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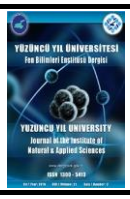

Research Article

Effects of Pharmaceuticals Used Against COVID-19 on HDAC Activity and Inflammation in Cell Line Obtained from a Colorectal Adenocarcinoma Patient

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Abstract: Different doses of various drugs were used in the treatment of the Coronavirus Disease 2019 (COVID-19) pandemic that affected the whole world. Since these drugs are not directly related to COVID-19, it was very important to elucidate their effects. Studies have shown that COVID-19 is closely related to colorectal cancer. Our study examines the effects of Lopinavir/ritonavir, hydroxychloroquine, favipiravir, and oseltamivir, as well as their active ingredients used during COVID-19 treatment, on the histone deacetylase enzyme (HDAC) in colorectal cancer cells. Thus, it is aimed to provide a different perspective on the relationship between pandemic and colorectal cancer. In this study, the effects of the applied drugs on cell viability were evaluated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis, acridine orange/propidium iodide (AO/PI) staining and histone deacetylase enzyme activity was evaluated in colorectal adenocarcinoma cell line (Caco-2) with HDAC assay. Using an enzyme-linked immunosorbent assay (ELISA), the inflammatory effects on the cells due to the application were assessed based on the expression levels of interleukin 10 (IL-10), interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF-α). Experimental groups showed continued cell viability, and an increase in HDAC enzyme activity was observed. This increase was found to effectively influence the IL-6 expression, which is believed to enhance inflammation.

Keywords: Favipiravir, HDAC, Hydroxychloroquine, Lopinavir/ritonavir, Oseltamivir, Valproic acid

COVID-19'a Karşı Kullanılan Farmasötiklerin Kolorektal Adenokarsinom Hastasından Elde Edilen Hücre Dizisinde HDAC Aktivitesi ve Enflamasyon Üzerindeki Etkileri

Öz: Koronavirüs Hastalığı 2019 (COVID-19) pandemisinin tedavisinde çeşitli ilaçların farklı dozları kullanıldı. Bu ilaçlar doğrudan COVID-19 ile ilişkili olmadığından oluşturacakları etkilerin aydınlatılması oldukça önem taşımaktaydı. Yapılan araştırmalarla COVID-19'un kolorektal kanser ile yakından ilişkili olduğu ortaya konulmuştur. Gerçekleştirdiğimiz çalışmada kolorektal kanser hücre soyunda (Caco-2), COVID-19 tedavisinde kullanılan lopinavir/ritonavir, hidroksiklorokin, favipiravir, oseltamivir ilaç ve etken maddelerinin histon deasetilaz enzimi (HDAC) üzerindeki etkinlikleri ve enflamasyona katkıları araştırılmıştır. Böylece pandeminin kolorektal kanserle ilişkisine farklı bir bakış açısı kazandırmış olmak hedeflenmiştir. Bu çalışmada, uygulanan etken maddelerin Caco-2 hücrelerinin hücre canlılığı üzerindeki etkileri; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromid (MTT) analizi, akridin turuncusu/propidyum iyodür (AO/PI) boyaması kullanılarak belirlendi ve histon deasetilaz enzim aktivitesi HDAC testi ile saptandı. Ayrıca enflamatuvar etki, enzim bağlantılı immünosorbent testi (ELISA) kullanılarak interlökin 10 (IL-10), interlökin 6 (IL-6) ve tümör nekroz faktörü alfa (TNF-α) ekspresyon seviyelerinde meydana gelen değişiklere göre değerlendirildi. Uygulama yapılan gruplarda hücre canlılığı devam ederken, HDAC enzim aktivitesinde artış meydana geldi. Bu artışın IL-6 ekspresyonu üzerinde etkili olduğu gösterilerek enflamasyona neden olduğu düşünülmektedir.

Anahtar Kelimeler: Favipiravir, HDAC, Hidroksiklorokin, Lopinavir/ritonavir, Oseltamivir, Valproik asit

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1. Introduction

Since appeared on December 31, 2019, coronavirus-2 syndrome (SARS-CoV-2), referred to as COVID-19, has rapidly evolved into an expanding pandemic (Page et al., 2021). Drugs repurpose is the technique of using an existing drug or drug candidate for a new treatment or medical condition for which it was not indicated before (Kulkarni et al., 2023). According to this principle, many existing antiviral drugs, such as Lopinavir/ritonavir (Lop/r) (Cao et al., 2020), chloroquine (Wang et al., 2020), hydroxychloroquine (Gautret et al., 2020), and favipiravir (Cai et al., 2020), which were previously used to treat Severe Acute Respiratory Syndrome (SARS), Middle East Respiratory Syndrome (MERS), Human Immunodeficiency Virus (HIV/AIDS), and malaria, have been considered potentially effective in treating COVID-19 and approved for use in patients. Some of these drugs are still in the clinical trial phase (Li & De Clercq, 2020; Sanders et al., 2020).

Lop/r is an antiretroviral agent. *In vivo* studies have shown that it may be effective against MERS, and there is limited evidence that it may also be effective in MERS/SARS patients. In Singapore, 2 out of 5 COVID-19 patients who began treatment with Lop/r deteriorated, suggesting that using a low dose of Lop/r may be effective (Young et al., 2020). In a retrospective analysis of 134 cases COVID-19 in China, 52 patients were given Lop/r, 34 patients were given arbidol, and 48 patients were considered controls; as a result, no differences were observed between the groups regarding symptom improvement and viral load reduction. (Yao et al., 2020).

The positive effects of chloroquine/hydroxychloroquine in the treatment of the SARS-CoV pandemic in 2002-2003 made it a candidate drug for use in the treatment of SARS-CoV-2. Hydroxychloroquine is thought to act through various mechanisms, prevents the virus from binding to the host cell by disrupting the terminal glycosylation of the angiotensin-converting enzyme-2 (ACE-2) receptor, which is the receptor of the SARS-CoV-2 virus that has accumulated there by increasing the pH of the endosomes and lysosomes. An evaluation of data from 100 patients who participated in multicenter clinical trials receiving chloroquine treatment in China indicated that chloroquine phosphate was more effective than the control group in suppressing pneumonia exacerbations, enhancing lung imaging findings, and reducing the duration of the disease (Gao et al., 2020). In a study conducted in France; hydroxychloroquine was given to 20 patients diagnosed with COVID-19 and compared with 16 patients control, who did not receive treatment. The patients' nasopharyngeal samples were examined by polymerase chain reaction (PCR) for daily nasopharyngeal virus detection, and it was found that the negative PCR test rate on days 3, 4, 5, and 6 of treatment in the hydroxychloroquine group was significantly different from that of control patients (Gautret et al., 2020).

Favipiravir inhibits ribonucleic acid (RNA)-dependent RNA polymerase of RNA viruses and prevents the virus from multiplying. This agent, previously used in the treatment of resistant influenza and Ebola virus infection, has also been used in coronavirus infection due to its mechanism of action (Jordan et al., 2018; Shiraki & Daikoku, 2020; Shiraki et al., 2022). In Shenzhen, 35 patients treated with favipiravir achieved negative results in a median of 4 days, while the duration of the disease in the control group of 45 patients who did not receive favipiravir lasted 11 days. The study conducted in 2021 showed that the probability of clinical recovery was 24% higher when favipiravir was administered within the first seven days of hospitalization; However, there was no statistically significant reduction in mortality in hospitalized patients (Hassanipour et al., 2021; Swift et al., 2021).

Oseltamivir is a neuraminidase inhibitor approved for the treatment and prophylaxis of influenza A and B (Agrawal et al., 2020; Yousefi et al., 2021). In a cohort study involving 285 patients COVID-19, the severity of the disease was greater in patients treated with oseltamivir compared to the control group (5.02 vs. 4.2, $p = 0.01$). Additionally, both recovery time and hospital stay were significantly shorter for patients in the oseltamivir group (4.9 vs. 6.6 days, $p < 0.001$). Similarly, the mortality rate was lower in the oseltamivir group (1.7% vs. 6.7%, $p = 0.06$) (Zendehdel et al., 2022).

HDACs are essential in numerous biological processes in living organisms, including transcription, chromatin remodeling, the cell cycle, signal transduction, and gene expression regulation. The regulation of gene expression occurs via the acetylation and deacetylation of transcription factors. Histone tails carry a positive charge due to the presence of amine groups in the amino acid lysine and arginine, facilitating interaction with the negatively charged phosphate groups in the DNA backbone. Histone acetylation, performed by histone acetyltransferases, neutralizes this positive charge by

converting amines to amides, thereby reducing the electrostatic interaction between histones and deoxyribonucleic acid (DNA), which allows chromosomes to undergo transcription more readily (Khangura et al., 2017). Given these properties, they present potential as drug targets. HDAC inhibitors bind to the active sites of HDAC enzymes, resulting in histones remaining in their acetylated form and altering gene expression. These inhibitors impact fundamental cellular processes such as cell division, apoptosis, and differentiation, and are being explored for the treatment of various diseases. HDAC inhibitors have gained prominence in studies focused on treating diverse disease groups, particularly cancer, spinal muscular atrophy, Alzheimer's disease, diabetes, psychiatric disorders, and parasitic infections. Valproic acid, one of the HDAC inhibitors utilized in clinical practice, inhibits HDAC activity both in vitro and in vivo, leading to histone modification (Kucukoglu, 2013). It has been demonstrated to significantly reduce tumor growth and metastasis in animal studies. Consequently, valproic acid may serve as an effective therapeutic agent for cancer treatment.

In this study, conducted with cell line Caco-2, the effects of Lop/r, hydroxychloroquine sulfate, favipiravir, oseltamivir on the activity of HDAC, which play a role in various cellular regulatory processes such as gene transcription, cell growth, apoptosis, necrosis, survival, and cellular proliferation, were investigated (Gallinari et al., 2007). Some drugs that target HDAC enzymes affect various cellular events, and compounds that inhibit these enzymes are used in treatment protocols by participating in these processes (Singh et al., 2018; Tsai et al., 2020).

In the actual literature, there are no studies evaluating the effects of Lop/r, hydroxychloroquine, favipiravir, oseltamivir pharmaceuticals on the activation and inhibition of HDAC, which plays an important role in epigenetic mechanisms in Caco-2 cells. In our study, we investigated the effects of Lop/r, hydroxychloroquine (HYC), favipiravir (FAV), oseltamivir (OSM) active ingredients on HDAC activity by applying them to Caco-2 cell line for 24, 48, 72 hours. To determine the viability of Caco-2 cells, MTT analysis, AO/PI staining were performed.

HDAC activity was measured in cell lysates obtained using NETN solution with the Bio Vision HDAC Activity Colorimetric Assay Kit. Additionally, valproic acid (VPA), a HDAC inhibitor, served as the control group of our study. This approach allowed us to evaluate the cellular activities of the drugs on HDAC at specific time intervals.

Enterocytes regulate intestinal maintenance and defense by secreting and responding to inflammatory mediators and regulating intestinal epithelial permeability. The expression and secretion of cytokines such as IL-6, IL-10 and TNF-α, which play an important role in inflammatory processes, in Caco-2 cells are closely related to intestinal inflammation, inflammatory bowel diseases and colon cancer. In inflammatory effects, IL-6 and TNF-α aggravate inflammation, while IL-10 has an inflammatory effect. Its effectiveness on the intestinal barrier depends on the overproduction of these cytokines. In colon cancer, IL-6 and TNF-α pro-inflammatory cytokines have been shown to be at high levels. These cytokines may play a crucial role in processes such as tumor growth, metastasis, and angiogenesis. The expression and secretion of IL-6, IL-10 and TNF-α cytokines in Caco-2 cells have a very complex regulatory network. This regulation is affected by intracellular transcription factors, epigenetic regulations, microRNAs, extracellular bacterial products, viral infections, other cytokines, oxidative stress, nutritional factors. In our study, we evaluated the relationship between the inflammatory effects on the cells resulting from the application and the HDAC enzyme activity according to the IL-10, IL-6 and TNF-α expression levels using ELISA method (Vitkus et al., 1998; Kany et al., 2019).

2. Material and Methods

2.1. Cell culture

Caco-2 (HTB-37) cells (from ATCC) were cultured in DMEM (Sigma) medium supplemented with 15% FBS (Fetal Bovine Serum, Sigma Aldrich), 2 mM L-glutamine (Multicell), and 100 U/mL penicillin-streptomycin (Gibco) at 37 °C in a 5% CO_2 environment. A total of 3.2 x 10^4 Caco-2 cells per well were seeded in 96-well culture dishes for MTT assay, AO/PI staining, HDAC activity assessment and ELISA analysis. The experimental procedures were performed in triplicate.

2.2. Pharmaceuticals application to cell cultures

According to the IC50 doses in the literature, FAV at a final concentration of 62 µM (4 mg/ml), VPA at 0.5 mM (10 mg/ml), Lop/r at 400mg/100ml, HYC at 200mg and OSM at 15 µM/ml were applied to Caco-2 cell cultures (Cvetkovic & Goa 2003; Terzioglu et al., 2020; Kaya et al., 2024). The instructions for use of the drugs are extended for a maximum of 5 days for FAV and OSM, and for Lop/r and HYC according to the course of the disease. In our study, we applied the pharmaceuticals to Caco-2 cells at 0h-24h-48h-72h time intervals. This point was chosen because it was the most appropriate period to evaluate the duration of efficacy of the pharmaceuticals in the four groups (Table 1).

Table 1. Experimental groups

Group No	Application	Application Period	Application Amount
1		24 h	
2	HYC	48 h	5 µg/ml
3		72 h	
4		24 h	
5	FAV	48 h	52 µg/ml
6		72 h	
7		24 h	
8	OSM	48 h	0.05 µg/ml
9		72 h	
10		24 h	
11	Lop/r	48 h	6000 µM Lop+1400µM r
12		72 h	
13		24 h	
14	VPA	48 h	43 µM
15		72 h	
16		24 h	
17	Control (No application)	48 h	-
18		72 h	

2.3. Cell viability, toxicity, and proliferation analyses were performed using MTT assay

The experimental groups of changing in cell viability was evaluated using by the Vybrant MTT Cell Proliferation Assay [Catalog No. V13154, Thermo Fisher Scientific, Waltham, MA, USA]. Cellular proliferation and inhibition of proliferation were determined according to the optical density (OD) values of the experimental groups at a wavelength of 540 nm. The OD of the control group was considered as 100% viable and all experimental groups were calculated according to this percentage (Guzelel et al., 2024).

2.4. Cell Staining with AO, PI

Firstly, the surface morphology of Caco-2 cells was examined using an inverted light microscope without any treatment applied to the experimental groups.

To assess the accuracy of MTT data and cell viability, nucleic acid binding dyes were utilized and visualized under a fluorescence microscope (Leica DM2500, DH Life Sciences, USA). Cells were labeled with AO and PI dyes; AO stains all nucleated cells and produces green fluorescence, while PI penetrates cells with disrupted membrane integrity and stains dead cells, producing red fluorescence (Kaya et al., 2020).

2.5. Obtaining cell lysates by NETN solution

At the end of the experimental period, cell supernatants were removed, cell dishes were placed on ice plates and NETN solution [250 mM NaCl, 5 mM ethylene diamine tetra acetic acid (EDTA), 50 mM HCl, 0.5% Octylphenyl polyethylene glycol (IGEPAL), 1X Halt Protease Phosphatase Inhibitor Cocktail] was added. Cells were collected from cell culture dishes by scraping and transferred to test

tubes. After incubation on ice for half an hour, the tubes were centrifuged at 13000g. The supernatant was transferred to new test tubes and cell lysates were obtained.

2.6. Protein quantification

The protein amounts in Caco-2 cellular were determined using the Coomassie (Bradford) Protein Assay Kit. Protein quantification was evaluated with Bovine Serum Albumin (BSA) standart graphs prepared using the Sigma Plot 10.0 program.

2.7. HDAC activity test

The manufacturer's instructions were followed for HDAC activity test (Biovision, HDAC Activity Colorimetric Assay Kit, Catalog no: K331). A protein amount of 4 mg from the quantified cell lysates was used for each test. 10 µl of 10X HDAC Assay Buffer and 5 µl of HDAC colorimetric substrate were added to the wells of the 96-well plate, to which the samples were added. The assay plate was incubated at 37° C for 2 hours. Then, 10 µl of Lysine Enhancer was added to the wells to stop the reaction and the plate was incubated again for 30 minutes at 37° C. After incubation, the OD values of the wells were determined by the plate reader at a wavelength 405 nm.

2.8. Determined IL-10, TNF-α, IL-6 expressions levels with ELISA test

IL-10, IL-6, TNF-α expression levels were determined using a commercial booster ELISA kit (IL-6, catalog no: ek0410; IL-10, catalog no: ek0416; TNF-α, catalog no: ek0525). Analyses were performed according to the manufacturer's instructions. Standard protein curves in the kit were created separately for each protein. IL-10, IL-6, TNF-α expression levels were determined using the supernatants obtained at the end of the culture periods. Evaluation: the value of the control group was accepted as 100% and protein amounts were calculated as percentages by comparing between groups. 100 ml of supernatant sample was added to the ELISA plate wells. The plate was incubated at 37 ˚C for 90 minutes. Then, the content of the wells was discharged and 100 µL of 1× biotinylated antihuman IL-6, IL-10 or TNF-α was added to the relevant wells and incubated at 37°C for 60 min. After incubation, the content of the wells was discharged, and the wells were washed 3 times with 1× wash buffer. 100 µL of 1× avidin biotin peroxidase complex was added, and incubation was carried out at 37°C for 30 min. Then, 100 µL of 1× avidin biotin peroxidase complex was added to the wells that were washed 5 times with 1× wash buffer and the plate was incubated at 37°C for 30 min. At the end of the period, the wells were washed 5 times and 90 µL of color development reagent was added and incubation was carried out at 37°C for 25 min. Finally, 100 µL of stop solution was added to each well and OD values were measured at 450 nm absorbance in an ELISA microplate reader.

2.9. Statistical analysis

Statistical evaluation between the treatment groups of our study; one way ANOVA test, which is used to determine whether the mean differences between three or more groups are significant, dependent t-test, which determines the relationship between the dependent sample and the variable sample, and regression test, which measures the relationship between two or more variables, were used to perform (p value <0.05).

3. Results

3.1. Cell viability analysis

The effects of drugs applied to Caco-2 cells on cell viability were calculated which the control group was accepted as 100% in terms of density depending on time (Table 2).

Table 2. Cell viability (%) of experimental groups

Group No	Application	Application Period	% Cell Viability
1		24 h	111.45
2	HYC	48 h	129.07
3		72 h	107.26
4		24 h	88.76
5	FAV	48 h	76.65
6		72 h	93.39
7		24 h	124.67
8	OSM	48 h	113.87
9		72 h	94.93
10		24 h	96.25
11	Lop/r	48 h	70.70
12		72 h	64.09
13		24 h	111.45
14	VPA	48 h	95.37
15		72 h	124.67
16		24 h	100
17	Control (No application)	48 h	100
18		72 h	100

The cell viability of the drug application groups are as follows: 11.45% increase at 24 hours, 29.07% at 48 hours and 7.06% at 72 hours in HYC group; 11.2% decrease at 24 hours, 23.3% at 48 hours and 6.6% at 72 hours in FAV group; 24.67% increase at 24 hours, 13.87% at 48 hours and 5.06% decrease at 72 hours in OSM group; 3.75% decrease at 24 hours, 29.3% at 48 hours and 35.91% decrease at 72 hours in Lop/r group; in VPA acid group, an increase of 11.45% in 24 hours and 24.64% in 72 hours and a decrease of 4.63% in 48 hours were observed compared to the control group. Although the viability percentages changed between the groups at the end of the application periods, no significant difference was observed in cell viability rates between the groups according to the ANOVA statistical test data. This data revealed that the applied HYC, FAV, OSM, Lop/r drug doses did not have a significant effect on cell viability and the cells continued to proliferate in some groups. AO/PI staining was performed on all samples treated with HYC, FAV, OSM, Lop/r groups determine whether cell survival or death (if any) was apoptotic and the existing relationship with MTT analysis. The experimental groups were morphologically visualized and evaluated at 0, 24, 48 and 72 hours according to the application hours (Figure 1). It was observed that the applied doses of HYC, FAV, OSM, Lop/r, VPA groups did not affect the morphology of Caco-2 cells, and the cells continued to proliferate while maintaining their viability. AO/PI staining shows that dead cells are stained red and live proliferating cells are stained green. Figure 1 is shown respectively, Figure 1.A: control group; Figure 1.B: Unstained Caco-2 cell morphology; Figure 1.C: HYC group 5µg/ml at 24 hours; Figure 1.D: Lop/r group 6000 µM Lop+1400 µM r at 24 hours; Figure 1.E: FAV group 50 µg/ml at 24 hours; Figure 1.F: OSM group 0.05 µg/ml at 24 hours; Figure 1.G: VPA group 43 µM at 24 hours; Figure 1.H: HYC group 5µg/ml at 48 hours; Figure 1.I: Lop/r group 6000 µM Lop+1400 µM r at 48 hours; Figure 1.J: FAV group 50 µg/ml at 48 hours; Figure 1.K: OSM group 0.05 µg/ml at 48 hours; Figure 1.L: VPA group 43 µM at 48 hours; Figure 1.M: HYC group 5µg/ml at 72 hours; Figure 1.N: Lop/r group 6000 µM Lop+1400 µM r at 72 hours; Figure 1.O: FAV group 50 µg/ml at 72 hours; Figure 1.P: OSM group 0.05 µg/ml at 72 hours; Figure 1.R: VPA group 43 µM at 72 hours.

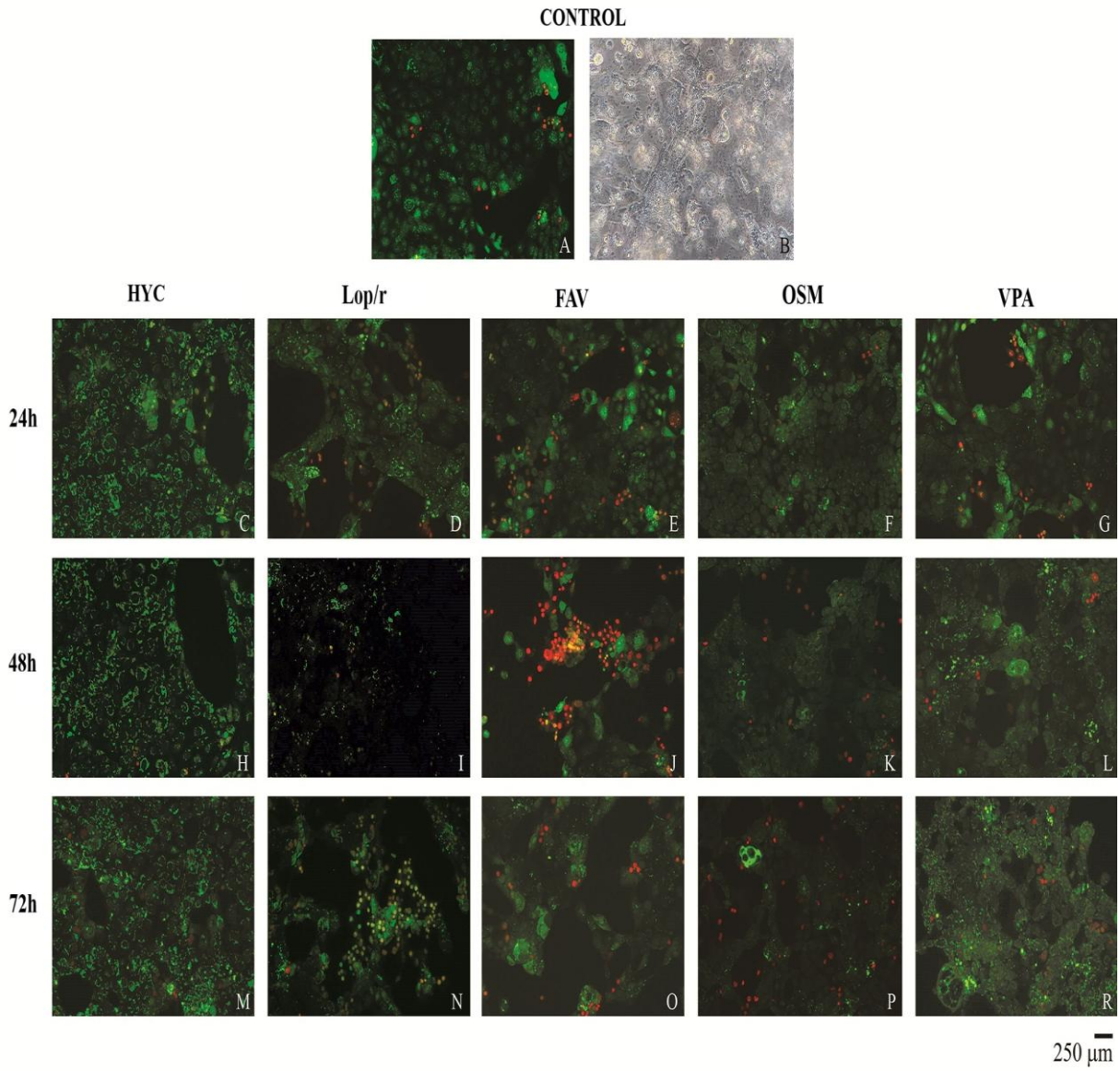

Figure 1. The microphotographs of the AO/PI staining of 0h, 24h, 48h 72h HYC (hydroxychloroquine) 5µg/ml; FAV (favipiravir) 50µg/ml; OSM (oseltamivir) 0.05µg/ml, Lop/r (Lopinavir/ritonavir) 6000 µM Lop+1400µM r; VPA (valproic acid) 43µM application. Dead cells are stained red and live proliferating cells are stained green.

3.2. HDAC activity

The time-dependent effects of drugs on HDAC activity were determined (Table 3). In our study, HDAC activity was evaluated by considering the values of the VPA group, which constituted the technical negative group. In addition, the OD value of the control group without any application was accepted as 100% and the percentage of HDAC activity of other groups was determined. HDAC activity of drug application groups increased by 34.40% in HYC group in 24 hours, 54.77% in 48 hours and 38.83% in 72 hours; 10.87% in FAV group in 24 hours, 77.75% in 48 hours and 83.63% in 72 hours; 7.28% in OSM group in 24 hours, 13.28% in 48 hours and 28.50% in 72 hours; 1.79% increase in Lop/r group in 24 hours, 2.51.91% in 72 hours and 20.03% decrease in 48 hours; In VPA group, a 40.85% decrease was seen in 24 hours, a 37.84% decrease in 48 hours, and a 36.25% increase in 72 hours; all were compared to the control group.

Table 3. HDAC activity (%) of experimental groups

Group No	Application	Application Period	% HDAC Activity
1		24 h	137.40
2	HYC	48 h	154.77
3		72 h	64.17
4		24 h	110.84
5	FAV	48 h	177.75
6		72 h	183.63
7		24 h	107.25
8	OSM	48 h	113.28
9		72 h	128.50
10		24 h	101.79
11	Lop/r	48 h	79.97
12		72 h	102.51
13		24 h	59.15
14	VPA	48 h	62.16
15		72 h	136.25
16		24 h	100
17	Control (No application)	48 h	100
18		72 h	100

Statistical evaluation of HDAC activity between each application group was performed using the ANOVA test. A significant difference was observed between VPA (p=1.89x10^{-12}), OSM (p=0.0014), HYC (p=4.14x10^{-7}), Lop/r (p=3.3x10^{-7}), FAV (p=2.13x10^{-8}) groups. The HDAC activities of different groups at the same application times were statistically evaluated using a paired t-test. There was a statistically significant difference among all groups at 24h (p<0.05) and 48h (p<0.05). However, while OSM group at 72h was not statistically significant compared to the other groups (p=0.384); there was a significant difference between HYC, Lop/r, FAV groups (p<0.05).

3.3. ELISA results

IL-6 expression levels; at 24 h, HYC group approximately 159% and FAV group reached 158% showed a significant increase, VPA control group showed an increase of 223%, OSM group reached 162% and Lop/r group reached 166%. Considering the 24h data, it was shown that there would be an inflammatory response for the drug groups. At 48h, FAV group 205% OSM group 227% increases, Lop/r group reached a peak increase of 451%, indicating a strong inflammatory response in these groups. However, the VPA group showed a lower increase at this point, reaching approximately 127%. At the 72 hours mark, the HYC, FAV, and OSM groups showed varying responses, with HYC group reaching approximately 265%, FAV group reaching 477%, and OSM group 216%. Lop/r group showed persistently high IL-6 levels, while VPA group decreased to approximately 69%, indicating a return to baseline IL-6 levels.

TNF-α expression levels; according to the results of 24h application, HYC group showed a slight decrease to approximately 82%, while FAV group decreased to 42%. However, the OSM group showed an increase to approximately 139%, Lop/r group showed a decrease to 55%, while VPA group reached a peak at 372%, indicating a significant increase in TNF-α levels of the control at this time. 48h, HYC group and FAV group 92% and 43%, respectively, while OSM group increased to 206%. Lop/r group showed a decrease to approximately 46%, and the TNF-α level of VPA group decreased to approximately 255%. At 72h, HYC group and FAV group experienced decreases in TNF-α levels to approximately 61% and 53%. OSM group at 135% and Lop/r group showed 74%. The VPA group showed a decrease to approximately 140%, indicating a return to baseline TNF-α levels. These observations suggest that as TNF-α levels varied between treatments, VPA group consistently showed high TNF-α responses and served as a reliable control for the baseline inflammatory response.

IL-10 expression levels were at 24 hours in HYC group 92%, FAV group 77%, OSM group 115%, Lop/r group 98%, VPA group 125%; at 48 hours in HYC group 88%, FAV group 71%, OSM group 133%, Lop/r group 126%, VPA group 131%; at 72 hours in HYC group 114%, FAV group 108%, OSM group 101%, Lop/r group 131%, VPA control group 143%. IL-10 levels were slightly lower than

the VPA group but remained relatively constant throughout the experiment. The results showed minimal fluctuations in IL-10 levels, suggesting that the HYC group did not significantly affect IL-10 production compared to the VPA control. Statistically, by applying one-way ANOVA test, a significant difference was found between the expression levels of IL-6, IL-10, TNF-α in the Lop/r and OSM application groups ($p<0.05$). This significant difference was determined to be due to the negative correlation between the expression levels of IL-6 and IL-10 in the two groups.

4. Discussion and Conclusion

Many existing antiviral drugs used to treat SARS, MERS, HIV/AIDS, and malaria with the principle of drug repositioning for the management of the COVID-19 pandemic, such as Lop/r (Cao et al., 2020), chloroquine (Wang et al., 2020), HYC (Gautret et al., 2020), and FAV (Cai et al., 2020), were used for potential COVID-19 treatments (Li & De Clerq, 2020; Sanders et al., 2020).

There are no studies in the literature about the epigenetic changes caused by Lop/r, HYC, FAV, OSM pharmaceuticals used worldwide in the treatment of COVID-19 through HDAC activity in intestinal enterocyte cells. This study determined the changes in HDAC activity caused by the application of Lop/r, HYC, FAV, OSM drugs at certain time intervals in Caco-2 cells at the usage doses determined in previous studies. Morphological evaluations, AO/PI staining and MTT analysis results were consistent and showed that the application groups did not cause cytotoxicity. In the presence of the drugs, Caco-2 cells maintained their viability and continued to proliferate. In addition, this study aimed to determine the relationship between cellular responses in cells resulting from the applications and HDAC activation. HDAC activity controls many biological events such as DNA packaging, replication, repair, and gene expression (Zhao & Shilatifard, 2019). Compounds that can inhibit HDAC enzymes (HDAC inhibitors) have been revealed to be effective in suppressing cell division, inducing apoptosis, differentiation, and other biological activities. The effect of these compounds in the treatment of diseases is one of the important areas of pharmacology. Loss of acetylation condenses the chromatin structure and suppresses gene expression. Studies have revealed a direct relationship between DNA methylation and histone modifications (Hu et al., 2020).

Various studies have been conducted on the effects of COVID-19 on cancer patients (Al-Quteimat & Amer, 2020). According to these, cancer was found to be associated with a 2.84-fold increased risk of severe disease and a 2.60-fold increased risk of death in COVID-19 patients. Colorectal cancer ranks second in cancer deaths and is the third most common malignancy in the world. A subgroup analysis based on place of origin showed that Chinese colorectal patients were at significant risk of infection and the burden of COVID-19 infection was 45.1% (Song et al., 2023). This study allowed us to evaluate the epigenetic changes caused by the drugs used in the treatment of COVID-19 (Lop/r, HYC, FAV, OSM) via HDAC activity in the colorectal adenocarcinoma cell line Caco-2, which constitutes the colorectal cancer model. It is well documented in the literature that HDAC activation is high in cancer patients and the importance of using HDAC inhibitors to treat cancer (Roostaee et al., 2015).

Histone deacetylase inhibitors (HDACi) signify a new class of cancer therapies with different effects, such as apoptosis or autophagic cell death. These agents selectively inhibit the activity of HDACs and thereby disrupt the balance of acetylation of histones and other proteins, modifying gene transcription. HDACi can reverse epigenetic abnormalities associated with carcinogenesis and thereby restore intrinsic antitumor responses, such as cell cycle arrest and in vitro cell differentiation. This study shows that HDACi inhibits gene expression in Caco-2 cells.

In our study, the cytotoxic effects of Lop/r, HYC, FAV, OSM pharmaceuticals on Caco-2 cell culture, their effects on HDAC activity, and the changes in proinflammatory IL-6, TNF-α, and anti-inflammatory IL-10 expression caused by this effect were determined. The data we obtained at the end of our study are consistent with the literature, and the application of Lop/r, HYC, FAV and OSM drugs in individuals with colorectal cancer increased HDAC activation. It has been shown that HDACi (VPA) used as the control group decreased HDAC activation in groups without application. A significant difference was observed between the VPA ($p=1.89x10-12$), OSM ($p=0.0014$), HYC ($p=4.14x10^{-7}$), Lop/r ($p=3.3x10^{-7}$) and FAV ($p=2.13x10^{-8)}$ groups. A statistically significant difference was found between all groups at 24 hours ($p<0.05$) and 48 hours ($p<0.05$). In the current study, a specific HDAC family member was not targeted. Instead, we evaluated total HDAC activity and found that the application of the HDAC inhibitor VPA group suppressed HDAC activity in these cells, while all other

drug applications changed HDAC activity in the cultures. As a result, it was revealed that the increase in HDAC activation was applied to Caco-2 cells may have caused some epigenetic changes.

The effectiveness of the application groups on inflammation; in the HYC group, an increase in IL-6 expression was also observed in the 24–48-hour applications. In the FAV group, it was determined that the increase in HDAC activation depending on the application hours was related to the increase in IL-6 levels. In the OSM group, it was statistically determined that the increase in HDAC activation at the 24 and 48 hours caused a significant difference between the expression levels of IL-6, IL-10 and TNF-α ($p<0.05$). In the Lop/r group, difference was found between the expressions of IL-6 and IL-10. Therefore, it was shown that the increase in HDAC enzyme activity in the Lop/r and OSM groups was effective on the inflammatory response. In all groups, the change in IL-10 expression did not affect cell viability and continuous proliferation was achieved. While TNF-α levels showed variable values among all applications, high TNF-α responses were obtained in the VPA group. This suggests that the VPA group serves as a reliable control for the baseline inflammatory response.

This is the first study to provide evidence to determine the effects of HYC, FAV, OSM, Lop/r groups on Caco-2 cell viability, HDAC activity, and inflammatory response. The effects of changes in HDAC enzyme levels on inflammation have been revealed. Thus, a new perspective has been gained on the effectiveness of HYC, FAV, OSM, Lop/r drugs on Caco-2 cells.

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