The effects of cetuximab with agomelatine on gene expression in colon cancer cells

ABSTRACT

This study investigated the combined effects of agomelatine, a melatonergic antidepressant, and cetuximab, an EGFR inhibitor, on the colorectal cancer cell line (Caco-2). Caco-2 cells were treated with agomelatine (0.3 µg/ml and 3 µg/ml) and cetuximab (50 µg/ml), individually and in combination, for 24 and 48 hours. Cell viability was assessed using the MTT assay. Gene expression analysis of *EGFR*, *BCL2*, *PIK3CA*, *BAX*, *mTOR*, and *AKT3* was performed using real-time PCR. All treatment groups showed significant decreases in cell viability compared to the control ($p<0.05$), with enhanced effects in combined treatments. *EGFR* expression was significantly reduced in drug-treated groups, particularly with cetuximab ($p<0.05$). While changes were observed in *BCL2*, *PIK3CA*, *BAX*, *mTOR*, and *AKT3* expression, these were not statistically significant ($p>0.05$). This study demonstrates the potential synergistic cytotoxic effects of agomelatine and cetuximab on Caco-2 colorectal cancer cells. The significant reduction in *EGFR* expression suggests a potential mechanism of action. These findings provide insights into combining chemotherapeutic agents with drugs addressing circadian rhythm disorders in CRC treatment strategies. Further research is warranted to elucidate the clinical implications of these observations.

Keywords: Agomelatine, cetuximab, colorectal cancer, gene expression, chemotherapy

INTRODUCTION

Colorectal cancer (CRC) remains one of the most prevalent and lethal malignancies worldwide, demonstrating a substantial burden on global public health (Xi and Xu, 2021). Characterized by unregulated cell growth in the colon or rectum, CRC has been intricately associated with genetic mutations, environmental influences, and various pathophysiological mechanisms (Alharbi et al., 2022). A significant percentage of CRC cases have been identified to exhibit overexpression of the Epidermal growth factor receptor (EGFR), which is intricately involved in cellular proliferation, apoptosis, and differentiation (Han et al., 2022; Ogrodnik, 2021).

Targeted therapies, particularly employing monoclonal antibodies, have emerged as a pivotal approach in CRC treatment. Cetuximab (CTX), an EGFR antagonist, has been widely utilized due to its ability to inhibit ligand-induced phosphorylation and activation of receptor-associated kinases, thus interfering with downstream signaling pathways implicated in cancer progression (Moreno-SanJuan et al., 2023). However, despite the initial response, resistance to cetuximab invariably develops, necessitating alternative or adjunctive therapeutic strategies.

Agomelatine, primarily recognized for its utility in managing depressive disorders by modulating circadian rhythms, has recently garnered attention in oncology (Fekry and Eckel-Mahan, 2022).

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Research Article

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Intriguingly, disturbances in circadian rhythms have been implicated in the pathogenesis and progression of several cancers, including CRC, through mechanisms involving cell cycle regulation, DNA damage response, and metabolism (Aghamiri et al., 2019; Hossain et al., 2022; Wathoni et al., 2020). The potential impact of agomelatine on CRC, particularly in conjunction with cetuximab, remains a fertile ground for exploration, potentially unraveling novel insights into the intricate web of CRC pathophysiology and therapeutic resistance.

This research seeks to illuminate the effects of cetuximab and/or agomelatine on cell proliferation and apoptosis in Caco-2 colorectal cancer cells. Through a meticulous investigation of cellular and molecular responses to these agents, this study endeavors to elucidate the underlying mechanisms and pathways, thereby contributing to the burgeoning field of targeted cancer therapy and paving the way toward more effective and sustainable therapeutic strategies in CRC management.

MATERIALS AND METHODS

Cell culture

The colorectal cancer cell line (Caco-2) used in this study is preserved at -196°C in a nitrogen storage tank at the Molecular Cancer Biology Laboratory, Erzurum Technical University. Cell culture medium was prepared by adding 10% fetal bovine serum (FBS), 1% ml of penicillin-streptomycin (Pen-Strep) to DMEM. Parenteral Caco-2 monolayer cell lines were incubated in a cell culture medium at 37°C with 5% carbon dioxide (CO2) and 95% humidity in an incubator (Esco Co., Korea) in 25 cm² flasks under sterile conditions to facilitate cell proliferation.

Drug treatment

Cetuximab and agomelatine treatments

Caco-2 colorectal cancer cells were exposed to treatments with cetuximab (IMC-C225, Erbitux) and/or agomelatine (Valdoxan, Thymanax, AG0178) to evaluate their effects on cellular proliferation and apoptosis related genes. Specifically, agomelatine was prepared in cell medium and administered at dosages of 0.3 µg/ml and 3 µg/ml, whereas cetuximab was utilized at a dosage of 50 µg/ml, both independently and in combination with agomelatine.

For viability analyses, 1500 cells were seeded into each well of a 96-well plate, and interventions were administered as outlined in Table 1 of the original study. Subsequent to the treatments, cells were incubated for 24 and 48 hours in a 37°C, 5% CO_2 incubator, followed by an MTT cytotoxicity analysis utilizing an appropriate MTT assay kit.

Table 1. Primer sequences used in RT-PCR analysis

Gene	Forward Primer	Reverse Primer
BAX	CGCATCCTGAGGCACCGG	TTTCATCCAGGATCGAGCAGGG
EGFR	TCGTTGGACAGCCTTCAAGACC	AACACCCTGGTCTGGAAGTACG
BCL2	CGCATCCTGAGGCACCG	TTTCATCCAGGATCGAGCAGGG
AKT3	GGAAGAATGGACAGAAGCTATTCCA	TCCACTTGCCTTTCTCTCGAAC
MTOR	GTCAGTGGGACAGCATGGAAG	CCCATATGCCCGACTGTAACTC
PIK3CA	TGGATGCTTCACAGGGCTTTCT	TATCTTGCCGTAAATCATCCCCCA

MTT assay for cell viability

Assay procedure

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was employed to assess cell viability post-drug treatments. Cells were seeded at a density optimized for the detection of cellular metabolic activity and subsequently treated with the drugs. Post-treatment, MTT solution was added, and cells were incubated to facilitate the formation of

formazan crystals. The crystals were dissolved, and the absorbance was measured at 570 nm spectrophotometrically. Data was normalized and analyzed to determine the effects of treatments on cellular viability.

Quantitative real-time PCR (qRT-PCR) analysis

RNA isolation

Total RNA was isolated from the Caco-2 cells treated with the drugs using a commercial kit, adhering to the manufacturer's guidelines (Ambion RNA Mini Kit, USA). The RNA isolation procedure involved several steps including cell lysis, homogenization, and purification, following a detailed protocol to ensure the integrity and purity of the isolated RNA. The concentration and purity of the extracted RNA were determined spectrophotometrically using a Nanodrop device (EPOCH Take3 Plate, Biotek), and RNA samples were stored at -20°C until further use.

cDNA synthesis

Following RNA extraction, cDNA synthesis was conducted using the Maxime RT Premix kit. The synthesis protocol involved combining 5 µl of RNA with 15 µl of RNase-free water, and the reaction was performed using a Veriti 96 Well Thermal Cycler (Applied Biosystem) with the temperature settings set at 45°C for 60 minutes and 95°C for 5 minutes. Subsequent to synthesis, cDNA samples were quantified spectrophotometrically and stored at -20°C.

qRT-PCR analysis

Gene expression analyses of *AKT3*, *PIK3CA*, *EGFR*, *Bcl-2*, *Bax*, *MTOR*, and *GAPDH* genes were performed using qRT-PCR with specific primers designed for each gene (Table 1). The amplification, detection, and data analysis were conducted using the Qiagen Rotor-Gene Real Time PCR System (Rotor-Gene Q 5plex HRM System), ensuring specificity, efficiency, and reproducibility of the results. The amplification conditions were set at 95°C for 3 minutes for enzyme activation, 95°C for 5 seconds for denaturation (40 cycles), and 60°C for 10 seconds for amplification (40 cycles). Relative gene expression was calculated using the ΔΔCT method, providing insights into the molecular mechanisms underlying the cellular responses to drug treatments (Livak and Schmittgen, 2001).

Statistical analysis

Statistical analysis of the cell viability data, obtained from four replicates, was performed using Microsoft Office Excel. Data were presented as mean ± standard deviation. IBM SPSS Statistics 22.0 software was used for significance analysis. Differences between groups were evaluated using one-way analysis of variance (ANOVA) followed by Duncan's post-hoc test. Statistical significance was set at $p<0.05$.

RESULTS

Cytotoxicity analysis

The cytotoxic effects of agomelatine and cetuximab, individually and in combination, were evaluated on Caco-2 colorectal cancer cell lines through comprehensive analyses. Table 2 presents the cytotoxicity results for Caco-2 cells incubated for 24 and 48 hours, following treatment with various dosages of agomelatine and cetuximab.

Table 2. Cytotoxicity results for CACO-2 colorectal cancer cells treated with different doses of agomelatine and cetuximab and incubated for 24 and 48 hours.

Groups	24h	48h
Control	0.250± 0.022	0.281± 0.023
Ago-0.3	0.139± 0.011	0.190 ± 0.020
Ago-3	0.143± 0.011	0.205± 0.053
Cet-50	0.143± 0.012	0.183± 0.007
Ago-0.3+Cet-50	0.152 ± 0.008	0.173± 0.009
Ago-3+Cet-50	0.142 ± 0.005	0.182± 0.012

The results indicate a discernible decrease in cell viability across all treatment groups compared to the control, with distinct dose-dependent and time-dependent variations ($p<0.05$). Particularly, the administration of agomelatine at dosages of 0.3 µg/ml (Ago-0.3) and 3 µg/ml (Ago-3) demonstrated a significant

reduction in cell viability after 24 and 48 hours of incubation ($p<0.05$). Likewise, cetuximab at a dosage of 50 µg/ml (Cet-50) exhibited potent cytotoxic effects, further pronounced when combined with agomelatine at both aforementioned dosages ($p<0.05$).

The combined application of agomelatine and cetuximab indicated a notable synergistic effect, particularly pronounced in the 48-hour incubation period ($p<0.05$). The cytotoxicity was not merely additive but exhibited an enhanced effect, suggesting an interaction in the apoptotic and proliferative pathways influenced by the two drugs.

MTT assay results

The viability of Caco-2 cells, subsequent to treatment with varying concentrations of agomelatine and cetuximab, was scrutinized utilizing the MTT assay. Results manifested discernible alterations in cell viability in response to both singular and combined drug treatments across the distinct incubation periods 24h and 48h (Figure 1).

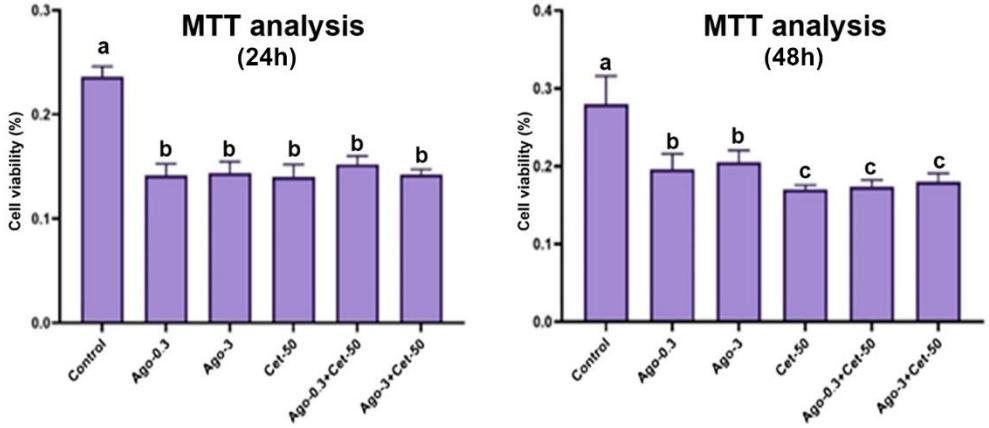

Figure 1. Cytotoxicity results of CACO-2 colorectal cancer cell lines incubated for 24 and 48-hours following treatment with different doses of agomelatine and/or cetuximab. Different letters (a, b, c) indicate statistically significant differences between groups ($p<0.05$). Data is presented as mean ± SD of four independent experiments.

Invert microscopic analysis

Subsequent observations at 24 and 48h further elucidated the cellular dynamics and morphological changes induced by the drug treatments, as depicted in Figure 2. A detailed analysis revealed a significant reduction in viability in groups subjected to agomelatine and cetuximab treatments, with pronounced effects observed at specific dosage levels ($p<0.05$). Furthermore, the results elucidated potential dose-dependent and time-dependent cytotoxic effects of the administered drugs.

Real time-PCR analysis results

This study investigated the effects of agomelatine and cetuximab on gene expression in Caco-2 colon cancer cells. Real-time PCR analysis was conducted to examine the expression of *EGFR*, *BCL2*, *PIK3CA*, *BAX*, *mTOR*, and *AKT3* genes under various treatment conditions and incubation periods of 24 and 48 hours. The results are presented in Figure 3.

***EGFR* gene expressions**

EGFR gene expression analysis revealed significant differences between the control group and various treatment conditions (Figure 3). The control group exhibited the highest *EGFR* expression levels across both 24-hour and 48-hour incubation periods ($p<0.05$). Groups treated with drug combinations, particularly Cet-50 and Ago-0.3+Cet-50, showed reduced *EGFR* mRNA expression, with ratios approaching 1.01. The inhibitory effect on *EGFR* expression was most pronounced in the Cet-50 group during the 48-hour incubation period ($p<0.05$).

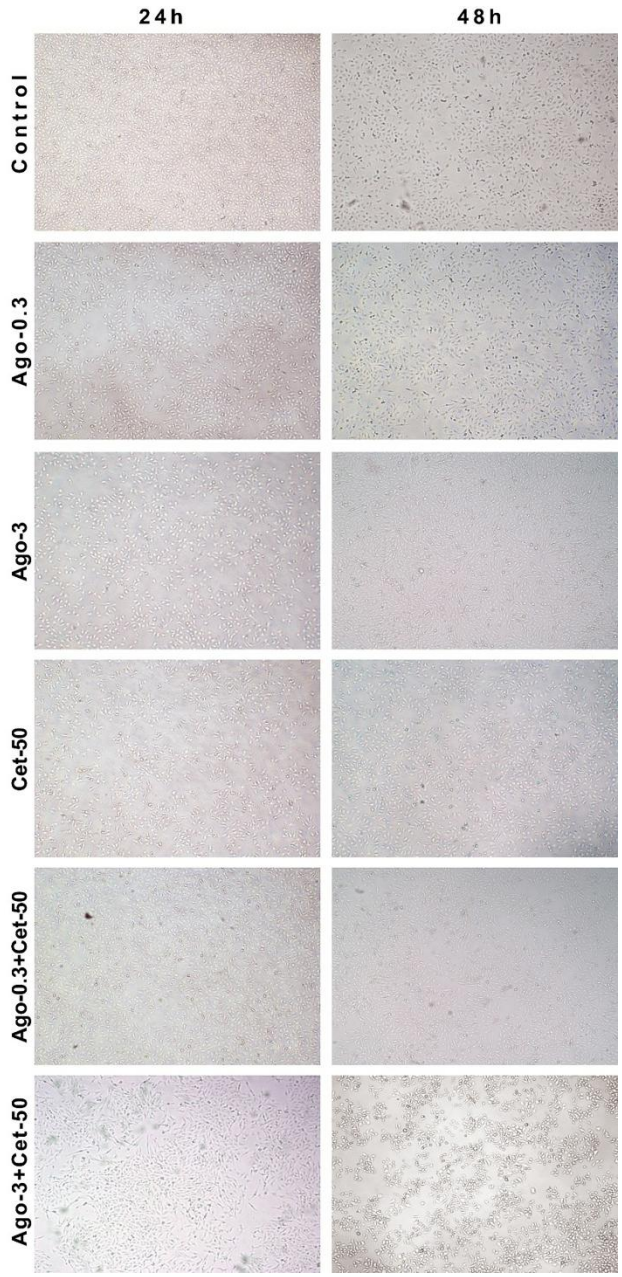

Figure 2. Inverted microscope images of Caco-2 cells for various groups after drug application at 24h and 48h.

BCL gene expression

BCL2 expression patterns, as shown in Figure 3, remained relatively stable across treatment groups and incubation periods. At 24 hours, the Cet-50 group showed a slight increase in *BCL2* expression compared to the control, but this difference was not statistically significant ($p>0.05$). At 48 hours, all groups exhibited similar *BCL2* expression levels.

PIK3CA gene expression

PIK3CA expression, depicted in Figure 3, showed some variability among groups. At 24 hours, Ago-3 and Cet-50 groups showed slightly higher expression compared to the control. At 48 hours, Ago-3 and Ago-3+Cet-50 groups exhibited a minor increase in *PIK3CA* expression compared to other groups. However, these differences did not reach statistical significance ($p>0.05$).

BAX gene expression

BAX gene expression results are presented in Figure 3. A trend towards upregulation was observe in drug-treated groups at 24 hours, with Ago-3 and Ago-0.3+Cet-50 groups showing slight elevations. At 48 hours, the Ago-0.3 group demonstrated a minor increase in BAX expression. Despite these observations, the differences were not statistically significant when compared to the control group ($p>0.05$).

mTOR gene expression

mTOR gene expression, as illustrated in Figure 3, showed some fluctuations across treatment groups and time points. The Ago-3 group showed a slight elevation in *mTOR* expression at 24 hours. At 48 hours, the Cet-50 group displayed a minor increase in *mTOR* expression. However, these changes were not statistically significant relative to the control group ($p>0.05$).

AKT3 gene expression

AKT3 expression levels, presented in Figure 3F, remained relatively consistent across most treatment conditions. A slight increase was observed in the Ago-0.3+Cet-50 group at 48 hours, but this change did not reach statistical significance ($p>0.05$).

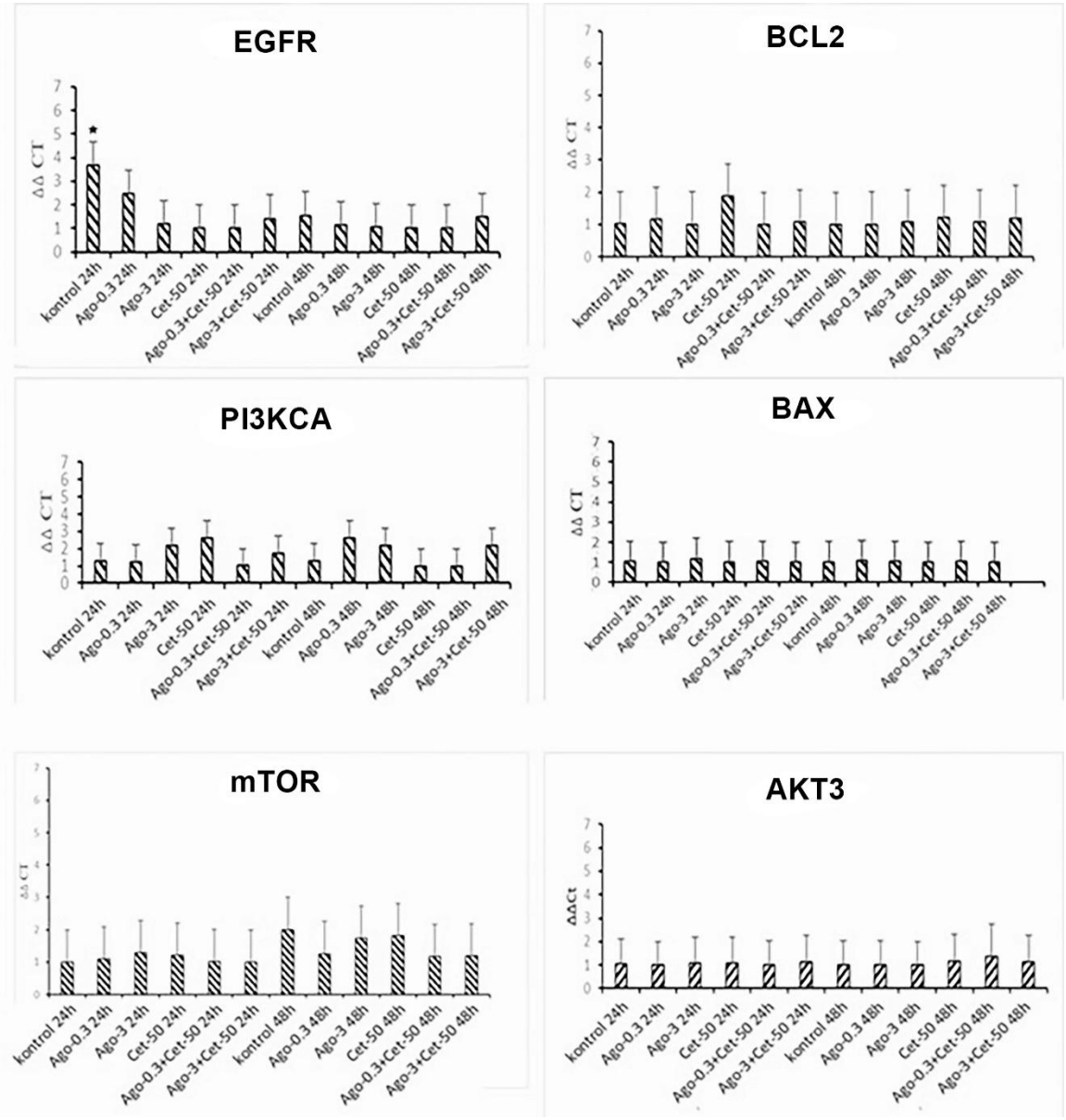

Figure 3. Gene expression values of *EGFR*, *BCL*, *PIK3CA*, *BAX*, *PIK*3CA, *mTOR*, and *AKT3* in Caco-2 colon cancer cell lines Incubated for 24 and 48 hours with various doses of agomelatine and cetuximab. The astarix indicate the Statistical significance between the groups. Statistical differences were determined with $p<0.05$ compared to the other groups. Data is presented as mean ± SD of four independent experiments.

DISCUSSION

CRC ranks as the third most common type of cancer worldwide, witnessing a continual rise in new case rates year after year. The treatment of colorectal cancer is contingent upon the stage of the cancer and the patient's overall condition, offering a range of chemotherapy drugs and treatment modalities (Li et al., 2020). Cetuximab, employed in this study, acts as an antagonist of EGF and a monoclonal antibody, finding pervasive use in CRC treatment (Giordano et al., 2019). Nevertheless, cancer patients often face significant challenges in treatment progression due to circadian rhythm and mood disorders, necessitating, at times, the incorporation of antidepressant agents into the cancer treatment regimen (Kılıç and Erbaş, 2021) . In this context, recent studies advocate the use of agomelatine, a melatoninergic agonistic analog, renowned for its chronobiotic, anxiolytic, and antidepressant effects and its capacity to expedite the resynchronization of

fundamental biological circadian rhythms (Moreno-SanJuan et al., 2023).

In essence, individuals diagnosed with cancer are commonly administered chemotherapy drugs and/or medications like chronobiotics, anxiolytics, and antidepressants for treatment purposes and to manage psychopathological disorders stemming from the disease, respectively (Chang and Shen, 2019; Naser et al., 2021). Consequently, the combined use of a chemotherapeutic agent and an antidepressant in this study explores a potential treatment approach and examines the effects of cetuximab and agomelatine in cancer treatment, an area that has not been extensively studied. This research protocol aims to contribute to the literature by assessing the effects of a chemotherapeutic agent along with agomelatine, a melatonin analog, in a model that aligns with the pathophysiology of the disease.

This study investigated the effects of agomelatine and/or cetuximab on cell cytotoxicity, apoptotic cell death, and the expression levels of regions related to genes associated with proliferation in the Caco-2 cancer cell line under in vitro conditions. To this end, Caco-2 cells were incubated for 24 and 48 hours with agomelatine at concentrations of 3 µg/ml and 0.3 µg/ml, and cetuximab at a concentration of 50 µg/ml. Cell cytotoxicity was determined using the MTT method, while the expression levels of *BAX*, *EGFR*, *BCL2*, *AKT3*, *mTOR*, and *PIK3CA* genes were analyzed through quantitative real-time PCR.

EGFR plays a pivotal role in cancer development as a key protein, its overexpression being prevalent in numerous tumors. It has been targeted for treatment via small molecule inhibitors and monoclonal antibodies, with the latter playing a role in the treatment of metastatic disease (Alharbi et al., 2022; Amodio et al., 2020; Giordano et al., 2019). CTX, being an EGFR antagonist and a monoclonal antibody, is widely employed in CRC treatment. It can reduce receptor activation in some cancer cells by inhibiting the binding of the ligand to the respective receptor and can activate apoptosis (Cho et al., 2010). The combination of anti-EGFR therapy and cytostatics, which cause DNA damage, has been shown to exert an anti-tumor effect by inhibiting cell cycle progression and activating apoptosis. Cetuximab augments apoptosis while suppressing cell proliferation, angiogenesis, and metastasis by blocking downstream signaling (Hanck-Silva et al., 2020). In our study, we observed changes in EGFR expression in Caco-2 cells treated with CTX and agomelatine. The control group exhibited the highest EGFR expression levels, while groups treated with drug combinations, particularly Cet-50 and Ago-0.3+Cet-50, showed reduced EGFR mRNA expression. These observations align with existing research that suggests CTX's ability to affect receptor activation (Cunningham et al., 2004). The simultaneous application of CTX and agomelatine appeared to influence EGFR expression, which may be related to the approach of combining anti-EGFR therapy with other agents. Previous studies have shown that such combinations can affect cell cycle progression and apoptosis (Sartore-Bianchi et al., 2016). These findings may contribute to our understanding of EGFR-focused interventions in CRC and suggest potential areas for further research into combined therapeutic approaches in oncology.

The PI3K/Akt/mTOR pathway, a central signaling stream system, plays a crucial role in vital physiological events such as the cell cycle, cell life, protein synthesis, growth, metabolism, and angiogenesis (Miricescu et al., 2020). AKT, a serine/threonine kinase and a central mediator in the PI3K pathway, governs key cellular events, stimulating protein synthesis and cell growth by activating mTOR (Revathidevi and Munirajan, 2019). 24-hour application of drugs at concentrations of 0.3 µg/ml and 0.3 µg/ml+50 µg/ml suppressed the proliferation of *PIK3CA*, *EGFR*, and *AKT* genes, but not *mTOR*. This

finding is interesting when compared with existing literature, which often highlights the central role of mTOR in driving cell growth and proliferation in the context of the PI3K/Akt pathway. The selective suppression of upstream components like *PIK3CA* and *EGFR* may indicate potential for targeted inhibition strategies in colorectal cancer. For example, studies have shown that targeting *EGFR* can effectively disrupt cancer progression (Ayati et al., 2020; Li et al., 2022), and our findings seem to support this strategy, particularly in the early stages of drug treatment. The results showing that a 48-hour application of drugs at concentrations of 50 µg/ml and 0.3 µg/ml+50 µg/ml suppressed *mTOR*, *PIK3CA*, and *EGFR* genes, but not *AKT*, suggest a time-dependent response in the pathway's components. This aligns with the understanding that prolonged drug exposure can lead to different cellular responses. In the context of colorectal cancer, studies have indicated that sustained inhibition of mTOR can be more effective over time (Faivre et al., 2006; Fasolo and Sessa, 2008).

On the apoptosis mechanism, proteins like caspase-3, caspase-9, bax, bcl-2, and p53 play a key role. Some members of the Bcl-2 protein family are pro-apoptotic, while others are anti-apoptotic (Choudhury et al., 2012). This protein, localized on the mitochondrial membrane, controls the permeability of mitochondrial pores and has an anti-apoptotic effect. BAX protein, a proapoptotic protein localized on the mitochondria, plays a crucial role in facilitating apoptosis (Dadsena et al., 2021). In our study, we examined *BAX* and *BCL2* gene expressions in colorectal cancer cells. We observed changes in gene expression in groups treated with 3 µg/ml and 0.3 µg/ml+50 µg/ml drug combinations for 24 hours, particularly noting changes in *BAX* expression in the Ago-3 and Ago-0.3+Cet-50 groups. These observations may be considered in the context of recent literature exploring the impact of melatonin on cancer cells. Studies have demonstrated that melatonin can affect stress-induced insulin resistance and cellular responses to apoptotic signals by influencing COX expression and the Bax/Bcl-2 ratio (Bu et al., 2017). While our study used agomelatine rather than melatonin, these findings may suggest areas for further investigation. The effects of MEL on apoptosis and autophagy appear to be cell-type dependent. For instance, Tran et al. (2021) reported that melatonin synergizes with doxorubicin to activate apoptosis in breast cancer cells and enhances the therapeutic effect of doxorubicin by inducing autophagy. In the context of colorectal cancer, Zhao et al. (2022) found a synergistic anti-tumor effect of melatonin and *Andrographis paniculata* in reducing the viability of colon cancer cells and stimulating apoptosis. They also noted that this combination inhibited autophagy by affecting the expression of autophagy-related genes such as NR4A1, CTSL, and Atg12 (Ma et al., 2020). Similarly, Chok et al. (2021) observed that melatonin increased colorectal cancer cell death, oxidative stress, and autophagic vacuole formation in a dose-dependent manner. These studies highlight the complex interplay between pro-apoptotic and anti-apoptotic mechanisms in cancer cells, and the role of external agents like melatonin in modulating these processes. The parallels between these findings and our own suggest that the regulation of *BAX* and *BCL2* expression is a critical factor in the effectiveness of cancer therapies and underscore the potential of targeting these pathways in colorectal cancer treatment.

CONCLUSION

In conclusion, this study demonstrates the potential cytotoxic effects of agomelatine and cetuximab, both individually and in combination, on Caco-2 colorectal cancer cells. The MTT assay revealed significant reductions in cell viability across treatment groups, with

pronounced synergistic effects observed in combined treatments, particularly after 48 hours of incubation. Gene expression analysis showed a significant decrease in *EGFR* expression in drug-treated groups, especially with cetuximab, suggesting a potential mechanism of action. While changes were observed in the expression of *BCL2*, *PIK3CA*, *BAX*, *mTOR*, and *AKT3* genes, these were not statistically significant. These findings provide insights into the molecular effects of agomelatine and cetuximab on colorectal cancer cells and suggest potential avenues for further research in combining chemotherapeutic agents with drugs addressing circadian rhythm disorders in cancer treatment strategies.

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Author contributions: Concept - RK, AK; Supervision –AK, HÜ; Materials- RK, KA, AK; Data Collection and/or Processing- RK, HÜ, EE, KA, AK; Analysis and/or Interpretation - RK, EE, KA, AK; Writing – RK, HÜ, AK.

Availability of data and materials: Data and materials related to this study are available from the corresponding author upon reasonable request.

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