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

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Running title: MCM Reduction Chemosensitizes PDAC Cells

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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 (5-FU) 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 back-up initiation sites under conditions of replicative stress. The current study provides definitive evidence that co-suppression of the excess/back-up MCM complexes sensitizes PDAC tumor lines to both gemcitabine and 5-FU, leading to increased loss of proliferative capacity compared to 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 non-tumor, immortalized epithelial cells. Similarly, colon tumor cells are rendered less viable when co-suppression of MCM complexes occurs during exposure to the crosslinking agent oxaliplatin or topoisomerase inhibitor etoposide.

Implications: These studies demonstrate that suppressing the back-up 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.
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) (1, 2). Gemcitabine and 5-FU are pyrimidine analogs 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 heterohexameric 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 ~3-10X the number of MCM complexes than are needed (3, 5, 14). This excess of MCMs serves as a back-up 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 back-up MCMs that had loaded in G1-phase, since no new MCMs can be loaded in S-phase (3-7, 15, 16).

Studies have shown that reduction/loss of the back-up complement of MCMs causes human tumor cells to become sensitized to drugs that inhibit replication fork progression. Depletion of Mcm5 to ~25% of normal levels (effectively removing the back-ups) in U2OS osteosarcoma cells does not adversely affect cell growth on its own, but in combination with aphidicolin (DNA polymerase inhibitor) or hydroxyurea (HU; ribonucleotide reductase inhibitor) proliferation is more impaired than with drugs alone (5). A similar anti-proliferative sensitizing effect was observed when Mcm5 suppression occurred in combination with exposure to camptothecin (topoisomerase inhibitor) (5). Reduction of Mcm2 or Mcm3 to ~5-10% of normal levels in HeLa cells also causes hypersensitivity to aphidicolin and HU 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 HU 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 anti-neoplastic
therapy. As a prediction, co-suppression of MCM function offers the potential to enhance cytotoxicity and effectiveness of anti-neoplastic 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 HU) 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 co-suppression 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 back-up 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 anti-neoplastic drug. Importantly, non-transformed epithelial cells are not sensitized to similar doses of drug and MCM reduction compared to PDAC cells. We also show that MCM co-suppression 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 anti-tumor 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 anti-neoplastic 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 non-tumorigenic and keratinocyte in derivation (22). Panc1, Colo357, and HaCaT cells were cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum (Hyclone), and SW480 cells were cultured in RPMI medium containing 10% fetal bovine serum. 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-hr) with 1-3nM of siRNA targeting HsMcm7 or HsMcm4 (siGenome Smartpools, Thermo Scientific), or with non-specific control siRNA (siGenome control, Thermo Scientific). Exposure to siRNA pools occurred for 120 hrs (for clonogenic assays), or for 72 hrs (for SW480 apoptosis assays and Panc1 recovery assays). Exposure to drugs occurred concurrent with siRNA treatment from 72-120 hrs (for clonogenic assays) or 24-72 hrs (SW480 apoptosis assays). Aphidicolin, gemcitabine, 5-fluorouracil, 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 hrs of knockdown were compared to 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), 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 35mm culture dishes, using transfection regimens described above and in the figures. Prior to drug exposures, cells were reduced in density 48 hrs after siRNA transfections by splitting 1:1000 into new 35mm plates (to allow better colony visualization). SW480 cells assayed for apoptosis were not split after siRNA transfections. Transfected and non-transfected cells were treated with the indicated drugs and doses as shown in the figures, and allowed to culture for the remainder of two weeks. Cells were then fixed and stained with Giemsa to quantify surviving colony numbers. Apoptosis assays on SW480 cells 72 hrs after siRNA transfections were conducted using an Annexin-V staining kit (BD Biosciences) and flow cytometric analyses according to manufacturer methods.

**DNA replication recovery assays.** Equal cell numbers of asynchronous Panc1 cells in 24-well dishes were transfected with 100nM siRNA against Mcm7 or Mcm4, or with an equal amount of
si-control for 72 hrs. This achieved an ~ 90-95% 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 100nM gemcitabine for 6 hrs, 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 (TCA) 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-fluorouracil following reduction of back-up MCM complexes.** Co-suppression of back-up (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-fluorouracil (5-FU) are often used in chemotherapy regimens against PDAC, although their anti-proliferative efficacy is very limited in this particular disease (2). We determined if reduction of the back-up complement of MCMs could render PDAC tumor lines (Panc1 and Colo-357) hypersensitive to the anti-proliferative effects of gemcitabine and/or 5-FU.

The collective findings of several studies have indicated that mammalian cells contain ~3-10X the number of MCM complexes than are necessary for normal S-phase progression, indicating that ~70-90% of total MCM complexes are considered excess and provide back-up 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 six MCM genes are essential for yeast viability (25-27). Here, we intentionally wanted to obtain a partial knockdown of the back-up 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 200nM concentration (by Dharmacon) caused a severe loss of Mcm7
protein and suppression of cell proliferation (data not shown). However, siRNA concentrations between 1-100nM produced a partial, but significant, reduction of Mcm7 protein ranging from ~70-95% reduction that did not reduce cell growth on its own (see below). Figure 1A shows that 3nM siMcm7 reduces Mcm7 protein to ~30% levels in Panc1 cells. This is shown by comparing the level of Mcm7 protein after 72 hrs of knockdown to serially-titrated dilutions of untreated Panc1 cells via an immunoblot of whole cell lysates. Our ability to reduce Mcm7 by ~70% indicates that we have significantly suppressed the levels of back-up MCM complexes in Panc1 cells using our conditions. This 1-100nM 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 (Figure 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 (Figure 1B, bottom).

We next wanted to determine if this partial reduction of Mcm7 (and consequently back-up 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 stressing drugs.
concurrent with Mcm7 protein reduction. At the start of the assay cells were transfected with siRNA against Mcm7 or control siRNA (Figure 1C). Mcm7 knockdown occurs by 24 hrs (data not shown) and endures as long as 120 hrs (see below). After 48 hrs of siRNA treatment, cells were transferred to dishes at a lower density. At 72 hrs, cells were exposed to various levels of drugs for 2 days, then allowed to culture for the remainder of two 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 hrs to 120 hrs (Figure 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 Figure 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, co-suppression of the back-up MCM complexes concurrent with drug exposure resulted in a significant sensitization to the anti-proliferative effects of aphidicolin, consistent with other reports (3, 5). Representative fields for conditions of 0.5 µM aphidicolin exposure +/- siRNA against Mcm7 or control siRNA are shown in Figure 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, co-suppression of the excess back-up MCM complement alongside drug exposure increased the anti-proliferative effects of the compounds (Figure 1G&H). Noteworthy, co-suppression of MCM complexes with 5 nM gemcitabine reduced proliferative capacity more than gemcitabine alone at double the dose of 10 nM (Figure 1G).

To confirm and validate the above findings, we next assessed if co-reduction 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 hrs after siRNA transfection compared to titrated amounts of whole cell lysate from Panc1 cells (Figure 2A). An experiment similar to that described above for Mcm7 assessment was performed (Figure 2B). Successful Mcm4 reduction by siRNA during the experiment was verified by immunoblotting Panc1 lysates at 72 hrs and 120 hrs, the times bordering the duration of 5-FU exposure (Figure 2C). Reduction of Mcm4 in the absence of drug exposure did not adversely affect cell proliferation, but as above for Mcm7 loss, co-suppression of MCM back-up complexes via Mcm4 loss produced a noticeable chemosensitizing effect in conjunction with 5-FU exposure (Figure 2D). Representative fields for conditions of 2 µM 5-FU exposure +/- siRNA against Mcm4 or control siRNA are shown in Figure 2E. We conclude from these experiments that co-suppression of the back-up MCM complement by reduction of Mcm7 or Mcm4 during exposure to gemcitabine or 5-FU chemosensitizes Panc1 PDAC tumor cells to the anti-proliferative effects of both drugs.

**Reduction of the back-up 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 (Figure 3A). We verified that Mcm7 was partially reduced by siRNA treatment at 72 hrs and 120 hrs, both times of which border the exposure to 5-FU (Figure 3B). Partial loss of Mcm7 in Colo-357 cells in the absence of drug exposure does not reduce proliferative capacity of the population (Figure 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 (Figure 3C). Representative fields for conditions of 5 μM 5-FU exposure +/- siRNA against Mcm7 or control siRNA are shown in Figure 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 co-suppression 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 back-up complexes are co-suppressed in availability.

MCM reduction suppresses recovery of DNA replication following drug exposure.

We next determined the mechanism by which a loss of back-up MCMs causes problems for PDAC cells exposed to gemcitabine. Other studies have shown that HeLa and U2OS cells exposed to aphidicolin or HU 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; (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 HU 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 back-up MCMs have been shown by single-fiber DNA strand analyses to be required for dormant origin activation during replicative stress (3, 5). An experiment was designed as shown in Figure 4A, where Mcm7 or Mcm4 were partially suppressed in Panc1 cells for 72 hrs, then cells were exposed to gemcitabine for 6 hrs, followed by drug removal and acute assessment of DNA replication recovery. Partial suppression of Mcm7 and Mcm4 protein expression was verified by immunoblotting (Figure 4B). Quantitative analysis using tritiated-thymidine incorporation showed that partial MCM suppression for 72 hrs did not reduce the basal rates of DNA replication in the absence of drug (Figure 4C), consistent with the suppressed MCMs providing a back-up 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 (Figure 4D) or Mcm4 (Figure 4E) caused a significant suppression in DNA replication at each time interval relative to untreated and si-control treated samples. Interestingly, although overall amounts of DNA replication were reduced by MCM loss, the slope of the curves were generally similar, indicating that existing forks were likely progressing at similar rates, but that there were fewer overall forks in the cells. This is consistent with the observation that MCM loss blocks activation of dormant origins on DNA fiber analysis (3, 5). We conclude that PDAC cells exposed to gemcitabine have a diminished ability to recover efficient DNA replication under conditions of MCM suppression.

Non-transformed epithelial cells and PDAC cells display differential sensitivity to
**MCM loss and gemcitabine exposure.** We next determined if PDAC cells were more sensitive to gemcitabine exposure and MCM reduction relative to non-transformed 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, non-tumorigenic 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 Figure 1, and Mcm7 levels were partially and similarly reduced by siRNA treatment in both Panc1 and HaCaT cells during the 72-120 hrs overlapping gemcitabine treatment (Figure 5A&B). The results of Figure 5C&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 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 hrs to ~30% of its normal levels in SW480 cells (Figure 6A). We first performed
clonogenic assays as diagrammed in Figure 6B. Partial reduction of Mcm7 protein in SW480 cells during the drug interval of 72-120 hrs was verified by immunoblotting (Figure 6C). Exposure of SW480 cells to oxaliplatin or etoposide under conditions of MCM co-suppression reduced the proliferative potential of the tumor cells at all doses tested (Figure 6D&E).

We next determined if 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 hrs, and drugs were added during the last 48 hrs, followed by assessment of apoptotic index using Annexin-V staining and flow cytometry (Figure 6F). Knockdown of Mcm7 protein occurred from 24-72 hrs (data not shown, but 72 hrs shown in Figure 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 (Figure 6H&I). However, upon exposure to oxaliplatin (Figure 6H) or etoposide (Figure 6I), co-suppression of MCM complexes produced a significant sensitization to both drugs, resulting in higher apoptotic indices relative to si-control or no siRNA treatment. Interestingly, co-suppression of MCMs appeared to almost double the effectiveness of both drugs at the highest concentrations tested (Figure 6H&I). We conclude from these experiments that, similar to what was observed for gemcitabine and 5-FU, reduction of the back-up 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 back-up 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 anti-proliferative effects of both gemcitabine and 5-FU. A similar effect is observed for a colon tumor line exposed to oxaliplatin or etoposide concurrent with co-suppression 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 back-up 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, non-transformed 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 co-targeting 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 co-suppression being more advantageous. Alternatively, chemotherapy alternating with shorter anti-MCM regimens may be more effective and less likely to produce genomic damage in non-tumor tissue.
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Figure legends

**Figure 1:** Reduction of back-up MCM complexes via downregulation of Mcm7 sensitizes PDAC cells to gemcitabine and 5-FU. (A) Immunoblotting demonstrates that siRNA treatment (3nM) reduces Mcm7 protein to 30% of normal levels. Lysates from Panc1 cells treated with si-Mcm7 for 72 hrs were compared to non-transfected Panc1 lysates, serially titrated to amounts indicated. (B) siRNA-mediated suppression of Mcm7 or Mcm4 causes reduction of Mcm7 and Mcm4, both in total levels and on chromatin. Total protein lysates and chromatin-enriched fractions were analyzed by immunoblotting. Actin and Orc4 probings are loading controls. (C) Diagram showing experimental design for cell proliferation assays. (D) Immunoblot verifying that Mcm7 protein was partially reduced in Panc1 cells during the entirety of drug exposure from 72-120 hrs. (E) Cell proliferation experiment (clonogenic assay) demonstrating that loss of Mcm7 chemosensitizes Panc1 cells to aphidicolin. Note that si-Mcm7 alone (i.e., loss of back-up MCM complexes without drug exposure) does not cause loss of proliferative capacity of the population. Duplicate plates for each condition were scored on three fields each, and the results averaged +/- 1s.d. For statistics, a single asterisk represents p-value less than or equal to 0.05, and two asterisks represent p-value less than or equal to 0.01. (F) Representative fields of the results from the experiment in panel E. Note that, in the absence of aphidicolin and presence of si-Mcm7, colony growth is similar in number and robustness to cells exposed to control-siRNA. (G) Clonogenic assay demonstrating that loss of Mcm7 chemosensitizes Panc1 cells to gemcitabine. Duplicate plates for each condition were scored on three fields each, and the results averaged +/- 1s.d. (H) Cell proliferation experiment demonstrating that loss of Mcm7 chemosensitizes Panc1 cells to 5-FU. Duplicate plates for each condition were scored on three fields each, and the results averaged +/- 1s.d.
Figure 2: Reduction of another member of the MCM complex, Mcm4, also sensitizes Panc1 cells to 5-FU.  
(A) Immunoblotting demonstrates that siRNA treatment (3nM) reduces Mcm4 protein to 30% of normal levels. Lysates from Panc1 cells treated with si-Mcm4 for 72 hrs were compared to non-transfected Panc1 lysates, serially titrated to amounts indicated. Partial reduction by si-Mcm4 therefore yields loss of ~70% of normal Mcm4 protein level, or the majority of back-up MCM complexes, similar to loss of Mcm7 in Figure 1. (B) Diagram showing experimental design for cell proliferation assay. (C) Immunoblot verifying that Mcm4 protein was partially reduced in Panc1 cells during the entirety of drug exposure from 72-120 hrs. (D) Cell proliferation experiment demonstrating that loss of Mcm4 chemosensitizes Panc1 cells to 5-FU. Note that si-Mcm4 alone (i.e., loss of back-up MCM complexes without drug exposure) does not cause loss of proliferative capacity of the population. Duplicate plates for each condition were scored on three fields each, and the results averaged +/- 1s.d. (E) Representative fields of the results from the experiment in panel D. Note that, in the absence of 5-FU and presence of si-Mcm4, colony growth is similar in number and robustness to cells treated with control-siRNA.

Figure 3: Reduction of Mcm7 sensitizes Colo357 PDAC cells to 5-FU.  
(A) Diagram showing experimental design for cell proliferation assay. (B) Immunoblot verifying that Mcm7 protein was partially reduced by siRNA (3nM) in Colo357 cells during the entirety of drug exposure from 72-120 hrs. (C) Cell proliferation experiment demonstrating that loss of Mcm7 chemosensitizes Colo357 cells to 5-FU. Note that si-Mcm7 alone (i.e., loss of back-up MCM complexes without drug exposure) does not cause loss of proliferative capacity of the population.
Duplicate plates for each condition were scored on three fields each, and the results averaged +/- 1s.d. (D) Representative fields of the results from the experiment in panel C. Note that, in the absence of 5-FU and presence of si-Mcm7, colony growth is similar in number and robustness to cells treated with control-siRNA.

Figure 4: MCM reduction suppresses recovery of DNA replication following gemcitabine exposure in Panc1 cells. (A) Diagram showing experimental design. (B) Immunoblots showing partial reduction of Mcm7 and Mcm4 in Panc1 cells at time of drug exposure (72 hrs). (C) Basal DNA replication rates were determined prior to drug addition. Tritiated-thymidine was pulsed for 30 minutes after 72 hrs of treatment with Mcm7 siRNA, si-control, or no transfection. (D&E) Tritiated-thymidine was pulsed for 30 minutes in cells at the times indicated after drug removal at time 0. For panels C-E, incorporation of thymidine was measured in duplicate or triplicate samples via scintillation counting. Results were averaged and plotted, +/- 1 s.d.

Figure 5: PDAC cells are selectively chemosensitized to gemcitabine by MCM reduction. Experimental design was the same as for clonogenic assays in Figure 1. (A) Immunoblot showing partial Mcm7 reduction (3nM siRNA) in Panc1 PDAC cells during the 72-120 hrs overlapping drug exposure. (B) Immunoblot showing partial Mcm7 reduction by siRNA (3nM) in immortalized HaCaT keratinocytes cells during the 72-120 hrs overlapping drug exposure. (C) Clonogenic assay performed with Mcm7 knockdown and gemcitabine doses indicated on Panc1 cells. (D) Clonogenic assay performed with Mcm7 knockdown and gemcitabine doses indicated on HaCaT cells. Duplicate plates for each condition in clonogenic assays were scored on two fields each, and the results averaged +/- 1s.d.
**Figure 6: Reduction of back-up MCM complexes via Mcm7 downregulation sensitizes SW480 colorectal carcinoma cells to oxaliplatin and etoposide.** (A) Immunoblot of SW480 cells treated with 1nM siRNA against Mcm7 for 72 hrs, compared to titrated non-transfected cells. Mcm7 protein was reduced to ~30% normal levels. (B) Design of experiments in panels C-E. (C) Mcm7 protein was partially reduced in SW480 cells during the 72-120 hrs 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 +/- 1s.d. (F) Design of experiments in panels G-I. (G) Immunoblot verifying that Mcm7 protein was partially reduced in SW480 cells at 72-hrs. (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 +/- 1s.d. (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 +/- 1s.d.
Bryant, et al., Figure 5

A

Mcm7 siRNA

-  
-  
+  
+  

Mcm7  Actin

72 hrs  120 hrs

Panc1

B

Mcm7 siRNA

-  
-  
+  
+  

Mcm7  Actin

72 hrs  120 hrs

HaCaT

C

Panc1

control siRNA  Mcm7 siRNA

Average number of colonies +/- lsd

no drug  1 nM  2 nM

D

HaCaT

control siRNA  Mcm7 siRNA

Average number of colonies +/- lsd

no drug  1 nM  2 nM
Suppression of Reserve MCM Complexes Chemosensitizes to Gemcitabine and 5-Fluorouracil

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