Synthetic Lethality by Lentiviral Short Hairpin RNA Silencing of Thymidylate Kinase and Doxorubicin in Colon Cancer Cells Regardless of the p53 Status

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Abstract

Intracellular supply of dTTP is a highly regulated process and has been a key target for chemotherapeutic drug development. Thymidylate kinase (TMPK) is the key enzyme for dTTP formation in both de novo and salvage pathways. In this study, we used lentiviral-based small hairpin RNA to silence TMPK expression in p53(+/+) and p53(−/−) HCT-116 colon cancer cells. This approach was sufficient to decrease the dTTP pool gradually without affecting p53 expression and generating cytotoxicity. TMPK knockdown significantly increased doxorubicin sensitivity dramatically in p53-proficient, p53-null HCT-116, and LoVo colon cancer cells. The decrease in the dTTP pool using this approach augmented the DNA damage response and enhanced apoptotic induction to low-dose doxorubicin, leading to cell death. In contrast, silencing of thymidylate synthase which blocks the de novo pathway was incapable of sensitizing p53-null HCT-116 cells to doxorubicin-induced apoptosis because of the compensation by the salvage pathway. Our results suggest the lentiviral delivery of small hairpin RNA targeting TMPK in combination with a low dose of doxorubicin as a new approach to kill colon cancer cells regardless of p53 status. [Cancer Res 2008;68(8):2831–40]

Introduction

There are two major pathways controlling dTTP synthesis in cells, a de novo and a salvage pathway. In the de novo pathway, thymidylate synthase (TS) catalyzes the rate-limiting step of dUMP conversion to dTMP (1). In the salvage pathway, thymidine kinase (TK) is a key enzyme, transferring the terminal phosphate of ATP to the 5'-hydroxyl group of thymidine to form dTMP (2). Subsequent phosphorylation of dTMP by thymidylate kinase (TMPK) gives dTDP, which is then anabolized to dTTP by dNDP kinase for DNA synthesis (1). Therefore, TMPK is a key enzyme for dTTP formation via both the salvage and de novo pathways.

The high demand for deoxynucleotide triphosphate supply in DNA synthesis in cancer cells makes dTTP metabolism a key target for chemotherapeutic drug development. 5-Fluorouracil (5-FU) and 5-fluoro-2-deoxyuridine have been the most commonly used chemotherapeutic agents (3). 5-FU-based treatment inhibits cancer cell growth and initiates apoptosis by inhibiting TS in the de novo pathway and by direct misincorporation of fluoronucleotides into RNA and DNA (3). Multiple cellular factors can influence the sensitivity of cancer cells to 5-FU (4, 5), including the expression levels of genes involved in the metabolism of 5-FU (6, 7), its targeted enzyme TS (8–10), the genetic status of p53 (11, 12), and DNA mismatch-repair genes (13). In particular, the loss of p53 function (due to its high frequency of mutation) decreases the 5-FU responsiveness in cancer cells (14, 15). Because >50% of tumors harbor p53 mutations (16), it would be important to develop a strategy that can specifically perturb the synthesis of dTTP and damage cancer cells regardless of the p53 status and the cellular differences in the metabolic conversion of these anticancer agents.

TMPK is the key enzyme for catalyzing the reaction converting dTMP to dTDP (17). Therefore, it controls dTTP synthesis from either the de novo or salvage pathway. In this study, we used RNA interference (RNAi) to deplete TMPK in isogenic p53(+/+) and p53(−/−) colon cancer HCT-116 cells (18). Because the lentiviral system is a feasible approach to apply RNAi technology in molecular therapy (19–21), we generated lentivirus expressing TMPK shRNA to silence TMPK expression in p53(+/+) and p53(−/−) HCT-116 cells and to examine its effect on the dTTP pools. In addition, we assessed whether this strategy would have therapeutic potential in combination with genotoxic agents in cell killing.

Doxorubicin, an inhibitor of DNA topoisomerase II, has been widely used as a chemotherapeutic agent which acts by introducing double-strand breaks on DNA in proliferating cells to trigger apoptosis (22). The use of doxorubicin in cancer therapy has been limited by its cytotoxicity and the development of cellular resistance. Lack of p53 function is also associated with doxorubicin resistance (15). Therefore, it is more attainable to use a lower dosage of doxorubicin in combination with the new drug in triggering apoptosis regardless of tumor p53 status for cancer therapy. In this study, we sought to impair the DNA repair process by depleting dTTP, thus sensitizing cancer cells to doxorubicin-induced death regardless of the genetic p53 status. Our experimental results show that specific depletion of TMPK expression decreases the dTTP pool in HCT-116 cells, rendering colon cancer cells susceptible to doxorubicin-induced death.

Materials and Methods

Materials. Anti-hTMPK and anti-hTK1 polyclonal antibodies were prepared as described previously (23, 24). Anti-ITIS antibody (clone 4H8/1) and anti-p53 monoclonal antibody (ab-6) were purchased from Zymed and Calbiochem, respectively. Anti-β-tubulin, anti-mouse tetramethylrhodamine isothiocyanate (TRITC) antibody, doxorubicin, and thymidine were from Sigma. Anti-yH2AX (Ser139) antibody was from Cell Signaling. Anti-ATM antibody (2C1) was purchased from Santa Cruz. Anti-phosphorylated Ser1981 ATM was from Rockland. hTK1 small interfering RNA (siRNA) was purchased from Rockland.
HCT-116 cells were infected with lentiviral TMPKshRNA or scrambleshRNA (Invitrogen). Nucleotides 509 to 527 of hTMPK open reading frame and nucleotides 1017 to 1036 in the 3' untranslated region of the hTS gene were chosen as the target sequence, and the random sequences derived from hTMPK and hTS were scrambled. We synthesized one strand of oligonucleotide containing the target sequence followed by a 7-nucleotide short loop and sequence that was the reverse complement of the initial target sequence. The oligonucleotides in pairs were annealed and inserted into pENTR/U6 RNAi cassette to generate an entry construct. The lentiviral constructs were then individually cloned by recombination of the U6 RNAi cassette into the pLent6/BLOCK-iT-DEST vector.

Lentiviral shRNA production for infection. 293FT producer cells (6 × 10^5 cells) were cotransfected with the ViraPower packaging Mix (containing a mixture of the pLP1, pLP2, and pLP/VSVG plasmid) and pLent6(scramble)shRNA, TPMPKshRNA, or TSshRNA plasmid by Lipofectamine 2000 (Invitrogen). After 24 h, the supernatant containing lentivirus was collected and concentrated. The titer of lentivirus was determined by transducing HCT-116 cells with the virus stock at serial dilution for colony formation in culture medium containing blasticidin (5 µg/mL). The lentiviral scramble(shRNA), TPMPK(shRNA), and TS(shRNA) stocks in 1 mL of medium containing 8 µg/mL of polybrene were used to infect 2.5 × 10^5 cells with a multiplicity of infection of 18, 17.2, and 20, respectively, for 6 h, after which the supernatants were replaced with complete medium for the subsequent assays.

TK1 siRNA transfection. After 1 day of lentiviral scramble(shRNA) or TS(shRNA) infection, cells were transfected with or without 100 pmol of hTK1 siRNA with Lipofectamine 2000 for 6 h.

MTS cytotoxicity assay. After 48 h of lentiviral shRNA infection, 4 × 10^5 cells per well were seeded onto 96-well plates and allowed to grow for 24 h prior to doxorubicin treatment for a further 24 h. Cell viability was then measured by MTS assay (Promega; ref. 25).

Doxorubicin uptake measurement. The cellular uptake of doxorubicin was quantified by measuring the fluorescence intensity using a spectrofluorometer. After lentiviral scramble(shRNA) or TPMPK(shRNA) infection for 48 h, 3 × 10^4 cells were seeded on six-well plates overnight then treated with doxorubicin at different concentrations for 1 h. Cells were then trypsinized and lysed in 50 µL of 0.4% SDS followed by the addition of 1 mL of butanol and centrifugation at 3,000 rpm for 20 min. The supernatants were subjected to fluorescence measurement using a spectrofluorometer at wavelengths of 470 nm (excitation) and 590 nm (emission).

Whole-cell dTTP pool extraction and pool size determination. Cells (1 × 10^4) were washed twice with 1 mL of cold PBS and extracted with 1 mL of ice-cold 60% methanol at −20 °C overnight, followed by centrifugation for 30 min at 16,000 × g. The supernatant was transferred to a fresh tube and dried under vacuum. The residue was dissolved in sterile water and stored at −20°C for dTTP measurement based on the method of Sherman and Fye (26).

Apoptosis analysis. An Annexin V-FITC apoptosis kit (Oncogene Research Products) was used to detect apoptosis. Cells were trypsinized and suspended in PBS at ~1 × 10^6 cells/mL prior to staining with Annexin V-FITC for fluorescence microscopy.

Colonies formation assay. Cells were seeded at 4,000 cells/100 mm dish. After treatment, cells were washed with PBS and refreshed with growth medium. After culture for 14 days, surviving colonies were fixed with 100% ice-cold methanol, stained by crystal violet for counting.

Immunostaining of γH2AX foci. Cells were fixed with 3% paraformaldehyde for 30 min then blocked with 5.5% goat serum for 1 h. Following blocking, cells were stained with anti-γH2AX antibody (1:500) for 2 h at room temperature then stained with anti-mouse TRITC antibody (1:100) and 4′,6-diamidino-2-phenylindole for 1 h. After mounting overnight, γH2AX foci were observed by fluorescent microscopy.

Results

Silencing of TMPK decreases the dTTP pool in p53(+/-) and p53(−/−) HCT-116 cells. p53(+/-) and p53(−/−) HCT-116 cells were infected by lentivirus expressing TMPK and scrambled shRNA, and the expression levels of TMPK, TK1, TS, and dTTP pool and cell growth were determined in each day after infection. In both cell lines, the level of TMPK was significantly decreased after 1 day of TMPK(shRNA) lentiviral infection with a persistent silencing effect for 3 days without changing the expression level of TK1, TS, and p53 (Fig. 1A). In parallel samples, we found that the steady state level of dTTP was gradually reduced (Fig. 1B). TK1, TS, and p53 (Fig. 1C). Because the basal level of dTTP pool was much higher in p53(−/−) HCT-116 cells, it is possible that the decrease of dTTP pool by silencing of TMPK in p53-null cells did not reach a range that would severely affect DNA replication for cell proliferation. We further examined the effect of TMPK depletion on cell cycle distribution. After lentiviral infection for 3 days, p53(+/-) and p53(−/−) HCT-116 cells were subjected to flow cytometric analysis. There were 2-fold more S phase cells in p53(−/−) cells than in p53(+/-) cells, which was well correlated with the difference in dTTP pool size. TMPK knockdown caused an 8% and 11% increase of G2/M fraction in p53(+/-) cells and p53(−/−) cells, respectively (Fig. 1D), indicating that the G1-S progression was affected by the decrease of dTTP level. Because we did not detect the sub-G1 population, the growth retardation in cells depleted of TMPK was unlikely to be due to cytotoxicity.

Sensitization of HCT-116 cells to doxorubicin by TMPK depletion in colon cancer cells. These infected and control cells were then treated with doxorubicin at various concentrations, from 0.001 to 10 µmol/L for 24 hours, and cell viability was measured. The IC50 of doxorubicin was ~6.9 and 8.8 µmol/L in scrambled shRNA-infected p53(+/-) and p53(−/−) HCT-116 cells, respectively. Infection with virus expressing TPMPK(shRNA) dramatically
decreased the doxorubicin IC\textsubscript{50} to 0.7 and 0.5 μmol/L in p53(+/+) and p53(−/−) cells, respectively (Fig. 2A). Because TMPK depletion did not change doxorubicin transport in cells at various dosages of doxorubicin treatment (Fig 2B), the significant increase of doxorubicin sensitivity by TMPK depletion was not due to more doxorubicin uptake in both p53-proficient and p53-null colon cancer cells. Colonogenic assays were also performed in cells infected with or without lentivirus delivering shRNA against TMPK with exposure to 0.5 μmol/L of doxorubicin for 24 hours. After refreshment with drug-free growth medium, the ability of p53-proficient and p53-null cells with TMPK knockdown alone exhibited 20% to 30% reduction of the colony formation efficiency, whereas exposure to 0.5 μmol/L of doxorubicin alone suppressed the clonogenicity of p53-proficient but not p53-null cells (Fig 2C).

TMPK knockdown in combination with doxorubicin treatment drastically reduced the number of surviving colonies in both cell lines. Clearly, TMPK depletion significantly enhances the cell killing effect by low-dose doxorubicin exposure. Of note, a 3-fold difference in colony formation was still seen in the p53-proficient and p53-null cells depleted of TMPK with doxorubicin treatment, suggesting the contribution of p53 in suppressing cell survival. Silencing of TMPK also increased doxorubicin sensitivity ~8-fold in another human colon cancer cell line, LoVo (Fig 2D). Thus, lentivirus expressing TMPK\textsuperscript{shRNA} should be considered as a chemosensitizer for doxorubicin in killing colon cancer cells.

Amplification of doxorubicin-induced DNA damage response by TMPK depletion. We then investigated whether the decrease in the dTTP pool by TMPK knockdown augments DNA damage response induced by doxorubicin by examining histone γH2AX foci formation, which is a marker for persistent DNA damage due to double-strand breaks (27, 28). Treatment with low-dose doxorubicin (0.5 μmol/L) was unable to clearly cause γH2AX foci formation in p53(+/+) cells and p53(−/−) cells (Fig 3A). With TMPK shRNA viral infection, both p53(+/+) and p53(−/−) cells displayed strong γH2AX foci after treatment with low-dose doxorubicin. Because TMPK depletion by itself did not induce γH2AX foci formation in these cells (Fig 3A), these results suggest that TMPK depletion is able to intensify DNA damage lesions induced by doxorubicin. The Western blot analysis further showed specific induction of p53 by doxorubicin treatment, and the extent of p53 induction was further increased in cells with TMPK\textsuperscript{shRNA} virus infection (Fig 3B). Apparently, dTTP reduction enhances p53 induction in response to DNA double-strand break damage. Consistent with p53 induction, p21 expression was increased by doxorubicin treatment; however, TMPK knockdown did not further promote the extent of p21 induction, probably because apoptosis, instead of cell cycle arrest, predominates in p53(+/+) cells depleted of TMPK. In p53-null cells, TMPK knockdown markedly enhanced doxorubicin-induced ATM activation in contrast to the moderate amount of phosphorylated ATM detected in p53-proficient cells, indicating that a reduction in the dTTP pool enables p53-null cells to synergize with doxorubicin-induced DNA damage in the activation of the ATM checkpoint pathway.

To know whether there is an increase of dTTP synthesis to coordinate with DNA repair after DNA damage, HCT-116 p53(+/-) and p53(−/−) cells were treated with doxorubicin for 24 hours and harvested for dTTP pool determination (Fig 3C). It turned out that the dTTP level in p53-null cells was increased 3-fold to 3.5-fold after doxorubicin treatment as compared with a moderate 1.6-fold to 2.6-fold increase in p53-proficient cells. These results not only show the increase of dTTP level being associated with DNA damage response but also suggest that the cellular p53 context affects the magnitude of dTTP synthesis in response to doxorubicin treatment.

Lack of doxorubicin sensitization effect by TS knockdown in p53-null cells. It has been shown that silencing TS expression by small double-stranded RNAs in combination with TS inhibitor compounds was able to increase cell killing in a K0 variant expressing high levels of TS (29). In this study, we also tested the effect of TS depletion on doxorubicin sensitization by generating lentivirus expressing TS\textsuperscript{shRNA} for infecting p53(+/-) and p53(−/−) HCT-116 cells. Three days after infection, cells were harvested for Western blot analysis. Expression of TS\textsuperscript{shRNA} specifically depleted TS protein in both cell lines (Fig 4A). Interestingly, we found that TS depletion resulted in the elevation of TK1 expression in both cell lines and a concomitant increase of p53 level in p53(+/-) cells. We then treated these cells with doxorubicin at a concentration range of 0.001 to 10 μmol/L. The IC\textsubscript{50} of doxorubicin was 6.79 and 2.13 μmol/L for p53(+/-) and p53(−/−) HCT-116 cells infected by lentiviral scramble\textsuperscript{shRNA} and TS\textsuperscript{shRNA} respectively. As for p53(−/−) cells, TS depletion had no effect on doxorubicin sensitivity (Fig 4B). Thus, unlike TMPK knockdown, the increased susceptibility of HCT-116 cells to doxorubicin following TS depletion requires the participation of p53.

Differential effects of TMPK and TS depletion on apoptotic induction after low-dose doxorubicin treatment in p53-null cells. We further compared the effect of TS and TMPK knockdown on doxorubicin-induced apoptosis by FITC-labeled Annexin V staining. It seems that low-dose doxorubicin or TMPK knockdown alone had no effect on apoptotic induction (Fig 5A). Depletion of TMPK followed by doxorubicin treatment resulted in essentially all p53-null and p53-proficient cells becoming Annexin V-positive, an indication of extensive apoptosis (Fig 5A). In contrast, TS knockdown caused p53(+/-) but not p53(−/−) HCT-116 cells to undergo apoptosis following 1 μmol/L of doxorubicin treatment (Fig 5B). In conclusion, the combination treatment of low-dose doxorubicin with TMPK knockdown was more effective than with TS knockdown in inducing the apoptosis of colon cancer cells deficient of p53.

Figure 2. Alteration of doxorubicin sensitivity by TMPK depletion in colon cancer cells. A, p53(+/-) and p53(−/−) HCT-116 cells infected by lentiviruses delivering TMPK\textsuperscript{shRNA} or scramble\textsuperscript{shRNA} as described in the legend to Fig 1 were collected at 48 h, and were plated into a 96-well plate at 4 × 10\textsuperscript{3} cells per well. Following overnight culture, cells were treated with various concentrations of doxorubicin as indicated for 24 h, and cell viability were measured by MTS assay (points, mean of four to six experiments; bars, SE). B, after infection for 48 h, cells were plated at 3 × 10\textsuperscript{5} cells/well and treated with doxorubicin at the concentrations indicated. After 1 h, cells were harvested to measure doxorubicin uptake (columns, mean of six experiments; bars, SE). C, cells post-infected for 72 h were seeded in 100-mm dishes at 4,000 cells/dish. Following treatment with or without 0.5 μmol/L of doxorubicin for 24 h, cells were washed with PBS and refreshed with growth medium. After 14 days of culture, surviving colonies were fixed, stained by crystal violet, and counted (columns, mean of three independent experiments; bars, SE). *P < 0.05; **P < 0.01; ***P < 0.001 (based on a two-tailed Student’s t-test). D, LoVo cells were infected without or with lentiviruses delivering TMPK\textsuperscript{shRNA} or scramble\textsuperscript{shRNA} (SC) and were split into two pools. One pool, after transfection for 72 h, was used for Western blot analysis with anti-p53, hTMPK, and β-tubulin antibodies. Another pool of cells was plated into a 96-well plate at 4 × 10\textsuperscript{3} cells per well for the determination of doxorubicin sensitivity as described (points, mean of six experiments; bars, SE).
Contribution of the salvage pathway in compromising TS knockdown. Notably, TK1 protein expression level was elevated in cells depleted of TS and the steady-state level of dTTP was significantly increased after doxorubicin treatment. Therefore, one possible explanation for p53-null cells irresponsive to TS knockdown in doxorubicin sensitization is that dTTP synthesis via the salvage pathway might be able to compensate for the loss of de novo synthesis. To verify this speculation, we then depleted both TS and TK1 in p53-null cells followed by doxorubicin sensitivity measurement. Concordant with the results observed in silencing of TMPK, simultaneous knockdown of TK1 and TS was able to sensitize p53-null cells to doxorubicin-induced death (Fig. 6A). Furthermore, to substantiate the role of the salvage pathway, we used dialyzed serum to deprive exogenous thymidine supply and incubated cells depleted of TS for doxorubicin treatment. As a comparison, a parallel set of cells were treated with doxorubicin in the dialyzed-serum medium containing 10 μmol/L of thymidine. We found that TS knockdown decreased the doxorubicin IC₅₀.

Figure 3. Changes of doxorubicin-induced DNA damage response by TMPK depletion. A. p53(+/+ and p53(--/−) HCT-116 cells were infected with or without lentivirus delivering TMPK shRNA for 72 h with a subsequent 0.5 μmol/L of doxorubicin treatment as indicated. Cells were fixed with 3% paraformaldehyde and immunostained with anti-γH2AX antibody (1:500) and anti-mouse TRITC (1:100). The formation of γH2AX foci was observed by fluorescent microscopy. B, Western blot analysis of cell samples from the experiment described above. C, cells were treated with doxorubicin at different concentrations as indicated. After 24 h, cells were harvested for dTTP measurement. Points, averages from three independent experiments.

* P < 0.005; ** P < 0.001 based on a two-tailed Student’s t test.
from 9.1 to 1.3 μmol/L in p53(-/-) cells in medium containing
dialyzed serum, whereas the presence of thymidine abolished the
doxorubicin sensitization by TS depletion (Fig. 6B). Clearly, dTTP
synthesis via the thymidine-dependent salvage pathway contributes
to the insensitivity of p53-null cells to TS depletion with
doxorubicin treatment.

Discussion

De novo synthesis of dTTP has been an important targeted
pathway for chemotherapy. Because intracellular synthesis of
dTTP is also dependent on the salvage pathway, in this study,
we disrupted both de novo and salvage pathways in dTTP supply
simultaneously by depleting the expression of TMPK using
lentiviral delivery of shRNA targeting TMPK. Our results showed
that this approach effectively decreased the dTTP pools in
p53(+/-) and p53(-/-) HCT-116 cells without inducing cytotox-
icity. With exposure to genotoxic agent doxorubicin, we found
that TMPK knockdown was capable of sensitizing p53-null and
p53-proficient HCT-116 cells to doxorubicin killing. Because p53
is not functional in 50% of tumors (16), and drug resistance to
chemotherapy has been largely attributed to the status of p53
(15), our data suggest that lentiviral-based shRNA targeting
TMPK has the potential to improve the chemotherapeutic
efficacy of low-dose doxorubicin treatment for colon cancer
cells regardless of the p53 status.

In this study, we also tested the effect of TS depletion on
sensitizing HCT-116 cells to doxorubicin. Although TS knockdown
increased doxorubicin sensitivity ~ 3-fold in p53(+/-) cells, the
sensitization effect was much less than TMPK depletion. In
particular, it was noted that knockdown of TS but not of TMPK
by itself already increased the p53 level. This result is related to
the previous findings showing that p53 is induced upon inhibiting
TS by 5-FU or antifolate treatment due to the misincorporation of
dUTP into DNA (30). Considering the alteration in expression
levels of TK1 and p53, the molecular effect of TS knockdown
seems to be quite distinct from that of TMPK depletion. The
difference is probably because the TS knockdown, like 5-FU
treatment, leads to an increase of dUTP formation which in turn
generates a DNA damage signal (31), whereas TMPK depletion
might simply result in dTMP accumulation and a decrease in the
dTTP supply. In this report, we did not assess the effect of TS

Figure 4. Doxorubicin sensitization by TS knockdown dependent
on p53. A, p53(+/-) and p53(-/-) HCT-116 cells were treated
without or with lentiviral TShRNA or scrambleTShRNA (SC). After
72 h, cells were subjected to Western blot analysis with anti-p53,
hTS, hTK1, hTMPK, and β-tubulin antibodies. B, a proportion
of cells after lentiviral TShRNA or scrambleTShRNA infection and
without infection was collected for doxorubicin sensitivity
determination as described in the legend to Fig. 2.
depletion on the cellular dTTP and dUTP levels because our assay method for dTTP measurement was unable to distinguish dUTP from dTTP. Therefore, it remains to be determined whether sensitization of p53(+/+) HCT-116 cells to doxorubicin by silencing of TS is accomplished due to a decrease of the dTTP pool or due to dUTP-mediated DNA damage effect.

Very interestingly, we observed that up-regulation of TK1 was associated with TS knockdown in p53(+/+) and p53(−/−) HCT-116 cells. In contrast with p53(+/+) cells, TS depletion in p53(−/−) cells had no effect on increasing doxorubicin sensitivity. In this study, we showed that simultaneous silencing of TK1 and TS significantly increased doxorubicin sensitivity in p53-null cells. Moreover, TS knockdown was also able to sensitize p53-null cells to doxorubicin in dialyzed-serum medium devoid of exogenous thymidine. These data suggest that TS depletion up-regulates the expression of TK1 in the salvage pathway to provide sufficient dTTP in p53-null cells, thereby allowing these cells the capability to repair DNA for survival during doxorubicin treatment.

Figure 5. Differential effects of TMPK and TS depletion on doxorubicin-induced apoptosis in p53-null cells. FITC-labeled Annexin V apoptosis assay. p53(+/+) and p53(−/−) HCT-116 cells were infected with or without lentiviral TMPKshRNA for 72 h, treated with 0.5 μmol/L of doxorubicin for 24 h (A), or infected with or without lentiviral TSshRNA for 72 h with a subsequent 1 μmol/L doxorubicin treatment for 24 h as indicated (B). Cells were then stained with FITC-labeled Annexin V and observed by fluorescent microscopy.
Consistent with the role of TMPK in catalyzing the merging reaction from the salvage and de novo pathways for dTTP formation, we showed that dTTP reduction was effectively achieved by silencing TMPK in HCT-116 cells. Unlike TS knockdown, silencing of TMPK did not significantly increase the p53 level, even though the pool size of dTTP was reduced to half. Therefore, the reduction of dTTP pool by itself was insufficient to induce DNA damage response. In response to DNA double-strand breaks, cells activate the ATM pathway to arrest the cell cycle and initiate the repair process (32–34). In p53-null cells, the extent of ATM phosphorylation induced by doxorubicin exposure was markedly increased by the silencing of TMPK, indicating that the change in the dTTP pool amplifies the signal for ATM-mediated checkpoint pathways. As for p53(+/+) cells, silencing of TMPK increased the magnitude of p53 expression induced by doxorubicin treatment with a rather moderate increase in ATM activation. It is not yet clear whether the difference of the cell cycle distribution in p53-proficient and p53-null cells contributes to the differential effect of dTTP reduction on the extent of doxorubicin-induced ATM activation. Despite these molecular differences in response to TMPK depletion in combination with low-dose doxorubicin treatment, p53(+/+) and p53(−/−) HCT-116 cells all displayed intensified DNA damage lesions, as shown by a marked increase of γH2AX staining, and underwent apoptosis. At present, it is reasonable to assume that the decrease in the cellular dTTP pool might impair the repair process necessary for cell survival, thereby severing the extent of DNA damage and triggering apoptotic activation through the p53-dependent and p53-independent pathways.

Because TMPK knockdown was unable to sensitize normal IMR-90 fibroblasts to doxorubicin (data not shown), it is likely that
cancer cells with high proliferative rates are more susceptible to the synthetic lethality of TMPK depletion and low doses of doxorubicin. In conclusion, our study data showed several advantages of using lentiviral shRNA targeting TMPK in combination with doxorubicin in cancer treatment. First, it has a low cytotoxicity toward normal cells. Second, it activates apoptosis effectively in cancer cells regardless of the p53 status. Third, this approach has a therapeutic potential in treating colon cancer cells that are resistant to 5-FU due to a variety of cellular factors that affect the metabolism of 5-FU.

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References

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