threshold, there is no further increase in rereplication with more Cdt1. In addition, other substrates/pathways affected by MLN4924 may make cells die, thereby decreasing the extent of rereplication observed.

In contrast to the lack of synergy in induction of rereplication by siCdt1 + MLN4924, siGeminin (to activate Cdt1 in S phase) + MLN4924 caused more rereplication than either agent alone. This is particularly evident when one considers the proportion of cells with >6N DNA content (Fig. 2B).

These results suggest that removal of an inhibitor of Cdt1 (Geminin) will act additively with stabilization of Cdt1 by MLN4924 to cause more rereplication. In contrast, inhibition of CRL4Cdt2 by siCdt2 and MLN4924 does not additively cause more rereplication, even though there was more stabilization of Cdt1. This result is consistent with the hypothesis that MLN4924 causes rereplication primarily through the inhibition of CRL4Cdt2.

**Transient exposure of HCT116 cells to MLN4924 induces rereplication**

One advantage of using MLN4924 treatment to induce rereplication lies in its ability to act rapidly. Previous studies have shown that in as little as 5 minutes following MLN4924 treatment, the NEDD8 pathway is inhibited concurrent with the accumulation of Cdt1 protein (20, 21). Therefore, we wanted to test whether transient treatment of MLN4924 is sufficient to induce rereplication in HCT116 cells.

Surprisingly, as shown in Figure 3A, 1-hour exposure to MLN4924 was sufficient to induce rereplication in 40% of the cells. The percentage of cells with rereplication increased with longer pulse of MLN4924 but was close to its maximum after 4 or 8 hours of treatment, with 60% of cells rereplicating their DNA.

To determine the rate of Cdt1 turnover following MLN4924 washout, we treated HCT116 cells with MLN4924 for 4 hours and harvested the cells after different time periods. Cdt1 protein level increased following MLN4924 treatment, the NEDD8 pathway is inhibited concurrent with the accumulation of Cdt1 protein (20, 21). Therefore, we wanted to test whether transient treatment of MLN4924 is sufficient to induce rereplication in HCT116 cells.

In contrast to the lack of synergy in induction of rereplication by siCdt1 + MLN4924, siGeminin (to activate Cdt1 in S phase) + MLN4924 caused more rereplication than either agent alone. This is particularly evident when one considers the proportion of cells with >6N DNA content (Fig. 2B).

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**S-phase cells are more susceptible to MLN4924-induced rereplication**

Because a mere 4-hour pulse treatment of MLN4924 leads to rereplication, we decided to test whether the portion of the cell cycle was more susceptible to the drug exposure. HCT116 cells synchronized by nocodazole block/mitotic shake-off were exposed to MLN4924 at the indicated times postmitosis (Fig. 4A). Cells were then collected for flow...
Both checkpoint and apoptosis pathways are activated upon short exposure of cells to MLN4924

Rereplication induces DNA damage and checkpoint activation (24, 26, 34). The initiation of DNA rereplication by a short exposure to MLN4924 led us to test whether checkpoint pathways were similarly activated in those cells. We treated HCT116 cells with MLN4924 for 8 hours and harvested cells 24 or 72 hours after washout. Chk1 was activated 24 hours after drug washout, whereas DNA rereplication was seen in 30% to 55% of cells (at 1 and 3 μmol/L MLN4924, respectively). The DNA damage checkpoint pathway still persisted even at 72 hours after washout, when rereplication was observed in 10% to 25% of cells (Fig. 5A and B). In addition, we noticed that PARP cleavage happened only at the later time point, suggesting that apoptosis was not activated until 72 hours after washout. This was further confirmed by the increase in sub-G1 population cells (Fig. 5B). Overall, these results were consistent with the idea that even transient exposure of MLN4924 leads to rereplication, activates checkpoint pathways, and eventually induces apoptosis following irreparable DNA damage.

Senescence is induced after transient exposure to MLN4924 through the induction of p53 and p21

When culturing cells after transient exposure to MLN4924, we noticed changes in cell morphology starting approximately 72 hours post-washout, including an increase in cell size, intracellular vesicle accumulation, and flatness. As shown in the top panel of Figure 6A, after 48 hours or more of continuous exposure to MLN4924, cells shrank and became round, suggesting that these cells were undergoing apoptosis. However, after a transient 8-hour exposure to MLN4924, cells exhibited the flattened, vesiculated morphology, often noticed when cellular senescence pathway is activated.

Senescence is marked by permanent withdrawal from the cell cycle. To test whether MLN4924 induces senescence, we first conducted colony formation assays to determine the clonogenicity of the cells (35). We added MLN4924 to HCT116 cells for 8 hours and cultured cells for 7 days after washout for colony formation as measured by crystal violet staining (Supplementary Fig. S2A). Quantitation of the optical density of staining (Fig. 6B) showed that MLN4924 treatment suppressed the clonogenicity, a characteristic of senescent cells.

Senescence associated-β-gal (SA-β-gal) staining is a well-accepted biomarker of senescence (36). Transient treatment of cells with MLN4924 increased the percentage of rereplicating cells (Fig. 3A) and the percentage of SA-β-gal staining (Fig. 6C and D). This suggested that the senescence pathway was activated upon transient exposure to MLN4924. These results were consistent with the earlier findings that rereplication can activate the DNA damage response leading to cellular senescence (37).

We next examined whether reduction of rereplication by Cdt1 depletion could decrease senescence following MLN4924 treatment. We conducted a similar assay as that displayed in Figure 1A, except that the HCT116 cells were exposed to MLN4924 for only 8 hours and cells collected for FACS after...
24 hours or SA-β-gal staining after 72 hours (Fig. 6E). Consistent with our hypothesis, Cdt1 depletion reduced both rereplication and senescence to a similar degree. Together, these data suggest that rereplication induced by transient exposure to MLN4924 leads to senescence. Eight-hour exposure to MLN4924 also induced apoptosis, as measured by the cleavage of PARP (Fig. 5A). Thus, transient treatment with MLN4924 induces senescence or apoptosis (also evident in Fig. 6A, 72 hours per washout), whereas continuous treatment with the drug leads mostly to apoptosis (Fig. 6A, 72 hours).

Both p53 and p21 can have a function in the cellular senescence pathway (38, 39). We therefore examined protein expression levels of p53 and p21 over 8 days following an 8-hour treatment with MLN4924. Both p53 and p21 were induced 24 hours post-washout and their expression persisted thereafter for the entire time course (Fig. 6F). We then conducted the SA-β-gal staining assay in p53−/−/C0 or p21−/−/C0 HCT116 cells to determine the level of senescence in the absence of these proteins. As shown in Figure 6G, the number of SA-β-gal−stained cells was only half in the p21−/−/C0 HCT116 compared with those of WT HCT116, indicating that p21 plays an important role in the senescence pathway. p53 seemed to be less essential than p21, which was consistent with results from other studies (40). The p16 gene is silenced in these cells (41), so the residual senescence in the p21−/−/C0 cells was most likely by a p21- and p16-independent pathway.

**p21- and p53-deficient HCT116 cells are more sensitive to transient treatment with MLN4924**

Because transient exposure to MLN4924 causes both senescence and apoptosis, but senescence is attenuated in the p21−/− cells, we could ask how important is the senescence for the toxicity of MLN4924 on cancer cells. We noticed that although p21- and p53-deficient cells exhibited less...
respectively (Supplementary Fig. S4A). Newly, we compared IC50 of WT, p53−/−, and p21−/− cells, respectively (Fig. S4B). Similar results were obtained when we compared IC50 in 2 lung cancer cells (Fig 7B): NCI-H23 mutant p53 (IC50 = 0.28 µmol/L) and NCI-H460 (wild type p53 = IC50 1.5 µM). Intriguingly, when we compared IC50 in more than 20 cancer cell lines upon 72 hours MLN4924 treatment, after dividing them into p53 WT (wild type) group and p53 MT (mutant) group, we found the median value of WT group was significantly higher than MT group (465 nmol/L vs. 280 nmol/L; Supplementary Fig. S4D) despite all the different genetic backgrounds. It must be noted, however, that there are other genetic factors that affect MLN4924 sensitivity besides p53 status. For example, we obtained opposite results when comparing MCF7 (WT p53) with MDA-MB-231 (MT p53) cells, whereby the MCF7 cells were more susceptible to MLN4924 (Supplementary Fig. S4D). Despite this exception, our data suggest that p53 mutant cells are generally more susceptible to MLN4924.

Thus, there is a clear therapeutic advantage of transient MLN4924 treatment, particularly considering that the p53 mutant cells are more susceptible to cell death than the p53 WT cells. Because up to 50% of human tumors have a mutant p53 gene, our results suggest that the kinetics of MLN4924 administration might alter the therapeutic index.

### Discussion

As a potential anticancer drug, MLN4924 was discovered to inhibit NAE activity, inhibit cullins, increase the expression of CRL substrates, induce rereplication, and cause cell death (21). In this article, we showed that the regulation of Cdt1 protein level is the rate-limiting step for the induction of rereplication upon MLN4924 treatment. It is noteworthy that even transient exposure of HCT116 colon cancer cells to MLN4924 leads to DNA rereplication. Once rereplication is induced, DNA damage checkpoint pathways are activated, which then lead to apoptosis and cellular senescence. We found that p53−/− and p21−/− HCT116 cells were both more sensitive to MLN4924 exposure than wild type cells, indicating that cancer cells with p53 mutations were likely more susceptible to transient exposure to the drug.

Various CRL substrates accumulate upon MLN4924 treatment, including Cdt1, p27, NRF2 (21), and possibly Cdc6 (Supplementary Fig. S1C). However, our data suggest that the deregulation of Cdt1 protein level plays an essential role in DNA rereplication induction, as shown by the decline in rereplication when Cdt1 is knocked down. That depletion of Orc2, MCM7, and Cdc6 did not prevent rereplication should not be interpreted to say that prereplicative complex components are not required for rereplication. The more likely hypothesis is that these proteins are in vast excess and so do not become rate limiting for rereplication after siRNA depletion.

We noticed a high G2 peak and a residual 15% of cells rereplicating after MLN4924 treatment in Cdt1-depleted cells (Supplementary Fig. S1C). The 15% of cells labeled as senescence after transient treatment with MLN4924, the total cell numbers observed were much less than in WT cells, which suggested that p53−/− or p21−/− HCT116 cells might be more susceptible to overall cell death or growth inhibition by MLN4924. Colony formation assays following an 8-hour treatment of MLN4924 in all 3 cell lines confirmed this (Fig 7A). Consistent with our previous results (Fig. 6B), colony formation was decreased by MLN4924 treatment in a dose-dependent manner in WT HCT116 cells, but the p53- and p21-deficient cells formed fewer colonies than WT cells, indicating that the absence of p53 or p21 sensitized the cells to the drug. As senescence is attenuated in the mutant cells (Fig. 6G), the result suggests that apoptosis pathways are important for cell killing after transient exposure to MLN4924.

We also conducted an MTT cell growth assay to compare cell survival rate upon either transient or 72 hour continuous exposure to MLN4924. After 8-hour treatment, the IC50 for WT, p53−/−, and p21−/− cells were 0.9, 0.18, and 0.25 µmol/L, respectively (Fig 7B), which was consistent with the results of the colony formation assays. However, the difference was much larger upon 72-hour continuous exposure: IC50 of 0.08, 0.07, and 0.07 µmol/L in WT, p53−/−, and p21−/− cells, respectively (Supplementary Fig. S4A).
rereplicating could arise from the tail of the large G2/M peak observed and may not be real rereplication that leads to DNA damage, as there was no activation of either Chk1 or Chk2 in these cells (Fig. 1B). Taken together, these data show that MLN4924 cause a G2/M arrest, consistent with the report that siCdt2 can induce G2/M arrest (13). This hypothesis was further confirmed by the increased phosphorylation of Cdc2 on Y15 and the loss of phosphorylation of H3 on S10 in cells treated with MLN4924 (Supplementary Fig. S1E), indicating that cells cannot enter mitosis. None of these changes, increase in G2 population, increase in Cdc2-P-Y15, and decrease in H3 phosphorylation, were relieved by decreasing Cdt1. Thus, unlike rereplication, the G2/M block seen with MLN4924 may be due to stabilization of substrates other than Cdt1.

In vivo data suggested that Cdt1 protein level peaked at 2–4 hours after injection of MLN4924 into tumor-bearing mice and started to decrease by 4–8 hours postinjection (21). Therefore, we hoped to evaluate the effect of transient exposure of cancer cells to MLN4924. Amazingly even 1-hour exposure was sufficient to induce rereplication in 40% of a colon cancer cell population in culture. With short treatment, we discovered that S-phase cells were more susceptible to MLN4924-induced rereplication, which is consistent with the idea that S-phase cells have already licensed origins (and fired many of them), so that relicensing by transient stabilization of Cdt1...
would cause rereplication. The observations that transient exposure can lead to rereplication and S-phase cells are more susceptible to this exposure are positive indicators for the clinical usefulness of this compound.

In addition to activation of apoptosis, we observed activation of senescence pathway after transient exposure of MLN4924. As previously stated, this was not due to a reduction in rereplication, as HCT116 cells displayed an equivalent increase in cells with a >4N DNA content even after short treatment with MLN4924 compared with continuous treatment (Fig. 3). This rereplication subsequently led to DNA damage and activated checkpoint and apoptosis pathways (Fig. 5). Unexpectedly, we observed that the senescence phenotype did not appear in continuously treated cells (Fig. 6A). Although there was barely any difference in the extent of rereplication between the 2 treatments, DNA damage signals (DDS) were possibly different, resulting in a different choice of cell fate between apoptosis and senescence (42). Upon short exposure, no new DDS occurred from persistent origins, which likely occurred in the continuously treated cells. This lower level of DNA damage signaling perhaps is not great enough in duration or extent to trigger cells apoptosis, though it is sufficient to induce p21 and p53.

One remaining question is what activates senescence. Is it related to rereplication-induced DNA damage? Previous articles suggested DNA damage caused by rereplication could
activate the senescence pathway (37, 43, 44). In our hands, depletion of Geminin or Emi1 in HCT116 cells similarly induced senescence after 3–4 days (data not shown). Consistent with this idea, decrease in re-replication by depletion of Cdt1 reduced cellular senescence (Fig. 6E). Thus, the senescence is triggered by the re-replication-induced DNA damage. Another intriguing question is, once cell fate has been determined, is it reversible? We treated cells with Z-VAD-FMK together with MLN4924 to inhibit cells from entering apoptosis (Supplementary Fig. S3). However, there was no significant increase in senescence, which suggested an irreversible commitment to apoptotic, non-senescent pathways.

It has already been suggested that p53 and p21 levels are increased during cellular senescence (45, 46). Multiple studies have shown that the p53–p21 pathway is critical for senescence to occur in human fibroblasts and cancer cells (38, 42, 47, 48). However, some researchers have observed that although p53 and p21 are positive factors in senescence, they are not necessary (49). Our results suggest that p53 and p21 have important functions in initiating cellular senescence upon MLN4924 treatment in tumor cells, but they are dispensable given that p53−/− or p21−/− cells showed decreased but not absent SA-β-gal staining (Fig. 6G).

Although both p53−/− and p21−/− HCT116 cells underwent less senescence than WT cells, both were more susceptible to cell death after transient treatment with MLN4924 (Fig. 7), suggesting a shifting of the balance toward a more apoptotic phenotype upon intermittent treatment in those cells. This p53-independent susceptibility to MLN4924 is potentially critical for clinical applications, where nearly half of human tumors have mutated their p53 gene. Conventional chemotherapy is less effective in p53 mutant cells. Thus, MLN4924 is exceptional in its ability to target p53 mutant tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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