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Article

Novel anticancer effects of melatonin and berberine via signaling pathways in colorectal cancer and lymphoma

Amber Lin[1], Wayne Lin[2], Wei Zhu[3]

[1] TVT Community Day School, Irvine, California
[2] Chint Power Systems America, Irvine, California
[3] SCI Research Institute, Jericho, New York

SUMMARY

Colorectal cancer (CRC) and lymphoma are leading causes of death worldwide. Berberine, a component of the Chinese herb Coptis chinensis, and melatonin, produced endogenously by humans and obtained in the diet, have similar anticancer effects of inducing apoptosis and modifying signaling pathways involved in cancer. Current cancer treatments like radiotherapy are toxic, costly, and have a limited number of targets. We hypothesized that berberine and melatonin would have synergistic anticancer effects on CRC and lymphoma cancer cells through inducing apoptosis and mitigating metastasis. Different dilutions of melatonin and berberine were used to treat cancerous COLO320 (CRC) and U937 (lymphoma) cells, with healthy CCD18 colon cells as a control. Molecular docking was conducted between melatonin and berberine with genes in major cancer pathways. There was a significant, dose-dependent decrease in cancer cell proliferation after 24-hour and 96-hour berberine-melatonin combined treatment. There was a significant decrease in cell adhesion. Also, there was a significant increase in the activity of caspase by a factor of 5x, which led to the induction of apoptosis in cancer cells. Furthermore, berberine and melatonin caused a significant downregulation of BDNF and MMP9 expression. In the molecular docking results, berberine and melatonin bound very strongly to MMP9 at the same location, correlating with the ELISA assay results and suggesting their synergistic properties. In conclusion, the hypothesis that berberine and melatonin have potential synergistic anticancer effects compared to berberine and melatonin alone is supported. In the future, melatonin and berberine could be used to strengthen or as an alternative to conventional cancer treatments.

INTRODUCTION

Colorectal cancer (CRC), a leading global cause of death, is rising in patients under 50 (1). While cases decline in high-income countries like the UK and Canada, they continue to rise in lower-income nations such as Mexico and Chile due to aging populations (1, 2). Standard CRC treatments include surgery, radiotherapy, and chemotherapy (2). Matrix metalloproteinase 9 (MMP9), elevated in CRC patients, is a key drug target (3). We used MMP9 to assess the effects of berberine and melatonin on CRC cells.

Lymphoma, the ninth most common cancer in the U.S., affects various organs and includes Hodgkin and non-Hodgkin types—the latter being more prevalent (4, 5). High-grade non-Hodgkin lymphomas like B- and T-cell subtypes are especially common in low-income countries (5). Like CRC, lymphoma is treated with costly and harmful radiotherapy and chemotherapy (6). Given the higher burden of advanced-stage disease in low-income settings, safer, more affordable treatments are urgently needed.

Berberine, a plant-derived metabolite from *Coptis chinensis*, has been used in traditional Chinese medicine for fever reduction and detoxification (1, 7). It enhances human cell metabolism, such as activating brown adipose tissue (8). It exerts anticancer effects by inducing apoptosis, regulating signaling pathways, controlling the cell cycle, and reducing oxidative stress (9). At 200 μM, berberine promotes apoptosis in cancer cells by inducing AIF nuclear translocation and increasing caspase-3, 7, 8, and 9 activity, impacting cancers like pancreatic, leukemia, and CRC (9). In CRC, it also reduces telomerase activity and arrests the cell cycle at G0/G1 (10). However, its poor bioavailability—around 80% metabolized in the liver and excreted—limits its effectiveness (7–11). Despite this, its natural origin makes it a promising adjunct to traditional treatments for CRC and lymphoma.

Melatonin, produced by the pineal gland, is amphiphilic and crosses membranes like the blood-brain barrier (12). It is also available in synthetic form as a supplement (13). Its receptors, MT1 and MT2, are GPCRs that inhibit cAMP and cGMP signaling (14). Melatonin exerts anticancer effects by inducing apoptosis, modulating signaling pathways, and regulating the cell cycle (12). It has been shown to increase pro-apoptotic proteins (BAX/BAK, Apaf-1, caspases, p53) (12). Also, it reduces estrogen receptor alpha (ERα), which may inhibit estrogen-driven cancers like breast cancer (15, 16).

We hypothesized that combining berberine and melatonin would synergistically inhibit CRC and lymphoma cells by targeting caspase-3 (apoptosis) and MMP9 (metastasis). Molecular docking confirmed their interaction with key

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proteins prior to in vitro testing. Combined treatment significantly reduced cancer cell proliferation and adhesion in a dose-dependent manner, and downregulated biomarkers including caspase-3, BDNF, and MMP9. These findings support further exploration of berberine and melatonin as adjuncts to conventional cancer therapies.

RESULTS

Molecular docking of cancer-related proteins with berberine and melatonin

We conducted molecular docking using PyRx and BIOVIA Discovery Studio Visualizer to see the binding affinities between berberine, melatonin, and different major proteins involved in cancer pathways. The proteins we chose were researched as commonly found in colorectal cancer and lymphoma cancer pathways.

From the docking, we produced binding affinity values, all between 5.0 and 8.0 (**Table 1**). In most 2D binding diagrams of proteins of cancer pathway genes binding to berberine and melatonin, these molecules bound different locations on the target protein. However, each macromolecule's alteration of the protein most likely combines to be synergistic, given the synergistic effects of berberine and melatonin shown in the other assays. In one case only, both melatonin and berberine bound to MMP9 at phenylalanine (A:3412) with a Pi-Pi-T-shaped bond (**Figure 1**). This result correlated with the high binding affinity as well as the ELISA assay of MMP9, showing berberine and melatonin were able to have a pharmacological impact on MMP9, a genetic target of cancer. Thus, in addition to the data mentioned earlier in assays that demonstrated cancer cells' decrease in survival after berberine and melatonin treatment, berberine and melatonin had potential anticancer effects on targets of CRC and lymphoma.

Berberine and melatonin reduce colorectal and lymphoma cancer cell survival

We conducted 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) cell proliferation assays to determine berberine and melatonin's effect on colorectal and lymphoma cancer cell survival.

We first looked at COLO230 cells viability after 24-hour treatment (**Figure 2a**). A high concentration of berberine (149 μM) showed a 20.86% cell viability decrease ($p = 0.0022$, one-way ANOVA). A low concentration of melatonin (0.215 μM) showed an 11.88% decrease in cell viability ($p = 0.0317$, one-way ANOVA). When treated with a high concentration of berberine (149 μM) and high concentration of melatonin (21.5 μM), the COLO320 cells showed a 21.12% cell viability decrease ($p = 0.0004$, one-way ANOVA). In addition, cell viability decreased in a dose-dependent manner for the groups receiving berberine when given alone and with melatonin.

Gene	Highest binding energy to BBR (kcal/mol)	Highest binding energy to M (kcal/mol)
TGF-β1	-6.9	-5.7
STAT3	-7	-5.8
ESR1	-7.5	-6.1
HS90AA1	-7.1	-5.9
MMP9	-7.6	-5.8
VEGFA	-7.4	-5.2

Table 1: Binding energies of genes with Berberine (BBR) and Melatonin (M). BBR and M have high binding values ranging from -5.2 to -7.6 kcal/mol. Molecular docking using PyRx was conducted between BBR and M individually with protein products of genes found in common cancer pathways. Bolded genes indicate molecular docking results with the highest binding affinities.

Next, we looked at U937 cell viability after 24-hour treatment (**Figure 2b**). A high concentration of berberine (149 μM) showed a 60.10% cell viability decrease ($p < 0.0001$, one-way ANOVA). A high concentration of melatonin (21.5 μM) showed a 57.97% cell viability decrease ($p < 0.0001$, one-way ANOVA). When treated with medium concentration of berberine (14.9 μM) and medium concentration of melatonin (2.15 μM), the U937 cells showed a 70.63% cell viability decrease ($p < 0.0001$, one-way ANOVA). The greater percentage decrease in cell viability in the combined treatment showed that berberine and melatonin may have a synergistic effect on U937 cells. Cell viability decreased in a dose-dependent manner for berberine alone and melatonin alone treatment.

We also looked at cell viability in noncancerous CCD18 cells after 24-hour treatment (**Figure 2c**). A medium concentration of berberine (14.9 μM) showed a 49.69% cell viability increase ($p < 0.0001$, one-way ANOVA). For the rest of the treatments, there were mostly no other significant values when compared to the control. Therefore, we conducted the rest of the cell assays in U937 cells and COLO320 cells only. Overall, the results indicated different concentrations of berberine and melatonin can inhibit different types of cancer cell survival.

We looked at COLO320 cell viability after 96-hour treatment (**Figure 3a**). A high concentration of berberine (149 μM) showed a 54.52% decrease ($p < 0.0001$, one-way ANOVA). A low concentration of melatonin (0.215 μM) showed a 31.02% decrease ($p < 0.0001$, one-way ANOVA). When treated with a medium concentration of berberine (14.9 μM) and medium concentration of melatonin (2.15 μM), the COLO320 cells showed a 41.68% decrease ($p < 0.0001$, one-way ANOVA). For berberine, there was a dose-dependent upward trend, while there was a dose-dependent downward trend for melatonin.

We looked at U937 cell viability after 96-hour treatment (**Figure 3b**). A high concentration of berberine (149 μM) showed a 77.34% decrease ($p < 0.0001$, one-way ANOVA). A medium concentration of melatonin (2.15 μM) showed a 53.31% decrease ($p < 0.0001$, one-way ANOVA). When treated with a high concentration of berberine (149 μM) and a high concentration of melatonin (21.5 μM), the U937 cells showed a 56.34% decrease ($p < 0.0001$, one-way ANOVA). For the treatment of berberine and the combined treatment, there was a dose-dependent upward trend.

Berberine and melatonin inhibit COLO320 cell migration

We then conducted cell migration assays to determine whether berberine and melatonin treatment would decrease cell migration of COLO320 cells. We seeded COLO320 cells and treated them with various berberine and melatonin concentrations. Then, we made rifts to simulate wounds and recorded rift widths (**Figure 4a**). The shorter the length of the rift, the more the COLO320 cells grew back, indicating an increase in cell migration, correlating with cancer metastasis. The medium concentration of berberine (14.9 μM) and high concentration of melatonin (21.5 μM) combined treatment showed a 40.76% decrease ($p < 0.0001$, one-way ANOVA).

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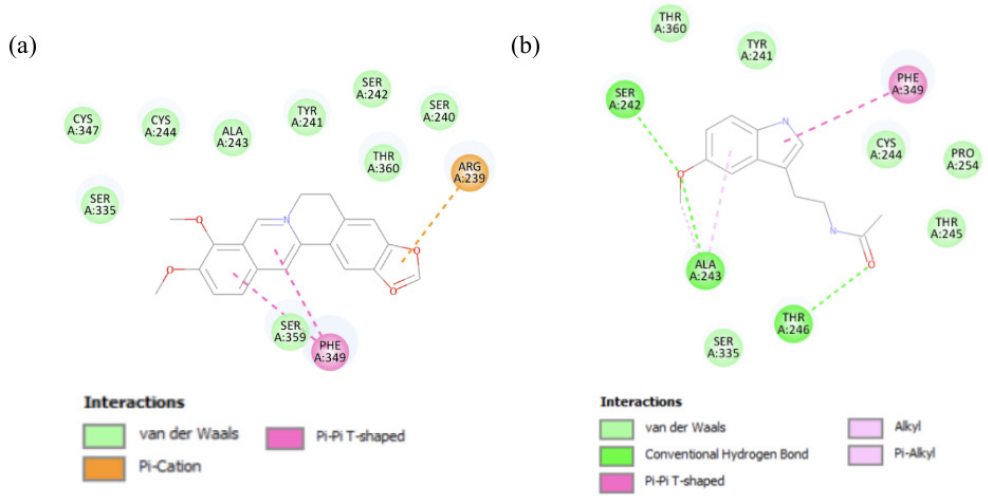

Figure 1. 2D binding diagrams of MMP9. a) Berberine and **b)** Melatonin bind to many of the same amino acids in MMP9. The BIOVIA Discovery Visualizer was used to create 2D binding diagrams of BBR and M to the proteins of common cancer pathway genes.

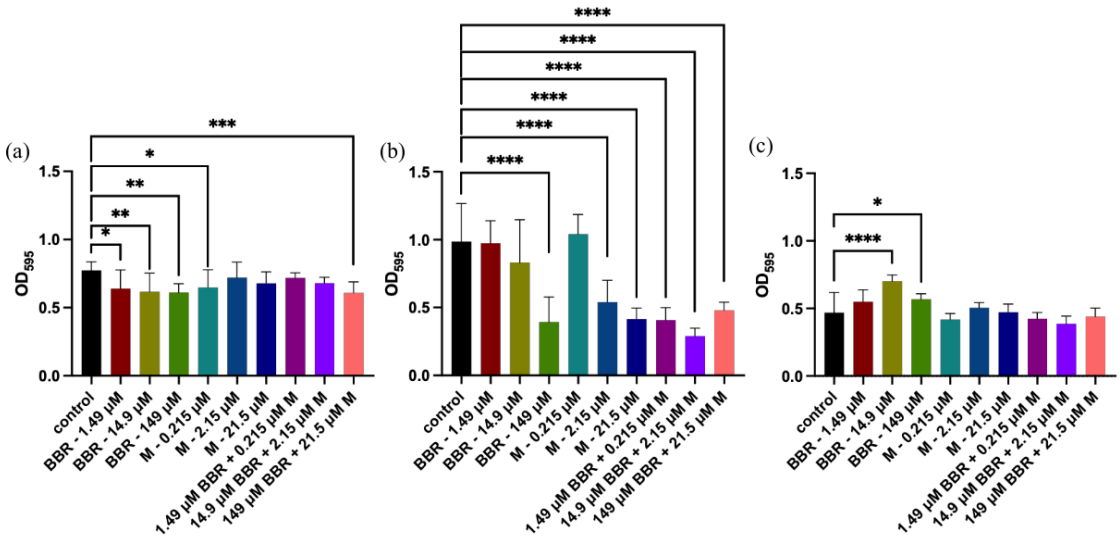

Figure 2. Cancer cell survival rates under short-term Berberine (BBR) and Melatonin (M) treatments were measured by MTT cell proliferation assay. Optical density (OD) for various cell lines treated with BBR and M over 24 hours. An MTT assay was conducted, then absorbance was measured at 595 nm. Increased OD value correlates with increased cell viability, and vice versa. **a)** COLO320, **b)** U937 cells, and **c)** CCD18 cells. Data is presented as mean ± s.d. (n=3). Experiments were repeated 3 times with similar results.
*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001

All treatments were able to increase the rift width, which is the length between two sides of the wound, indicating that they decreased cell migration. A medium concentration of berberine (14.9 µM) added to a high concentration of melatonin (21.5 µM) caused the greatest rift width increase (62.16 µm), showing that berberine and melatonin could have synergistic effects on COLO320 cell migration. In comparison, the control treatment had a much smaller rift width.

We took photos of COLO320 cells (**Figure 4b**). These photos corresponded to the cell migration assay results, with a medium concentration of berberine (14.9 µM) added to a high concentration of melatonin (21.5 µM) treatment having the longest rift width, indicating a decrease in cell migration (**Figure 4a**). Furthermore, the color of the cells was significantly lighter than the control, indicating that this treatment decreased cell density as well. This result showed berberine and melatonin's potential apoptotic effects on COLO320 cells.

Berberine and melatonin inhibit U937 cell adhesion

We conducted a cell adhesion assay on U937 cells. We first coated the cell plate with Human Collagen Type IV and stained it. 12-O-Tetradecanoylphorbol 13-acetate (TPA) is a tumor promoter that stimulates cell adhesion found in cancer cells, so TPA was used to treat certain U937 cells to facilitate cell adhesion (17).

We calculated the cell number of each cell well as an indicator of cell adhesion (**Figure 5a**). For medium concentration of berberine (5.95 µM) added to 10 µM TPA, the U937 cells had a 66.12% cell adhesion decrease ($p < 0.0001$, one-way ANOVA). Even with stimulation of TPA, berberine decreased cell adhesion at a higher percentage

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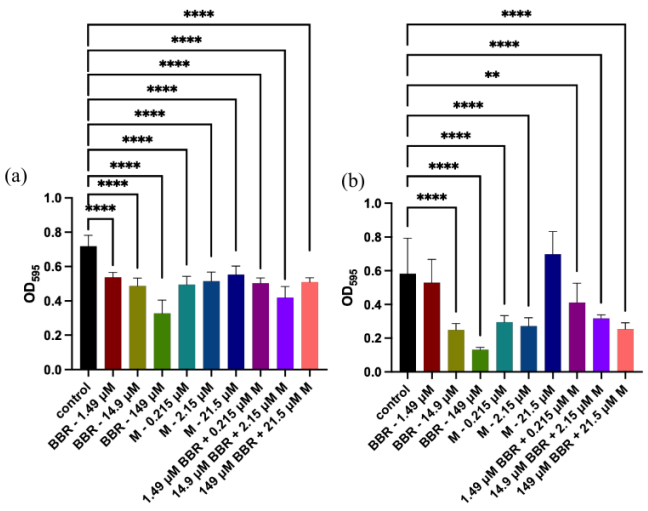

Figure 3. Cancer cell survival rates under long-term Berberine (BBR) and Melatonin (M) treatments were measured by MTT cell proliferation assay. Optical Density (OD) for various cell lines treated with BBR and M over 96 hours. An MTT assay was conducted, then absorbance was measured at 595 nm to produce OD values. Increased OD value correlates with increased cell viability, and vice versa. **a)** COLO320 and **b)** U937 cells. Data is presented as mean ± s.d. (n=3). Experiments were repeated 3 times with similar results. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001

than with no TPA. A medium concentration of melatonin (0.861 µM) showed a 58.66% cell adhesion decrease ($p < 0.0001$, one-way ANOVA). Melatonin wasn't able to decrease cell adhesion as much with TPA added, only 38.89%. In addition, for a medium concentration of berberine (5.95 µM) added to a medium concentration of melatonin (0.861 µM) and 10 µM TPA, the U937 cells showed a 76.82% cell adhesion decrease ($p < 0.0001$, one-way ANOVA). The percentage of the combined treatment with TPA is higher compared to both melatonin added to 10 µM TPA and berberine added to 10 µM TPA. This suggested that melatonin and berberine can act synergistically on U937 cells, similar to the MTT assay on U937 cells and the cell migration assay results.

We took photos of U937 cells with treatment (**Figure 5b**). The largeness and amounts of "dots" (cells) in each photo indicated the cell density and, therefore, cell adhesion of U937 cells after treatment. All of the treatments showed smaller and fewer cells, indicating decreased cell adhesion. In addition, the relative cell adhesions displayed in the photos correlated with cell adhesion assay results (**Figure 5a**). For the combined treatment of medium concentration of berberine (5.95 µM) and medium concentration of melatonin (0.861 µM) added to 10 µM TPA, the U937 cells had the least amount of cells, signifying the most decrease in cell adhesion. For a medium concentration of berberine (5.95 µM) added to 10 µM TPA, it had relatively the second least amount of cells, meaning the second most decrease in adhesion.

Berberine and melatonin effect on MMP9 and BDNF expression

We determined the protein levels of MMP9 and BDNF in the cell media of CCD18 and COLO320 cells by ELISA assays. MMP9 has been found to be a biomarker for CRC, and BDNF is a biomarker for lymphoma, making them detectors for cancer (18, 19).

We measured MMP9 expression in COLO320 cells (**Figure 6a**). A medium concentration of melatonin (2.15 µM) upregulated MMP9 by 24.43% ($p < 0.0001$, one-way ANOVA). A medium concentration of berberine (14.9 µM) added to a high concentration of melatonin (21.5 µM) downregulated MMP9 by 0.76%, which made it the only treatment to downregulate MMP9.

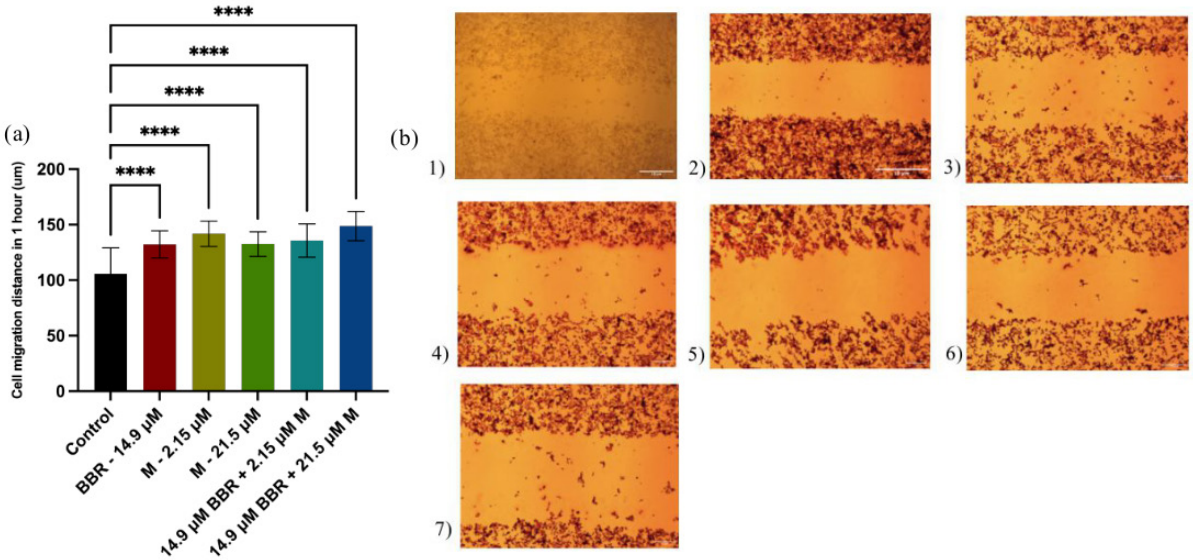

Figure 4. Berberine (BBR) and Melatonin (M) effect on COLO320 cell migration. Cell migration distance of COLO320 cells, treated with BBR and M, through 1 hour. A cell migration assay was conducted, then cell migration distance was analyzed using ImageJ. **a)** Cell migration distances of COLO320 cells after one hour. **b)** Photos of COLO320 cell migration assay. **b1)** Scratch marks at 0 minutes, control. **b2)** Control at 1 hour. **b3)** 14.9 µM BBR treatment at 1 hour. **b4)** 2.15 µM M treatment at 1 hour. **b5)** 21.5 µM M treatment at 1 hour. **b6)** 14.9 µM BBR + 2.15 µM M treatment at 1 hour. **b7)** 14.9 µM BBR + 21.5 µM M treatment at 1 hour. Data is presented as mean ± s.d. (n=3). Experiments were repeated 3 times with similar results. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001

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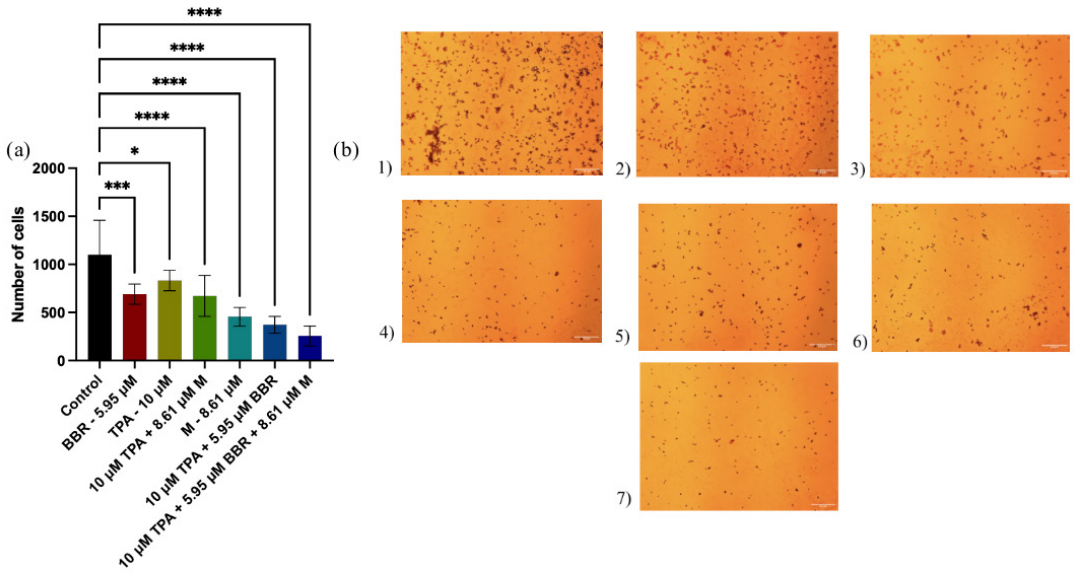

Figure 5. Berberine (BBR) and Melatonin (M) effect on U937 cell adhesion. Number of U937 cells that adhere to the cell plate. A cell adhesion assay was conducted by coating cell plates with Human Collagen Type IV then staining the cells. **a)** Cell adhesion assay results of U937 cells. **b)** Photos of cell adhesion assay on U937 cells. **b1)** Control treatment did not change U937 cell adhesion percentage. **b2)** 10 µM TPA treatment decreased cell adhesion by 25.14%. **b3)** 5.95 µM BBR treatment decreased cell adhesion by 37.20%. **b4)** 5.95 µM BBR + 10 µM TPA treatment decreased cell adhesion by 66.12%. **b5)** 0.861 µM M treatment decreased cell adhesion by 58.66%. **b6) 0**.861 µM M + 10 µM TPA treatment decreased cell adhesion by 38.89%. **b7)** 5.95 µM BBR + 0.861 µM M + 10 µM TPA treatment decreased cell adhesion by 76.82%. Data is presented as mean ± s.d. (n=3). Experiments were repeated 3 times with similar results. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001

We measured MMP9 expression in CCD18 cells (**Figure 6b**). A medium concentration of melatonin (2.15 µM) upregulated MMP9 by 16.69% ($p < 0.01$, one-way ANOVA), making it the only treatment that upregulated MMP9 in CCD18 cells. A high concentration of melatonin (21.5 µM) downregulated MMP9 by 26.90% ($p < 0.0001$, one-way ANOVA). A medium concentration of berberine (14.9 µM) added to a high concentration of melatonin (21.5 µM) downregulated MMP9 by 7.93%. The molecular docking results for MMP9, specifically for melatonin, correlated with the ELISA assay of MMP9 results.

We measured BDNF expression in COLO320 cells, in which all treatments caused BDNF downregulation (**Figure 6c**). The treatment of a high concentration of melatonin (21.5 µM) had a BDNF downregulation of 44.87% ($p < 0.0001$, one-way ANOVA), the greatest downregulation out of all treatments.

We measured BDNF expression in CCD18 cells, in which all treatments caused significant downregulation of BDNF, similar to COLO320 cells (**Figure 6d**). The treatment of a high concentration of melatonin (21.5 µM) downregulated BDNF by 61.03% ($p < 0.0001$, one-way ANOVA), the greatest downregulation out of all treatments. The effects of a high concentration of melatonin (21.5 µM) were seen in the downregulation of MMP9 in CCD18 cells as well as the downregulation of BDNF of COLO320 cells.

Berberine and melatonin effect on caspase-3 activity

We conducted caspase assays to determine the caspase-3 levels within COLO320 cells and U937 cells. Caspases are associated with apoptosis of cancer cells, so they were measured to see if berberine or melatonin treatment changed their levels (9). We seeded COLO320 and U937 cells and treated them with various berberine and melatonin concentrations before we conducted a caspase assay using the Caspase Colorimetric Apoptosis Assay kit. We further diluted melatonin and berberine using media.

We measured the optical density (OD) value to indicate regulation of caspase-3 levels in COLO320 cells (**Figure 6e**). A medium concentration of berberine (1.49 µM) added to a medium concentration of melatonin (0.215 µM) caused a 5.08-fold increase in caspase-3, the greatest upregulation of all treatments on COLO320 cells. A medium concentration of berberine (1.49 µM) added to a high concentration of melatonin (2.15 µM) caused a 1.83-fold increase in caspase-3, the second greatest upregulation, suggesting that berberine and melatonin may have a synergistic effect. The other treatments of a medium concentration of berberine (1.49 µM), a medium concentration of melatonin (0.215 µM), and a high concentration of melatonin (2.15 µM) had caspase-3 downregulations of 1.33, 1.50, and 1.83-fold, respectively.

We also measured the OD value in U937 cells (**Figure 6f**). A medium concentration of berberine (1.49 µM) added to a high concentration of melatonin (2.15 µM) caused a 1.33-fold increase of caspase-3, the greatest upregulation out of all treatments, showing the likely synergistic effects of berberine and melatonin. A medium concentration of berberine (1.49 µM) added to a medium concentration of melatonin (0.215 µM) upregulated caspase-3 by 55.56%, the second greatest percentage. The other treatments of a medium concentration of berberine (1.49 µM), a medium concentration of melatonin (0.215 µM), and a high concentration of melatonin (2.15 µM) upregulated caspase-3 by 11.11%, downregulated by 33.33%, and caused 0% change, respectively.

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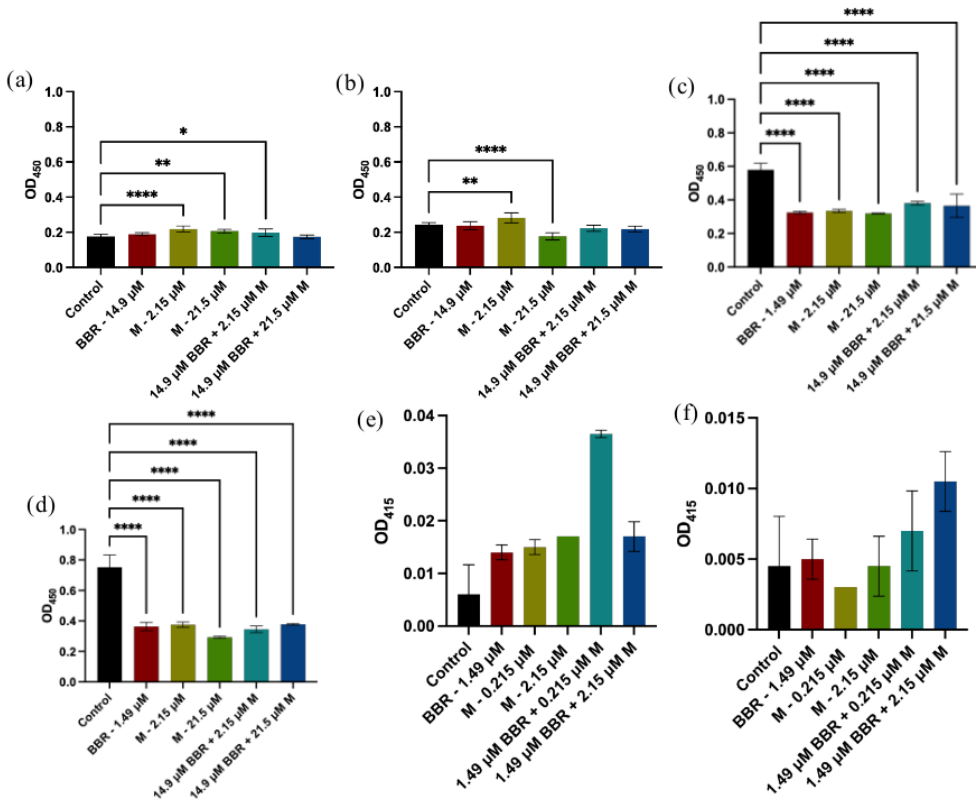

Figure 6. Expression of MMP9, BDNF, and caspase-3 in different cancer cells measured by ELISA assay and caspase assay. MMP9, BDNF, and caspase-3 levels in COLO320, CCD18, and U937 cells treated with Berberine (BBR) and Melatonin (M). An ELISA assay was done on CCD18 and COLO320 cells treated with BBR and M, then absorbance was measured at 450 nm. A caspase assay was done on COLO320 and U937 cells, then absorbance was measured at 415 nm. **a)** Expression of MMP9 in COLO320 cells. **b)** Expression of MMP9 in CCD18 cells. **c)** Expression of BDNF in COLO320 cells. **d)** Expression of BDNF in CCD18 cells. **e)** Expression of caspase-3 in COLO320 cells. **f)** Expression of caspase-3 in U937 cells. Data is presented as mean ± s.d. (n=3). MMP9 and BDNF experiments were repeated 3 times, and the caspase-3 experiments were repeated 2 times. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001

DISCUSSION

We aimed to assess the anticancer potential of melatonin and berberine as non-invasive, targeted therapies, addressing limitations of current treatments. We hypothesized that their combination could modulate key cancer-related biomarkers, including caspase-3, MMP9, and BDNF. Our findings provide insight into their effects on COLO320 and U937 cells, as well as MMP9 and BDNF expression.

Both berberine and melatonin have been reported to have synergistic anticancer effects with other compounds. Berberine has been used to amplify the anticancer effects of the drug Lovastatin, decreasing the growth of pancreatic cancer cells (9). Melatonin has synergistic effects with other antioxidants, such as increasing vitamin E, glutathione, and vitamin C's ability to protect against lipid peroxidation (12). In addition, both berberine and melatonin are economically beneficial since they cost less, with few side effects (20). Berberine and melatonin showed strong binding affinities with MMP9 and shared the same binding site (phenylalanine A:3412), suggesting potential synergy (**Table 1, Figure 1**). Given their known synergy with other molecules, this may help explain their combined anticancer effects (9, 12).

In short-term MTT assays on COLO320 cells, high-dose berberine significantly reduced cell viability (**Figure 2a**), consistent with previous U937 results showing dose-dependent effects (21, 22). Melatonin's effect varied by cell line, but the combined treatment consistently reduced viability more than either alone. No significant effects were seen in healthy CCD18 cells, indicating melatonin's harmlessness (**Figure 2c**).

In COLO320 migration assays, berberine greatly reduced cell migration, in line with previous findings on its anti-metastatic effects via TGFBR1 and cell-matrix interaction regulation (7, 9). Both compounds also reduced cell adhesion (**Figure 5**), consistent with earlier studies, further supporting synergy (23, 24).

In COLO320 cells, most berberine-melatonin combinations did not significantly alter MMP9 expression, and melatonin alone upregulated it (**Figure 6a**), likely due to low concentrations—a study limitation. ELISA showed stronger effects, as it directly measured CRC biomarkers. In CCD18 cells, all treatments except melatonin reduced MMP9 (**Figure 6b**), suggesting anti-inflammatory effects without synergy. All treatments downregulated BDNF in COLO320 cells (**Figure 6c**), and nearly all increased caspase-3 in both COLO320 and U937 cells (**Figures 6e, 6f**), supporting the hypothesis that berberine and melatonin synergistically enhance anticancer activity.

In future studies, more ELISA assays could be done on other proteins with high binding affinities from the molecular docking. First, the binding affinities of *ESR1* with berberine and melatonin, 7.5 and 6.1, respectively, were one of the highest binding affinities observed in our experiment (**Table 1**). Estrogen receptor alpha (ERα), encoded by *ESR1*, could

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increase cell proliferation in breast cancer (25). Berberine has the ability to inhibit ERα36 in breast cancer cells to enhance tamoxifen, an antagonist of ERα (26). In addition, melatonin could block estrogen from binding to ERα and control the proliferation of breast cancer cells (27). Most other proteins also had high binding affinities with both berberine and melatonin. For example, HS90AA1 had binding affinities of 7.1 and 5.9 to berberine and melatonin, respectively. STAT3 had similar affinities of 7 and 5.8 to berberine and melatonin, respectively. A few proteins had stronger binding to either berberine or melatonin, such as VEGFA having binding affinities of 7.4 and 5.2 to berberine and melatonin, respectively. All of these binding affinities are high and demonstrate the potential pharmacological effects of berberine and melatonin on these proteins.

Besides more ELISA assays, it would be beneficial to treat cells with a greater range of melatonin and berberine combined in smaller increments to find the optimal concentration of both berberine and melatonin. Another step would be to improve berberine bioavailability to increase its efficacy by reducing its particle size using nanoization techniques (28).

In conclusion, we saw that melatonin and berberine have likely synergistic anticancer effects. Berberine alone significantly reduced cell viability more than melatonin, but the combination of both had greater dose-dependent synergistic effects in reducing cell viability for both of the cell lines tested. Short- and long-term MTT assays, as well as a cell migration assay, showed the greatest decrease in cell viability and migration with the combined treatment. In the cell adhesion assay, the combination decreased cell adhesion second most, after berberine alone. ELISA assays showed significant decreases in BDNF in both COLO320 and CCD18 cells with the combined treatment. Molecular binding evidence indicated that berberine and melatonin shared binding sites with high affinity. The cell caspase assay showed the highest decrease in caspase-3 with the combined treatment. This likely synergy supports further study of berberine and melatonin's synergistic anticancer effects, with the end goal being use of these drugs together as cost-effective, safer alternatives to current cancer treatments.

MATERIALS AND METHODS

Molecular docking

Melatonin and berberine SDF files were downloaded from databases found on the PubChem website. PDB files of proteins were downloaded from the Protein Data Bank: CD44, ESR1, ESR2, LCP2, and TGF-β1. The following proteins were downloaded from the AlphaFold Protein Structure Database: STAT3, TP53, EGFR, ESR1, HSP90AA1, MMP9, TNF, and VEGFA. Using PyRx, the downloaded proteins were opened and autodocked, and the "Make Macromolecule" button was clicked. Berberine and melatonin were individually docked as ligands. The binding affinity table and the 3D structure of the macromolecule-protein complex were downloaded. The downloaded 3D structure was opened in the BIOVIA Discovery Visualizer application, and the berberine or melatonin molecule was made the ligand. The binding sites' 2D diagram was screenshotted.

Dilution of melatonin and berberine

Melatonin (Sigma-Aldrich) and berberine (Sigma-Aldrich) were solvated in distilled water. For the MTT cell proliferation assay, cell migration assay, and ELISA assay, solutions were further diluted using media into 21.5, 2.15, and 0.215 μM melatonin and 149, 14.9, and 1.49 μM berberine. For the cell adhesion assay, solutions were further diluted using media to 8.61, 0.861, and 0.0861 μM melatonin and 59.5, 5.95, and 0.595 μM berberine. For the caspase assay, melatonin and berberine were further diluted using the cellular media to 2.15, 0.215, and 0.0215 μM melatonin and 14.9, 1.49, and 0.149 μM berberine. All controls were Minimum Essential Media (MEM).

MTT cell proliferation assay

COLO320 (Cat#: ATCC-CCL-220.1, ATCC) (CRC), U937 (Cat#: CRL-3253, ATCC) (lymphoma) and CCD18 cells (Cat#: CRL-1459, ATCC) (normal colon as a healthy control) were seeded at $1x10^5$ cells for all types of cells and treated with 5.0 μL of various berberine and melatonin concentrations over 24 hours. As an adherent cell type, U937 cells were centrifuged and resuspended in fresh MEM. After incubation with an incubator, cell proliferation was assessed using an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich) assay as previously described (29). However, optical density (OD) was measured at 595 nm. An increase in OD value correlates with increased cell viability, and a decrease in OD value correlates with decreased cell viability. This process was repeated for one plate of COLO320 cells and one plate of U937 cells, which were treated with berberine and melatonin for 96 hours to simulate long-term effects.

Cell migration assay

COLO320 cells, seeded at 1x105 cells per well. A migration assay was conducted using the protocol of a previous paper on the procedures of *in vitro* scratch assays, with minor modifications such as treating the cells with various berberine and melatonin treatments (30). Horizontal scratches were generated using a 0.5-10 μL micropipette tip in each well. Images were taken at the 0-hour time point. ImageJ was used to analyze six microscope pictures of each treatment after 24 hours. The number of cells was then counted using PixelArt by dragging a line across the width of each mark and measuring the number of pixels.

ELISA assay

CCD18 cells and COLO320 cells were treated with various melatonin and berberine solutions (control, 14.9 μM berberine, 2.15 μM melatonin, 21.5 μM melatonin, 14.9 μM berberine added to 2.15 μM melatonin, and 14.9 μM berberine added to 21.5 μM M) and incubated overnight at 37 ˚C. 0.25% trypsin (Thermo Fisher Scientific) was used to detach the cells. The plate rested for three minutes, 1 mL of MEM was added to each well and cells were concentrated by centrifugation and frozen for future assays. Following the protocol of the Human MMP-9 ELISA Kit (Cat#: EK0465, Boster Bio), MMP9 levels and the BDNF levels were measured using a microplate reader at 450 nm.

ELISA results were normalized to total protein. Absorbance/OD was measured at 450 nm. An increase in OD value correlates with increased cell protein levels, and a decrease in OD value correlates with decreased cell protein levels.

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Cell adhesion assay

U937 cells were treated with various melatonin, berberine, and 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma-Aldrich) solutions. The plate was coated with 3 µg of Human Collagen Type IV (Cat#: CC076, Sigma-Aldrich, St. Louis, MO). The cells were stained with Hema-Quik II and then washed with distilled water. The cells were observed with a microscope at 40x. In ImageJ, the Image-Based Tool for Counting Nuclei (ITCN) plugin was used to calculate the cell number and density of each cell well.

Cell caspase assay

COLO320 cells and U937 cells were collected in MEM. Caspase activity was measured using the Caspase Colorimetric Apoptosis Assay kit (Cat#: K11900, Fisher Scientific) per the manufacturer's instructions. Microplate reader readings to measure absorbance were done with 415 nm at 0, 15, 45, and 60 minutes. The percent change of the caspase activity through an hour was calculated.

Statistical analysis

To prepare data for analysis, the microplate readings for all assays were inputted into an Excel spreadsheet to calculate the percentage change in data. Then, the data was pasted in the application Prism to conduct one-way ANOVA tests.

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