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Defensin γ-thionin from *Capsicum chinense* improves butyrate cytotoxicity on human colon adenocarcinoma cell line Caco-2

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ABSTRACT

Background: Butyrate is a histone deacetylase inhibitor that induces apoptosis and inhibits cell proliferation of colorectal cancer cells. To improve its anticancer activity, butyrate has been evaluated mixed with drugs and different molecules. Plant antimicrobial peptides are attractive anticancer alternative molecules because they show selective cytotoxic activity against different cancer cell lines. In this work, we explore if the plant defensin γ-thionin (*Capsicum chinense*) can improve butyrate activity on Caco-2 cell line and we also determined the mechanism of death activated.

Results: The combined treatment of γ-thionin (3.5 μM) and butyrate (50 mM) showed higher cytotoxicity on Caco-2 cells with respect to single treatments. Also, the combined treatment reduced cell proliferation and exhibited a higher rate of apoptosis than single treatments. Combined treatment induced caspases 8 and 9 activation to an extent comparable with that of butyrate while γ-thionin did not activate caspases. Additionally, reactive oxygen species generation preceded the onset of apoptosis, and superoxide anion production was higher in cells treated with the combined treatment.

Conclusions: The γ-thionin from Habanero chili pepper improved the butyrate cytotoxicity on Caco-2 cells. This effect occurred through apoptosis induction associated with reactive oxygen species production. Therefore, the combination of butyrate with cytotoxic antimicrobial peptides could be an attractive strategy for cancer therapy.

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1. Introduction

Cancer is a serious health problem worldwide [1]. Colorectal cancer (CRC) stands out because it is the third most common cancer in men and the second in women worldwide [2]. Some types of CRC are inherited but most of them have been associated with lifestyle factors such as diet [3]. Diet composition influences the production of short-chain fatty acids (SCFA), the major fermentation products of dietary fiber in the gut. The principal SCFA are acetate, propionate, and butyrate whose concentrations range from 50 to 150 mM in the colon where they have nutritional, regulatory, and immunomodulatory functions [4].

Butyrate is the main source of energy for colonocytes and plays an important role in the homeostasis of the colonic epithelium [5,6]. Also, butyrate is a histone deacetylase (HDAC) inhibitor that epigenetically regulates gene expression and inhibits cell proliferation, and induces apoptosis in cancer cells [7]. For the above-mentioned, butyrate and other HDAC inhibitors have been evaluated as alternative treatments against different types of cancer. Also, to improve the butyrate anticancer activity, this SCFA has been assessed mixed with drugs currently used in the antitumor therapy, increasing their effect with respect to the single therapies [8,9,10].

In recent years, antimicrobial peptides (AMPs) have been considered as an attractive alternative for cancer therapy [11]. AMPs are relatively small molecules with 12–100 aa residues, mainly cationic, and involved in host defense against pathogens [12,13]. Today, in the antimicrobial database (http://aps.unmc.edu/AP/main.php) exist more than 3200 AMPs reported, of which ~7% showed anticancer activities mainly associated with necrosis [14] or apoptosis induction [15]. A group of attractive AMPs with anticancer activities corresponds to the plant antimicrobial peptides

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(PAP) [13]. They are grouped in families, the plant defensins being the most abundant. Some members of this family have shown specific cytotoxicity against cancer cell lines; thus, reducing undesirable side effects [16]. However, diverse factors such as the high cost of large-scale production, toxicity, and poor bioavailability have limited the AMPs development as therapeutic agents. One strategy to overcome these disadvantages is searching for drug interactions that improve their effects [17,18]. A possibility is to use a combination of SCFA, such as butyrate, with PAPs.

Recently, we have shown that defensin γ-thionin from Habanero chili pepper (*Capsicum chinense*) showed immunomodulatory effects in bovine mammary epithelial cells [19]. Also, we reported the cytotoxic activity of this defensin against MCF-7 breast cancer cell line [20]. However, it is unknown if this PAP could improve the effects of other cytotoxic molecules. To explore if γ-thionin can improve the butyrate activity, in this work the cytotoxicity of both combined molecules on human colon adenocarcinoma cell line Caco-2 was evaluated.

2. Materials and methods

2.1. Caco-2 cell culture

The human colon adenocarcinoma cell line Caco-2 (ATCC® HTB-37™) was cultured in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 Ham (DMEM-F12, Sigma), supplemented with 10% fetal bovine serum (Corning), 100 U/ml penicillin, 100 μg/ml streptomycin (Corning) and 250 ng/ml amphotericin B (Sigma), and maintained in an atmosphere of 5% CO_2 at 37°C. Cells (from passages 30–35) at ~90% of confluence were used in all the experiments.

2.2. γ-Thionin peptide and butyrate

The chemically synthesized peptide γ-thionin (50 aa) used in this work was obtained from Invitrogen and corresponds to the mature region (NH2-QNNICKTTSKHFKGLCFADSKCRKVCIQEDK FEDGHCSKLQRKCLCTKNC-COOH) (Genbank AF128239). The formation of disulfide bonds was accomplished as described by Guzman-Rodriguez et al. [15]. For all experiments, a final concentration of vehicle dimethyl sulfoxide (DMSO) 0.4% was employed. Stocks were maintained at −20°C. The concentrations used were 0.8, 1.7, and 3.5 μM, which were selected from preliminary screening.

Sodium butyrate was acquired from Sigma. Butyrate stocks were prepared in sterile water and the concentrations evaluated ranging physiological levels (10, 50, and 75 mM). Stocks were maintained at −20°C.

2.3. Inhibition assays

The cytotoxic effects of γ-thionin, butyrate, and combined treatments on Caco-2 cell viability were evaluated by MTT [3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] and trypan blue assays as described in Lara-Marquez et al. [21]. Briefly, 2×10^4 cells were cultured in 96-well flat-bottom plates (Costar) and synchronized in DMEM-F12 without serum (24 h). The treatments evaluated were: (1) 0.8, 1.7 and 3.5 μM γ-thionin; (2) 10, 50, and 75 mM butyrate. Measurements were carried out at 24, 48 or 72 h. For combined treatments, cells were pretreated with γ-thionin 24 h, and then butyrate was added and measurements were made after 24 and 48 h. The absorbance was measured in a microplate reader iMark (Bio-Rad) at 595 nm. Trypan blue assays were made in an automatic cell counter (Bio-Rad). Actinomycin D (0.5 μM, Sigma) was used as a positive control of cell death.

2.4. Nuclei staining

For nuclei staining, 1×10^5 Caco-2 cells were seeded on coverslips pretreated for 1 h with rat-tail type I collagen (Sigma). Cells were synchronized and treated as indicated in inhibition assays. After treatments, the cells were washed with PBS and fixed with 2% paraformaldehyde for 10 min. The fixed cells were washed with PBS and staining with Syto 9 (1.34 μM, Thermo Fisher Scientific) for 30 min at room temperature in the dark. After, cells were washing with PBS to remove the unbound dye and then photographed using a Leica 3000 fluorescence microscope.

2.5. Membrane depolarization assay

This assay was carried out as described by Lara-Marquez et al. [21]. Briefly, 2×10^4 cells were cultured in 96-well black plates (Thermo Scientific Nunc) pretreated with rat-tail type I collagen (Sigma). Then, cells were washed twice with Hanks' HEPES buffer and incubated with 0.2 mM of 3,3′-dipropylthiadicarbocyanine iodide, $DiSC_3(5)$ (Sigma) in Hanks' HEPES buffer for 30 min at 37°C in a CO_2 incubator. After four baseline determinations were made and the treatments (vehicle, butyrate, γ-thionin, and valinomycin) were added. In the combined treatment, γ-thionin was first added and fifteen readings were obtained, further butyrate was added and new measurements were made. Fluorescence was monitored by 2 h in a Varioskan Flash reader (Thermo Scientific). The K^+ ionophore valinomycin (0.4 mM, Sigma) was used as a positive depolarization control.

2.6. Calcium flux assessment

Calcium levels were evaluated using a Calcium Assay Kit (BD Biosciences) according to the manufacturer's instructions [21]. Briefly, confluent cells were washed with PBS and incubated in a medium without supplements for 24 h. Cells (1×10^6 cells/ml) were collected and resuspended in the 1× signal enhancer (1× calcium assay buffer and 1 mM probenecid) with the calcium indicator (1 μl/ml) (BD Biosciences), and incubated for 1 h at 37°C. Samples were left at room temperature for 20 min before reading, and then fluorescence was monitored in a BD Accuri™ C6 flow cytometer (BD Biosciences). Determination of basal fluorescence was performed for 80 s, at the end of this time, treatments were added directly to the cell suspension without interrupting the data collection. In combined treatment, butyrate was added 1 min later. Fluorescence was monitored for 280 s. Phorbol 12-myristate 13-acetate (3 μM PMA, Sigma) was used as a positive control. Data were analyzed using the FlowJo Software v. 10.4.2 (TreeStar, Inc.).

2.7. Apoptosis and caspase activity analysis

Apoptosis was evaluated as reported by Flores-Alvarez et al. [22]. For this, cells (2×10^4 cells) treated as described in inhibition assays were harvested by trypsinization at the end of pretreatment (t_0) and after 6 (t_6) or 24 h (t_{24}) for combined treatment, washed with PBS, and then stained with Annexin V (Alexa Fluor® 488 annexin V conjugate, Thermo Fisher Scientific) and 7-aminoactinomycin D (7AAD, BioLegend) according to the manufacturer's instructions. A total of 10,000 events were collected using a BD Accuri™ C6 flow cytometer (BD Biosciences) and data were analyzed using the FlowJo Software v. 10.4.2 (TreeStar, Inc.). Actinomycin D 0.5 μM was used as a positive control for apoptosis.

The activity of the representative caspases of apoptosis pathways was assessed at t_{24} by luminescence using the caspase-Glo®

8 and 9 assay kits from Promega according to the manufacturer's instructions. Cells were cultured in 96-well white-walled plates with a clear bottom (Costar 3610) and treated as described. After treatment (24 h) the medium was removed and a mixture 1:1 medium/reagent (prepared according to the manufacturer's instructions) was added to each well. Plates were shaken for 1 min and incubated for 1 h in the dark. Luminescence was measured using a Varioskan Flash reader (Thermo Scientific).

2.8. Measurement of reactive oxygen species (ROS) production

ROS production was analyzed as described in Tarpey and Fridovich [23]. 2×10^4 cells cultured in 96-well plates were treated for 45 min (for combined treatment cells were pretreated 15 min with γ-thionin, then butyrate was added and were left for 30 min with both compounds). Further, cells were recovered by centrifugation at 1500 rpm for 5 min and resuspended in 20 μM dihydroethidium (DHE, Sigma) or 20 μM 2′,7′-dichlorodihydrofluor escein diacetate (H2DCFDA, Sigma). Cells were incubated protected from light for 30 min at 37°C and 5% CO_2. Fluorescence of 10,000 events was measured in a BD Accuri™ C6 flow cytometer (BD Biosciences). $FeCl_3$ 30 μM was used as a positive control.

2.9. Cell cycle progression analysis

DNA content was analyzed by flow cytometry using the BD Cycletest Plus DNA reagent kit. To this, 8×10^4 cells were cultured in 24-well plates (Costar) and synchronized by serum deprivation for 24 h. Treatments (as indicated in inhibition assays) were carried out in a medium containing 10% heat-inactivated FBS. Cells were harvested and centrifuged for 5 min at 300 g at room temperature. The pellet recovered was resuspended in buffer solution by mixing at low speed and adjusted at a density of 1×10^6 cells/ml. Cells were centrifuged again and treated with solutions A (trypsin), B (trypsin inhibitor and ribonuclease A), and C (propidium iodide) as indicated in the manufacturer's instructions. A total of 20,000 events were collected using a BD Accuri™ C6 flow cytometer (BD Biosciences).

2.10. Data analysis

The normality tests were done with the Shapiro-Wilk test. The data were compared with one-way analysis of means comparisons using one-way ANOVA *post hoc* Tukey ($p < 0.05$) in the JMP 6.0 software. Results are reported as mean ± standard deviation (SD).

3. Results

3.1. γ-Thionin improves the butyrate cytotoxicity on Caco-2 cells

Cells were treated with 10, 50, and 75 mM butyrate or 0.8, 1.7, and 3.5 μM γ-thionin, and cytotoxicity was assessed at 24, 48, and 72 h by MTT. According to results, butyrate essentially did not affect the cells at 24 h, only 75 mM reduced ~20% the cell growth. However, at 48 h a significant reduction in cell growth was observed, which increased at 72 h (~70%) with 50 and 75 mM butyrate (Fig. 1a). On the other hand, the γ-thionin inhibitory effect on Caco-2 cells growth was slight, being not more than ~20% in all the times tested (Fig. 1b). From these results, we selected the following concentrations for the next experiments: 50 and 75 mM butyrate, 1.7 and 3.5 μM γ-thionin.

Then, we evaluated whether the antimicrobial peptide γ-thionin may improve the effect of butyrate on Caco-2 cells. For this, the cells were pretreated 24 h with the different concentrations of γ-thionin, and then, butyrate treatments were added and cell viability was evaluated by trypan blue assay. The combined treat-

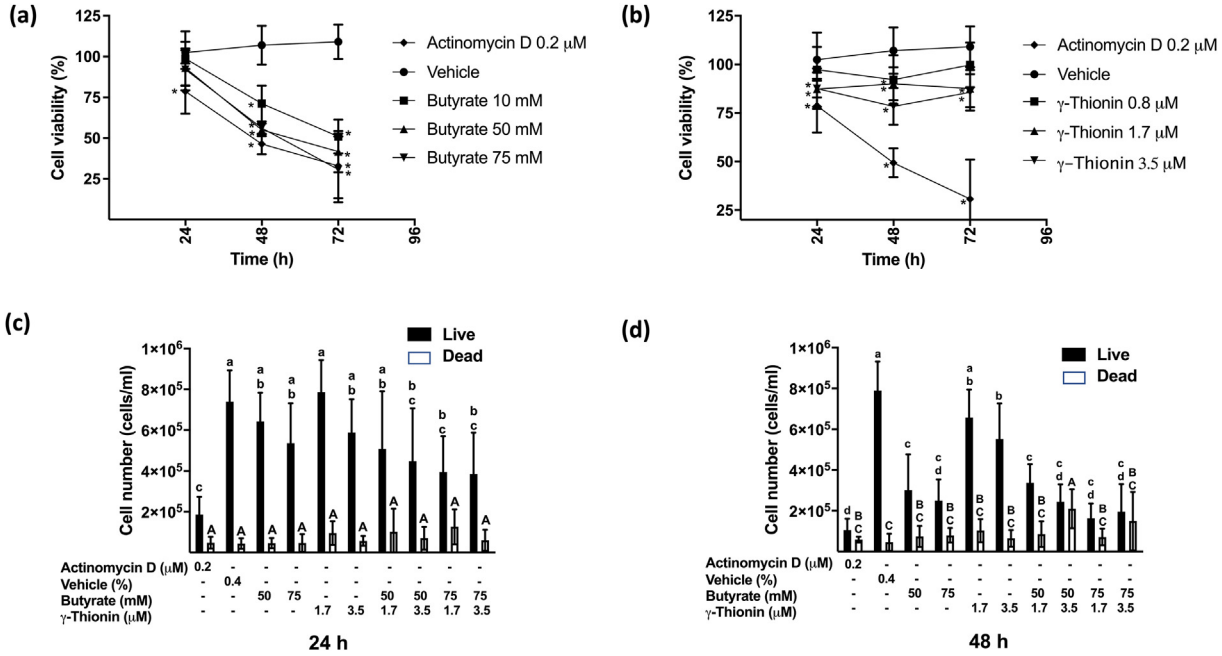

Fig. 1. γ-Thionin improves butyrate cytotoxicity in Caco-2 cells. Individual cytotoxicity of butyrate (a) and γ-thionin (b) is shown. Cells were treated with different concentrations of butyrate or γ-thionin for 24, 48, and 72 h and cell growth was analyzed by MTT assay. Untreated cells were established as 100% viability. Actinomycin D was used as a positive control. (*) Represents statistically significant difference with respect to the vehicle DMSO 0.4% (One-way ANOVA *post hoc* Tukey). Graphs represent mean ± SD from three experiments in triplicate. Cytotoxicity of combined treatment of γ-thionin and butyrate. Cells were pretreated with 3.5 μM γ-thionin 24 h and then 50 mM butyrate was added. Evaluations were carried out at other 24 h (c) or 48 h (d) and viability was analyzed by trypan blue assays. Graphs represent mean ± SD from three experiments in triplicate. Statistical analysis was performed by comparing live and dead cells separately. The lower case and capital case letters indicate significant changes within the parameter analyzed (live or dead cells, respectively). Different letters above the bars represent a statistically significant difference between treatments within the same parameter analyzed (One-way ANOVA *post hoc* Tukey, $p < 0.05$).

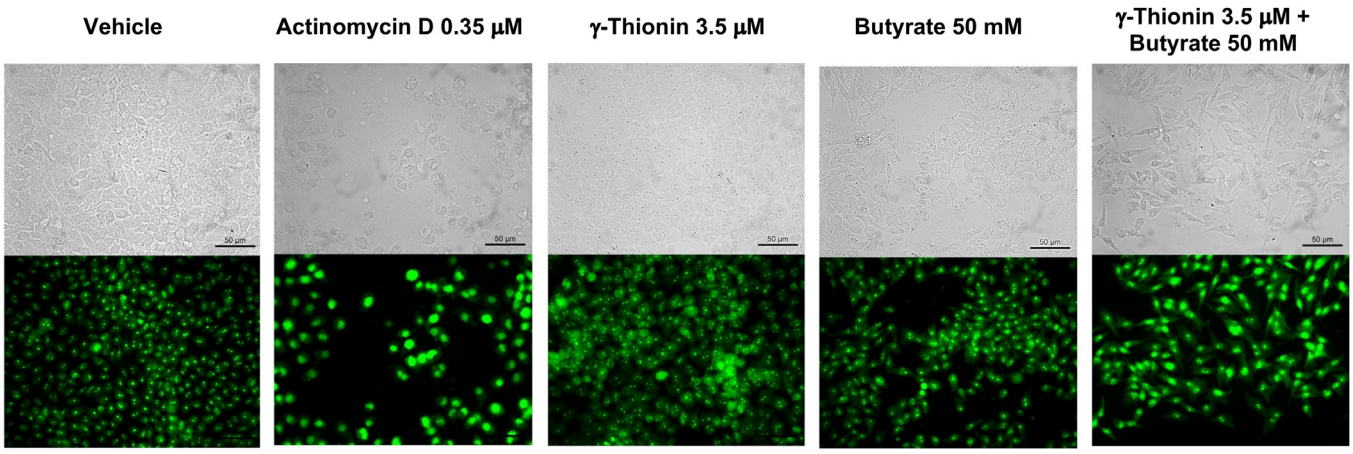

Fig. 2. Caco-2 cells treated with butyrate and γ-thionin shown morphological changes. Cells were treated as indicated in Materials and Methods, and then subjected to Syto 9 staining for 30 min at room temperature in the dark. Bright-field and fluorescence photographs are shown. Bar = 50 µM.

ments showed a reduction in the total number of cells with respect to individual treatments since 24 h (Fig. 1c), being higher the effect at 48 h (~75%) (Fig. 1d), which suggests an inhibition of proliferation. Also, we observed an increase in the number of dead cells in the combined treatments of 50 and 75 mM butyrate with 3.5 µM γ-thionin, reaching similar values of live and dead cells (~50%) (Fig. 1d). These results show that the pretreatment of Caco-2 cells with γ-thionin improves the butyrate cytotoxicity. According to these results, the combination of 50 mM butyrate and 3.5 µM γ-thionin was selected to analyze the effects on cell death.

3.2. The combined treatment of γ-thionin and butyrate affect the Caco-2 cell morphology

By Syto 9 staining, we observed small round, well-defined green dots in cells treated with the vehicle (Fig. 2), while with actinomycin D the nuclei were diffuse and a notable increase in the fluorescence intensity was observed. Also, γ-thionin and butyrate treatments showed similar behavior, only a small number of cells with similar characteristics to actinomycin D was observed. Besides, in the combined treatment most of the cells showed diffuse nuclei and exhibited a fibroblast-like morphology suggesting an epithelial-mesenchymal transition phenotype.

3.3. The combined treatment of γ-thionin and butyrate does not affect the plasmatic membrane integrity of Caco-2 cells

To find the mechanism of dead activated by the treatments, the membrane depolarization was evaluated with the dye $DiSC_3(5)$. Only the positive control valinomycin 0.4 mM induced changes in fluorescence intensity, which indicates membrane depolarization (Fig. 3a). Further, the levels of intracellular calcium were measured. Only cells treated with PMA 50 mM showed an increase in fluorescence intensity (Fig. 3b–f). According to these results, cell membrane integrity was not affected by the treatments with 3.5 µM γ-thionin, 50 mM butyrate, or both, which suggest that the mechanism involved in the cell death activated does not involve membrane damage.

3.4. γ-Thionin increases the butyrate-induced apoptosis in Caco-2 cells but the caspases activity was not affected

Further, we evaluated if apoptosis was induced in cells treated with γ-thionin, butyrate or both (Fig. 4). The first evaluation was carried out at the end of the pretreatment (24 h) with γ-thionin, which was designated t_0. At this time, cells treated with the vehicle or the peptide showed ~5% and 9% apoptosis, respectively (Fig. 4f). Then, butyrate was added and apoptosis determinations were made at 6 and 24 h and were designated as t_6 and t_{24}, respectively. At these times, cells treated with the vehicle maintained a reduced rate of apoptosis (~8%) (Fig. 4a). At t_6 neither butyrate nor actinomycin D showed a significant difference with relation to the vehicle. However, a significant increase in apoptosis was observed in cells treated with γ-thionin (~30%) and the combined treatment of γ-thionin and butyrate (~50%) (Fig. 4f). Moreover, at t_{24} the proportion of cells treated with γ-thionin in late apoptosis was increased; besides, a significant percentage of apoptotic cells was detected in butyrate-treated cells (~20%); while the actinomycin D and the combined treatment exhibited the greatest effect (~65 and 55%, respectively) (Fig. 4f). These results showed that the combination of γ-thionin and butyrate activated apoptosis in a shorter time than single treatments and increased apoptosis rate in Caco-2 cells.

Then, we measured the activation of caspases 8 and 9, representative of extrinsic and intrinsic apoptosis pathways, respectively. Cells treated with vehicle or γ-thionin at t_0 showed similar basal activation of both caspases, which was not modified at t_{24} (Fig. 5). However, at t_{24} butyrate and actinomycin D increased the activation of both caspases. Also, the activation of both caspases in cells with the combined treatment of γ-thionin and butyrate was like that of cells treated only with butyrate.

3.5. The combined treatment of γ-thionin and butyrate increases superoxide production in Caco-2 cells

ROS production has been associated with the activation of apoptosis and was evaluated in this work. All of the treatments similarly stimulated the generation of ROS in relation to the vehicle, accordingly with the general indicator H2DCFDA (Fig. 6a). However, when the superoxide anion was detected using the DHE indicator (Fig. 6b), the combined treatment of 3.5 µM γ-thionin and 50 mM butyrate showed a significant induction, which was similar to $FeCl_3$. This increase correlates with the increase in apoptosis induced by the combined treatment of γ-thionin and butyrate.

3.6. The combined treatment of γ-thionin and butyrate induces cell cycle arrest in the G1 phase in Caco-2 cells

Cell cycle progression analysis showed that butyrate treated cells showed a slight increase in the proportion of cells in G1 and

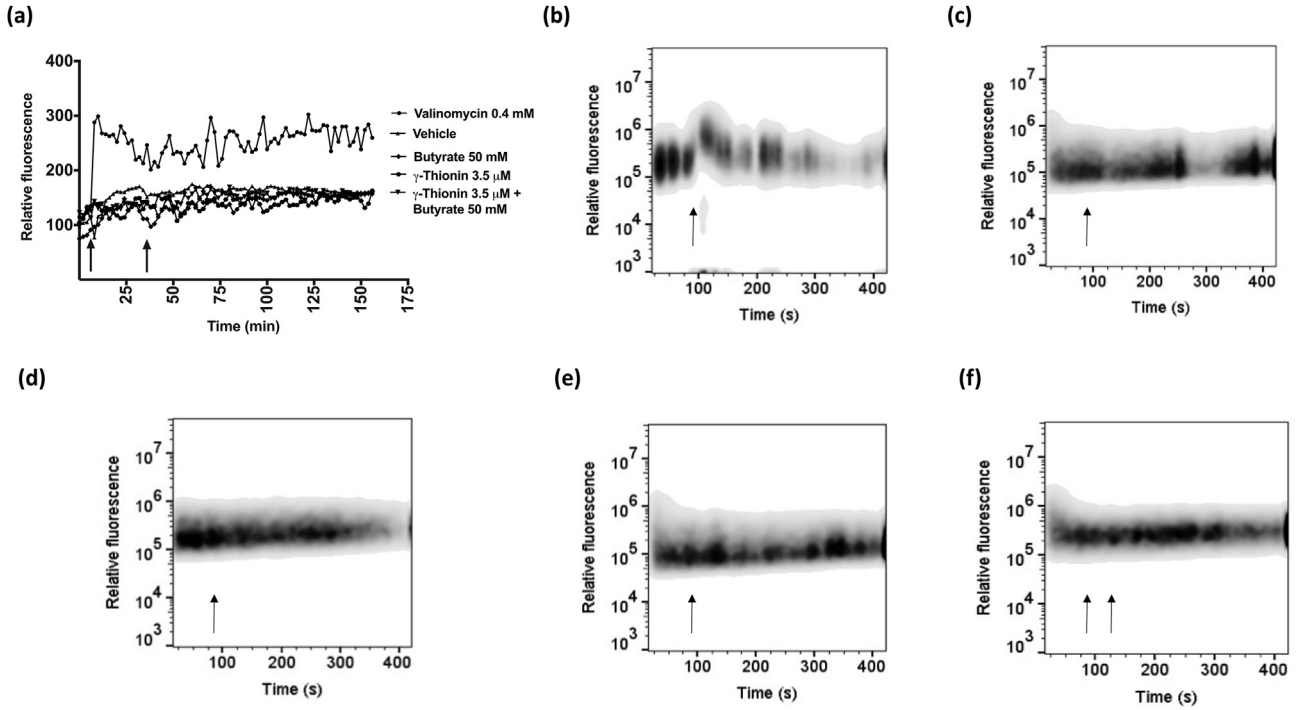

Fig. 3. γ-Thionin and butyrate does not affect cell membrane integrity of Caco-2 cells. (a) Changes in the membrane potential were measured using $DiSC_3(5)$ dye. Four fluorescence baseline determinations were made and the first treatments (vehicle, γ-thionin, butyrate, and valinomycin) were applied. In the first combined treatment, fifteen determinations were performed, and then the second treatment was applied. Fluorescence was monitored for 120 min. Valinomycin was used as positive depolarization control. Lines represent the means of two experiments performed in triplicate. Calcium mobilization was monitored by flow cytometry in cells treated with PMA (b), vehicle (c), γ-thionin (d), butyrate (e), and combined treatment (f) 3.5 μM γ-thionin and 50 mM butyrate. Determination of basal fluorescence was performed for 80 s; at the end of this time treatments were added directly to the cell suspension without interrupting the data collection. Butyrate was added 60 s later to combined treatment and fluorescence was monitored 280 s. PMA 50 μM was used as a positive control. Arrows indicate the treatment addition.

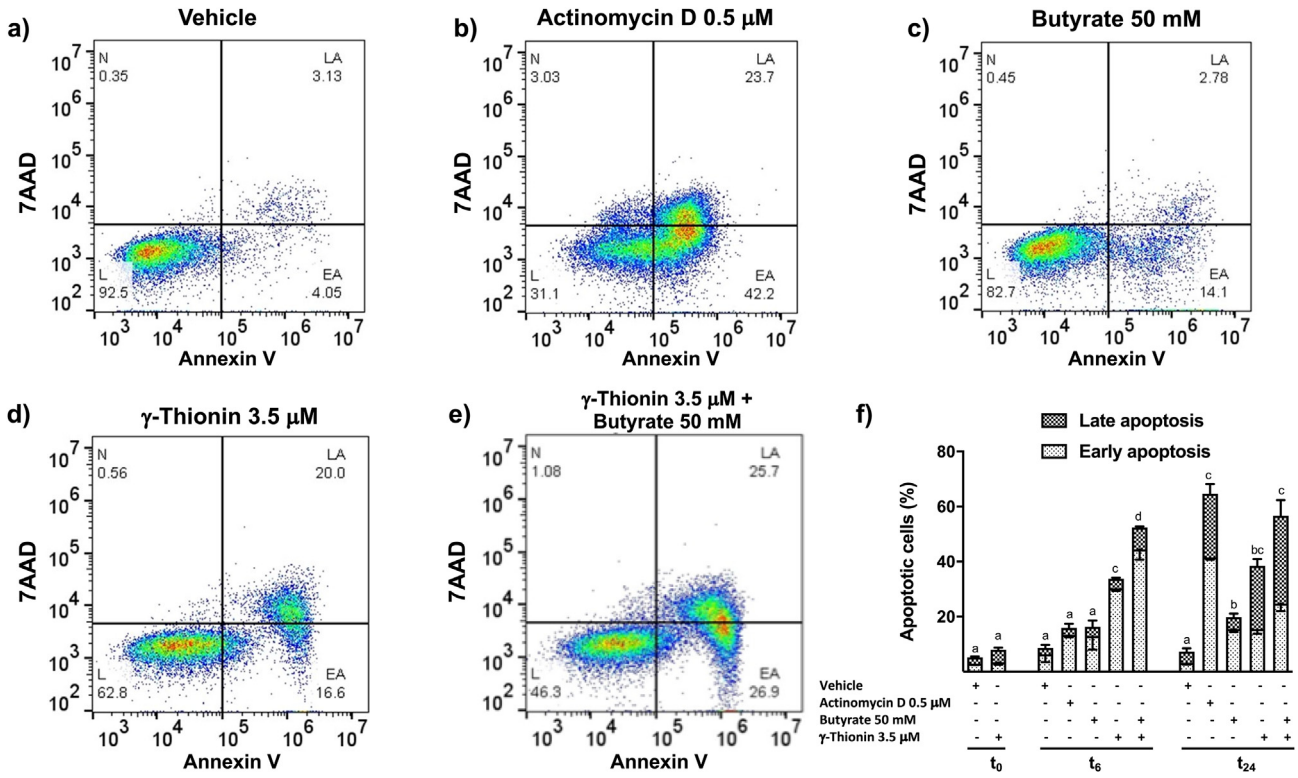

Fig. 4. γ-Thionin increases the apoptosis induced by butyrate in Caco-2 cells. The cells were exposed to the different treatments and then stained with Annexin V and 7AAD and subjected to flow cytometry. Apoptosis was measured at t_0, t_6, and t_{24} times. Actinomycin D 0.5 mM was used as a positive control. (a–e) Representative dot plots of t_{24} are shown (~10,000 events/plot). L- live cells, N- necrotic cells, EA- early apoptotic cells, and LA- late apoptotic cells. (f) The graph shows the percentage of apoptosis in the times evaluated. Bars represent mean ± SD of two experiments performed in triplicate. Statistical analysis was performed by comparing the treatments within each time evaluated. Different letters above the bars represent a statistically significant difference between treatments within the same time analyzed (One-way ANOVA *post hoc* Tukey, $p < 0.05$).

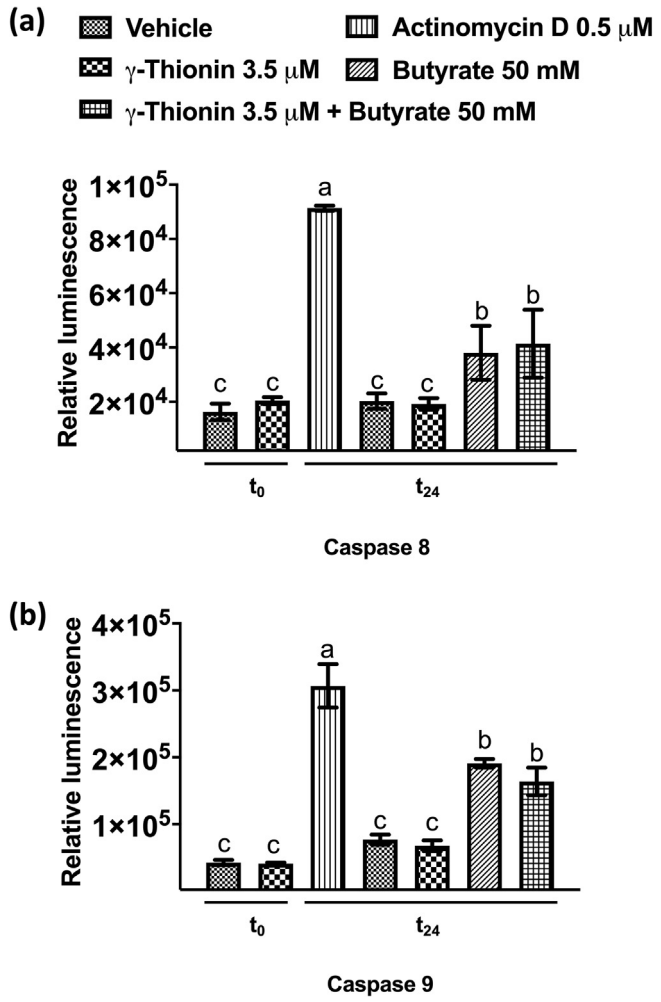

Fig. 5. γ-Thionin does not change the caspase activity induced by butyrate in Caco-2 cells. Caspases 8 (a) and 9 (b) activity was measured using the Caspase-Glo kits from Promega. Luminescence reflects caspase activity. Determinations were made at t_0 and t_{24} times. Actinomycin D was used as a positive control. Comparisons were made using one-way ANOVA *post hoc* Tukey for each caspase independently. Bars with different letters are significantly different ($p < 0.05$).

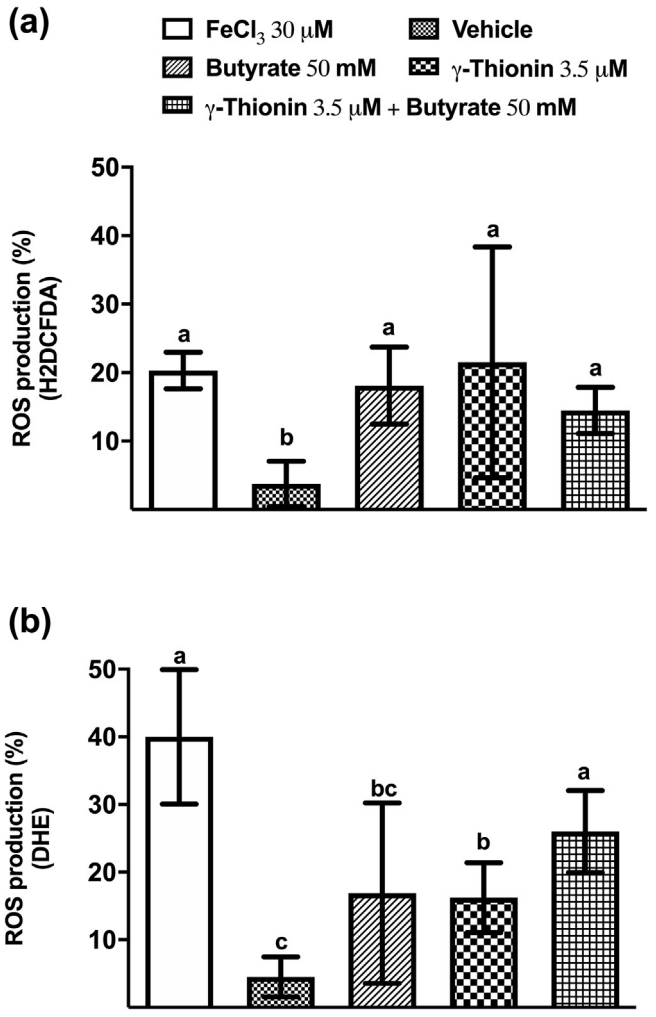

Fig. 6. γ-Thionin in combination with butyrate induces ROS generation in Caco-2 cells. Cells were stained with the general indicator H2DCFDA (a) or the superoxide-specific indicator DHE (b) and subjected to flow cytometry. The graphs represent the mean ± SD of the percentage of ROS production of 10,000 events. The comparisons were performed using one-way ANOVA *post hoc* Tukey ($p < 0.05$). Different letters above the bars represent a statistically significant difference.

a reduction in S but these effects were not statistically significant with respect to the vehicle. Also, a similar result was observed with 3.5 μM γ-thionin (Fig. 7). However, the combined treatment of 3.5 μM γ-thionin and 50 mM butyrate produced a significant arrest in the G1 phase (~90%) compared with the vehicle (~78%), and a significant reduction in the S phase (~6%) also with respect to control cells (~18%). Therefore, cell cycle arrest may give to the increase in cytotoxicity of combined treatment of γ-thionin and butyrate.

4. Discussion

Butyrate inhibits cell proliferation and stimulates apoptosis in tumor-derived cell lines from different origins [24,25]. The cytotoxicity of butyrate on colon carcinoma cell lines is variable; in particular, Caco-2 cells show intermediate sensitivity [26]. For this reason, the search for interactions with other molecules that improve its cytotoxicity is attractive. In this study, we show that γ-thionin, an antimicrobial peptide from Habanero chili pepper fruit, improves the butyrate cytotoxicity through the increase in apoptosis.

Butyrate shows cytotoxicity on Caco-2 cells in a range of concentrations (0.01–100 mM) and exerts a proapoptotic effect above a threshold concentration of 5 mM [27]. In agreement, we observed a significant reduction in Caco-2 cell viability (~50%) and inhibition of cell proliferation after 48 h of treatment with the different concentrations tested (10, 50, and 75 mM) (Fig. 1a). The butyrate cytotoxic concentrations are in the physiological levels because luminal concentrations have been estimated in a range of 10–70 mM [28,29].

On the other hand, γ-thionin showed a cytotoxic effect on Hela cells when the conditioned medium from clones of transfected bovine endothelial cells that expressed γ-thionin was used [30]. Moreover, the synthetic peptide exhibited cytotoxicity against the breast cancer cell line MCF-7 [20]. In this work, we used lower concentrations of γ-thionin (0.8–3.5 μM) to diminish its cytotoxic effect and to allow us to study the combined effect with butyrate. As expected, γ-thionin showed a slight reduction in cell viability of ~20% (Fig. 1b). Also, γ-thionin (3.5 μM) did not affect the viability of human fetal non-malignant colon cells CRL-1790 (data not shown); however, further experiments are required to determine the combined effect with butyrate.

Despite their advantages as anticancer agents, both butyrate and AMPs has some therapeutic challenges, as poor stability and

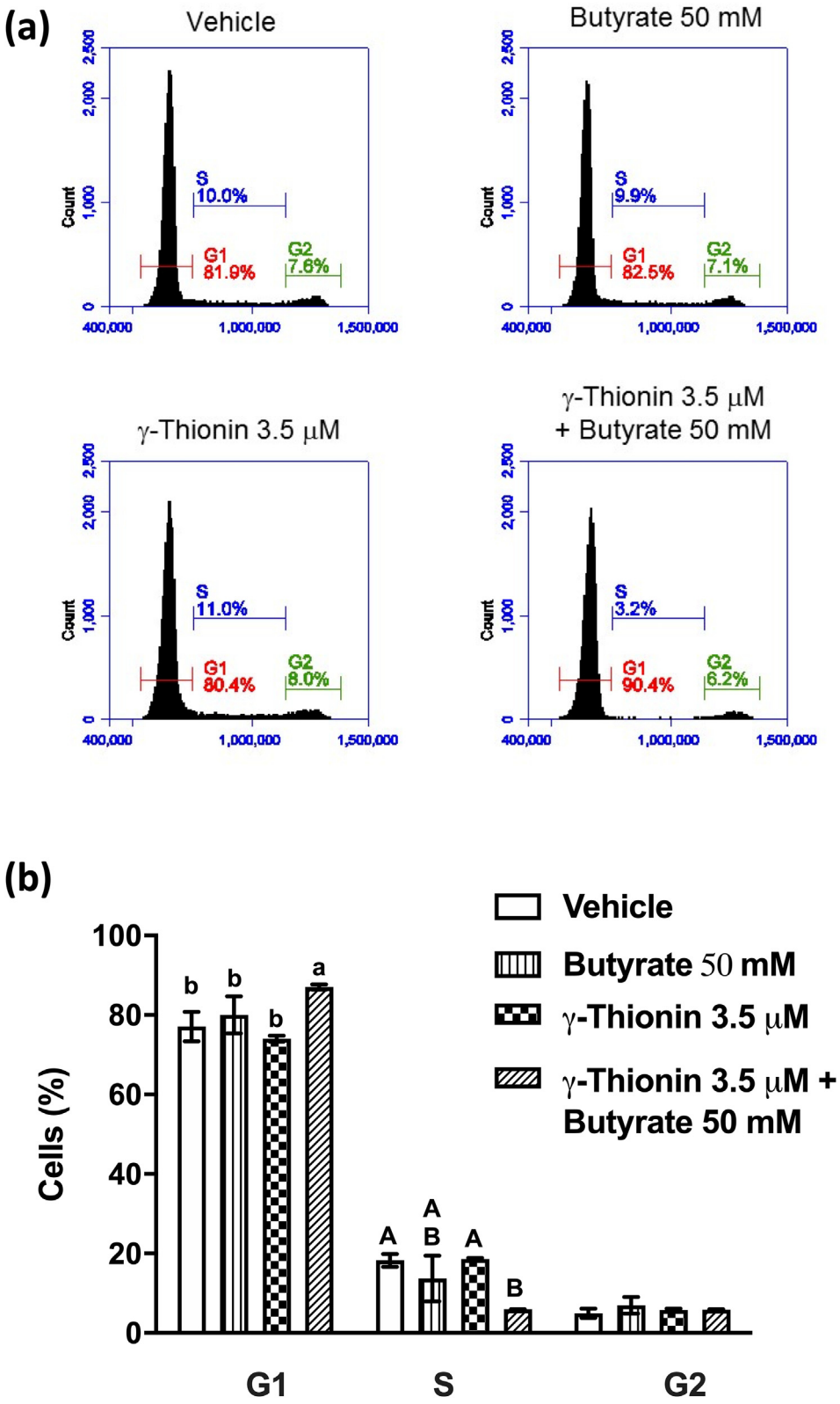

Fig. 7. γ-Thionin in combination with butyrate induces cell cycle arrest in Caco-2 cells. Nuclei were stained with propidium iodide (BD Cycletest Plus DNA reagent) and analyzed by flow cytometry to determine the cell cycle progression. Representative plots (a) and graph of the different cell cycle phases (b). Graph represent mean ± SD of one experiment performed in triplicated. A total of 20,000 events were collected using a BD Accuri™ C6 flow cytometer (BD Biosciences). The analysis was performed by comparing the treatments within each cell cycle phase. Different letters above the bars represent a statistically significant difference between the treatments within the cell cycle phase evaluated (One-way ANOVA *post hoc* Tukey, $p < 0.05$).

bioavailability. One strategy to overcome these disadvantages is their use in combination with other anticancer drugs [31,32]. The possible favorable outcomes for drug combination include an improved effect decreasing the concentration and minimizing the

development of drug resistance [33]. In this work, the combined treatment of γ-thionin and butyrate induced cytotoxicity in an additive way (~30%), and a lower number of live cells was recovered from combined treatment than from individual treatments (Fig. 1). These observations are in agreement with the results of cell cycle progression analysis and apoptosis. A cell cycle arrest in G1 phase after 24 h of combined treatment was observed and fewer cells were recovered from this treatment. Butyrate also increased the proportion of cells in G1 phase, but this was not statistically significant; probably in longer times of treatment, we could detect a greater effect because it has been shown that butyrate cell cycle arrest in G1 phase is time and concentration-dependent [34]. In accordance, co-treatment of the cyclic β-sheet AMP tachyplesin and butyrate in BGC-823 cells increased the proportion of cells in G1 phase and reducing S phase [35].

Many antimicrobial peptides induce membrane permeabilization as part of their cytotoxic mechanism, which is accompanied by loss of the membrane potential [36,37]. These characteristics were evaluated in the present work measuring the intracellular calcium mobilization and changes in the membrane potential (Fig. 3). Results showed that 3.5 μM γ-thionin did not affect the cell membrane integrity of Caco-2 cells. It has been reported that the effect caused by AMPs depends on the concentration since the same peptide can cause cell lysis at higher concentrations or induces apoptosis at lower concentrations such as the cathelicidin BMAP-28 [38,39]. However, there was no evidence of membrane lysis induced by γ-thionin in MCF-7 cells with a concentration six times higher than the concentration used here [20]. Otherwise, it is known that butyrate induces intracellular calcium release by binding to G-protein-coupled receptors, mainly GPR43 [40]. However, this receptor is often lost in colon cancer cells and its expression has not been detected in Caco-2 cells by RT-PCR [41], this is in agreement with our results in which calcium changes were not observed. These results suggest that the effect of butyrate and combined treatments may be due to an intracellular action.

It is known that cytotoxicity of butyrate and AMPs may occur through apoptosis induction [20,27]. In agreement, 3.5 μM γ-thionin or butyrate induced apoptosis after 24 h of pretreatment (t_0) (Fig. 4). Besides, cells treated with the combined treatment reached ~55% of apoptosis after 24 h, which indicates an additive effect. Also, we do not observe a difference between the number of dead cells at 24 h, but the number of cells that have activated their apoptosis is different, which coincides with the increase in dead cells at 48 h in the combined treatment, where approximately 50% of cell death is reached. An earlier report of combined treatment of butyrate (5 mM) and epigallocatechin gallate (10 μM) during 48 h in RKO CRC cells showed a similar result with significant apoptosis (~13%) in comparison with the individual treatments (~8%) [42]. In the same way, the treatment of Caco-2 cells with 10 mM butyrate and 50 ng/ml TNF-α during 24 h also showed a significant apoptosis induction (~17%) in comparison with the individual treatments (~2%) [43]. However, the apoptosis induction in the present work was higher. Likewise, we have evaluated the cytotoxicity of combined treatment on HCT-116 cells (human colon carcinoma). The pretreatment of these cells for 24 h with 3.5 μM γ-thionin and then with 5 mM butyrate (24 h) increased cell death compared with individual treatments (data not shown). These results suggest that the combined treatment could improve the butyrate activity on colon cancer cells with different genetic backgrounds.

In agreement with the apoptosis induction, 50 mM butyrate treatment (24 h) induced activity of caspases 8 and 9 (Fig. 5), suggesting the activation of the intrinsic and extrinsic apoptotic pathways. Some reports only showed activation of caspase 9 in Caco-2 cells treated with butyrate; however, other studies support our findings [44,45]. These discrepancies could be attributed to differences in concentration, time, and detection methods. Otherwise, 3.5 μM γ-thionin did not induce activation of any caspase. Therefore, apoptosis induced by γ-thionin could be mediated through elements different from caspases, which require further investigation.

Generation of ROS is one of the earliest intracellular events occurring during apoptotic cell death induction [46]. Treatments with 3.5 μM γ-thionin, 50 mM butyrate, and both agents together induced similar amounts of ROS (Fig. 6). Also, the combined treatment showed higher production of superoxide anion than single treatments, which is an important molecule for apoptosis induction. This supports the major apoptotic effect induced by the combined treatment. Similar results were reported to the AMP cecropin A, which induces ROS and activates caspase independent apoptosis in the promyelocytic cell line HL-60 [47].

5. Conclusions

The AMP γ-thionin in combination with butyrate exhibited higher cytotoxicity on Caco-2 cells than individual treatments. The combined treatment increased apoptosis rate, which was associated with the increase in ROS production. Therefore, the combination of butyrate with cytotoxic AMPs could be an attractive strategy for cancer therapy.

Conflict of interest

The authors declare that there are no conflicts of interest.

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