Suppression of Reserve MCM Complexes Chemosensitizes to Gemcitabine and 5-Fluorouracil

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Abstract
Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest forms of cancer and is very difficult to treat with conventional chemotherapeutic regimens. Gemcitabine and 5-fluorouracil are used in the management of PDAC and act by indirectly blocking replicative forks. However, these drugs are not highly effective at suppressing disease progression, indicating a need for the development of innovative therapeutic approaches. Recent studies indicate that suppression of the MCM helicase may provide a novel means to sensitize cancer cells to chemotherapeutic agents that inhibit replicative fork progression. Mammalian cells assemble more MCM complexes on DNA than are required to start S-phase. The excess MCM complexes function as backup initiation sites under conditions of replicative stress. The current study provides definitive evidence that cosuppression of the excess backup MCM complexes sensitizes PDAC tumor lines to both gemcitabine and 5-FU, leading to increased loss of proliferative capacity compared with drugs alone. This occurs because reduced MCM levels prevent efficient recovery of DNA replication in tumor cells exposed to drug. PDAC tumor cells are more sensitive to MCM loss in the presence of gemcitabine than are nontumor, immortalized epithelial cells. Similarly, colon tumor cells are rendered less viable when cosuppression of MCM complexes occurs during exposure to the crosslinking agent oxaliplatin or topoisomerase inhibitor etoposide.

Implications: These studies demonstrate that suppressing the backup complement of MCM complexes provides an effective sensitizing approach with the potential to increase the therapeutic index of drugs used in the clinical management of PDAC and other cancers. Mol Cancer Res; 13(9); 1–10. ©2015 AACR.

Introduction
Pancreatic ductal adenocarcinoma (PDAC) is often diagnosed in its late stages and is associated with high mortality rates. Chemotherapeutic intervention for advanced pancreatic cancer involves treatment regimens with gemcitabine, or a combination chemotherapy regimen including 5-fluorouracil (5-FU; refs. 1, 2). Gemcitabine and 5-FU are pyrimidine analogues that negatively affect the deoxyribonucleotide pool required for DNA replication, thereby producing an indirect suppression of replicative fork functioning and S-phase stress. Unfortunately, these drugs demonstrate only modest efficacy against PDAC disease progression and are associated with toxic side effects (2). As such, the development of innovative approaches that increase the effectiveness of these drugs, ideally using lowered doses of gemcitabine and/or 5-FU, would be beneficial to pancreatic cancer patient management due to a better therapeutic index of the drugs in clinical practice.

Recent studies have suggested that suppression of the MCM complex (mini-chromosome maintenance) may provide for a novel means of increasing the effectiveness of chemotherapeutic drugs that slow or inhibit replication fork function (3–5). The MCM complex (comprised of Mcm2-7 subunits) is the hexameric ATPase core of the replicative helicase that is loaded onto DNA at multiple sites only during G1 phase in a process called licensing, which allows for one round of DNA replication per cycle (6–8). Certain MCM complexes are chosen to function as active helicases upon recruitment of Cdc45 to MCM cores, along with the GINS complex, producing what is referred to as a CMG helicase (9–11). The active CMGs represent the functional origins of DNA replication and proceed with the forks during elongation (9, 11–13). Mammalian cells load more MCM complexes onto DNA during G1 phase than are required to start and complete S-phase (called dormant origins), and estimates from several studies indicate that cells load approximately 3 to 10 times the number of MCM complexes than are needed (3, 5, 14). This excess of MCMs serves as a backup for cells when they are subjected to replication stresses during S-phase, in particular following exposure to fork-slowing agents (3, 5). Recovery from such agents requires the excess backup MCMs that had loaded in G1 phase, as no new MCMs can be loaded in S-phase (3–7, 15, 16).

Studies have shown that reduction/loss of the backup complement of MCMs causes human tumor cells to become sensitized...
to drugs that inhibit replication fork progression. Depletion of Mcm5 to approximately 25% of normal levels (effectively removing the backups) in U2OS osteosarcoma cells does not adversely affect cell growth on its own, but in combination with aphidicolin (DNA polymerase inhibitor) or hydroxyurea (ribonucleotide reductase inhibitor) proliferation is more impaired than with drugs alone (5). A similar antiproliferative sensitizing effect was observed when Mcm5 suppression occurred in combination with exposure to camptothecin (topoisomerase inhibitor, ref. 5). Reduction of Mcm2 or Mcm3 to approximately 5% to 10% of normal levels in HeLa cells also causes hypersensitivity to aphidicolin and hydroxyurea in terms of loss of proliferative capacity (3). Finally, reduction of ORC (origin recognition complex) or Cdc6 proteins, both involved in assembly of MCM complexes onto DNA, also sensitizes U2OS, HeLa, and MDA-MB-231 tumor cells to hydroxyurea and hydrogen peroxide as assessed by viability and proliferative capacity assays (17).

The studies described above indicate that a full complement of MCM complexes are required for cells to remain viable after exposure to certain drugs that slow or block replication fork progression and thereby cause S-phase stress. Reduction of MCM levels produces hypersensitivity to such drugs and decreases proliferative capacity of the population. Such findings have important implications for clinical application of this concept to antineoplastic therapy. As a prediction, cosuppression of MCM function offers the potential to enhance cytotoxicity and effectiveness of antineoplastic compounds that similarly affect replication fork activity, for which many exist in the clinical arsenal. However, with the exception of camptothecin, the drugs used thus far to investigate this chemosensitization concept (aphidicolin and hydroxyurea) are not indicated for clinical management of human cancer (3, 5). Gemcitabine and 5-FU are used in clinical management of PDAC and function by indirectly blocking replication fork activity (2). Given the results of these prior studies, it can be inferred that the effectiveness of gemcitabine and 5-FU against PDAC cell proliferation might also be increased in combination with MCM loss. However, a conclusive demonstration of this remains to be shown.

Using PDAC tumor lines as a model system for questions related to pancreatic cancer treatment efficacy, we show here definitive evidence that cosuppression of excess MCM complexes does indeed chemosensitize PDAC lines to both gemcitabine and 5-FU. We demonstrate that partial loss of MCMs, consistent with loss of the backup complement of MCMs, noticeably reduces the proliferative capacity of PDAC lines beyond that achieved with gemcitabine or 5-FU alone. Mechanistically, MCM reduction renders the tumor cells less capable of recovering DNA replication activity after exposure to antineoplastic drug. Importantly, nontransformed epithelial cells are not sensitized to similar doses of drug and MCM reduction compared with PDAC cells. We also show that MCM cosuppression causes an increase in the apoptotic index of colorectal cancer cells exposed to oxaliplatin or etoposide, two chemotherapeutic agents that similarly function via replication fork slowing mechanisms and are used in the management of colon cancer and other tumor types (18). Given that these chemotherapeutic compounds are known to be only modest in antitumor activity (e.g., for PDAC therapy) and/or display side effects that limit patient tolerance (1, 2, 18–21), our work provides a proof-of-principle that the development of anti-MCM drugs in the future has the potential to increase the therapeutic index of these existing antineoplastic drugs.

Materials and Methods

Cell culture, transfections, and drugs

Panc1 and SW480 cells (both from ATCC) were cultured less than 8 weeks from frozen stocks for these studies. Colo357 were obtained from Dr. Said Sebti (Moffitt Cancer Center, Tampa, FL), and cultured less than 8 weeks from frozen stocks. HaCaT cells were obtained from Dr. Petra Boukamp (University of Heidelberg, Germany), and were cultured less than 8 weeks from frozen stocks for this study. HaCaT were generated and characterized as nontumorigenic and keratinocyte in derivation (22). Panc1, Colo357, and HaCaT cells were cultured in DMEM supplemented with 10% FBS (Hyclone), and SW480 cells were cultured in RPMI medium containing 10% FBS. Transfections utilized Dharmafect reagent according to manufacturer methods (Thermo Scientific). For clonogenic and apoptosis assays, cells were seeded at 50% density and transfected the next day (time, 0 hour) with 1 to 3 nmol/L of siRNA targeting HsMcm7 or HsMcm4 (siGenome Smartpools, Thermo Scientific), or with nonspecific control siRNA (siGenome control, Thermo Scientific). Exposure to siRNA pools occurred for 120 hours (for clonogenic assays), or for 72 hours (for SW480 apoptosis assays and Panc1 recovery assays). Exposure to drugs occurred concurrent with siRNA treatment from 72 to 120 hours (for clonogenic assays) or 24 to 72 hours (SW480 apoptosis assays). Aphidicolin, gemcitabine, 5-FU, oxaliplatin, and etoposide were purchased from Sigma-Aldrich and used at concentrations indicated.

Immunoblotting and antibodies

For verifying successful siRNA knockdowns of MCM proteins by immunoblotting, equal numbers of cells were lysed and boiled in 1X Laemmli loading dye and analyzed by standard immunoblotting techniques (14). Chromatin enrichment of samples was done by isolating Triton X-100 detergent-resistant nuclear fractions as described (14, 23). To determine the degree of partiality of siRNA knockdown for Mcm7 and Mcm4, samples from 72 hours of knockdown were compared with serially titrated samples from control cells not exposed to siRNA. Percentages of the control lysates loaded are indicated in the figures, with 100% control lysate representing an equal amount of cell number lysate relative to the si-Mcm7 knockdown sample. Antibodies and concentrations used for immunoblots were mouse monoclonal anti-actin (1:10,000; Sigma), mouse monoclonal anti-Mcm7 (1:1,000; Santa Cruz Biotechnology), and chicken polyclonal anti-Mcm4 (1:1,000) generated by Aves Labs (14). Mouse monoclonal anti-Orc4 (1:500) was from BD Transduction Laboratories.

Cell proliferation and apoptosis assays

Cell proliferation (clonogenic) assays were conducted in duplicate on 35 mm culture dishes, using transfection regimens described above and in the figures. Prior to drug exposures, cells were reduced in density 48 hours after siRNA transfections by splitting 1:1,000 into new 35 mm plates (to allow better colony visualization). SW480 cells assayed for apoptosis were not split after siRNA transfections. Transfected and nontransfected cells were treated with the indicated drugs and doses as shown in the figures, and allowed to culture for the remainder of 2 weeks. Cells were then fixed and stained with Giemsa to quantify surviving colony numbers. Apoptosis assays on SW480 cells...
72 hours after siRNA transfections were conducted using an Annexin V staining kit (BD Biosciences) and flow cytometric analyses according to manufacturer’s methods.

DNA replication recovery assays

Equal cell numbers of asynchronous Panc1 cells in 24-well dishes were transfected with 100 nmol/L siRNA against Mcm7 or Mcm4, or with an equal amount of si-control for 72 hours. This achieved an approximately 90% to 95% partial reduction of Mcm7 protein (data not shown) for acute testing of DNA replication recovery. Parallel plates were not transfected as comparisons. Cells were exposed to 100 nmol/L gemcitabine for 6 hours, and medium was changed (at time 0) to remove drug and siRNAs. Cultures were pulsed 30 minutes in duplicate or triplicate with 3μCi/mL tritiated thymidine at the times indicated. Trichloroacetic acid precipitable material was processed for scintillation counting as described (24). The average counts per minute (cpm) were determined and plotted to measure DNA replication rates for each condition and time after drug removal.

Results

Hypersensitivity of Panc1 PDAC cells to gemcitabine and 5-FU following reduction of backup MCM complexes

Cosuppression of backup (excess) MCM complexes causes osteosarcoma and HeLa tumor lines to become sensitized to the growth-suppressive effects of aphidicolin or hydroxyurea (3, 5). Both drugs act by directly or indirectly blocking replication fork progression during S-phase. A prediction from this is that chemotherapeutic drugs indicated for specific cancer management in the clinic, and which similarly slow or inhibit replication fork activity, will become more effective in their growth-suppressive abilities when MCM levels are concurrently reduced. We tested this prediction using pancreatic ductal adenocarcinoma tumor lines as a model system. Gemcitabine and 5-FU are often used in chemotherapy regimens against PDAC, although their antiproliferative efficacy is very limited in this particular disease (2). We determined whether reduction of the backup complement of MCMs could render PDAC tumor lines (Panc1 and Colo-357) hypersensitive to the antiproliferative effects of gemcitabine and/or 5-FU.
The collective findings of several studies have indicated that mammalian cells contain approximately three to ten times the number of MCM complexes than are necessary for normal S-phase progression, indicating that approximately 70% to 90% of total MCM complexes are considered excess and provide backup functionality as dormant origins (3, 5, 14). Severe reduction of a single MCM subunit limits the ability of mammalian cells to proliferate (3), consistent with the fact that all 6 MCM genes are essential for yeast viability (25–27). Here, we intentionally wanted to obtain a partial knockdown of the backup complement of MCM complexes such that the partial MCM reduction on its own did not adversely affect cell proliferation. Using siRNA against Mcm7 in transfections of Panc1 cells at the recommended 200 nmol/L concentration (by Dharmacon) caused a severe loss of Mcm7 protein and suppression of cell proliferation (data not shown). However, siRNA concentrations between 1 and 100 nmol/L produced a partial, but significant, reduction of Mcm7 protein ranging from approximately 70% to 95% reduction that did not reduce cell growth on its own (see below). Figure 1A shows that 3 nmol/L siMcm7 reduces Mcm7 protein to approximately 30% levels in Panc1 cells. This is shown by comparing the level of Mcm7 protein after 72 hours of knockdown to serially titrated dilutions of untreated Panc1 cells via an immunoblot of whole-cell lysates. Our ability to reduce Mcm7 by approximately 70% indicates that we have significantly suppressed the levels of backup MCM complexes in Panc1 cells using our conditions. This 1 to 100 nmol/L concentration of siRNA treatment was used throughout our experimental approaches to achieve similar partial reductions of MCM subunit expression across cell types.

Reduction of a single MCM subunit in mammalian cells by siRNA-mediated approaches causes suppression of functional MCM complexes due to the hexameric dependency of the MCM complex for helicase activity (3, 5, 9, 11). Cells also appear to have a sensing mechanism that maintains equal MCM subunit stoichiometry (3, 14, 28, 29). This is evident in Panc1 cells, where partial reduction of Mcm7 or Mcm4 by siRNA also causes a partial reduction of the other subunit (Fig. 1B, top). Partial reduction of Mcm7 or Mcm4 also results in both subunits becoming diminished on chromatin, indicating that suppression of a single subunit causes a partial reduction of MCM hexamers on chromatin (Fig. 1B, bottom).

We next wanted to determine if this partial reduction of Mcm7 (and consequently backup MCM complexes) in Panc1 cells was not itself detrimental to cell growth, and more importantly, if such a reduction of Mcm7 caused Panc1 cells to become hypersensitive to gemcitabine and/or 5-FU. An assay was performed that assessed...
the ability of Panc1 cells to proliferate and form colonies (or not) following exposure to DNA replication stressors drugs concurrent with Mcm7 protein reduction. At the start of the assay cells were transfected with siRNA against Mcm7 or control siRNA (Fig. 1C). Mcm7 knockdown occurs by 24 hours (data not shown) and endures as long as 120 hours (see below). After 48 hours of siRNA treatment, cells were transferred to dishes at a lower density. At 72 hours, cells were exposed to various levels of drugs for 2 days, then allowed to culture for the remainder of 2 weeks. Cells were then fixed, stained, and assessed for resulting colony numbers under each condition. We verified by immunoblotting that the siRNA was effective against Mcm7 during the entirety of the drug exposure from 72 to 120 hours (Fig. 1D).

To relate our work to previously published studies (3, 5), sensitization of Panc1 cells to aphidicolin by Mcm7 suppression was determined. As can be seen in Fig. 1E, suppression of the excess MCM complement (via Mcm7 loss) was not itself detrimental to proliferative capacity of the population in the absence of drug exposure. However, cosuppression of the backup MCM complexes concurrent with drug exposure resulted in a significant sensitization to the antiproliferative effects of aphidicolin, consistent with other reports (3, 5). Representative fields for conditions of 0.5 µmol/L aphidicolin exposure +/- siRNA against Mcm7 or control siRNA are shown in Fig. 1F. Using the same experimental approach, we then asked if a similar chemosensitizing effect could be observed for gemcitabine and 5-FU exposure. In both cases, cosuppression of the excess backup MCM complements alongside drug exposure increased the antiproliferative effects of the compounds (Fig. 1G and H). Noteworthy, cosuppression of MCM complexes with 5 nmol/L gemcitabine reduced proliferative capacity more than gemcitabine alone at double the dose of 10 nmol/L (Fig. 1G).

To confirm and validate the above findings, we next assessed if coreduction of a different MCM subunit, Mcm4, could also chemosensitize Panc1 cells to 5-FU. Similar to that obtained for Mcm7, siRNA treatment against Mcm4 achieved a significant knockdown of Mcm4 protein to ~30% normal levels. The latter was demonstrated using immunoblotting for Mcm4 protein 72 hours after siRNA transfection compared with titrated amounts of whole-cell lysate from Panc1 cells (Fig. 2A). An experiment similar to that described above for Mcm7 assessment was performed (Fig. 2B). Successful Mcm4 reduction by siRNA during the experiment was verified by immunoblotting Panc1 lysates at 72 hours and 120 hours, the times bordering the duration of 5-FU exposure (Fig. 2C). Reduction of Mcm4 in the absence of drug exposure did not adversely affect cell proliferation, but as above for Mcm7 loss, cosuppression of MCM backup complexes via Mcm4 loss produced a noticeable chemosensitizing effect in conjunction with 5-FU exposure (Fig. 2D). Representative fields for conditions of 2 µmol/L 5-FU exposure +/- siRNA against Mcm4 or control siRNA are shown in Fig. 2E. We conclude from these experiments that cosuppression of the backup MCM complement by reduction of Mcm7 or Mcm4 during exposure to gemcitabine or 5-FU chemosensitizes Panc1 PDAC tumor cells to the antiproliferative effects of both drugs.

Reduction of the backup MCM complement chemosensitizes Colo-357 PDAC cells to 5-FU

We wanted to extend these observations to a different PDAC tumor line to confirm that the results were not cell type specific. Colo-357 PDAC tumor cells were analyzed in an experiment in which Mcm7 was partially suppressed concurrent with 5-FU drug exposure, and proliferative capacity determined for each condition (Fig. 3A). We verified that Mcm7 was partially reduced by siRNA treatment at 72 hours and 120 hours, both times of which border the exposure to 5-FU (Fig. 3B). Partial loss of Mcm7 in Colo-357 cells in the absence of drug exposure does not reduce proliferative capacity of the population (Fig. 3C). However,
partial loss of Mcm7 does result in a noticeable increase in the sensitivity of Colo-357 cells to concurrent exposure to 5-FU at any dose tested (Fig. 3C). Representative fields for conditions of 5 μmol/L 5-FU exposure/C6 siRNA against Mcm7 or control siRNA are shown in Fig. 3D. Strangely, Colo-357 cells were particularly resistant to inhibitory effects of gemcitabine (data not shown), and we were thus unable to assess the effects of MCM cosuppression with gemcitabine in these tumor cells. Nonetheless, we conclude from these experiments that, similar to Panc1 findings, Colo-357 PDAC tumor cells are also rendered chemosensitive to 5-FU exposure if MCM backup complexes are cosuppressed in availability.

MCM reduction suppresses recovery of DNA replication following drug exposure

We next determined the mechanism by which a loss of backup MCMs causes problems for PDAC cells exposed to gemcitabine. Other studies have shown that HeLa and U2OS cells exposed to aphidicolin or hydroxyurea in the presence of diminished MCM levels display signs of checkpoint activation and DNA damage responses. Biochemical indicators have included increased Chk1 and Chk2 activation, H2AX phosphorylation, or p53 phosphorylation (3, 5), consistent with replication fork stress. Interestingly, using multiple experimental approaches that manipulated drug doses, timing, and MCM knockdown levels, we were unable to detect changes in Chk1, p53, p53-p, H2AX-p, or the Blm protein (fork stress responder; ref. 30) in PDAC cells exposed concurrently to MCM reduction and gemcitabine (data not shown). Similar results were obtained by others in U2OS cells, where Chk1 activation and H2AX-p changes are not observed even though p53 is altered upon MCM loss and hydroxyurea exposure (5). It has been shown that some cancer cells are apparently defective for fork stress/damage sensor mechanisms that involve ATR activation, resulting in sensitivity of such cancer cells to functional MCM loss in the absence of drug exposure (31). This suggests that tumor cells may differentially respond to MCM reduction and concurrent drug exposure dependent on the presence or absence of sensor pathways that ultimately produce changes in replication fork stress indicators.

Despite a lack of observable biochemical indicators for replication fork stress, we reasoned that PDAC cells were having difficulty recovering DNA replication after gemcitabine exposure under conditions of reduced MCM levels. This is because backup MCMs have been shown by single-fiber DNA

![Figure 4](Image URL)
strand analyses to be required for dormant origin activation during replicative stress (3, 5). An experiment was designed as shown in Fig. 4A, where Mcm7 or Mcm4 were partially suppressed in Panc1 cells for 72 hours, then cells were exposed to gemcitabine for 6 hours, followed by drug removal and acute assessment of DNA replication recovery. Partial suppression of Mcm7 and Mcm4 protein expression was verified by immunoblotting (Fig. 4B). Quantitative analysis using tritiated thymidine incorporation showed that partial MCM suppression for 72 hours did not reduce the basal rates of DNA replication in the absence of drug (Fig. 4C), consistent with the suppressed MCMs providing a backup function rather than being required for ongoing DNA replication in unperturbed cells. However, similar tritiated thymidine incorporation analysis after drug exposure and removal demonstrated that reduction of Mcm7 (Fig. 4D) or Mcm4 (Fig. 4E) caused a significant suppression in DNA replication at each time interval relative to untreated and si-control–treated samples. Duplicate plates for each condition in clonogenic assays were scored on two fields each, and the results averaged ± 1 SD.

Nontransformed epithelial cells and PDAC cells display differential sensitivity to MCM loss and gemcitabine exposure

We next determined whether PDAC cells were more sensitive to gemcitabine exposure and MCM reduction relative to nontransformed cells. The immortalized and non–tumor-derived cell line HaCaT was used in this analysis (22, 32, 33). We wanted to compare PDAC cells to this highly proliferative, nontumorigenic epithelial cell type for two reasons. First, this removed any bias due to slow-proliferating issues (such as with primary cells) that might render drug + MCM loss ineffective simply due to lack of cell cycling/proliferation. Second, chemotherapy tends to cause adverse effects especially on epithelial cells in patients, and HaCaT are keratinocytes in derivation (22). Clonogenic assays were performed in the same manner as in Fig. 1, and Mcm7 levels were partially and similarly reduced by siRNA treatment in both Panc1 and HaCaT cells during the 72 to 120 hours overlapping gemcitabine treatment (Fig. 5A and B). The results of Fig. 5C and D show clearly that Panc1 cells are sensitive to gemcitabine exposure
under conditions of MCM reduction, while HaCaT cells remain unaffected in their proliferative potential. This suggests that suppression of MCM function might provide a selective adverse effect toward tumor cells during exposure to replicative stressing drugs.

Colon carcinoma cells display chemosensitivity to oxaliplatin and etoposide when MCMs are suppressed

We predicted that the chemosensitization effect of MCM suppression was likely to extend beyond PDAC growth suppression. We tested SW480 colon carcinoma cells for their sensitivity to oxaliplatin and etoposide when MCMs are suppressed. We found that the chemosensitization effect of MCM suppression was likely to extend beyond PDAC growth suppression. We tested SW480 colon carcinoma cells for their sensitivity to oxaliplatin and etoposide when MCMs are suppressed. We found that the chemosensitization effect of MCM suppression was likely to extend beyond PDAC growth suppression.

Figure 6.
Reduction of backup MCM complexes via Mcm7 downregulation sensitizes SW480 colorectal carcinoma cells to oxaliplatin and etoposide. A, immunoblot of SW480 cells treated with 1 nmol/L siRNA against Mcm7 for 72 hours, compared with titrated nontransfected cells. Mcm7 protein was reduced to approximately 30% normal levels. B, design of experiments in C–E. C, Mcm7 protein was partially reduced in SW480 cells during the 72 to 120 hours of drug exposure, measured by immunoblotting. D, clonogenic assay performed on SW480 cells with Mcm7 knockdown and oxaliplatin exposure at doses indicated. E, clonogenic assay performed on SW480 cells with Mcm7 knockdown and etoposide exposure at doses indicated. Duplicate plates for each condition in clonogenic assays were scored on two fields each, and the results averaged ± 1 SD. F, design of experiments in G–I. G, immunoblot verifying that Mcm7 protein was partially reduced in SW480 cells at 72 hours. H, SW480 cells exposed to oxaliplatin concurrent with partial loss of Mcm7 display an increased apoptotic index relative to untreated cells or cells treated with control siRNA. Annexin V staining and flow cytometry was used to assess the apoptotic index. Conditions were performed in duplicate, and results averaged ± 1 SD. I, SW480 cells with partially reduced Mcm7 levels are more sensitive to etoposide exposure relative to untreated cells or cells treated with control siRNA. Annexin V staining was again used to assess apoptotic levels of the populations for each condition. Conditions were performed in duplicate, and results averaged ± 1 SD.
oxaliplatin and etoposide in the presence and absence of MCM partial suppression. Oxaliplatin and etoposide are both replication fork–blocking drugs, with oxaliplatin functioning as a DNA crosslinking agent and etoposide as a topoisomerase inhibitor. Using similar conditions as we did for PDAC lines, Mcm7 was partially knocked down by siRNA exposure for 72 hours to approximately 30% of its normal levels in SW480 cells (Fig. 6A). We first performed clonogenic assays as shown in Fig. 6B. Partial reduction of Mcm7 protein in SW480 cells during the drug interval of 72 to 120 hours was verified by immuno-blotting (Fig. 6C). Exposure of SW480 cells to oxaliplatin or etoposide under conditions of MCM cosuppression reduced the proliferative potential of the tumor cells at all doses tested (Fig. 6D and E).

We next determined whether exposure to the drugs during MCM suppression resulted in elevated apoptotic responses in the SW480 cells. An experiment was performed in which Mcm7 was suppressed by siRNA for 72 hours, and drugs were added during the last 48 hours, followed by assessment of apoptotic index using Annexin V staining and flow cytometry (Fig. 6F). Knockdown of Mcm7 protein occurred from 24 to 72 hours (data not shown, but 72 hours shown in Fig. 6G). Treatment with si-control or siRNA targeting Mcm7 in the absence of either drug produced a slight increase in apoptotic levels of the SW480 population (Fig. 6H and I). However, upon exposure to oxaliplatin (Fig. 6H) or etoposide (Fig. 6I), cosuppression of MCM complexes produced a significant sensitization to both drugs, resulting in higher apoptotic indices relative to si-control or no siRNA treatment. Interestingly, cosuppression of MCMs appeared to almost double the effectiveness of both drugs at the highest concentrations tested (Fig. 6H and I). We conclude from these experiments that, similar to what was observed for gemcitabine and 5-FU, reduction of the backup complement of MCM complexes also chemosensitizes colorectal carcinoma cells to the suppressive effects of oxaliplatin and etoposide.

**Discussion**

We provide here definitive evidence that reduction of the excess backup complement of MCM complexes increases the chemosensitivity of multiple human tumor cell types to several therapeutic compounds used in the clinic to manage human cancer. Partial reduction of MCM complexes via downregulation of Mcm7 or Mcm4 causes PDAC tumor cells to become more sensitive to the antiproliferative effects of both gemcitabine and 5-FU. A similar effect is observed for a colon tumor line exposed to oxaliplatin or etoposide concurrent with cosuppression of MCM complexes. All of the chemotherapeutic drugs tested herein function indirectly or directly by blocking DNA replication fork activity, and prior studies have suggested that the backup complement of MCM complexes are required to facilitate replication recovery following exposure to drugs that cause replicative stress (3, 5). However, given that these prior studies relied primarily on assessing chemosensitization and MCM suppression using aphidicolin or hydroxyurea (3, 5), our work extends this important concept in a conclusive manner to drugs used in clinical practice that function via replication fork interference. We also demonstrate herein that, following exposure to gemcitabine, the recovery of DNA replication in PDAC lines is indeed abrogated when MCM levels are reduced in cells. Furthermore, nontransformed epithelial cells are less sensitive to MCM loss with gemcitabine exposure relative to PDAC cells, indicating that a selective advantage against tumor cell proliferation exists in cotargeting MCMs during chemotherapy regimens.

PDAC tumor lines can be chemosensitized by MCM suppression to drugs directly relevant to the clinical management of pancreatic cancers (gemcitabine and 5-FU), but which are not highly effective on their own (2). This has important implications for future therapeutic approaches for the management of PDAC disease, and suggests that the development of drugs targeting MCM functionality have the potential to enhance the therapeutic efficacy of current drugs used in PDAC regimens. Colon cancer management often involves the use of oxaliplatin in chemotherapy. However, oxaliplatin is associated with neuropathy that limits the ability to treat patients with this compound (18, 19). Similarly, etoposide is indicated for use in the clinic against several cancer types, including breast cancer, but etoposide is known to produce cardiotoxic side effects many years after clinical exposure that limit its utility (20, 21). Our work suggests that the development and use of anti-MCM drugs has the propensity to increase the therapeutic index of these and similar chemotherapeutic compounds when used in combination regimens. This might also allow for a reduced administration of oxaliplatin or etoposide that could mitigate the toxic side effects associated with these drugs. Although the potential utility in suppressing MCM function is clear, an important problem must be considered in any clinical approach targeting MCM suppression over sustained time periods. Partial suppression of MCM function can result in reduced genomic stability and increased DNA damage (3, 5, 28, 31), and mice with sustained, partially defective MCM function display increased cancer risk (30). As such, extended MCM suppression during chemotherapy may be contraindicated for clinical management of cancer, with perhaps shorter durations of MCM cosuppression being more advantageous. Alternatively, chemotherapy alternating with shorter anti-MCM regimens may be more effective and less likely to produce genomic damage in nontumor tissue.

**Disclosure of Potential Conflicts of Interest**

T.J. Yeatman is chief scientist at The Center for Advancement of Science in Space. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

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Writing, review, and/or revision of the manuscript: V.L. Bryant, M.G. Alexandrow

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.G. Alexandrow

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