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In vitro investigation of the toxicological mechanisms of gemcitabine in colorectal cancer cells

Eşref TATLIPINAR[1,2], Tuğçe BORAN[3], Özge Sultan ZENGIN[1], Gül ÖZHAN[1*]

1 Istanbul University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology, Istanbul, Türkiye
2 Istanbul University, Institute of Graduate Studies in Health Sciences, Istanbul, Türkiye
3 Istanbul University Cerrahpasa, Faculty of Pharmacy, Department of Pharmaceutical Toxicology, Istanbul, Türkiye

ABSTRACT

Colon cancer is the third most common cancer type in the world. Gemcitabine (2'deoxy-2'2'-difluorocytidine monohydrochloride) was found to be very effective against small cell lung cancer, pancreatic cancer, bladder cancer and breast cancer and was approved for the treatment for these cancers. Although it is similar to cytosine arabinoside (Ara-C) in terms of structure, metabolism and mechanism of action, the spectrum of antitumor activity of gemcitabine is much wider. Autophagy is a general term that refers to the degradation of cytoplasmic components within lysosomes. Autophagy plays a critical role in many disorders such as neurodegenerative diseases, cancer, infection, cardiovascular, metabolic, pulmonary diseases and aging. In this study, we evaluate the gemcitabine alone and its' combinations with autophagy inhibitor (chloroquine) and activator (rapamycin) effect on cell cycle, apoptosis and autophagy on human colorectal cancer cell line (HCT-116). We exposed the cells to gemcitabine (0,625 mM, 12,5 mM, 2,5 mM, 5 mM), rapamycin (0,5 μM) and chloroquine (20 μM) for 24 hours. Gemcitabine, alone or in combination with chloroquine caused cell cycle arrest at G1 and G2. However, the combination with rapamycin doesn't cause any significant change in the cell cycle of the exposed

*Corresponding author: Gül ÖZHAN
E-mail: gulozhan@istanbul.edu.tr
ORCIDs:
Eşref TATLIPINAR: 0000-0001-8422-3188
Tuğçe BORAN: 0000-0003-4302-1947
Özge Sultan ZENGİN: 0009-0006-9301-7371
Gül ÖZHAN: 0000-0002-6926-5723
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cells. Gemcitabine-chloroquine group also significantly increased the apoptosis. Consequently, combining gemcitabine with chloroquine increased the cell death when comparing gemcitabine alone. Therefore, gemcitabine and chloroquine combination could increase the efficacy of chemotherapeutic treatment of colorectal cancer.

Keywords: colorectal, cancer, gemcitabine, autophagy, cytotoxicity

INTRODUCTION

Colon cancer is the third most common cancer type in the world[1,2]. In Europe, 250,000 new cases of colon cancer are diagnosed each year, accounting for 9% of all diseases. The incidence of colon cancer is increasing with industrialization and urbanization. The incidence of colon cancer in individuals under the age of 45 is very rare (2 per 100,000 people per year). While it is 20 per 100,000 people between the ages of 45-54, this rate increases much more as the age increases to be 55 per 100,000 for the 55-64 age group, 150 per 100,000 for the 65-74 age group, and 250 per 100,000 after the age of 75[3].

Surgical intervention is considered the first in patients with colorectal cancer who have a chance of recovery. Adjunctive therapy is a systemic therapy used to reduce the risk of cancer recurrence and death. The risk of recurrence can be estimated by pathological staging[4]. While adjuvant chemotherapy has become the standard for third-stage patients, it does not play much of a role in the second stage. In the case of metastatic cancer, the most important goal of chemotherapy is to prolong and improve life expectancy[4].

Chemotherapeutic treatment of colorectal cancer contains several drugs like fluoropyrimidine, irinotecan, oxaliplatin, bevacizumab, cetuximab, panitumumab, capecitabine[5]. Generally, first-line chemotherapy includes fluorouracil (5-FU) or capecitabine or combining them with leucovorin (LV) or oxaliplatin for alleviating the symptoms and increasing the quality of life. In second-line chemotherapy, patients will be selected based on resistance of chemotherapeutic drugs[6]. Gemcitabine (2' deoxy-2'2'-difluorocytidine monohydrochloride) was approved for the treatment of small cell lung cancer[7], pancreatic cancer[8], and breast cancer[9].

Gemcitabine inhibits DNA synthesis at G1/S cell cycle and represses cell proliferation. Gemcitabine is not approved for the colorectal treatment, however, some studies reported that some chemotherapeutics like capecitabine, oxaliplatin in combination with gemcitabine can be a therapeutic option for refractory

advanced or progressive colorectal cancer[10,11]. In addition, gemcitabine has been found to be effective in oxaliplatin-resistant colorectal cells an *in vitro* study[12].

Gemcitabine (Gem) is a potent and specific analogue of deoxycytidine. After being taken up by malignant cells, gemcitabine is phosphorylated by deoxycytidine kinase to form gemcitabine monophosphate. This monophosphate form is then converted to gemcitabine diphosphate and gemcitabine triphosphate, which are the active metabolites of gemcitabine. Consequently, these active metabolites are responsible for the antitumor activity of gemcitabine[13].

Autophagy is a general term that refers to the degradation of cytoplasmic components within lysosomes. Autophagy plays a critical role in many disorders such as neurodegenerative diseases including cancer, infection, cardiovascular, metabolic, pulmonary diseases and aging[14]. Autophagy has effects on carcinogenesis that can go both ways. While autophagy helps to prevent the transformation into malignancy by removing damaged organelles, accumulated proteins on normal cells, reducing DNA damage, reactive oxygen derivatives (ROS) and mitochondrial abnormalities; it also contributes to tumor formation by enabling the tumor cell to reach nutrients, prevent cellular death and increase drug resistance[15,16]. The response of cells to autophagy during cancer metastasis is phase-of-cancer dependent. In the early stages, autophagy inhibits tumor cell metastasis by producing inflammatory responses against tumors. In addition, autophagy limits tumor necrosis and transformation of dormant cancer cells into micro-metastases[17]. In advanced stages, autophagy increases the survival of metastatic cells in the extracellular matrix and promotes the spread of cancer cells to distant organ sites[17,18]. Some studies showed that both activation and inhibition of autophagy with the specific chemicals increased anticancer activity of the chemotherapeutics[19-24].

In this study, it is aimed to determine the effects of gemcitabine and its' combinations (autophagy inhibitor, chloroquine and autophagy activator, rapamycin) on HCT-116 human colorectal cancer cell line and to investigate the role of autophagy in the anticancer activity of gemcitabine in colorectal cancer cells.

METHODOLOGY

Cell culture

HCT-116 (Human colorectal carcinoma-CCL-247) cell line was purchased from ATCC, USA. Cells were cultured in RPMI-1640 medium contained 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic and 1% non-essential amino acid. Cells were subcultured when they reach 60-70% confluence.

Drug treatment

Cells were exposed to gemcitabine for 24 hours. 1.25 mM gemcitabine was used for the combination studies. In the study, rapamycin (RAPA) was used as an autophagy activator, and chloroquine (CQ) was chosen as an autophagy inhibitor. The concentration of the drugs was determined as 0.5 μM for rapamycin[25,26,27] and 20 μM for chloroquine[28,29,30] according to the literature. To validate autophagy activation/inhibition in our conditions LC3B II/I protein expression level was investigated with western blot after 0.5 μM rapamycin and 20 μM chloroquine exposure for 24 hours. After 0.5 μM rapamycin and 20 μM chloroquine exposure LC3B II/I protein expression enhanced significantly, and the increase was found significantly higher after chloroquine exposure than rapamycin (Figure 1). Chloroquine impairs autophagosome degradation by affecting autophagosome-lysosome fusion, so LC3B II accumulates in the cell[31], which is supported by other studies[28,32,33]. However, the cell viability was not affected significantly (data not shown) at these concentrations. Inhibitor of autophagy, CQ induced the formation of the autophagosome, but inhibited the degradation of autophagosome in the last stage of autophagy[34].

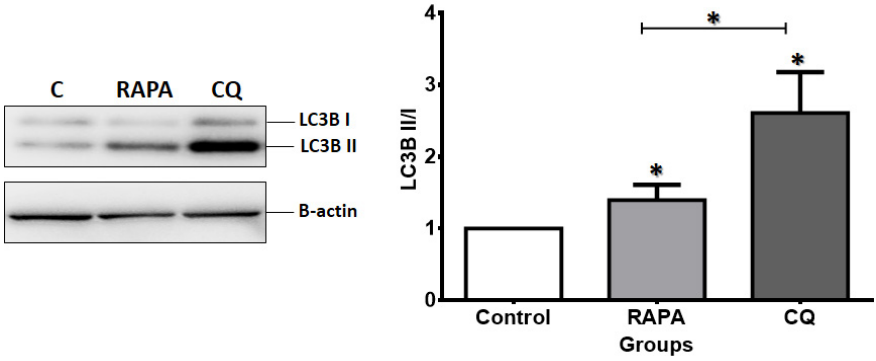

Figure 1. Changes in LC3B II/I expression after 0.5 nM RAPA (rapamycin) and 20 μM chloroquine (CQ) exposure for 24 h
*p<0.05

Cell viability assay

Cell viability was evaluated with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. HCT-116 cells were treated with gemcitabine only (1.562-50 mM), combinations of gemcitabine 1.25 mM) with chloroquine (20 μM) and rapamycin (0.5 μM), for 24 hours. At the end of exposure time, MTT (5 mg/mL) solution was added to each well and incubated at 37°C

for 3 hours. Then, the medium and MTT dye in the wells were removed and formazan was dissolved with DMSO in, Optical density (OD) was measured at 590 nm using a multiwell plate reader (Biotek, Bad Friedrichshall, Germany).

Detection of apoptosis

Apoptosis was determined with a commercial kit (Biolegend, California, USA) following the manufacturer's rules. After 24 hours exposure, the cells were collected with trypsin and resuspended in Annexin V binding buffer (100 µL). Then Annexin V (5 µL) and PI (10 µL) dye solutions were added to the cell suspension and incubated at room temperature. After 15 min incubation, 400 µl of Annexin V Binding Buffer was added and fluorescence signals were determined in the FITC channel (FL-1) and PE channel (FL-2) by ACEA flow cytometry (Agilent, California, USA). The results were analyzed with Novoexpress software (Agilent, California, USA).

Cell cycle analysis

Cell cycle analysis was performed with a commercial kit (Elabscience Biotechnology, Houston, USA) following the manufacturer's instructions. After drug treatments, cells were collected by trypsinization and washed with PBS. Then, cells were transferred to tubes containing 1.2 mL absolute ethanol and incubated at -20 °C for 1 hour. After centrifugation and washing steps, 100 µL of RNase A reagent was added to each tube and incubated in a water bath at 37 °C for 30 minutes. At the end of the incubation time, 400 µL propidium iodide (PI) staining solution was added to each tube and incubated at 2-8°C for 30 minutes. Fluorescence intensity was determined by a flow cytometry in FL-2-A channel (Agilent, California, USA) and results were calculated using Novoexpress software (Agilent, California, USA).

Statistical analysis

Data were analyzed using GraphPad prism software (version 6) with one-way ANOVA followed by Tukey test. $p<0.05$ values were considered as statistically significant. All data were represented as mean ± standard deviation (SD).

RESULTS and DISCUSSION

Cell viability

According to cell viability assay, gemcitabine decreased cell viability starting from 2.5 mM dose (cell viability 71.16% ± 1.86) and IC_{50} value of gemcitabine was calculated to be 5.50 mM ± 0,2. Cell viability decreased in gemcitabine (1.25 mM)-rapamycin (0.5 µM) combination at the same doses comparing to the control group, however it was not found to be statistically significant differ-

ence when comparing with gemcitabine group. Gemcitabine (1.25 mM)-chloroquine (20 μM) combination inhibited cell viability more than gemcitabine group at 1.25 mM, 2.5 mM and 5 mM concentrations (Figure 2).

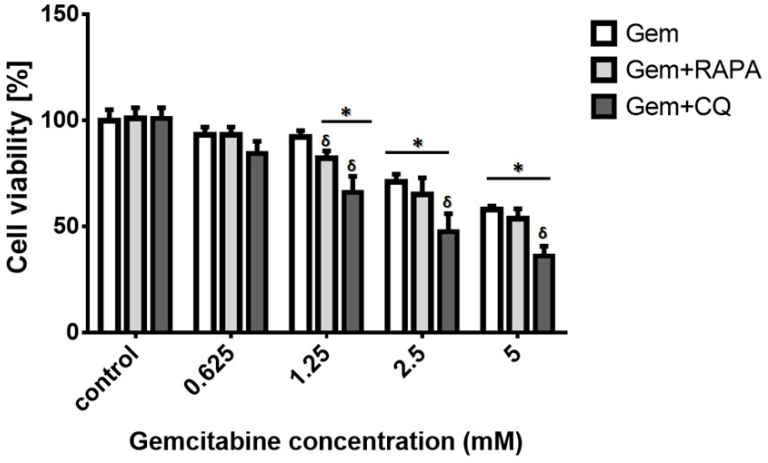

Figure 2. Changes in the cell viability following 24h Gem, Gem+RAPA and Gem+CQ exposures *p<0.05 versus control group, $^{\delta}$p<0.05 versus Gem group. Gem: Gemcitabine, RAPA: Rapamycin, CQ: Chloroquine

Apoptotic and necrotic cell death

Gemcitabine (1.25 mM) induced 1,5-fold more apoptotic cell death (p<0.05) but not necrotic cell death. Apoptotic cell death was 1.36-fold less in gemcitabine-rapamycin (0.5 μM) group comparing to gemcitabine group. Apoptosis significantly increased (1,5-fold) in gemcitabine-chloroquine (20 μM) group in comparison with the control group but there was no significant difference with the gemcitabine-alone group (Figure 3).

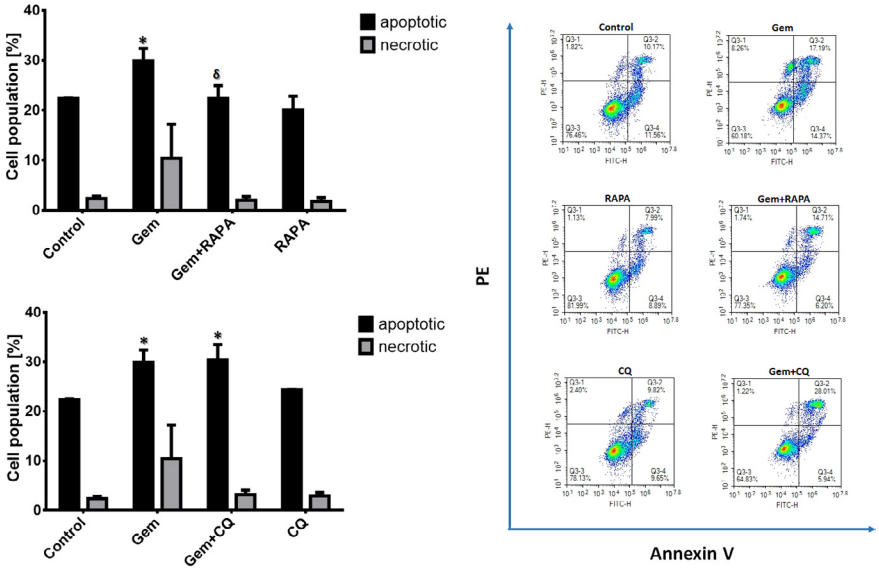

Figure 3. Changes in apoptotic and necrotic cell population following 24 hours Gem (1.25 mM), Gem+RAPA (0.5 μM) and Gem+CQ (20 μM) exposures *p<0.05 versus control group, $^{\delta}$p<0.05 versus Gem group. Gem: Gemcitabine, RAPA: Rapamycin, CQ: Chloroquine.

Cell cycle analysis

It was observed that gemcitabine led to G1 and G2 arrest in HCT-116 cell line. Although rapamycin (0.5 μM) also induced G2 arrest compared to the control group (1.25-fold), there were no significant changes in gemcitabine-rapamycin combination group in comparison with gemcitabine group. However, G1 and G2 arrest was exacerbated (1.5-fold) in chloroquine (20 μM)-gemcitabine (1.25 mM) combination compared to the control group and gemcitabine-alone (1.25 mM) group also cause to the cell cycle arrest, but arrest of gemcitabine-chloroquine combination group was higher than chloroquine-alone group probably due to the synergistic effect (Figure 4).

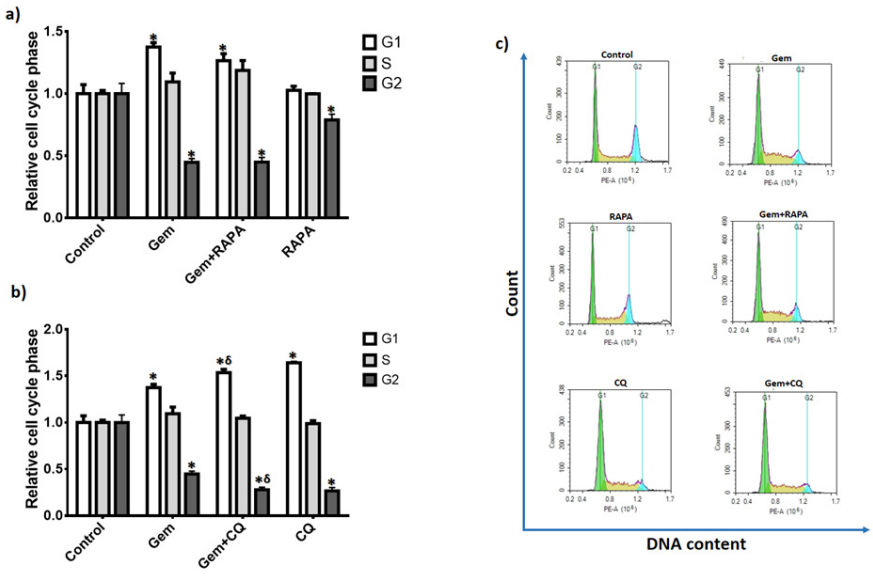

Figure 4. Changes in G1, S and G2 cell cycle phases following 24 hours Gem (1.25 mM), Gem+RAPA (0.5 μM) and Gem+CQ (20 μM) exposures *p<0.05 versus control group, $^{\delta}$p<0.05 versus Gem group. Gem: Gemcitabine, RAPA: Rapamycin, CQ: Chloroquine.

Colorectal cancer is the third most common and fourth deadliest cancer type in the world[1,2,35]. In 1996, Food and Drug Administration (FDA) approved gemcitabine for first line treatment of advanced and metastatic pancreas[36]. Although gemcitabine has no FDA approval in the treatment of colorectal cancer, some studies suggest that adding gemcitabine into the traditional chemotherapy improves the anticancer effect of treatments[8,9]. Autophagy activators/inhibitors that added to conventional chemotherapy has been shown to increase anticancer effect of the chemotherapy[37,38]. Besides, the combination therapies can be used to overcome chemotherapy resistance[39]. In the study, anticancer activity of gemcitabine was investigated in the presence of rapamycin (autophagy activator) and chloroquine (autophagy inhibitor) using HCT-116 cell line.

According to the findings, both rapamycin and chloroquine combinations decreased cell viability compared with gemcitabine group and the decrease was found statistically significant for chloroquine combination but not for rapamycin.

Apoptosis is a programmed cell death that protects the entire organism against more serious damages, such as cancers. In normal cell, when there is a damage on DNA and it can't be repaired, apoptosis is triggered, and the abnormal cell dies as programmed. When there is a problem in apoptosis induction, the

abnormal cell continues to proliferation and finally, cancer cells will occur. In cancer, there is an imbalance between proliferation and programmed cell death. Most treatments like chemotherapy, radiation, hormonal treatments generally aim to create an irreparable cellular damage and trigger their apoptosis[40]. Gemcitabine has been shown to induce apoptosis on many cancer cell lines like, pancreatic, breast, and human osteosarcoma cells[41,42,43]. Similarly, it was also found that gemcitabine increased the apoptotic cell death after 24 h exposure in the study. It has been noted that gemcitabine-induced autophagy has been shown to prevent apoptosis in lung cancer cells. Thus, adding of autophagy inhibitors to gemcitabine treatment increased apoptosis in lung cancer cells[38]. Another study also showed that autophagy inhibition increased hypoxia-induced apoptosis in HCT-116 cells[44]. Similarly, gemcitabine and chloroquine combination induce apoptotic cell death in HCT-116 cell comparing to gemcitabine group in the present study.

Activating the autophagy with rapamycin (50 nM, for 24 hours exposure) has been reported to induce the apoptosis in human osteosarcoma cells[19]. Furthermore, anticancer effect was increased the anticancer drug efficiency when rapamycin (10.3 nM, for 48 hours exposure) was added to the regimen through stimulating autophagy, apoptosis and cell cycle arrest in breast cancer cells[21]. In our study, rapamycin did not increase apoptotic cell death in HCT-116 cells and autophagy activation by rapamycin treatment alleviated the gemcitabine-induced apoptotic cell death in HCT-116 cells.

Eukaryotic cell division is regulated by different mechanisms to prevent uncontrolled cell proliferation under physiological conditions. interphase and M phase are major components of the mitotic cell division. After all, separation of cellular content duplication during interphase occurs and two genetically identical daughter cells are formed. DNA replication is performed in S phase. The phase that separates end of mitosis from S phase is G1 and separates S phase from M phase is G2, which are also called gap phases since they have been considered as gaps between DNA duplication and DNA segregation. Additionally, these phases play crucial role for the regulation of cell cycle[45,46]. It is known that the regulation of the cell cycle plays a crucial role in influencing the proliferation, metastasis, and recurrence of tumor cells. In cancer treatment, many chemotherapeutic drugs show anticancer effect via inducing cell cycle inhibition[47]. It has been reported that gemcitabine (30 nM for 24-48 hours exposure) caused to cell cycle arrest at G1, S and G2 phases in some cancerous cell lines[48,49]. In the present study, gemcitabine (1.25 mM) also induces cell cycle disruption at G1 and G2 phases in HCT-116 cells.

The antimalarial drug chloroquine has demonstrated anticancer effects on some cancer cells[50,51]. In the present study, chloroquine caused to disruption of G1 and G2 phases on colorectal cancer cells. Furthermore, when used in combination with conventional chemotherapies, Chloroquine has been found to enhance the anticancer effect of the treatment and sensitized the tumor cells to chemotherapeutic agent or radiotherapy[24,52,53]. Similar to these studies, chloroquine combination potentiates gemcitabine-induced G1 and G2 arrest when compared with gemcitabine group.

Our findings indicate that combining gemcitabine with chloroquine results in a higher rate of HCT-116 cell death compared to using gemcitabine alone, likely due to disturbance in the cell cycle, but there is no significant change in cell death for gemcitabine-rapamycin group. Therefore, gemcitabine and chloroquine combination could be a therapeutic option in the treatment of colorectal cancer.

STATEMENT OF ETHICS

This study does not require any ethical permission.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Design: G.Ö., T.B., E.T., Ö.S.Z. Acquisition of data: T.B., E. T., Ö.S.Z. Analysis of data: T.B., E. T., Ö.S.Z. Drafting of the manuscript: E.T. Supervision: G.Ö. Statistical analysis: T.B.

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