Radiosensitization of Human Pancreatic Cancer Cells by MLN4924, an Investigational NEDD8-Activating Enzyme Inhibitor

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Abstract

Radiotherapy is used in locally advanced pancreatic cancers in which it can improve survival in combination with gemcitabine. However, prognosis is still poor in this setting in which more effective therapies remain needed. MLN4924 is an investigational small molecule currently in phase I clinical trials. MLN4924 inhibits NAE (NEDD8 Activating Enzyme), a pivotal regulator of the E3 ubiquitin ligase SCF (SKP1, Cullins, and F-box protein), that has been implicated recently in DNA damage and repair. In this study, we provide evidence that MLN4924 can be used as an effective radiosensitizer in pancreatic cancer. Specifically, MLN4924 (20–100 nmol/L) effectively inhibited cullin neddylation and sensitized pancreatic cancer cells to ionizing radiation in vitro with a sensitivity enhancement ratio of approximately 1.5. Mechanistically, MLN4924 treatment stimulated an accumulation of several SCF substrates, including CDT1, WEE1, and NOXA, in parallel with an enhancement of radiation-induced DNA damage, aneuploidy, G2/M phase cell-cycle arrest, and apoptosis. RNAi-mediated knockdown of CDT1 and WEE1 partially abrogated MLN4924-induced aneuploidy, G2/M arrest, and radiosensitization, indicating a causal effect. Furthermore, MLN4924 was an effective radiosensitizer in a mouse xenograft model of human pancreatic cancer. Our findings offer proof-of-concept for use of MLN4924 as a novel class of radiosensitizer for the treatment of pancreatic cancer.

Introduction

Pancreatic ductal adenocarcinoma is the fourth leading cause of cancer-related deaths in the United States and one of the deadliest human malignancies, with an overall 5-year survival rate of approximately 5% (1). Although gemcitabine is the standard systemic therapy for pancreatic cancer (2), the finding that as many as 1/3 of pancreatic cancer patients die of locally destructive disease underscores the importance of radiotherapy for local disease control (3). Indeed, the combination of radiation with gemcitabine has been shown to significantly prolong survival compared with gemcitabine alone (4), and intensifying radiation also seems to improve survival (5). Despite these improvements, the prognosis for patients with pancreatic cancer is still very poor, and new combinational therapies are urgently needed. Strategies utilizing small molecule inhibitors to sensitize pancreatic tumor cells to radiation (and gemcitabine) are well underway. We have recently shown that small molecule inhibitors of CHK1 (checkpoint kinase 1), sensitized pancreatic tumor cells and xenografts to radiation by G2 checkpoint abrogation and homologous recombination repair inhibition (6). Of the small molecule inhibitors tested in phase III clinical trials in pancreatic cancer, erlotinib, an inhibitor of epidermal growth factor receptor with only very modest activity in pancreatic cancer, has been shown to improve survival (7, 8). Thus, there is a demand for novel and more effective therapies. Our laboratory has recently shown that SCF E3 ubiquitin ligases are promising radiosensitizing targets in cancer cells (9, 10). Whether inhibition of SCF E3 ubiquitin ligases might also be a useful strategy for sensitizing pancreatic cancers to radiation has not previously been tested.

The SCF (SKP1-Cullin-F-box proteins) E3 ubiquitin ligases, consisting of an adaptor protein SKP1, a scaffold protein cullin, a substrate receptor F-box protein, and a RING protein RBX1 or RBX2, are the largest multiunit ubiquitin ligases (11, 12). The SCF E3s, therefore, regulate numerous biological processes, including cell-cycle progression, signal transduction, and DNA replication, among others (11, 12). It is well established that the core of SCF E3 ligase is a cullin-RING finger protein complex (14). In human and mouse, there are 7 cullins (cullins 1–3, 4A, 4B, 5, and 7) and 2 RING family members, RBX1

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(RING box protein-1) and RBX2, also known as SAG (Sensitive to Apoptosis Gene; refs. 11, 12, 15). The ligase activity of the SCF requires (i) RBX1/RBX2, which binds to ubiquitin-loaded E2, and (ii) cullin neddylation (16–19), the addition of a ubiquitin-like protein NEDD8 to cullin, catalyzed by NEDD8-activating enzyme E1 (NAE), NEDD8-conjugating enzyme E2 (UBC12), and NEDD8-E3 ligase (20).

We have recently shown that inactivation of SCF E3 ligase by siRNA knockdown of either RBX1 or RBX2/SAG induced apoptosis and senescence, and sensitized human cancer cells to radiation (9, 10, 21), and that SAG knockout also sensitized mouse embryonic stem cells to radiation via inducing apoptosis (22). Because MLN4924 effectively inactivates SCF E3 by blocking cullin neddylation (13), we tested our working hypothesis that MLN4924 could sensitize pancreatic cancer cells to radiation. We reported here that MLN4924 indeed acts as a radiosensitizing agent by enhancing radiation-induced DNA damage, aneuploidy, G2/M arrest, and apoptosis via mechanism including accumulation of CDT1 and WEE1, 2 well-known substrates of SCF E3 ubiquitin ligase.

Materials and Methods

Cell culture

Two human MiaPaCa-2 and BxPC-3 pancreatic cancer lines, and human H1299 lung cancer cell line were purchased from American Type Culture Collection. MiaPaCa-2 and H1299 cells were grown in DMEM with 10% FBS. BxPC-3 and human lung fibroblast MRC5 cells (a gift from Dr. A. Rehemtulla) were cultured in RPMI1640 with 10% FBS. All lines were tested and were free of Mycoplasma contamination.

ATPlite growth assay and IC50 determination

Cells were seeded in 96-well plates in triplicate and treated with MLN4924 (13), in various doses for 7 days. Cell viability was measured with ATPlite Kit (Perkin Elmer; ref. 23).

MLN4924 preparation for in vitro and in vivo assays

MLN4924 was synthesized by Millennium Pharmaceuticals, Inc. (13) and was dissolved in dimethyl sulfoxide to make a 10 mmol/L stock solution and kept in −20°C before use. For in vivo experiment, MLN4924 was freshly made every week as follows: To make an 8.32 mg/mL solution, the compound was dissolved in 10% 2-hydroxypropyl-β-cyclodextrin (HPBCD) in sterile water. The pH value was adjusted to 5.0 with 1 mol/L KOH. The solution of MLN4924 was stored at room temperature before use.

Radiation exposure and clonogenic assay

Cells were seeded in 60-mm dishes in duplicate and exposed to different doses of radiation (Philips RT250, Kimtron Medical) after pretreatment with MLN4924 for 6 hours (at 100 mmol/L) or 24 hours (at 20–30 mmol/L). MLN4924 was either washed away (at 100 mmol/L) or stayed in the medium (at 20–30 mmol/L) afterwards, followed by incubation at 37°C for 7 to 9 days. Survival curves were fitted using the linear-quadratic equation, and the mean inactivation dose was calculated (24).

siRNA knockdown of CDT1 and WEE1

Two independent sets of siRNA oligonucleotides were used to target CDT1 or WEE1, respectively. Their sequences are as follows: siCDT1-1 (5’-CGUGGAAGAUAACCCGACUU-3’) and siCDT1-2 (5’-GCAUGUUCGCGCAGAAU-3’), and siWEE1-1 (5’-AGGCGUGAAGGAUGCACUUU-3’) and siWEE1-2 (5’-CUCCGGGUGAUCUCUCUUU-3’), and scrambled control siRNA (Si-Ctrl: 5’-ATTGTATGCGATCGCA-GACT-3’). All oligos were purchased from Dharmacon. Cells were transfected with siRNA using X-tremeGENE (Roche) and split 48 hours later. One portion was used for clonogenic assay and the other portions for immunoblotting (IB) or fluorescence-activated cell sorting (FACS) profile (23).

Immunoblotting

Cells were exposed to various treatments and harvested for IB as described (23) using antibodies against CDT1, WEE1, CUL1, total CDC2 (Santa Cruz Biotechnology), ORC1, p21, p27 (BD Biosciences), NOXA, BIM1, phospho-CHK1 (S345), phospho-CHK2 (T68), γ-H2AX (Ser139), phospho-CDK2 (Y15), phospho-IκB, Cyclin B1 (Cell Signaling), and β-actin (Sigma).

Fluorescence-activated cell sorting

Cells were treated with MLN4924, or exposed to radiation alone or in combination. Cells were harvested 24 or 48 hours postradiation and analyzed by flow cytometry (23).

Immunofluorescence staining

Cells after exposure to various treatments were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, blocked with 0.5% bovine serum albumin, and incubated with primary antibody against γ-H2AX (Millipore) at 1:1,000, followed by incubation with Alexa Fluor 594 goat anti-mouse IgG at 1:2,000. Cellular nuclei were stained with 4′,6-diamidino-2-phenylindole, as described (9). The stained cells were observed under fluorescent microscope (Olympus 1 × 71), using Olympus LCP Ian F1 lens and Olympus DP70 cameras. The acquisition software used was Olympus DP Controller 2002 (Olympus Optical Co. Ltd).

In vivo antitumor study

All animal studies were conducted in accordance with the guidelines established by the University Committee on Use and Care of Animals. Five million MiaPaCa-2 cells were inoculated subcutaneously in both flanks of nude mice. The mice were randomized and the treatment started when the tumor size reached 100 mm³ at 14 days after inoculation. MLN4924 (30 mg/kg, s.c.) and radiation (1 Gy) were given once a day, 5 days a week for 3 weeks. Radiation was delivered directly to the tumor with the rest of the animal shielded. For combination treatment, MLN4924 was given 2 hours prior to radiation exposure with the same schedule as for individual treatments. The growth of tumors (8 for control group, 12–16 for the other groups) was measured twice a week and average tumor volumes were calculated, as estimated from the formula (L × W²)/2. Radiation was conducted in the University of Michigan Comprehensive Cancer Center Experimental Radiation Core.
Statistical analysis

ANOVA models were used with SPSS software for statistical comparisons involving multiple groups, followed by an SNK post hoc test to determine significance of each of 2 groups (P < 0.05). Tumor volume doubling (tripling) was determined for each xenograft by identifying the earliest day on which it was at least twice (3 times) as large as on the first day of treatment. A cubic smoothing spline was used to obtain the exact time of doubling (tripling). The Kaplan–Meier method was used to analyze the doubling (tripling) times derived from the smoothed growth curves, and the Cox regression model was used for comparisons between any 2 treatment groups after adjusting the initial tumor sizes. Bayesian hierarchical changepoint model was conducted to estimate the tumor growth profile characterized by a prenadir regression rate, a regression period, a nadir volume, and a postnadir regrowth rate. The 90% highest probability density (HPD) CIs were calculated to compare these features between different treatments. If the HPD interval of the difference in any feature between two treatments covers zero, the two treatments are not significantly different on that feature at the significant level of 0.1 (25).

Results

Sensitivity of pancreatic cancer lines to MLN4924 as a single agent

Our recent studies showed that inactivation of SCF E3 ubiquitin ligase by siRNA knockdown of its RING component, RBX1 or RBX2/SAG, sensitized human cancer cells to radiation (9, 10), whereas Sog knock out also sensitized mouse embryonic stem cells to radiation by inducing apoptosis (22), suggesting that SCF E3 is an attractive radiosensitizing target. Here, we tested our working hypothesis that MLN4924, a small molecule inhibitor of NEDD8 activating enzyme (NAE), which suppresses cancer cell growth by inhibiting activity of SCF E3 RBX1 or RBX2/SAG, sensitized human cancer cells to radiation with a sensitivity enhancement ratio (SER) of 1.43 to 1.55, respectively (Fig. 1D). In the second regimen (lower MLN4924 dose with longer exposure time), we pretreated cells with MLN4924 at a lower concentration (20 nmol/L for MiaPaCa-2 and 25 nmol/L for BxPC-3) for 24 hours, followed by radiation at different doses up to 8 Gy. Cells were then cultured for additional 7 to 9 days in the presence of MLN4924 at these low doses (without medium change). Under this condition, we confirmed that cullin-1 was deneddylated throughout the experimental periods (Supplementary Fig. S2A) and observed MLN4924 radiosensitization with SER of approximately 1.4 (Supplementary Fig. S2B). Thus, we conclude that MLN4924 is a potent radiosensitizer in pancreatic cancer cells.

To determine whether radiosensitization by MLN4924 is tumor cell selective, we assessed the ability of MLN4924 to radiosensitize H1299 lung cancer cells, paired with human lung fibroblast MRC5 cells, because there are no “normal” pancreatic cells capable of forming colonies in standard clonogenic assays. We found that in H1299 cancer cells, but not in MRC5 normal cells, that cullin-1 is highly neddylated, which is completely inhibited by MLN4924 at 100 nmol/L (Supplementary Fig. S3A). Consistent with targeting cullin neddylation, MLN4924 was 5-fold more potent in growth suppression of H1299 cancer cells (IC50 = 83 nmol/L) than MRC5 normal cells (IC50 = 429 nmol/L; Supplementary Fig. S3B) and was 4-fold more potent in inhibition of clonogenic survival of H1299 cells (IC50 = 50 nmol/L; Supplementary Fig. S3B) and was 4-fold more potent in inhibition of clonogenic survival of H1299 cells (IC50 = 200 nmol/L; Supplementary Fig. S3C). Likewise, MLN4924 at as low as 15 nmol/L sensitized radiosensitive cells (23) to radiation with a SER of 1.39 but had little effect on MRC5 cells (SER = 1.13; Supplementary Fig. S3D). Thus, MLN4924 seems to selectively target cancer cells with high levels of cullin neddylation and SCF E3 ligase activity.

Radiosensitization by MLN4924 is attributable to enhanced G2/M arrest, aneuploidy, and induction of apoptosis

To determine the nature of MLN4924 radiosensitization, we carried out cell-cycle profile of 2 pancreatic cancer cell lines treated with MLN4924, radiation, or MLN4924 in combination with radiation (Fig. 2A), and found that MLN4924 remarkably enhanced the radiation-induced G2/M arrest in MiaPaCa-2 (IR at 34% vs. IR plus MLN4924 at 68%) and BxPC-3 cells (IR at 52% vs. IR plus MLN4924 at 65%) (Fig. 2B). MLN4924 also increased the aneuploid cell population (DNA content greater than 4N) from 10% in response to radiation alone to 40% in response to radiation plus MLN4924 in MiaPaCa-2 and from 4% to 24% in
in BxPC-3 cells, respectively (Fig. 2C). In addition, MLN4924 increased the radiation-induced sub-G1 apoptotic population at later time points (48 hours; Fig. 2D). These results suggested that radiation-induced disruption of cell-cycle progression and genomic instability, followed by apoptotic cell death, can be further enhanced by MLN4924.
MLN4924 enhanced radiation-induced DNA damage

Because the major cellular effect of ionizing radiation is to cause DNA damage and trigger the DNA damage response (26), we, therefore, examined whether MLN4924 treatment would enhance radiation-induced DNA damage and interfere with the DNA damage repair process. We determined DNA double-strand breaks (DSB) by measuring the overall levels of γ-H2AX protein and formation of nuclear foci upon radiation and MLN4924 treatment, alone or in combination. We found the levels of γ-H2AX increased regardless of single or combination treatment at an early time point (4 hours). However, a higher protein level of γ-H2AX was seen in the combination treatment group at 24 hours for both pancreatic cancer cell lines (Fig. 3A). Consistently, we observed a significant increase in the population of γ-H2AX foci positive cells in combination treatment group (Fig. 3B and C). We also determined the DNA damage response upon MLN4924 radiation treatment by measuring phosphorylation of CHK1 and CHK2 and found that although MLN4924 did not enhance CHK2 phosphorylation, MLN4924 did enhance CHK1 phosphorylation in response to radiation.

Figure 2. MLN4924 alters cell-cycle progression and induces apoptosis. Cells were treated with MLN4924 at 100 nmol/L for 24 hours (A–C) or up to 48 hours (D), alone or in combination with radiation (6 Gy), followed by FACS analysis for cell-cycle profiling (A), cell-cycle distribution (B), cell population with more than 4N DNA content (C), and sub-G0 population for apoptosis (D). Shown is mean ± SEM (n = 3). * P < 0.05. One representative result is shown for A. DMSO, dimethyl sulfoxide; PI, propidium iodide.
Figure 3. MLN4924 induces DNA damage and prolongs DNA damage response. Cells were treated with MLN4924 (100 nmol/L) alone or in combination with radiation (6 Gy) for indicated periods of time, followed by IB analysis (A and D), or by immunofluorescence staining for γ-H2AX foci (B and C, left panels). Cells with more than 10 foci were counted and quantified data is plotted (B and C, right panel). Shown is mean ± SEM (n = 5); *, P < 0.05. DAPI, 4',6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide.
particular at the later 24-hour time point (Fig. 3D). Similar enhancement of radiation-induced γ-H2AX levels and CHK1 phosphorylation by MLN4924 was also observed in H1299 lung cancer cells, which are sensitive to MLN4924 radiosensitization, but not in MRC5 lung fibroblast, which are resistant to MLN4924 radiosensitization (Supplementary Fig. S4). Taken together, these results suggested that MLN4924 enhances radiation-induced DNA damage and prolongs the process of DNA repair, which likely contributes to its radiosensitizing effects.

**MLN4924 caused accumulation of SCF E3 ligase substrates, which is responsible for G_2/M arrest and DNA damage response**

To identify the mediators of MLN4924 which lead to the enhanced radiation effects, we measured the levels of proteins known to be (i) the substrates of SCF E3 ligases and (ii) involved in the regulation of cell-cycle progression, DNA damage response, and apoptosis (10, 12, 27). We found that as expected, the levels of cell-cycle regulators, including p21, p27, WEE1, DNA licensing proteins CDT1 and ORC1, and apoptosis regulators NOXA, BIM EL, and pIkB, increased substantially upon treatment with MLN4924, but not radiation (Fig. 4). The MLN4924–radiation combination further increased the levels of CDT1 and NOXA (Fig. 4), as well as WEE1 activity, as reflected by enhanced phosphorylation of its substrate, CDC2 (Y15; Fig. 4, bottom panels), suggesting these 3 proteins may contribute to MLN4924 radiosensitization.

[Figure 4. MLN4924 induces accumulation of SCF E3 ligase substrates. Subconfluent cells were treated with MLN4924 (100 nmol/L) or radiation (6 Gy) alone or in combination for 24 hours, followed by IB analysis using indicated antibodies.]

**Partial rescue of aneuploidy, G_2/M arrest, and radiosensitization by siRNA knockdown of CDT1 and WEE1**

Because apoptosis seems to be a late and secondary effect of MLN4924 radiation (Fig. 2D), likely resulting from enhanced DNA damage and prolonged G_2/M arrest, we focused our attention on CDT1, a DNA licensing protein whose overexpression is known to cause DSB and trigger DNA damage response (9, 28), and WEE1, a protein tyrosine kinase that phosphorylates CDC2 on Tyr15 to arrest cells at the G2 phase of cell cycle (29, 30). We reasoned that if CDT1 and/or WEE1 were critical to MLN4924-induced enhancement of radiation effects, we should be able to abrogate these effects, at least in part, by their simultaneous knockdown. Indeed, in MLN4924–radiation–treated cells, CDT1 knockdown, using 2 independent siRNA oligos abrogated the radiation enhancement of radiation-induced DNA damage and prolonged G_2/M arrest, which likely contributes to its radiosensitizing effects.

Finally, we assessed MLN4924-mediated radiosensitization in vivo using the MiaPaCa-2 xenograft model. To ensure that MLN4924 “hits” its target cullin-1 in vivo tumors and to determine when radiation should be delivered post MLN4924 administration, we measured the levels of MLN4924-conjugated NEDD8, neddylated cullin-1, SCF substrates, WEE1, and NRF2 at the indicated times after a single administration of vehicle, MLN4924, radiation, or MLN4924 plus radiation (2 representative tumors per group). As shown in Fig. 6A, MLN4924–NEDD8 adduct was detectable only in groups with MLN4924 treatment, alone (lanes 5 and 6) or in combination (lanes 13 and 14) at the early time point (4 hours), which nearly disappeared at 24 hours posttreatment (lanes 7 and 8 and 15 and 16). Similarly, cullin-1 neddylation was inhibited with a corresponding increase in the SCF E3 substrates WEE1 and NRF2 in MLN4924-treated groups at the early 4-hour time point (lanes 5 and 6 and 13 and 14). At later time point (24 hours), both neddylated cullin-1 and NRF2 levels returned to the control levels with WEE1 level remaining slightly higher (lanes 7 and 8 and 15 and 16). As expected, radiation alone had no effect on cullin-neddylation or the levels of selected SCF E3 substrates (lanes 9–12). Our results clearly
show that MLN4924 indeed “hits” its target and suggest that radiation should be delivered 2 hours postadministration of MLN4924, when SCF E3 is inactivated due to cullin neddylation.

We next determined the in vivo radiosensitizing activity of MLN4924. As shown in Fig. 6B and C, administration of MLN4924 alone at a dose of 30 mg/kg s.c./d, 5 d/wk for 3 weeks had a moderate inhibitory effect on tumor growth in nude mice. Radiation treatment at a clinically relevant dose of 1 Gy/d, 5 d/wk for 3 weeks also had moderate antitumor activity. In response to treatment with the combination of MLN4924 and radiation, tumor growth was significantly inhibited compared with either treatment alone (at day 60; Fig 6B). Similarly, the time required for tumor volume doubling or tripling was significantly increased in response to MLN4924 radiation treatment compared with vehicle or radiation treatment (Fig. 6C). Consistent with this, we also observed a significantly reduced tumor volume nadir corresponding with a marginally significant increase in the tumor regression rate in response to MLN4924 radiation (compared with
radiation; Supplementary Table S1). Importantly, the combination treatment was well tolerated by the animals with a minimal loss of body weight (Fig 6D). Taken together, our results showed that MLN4924 is a radiosensitizer in pancreatic cancer, as assessed in both in vitro cell culture and in vivo tumor xenograft models, likely by causing accumulation of CDT1 and WEE1, among with other substrates of SCF E3 ubiquitin ligase.

Discussion

Our previous work has shown that SCF E3 ubiquitin ligase is an attractive anticancer target (for review, see refs. 15, 31–33). However, because of the complexity of establishing a high-throughput (HTS) screen for multi-components SCF E3 ubiquitin ligase, there is no single small molecule discovered that directly inhibits its ligase activity, although a number of
HTS methods have been established and optimized to screen for small molecule inhibitors of single peptide E3 ligases (31, 34). MLN4924 is a newly discovered small molecule inhibitor of NAE (13). MLN4924 binds to NAE at its active site to create a covalent NEDD8–MLN4924 adduct, which resembles NEDD8 adenylate, but cannot be further utilized in subsequent intraenzyme reactions, thus inhibiting NAE activity and blocking cullin neddylation (13, 35). Because cullin neddylation is required for SCF E3 activity (16, 18, 19), MLN4924 becomes the first of a class of “indirect” inhibitors of SCF E3. By inhibiting SCF E3 ligase activity, MLN4924 causes the accumulation of a number of SCF E3 substrates to suppress the growth of acute myeloid leukemia (36), diffuse large B-cell lymphoma (37), and many cancer cell lines derived from solid tumors both in vitro and in vivo by inducing apoptosis (13, 36, 37) and senescence (38–40). Importantly, in vivo xenograft assays showed that MLN4924 was well tolerated in mice at various doses and treatment regimens (13), showing a selective killing of cancer cells. With all these preclinical efficacies, MLN4924 has been advanced to several phase I clinical trials for solid tumors and hematologic malignancies (41).

In this study, we tested our hypothesis that MLN4924 is a potent radiosensitizing agent for pancreatic cancer cells, based upon our target validation work using both siRNA knockdown and gene knockout approaches for RBX1 and SAG, the RING components of SCF E3, required for its ligase activity (9, 10, 21, 22). For the first time, we showed that in vitro cell culture models, MLN4924 was a potent growth inhibitor as a single agent, as well as a radiosensitizer in 2 pancreatic cancer cell lines and 1 lung cancer cell line, but not normal lung fibroblast cells. The resistance of normal fibroblasts to MLN4924 radiosensitization is likely due to the lack of cullin neddylation. We also showed in vivo, using a MiaPaCa-2 xenograft model, that MLN4924 inhibited tumor growth and conferred radiosensitization with minimal toxicity to the mice. Mechanistically, we showed that MLN4924 enhanced radiation-induced G2/M arrest, aneuploidy, and, eventually, apoptosis by inhibiting cullin neddylation to trigger the accumulation of a number of SCF E3 substrates, related to DNA damage response and cell-cycle regulation. Among these accumulated substrates, CDT1 and WEE1 were further increased when combined with radiation and were critical for enhanced DNA damage and aneuploidy or G2/M arrest, respectively, and eventually for MLN4924 radiosensitization, as evidenced by siRNA knockdown-based rescuing experiments.

Precise duplication of the genome at the S phase of each cell cycle requires initiation of DNA replication from thousands of origins. Initiation of too few origins would cause collapse of replication forks, leading to DNA damage and incomplete replication of the genome, whereas more than one initiation of DNA replication per cell cycle would cause DNA “hyperreplication” or “rereplication” and subsequent DNA damage (42), leading to genomic instability and cancer (43). CDT1 is a key licensing factor which, along with the protein CDC6, functions to form the prereplicative complex (pre-RC) to initiate DNA replication (44). It was recently reported that overexpression of Double-arked (Dup), the Drosophila ortholog of CDT1 caused DNA rereplication and DNA damage, followed by caspase activation and apoptotic cell death (45). In human U2-OS cancer cells, CDT1 overexpression caused double strand breaks to trigger DDR, followed by induction of senescence and apoptosis (28). Induction of CDT1 accumulation by MLN4924 was found to be attributable to DNA rereplication, aneuploidy, and apoptotic death in HCT116 cells (13, 40, 46). Our data presented here indicated that CDT1 accumulation is also found in 2 pancreatic cancer cell lines and is critical for enhanced aneuploidy and MLN4924 radiosensitization.

The WEE1 kinase negatively regulates G2/M transition by directly phosphorylating Tyr15 of CDC2, which inhibits the CDC2 activity (30). We have previously shown that WEE1 can directly phosphorylate Tyr15 of CDC2, this inhibits the CDC2 activity (30). We have previously shown that WEE1 can directly phosphorylating Tyr15 of CDC2, which inhibits the CDC2 activity (30). We have previously shown that WEE1 can directly phosphorylating Tyr15 of CDC2, which inhibits the CDC2 activity (30). We have previously shown that WEE1 can directly phosphorylating Tyr15 of CDC2, which inhibits the CDC2 activity (30). We have previously shown that WEE1 can directly phosphorylating Tyr15 of CDC2, which inhibits the CDC2 activity (30). We have previously shown that WEE1 can directly phosphorylating Tyr15 of CDC2, which inhibits the CDC2 activity (30). We have previously shown that WEE1 can directly phosphorylating Tyr15 of CDC2, which inhibits the CDC2 activity (30). We have previously shown that WEE1 can directly phosphorylating Tyr15 of CDC2, which inhibits the CDC2 activity (30). We have previously shown that WEE1 can directly phosphorylating Tyr15 of CDC2, which inhibits the CDC2 activity (30). We have previously shown that WEE1 can directly phosphorylating Tyr15 of CDC2, which inhibits the CDC2 activity (30). We have previously shown that WEE1 can directly phosphorylating Tyr15 of CDC2, which inhibits the CDC2 activity (30). We have previously shown that WEE1 can directly phosphorylating Tyr15 of CDC2, which inhibits the CDC2 activity (30). We have previously shown that WEE1 can directly phosphorylating Tyr15 of CDC2, which inhibits the CDC2 activity (30). We have previously shown that WEE1 can directly phosphorylating Tyr15 of CDC2, which inhibits the CDC2 activity (30). We have previously shown that WEE1 can directly phosphorylating Tyr15 of CDC2, which inhibits the CDC2 activity (30).
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