Resveratrol Enhances Mitomycin C-mediated Suppression of Human Colorectal Cancer Cell Proliferation by Up-regulation of p21$^{WAF1/CIP1}$

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Abstract. *Background/Aim: Studies have shown that natural products could potentially be employed in combination therapies to decrease toxicity to healthy tissues by chemotherapy drugs. No studies however, have investigated the potential modulatory role of resveratrol (RV) on mitomycin C (MMC)-mediated effects on colorectal cancer. The aim of the present study was to investigate the impact of RV on MMC-mediated inhibition of colorectal cancer cell proliferation and to assess the potential mechanisms for such effects. Materials and Methods: Primary cell lines generated from resected colorectal tumor specimens were treated with RV, MMC or RV+MMC and cell proliferation and gene expression analyses were performed. Results: Suppression of cell proliferation by RV+MMC was significantly greater than individual treatments. RV+MMC synergistically modulated several genes but the up-regulation of p21$^{WAF1/CIP1}$ was several-fold greater. Conclusion: The up-regulation of p21$^{WAF1/CIP1}$, which inhibits the cell cycle at G_0/G_1 and G_2/M phases, may represent the predominant mechanism for enhancement of MMC-mediated anti-cancer effects by resveratrol.*

The relative lack of specificity of the majority of chemotherapeutic drugs for malignant cells is largely responsible for the toxicity to normal tissues experienced by patients (1). Furthermore, while successful in controlling tumor progression and prolonging survival in most cancer patients, chemotherapy-alone is rarely curative (2-4). Because of the dual problem of limited effectiveness against malignant cells and significant toxicity to normal tissues, numerous approaches to enhance the clinical effectiveness of chemotherapy without increasing its toxicity have been tested. One such approach that has been investigated for decades is to combine extracts of different natural substances with different conventional cancer treatments in an "integrative" model of care.

Justification for combining natural products with anti-cancer therapy is based on the demonstration that a wide variety of natural products exert anti-tumor effects in different systems, while being well-tolerated *in vivo* (5-8). However, a standard approach for designing combinations of natural products and cancer chemotherapy, that reliably improves clinical effectiveness compared to chemotherapy-alone, has yet to be developed. The exponential increase in our knowledge of the cellular and molecular changes responsible for the malignant phenotype, coupled with the understanding of how natural products and cancer drugs affect these processes, should provide guidance for designing maximally-effective combinations of these agents. With respect to enhancement of specific anticancer drugs, one approach might be to identify natural products that can exert additive or even synergistic effects against critical molecular pathways targeted by the chemotherapy agent. Another approach might be to employ natural products whose mechanism of action favorably affects a molecular feature of malignancy not affected by the chemotherapy agent (*i.e.* a collaborative or complementary approach).

The current study was designed to assess the capacity of a widely used natural product, resveratrol (RV), to increase the effectiveness of a chemotherapy agent, mitomycin C (MMC), in inhibiting the proliferation of human colorectal cancer cells. This model was chosen in part because of the extensive knowledge of how each substance affects tumor cell growth in other systems, as well as the relative availability of human colorectal cancer tissues. RV is a naturally-occurring polyphenol present in the skin of grapes and other foods (9), which is typically administered orally. Its pluripotent anticancer properties and low toxicity (10) make it an ideal candidate for use in combination with standard

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chemotherapy drugs. MMC is one of the families of mitomycins considered as anti-tumor antibiotics because of their capacity to inhibit bacterial DNA synthesis. They were originally isolated by extraction from different *Streptomyces* strains and subsequently chemically-purified. Their principal mechanism of action is as a DNA cross-linker. In cancer patients, MMC can be given either intravenously or topically to treat upper gastro-intestinal (*e.g.* esophageal carcinoma), peritoneal cancers of gastro-intestinal origin, anal cancers, breast cancers and bladder cancers, where it can be given by bladder instillation for superficial tumors (11-15). In addition, MMC is used to perfuse the peritoneal cavity following surgical de-bulking of patients with peritoneal carcinomatosis of gastrointestinal origin in the HIPEC (hyperthermic intraperitoneal chemotherapy) procedure (16).

The present study was conducted to determine whether RV's pluripotent effects on cancer cells can potentiate MMC-mediated effects on colorectal cancer cell proliferation and whether this modulation involved cell-cycle genes. Moreover, to assess the consistency of any effects observed with complex, heterogeneous malignant tissues, the current study employed cells prepared from resected tumors obtained from cancer patients at the time of their treatments. The results show that RV augments the anti-proliferative effect of MMC on colorectal cancer cells suggesting that RV may enhance the clinical effectiveness of MMC therapy.

Materials and Methods

Study design. Short term, primary cell lines were established from resected colorectal tumor specimens, classified and characterized by the attending surgical pathologist on each case. The tumors were obtained under an institutional review board approved protocol, from five female patients (age range=44-64 years) who were undergoing treatment for colorectal adenocarcinoma. These cell lines were treated with MMC, RV or their combination (RV+MMC). Cell proliferation assays were performed at 72 h. RNA isolation for quantitative polymerase chain reaction gene expression array analysis was also performed at 72 h.

Cell culture. Tumor lines were passaged in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Corning, Manassas, VA, USA) and 50 μg/ml Gentamicin (Sigma-Aldrich Corp., St. Louis, MO, USA). Tumor specimens received from surgery were dissected into 1mm slices and subjected to a collagenase/DNase (0.14/0.1%, Sigma) digestion at 37˚C for 1-2 h. After digestion, specimens were strained, washed in HBSS (Sigma), and put into culture.

Gene expression arrays. Transcriptional expression of genes was determined on the extracted RNA using 96-well real-time PCR arrays. cDNA was synthesized using the RT² First Strand cDNA Kit (SABiosciences Corp., Frederick, MD, USA) according to the manufacturer's instructions. Transcriptional gene expression was performed using the Human Cell Cycle RT² Profiler PCR Array System (SABiosciences Corp., Frederick, MD, USA), according to the manufacturer's instructions. Real-time PCR was performed using the MyiQ Real-time PCR detection system (Bio-Rad Laboratories Inc., Hercules, CA, USA). All transcriptional gene expression analyses were performed on the SABiosciences Corp. web portal using the RT² Profiler PCR Array Data Analysis program (version 3.2). Transcriptional gene expression, which is subsequently referred to as gene expression, was defined as fold-change *versus* media controls.

Cell proliferation assay. Cell proliferation was measured by a standard MTS Assay (Promega, Madison, WI, USA). A total of 5,000 cells in logarithmic growth phase were inoculated into treatment wells in quadruplicate. Cells were subjected to treatments with RV (5 μg/ml), MMC (100 ng/ml) or RV+MMC and incubated for 72 h. Three hours before the end of the incubation period, MTS reagent was added to the plates and absorbance readings were performed on a spectrophotometer at 490 nm (BioTec Instruments, Winooski, VA, USA).

Statistical analyses. The data pooled from the 5 primary colorectal cancer cell lines are expressed as means±standard error of the means (S.E.M). One-way analysis of variance (ANOVA) with *post hoc* pairwise multiple comparisons using the Student-Newman-Keuls method was performed to determine significance of differences between treatments.

Results

Assessment of RV's ability to impact MMC-mediated effects on proliferation of colorectal cancer cells. To establish optimal conditions for *in vitro* treatment of cells, one of the primary cell lines was used in the *in vitro* proliferation assay. The results (not presented) demonstrate the capacity of RV to inhibit proliferation in a concentration-dependent manner. In the same cultures, inhibition mediated by MMC alone (100 ng/ml) was enhanced by RV in a concentration-dependent manner. Optimal enhancement for the combination was exhibited with a RV concentration of 5 μg/ml combined with 100 ng/ml MMC; therefore, these conditions were used for all subsequent studies.

Effect of RV and MMC on colorectal cancer cell proliferation. Treatment of the 5 primary colorectal cancer cell lines with RV, MMC and RV+MMC significantly and consistently decreased cell proliferation; mean values were 56% ($p<0.001$), 43% ($p<0.001$) and 82% ($p<0.001$), respectively, relative to media controls (Figure 1). The suppression of colorectal cancer cell proliferation by the combination treatment was significantly greater than RV ($p<0.025$) and MMC ($p<0.005$).

Effect of RV and MMC on the expression of genes related to the cell cycle. The expression of cell-cycle regulating genes was differentially affected by treatment with RV, MMC or the combined treatment. Some genes were not affected while others were either increased or decreased by these treatments. There was, however, a group of nine genes that

were affected significantly and consistently in all 5 primary cell lines whereby the effect of the MMC treatment was enhanced with the addition of RV as described here.

Effect of RV and MMC on the expression of cyclins. The expression of cyclin B1 and B2 was suppressed by all treatments relative to untreated cells (Figure 2). Cyclin B1 expression was suppressed in comparison to control cells by 1.91 folds and 5.05 folds by RV ($p<0.05$) and MMC ($p<0.05$), respectively. The combined treatment elicited synergistic suppression greater than 18 folds ($p<0.05$ *vs*. control) which was significantly greater than RV ($p<0.05$) or MMC ($p<0.05$) alone (Figure 2).

Cyclin B2 expression was suppressed by RV ($p<0.05$) and MMC ($p<0.05$) compared to untreated cells. The expression by MMC was significantly different from that observed with RV ($p<0.05$). Cyclin B2 was also suppressed in a synergistic manner by the combined treatment. The 13-fold decrease observed in the combined treatment was significantly greater than that observed with either RV-alone (1.56-fold; $p<0.05$) or MMC-alone (4.1-fold; $p<0.05$) (Figure 2).

Effect of RV and MMC on the expression of cyclin-dependent kinase. The expression of cyclin-dependent kinase 1 (CDK1) was significantly decreased by both RV ($p<0.05$) and MMC ($p<0.05$) relative to untreated cells but these levels were not different relative to each other (Figure 2). The combination treatment of RV+MMC resulted in synergistic suppression of 13-fold, which was significantly greater than that by either RV-alone (1.4-fold; $p<0.05$) or MMC-alone (3.2-fold; $p<0.05$).

Effect of RV and MMC on the expression of genes regulating cyclin-dependent kinases. The expression of cyclin-dependent kinase inhibitor 1A (p21$^{WAF1/CIP1}$, CDKN1A) was increased by all treatments relative to untreated cells ($p<0.05$; Figure 3). The increased p21$^{WAF1/CIP1}$ expression elicited by RV (10-fold; $p<0.05$) and MMC (13-fold; $p<0.05$) individually was comparable with no significant difference between the 2 agents. With the combined treatment, however, a synergistic increase in p21$^{WAF1/CIP1}$ expression of 34-fold relative to untreated cells ($p<0.05$) was observed, which was significantly greater than that observed with either RV alone or MMC alone.

The effect on the expression of CDKN3 was diametrically opposite to that observed for p21$^{WAF1/CIP1}$. The expression of CDKN3 was significantly decreased in all treatments relative to untreated cells ($p<0.05$) (Figure 3). The suppression of CDKN3 by MMC was significantly different form RV ($p<0.05$). The expression of CDKN3 was also affected in a synergistic manner by the combined treatment. It was suppressed by 13-fold relative to untreated cells, which was significantly greater than that observed with RV (1.5-fold; $p<0.05$) and MMC (3.6-fold; $p<0.05$).

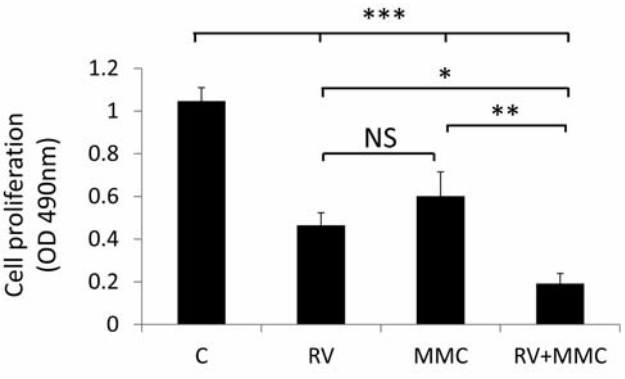

Figure 1. *Effect of Resveratrol (RV) and Mitomycin C (MMC) on proliferation of colon cancer cells. Cell proliferation was determined in colorectal cancer cells treated with RV or MMC or a combination of RV+MMC. MTS reagent was added in the last 3h of a 72h period and OD (490nm) values were taken. The data are expressed as means±S.E.M. of 5 distinct colorectal cancer cell lines. Statistical analysis: One-way ANOVA with post hoc pairwise multiple comparisons using the Student-Newman-Keuls method. *p<0.025; **p<0.005; ***p<0.001; NS: not statistically significant; n=5.*

Effect of RV and MMC on the expression of genes regulating various cell cycle functions. The expression of Checkpoint kinase 1 (CHEK1) was significantly decreased by all treatments relative to untreated cells ($p<0.001$) (Figure 4). The 3.0-fold decrease in the combination treatment was significantly greater than that by RV (1.4-fold; $p<0.001$) and MMC (1.9-fold; $p<0.002$) treatments alone.

RV+MMC also had a synergistic effect on the expression of Karyopherin Alpha 2 (*KPNA2*) a gene for the nuclear importer protein KPNA2 (Figure 4). Its expression was suppressed 5.4-fold in the combination treatment, which was significantly greater than that observed for RV-alone (1.9-fold; $p<0.001$) and MMC-alone (3.0-fold; $p<0.01$).

Similarly, a mitotic spindle assembly checkpoint protein, MAD2 Mitotic Arrest Deficient-Like 1 (MAD2L1) was also suppressed in all treatments ($p<0.05$; Figure 4). The combination treatment had a synergistic effect and suppressed MAD2L1 expression by 7.6-fold, which was significantly greater than the suppression elicited by RV-alone (1.4-fold; $p<0.05$) and MMC-alone (3.0-fold; $p<0.05$).

The combination treatment also increased the expression of SERTA Domain Containing 1 (SERTAD1), a CDK4-binding protein that renders the activity of cyclin D1/CDK4 resistant to the inhibitory effects of p16 (INK4a, cyclin-dependent kinase inhibitor 2A), by 4-fold relative to untreated cells ($p<0.001$), which was significantly greater than that observed with RV (2.7-fold; $p<0.002$) or MMC (2.1-fold; $p<0.001$, Figure 4) treatments.

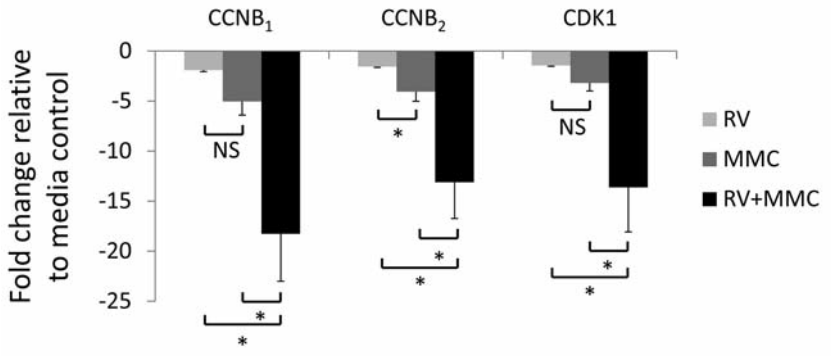

Figure 2. *Effect of Resveratrol (RV) and Mitomycin C (MMC) on the expression of cyclin and cyclin-dependent kinase. Colorectal cancer cells were treated with RV or MMC or a combination of RV+MMC for a period of 72h and transcriptional gene expression analysis was performed. The data are expressed as means±S.E.M. of fold change relative to media control from 5 distinct colorectal cancer cell lines. CCNB1, Cyclin B1; CCNB2, Cyclin B2; CDK1, cyclin-dependent kinase 1. Statistical analysis: One-way ANOVA with post hoc pairwise multiple comparisons using the Student-Newman-Keuls method. *p<0.05; NS: not statistically significant; n=5.*

The role that some of these genes play on the cell cycle is depicted in Figure 5A and the potential effect of their modulation on the cell cycle is displayed in figure 5B.

Discussion

The aims of this study were to determine whether RV favorably modulates the anti-tumor effects of MMC on colorectal cancer cell proliferation and if this modulation is associated with specific effects on cell-cycle genes. This study shows that combining RV with MMC additively or synergistically modulates specific cell-cycle genes in favor of anti-proliferation. Consistent effects across 5 different primary tumor cell lines from surgical specimens were shown for the down-regulation of *CCNB1, CCNB2, CDK1, CDKN3, CHEK1, KPNA2* and *MAD2L1* gene transcription, and the up-regulation of $p21^{WAF1/CIP1}$ and *SERTAD1* gene transcription. When one considers the function of each gene modulated, enhanced suppression of human colorectal cancer proliferation is consistent with the findings presented. Thus, a biologically active natural product, RV, can potentiate the anti-proliferative effects of MMC on human colorectal cancer cells.

Dysregulation of the cell cycle, resulting in uncontrolled proliferation, is a common feature of malignant cells. It is this feature which is targeted most often by chemotherapy drugs such as MMC (17). RV also suppresses cancer cell proliferation by modulating cell cycle genes that retard cells at the G_1/S and G_2/M cell-cycle phases (10). It was reasonable to hypothesize, therefore, that RV might be an appropriate natural product to incorporate into combination therapies with chemotherapy drugs. Unfortunately, much of what is known currently about the mechanisms responsible for RV-mediated suppression of tumor cell proliferation is based on studies in animals or with commercially available cell lines (18). Neither of these settings adequately replicates the complexities of human tumors. To fully appreciate RV's potential for human cancer therapies, studies need to be performed with tumor cells obtained from cancer patients undergoing treatment.

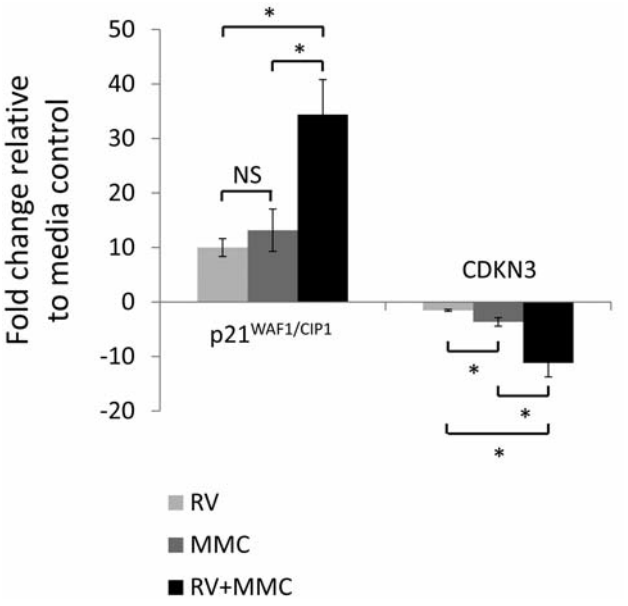

Figure 3. *Effect of Resveratrol (RV) and Mitomycin C (MMC) on the expression of cyclin-dependent kinase inhibitors. Colorectal cancer cells were treated with RV or MMC or a combination of RV+MMC for a period of 72h and transcriptional gene expression analysis was performed. The data are expressed as means±S.E.M. of fold change relative to media control from 5 distinct colorectal cancer cell lines. $p21^{WAF1/CIP1}$, Cyclin-dependent kinase inhibitor 1A; CDKN3, Cyclin-dependent kinase inhibitor 3. Statistical analysis: One-way ANOVA with post hoc pairwise multiple comparisons using the Student-Newman-Keuls method. *p<0.05; NS: not statistically significant; n=5.*

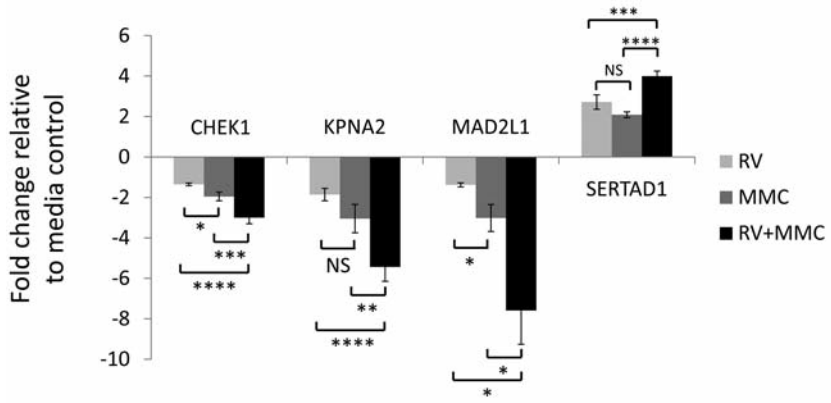

Figure 4. *Effect of Resveratrol (RV) and Mitomycin C (MMC) on the expression of genes regulating various cell cycle functions. Colorectal cancer cells were treated with RV or MMC or a combination of RV+MMC for a period of 72h and transcriptional gene expression analysis was performed. The data are expressed as means±S.E.M. of fold change relative to media control from 5 distinct colorectal cancer cell lines. CHEK1, Chekpoint kinase 1; KPNA2, Karyopherin alpha 2; MAD2L1, MAD2 Mitotic Arrest Deficient-Like 1 (Yeast); SERTAD1, SERTA Domain Containing 1. Statistical analysis: One-way ANOVA with post hoc pairwise multiple comparisons using the Student-Newman-Keuls method. *p<0.05; **p<0.01; ***p<0.002; ****p<0.001; NS: not statistically significant; n=5.*

In vitro testing of active natural products on cells from resected human tumors is considered a legitimate pre-clinical method for assessing the possible effectiveness of these products in combination therapies with standard chemotherapy drugs. The current model which employs short-term tumor lines established from specimens is considered superior to tests with established, long-term cultured cell lines, which are less able to represent the complexities and heterogeneity of human tumors. The results presented herein not only suggest that combining the natural product RV with a cytotoxic cancer drug such as MMC could enhance the clinical effectiveness of MMC, but also provide a mechanism whereby to explain such enhancement if that was found clinically. It also suggests that other natural products, chemotherapy drug combinations with similar mechanisms of action, may demonstrate comparable effects *in vitro* and *in vivo*.

The role played by each gene product found to be modulated in these studies is depicted in Figure 5A (developed from Reference 19). The cell cycle is regulated by several proteins that are expressed in a cell cycle phase-dependent manner such as the cyclins. Cyclin B1 and B2 are expressed during the G2/M phase of cell cycle (19). Cyclin B1 and B2 form complexes with cyclin-dependent kinase 1 (CDK1) and regulate the passage of cells through the M phase (19). CDK1 also binds to cyclin A and this complex is necessary for the cells to pass through the G_2 phase (19). Cyclin-dependent kinase inhibitor p21$^{WAF1/CIP1}$ regulates the activity of the CDK1 and inhibits the cyclin A/CDK1 and cyclin B/CDK1 complexes resulting in the retardation of cell cycle at the G_2/M phase (19). p21$^{WAF1/CIP1}$ also suppresses the activity of cyclin D/CDK4, 6 complexes, which retard the cells from entering the G1 phase of the cell cycle (19).

The suppressed expression of Cyclin B1, B2 and CDK1 with concomitant increase in the expression of p21WAF1/CIP1, as determined in this study, would lead to retardation of the cell cycle. However, as shown in Figure 5B, the effect of p21$^{WAF1/CIP1}$ overexpression on the cell cycle supersedes the effects of the modulation of the other genes on the cell cycle. p21$^{WAF1/CIP1}$ up-regulation leads to inhibition of the cell cycle at the G_0/G_1 and G_2/M phases, which essentially retards the cells from entering and exiting the cell cycle, respectively. These results suggest that the upregulation of p21$^{WAF1/CIP1}$ is the pivotal mechanism for the enhanced suppression of colorectal cancer cell proliferation.

The suppression of cell proliferation via up-regulation of p21$^{WAF1/CIP1}$ is a prominent mechanism activated by several chemotherapy drugs that cause genotoxic stress (20). Up-regulation of p21$^{WAF1/CIP1}$ can occur in a p53-dependent or a p53-independent manner (20, 21). The mutational status of P53 significantly affects its ability to up-regulate p21$^{WAF1/CIP1}$. Standard chemotherapy drugs up-regulate p21$^{WAF1/CIP1}$ in a p53-dependent manner and therefore require functional wild type P53 (22). The inability to up-regulate p21$^{WAF1/CIP1}$ in cells with mutated P53 makes these cells resistant to these chemotherapy drugs (23). Due to the fact that *P53* mutations are found in 50% of the tumors (24), the ability of these chemotherapy drugs to utilize this mechanism to eradicate cancers is severely compromised and may limit the effectiveness of these drugs clinically.

With this in mind, it is relevant that active natural products such as RV have the ability to up-regulate p21$^{WAF1/CIP1}$ in both a p53-dependent and a p53-independent manner (25-

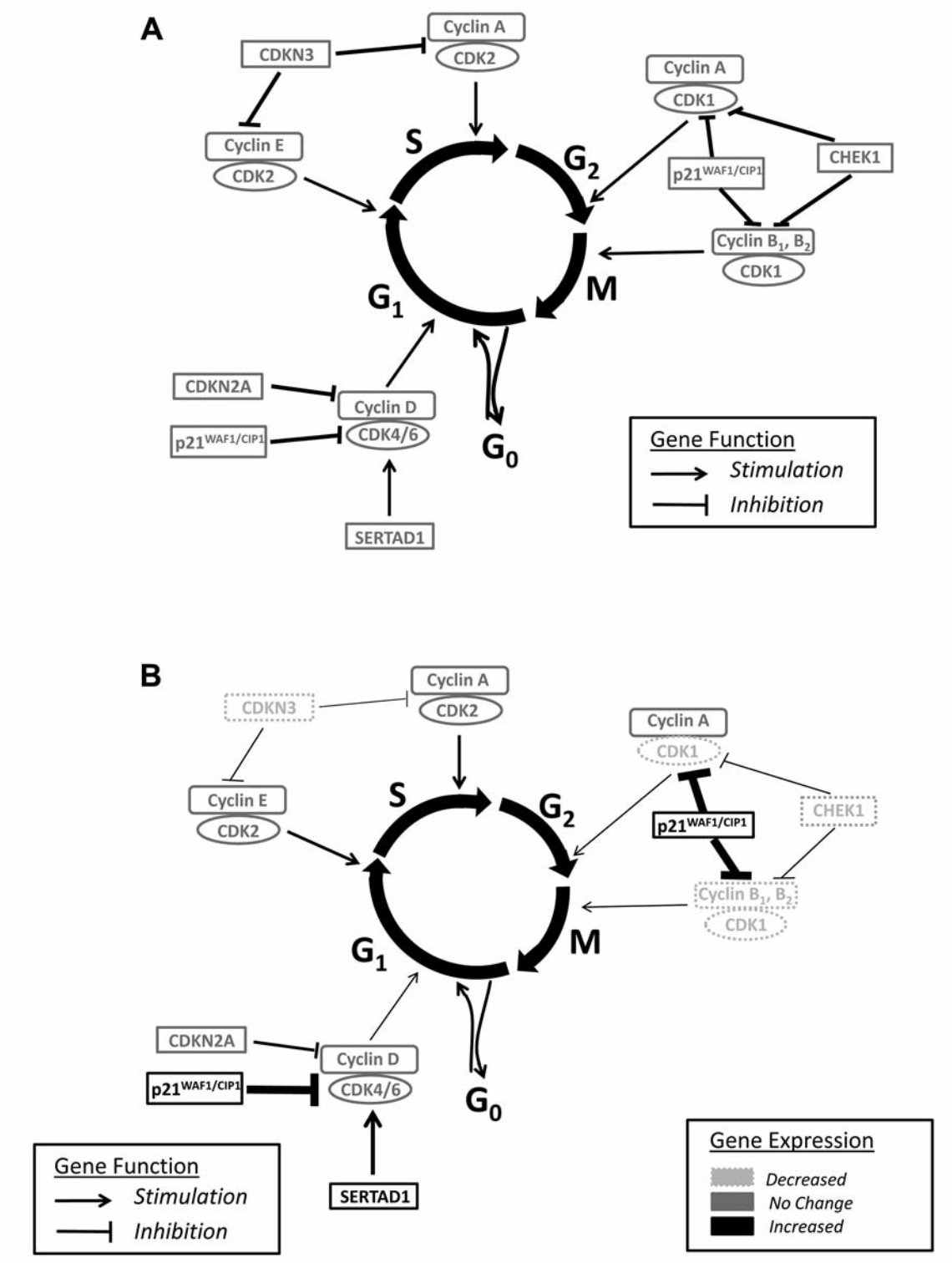

Figure 5. *Summary of the effects of RV+MMC on the expression of cell cycle genes in colorectal cancer cells. This schematic (developed from Reference 19) shows the potential impact of RV+MMC-mediated gene expression on the cell cycle in colorectal cancer cells. The cell cycle gene expression in media controls are shown in A and the RV+MMC treated are shown in B. The up-regulated genes are in black, the unchanged genes are in dark gray and the down-regulated genes are shown in light gray with dotted line boxes. The lines with arrows indicate stimulatory function and those with a blunt arrow indicate inhibitory function. The thickness of the lines indicates the degree of function with thicker lines indicating increased function and conversely the thinner lines indicating decreased function.*

27). Thus, such agents may provide a unique and tolerable means for augmenting the effects of the chemotherapy against cancer cells with or without *P53* mutations. Our study is the first to show that the treatment of colorectal cancer cells with RV+MMC leads to a synergistic up-regulation of p21$^{WAF1/CIP1}$. The mechanism for the synergistic up-regulation of p21$^{WAF1/CIP1}$ by RV+MMC in colorectal cancer cells has yet to be determined but it is conceivable that it could entail a combination of p53-dependent and p53-independent mechanisms.

One clinical treatment that may provide the opportunity to test this approach is the hyperthermic intraperitoneal chemotherapy (HIPEC) procedure employed in conjunction with de-bulking surgery in patients with peritoneal cancers. The intra-peritoneal instillation of RV, whose clinical activity is limited due to its rapid metabolism and clearance when given orally (28-30), may be a reasonable strategy for adding RV to MMC in cancer patients with peritoneal carcinomatosis. Thus, RV could be added to MMC at pharmacologically-relevant concentrations to investigate the potential for enhanced clinical effectiveness. This is especially attractive given the demonstration herein that one effect of RV is to up-regulate p21$^{WAF1/CIP1}$ in a p53-dependent or a p53-independent manner. The potential for testing whether enhanced tumor remission and extended tumor-free survival can be achieved by the combination therapy compared to what is seen in patients treated with MMC-alone (31, 32) is entirely feasible.

In conclusion, the present study shows that MMC-mediated suppression of colorectal cancer cell proliferation is enhanced by RV. This enhancement is correlated with synergistic up-regulation of p21$^{WAF1/CIP1}$ and may involve both p53-dependent and p53-independent mechanisms. The inability of MMC to induce p21$^{WAF1/CIP1}$ in cells with mutated *P53* makes RV a good candidate to be used in combination therapy with MMC because it can up-regulate p21$^{WAF1/CIP1}$ irrespective of the *P53* status. RV may be valuable in clinical settings where MMC is employed such as HIPEC.

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