The 1,2-Diaminocyclohexane Carrier Ligand in Oxaliplatin Induces p53-Dependent Transcriptional Repression of Factors Involved in Thymidylate Biosynthesis

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Abstract

Platinum-based chemotherapeutic drugs are widely used as components of combination chemotherapy in the treatment of cancer. One such drug, oxaliplatin, exerts a synergistic effect against colorectal cancer in combination with 5-fluorouracil (5-FU) and leucovorin. In the p53-proficient colorectal cancer cell line HCT116, oxaliplatin represses the expression of deoxuridine triphosphatase (dUTPase), a ubiquitous pyrophosphatase that catalyzes the hydrolysis of dUTP to dUMP and inhibits dUTP-mediated cytotoxicity. However, the underlying mechanism of this activity has not been completely elucidated, and it remains unclear whether factors other than downregulation of dUTPase contribute to the synergistic effect of 5-FU and oxaliplatin. In this study, we found that oxaliplatin and dachplatin, platinum-based drugs containing the 1,2-diaminocyclohexane (DACH) carrier ligand, repressed the expression of nuclear isoform of dUTPase (DUT-N), whereas cisplatin and carboplatin did not. Oxaliplatin induced early p53 accumulation, upregulation of primary miR-34a transcript expression, and subsequent downregulation of E2F3 and E2F1. Nutlin-3a, which activates p53 nongenotoxically, had similar effects. Introduction of miR-34a mimic also repressed E2F1 and DUT-N expression, indicating that this miRNA plays a causative role. In addition to DUT-N, oxaliplatin repressed, in a p53-dependent manner, the expression of genes encoding enzymes involved in thymidylate biosynthesis. Consequently, oxaliplatin significantly decreased the level of dITP in the dNTP pool in a p53-dependent manner. These data indicate that the DACH carrier ligand in oxaliplatin triggers signaling via the p53–miR-34a–E2F axis, leading to transcriptional regulation that ultimately results in accumulation of dUTP and reduced dITP biosynthesis, potentially enhancing 5-FU cytotoxicity. Mol Cancer Ther; 14(10); 2332–42. ©2015 AACR.

Introduction

Platinum-based chemotherapeutic drugs, such as cisplatin [cis-diaminedichloroplatinum (II)], carboplatin [cis-diaminecyclcoplatinum (I)], and oxaliplatin [cis-diaminocyclohexane (DACH)–platin(II)], are widely used in the treatment of various types of cancer, usually in combination with other types of drugs (e.g., antimitabolites, alkylating agents, or microtubule inhibitors; refs. 1, 2). The platinum-based drugs exert their cytotoxic effects primarily by producing platinum (Pt)–DNA adducts with similar complexities, but distinct structures. Cisplatin and carboplatin add the cis-diamine carrier ligand at DNA adducts, whereas oxaliplatin adds the 1,2-diaminocyclohexane (DACH) carrier ligand. In cisplatin, the cis-diamine and carboplatin induce distinct cellular responses. In the p53-proficient colorectal cancer cell line HCT116, cisplatin slows down the DNA replication phase and activates the G2-M checkpoint, whereas oxaliplatin activates the G1-S checkpoint and completely blocks the G2-M transition (Supplementary Fig. S2; ref. 3). However, the molecular mechanism by which the structural differences in Pt–DNA adducts cause different cellular responses and cytotoxicity is not fully understood.

Oxaliplatin exerts synergistic effects in combination with 5-fluorouracil (5-FU) and leucovorin. The combination of these drugs, called FOLFOX, is now a standard regimen for patients with advanced colorectal cancer (4). In addition to its RNA-mediated toxicity (5), 5-FU is metabolized to 5-fluoro-
dUTPase, which specifically suppresses the activity of thymidylate synthase (TS). Because TS plays an essential role in de novo dTTP synthesis by catalyzing the conversion of dUMP to dTMP, inhibition of this enzyme induces dUTP and/or dUTP accumulation. dUTP misincorporation into DNA and subsequent DNA repair processes mediated by uracil DNA glycosylases lead to massive DNA degradation and cause cytotoxicity (6). However, deoxuryridine triphosphatase (dUTPase), a pyrophosphatase that catalyzes the hydrolysis of dUTP to dUMP, eliminates dUTP from the dNTP pool and prevents dUTP misincorporation into DNA (7). Indeed, dUTPase is often overexpressed in tumors, and elevated expression of dUTPase in the nuclei of tumor cells is correlated with resistance to 5-FU-based chemotherapy in patients with metastatic colorectal cancer (8). Furthermore, siRNA-mediated knockdown of the gene encoding dUTPase (DUT) sensitizes various cancer cell lines to TS-targeted compounds, including 5-FU (9–11).

Despite the significant clinical advantages of oxaliplatin in combination with 5-FU-based chemotherapy, the underlying mechanism has not been fully elucidated (12). Downregulation of TS by oxaliplatin has been proposed as one possible mechanism (13). Furthermore, a previous study showed that expression of the gene encoding the nuclear isoform of dUTPase (DUT-N) is repressed by oxaliplatin in a p53-dependent manner (14). Detailed in vitro analysis revealed that DUT expression is regulated by the transcription factors E2F1 and Sp1, and oxaliplatin induces the enrichment of p53 at the DUT-N promoter with a concomitant reduction in the level of Sp1 (15). However, the contribution of E2F1 to this process has not been clearly demonstrated. The E2F pathway (E2F3 and E2F1) is also downregulated by miR-34a, a member of the miR-34 family (comprising miR-34a, b, and c; ref. 15). These microRNAs are induced by adriamycin in colon cancer cells in a p53-dependent manner (16) and by nutlin-3a, a small-molecule activator of p53 that disrupts the p53–MDM2 interaction by binding to the hydrophobic pocket of MDM2 even in the absence of genotoxic stress (17, 18). However, it remains unknown whether miR-34a is involved in transcriptional regulation under oxaliplatin-induced stress.

In this study, we found that oxaliplatin and dachplatin, but not cisplatin or carboplatin, induced p53-dependent repression of DUT-N expression, confirming the involvement of Pt-DNA adducts containing the DACH carrier ligand in this process. Oxaliplatin activated p53 via phosphorylation at Ser15, but the activation of the ATM-Chk2 and ATR-Chk1 signaling pathways was marginal. Like nutlin-3a, oxaliplatin-induced repression of DUT-N expression was preceded by early accumulation of p53 and upregulation of primary miR-34a transcript and subsequent downregulation of E2F3 and E2F1. Furthermore, several genes encoding dihydrofolate reductase (DHFR), thymidine kinase 1 (TK1), and TS, enzymes involved in thymidylate biosynthesis, were also repressed by oxaliplatin treatment. Consequently, the amount of dTTP in the dNTP pool was significantly decreased by oxaliplatin treatment in a p53-dependent manner. Taken together, our data indicate that DACH–Pt-DNA adducts induce oxaliplatin effectively stabilize p53 and upregulate miR-34a, resulting in repression of gene expression driven by the transcription factors E2F3 and E2F1. The oxaliplatin-triggered suppression of thymidylate biosynthesis may increase the cytotoxicity of TS inhibitors.

**Materials and Methods**

**Cell culture and reagents**

The colorectal cancer cell lines HCT116, LoVo, and SW480 were purchased from the ATCC in 2011. These cells were authenticated by short tandem repeat analysis in 2014. HCT-116 p53+/− cells were kindly provided by B. Vogelstein (Johns Hopkins University); they were confirmed to be p53-null by immunoblot analysis (Supplementary Fig. S3A), but otherwise were not authenticated. Cells were maintained in the supplier’s suggested culture media supplemented with 10% FBS. The following reagents were purchased from the indicated suppliers: cisplatin, carboplatin, and oxaliplatin (Tokyo Chemical Industry); dichloro(1,2-diaminocyclohexane)platinum(II) (dachplatin; Sigma-Aldrich); and nutlin-3a ([−]−nutlin-3) (Cayman Chemical).

**Western blotting and antibodies**

Whole-cell extracts were obtained by solubilizing cell pellets in NET-N buffer (20 mmol/L Tris–HCl [pH 8.0], 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP-40) containing protease and phosphatase inhibitor cocktail (Nacalai Tesque). Protein concentrations were measured using the BCA protein assay reagent (Thermo Fisher Scientific). For Western blotting, 10 or 20 μg of whole-cell extract was subjected to SDS-PAGE, and the following antibodies were used: anti-dUTPase (H00001854-M01; Abnova), anti–β-actin (A5316; Sigma), anti-p53 (M7001; Dako), anti–phospho-p53 [Ser15; #9284, Cell Signaling Technology (CST)], anti–p21 [sc-397; Santa Cruz Biotechnology (SCBT)], anti-Chk1 (sc-8408; SCBT), anti–phospho-Chk1 (Ser345; #2348; CST), anti-Chk2 [3440; CST], anti–phospho-Chk2 (Thr68; #2661; CST), anti–E2F-1 [3742; CST], and anti–E2F3 (sc-878; SCBT). Chemiluminescent signals were detected on an ImageQuant LAS 4000 Mini (GE Healthcare Life Sciences), and signal intensities were quantitated using the ImageQuant TL (GE Healthcare Life Sciences) or the ImageJ (NIH) software.

**RNA interference and ectopic expression of miRNA mimic**

RNAiMAX (Life technologies) was used for siRNA transfection. siRNA sequences were as follows: siCL2 (luciferase control) (19), CGGAGCGTGAGAGAACATGTT; siTS53 (p53); GAAGUUGCCUGUGACGAGUJU; siDUT (M7001; Dako); and siDUT-M (the mitochondrial isoform of dUTPase). CUACCAAUUUIUCUUACUCUUT (Sigma-Aldrich). Ectopic expression of miR-34a mimic transcript was performed using miRCURY LNA microRNA Mimic (Takara). The human TK1 gene was amplified from cDNA isolated from HCT116 p53+/− cells with the following primers: TK1_ATC_Fw, CACCATAGACCTGCAATACCTGCCC; TK1_Last_Rv, GGGCAGCATTGGAGCAGAAT. The TK1 cDNA was cloned into a pcDNA3.1 expression vector with the 3′-FLAG tag at the C-terminus using the Gateway cloning system (Invitrogen). X-tremeGENE HP DNA Transfection Reagent (Roche) was used for plasmid transfection.

**Quantitative real-time RT-PCR**

Total RNA was extracted from drug-treated cells using ISOGEN II (NIPPON GENE), and cDNA synthesis was performed using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo Life Science). Quantitative real-time PCR was carried out on an Mx3005P qPCR system (Agilent Technologies) using THUNDERBIRD qPCR Mix (Toyobo Life Science). Because of difficulty with the amplification, the KAPA SYBR Fast qPCR Kit (NIPPON
Samples for LC-QqQ-MS analysis were prepared as previously described (21). Statistical analysis was performed by the paired or unpaired t test.

**Quantitation of dTTP by LC-QqQ-MS**

Intracellular dTTP was quantitated using liquid chromatography/triple-stage quadrupole mass spectrometry (LC-QqQ-MS). Samples for LC-QqQ-MS analysis were prepared as previously described (22) with slight modifications. HCT-116 p53+/− cells or HCT-116 p53+/− cells, either non-treated or treated with 5 μmol/L oxaliplatin for 48 hours, were washed twice with ice-cold PBS and resuspended in 1 mL ice-cold 100% methanol containing 5 μmol/L (+/-)10-camphor sulfonic acid (10CS) per 1 × 10⁶ cells, incubated on ice for 3 minutes, collected by scraping with a rubber policeman, sonicated 10 times (each cycle consisted of 30 seconds of sonication and 30 seconds of cooling) with a BIORUPTOR (Cosmo Bio Co. Ltd.), and centrifuged at 21,500 × g for 5 minutes at 4°C. The supernatants were collected and mixed with chloroform and water at a ratio of 1:0.5:1, mixed well, and centrifuged for 5 minutes. The aqueous fraction was evaporated to dryness on a centrifugal evaporator (CVE-2000; EYELA) connected to a high vacuum pump (GCCD-051X; ULVAC) and a cold trap (UNI TRAP UT-1000; EYELA). Desiccated pellets were dissolved in water and filtered before LC-QqQ-MS analysis. The metabolites from the aqueous fraction were separated by ion-pair HPLC on a Synergi 2.5 μm Hydro-RP 100 Å (100 × 2.0 mm inside diameter, Phenomenex Inc.) and analyzed using an LCMS-8040 instrument (Shimadzu) as previously described (23). dTTP was detected with a optimized selective reaction–monitoring transitions in negative ionization mode as follows: precursor ion [m/z]/product ion [m/z] = 481/159, 481/383, and 481/120. Statistical analysis was performed by the unpaired t test.

**Results**

p53-dependent repression of DUT-N expression is induced by platinum-based drugs with the DACH carrier ligand

A previous study using isogenic HCT116 colon cancer cells showed that oxaliplatin-induced transcriptional repression of DUT-N, the nuclear isoform of dUTPase, is dependent on p53 (14). Consistent with these results, we found that dUTPase protein levels were markedly reduced 48 hours after oxaliplatin treatment in HCT116 p53+/− cells (Fig. 1A, top left), but not in HCT116 p53+/− cells (Fig. 1A, top right). The same effect was observed in LoVo cells, which express nonfunctional p53 harboring the R273H and P309S mutations (25). p53 accumulation and p21 induction were observed after 48 hours of oxaliplatin treatment in HCT116 p53+/− cells and LoVo cells, but not in HCT116 p53+/− cells or SW480 cells (Supplementary Fig. S3A). Furthermore, siRNA-mediated suppression of p53 expression alleviated the oxaliplatin-induced decrease in dUTPase protein in HCT116 p53+/− cells (Fig. 1B). These results confirm that oxaliplatin-induced transcriptional repression of DUT-N is dependent on functional p53 protein.

To identify the components of oxaliplatin that induce the repression of DUT-N expression, we used three additional structurally distinct platinum-based drugs (Supplementary Fig. S1). Of those, dachplatin repressed expression of DUT-N as effectively as oxaliplatin (Fig. 1C, bottom left). By contrast, cisplatin and carboplatin, which form cis-diammine-Pt–DNA adducts, did not repress DUT-N expression, even when we added these drugs at 5 μmol/L (Fig. 1C, top). After entering cells, oxaliplatin is metabolized to dachplatin and oxalate. Because oxaliplatin and dachplatin exerted similar effects on DUT-N expression, our data indicate that the DACH–Pt–DNA adducts formed by oxaliplatin treatment, rather than the oxalate, induce a distinct DNA-damage response and efficiently repress DUT-N expression.

Oxaliplatin-induced p53 activation is not associated with the activation of Chk1 and Chk2 kinases

The previous data indicate that DACH–Pt–DNA adducts may elicit some type of DNA-damage signaling that is not activated by platinum-induced DNA strand breaks (26). To investigate how DACH–Pt–DNA adducts activate p53 and repress DUT-N expression, we treated HCT116 p53+/− cells with 10 μmol/L cisplatin or 1 μmol/L oxaliplatin (the IC₅₀) for 48 hours and examined the status of the ATM–Chk2 and ATR–Chk1 signaling pathways. In cisplatin-treated cells, phosphorylation of Chk1 at Ser345 ([p-Chk1(S345)], the primary target of ATR kinase (27), was detectable at 3 hours, peaked at 24 hours, and largely disappeared by 48 hours (Fig. 2A and B, left). Phosphorylation of p53 at Ser345 ([p-p53(S15]), which leads to a reduced interaction of p53 with MDM2 and p53 accumulation (28), was also detectable in a similar kinetics (Fig. 2A and C, right). Phosphorylation of Chk2 at Thr68 ([p-Chk2(T68)], the primary target of ATM kinase (27), was detectable later at 24 and 48 hours (Fig. 2A and B, right), as was p21 accumulation (Fig. 2A and D, left). By contrast, p-Chk1(S345) and p-Chk2(T68) were barely detectable in oxaliplatin-treated cells (Fig. 2A and B, even though p53 accumulation and p-p53(S15; Fig. 2A and C) and p21 accumulation and DUT-N dissipation (Fig. 2A and D) could be observed. These results suggest that DACH–Pt–DNA adducts activate p53 via a mechanism distinct from the one activated by cis-diammine–Pt–DNA adducts, which trigger replication stress and DNA strand breaks (3).
other than DNA strand breaks. Hence, we next investigated whether p53 activation in the absence of genotoxic stress would still induce the repression of DUT-N expression. Nutlin-3a activates p53 by disrupting the p53–MDM2 interaction and inhibiting MDM2 E3 ligase activity toward p53 (17). As described previously (29), 5 μmol/L nutlin-3a suppressed the growth of HCT116 p53+/+ cells, as did 5 μmol/L oxaliplatin (data not shown). Under these conditions, nutlin-3a and oxaliplatin induced accumulation of p53 and p21 and repression of DUT-N expression to a similar extent (Fig. 3A and B). Of note, p-p53(S15) was detected only in oxaliplatin-treated cells, suggesting that oxaliplatin might induce unidentified DNA damage that triggers this phosphorylation. These events were also observed in p53-proficient LoVo cells (Fig. 3C and D). These results indicate that unidentified DNA damage induced by oxaliplatin activates p53 via its phosphorylation, but p53 phosphorylation itself is not necessary for the repression of DUT-N expression.

Figure 1.
DACH-Pt-DNA adducts induce p53-dependent downregulation of nuclear isoform of dUTPase (DUT-N). A, oxaliplatin-induced DUT-N downregulation. Colorectal cancer cells were treated with oxaliplatin (Oxali) for 24 or 48 hours. B, effect of p53 knockdown on oxaliplatin-induced DUT-N downregulation. HCT116 p53+/+ cells were treated with siRNA against luciferase (siGL2) or TP53 (siTP53), and then treated with oxaliplatin. C, effects of other platinum drugs on DUT-N expression. HCT116 p53+/+ cells were treated with cisplatin (Cis), carboplatin (Carbo), or dachplatin (Dach) for 24 or 48 hours. Equal amounts of protein (10 μg) were separated by SDS-PAGE. Levels of DUT-N protein, normalized against β-actin, are shown relative to the control for the corresponding time point (defined as 1). Arrowheads and asterisks, DUT-N and mitochondria isoform of dUTPase (DUT-M), respectively.
Expression of the DUT gene is regulated by the transcription factors Sp1 and E2F1 (14), and the expression of E2F1 is transcriptionally activated by E2F3 (30). Therefore, we next investigated whether these transcription factors are involved in the repression of DUT-N expression by nutlin-3a and oxaliplatin. Both nutlin-3a and oxaliplatin decreased E2F3 and E2F1 protein levels in HCT116 p53<sup>+/−</sup> cells (Fig. 3A and B) and LoVo cells (Fig. 3C and D). Importantly, a reduction in the levels of E2F3 and E2F1 was already detectable at 24 hours, when the reduction in the DUT-N level was marginal (Fig. 3B and D),
indicating that downregulation of these transcription factors precedes the decrease in expression of their target gene product, DUT-N.

**Oxaliplatin induces pri-miR-34a before repression of DUT-N expression**

MicroRNA-34a (miR-34a) is a direct target of p53 and a candidate tumor suppressor of neuroblastoma (31). Introduction of miR-34a downregulates E2F3 and E2F1 protein expression in HCT116 and RKO cells (16). Nutlin-3a treatment upregulates miR-34a expression and represses B-Myb and E2F1 in p53wild-type leukemic cells (18). Hence, we next investigated whether oxaliplatin upregulates miR-34a expression in HCT116 cells. A slight but significant increase in the level of the primary miR-34a transcript (pri–miR-34a), was detected at as early as 3 hours after oxaliplatin treatment, and the level of pri–miR-34a increased gradually thereafter, up to 14-fold by 24 hours (Fig. 4A). Inversely, DUT transcript started to decline as early as 12 hours and reached 40% of its initial level at 24 and 48 hours (Fig. 4B). The oxaliplatin-induced upregulation of pri–miR-34a expression and repression of DUT transcript was also observed in LoVo cells (Supplementary Fig. S4A and S4B). In HCT116 p53−/− cells, pri–miR-34a was induced later than 12 hours, but the level of induction was significantly lower than in HCT116 p53+/+ cells at all time points (Fig. 4A). Moreover, the repression of DUT transcript was very limited in HCT116 p53−/− cells (Fig. 4B). These results indicate that oxaliplatin induces miR-34a before repression of DUT transcript, which is mostly dependent on p53.

At the protein level, accumulation of p53 was already detectable by 3 hours after oxaliplatin treatment, whereas p21 was detected at a later time point (9 hours; Fig. 4C and D). On the contrary, the level of DUT-N protein was not reduced until 48 hours after oxaliplatin treatment, whereas E2F3 and E2F1 protein levels were clearly reduced by 24 hours (Fig. 4C and D). These results suggest that signaling via the p53–miR-34a–E2F axis triggers the repression of DUT-N expression following oxaliplatin treatment. We next tested whether miR-34a mimics repress the expression of E2F1 and DUT-N. Introduction of miR-34a mimic decreased the levels of the E2F1 and DUT-N proteins at 72 hours, whereas introduction of a control miRNA had no effect (Fig. 4E), indicating that miR-34a plays a causative role in the repression of downstream target gene expression.
Oxaliplatin represses the expression of thymidylate biosynthesis genes and suppresses dTTP biosynthesis

The thymidylate (dTTP) biosynthesis pathway is a target of fluoropyrimidine-type (i.e., 5-FU) or antifolate-type (i.e., raltitrexed and pemetrexed) chemotherapeutic drugs (32). TS and DHFR, which function in the de novo thymidylate biosynthesis pathway, are the primary targets of 5-FU and antifolate drugs, respectively. In addition, the salvage thymidylate biosynthesis pathway, which is initiated by TK1-mediated phosphorylation of thymidine, is another route for dTTP biosynthesis (33). We focused on three genes encoding enzymes of the thymidylate biosynthesis pathway, DHFR, TK1, and TS, whose promoters have architectures similar to those of genes involved in the G1–S transition, including DUT (34), and whose transcript levels during cell-cycle progression are regulated by Sp1 and E2F1 (35–37). The expression of DHFR (Fig. 5A), TK1 (Fig. 5B), and TS (Fig. 5C) was repressed by oxaliplatin treatment in HCT116 p53+/+ cells, but not in HCT116 p53+/- cells (Fig. 5A–C). Consistent with this, 48 hours oxaliplatin treatment caused a significant reduction (~40-fold) of intracellular dTTP in the dNTP pool in HCT116 p53+/+ cells, whereas no such reduction was observed in HCT116 p53+/- cells (Fig. 5D). Importantly, ectopic expression of TK1 (Supplementary Fig. S5A), which significantly increased dTTP in normal proliferating HCT116 p53+/+ cells (Fig. 5E, black bars), partially...
Suppression of Thymidylate Biosynthesis by Oxaliplatin

Oxaliplatin has greatly improved 5-FU-based chemotherapy (4). In the adjuvant setting, the FOLFOX regimen also significantly improved 5-year disease-free survival and 6-year overall survival in patients with stage III colon cancer relative to the 5-FU plus leucovorin regimen (38, 39). However, it remains unclear why oxaliplatin increases the antitumor activity of fluoropyrimidine-type antitumor drugs. One reasonable mechanism that would explain the synergistic effect is p53-dependent repression of expression of DUT-N, a nuclear isoform of critical sanitizing enzyme dUTPase that hydrolyzes cytotoxic dUTP to dUMP, in response to oxaliplatin-induced DNA damage (14). In this study, we found that p53-dependent repression of DUT-N expression by oxaliplatin was preceded by induction of pri–miR-34a expression and repression of expression of the transcription factors E2F3 and E2F1. Furthermore, we showed that oxaliplatin also repressed DAPI, TK1, and TS expression in a p53-dependent manner, and thereby suppressed dTTP biosynthesis (Fig. 5D). When combined with DUT-N suppression, an elevated dUTP/dTTP ratio may exacerbate dUTP misincorporation into DNA, resulting in DNA fragmentation and cytotoxicity (Fig. 6).

Our data indicate that unidentified DNA damage by the DACH carrier ligand of oxaliplatin activates p53, but it remains unclear how oxaliplatin-induced DACH–Pt–DNA adducts activate a p53-dependent DNA-damage signaling pathway that represses DUT-N expression. p-p53(S15) was clearly detected when HCT116 p53+/− or LoVo cells were treated with 5 μmol/L oxaliplatin for 24 hours (Fig. 3A and C), consistent with previous reports (40, 41). Under our experimental conditions, p53 accumulation was clearly detectable when HCT116 p53+/− cells were treated with 1 μmol/L oxaliplatin for 6 hours and this p53 accumulation was also associated with p-p53(S15) (Fig. 3A and C). However, the p-p53(S15)/total p53 protein ratio appeared to be lower in oxaliplatin-treated cells than in cisplatin-treated cells (Fig. 2C, right). In addition, p-Chk1(S345) by ATR and p-Chk2(T68) by ATM were barely detectable in oxaliplatin-treated cells (Fig. 2A and B). Oxaliplatin may induce p-p53(S15) in a distinct mechanism, which would be unveiled by identifying the responsible kinase for p-p53(S15) by oxaliplatin. Because p-p53(S15) weakens the p53–MDM2 interaction and contributes to p53 accumulation by inhibiting MDM2-mediated p53 degradation (28) and nutlin-3a also induced p53-mediated repression of DUT-N expression without p-p53(S15) (Fig. 3A and C), this posttranslational modification of p53 itself may not be required for p53 function as a repressor of DUT-N expression once the p53–MDM2 interaction was disrupted.

Our time-course analysis revealed that expression of pri–miR-34a was upregulated (Fig. 4A) in concert with p53 stabilization after as little as 3 hours of 5 μmol/L oxaliplatin treatment (Fig. 4C), consistent with the fact that p53 directly upregulates miR-34a precursor expression (31). By contrast, reductions in the levels of DUT transcript and DUT-N protein were not observed alleviated dTTP starvation even when cells were cultured in the presence of 5 μmol/L oxaliplatin for 48 hours (Fig. 5E, gray bar), without affecting their cell-cycle profile (Supplementary Fig. S5B). These results indicate that oxaliplatin represses the expression of factors involved in the thymidylate biosynthesis pathway and suppresses dTTP biosynthesis, largely dependent upon p53.

Figure 5.

Oxaliplatin suppresses the expression of genes implicated in the thymidylate biosynthesis pathway. A–C, mRNA expression. The amounts of DHPH (A), TK1 (B), and TS (C) mRNA, normalized against GAPDH mRNA, in HCT116 p53+/− and p53−/− cells after 5 μmol/L oxaliplatin (Oxali) treatment are shown as relative to the level in the control (Ctrl) at each time point (defined as 1). Error bars, SEs from three independent experiments. Asterisks, statistically significant differences (unpaired t test) between control- and oxaliplatin-treated samples: **, P < 0.01; † † †, P < 0.001. D and E, relative amount of dTTP in the dNTP pool. D, HCT116 p53+/− and p53−/− cells either untreated or treated with 5 μmol/L oxaliplatin for 48 hours. The relative amount was calculated as the level of dTTP normalized against (+)-10-camphor sulfonic acid (10CS) in the control of each cell (defined as 1). E, oxaliplatin-treated HCT116 p53−/− cells with ectopic TK1-3xFLAG expression. The relative amount was calculated as the level of dTTP, normalized against 10CS from HCT116 p53−/− cells transfected with empty 3xFLAG expression vector (defined as 1). Error bars, SEs from three independent experiments; † indicates that signals from two of the three experiments were below the detection threshold. Asterisks, statistically significant differences (unpaired t test); **, P < 0.01; †, P < 0.05.
until 12 hours (mRNA, Fig. 4B) and 48 hours (protein, Fig. 4C and D), respectively. Furthermore, downregulation of E2F1 protein was already detectable at 24 hours (Fig. 4C and D). In leukemic cells, nutlin-3a activates p53 and upregulates miR-34a, which directly suppresses the translation of E2F3 protein, and E2F1 expression is concomitantly downregulated (18). Transient introduction of miR-34a into two human colon cancer cell lines, HCT116 and RKO, causes complete suppression of cell proliferation and induces senescence-like phenotypes by downregulating E2F3 and E2F1 protein expression (16). We also reproduced this result, but the reduction in E2F1 or DUT-N protein was not as complete as that induced by nutlin-3a or oxaliplatin (Fig. 4E). In addition, the significant reduction of DUT transcript was also seen before E2F downregulation (Fig. 4B and Supplementary Fig. S4B). These results support the idea that oxaliplatin-induced DACH–Pt–DNA adducts are the critical activators of p53 and its tumor-suppressive target miR-34; however, miR-34a is not the sole factor. This conclusion is reasonable because the repression of DUT transcript is mediated by direct binding of p53 to, and exclusion of Sp1 from, the promoter region (14).

In addition to DUT-N (Fig. 1A; ref. 14) and TS (Fig. 5C; ref. 13), we found that the expression levels of DHFR and TK1 mRNA were clearly reduced in HCT116 p53+/+ cells after 24 hours of oxaliplatin treatment (Fig. 5A and B). DHFR catalyzes the reduction of dihydrofolate to tetrahydrofolate, which is an essential cofactor for thymidylate biosynthesis by TS (35), and TK1 is an essential kinase of the salvage pathway for thymidylate biosynthesis (33). Consistent with this, following oxaliplatin treatment we observed a significant decrease in the level of dTTP in the dNTP pool (Fig. 5D), which was partially alleviated by ectopic TK1 expression (Fig. 5E). The dTTP level also decreases when HCT116 p53+/+ cells are cultured in the presence of TS inhibitors, such as 5-FU, FdUrd, or pemetrexed (42). Because it targets genes other than TS, oxaliplatin may synergize with the suppressive effect on thymidylate biosynthesis and the cytotoxicity resulting from dTTP depletion. This scenario provides a possible explanation for the enhancement of 5-FU–based chemotherapy.

The combination of 5-FU and oxaliplatin is a standard chemotherapeutic regimen for patients with metastatic colorectal cancer (38, 39). Although the correlation between the clinical benefit of the fluoropyrimidine–oxaliplatin combination and p53 status remains controversial (43, 44), it is possible that unidentified oxaliplatin-induced DNA damage could exert some cytotoxic effects even on p53-deficient tumor cells, especially in combination with fluoropyrimidine. Further analysis will be necessary to evaluate such possibility and clarify its underlying mechanism.

Disclosure of Potential Conflicts of Interest
Y. Maehara reports receiving commercial research grants from Taiho Pharmaceutical Co. Ltd., Yakult Honsha Co. Ltd., Chugai Pharmaceutical Co. Ltd., and Ono Pharmaceutical Co. Ltd. H. Kitao reports receiving a commercial research grant from Taiho Pharmaceutical Co. Ltd. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: S. Kiyonari, H. Kitao
Development of methodology: S. Kiyonari, M. Iimori, D. Miura, H. Kitao
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Figure 6. Oxaliplatin represses thymidylate biosynthesis via the p53–miR-34a–E2F axis. DACH–Pt–DNA adducts induced by oxaliplatin treatment effectively stabilize p53, accompanied by phosphorylation at Ser15, by an unknown mechanism. Stabilized p53 transcriptionally activates cell-cycle regulatory genes (e.g., CDKN1A) and pri–miR-34a, and represses Sp1-regulated genes. The expression of E2F3 and E2F1 is suppressed by mature miR-34a. These events lead to the downregulation of genes encoding DUT-N and thymidylate biosynthesis enzymes, dUTP accumulation and dTTP starvation, possibly resulting in cytotoxicity caused by uracil-mediated DNA fragmentation.
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References


The 1,2-Diaminocyclohexane Carrier Ligand in Oxaliplatin Induces p53-Dependent Transcriptional Repression of Factors Involved in Thymidylate Biosynthesis

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