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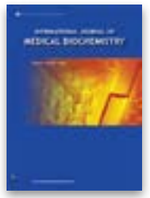

Research Article

Differential *in vitro* effects of oncogenic pathway inhibitors on carbonic anhydrase-IX, xanthine oxidase, and catalase in colorectal cancer

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Abstract

Objectives: PI3K/Akt/mTOR (The phosphatidylinositol-3-kinase/Protein Kinase B/Mammalian Target of Rapamycin) and Mitogene Activated Protein Kinase oncogenic signaling pathways play a role in the colon cancer development process by over-activating during carcinogenesis and proliferate cancer cells and stimulate metastasis. Carbonic Anhydrase-IX (CA-IX), one of the most important CA enzymes, is a tumor-associated enzyme and a transmembrane protein which reduce pH in the tumor microenvironment, leading to increased acidification. CA also increases the production of reactive oxygene species (ROS) and enables the activation of cancer signaling pathways. Due to the increase in ROS production, the antioxidant system is also activated. In this study, we aimed to investigate the effects of oncogenic pathway inhibitors on CA-IX and oxidant/antioxidant enzymes in colorectal cancer (CRC).

Methods: In this study, Acetazolamide (AZA)-CA inhibitor, Rapamycin (RAPA)-mTOR inhibitor, and Vemurafenib (VMF)-B-Raf inhibitor were applied to CRC cell line HT-29. The effects of AZA, RAPA, and VMF on oxidant/antioxidant system enzymes Xanthine oxidase (XO) and Catalase (CAT) activities were measured at 340 nm in Epoch™ Microplate Spectrophotometer. In addition, inhibition of CA-IX by AZA, RAPA, and VMF was determined using the Human CA IX ELISA Kit.

Results: According to the results of the CA-IX ELISA test, the CA-IX protein levels were 6.6 ng/µl and 4.6 ng/µl ($p<0.05$) in the HT-29 cell lines untreated and AZA-treated applied, respectively. CA-IX protein levels were 6.25 ng/µl and 5.92 ng/µl ($p\leq0.05$) in untreated and RAPA-treated HT-29 cell lines, respectively. CA-IX protein levels were found to be 3.04 ng/µl and 2.47 ng/µl ($p\leq0.05$) in untreated and VMF-treated HT-29 cell lines, respectively. AZA, RAPA, and VMF inhibitors applied to the HT-29 cell line caused a decrease in CAT activity ($p<0.001$) while an increase in XO activity ($p\leq0.001$).

Conclusion: This study will suggest a different perspective on colon cancer treatment. When our results are considered, an attitude can be taken to determine the treatment plan by taking into account the decreased antioxidant and CA-IX enzyme levels after the use of oncogenic inhibitors. Analyzing other antioxidant enzymes in the same way will add a different dimension to the study. In addition, the combined use of these inhibitors and the determination of the effects of different cancer pathway inhibitors on these enzymes will form the basis of our future studies.

Keywords: Antioxidant, CA-IX, colorectal cancer, MAPK, mTOR, oxidant, reactive oxygen species

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Colorectal cancer (CRC) is one of the most extensive cancers both in women and men, the incidence of CRC especially has been increasing over the last quarter-century [1]. There are some advanced treatment strategies for CRC such as endoscopic, oncologic, and surgical. Despite all treatments in CRC, patient survival remains low [2]. Thus, molecular and biological approaches for CRC become even more important. Oncogenic pathways have crucial roles in proliferation, angiogenesis, apoptosis and differentiation in CRC development. The most attractive signaling pathways in CRC development process are PI3K/Akt/mTOR and Mitogene Activated Protein Kinase (MAPK) pathways [3].

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PI3K/Akt/mTOR (The phosphatidylinositol-3-kinase/Protein Kinase B/Mammalian Target of Rapamycin) is a signal transduction pathway which is involved in cell proliferation, survival, and differentiation [4]. Multiple genetic changes in the PI3K/Akt/mTOR pathway play a role in the activation of CRC. mTOR, one of the most important proteins in PI3K/Akt/mTOR pathway, shown to be higly expressed in CRC cells [5]. MAPK pathway is stimulated by growth factors, oxidative stress, hormones, cytokines, and plays a prominent role in CRC development [6]. B-Raf (v-Raf Murine Sarcoma Viral Oncogene Homolog B1) protein which is encoded by BRAF gene acts as a controller in MAPK pathway. The missense mutation in the gene (BRAFV600E) leads to metastatic CRCs that generally show an insufficient response to treatments [7].

(CA, EC 4.2.1.1) catalyzes hydration of carbondioxide, generating bicarbonate ions and protons, involving in several metabolic process such as lipogenesis, gluconeogenesis, and ureagenesis [8]. Carbonic Anhydrase-IX (CA-IX), one of the tumor associated CA enzymes, is a transmembrane protein with five domains and consists of an intracytosolic tail (IC), transmembrane domain (TM), extracellular catalytic domain (CA), proteoglycan domain, and signal peptide [9]. It is suggested that after being phosphorylated, IC residues stimulate intracellular oncogenic signaling pathways, which significantly contribute to the carcinogenesis process [10, 11]. CA-IX strongly expressed in many cancers and has a potential role as a prognostic indicator, diagnostic marker/tumor therapeutic target [12]. Especially, CRC express the CA-IX isoform at high levels [13].

Antioxidants are the components that support the cells to maintain their integrity, reduce the oxidation capacity of oxidants, and inhibit their activities in the cell. They protect the cell against the damages caused by reactive oxygen species (ROS) [14, 15]. Xanthine Oxidase (XO) is a precursor enzyme to produce oxidants and is one of the most important cellular source of superoxide radicals. It catalyzes the reaction of hypoxanthine to xanthine and xanthine to uric acid. During this reaction, molecular oxygen is reduced, superoxide anion is formed, and hydrogen peroxide (H_2O_2) is produced [16, 17]. Catalase (CAT) acts as an antioxidant in counteract system to the damaging effects of ROS and it converts H_2O_2 to oxygene and water (O_2/H_2O); therefore, it eliminates high H_2O_2 concentrations. Thus, changes in the activity of CAT cause an alteration in the ROS balance of the metabolism, which lead to the stimulation of antitumorigenic signals and carcinogenesis [18].

On the contrary of normal cells, ROS production is increased in cancer cells. The changes in the levels of ROS play an important role in the process of tumorigenesis. In addition, extra high levels of ROS can also be harmful to cancer cells which tend to increase their antioxidant capacity to counteract the oxidative stress state [19]. There is a balance between ROS and antioxidants in metabolism. When the balance is disturbed, the formation of CRC is triggered due to the accumulation of ROS [20]. CRC development is promoted by oxygen radicals because ROS stimulate carcinogenic factors and cause metastatis in CRC [21, 22].

Metabolic antioxidant balance is also impaired in CRC in which the oncogenic signaling pathways play a prominent role. Tumor-associated enzyme CA-IX leads to decreased pH in the tumor microenvironment, leading to increased acidification. pH changes in cancer cells increase the production of ROS and enable the activation of cancer signaling pathways. In this study, we aimed to examine the effects of inhibitors of oncogenic signaling pathways on CA-IX enzyme, which is a remarkable marker in cancer, and oxidant/antioxidant enzyme levels.

Materials and Methods

Cell culture and reagents

Human CRC cells HT29 were obtained from ATCC (HTB-38™) and cultured in DMEM High Glucose Medium (Biowest, L0102) supplemented with 10% heat-inactivated FBS (Capricorn, FBS-HI-11A) at 37°C in a 5% CO_2 humidified atmosphere. AZA, Rapamycine (RAPA), and Vemurafenib (VMF) were purchased from Sigma-Aldrich, Cayman Chemical, and SelleckChem, respectively. All inhibitors were prepared as stock solutions by dissolving in DMSO at a concentration of 300 µM. Then, HT29 cells were plated into 6-well plate (0.3×10^6 cells/well) and treated with different concentrations of AZA, RAPA, and VMF (0, 2.5, 5, 10, 25, 50, 100, and 200) for 24 h and 48 h.

Cell viability assay (WST-1) and IC_{50} measurement

RAPA and VMF, which are the best known and approved inhibitors of PI3K/Akt/mTOR and MAPK pathway, were used as oncogenic pathway inhibitors in the study. Acetazolamide (AZA) was used as a control to confirm the inhibition of CA-IX. WST-1 assay was performed to determine the appropriate dose of AZA, RAPA and VMF on the HT-29 cells. First, HT29 cells were seeded into 96-well plates following incubation, AZA, RAPA, and VMF were prepared at concentrations of 0, 2.5, 5, 10, 25, 50, 100, and 200 µM and applied to the cells, respectively. The measurements were performed at 24th h and 48th h. Then, 10 µL of WST-1 solution (Cayman Chemical, 10008883) was added to each well and incubated at 37°C for 2–4 h [23]. To eliminate the effects of DMSO, it was added to the negative control wells at the rate that AZA, RAPA, and VMF were dissolved with the medium. After incubation, the appropriate inhibitor concentration for the cells was determined by measuring at 450 nm on an Epoch Microplate Reader (Winooski, VT, USA). The inhibitor doses (IC_{50}), which inhibited 50% of the cells, were calculated with Graphpad Prism 9.1.0.

Cell lysate preparation

To prepare the cell lysate, HT-29 colorectal cells were washed with phosphate buffered saline (PBS) and then the cells were lysed with lysis buffer containing 50 mM Tris, 150 µM NaCl, 1% NP-40 (Intron Biotechnology, IBS-BN015) and proteinase inhibitor factor (Intron Biotechnology, PIC001). After lysis, the cells were centrifuged (+4°C, 17000 g, 15 min). Then, the supernatant washed twice with PBS and was aliquoted.

Measurement of enzyme activities

XO and CAT activity

XO and CAT activity was measured spectrophotometrically by Epoch Microplate Reader. XO activity was assayed essentially according to the method described by Roussos [24]. Change in absorbance was recorded at 290 nm at 15 s interval for 1 min. Appropriate control was run simultaneously. Roussos has defined 1 U of activity as a change in absorbance at 290 nm in 1 min using 1 mL enzyme preparation. CAT activity was measured spectrophotometrically using the UV assay method. CAT-catalyzed decomposition of H_2O_2 was monitored for 5 min by measuring the decrease in absorbance at 240 nm, at 25°C. The results were expressed as units per liter (U/L) [24, 25].

CA-IX ELISA test

CA-IX ELISA test was used to determine the change in CA-IX levels of AZA in HT-29 cells. BT Lab Human CA IX ELISA Kit (E2273Ha) was employed. In the first step, standard solutions were prepared for the protocol. 40 µL of sample was added to the wells, then 10µL of biotinylated anti-CA-IX antibody was added. The next step was to add 50 µL of Streptavidin-HRP to the wells and an incubation at 37°C for 60 min. Following the incubation, the wells were washed 5 times with wash buffer and 50 µL of substrate solution A and 50 µL of substrate solution B were added to each well. It was incubated at 37°C for 10 min in the dark. Finally, 50 µL of stop solution was added to each well and then read at 450 nm on the Epoch Microplate Reader.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.1.0. All experiments were done in triplicate. One-way ANOVA test and Student's t-test were performed XO/CAT enzyme activity analyzes and CA-IX ELISA test, respectively. Statistical significance level was accepted as p≤0.05.

Results

Cell viability assay (WST-1)

AZA, RAPA, and VMF were applied to HT-29 cells at doses of 0, 2.5, 5, 10, 25, 50, 100, and 200 µM at the 24th and 48th h and the cytotoxic doses for the cells were determined. According to the WST-1 results, the viability percentages at 0, 2.5, 5, 10, 25, 50, 100, and 200 µM concentrations for HT-29 cells treated with AZA for 24 h were 100%, 100%, 94%, 87%, 50%, 35%, 30%, and 17%, respectively. The IC_{50} value for AZA is found to be 34.71 µM. Furthermore, viability percentages for HT-29 cells treated with RAPA for 24 h at concentrations of 0, 2.5, 5, 10, 25, 50, 100, and 200 µM were 100%, 99%, 92%, 73%, 39%, 39%, 28, and 20%, respectively. The IC_{50} value for RAPA is found to be 46.97 µM. Moreover, % viability percentages for HT-29 cells treated with VMF for 24 h at concentrations of 0, 2.5, 5, 10, 25, 50, 100, and 200 µM were 100%, 100%, 100%, 100%, 48%, 35%, 27%, and 18%, respectively. The IC_{50} value for VMF is found to be 35.84 µM (Fig. 1).

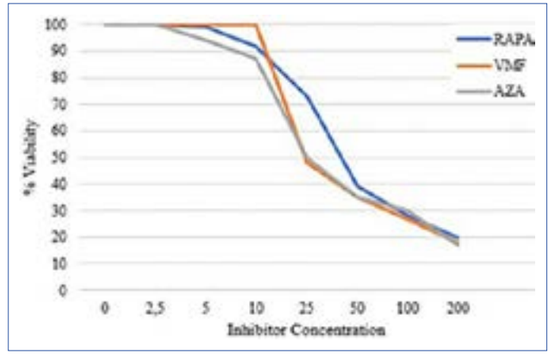

Figure 1. % viability for HT-29 cells treated with Acetazolamide (AZA), Rapamycin (RAPA) and Vemurafenib (VMF) for 24 hours at concentrations of 0, 2.5, 5, 10, 25, 50, 100 and 200 µM.

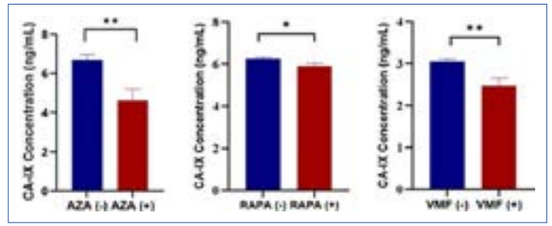

Figure 2. CA-IX concentration levels in AZA, RAPA and VMF treated and untreated HT-29 cells.

P=0.0055; p=0.0110; p=0.0070 for AZA, RAPA and VMF respectively. CA-IX: Carbonic anhydrase IX; AZA: Acetazclamide; RAPA: Rapamycin; VMF: Vemurafenib.

CA-IX ELISA

ELISA test was performed to determine the effects of AZA, RAPA, and VMF treatments on CA-IX enzyme in HT-29 cells. The changes in CA-IX levels were determined by measuring the absorbance values in cells with and without AZA, RAPA, and VMF treatment. As a result, the concentration of CA-IX was found to be 6.66 ng/mL in HT-29 cells without AZA, and 4.63 ng/mL in HT-29 cells with AZA. A dramatic decrease was detected in the CA-IX levels in HT-29 cells treated with AZA (p≤0.05). The concentration of CA-IX was found to be 6.25 ng/mL in HT-29 cells without RAPA and 5.92 ng/mL in HT-29 cells with RAPA. CA-IX levels were significantly decreased in HT-29 cells treated with RAPA (p≤0.05). In addition, the concentration of CA-IX was found to be 3.04 ng/mL in HT-29 cells without VMF and 2.47 ng/mL in HT-29 cells with VMF. CA-IX levels decreased significantly in HT-29 cells treated with VMF (p≤0.05) (Fig. 2).

Measurement of enzyme activities

To determine the effects of AZA, RAPA and VMF on XO and CAT in HT-29 cells, enzyme activities were measured spectrophotometrically. XO enzyme levels were increased significantly in HT-29 cells treated with AZA, RAPA, and VMF compared to the non-treated group (p≤0.05) (Fig. 3). CAT enzyme levels were

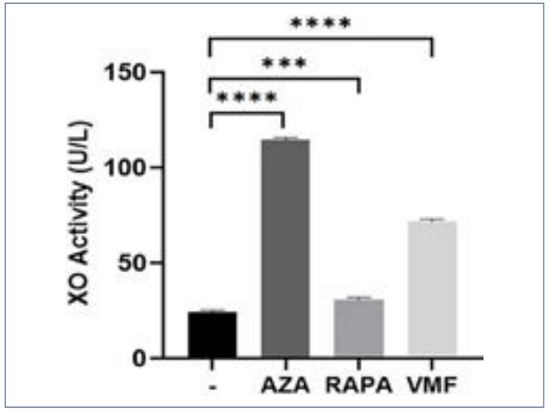

Figure 3. Xanthine oxidase (XO) activities in HT-29 cells treated with Acetazolamide (AZA), Rapamycin (RAPA), Vemurafenib (VMF) and untreated group.

***: p=0.0002; ****: p≤0.0001.

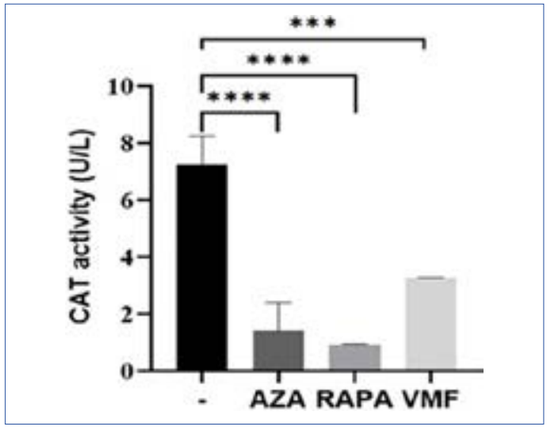

Figure 4. Catalase (CAT) activities in HT-29 cells treated with Acetazolamide (AZA), Rapamycin (RAPA), Vemurafenib (VMF) and untreated group.

***: p=0.0006; ****: p≤0.0001.

decreased significantly in HT-29 cells treated with AZA, RAPA, and VMF compared to the non-treated group (p≤0.05) (Fig. 4).

Discussion

When all cancer types considered, CRC has been reported to have the highest rate of morbidity and mortality. Chemotherapy and surgery are the primary treatment options for CRC patients. However, metastatic CRC has an unpredictable prognosis, which brings up the targeted therapy approach [26]. Oncogenic pathways, mTOR and MAPK, are remarkable mediators that cause deregulation of protein synthesis and lead to CRC which make these pathways as good candidates for targetted therapies [27]. mTOR signaling is associated with the clinical pathological parameters of human CRC. siRNA-mediated gene silencing of mTOR may be a novel therapeutic strategy for CRC. Zhang et al. [28] stated that mTOR signaling is associated with the pathology of CRC and silencing of this pathway may be a new therapeutic target for CRC. In addition, Fang and Richardson stated that the activation of the MAPK signaling pathway, which is another important signaling pathway in cancer, is widely detected in CRC and overactivation of this pathway plays an important role in CRC progression. They stated that this signaling pathway could be a molecular target for the treatment of CRC [29].

Studies have shown that single or combined use of mTOR and MAPK inhibitors (MEK1/2 inhibitor and PI3K inhibitor), which are frequently expressed in CRC, may be important tools for targeted therapy [30]. RAPA is a macrolide antibiotic known as the first inhibitor of mTOR. The antitumor effects of RAPA were first found in 2002. In addition to being a signaling pathway inhibitor, it has been reported to suppress tumor growth by inhibiting angiogenesis [5]. Studies have shown that RAPA regresses the development of CRC [31, 32]. VMF is an inhibitor used for the treatment of advanced melanoma that inhibits the increase in MAPK signaling pathway by inhibiting the BRAF V600E mutation [33]. However, Yang et al. [34] reported that VMF showed a dose-dependent inhibition effect in CRC, stopped cell proliferation in cell lines expressing BRAFV600, and inhibited tumor growth in xenograft models carrying BRAFV600E mutation. In addition, they stated that the use of VMF in combination with other MAPK pathway inhibitors resulted in increased antitumor activity and improved survival in xenograft models. Although the effects of RAPA and VMF on the development of CRC are known in the literature, their effects on CA-IX, which is an important enzyme and a transmembrane protein affecting the tumor microenvironment, as well as its effects on the antioxidant/oxidant mechanism in CRC, have not been investigated. The aim of this study was to investigate the effects of RAPA and VMF inhibitors on CA-IX and oxidant/antioxidant enzymes in CRC. In our study, the CA inhibitor AZA, the mTOR inhibitor RAPA, and the B-Raf inhibitor VMF were applied to the HT-29 colorectal cell line and the appropriate inhibitor doses were found to be 34.71, 46.97 and 35.84, respectively.

CA-IX which is highly expressed in CRC is associated with cancer signaling pathways because phosphorylation of IC tails stimulates these pathways. In our study, we investigated the effects of cancer signaling pathway inhibitors RAPA and VMF on CA-IX enzyme levels in CRC. The enzyme levels of CA-IX were decreased significantly after AZA, RAPA and VMF treatment. Tülüce et al. [35], showed that HT-29 cancer cells have more cytotoxic and apoptotic activity than normal cells through the production of ROS after the use of CA-IX inhibitor. CA-IX inhibitor was reported to be more effective in HT-29 cell line compared to HEK293 cell line. Huang et al. [36], reported the effects of CA-IX gene polymorphisms on the progression of colorectal malignancies. Kopacek et al. [37], stated that inhibition of the MAPK pathway in HeLa cells with U0126 caused

a decrease in the activity of the CA-IX promoter structure and a decrease in CA-IX protein levels. In addition, they indicated that the use of U0126 together with LY 294002, an inhibitor of the PI3K/Akt/mTOR pathway, caused a stronger inhibition.

Alternating ROS level may cause changes in biological activity through abnormal stimulation or repression of certain signaling pathways [38]. Increased production of ROS inside and outside the cell during oxidative stress may mediate the process of CRC by changing the activity of transcriptional factors. Oxidative stress causes cell damage and is involved in CRC through antioxidants by directly or indirectly affecting cell signaling pathways [39]. Due to excessive accumulation of ROS in CRC, the balance between ROS and antioxidants changes [20]. Crespo-Sanjuán et al. [40] stated that the oxidation process in CRC occurs at the polyp stages. Fruehauf et al. [41], indicated that ROS-induced DNA damage is a critical cause of CRC. Therefore, it is possible that the development of CRC occurs in a ROS-dependent way. We observed an increase in the XO enzyme, levels after the application of AZA, RAPA, and VMF to the HT-29 cell line and on the contrary, a decrease in the enzyme levels of CAT. Glorieux et al. [42] reported that CAT expression in MCF-7 breast cancer cells is dependent on the PI3K/Akt/mTOR signaling pathway. Although there are studies in the literature that decreased catalase activity activates the MAPK signaling pathway, there are no previous studies that indicates the effects of MAPK signaling pathway inhibitors on CAT [43]. Studies have shown that mTOR is necessary for XO activation. It was also stated that the p38 MAPK pathway is critical for XO activation in human myeloid leukemia cells [44, 45]. In addition, the effect of AZA, a CA inhibitor, on CAT and XO enzyme levels was also shown in our study. There are no previous studies on this topic in the literature. We observed that AZA increased the oxidant enzyme levels while decreasing the antioxidant enzyme levels in HT29 CRC cells.

Our shows that when inhibitors of oncogenic signaling pathways were used, CA-IX and antioxidant enzyme levels were decreased but oxidant enzyme levels were increased. This situation reveals the relationship between CA-IX and antioxidant enzyme levels. This preliminary study gives a novel perspective for designing new strategies on tumor associated enzymes and antioxidant/oxidant mechanisms. Moreover, the other antioxidant/oxidant enzymes also must be analyzed with further studies.

Conflict of Interest: The authors declare that there is no conflict of interest.

Ethics Committee Approval: Our research does not require ethics committee approval, as cell culture studies were conducted. This article does not include any studies with human participants or animals by any of the authors.

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Authorship Contributions: Concept – O.O.G.; B.E.O.B., E.T.; Design – B.E.O.B., E.T.; Supervision – O.O.G.; Funding – O.O.G.; Materials – O.O.G.; Data collection &/or processing – B.E.O.B., E.T.; Analysis and/or interpretation – O.O.G., B.E.O.B., E.T.; Literature search – B.E.O.B., E.T.; Writing – O.O.G., B.E.O.B., E.T.; Critical review – O.O.G.

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