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Impact of Andrographolide and Melatonin Combinatorial Drug Therapy on Metastatic Colon Cancer Cells and Organoids

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Abstract:	Background: The death rate (the number of deaths per 100,000 people per year) of colorectal cancer (CRC) has been dropping since 1980 due to increased screening, lifestyle-related risk factors, and improved treatment options; however, CRC is the third leading cause of cancer-related deaths in men and women in the US. Therefore, successful therapy for CRC is an unmet clinical need. The present study aimed to investigate the impacts of andrographolide (AGP) and melatonin (MLT) on CRC and the underlying mechanism. Methods: To investigate AGP and MLT anticancer effects, a series of metastatic colon cancer cell lines (T84, Colo 205, HT-29, and DLD-1) were selected. In addition, a metastatic patient-derived organoid model (PDOD) was used to monitor the anti-cancer effects of AGP and MLT. A series of bioassays including 3D organoid cell culture, MTT, colony formation, western blotting, immunofluorescence, and qPCR were performed. Results: The dual therapy significantly promotes CRC cell death, as compared with the normal cells. It also limits CRC colony formation and disrupts the PDOD membrane integrity along with decreased Ki-67 expression. A significantly higher cleaved caspase-3 and the endoplasmic reticulum (ER) stress proteins, IRE-1 and ATF-6 expression, by 48 h were found. This combinatorial treatment increased ROS levels. Apoptosis signaling molecules BAX, XBP-1, and CHOP were significantly increased as determined by qPCR. Conclusions: These findings indicated that AGP and MLT associated ER

	stress-mediated apoptotic mCRC cell death through the IRE-1/XBP-1/CHOP signaling pathway. This novel combination could be a potential therapeutic strategy for mCRC.

Impact of Andrographolide and Melatonin Combinatorial Drug Therapy on Metastatic Colon Cancer

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Abstract

Background: The death rate (the number of deaths per 100,000 people per year) of colorectal cancer (CRC) has been dropping since 1980 due to increased screening, lifestyle-related risk factors, and improved treatment options; however, CRC is the third leading cause of cancer-related deaths in men and women in the US. Therefore, successful therapy for CRC is an unmet clinical need. The present study aimed to investigate the impacts of andrographolide (AGP) and melatonin (MLT) on CRC and the underlying mechanism.

Methods: To investigate AGP and MLT anticancer effects, a series of metastatic colon cancer cell lines (T84, Colo 205, HT-29, and DLD-1) were selected. In addition, a metastatic patient-derived organoid model (PDOD) was used to monitor the anti-cancer effects of AGP and MLT. A series of bioassays including 3D organoid cell culture, MTT, colony formation, western blotting, immunofluorescence, and qPCR were performed.

Results: The dual therapy significantly promotes CRC cell death, as compared with the normal cells. It also limits CRC colony formation and disrupts the PDOD membrane integrity along with decreased Ki-67 expression. A significantly higher cleaved caspase-3 and the endoplasmic reticulum (ER) stress proteins, IRE-1 and ATF-6 expression, by 48 h were found. This combinatorial treatment increased ROS levels. Apoptosis signaling molecules BAX, XBP-1, and CHOP were significantly increased as determined by qPCR.

Conclusions: These findings indicated that AGP and MLT associated ER stress-mediated apoptotic mCRC cell death through the IRE-1/XBP-1/CHOP signaling pathway. This novel combination could be a potential therapeutic strategy for mCRC cells.

Introduction

The American Cancer Society (ACS) estimates that, in 2020 alone, there will be 104,610 new diagnoses of colon cancer in the United States. The majority of CRCs occur in adults ages 50 and older, 17,930 (12%) of cases are diagnosed in individuals younger than age 50, the equivalent of 49 new cases per day [1]. Chemotherapy, targeted therapy, and immunotherapy are the current treatment options for CRC, though each strategy has its own limitations [2-4]. Therefore, targeting mCRC cells via molecular targets remain important in colon cancer therapy.

Combination therapy, a treatment modality which combines two or more therapeutic agents, is a cornerstone of cancer therapy [5,6]. Moreover, this therapy is a promising strategy for synergistic anticancer treatment. It has different mechanisms of action that could reduce the dose of each agent, thus may reduce the individual drug-related toxicity [6]. The studies carried out if two combinatorial drug has an impact of metastatic colon cancer cells and stage III patient-derived organoids. Earlier studies have demonstrated that the plant metabolite andrographolide (AGP) induces CRC cell death due to apoptosis and is associated with the activation of IRE-1, an ER stress marker [7]. Additional studies showed that AGP induces apoptotic cell death through the induction of reactive oxygen species (ROS) which eventually play a role in down-regulating cell cycle progression and cell survival pathways [8]. AGP also induces cell death through the inhibition of angiogenic signaling, which is inversely related to the tumor suppressor gene expression, RASSF1A [9]. Co-treatment of AGP with other substances suggests its potential in combination therapies. In the context of cancer, AGP can diminish resistance to 5-Fluorouracil (5-FU), a first-line chemotherapeutic agent for CRC patients [10,11].

Recent studies have also shown that melatonin (MLT) has antimetastatic properties by modulating cell-cell and cell-matrix interactions, remodeling the extracellular matrix, and

suppressing angiogenesis [12-14]. In addition, extensive experimental data demonstrates that this chronobiotic agent exerts oncostatic effects throughout all stages of tumor growth, from initial cell transformation to mitigation of malignant progression and metastasis; additionally, MLT alleviates the side effects and improves the welfare of radio/chemotherapy-treated patients [15]. Moreover, it inhibits CRC proliferation by increasing reactive oxygen species (ROS) and inducing apoptosis, autophagy, and senescence [16]. Additionally, MLT also causes drug-resistant CRC cell death and inhibits CRC-colospheroids when it is co-administered with 5-FU [17]. In the present study, we tested the potential of AGP to inhibit CRC cell growth with reduced concentration compared to prior studies when combined with the safe, anti-cancer compound MLT. We demonstrate this dual compound has an impact on metastatic CRC cells (T84, Colo 205, HCT-15, HT-29, and DLD-1), and requires a concentration 3 times less than in previous studies. Moreover, these combinatorial therapies exert a little impact on normal cells.

Methods

Ethics Statement

All de-identified metastatic colon cancer tissues were received from the surgery of colon cancer patients with the approval the University of Maryland Institutional Review Board (HP-00066889-4). Written consent was obtained from all patients from whom discarded tissue was collected which included permission to publish results. A small piece of each tumor tissue was frozen for subsequent analysis and remaining tissue was processed for organoid cultures.

Generation and Propagation of Patient-Derived Organoid Cell Cultures (PDOD)

Organoid cells were generated from stage 3 metastatic cancer tissue and cultured as previously described [9,18,19]. The cultures were passaged when the aggregates reached a diameter of approximately 800 µm. Organoids were treated with 15 µM AGP, 0.5 mM MLT, or both for 48 h. Treated and untreated organoids were subjected to morphological analysis and immunofluorescence for Ki67 expression.

Cell Culture and Drug treatment

T84, and Colo 205 colon cancer cell lines were cultured as previously published [7-9,19]. HT-29, and DLD-1 were grown in RPMI-1640 nutrient media in a humidified incubator at 37°C with 5% CO_2. All media were supplemented with a 1X solution of antimicrobial reagents (10,000 U/ml penicillin, 10,000 streptomycin, and 25 µg/ml amphotericin B), 1X glutamine and 10% FBS. Mouse normal epithelial cells (GSM06) and prostate epithelial cells (RWPE1) cells were grown and cultured as previously described [19]. When cell was grown 80% confluent, media was replaced with media containing 2% FBS with AGP (Sigma Aldrich, St. Louis, MO) and MLT (a kind gift from Professor Russel J. Reiter) with indicated dose and time point. Stock AGP (100 mM) and MLT (1 M) were prepared in DMSO and control wells received DMSO at a final concentration of 0.01%.

Cytotoxicity Assay

Cytotoxicity assay in the presence or absence of AGP or, MLT was assessed using the MTT assay as previously described [7].

Clonogenic Assay

HT-29 and DLD-1 cells were seeded in 6-well plates (approximately 50, 0000/well). The clonogenic assay was performed as previously described [7].

Quantitative real-time polymerase chain reaction (qRT-PCR)

Gene expression was evaluated as previously described [7]. Primer sequences are listed in Supplementary Table 1. Relative gene expression changes were calculated using the $2^{-\Delta\Delta}$CT method and expression normalization was accomplished using the housekeeping gene, GAPDH.

Immunoblotting

Immunoblotting was performed as previously described [20]. The primary antibodies used were against Cleaved caspase-3 (#MAB835) from R&D Systems, Caspase 3 (#9662), IRE-1(#3294) from Cell Signaling, Cyclin B1(GNS1, #sc-245), p-PERK (#sc-32577), PRX (A-6) (#sc-137150P), TRX (#sc-271281) from Santa Cruz Biotechnology, ATF-6 (#MA5-16172) from Thermo Fisher Scientific, melatonin receptor 1B or MT2 (#NLS9320 from Novus Biologicals and GAPDH (G8795) from Sigma Aldrich. Blots were incubated with HRP-conjugated secondary antibodies followed by enhanced chemiluminescence (ECL) detection. All secondary antibodies were purchased from KPL, Gaithersburg, MD. Images were captured using a Syngene G Box digital imager (Frederick, MD, USA) and results were quantified by densitometry as previously described [19].

Immunofluorescence

Patient derived organoids were grown in 1:1 mixture of Matrigel and advanced DMEM:F12 (Life Technologies) supplemented with 1X penicillin/streptomycin, 1x glutamine, 1X N2, 1X B27, 1mM N-acetyl-L-cysteine, 20 ng/ml fibroblast growth factor and 50 ng/ml epidermal growth factor at 37°C on a 4-chambered glass slide. Fully grown organoids were treated with or without AGP

and MLT for 48 h. whole mount staining was carried out as previously described [9,21] with slight modifications. After desired time of treatment organoids were washed three times with 1X PBS and fixed with 4% paraformaldehyde at room temperature for 30 min, permeabilized with 0.2% Triton X-100 for 20 min, blocked with 2.5% horse serum (Vector; S-2012) and incubated with 1:40 dilution of Ki-67 (M-19) (#sc-7846) at 4°C for overnight. After incubation organoids were washed 3 times with PBS (10 mins/wash) and incubated with Alexa Flour 488 labeled donkey anti-goat IgG (H+L) (1:200) (ab 150129).

Fluorescence microscopy and image acquisition

Fluorescence microscopy and colony counting were performed using an inverted fluorescence microscope (Olympus IX-71, Pennsylvania, USA). Images for patient derived organoids (PDOD) were taken at 400 X magnification. Fluorescence intensity was quantified using ImageJ software version 1.39 (NIH). RGB composite images from control and treated groups were created using Axion Vision rel, 4.6 and analyzed. Images from five different fields were used for statistical analysis as previously described [8,9,21].

Statistical Analysis

Statistical analysis was performed with Graph Pad Prism for Macintosh 5.0c (Graph Pad Software Inc., San Diego, CA). The mean S.E. was calculated by one-way ANOVA. Significance between groups was analyzed using the post hoc Tukey's test and Bonferroni test. A $p<0.05$ was considered statistically significant.

Results

Co-treatment with AGP and MLT inhibits cell viability

Previous study have demonstrated that the IC_{50} value of AGP for T84 and Colo 205 is 45 μM [7], whereas IC_{50} value of MLT for CRC range from 1-2.5 mM [22,23]. To reduce the AGP concentration, T84, Colo 205, HT-29 and DLD-1 cells were co-treated with AGP (0-150 μM) and MLT (0.5 mM) for 24, 48 and 72 h to assess the effect on cell proliferation. MTT assays revealed co-treatment significantly reduced cell viability in a time and dose dependent manner (Fig. 1A-D). The IC_{50} was determined to be 15 μM for AGP and 0.5 mM for MLT at 48 h. This concentration was used for subsequent assays. Additional experiments were performed to determine the efficacy of this co-treatment on normal cells such as gastric surface mucous cell lines from transgenic mice GSM06 and normal prostate epithelial cells (RWPE-1). Co-treatment of normal epithelial cells with the same concentration of AGP and MLT had little effect on cell numbers (Fig. 1E-F). These data suggest that AGP and MLT co-treatment selectively inhibits CRC cells but not normal cells. The inhibitory properties of AGP and MLT on HT-29 and DLD-1 cells were also determined in a clonogenic assay and direct enumeration of stained colonies (Fig. 2). Co-treatment of cells for 48 h resulted in significantly fewer colonies compared with the untreated cells. Co-treatment significantly decreased the number of colonies ($P<0.001$) by 48 h.

Co-treatment with AGP and MLT induces apoptosis and apoptosis signaling is dependent upon ER stress

Earlier studies showed that AGP, at a concentration of 45 μM, causes apoptotic CRC cell death due to the unfolded protein response (*UPR*) mediated ER stress pathway [7-9]. To verify the function of a lower concentration of AGP on CRC cells, T84 and Colo 205 cells were co-treated with AGP and MLT as indicated. As shown in Figure 3A and 3B, co-treatment significantly increased the 17kDa cleaved Caspase 3 levels ($P<0.001$) as compared with the control and treatment with either AGP or MLT alone. The ratio of cleaved caspase 3 and total caspase 3 also significantly increased

(Fig. 3C; P<0.001). Additionally, co-treatment significantly upregulated pro-apoptotic BAX mRNA expression (Fig. 3D; P<0.001), but not mRNA of Bcl-2 (Fig. 3E T84). To verify the apoptotic induction with co-treatment of AGP and MLT is due to the *UPR*, T84 and Colo 205 mRNA levels were monitored for *UPR* signaling pathway initiators IRE-1, ATF-6 and PERK. Co-treatment resulted in a significant increase in IRE-1 and ATF-6 mRNA expression (~4.5-6-fold, P< 0.001) at 48 h (Fig. 4A, B). Consistent with IRE-1 activation, an increase in XBP-1 mRNA expression of over 3.5-fold for T84 and 1.5-fold for Colo 205 was observed at 48 h (Fig. 4C; P<0.001 and P<0.05). Expression of CHOP, which can be activated by XBP-1, was also significantly increased (Fig. 4D; P<0.05 for T84 and P<0.01 for Colo 205). An additional experiment was performed to monitor the ER stress protein level (IRE-1, p-PERK, and ATF-6) by western blot. ER stress protein analysis revealed increases only in IRE-1 in T84 and Colo 205 co-treated groups and ATF-6 expression in T84 co-treated group (Fig. 4E, F, and H). Taken together, the results indicate that co-treatment induced apoptosis is mediated via ER stress and the IRE-1 activation pathway.

Co-treatment induced G2/M cell cycle arrest and involvement of ROS molecules. AGP alone suppresses Cyclin B1 expression in T84 and Colo 205 cell lysates [8]. To monitor the Cyclin B1 expression in co-treatment groups, T84 and Colo 205 cells were treated with or without AGP and MLT. The cell lysates were analyzed for Cyclin B1 expression. Fig. 5A and 5A1 show AGP and MLT could effectively suppress Cyclin B1 expression (P<0.001) as compared to untreated control and AGP or MLT alone groups. Next, we monitored antioxidant protein expression (Prx, and Trx) by western blot. Peroxiredoxin (Prx) and Thioredoxin-1 (Trx-1) are upregulated in many human

cancers, including colon and rectum, and in some cancers, downregulation of Prx promotes apoptosis [24-26].

Our findings indicate co-treatment significantly downregulates Prx and Trx expression (Fig. 5B-B1; $P<0.05$-$P<0.001$) in both cell lysates. Taken together, the results depicted that co-treatment causes cell cycle arrest through downregulation of cyclin B1 and Prx- and Trx- mediated oxidative stress-induced cell apoptosis.

Impact of AGP and MLT on patient derived organoids

Patient derived organoids were generated as previously described [9] (Fig. 6A). Matured organoids were treated with or without AGP (15 µM) and MLT (0.5 mM) for 48 h. A loss of membrane integrity was found in the co-treatment group as compared with the AGP or MLT alone groups. Untreated group retained the structure of 3D organoids with intact membrane integrity (Fig. 6B). Immunofluorescence staining for Ki-67 expression was evaluated to measure the effect of AGP, MLT or co-treatment of AGP and MLT on organoids growth. Ki-67 was greatly reduced in all treatment groups as compared to the untreated group; maximum reduction was found in the co-treatment group (Fig. 6C).

Discussion

Colorectal cancer is the third most prevalent malignant tumor worldwide and the number of new cases may increase to nearly 2.5 million in 2035 [27,28]. The 5-year survival rate for CRC is ~64%, but drops to 12% for metastatic CRC, and therefore, additional treatment regimens are needed to develop effective approaches for medical intervention [29].

Previous studies have demonstrated that AGP alone causes inhibition of CRC cell proliferation in metastatic cell lines, and patient derived organoids at a concentration of 45 μM. The inhibition was shown to be due to ER stress mediated apoptosis [7-9]. Moreover, AGP displayed synergistic effect with chemotherapeutic drugs in CRC cells and hepatocellular carcinoma cells [30,31]. *In vitro and in vivo* studies have demonstrated the significant role AGP can have in re-sensitizing 5-Fu-resistant HCT116 (HCT116/5-FuR) cells to the cytotoxic effects of 5-Fu. AGP reverses 5-Fu resistance in human CRC through increasing the expression of BAX [11]. AGP, either alone or in combination with cisplatin, also induces CRC apoptotic cell death via increasing the expression of BAX and Bcl-2 and increasing the association of Fas and FasL [32]. Additional study provided the evidence its possible clinical application for enhancing the antitumor effort [33]. Recently, it is reported the synergistic cytotoxicity of AGP and MLT in mCRC cell lines, colospheroids and 5-FU drug resistance cells [34] and the molecular mechanism is apoptosis due to unfolded protein response mediated ER stress and angiogenic inhibition. In the present study, we have used a combination of AGP and MLT for metastatic CRC cell inhibition as both compounds have anti-angiogenic, apoptotic, cell cycle arrest dysregulation of various cancer signaling pathways and involved in regulation of immune function and tumor microenvironments [34]. Here, we examined the uses of a lower concentration of AGP when administered in combination with MLT (0.5 mM). The therapeutic concentration of MLT (0.5 mM) was selected because it modulates several signaling pathways which are considered likely anti-metastasis, anti-proliferative, and pro-apoptotic pathways in cancer cells [13,17,23,35,36]. Moreover, MLT does not show undesired side effects, even at extremely high doses [37,38]. However, on pharmacological grounds, MLT can be designated as a synergistic or potentiating effect and it may have a potential clinical implication in the treatment of several pathologies including neurodegerative diseases [39]

Additionally, it inhibits CRC stem cells by regulating the PrP^c-Oct4 axis. A synergistic effect has also been observed with MLT when combined with 5-Fu by inhibiting the stem cell markers Oct4, Nanog, Sox2 and ALDH1A1 through regulation of PrP^c [17].

We screened the potential effect of an AGP and MLT co-treatment on a panel of CRC cells and normal cells. The IC_{50} value of AGP in co-treatment is reduced 3-fold compared with AGP alone [7,8]. Co-treatment of AGP and MLT inhibits cell viability and has significantly less cytotoxicity in mouse normal epithelial cells and human prostate epithelial cells. Co-treatment induces an increase in mRNA and protein levels of IRE-1, one of the three major ER stress activated UPR proteins; an observation that is consistent between two cell lines. Increases in transcription of ATF6 mRNA were observed in T84 and Colo 205 cells, but protein levels only increased in T84 cell lysates at 48 h. Therefore, involvement of UPR protein at 48 h is associated with an increase in pro-apoptotic signaling and cell death.

Reactive oxygen species (ROS) generation can induce carcinogenesis by stimulating mutation and can inhibit tumor progression by inducing apoptotic signals [40]. Studies have demonstrated the importance of ROS in AGP-induced anti-cancer cell activities [8]. Among the Prx family of proteins, Prx-1 is the most prominent subtype with an increase in expression in tumor tissues [41]. Trx-1, a small redox protein, also has shown an increase in expression when observed in many human cancers including colon cancer. We have observed that co-treatment downregulates Prx-1 and Trx expression, which is consistent with the elevated expression of cleaved caspase 3, CHOP, and XBP-1 and the decreased expression of Cyclin B1.

Patient derived models are necessary to improve knowledge about CRC and to develop new therapeutic approaches. Our model represents an informative system employing both *in vitro* and *ex vivo* testing, support by a previous study [42]. Our data show the impact of AGP and MLT on the

inhibition of organoid morphology derived from third metastatic CRC patient tissue, which corroborates the inhibition of Ki67. A significant amount of Ki67 in organoids in the untreated group is consistent with another study which reports elevated Ki67 in $CD133^+$, $CD44^+/CD24^-$ and $ALDH^+$ CSCs [43]. Multiple studies have demonstrated that besides having a role in cell proliferation, Ki67 is also involved in metastasis and invasion of cancer cells [43,44]. These studies support a role for dual therapy using the natural products AGP and MLT against CRC.

Conclusion

This is the first report analyzing the potential of dual AGP and MLT treatment on CRC using CRC cell lines as well as patient-derived CRC organoids. The dual treatment was demonstrated to inhibit cell viability and promote cell cycle arrest as well as promote ER stress dependent apoptosis signaling in CRC cell lines. Cancer organoids derived from metastatic CRC were also shown to display membrane disruption and reduced proliferative activity in response to AGP and MLT treatment compared to control organoids. The molecular mechanisms that contribute to cell death in CRC cells in response to AGP and MLT remain unclear. Therefore, further investigation on this combination therapy will be necessary to delineate the molecular interactions within the CRC PDOD model.

Figure Legends

Figure 1. Impact of AGP and MLT on mCRC cell viability. A. T84, B. Colo 205, C. HT-29, D. DLD-1 E. GSM06 (gastric surface mice mucous), and F. RWPE-1 (prostate epithelial cells were

treated with indicated concentration of AGP (0-150 μM), and MLT (0.5 mM) for 24 h, 48 h, and 72 h. Cell viability was quantified using the MTT assay.

Figure 2. Co-treatment suppress clonogenicity in HT-29 and DLD-1 cells. HT-29 and DLD-1 cells were diluted and treated with AGP and MLT as indicated dose. Growth was measured by direct counting of clonal clusters stained in multiwell plates with crystal violet at 48 h. Representative micrographs are shown.

Figure 3. Co-treatment of AGP and MLT induced cell apoptosis. T84 and Colo 205 cells were treated with or without AGP and MLT at IC_{50} for 48 h and protein expression was determined by immunoblotting for Cleaved caspase 3 (A, upper lane), Caspase 3 (A, middle lane) and GAPDH (A, lower lane). Densitometry analysis was performed and normalized with GAPDH expression to determine significant upregulation of indicated proteins (Fig. 3B and 3C). The mRNA level for apoptosis associated genes was determined by qRT-PCR for BAX (D), Bcl-2 (E). Bar graphs show quantitative results normalized to GAPDH mRNA levels. Results are from three independent experiments. Statistical significance was determined using one way-ANOVA followed by Bonferroni test (*P<0.05, **P<0.01, ***P<0.001).

Figure 4. AGP and MLT induces ER stress-related IRE-1 and associated proteins. T84 and Colo 205 cells were treated as mentioned earlier. The transcriptional level of expression for ER stress associated genes was determined by qRT-PCR for A. IRE-1, B. ATF-6, C. XBP-1 and D. CHOP. Bar graphs show quantitative results normalized to GAPDH mRNA levels. The primary ER transducer translational level was determined by immunoblotting for E. IRE-1 (level 1), p-PERK (level-2), and ATF-6 (level-3). Densitometry analysis was performed and normalized with GAPDH (blot 4). Results are from three independent experiments. Statistical significance was

determined using one way- ANOVA followed by Bonferroni test. (*P<0.05, **P<0.01, ***P<0.001).

Figure 5. Co-treatment induced cell cycle arrest and decreased antioxidant protein expression in CRC cells. T84 and Colo 205 cells were treated with or without AGP (15 µM) and MEL (0.5 mM) for 48 h. Cell lysates were analyzed by immunoblot for cyclin B1 (A), PRX (B, upper lane), and TRX (B, middle lane) and quantified by densitometry for expression of A1. Cyclin B1, B1 (left) PRX and (right) TRX. Expression is normalized against GAPDH expression. Statistical significance was determined using one way-ANOVA followed by Bonferroni test (*P<0.05, **P<0.01, ***P<0.001).

Figure 6. Impact of AGP and MLT on patient-derived organoids (PDOD). A. Chronological development of PDOD in Matrigel droplet. Organoid structures were confirmed using an inverted light microscope. B. PDOD were treated with or without AGP or MLT and morphology was assessed by light microscopy. C. FITC labeled anti-Ki-67 and DAPI staining of PDOD in the presence or absence of AGP or MLT evaluated by fluorescence microscopy.

Availability of data and materials

The datasets generated and analyzed during the current study are not publicly available due to continuing the research but are available from the corresponding author on reasonable request.

Authors' contributions

T.G.B., S.J.C., A.B. Conceptualization. A.BF providing the surgical tissues. N.S., T.I., E.H, S.G. performed the experiments and analyzed the data. N.S., and A.B. writing-original draft

preparation, T.G.B., and S.J.C., A.BF reviewing and editing. A.B. supervision. All authors have read and approved the final manuscript.

Patient consent for publication

Not applicable.

Ethics approval and consent to participate

University of Maryland Institutional Review Board (HP-00066889-3

Competing interests

This study has been the subject of a patent application by TGB, SJC, and AB of the University of Maryland School of Medicine. The current status of the application is pending.

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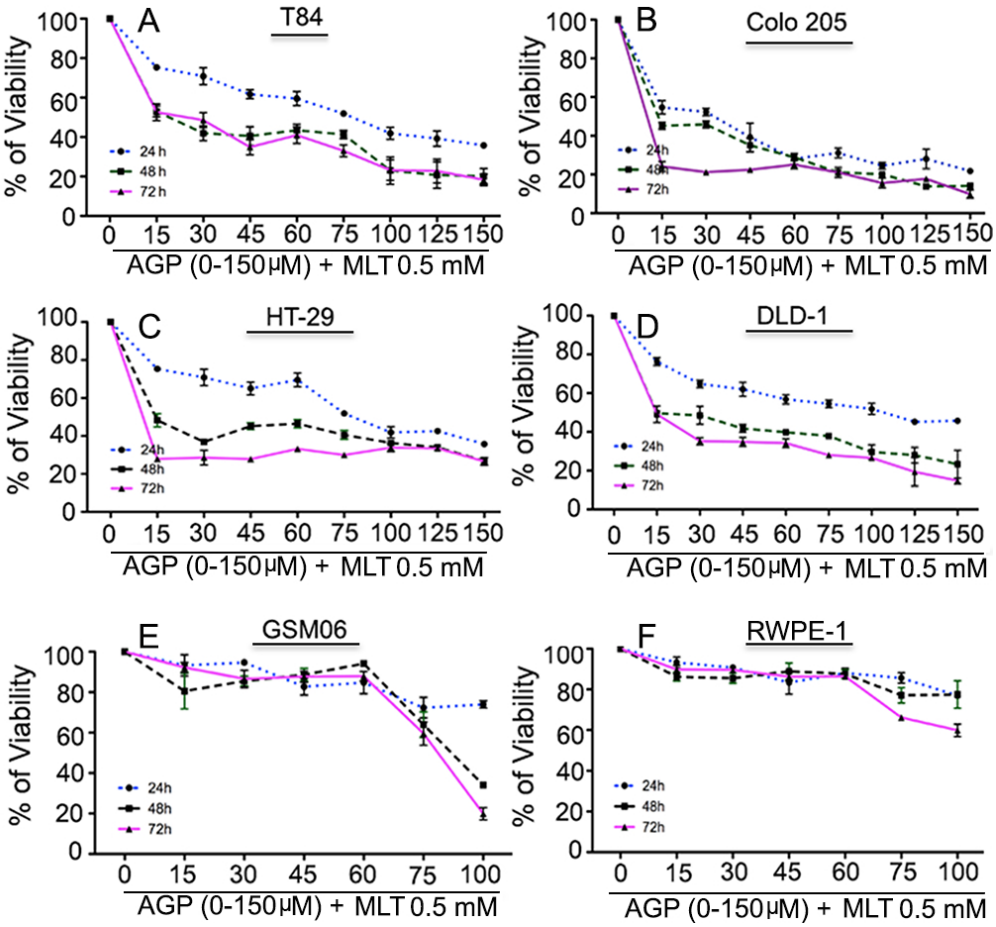

Figure 1. Impact of AGP and MLT on mCRC cell viability.

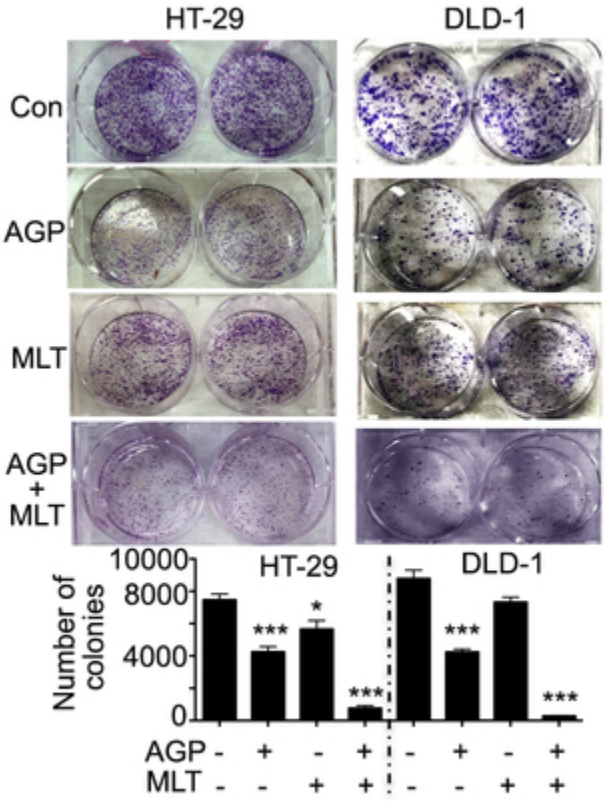

Figure 2. Co-treatment suppress clonogenicity in HT-29 and DLD-1 cells.

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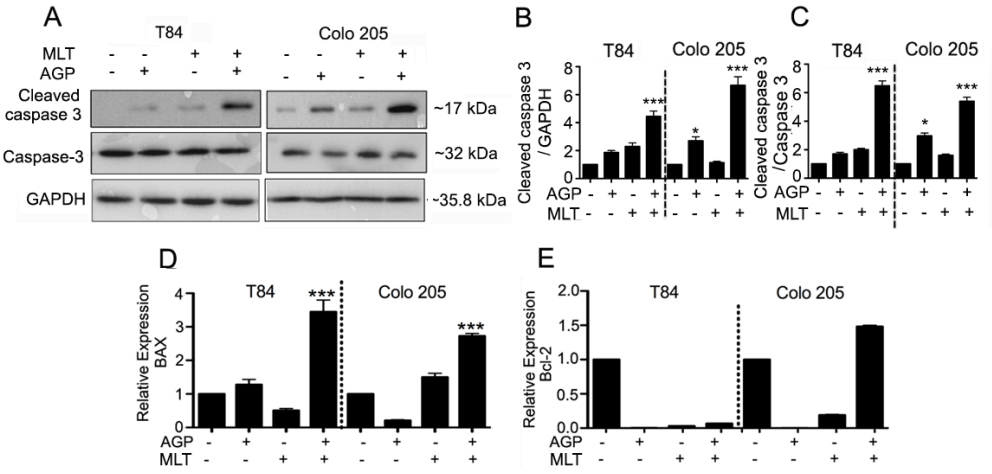

Figure 3. Co-treatment of AGP and MLT induced cell apoptosis.

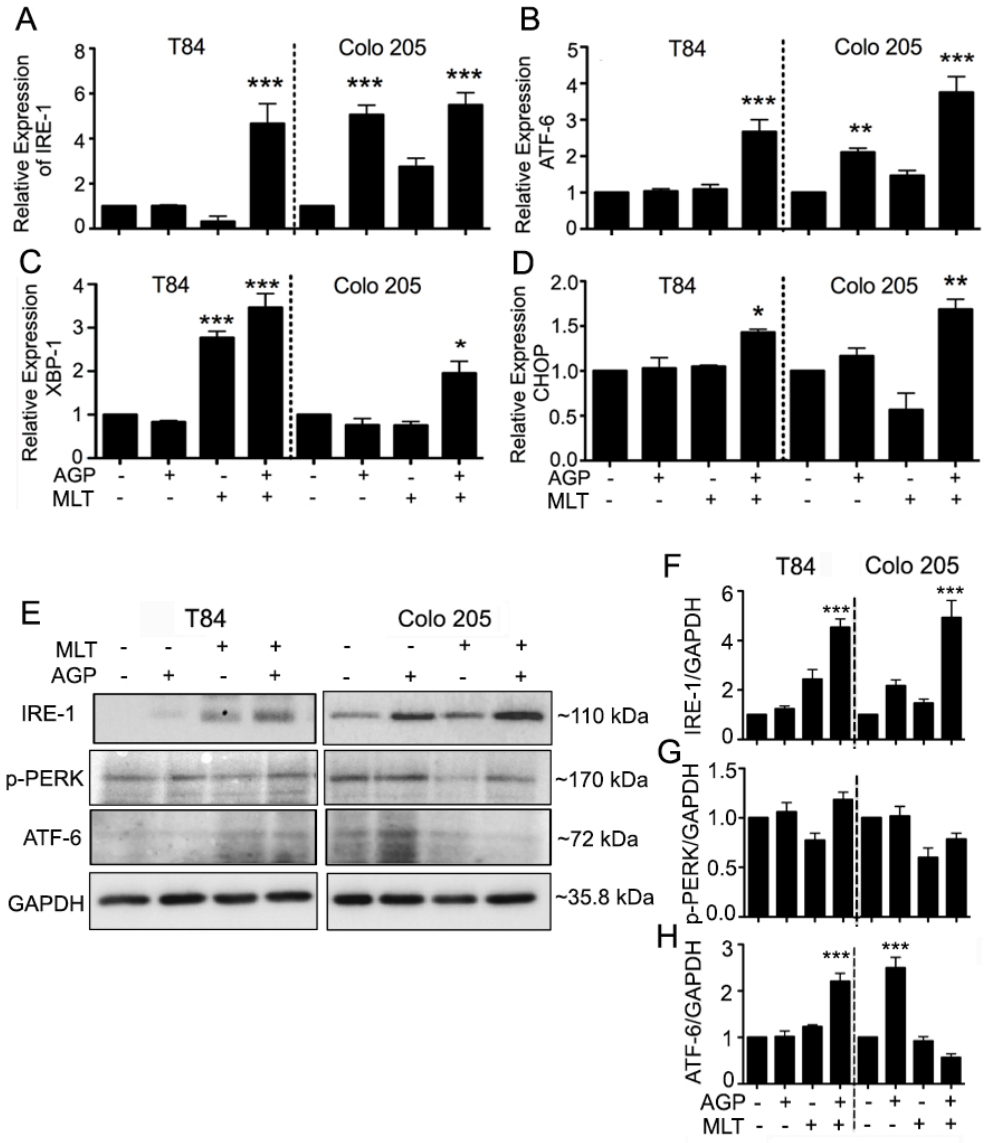

Figure 4. AGP and MLT induces ER stress-related IRE-1 and associated proteins.

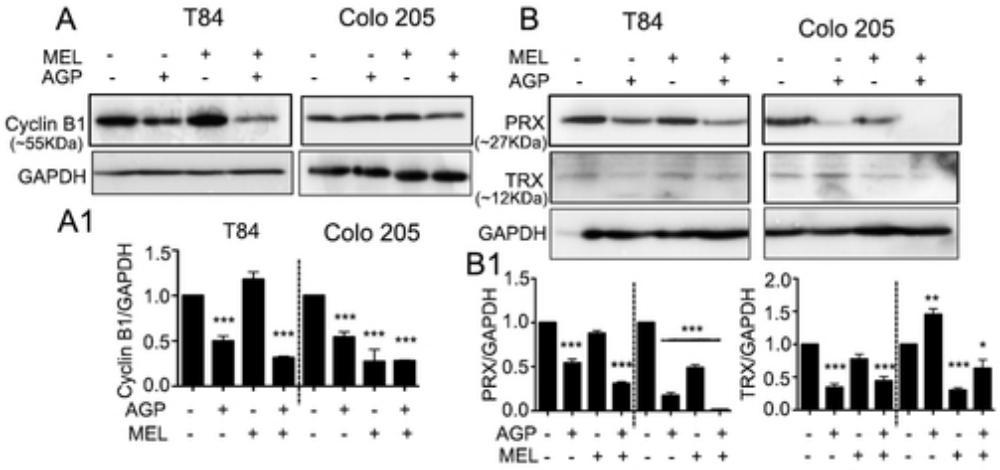

Figure 5. Co-treatment induced cell cycle arrest and decreased antioxidant protein expression in CRC cells.

42x20mm (300 x 300 DPI)

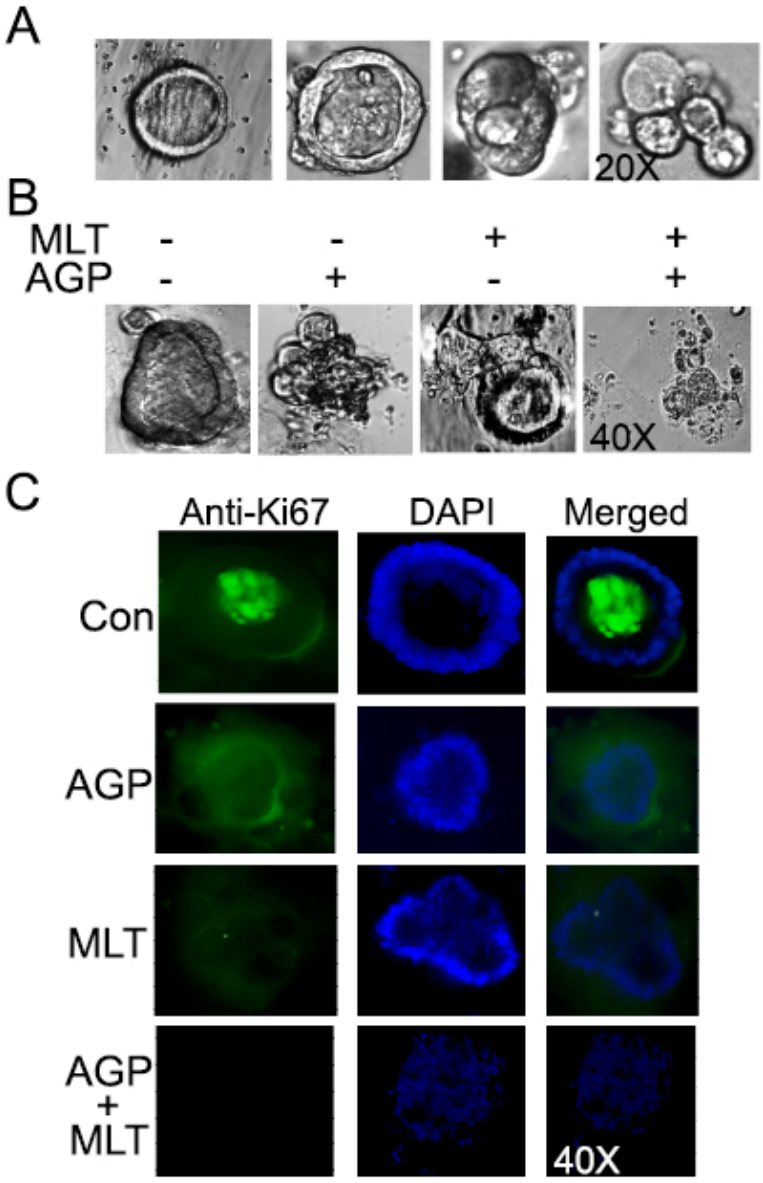

Figure 6. Impact of AGP and MLT on patient-derived organoids (PDOD).

Supplementary Table-1: qRT-PCR primers

Gene	Primer sequence forward	Primer sequence reverse
GAPDH	5′-CGACCACTTTGTCAAGCTCA-3′	5′-AGGGGAGATTCAGTGTGGTG-3′
IRE-1	5′-GGGAAATACTCTACCAGCCT-3′	5′-GAAATCTCTCCAGCATCTTG-3′
ATF6	5′-TCAGGGAGTGAGCTACAAGT-3′	5′-CTTGTGGTCTTGTTATGGGT-3′
CHOP	5′-TTCTCTGGCTTGGCTGACTG-3′	5′-CTGCGTATGTGGGATTGAGG-3′
Bcl-2	5′-GGAGGCTGGGATGCCTTT3′	5′-ACCCATGGCGGTGACCATGC-3′
Bax	5′-GAGAGGTCTTTTTCCGAGTGG-3′	5′-CCTTGAGCACCAGTTTGCTG-3′
XBP1	5′-AACCAGGAGTTAAGACAGCGCTT-3′	5′-CTGCACCCTCTGCGGACT-3′