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RESEARCH ARTICLE

THE CYTOTOXIC EFFECT OF BENZOIC ACID ON TEN DIFFERENT CANCER CELL LINES

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ABSTRACT

Cancer, having numerous types, is among the most dangerous and complex chronic diseases in the world affecting the wellbeing of humans, society and economy. The exploration and reassessment of effective chemicals, compounds, and natural products as potential agents for alleviating the adverse effects of cancer and its related symptoms continue on a global scale. This process involves an initial evaluation of the cytotoxic activities of potential drug candidates or treatment regimens on diverse cancer cell types in an ex vivo context. Benzoic acid (BA), an aromatic carboxylic acid that is widely available and used in the food industry, is one of the phenolic acids that may bear considerable anti-cancer potential. It is useful to find out the comparable effect of BA on various cancer types. Therefore, in this study, we tested the cytotoxicity of BA using MTT assay, on a number of ten different cancer cell lines and one normal cell type, namely prostate cancer (PC3), cervical cancer (HeLA), liver cancer (HUH7), colon cancer (CaCO2, HT29, SW48), bone cancer (MG63 and A673), pharyngeal cancer (2A3), lung cancer (CRM612) and kidney epithelial control cell line (Phoenix), respectively. IC50 (µg/ml) values after 48 and 72-hour exposure to BA were found to differ between 85.54±3.17 to 670.6±43.26, while the IC values for the control cell line Phoenix were 410.54±32.29 and 231.16±25.25, respectively. Taking into account of statistical evaluation of the IC50 values for BA on 11 cell types, we suggest that the molecular and omics approaches can be implemented in more details in order to find cellular and biochemical targets of BA as well as elucidating molecular mode of action, especially starting with the cancer cell lines of MG63, CRM612 and A673, in which the IC50 levels are relatively the lowest compared to those of the control cell line.

Keywords: Benzoic acid, Cytotoxic effect, Anticancer, MTT

1. INTRODUCTION

Cancer represents a cluster of diseases arising from genetic anomalies, including the loss of function in tumor suppressor genes, gene mutations, gene deletions in chromosomes and epigenetic changes in gene expression [1]. Transcriptional epigenetic modifications play an important role in the onset and progression of cancer pathogenesis [2]. Various factors, such as the rise in unhealthy eating habits, smoking, and aging of the population, contribute to the transformation of normal cells into cancerous ones [3].

The treatment process in cancer patients is related to the size of the tumor, the age of the patient, the presence of metastases, the susceptibility to chemotherapy, and the feasibility of surgical intervention [4]. The side effects associated with chemotherapy, radiotherapy, and commonly used surgical methods in cancer treatment, coupled with the development of chemotherapy resistance in patients and the tumor's potential to metastasize, impose limitations on treatment options [5]. Beyond these side effects, a prominent challenge in treatment lies in late diagnosis, which, along with economic costs and disease progression limitations, curtails the application of available therapeutic agents. Consequently, there is a

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pressing need to uncover novel drugs and treatment modalities that can enhance treatment success and mitigate side effects [6].

Phenolic compounds, secondary metabolites found in fruits, vegetables, grains, and legumes [7], have garnered interest due to their use in diets and minimal side effects [8]. These compounds play roles in plant defense mechanisms against ecological and physiological stress factors, synthesized in response to stressors such as pathogenic microorganisms, insects, and UV radiation [9]. Phenolic compounds are classified into various groups, including lignin, tannins, and simple phenols -primarily phenolic acids containing benzoic acid and cinnamic acid derivatives- based on the number of phenol groups in their structure and their substitution position [10-11-12]. Benzoic acid (BA), a natural compound in the hydroxybenzoic acid group, is widely available and utilized [13].

Benzoic acid is synthesized through natural and synthetic pathways. For instance, it is synthetically produced by the oxidation of toluene at high pressure and temperature [14]. Plants synthesize benzoic acid from phenylalanine or shikimate [15]. Several benzoic acid derivatives, such as ubiquinone (Coenzyme Q) and folic acid, are vital for plant metabolism. Ubiquinone is a benzoquinone involved in the mitochondria and electron transport system of some bacteria [16]. Salicylic acid is an herbal hormone found in the defense mechanism of plants. At the same time, some volatile benzoic acid derivatives found in plants are involved in pollination and protection of reproductive organs from bacterial infections [17]. Benzoic acid is naturally formed by the oxidation of benzaldehyde because of microbial degradation of hippuric acid and phenylalanine during the formation of fermented milk products [18]. *Streptomyces maritimus*, an aquatic microorganism, synthesizes benzoic acid by β-oxidation of L-phenylalanine while producing enterocin [19].

The fact that benzoic acid is a natural compound and can be produced synthetically makes it versatile to be used in many fields ranging from cosmetics to food industry. Studies have shown that benzoic acid has antibacterial, antioxidant, antifungal, and anticancer activities. One of the biggest problems encountered in livestock is gastrointestinal diseases. Intestinal flora in small cattle can be improved by using benzoic acid [20]. In another study with benzoic acid, the antibacterial effect against Enterohemorrhagic *Escherichia coli* (EHEC) bacteria, which causes hemorrhagic uremic syndrome and is transmitted from contaminated food and water, was investigated. The inclusion of benzoic acid in the diet has been shown to improve the intestinal microbiota [21]. In addition to all these activities, benzoic acid (E210) is used as a preservative in acidic or slightly acidic foods because it has antimicrobial activity [22].

In addition to the inhibitory effect observed on microbial organisms, benzoic acid is also recognized for its anticancer activity in cancer cells. The literature contains relatively limited number of studies examining the cytotoxic effect of benzoic acid specific to different cancer types. Phenolic compounds, which are an integral part of the daily human diet and are absorbed by the colon, have been studied in the context of colorectal cancer. In experiments where CaCo-2 colorectal cancer cells were exposed to benzoic acid and caffeic acid, the induction of apoptosis in cancerous cells was observed. Apoptosis induction occurred independently of mitochondrial DNA (mtDNA) [23]. This finding is promising, considering another study that established a relationship between changes in mtDNA levels in cells and resistance to anticancer drugs [24].

In some studies, aiming to determine the mechanism of the cytotoxic effect of benzoic acid, it has been suggested that benzoic acid and its derivatives may possess histone deacetyltransferase inhibitor (HDACi) activity. Acetylation of histones by the histone acetyltransferase (HAT) and deacetylation by the histone deacetyltransferase (HDAC) are the most well-known epigenetic mechanisms in the regulation of transcription [25]. An increase in the level of the HDAC enzyme may lead to the silencing of tumor regulator genes and contribute to the pathogenesis of cancer [26]. Benzoic acid, hydroxy benzoic acid, dihydroxy benzoic acid, and methylated forms of benzoic acid, as well as HDAC inhibition effects on breast (HeLA), cervical (SiHa), colon (HCT-116), and rectal (HCT-15) cancer types were

studied. Dihydroxy benzoic acid has been found to have a 50> HDAC inhibition activity [27]. In another study, it was reported that hydroxybenzoic acid inhibits the HDAC3 enzyme in breast cancer cells (MCF-7) and promotes apoptosis [28].

As a result of studies, it has been established that benzoic acid exhibits a cytotoxic effect on cancer cells. However, the literature indicates a shortage of studies focusing on different types within a cancer group (e.g., Colon cancer - CaCO2, HT29, SW48). In our conducted study, we investigated the cytotoxic effect of benzoic acid using 10 different cancer cell lines and 1 control one. The study aimed to determine the concentration of benzoic acid (IC50), which inhibits half of the population in the target cancer types. This study was conducted to unveil the anticancer effect of benzoic acid specific to various types of cancer.

2. MATERIALS and METHODS

2.1. Chemicals and Cell Lines

DMEM (Dulbecco's Modified Eagle's Medium) (Capricorn Scientific #DMEM-HA), RPMI (Roswell Park Memorial Institute) (Sigma #R8758), Trypsin/EDTA (Pan Biotech #P10-019100), Penicillin/Streptomycine (Capricorn #PS-B), FBS (Fetal Bovine Serum) (Gibco #A4766801), DMSO (Dimethyl sulphoxide) (Biofroxx #67-68-5), Trypan blue (Sigma #T8154), DPBS (Dullbecco's Phosphate Buffered Saline) (Sigma #D8537), MTT (Thiazoyl Blue Tetrazolium Bromide) (Sigma #M2128), Benzoic acid (Sigma #242381) were purchased from the indicated suppliers shown in parentheses.

The cell lines used in this study are prostate cancer (PC3), cervical cancer (HeLA), liver cancer (HUH7), colon cancer (CaCO2, HT29, SW48), bone cancer (MG63 and A673), pharyngeal cancer (2A3), lung cancer (CRM612) and kidney epithelial control cell line (Phoenix). HUH7 cancer cell line was obtained from Sigma Aldrich, CRM612 cancer cell line was obtained from Aydın Adnan Menderes University, Department of Medical Biology, and other cell lines were obtained from ATCC.

2.2. Cell Culture

Cells were grown at 37°C in a humidified environment with %5 CO_2. RPMI medium containing L-glutamine and sodium bicarbonate was used for the propagation of the PC3 cell line, and DMEM medium containing L-glutamine was used for the propagation of other cell lines. 10% FBS and %1.5 penicillin/streptomycin were added to the media. Cells were taken from flasks by trypsinization. Cells were seeded in 96-well cell culture plates at 10^5 cells per well. For the cells to adhere to the wells, they were incubated for 24 hours at 37°C in a %5 CO_2 incubator. The benzoic acid was prepared with Ethyl alcohol (EtOH) at a stock concentration of 125 mg/ml. In the experiments, 0, 23.44 µg/ml, 46.88 µg/ml, 93.75 µg/ml, 187.5 µg/ml, 375 µg/ml and 750 µg/ml benzoic acid were used. The experiment was replicated 3 times at each concentration tested.

2.3. MTT Test to Determine the Cytotoxicity of Benzoic Acid

After the cells were exposed to benzoic acid at given concentrations for 48 hours and 72 hours, the medium containing benzoic acid was removed and % 0.5 final volume of MTT solution was added to each well and incubated for 4 hours in the dark. After 4 hours, the MTT solution in the wells was removed from the wells and 100 µl of DMSO was added to each well to dissolve the formed formazan crystals. It was incubated for 15 minutes for the formation of purple color and absorbance values were measured at 570 nm in the spectrophotometer. Concentrations (IC50) at which benzoic acid inhibited each cell line by % 50 were calculated [29].

2.4. Statistical Analysis

Statistical analyses were performed using the GraphPad Prism 8.0.1 statistics program. Benzoic acid concentrations that inhibited the growth by 50% (IC50) were calculated. The MTT assay of each cell group was performed in triplicate. A t-test was performed following one-way ANOVA, and the statistically significant levels of IC50 values taken from two different time points were calculated. A p-value of less than 0.05 was considered significant. The calculations were carried out separately for each cell line for the 48- and 72-hour results.

3. RESULTS

The MTT test was conducted to assess the effect of benzoic acid on cell viability in various cancer cell lines. Cells were exposed to benzoic acid at concentrations of 0, 23.44 µg/ml, 46.88 µg/ml, 93.75 µg/ml, 187.5 µg/ml, 375 µg/ml, and 750 µg/ml for 48 and 72 hours. The Phoenix kidney epithelium-like cell line was included as a control.

As the concentration of benzoic acid increased, cell viability decreased depending on the dose and incubation time. Percentage cell viability (%) graphs were generated for each cell line separately based on increasing benzoic acid concentrations, with the no-treatment group normalized to 100. Figure 1 illustrates the cell viability graphs for HeLa, Huh7, CaCO2, and MG63 cells upon exposure to benzoic acid for 48 and 72 hours. Cell viability ratios for HT29, A673, SW48, and PC3 cells are presented in Figure 2. The effect of benzoic acid on the control cell line Phoenix- kidney epithelial-like cells, as well as the other two cancer lines, 2A3 and CRM612, is illustrated in Figure 3.

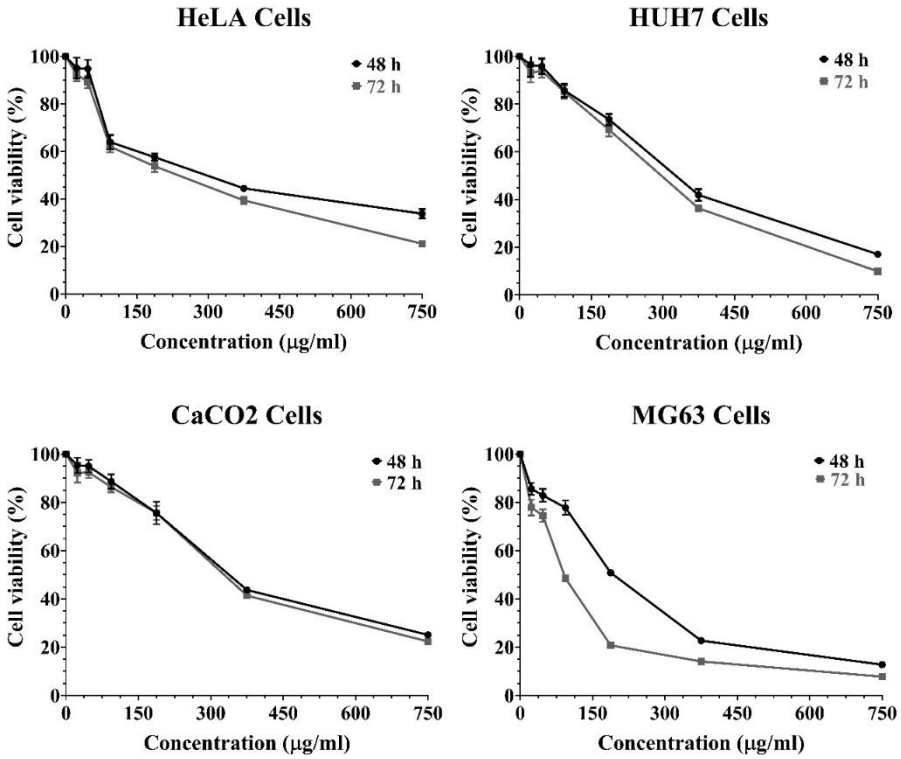

Figure 1. The cytotoxic effect of benzoic acid on HeLa, HUH7, CaCO2, and MG63 cancer cells. Percentage cell viability graphs are displayed for 48 and 72 hours of incubation. Benzoic acid concentrations are shown in µg/ml

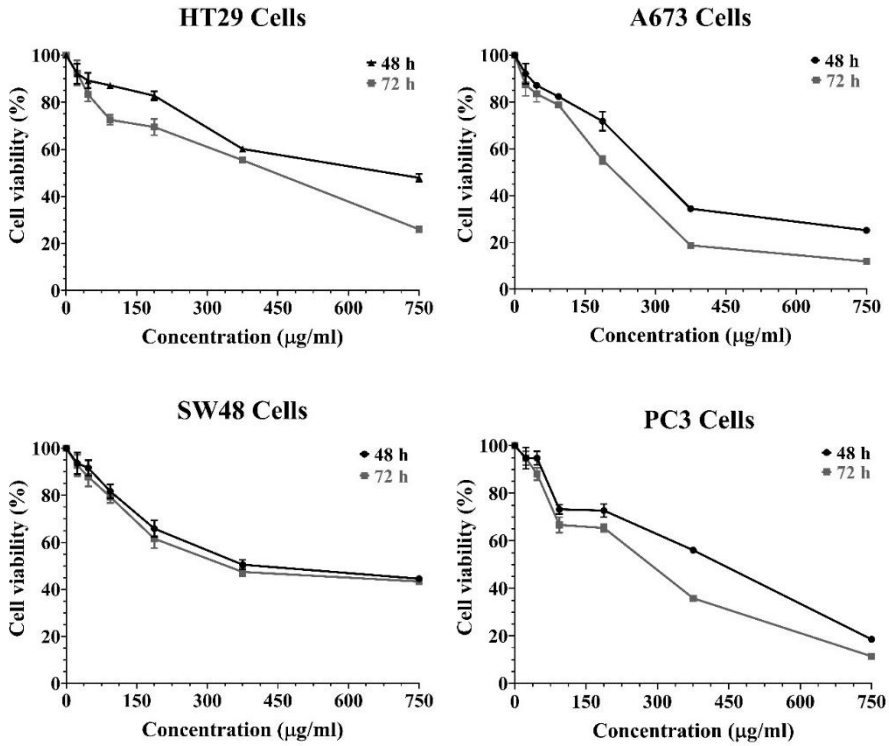

Figure 2. The cytotoxic effect of benzoic acid on HT29, A673, SW48 and PC3 cancer cells.

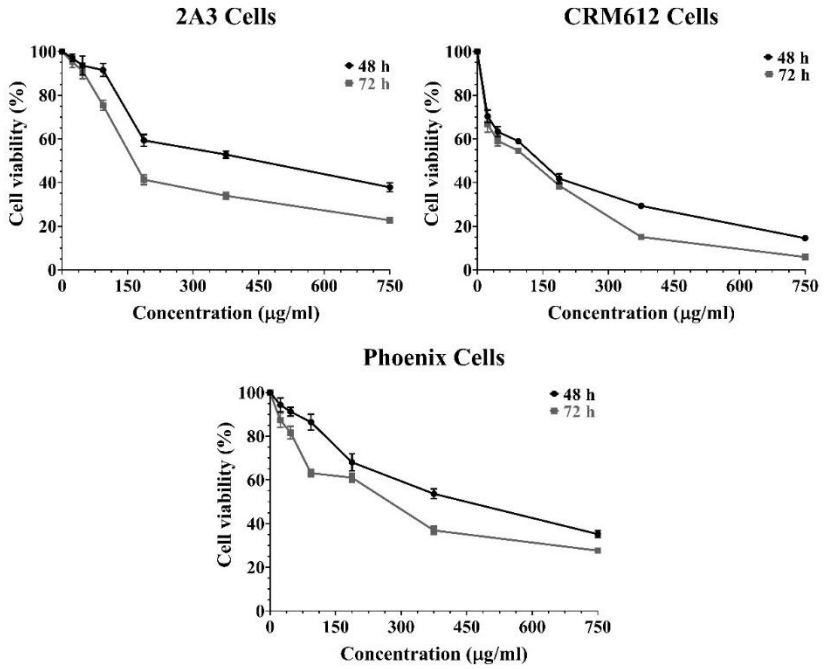

Figure 3. The cytotoxic effect of benzoic acid on 2A3, CRM612 cancer cells and Phoenix control cells.

IC50 values were calculated for each cell line from the cell viability graphs to determine the benzoic acid concentration that inhibited 50% of the cell population for both 48 and 72-hour exposure times.

These IC50 values are presented in Table 1. The range of IC50 values was observed to be from 85.54 ± 3.17 µg/ml to 670.6 ± 43.26 µg/ml, suggesting significant variation in the response of different cancer cells to BA, possibly attributable to various intrinsic tolerance mechanisms within the cells.

Statistical analysis of the findings from the 48-hour benzoic acid exposure revealed that HeLa, HUH7, MG63, A673, and CRM612 cells were significantly more affected ($p < 0.05$) (Figure 4) compared to the results obtained with the control cell line Phoenix. Subsequent statistical analyses performed after 72 hours of benzoic acid exposure revealed that MG63 and CRM612 cells were significantly more affected ($p < 0.05$) (Figure 5).

Among the cell lines affected by benzoic acid, CRM612 and MG63 stand out, particularly because these cell lines are inhibited by relatively low levels of BA compared to the control and other cell lines.

Table 1. IC50 values of the cancer cell lines used in this study and the Phoenix control cell line following exposure to benzoic acid for 48 and 72 hours

	IC50 (µg/ml)	
Cell line	**48 hour**	**72 hour**
HeLA	270.84 ± 14.22	219.61 ± 28.13
HUH7	317.83 ± 17.32	282.77 ± 7.62
CaCO2	331.07 ± 16.57	320.08 ± 12.22
MG63	195.21 ± 6.93	85.54 ± 3.17
HT29	670.6 ± 43.26	472.15 ± 10.09
A673	274.51 ± 14.11	199.31 ± 3.90
SW48	374.55 ± 33.11	321.58 ± 28.67
PC3	449.54 ± 13.12	260.84 ± 5.70
2A3	355.39 ± 16.95	193.06 ± 11.12
CRM612	108.18 ± 9.78	93.76 ± 7.49
Phoenix	410.54 ± 32.29	231.16 ± 25.25

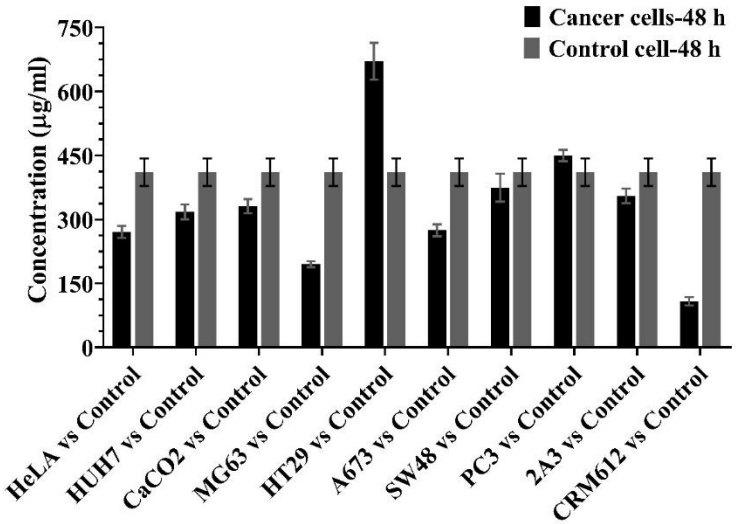

Figure 4. Comparison of IC50 values for the cancer cell lines and the control cell line following 48-hour exposure to benzoic acid

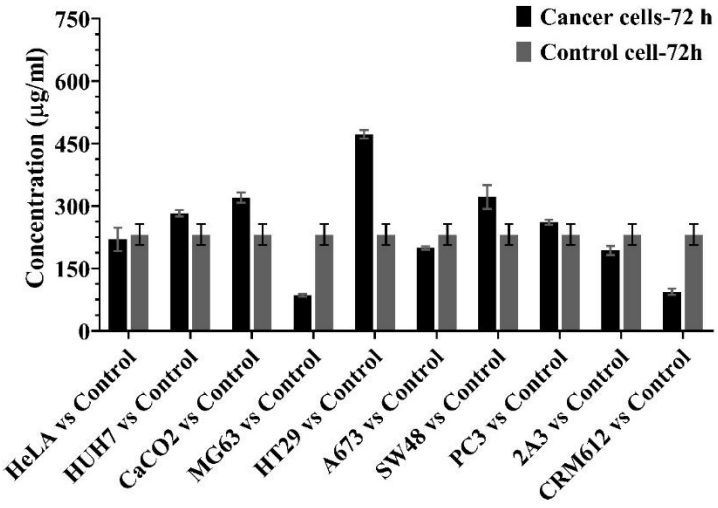

Figure 5. Comparison of IC50 values for the cancer cell lines and the control cell line following 72-hour exposure to benzoic acid

4. DISCUSSION AND CONCLUSION

Cancer is one of the leading causes of disease-related deaths globally, with its onset potentially triggered by lifestyle and dietary factors. Among the pivotal factors influencing cancer formation, along with genetic predisposition, is the escalation of Reactive Oxygen Species (ROS) within the cell due to environmental and physiological stress [30]. ROS instigates DNA damage by inducing mutations, causing structural alterations in DNA that result in aberrant cell proliferation and a decrease in apoptosis [31]. Furthermore, anomalies in post-translational epigenetic modifications in mammalian cells play a crucial role in the pathogenesis of cancer [26].

Epidemiological studies demonstrate that the consumption of foods rich in phenolic compounds is associated with a reduced risk of cancer [32]. The protective effects of these secondary metabolites may stem from their antioxidant and anti-inflammatory activities [33]. The antioxidant activity of phenolic acids is attributed to their capacity to inhibit reactive oxygen species and act as chelators of metal ions [34].

In the context of this study, we investigated the cytotoxic effects of increasing benzoic acid concentrations on 10 different cancer types and a control group cell line. We evaluated the decrease in cell viability resulting from 48- and 72-hour exposures to benzoic acid. Statistical analyses revealed that, especially at both time points, MG63 and CRM612 cancer cell lines were more sensitive to benzoic acid at lower concentrations compared to the control group ($p<0.05$). Notably, both A673 and MG63, the two analyzed bone cancer cell lines, exhibited sensitivity to benzoic acid for both 48 and 72 hours. However, the proliferation of MG63, in particular, was inhibited at lower concentrations (48 h - 195.21 µg/ml, 72 h - 85.54 µg/ml). Hence, there appears to be a mechanism in these bone cancer cell lines that is affected by benzoic acid. We suggest that further investigation into the effect of BA on A673 and MG63 may yield potentially useful information.

Osteosarcoma, a cancer originating in the bones, is an aggressive type that can manifest throughout the entire skeletal system in individuals of various ages. Its development is notably faster during adolescence [35]. Common treatments for this cancer involve chemotherapy and surgical intervention [6]. In cases where metastasis has not occurred, the patient's survival rate increases with the removal of the tumor area through surgical intervention [36]. Osteosarcoma typically metastasizes to the lungs,

exacerbating the clinical outcomes for patients [37]. The primary factors contributing to the lung metastasis rate of 80-90% in osteosarcoma include tumor-host signaling pathways, the structure of lung epithelial tissue, and innate adaptive immunity [38-39]. Investigations are underway for new drugs aimed at curing this type of cancer. We propose that the molecular structures associated with benzoic acid could serve as a promising starting point for the development of new candidates in anti-cancer drug design.

Based on the findings of our study, the cell line most affected by benzoic acid (BA) was the CRM612 cell line, associated with lung cancer. This discovery is intriguing and calls for further investigation, especially considering that lung cancer-related deaths rank highest among cancer types. Lung cancer is linked to factors such as the consumption of tobacco products, air pollution, and economic inadequacy [40]. Additionally, lung cancer can occur due to the propensity of bone cancer to metastasize to the lung [37].

Within the scope of the study, we included three colon cancer cell lines namely CaCO2, SW48 and HT29 cell lines and found the change in cell viability of three different colorectal cancer cells exposed to benzoic acid. Colorectal cancer (CRC) accounted for 1.93 million reported cases worldwide in 2020 [41]. There is believed to be a relationship between the development of colorectal cancer and nutrition. The occurrence of CRC differs between individuals consuming fiber-rich diets and those consuming diets high in animal protein and fat [42]. The development of CRC is hindered by the high consumption of meat products, affecting intestinal flora and structure. On the other hand, butyrate, released in the intestine because of fibrous food consumption, protects the intestinal structure [43].

The Epithelial Growth Factor Receptor (EGFR) is a tyrosine kinase receptor found in major synthesis pathways involved in cell proliferation, survival, and motility. This receptor is expressed in 25-80% of advanced colorectal cancer patients [44]. Cetuximab, an antibody inhibiting the EGFR signaling pathway, is employed in the treatment of colorectal cancer [45]. While SW48 and CaCO2 colorectal cancer cells exhibit sensitivity to cetuximab, HT29 cancer cells display resistance [46]. Our cytotoxicity studies with benzoic acid yielded similar findings to cetuximab. An analysis of cell viability changes in CaCO2, SW48, and HT29 colorectal cancer cell lines revealed an observed alteration in the viability of CaCO2 and SW48 cell lines following a 48-hour benzoic acid exposure ($p<0.05$).

The findings from cytotoxicity studies revealed variations in the inhibition concentration of benzoic acid among different types within the same cancer group. Literature studies suggest the existence of diverse inhibition mechanisms between cancer groups and types, with acetylation standing out as a particularly notable post-translational modification. Changes in the expression of HDAC, an enzyme involved in acetylation mechanisms, may contribute to the development of cancer pathogenesis. Research has demonstrated that benzoic acid and its derivatives exhibit HDAC inhibition activity in specific cancer types [27]. The intriguing aspect of benzoic acid being an inherent and natural component of the human diet, coupled with its observed cytotoxic activity in many of the analyzed cancer types within the study, further emphasizes the compound's significance.

As a result, the impact of benzoic acid on cancer cells should be further investigated, particularly to identify the biomolecules involved in the anticancer mechanism of the compound. This is especially pertinent for cervical, lung, colon, and bone cancer cell lines. In the present era, omics studies illuminate the mechanisms of action of various compounds on cells. Studies involving natural compounds with phenolic structures provide crucial examples of this [47-48]. More comprehensive studies using molecular genetics and omics approaches could be employed to decipher the genes, proteins, and metabolites associated with the effects of benzoic acid and its derivatives on cancer cells. Determining mRNA expression levels of target genes in specified cell lines or conducting proteomic analyses may lay the groundwork for future experiments. Focusing on the specific types of cancer, such as lung, colon, and bone cancers presented by this study, can offer valuable insights into the anticancer properties of

benzoic acid. In-depth studies in these areas may contribute to the development of potential therapeutic strategies or the identification of biomarkers associated with the anticancer activity of benzoic acid.

CONFLICT OF INTEREST

The authors stated that there are no conflicts of interest regarding the publication of this article.

AUTHORSHIP CONTRUBUTIONS

The author(s) contributed equally and stated that there are no conflicts of interest regarding the publication of this article.

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