Synergistic Cytotoxicity of Artemisinin and Sodium Butyrate on Human Cancer Cells

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Abstract. *Background: Butyric acid is a short chain fatty acid produced by large bowel bacterial flora. It serves as an anti-inflammatory agent and nutrient for normal colon cells. Butyric acid has also been shown to induce apoptosis in colon and many other cancer cells. Artemisinin is a compound extracted from the wormwood Artemisia annua L. It has been shown to selectively kill cancer cells in vitro and to be effective in treating animal and human cancer. We and others have found that the artemisinin analog, dihydroartemisinin (DHA), kills cancer cells by apoptosis. In the present study, the efficacy of a combined treatment of DHA and butyric acid at low doses in killing cancer cells was investigated. Materials and Methods: Molt-4 cells (a human lymphoblastoid leukemia cell line) and freshly isolated human lymphocytes, cultured in complete RPMI-1640 medium, were first incubated with 12 μM of human holotransferrin at 37˚C in a humid atmosphere of 5% CO_2 for one hour to enhance the iron concentration in the cells. Cells from each cell type were then divided into 20 flasks. These flasks were grouped into four sets of five cultures each. Zero, 5, 10 or 20 μM of DHA was added, respectively, to these sets and the cells were incubated at 37˚C for one hour. Zero, 1, 5, 10, or 20 mM of sodium butyrate was then added to the five cultures of each set, respectively. Thus, the treatments involved a combination of 4 doses of DHA and 5 doses of sodium butyrate. The cells were counted immediately before the addition of DHA, and at 24 and 48 hours after the addition of sodium butyrate. Results: DHA alone at the 24-hour time-point and 20 μM concentration significantly reduced the number of Molt-4 cells in the culture by approximately 40% (p<0.001, compared to non-treated control), whereas it did not significantly affect the number of normal human lymphocytes. Similarly, 1 mM sodium butyrate alone at 24 hours reduced the number of Molt-4 cells by approximately*

32% (p<0.001, compared to non-treated control), without significantly affecting normal human lymphocytes. The combination of 20 μM DHA and 1 mM sodium butyrate killed all Molt-4 cells at the 24-hour time-point and did not significantly affect lymphocytes. Conclusion: DHA in combination with butyric acid acts synergistically at low doses. The combination may provide a less toxic, inexpensive and effective cancer chemotherapy.

Artemisinin, a known anti-malarial drug (1) extracted from the wormwood *Artemesia annua L*, is a sesquiterpene lactone peroxide containing an endoperoxide moiety, which forms free radicals when it reacts with iron. Free radicals formed intracellularly could lead to cell death. Iron is needed for cell division and proliferation. It is transported from the plasma into cells by the iron-carrying protein transferrin. Compared to normal cells, cancer cells have a tumor aggressiveness-correlated higher concentration of cell surface transferrin receptors (2, 3). Thus, cancer cells would be selectively more susceptible to the cytotoxicity of artemisinin because of their higher rate of iron uptake. Previous studies showed the anticancer efficacy of artemisinin in cell cultures, rats and humans (4-7) and the work of other investigators (8, 9) supports this hypothesis. We and others have found that the analog of artemisinin, dihydroartemisinin (DHA), kills various types of cancer cells *via* apoptosis (10, 11).

Short chain fatty acids such as acetic acid, propionic acid and butyric acid are metabolic end products of the anaerobic bacterial fermentation of undigested carbohydrates (plant dietary fiber) in the colon (12, 13). There are more than $1x10^{11}$ bacteria per gram of colon contents (14). Colonic bacteria, weighing at least 175 grams, generate very high local concentrations (~16 mM) of butyric acid in the human colon (12). Roediger (15) described the beneficial role of these metabolic end products to colonocytes. Apart from providing a source of energy for colonocytes, these fatty acids prevent the apoptosis of colonocytes by enhancing the intracellular levels of glutathione (14, 16). Paradoxically, they also induced apoptosis in cancer cells of the colon *in vitro* (17)

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and have been shown to be effective against colon cancer in experimental animals (18-20). Kuefer *et al*. (21) used oral sodium butyrate to effectively treat mice with implanted human prostate cancer. Short chain fatty acids have been proposed to be useful for the prevention of colon cancer (13, 22). However, probably due to a rapid metabolism (23, 24) and delayed induction of apoptosis (25), short chain fatty acids used alone have limited effectiveness in the treatment of cancer. To enhance their effectiveness in killing cancer cells, some researchers have used the longer-acting butyric acid derivative AN -9. Rabizadeh *et al*. (26) reported that a combination of AN-9 and doxorubicin *in vitro* suppressed the *bcl-2* gene in cells from chronic lymphocytic leukemia patients. Recently, Rephaeli *et al*. (27) have shown the effectiveness of AN-9 in treating mice with implanted human prostrate cancer cells. Another longer-acting analog of butyric acid, isobutyramide (28), has been found to be effective against implanted human prostate cancer cells in nude mice.

DHA has been shown to selectively kill Molt-4 cells (a human lymphoblastoid leukemia cell line). The goal of this research was to investigate whether DHA and butyrate act synergistically in killing Molt-4 cells. The results gathered from such research on their interaction could provide information about the doses of DHA and butyrate that would be most effective against cancer cells with less toxic effect on normal cells.

Materials and Methods

Materials. Molt-4 cells and fetal bovine serum were purchased from ATCC (Rockville, MD, USA). RPMI-1640 culture medium was purchased from Life Technologies (GIBCO BRL, Rockville, MD, USA). Dihydroartemisinin (DHA) was purchased from the Calbiochem Novabiochem Corporation (EMB Biosciences, La Jolla, CA, USA). All other chemicals used in the research were purchased from the Sigma Chemicals Company (St. Louis, MO, USA).

Molt-4 cell culture. Molt-4 cultures were maintained in RPMI-1640 supplemented with 10% fetal bovine serum. The cells were cultured at 37˚C in 5% CO_2/95% air and 100% humidity, and were split 1:2 at a density of approximately 1×10^6 cells/ml.

Human lymphocyte culture. Human lymphocytes were isolated from fresh blood obtained from a healthy donor, using a modification of the Ficoll-hypaque centrifugation method of Boyum (29). In this method, 100 μl of whole blood, obtained from a finger prick, was mixed with 0.5 ml of ice-cold RPMI-1640 in a 1.5-ml heparinized microfuge tube (Kew Scientific Inc., Columbia, OH, USA). Using a Pipetman, 100 μl of cold lymphocyte separation medium (LSM) was layered at the bottom of the tube. The samples were centrifuged at 3,500 r.p.m. for 2 minutes in a microfuge (Sorvall, Microspin model 245) at room temperature. Lymphocytes in the upper portion of the Ficoll layer were pipetted out. The cells were washed twice with 0.5 ml of RPMI-1640 each by centrifugation for 2 minutes at 3,500 r.p.m. in a microfuge. The final pellet, consisting

of approximately 2×10^5 lymphocytes, was resuspended in RPMI-1640. Cell viability was determined before the experiments using trypan blue exclusion and found to be >95%.

Treatment of Molt-4 cells and human lymphocytes with dihydroartemisinin and sodium butyrate. A stock solution of DHA was made fresh in DMSO and a stock solution of sodium butyrate was made fresh in complete RPMI. The final concentration of DMSO was 1% in the cultures of both cell types.

Molt-4 cells and freshly isolated human lymphocytes, in complete RPMI-1640 medium, were first incubated with 12 μM of human holotransferrin at 37˚C in a humid atmosphere of 5% CO_2/95% air for one hour to enhance iron in the cells. Cells from each cell type were then divided into 20 flasks. These flasks were then grouped into four sets of five cultures each. Zero, 5, 10, or 20 μM of DHA was added, respectively, to these sets and the cells were incubated at 37˚C for an hour. The solvent DMSO was added to the cultures not treated with DHA at a final concentration of 1%. Zero, 1, 5, 10, or 20 mM of sodium butyrate was then added to the five cultures of each set, respectively. An equal volume of RPMI was added to cultures not treated with sodium butyrate. Thus, the treatment involved a combination of 4 doses of DHA and 5 doses of sodium butyrate. The cells were counted at 0 (defined as before the addition of DHA), and 24 and 48 hours after the addition of sodium butyrate. To minimize damage to the DHA and cells, minimum indirect light was maintained during these experiments. All experiments were performed three times.

Cell counts. Total cells were counted using a hemocytometer, as described by Lai and Singh (4). The total cell count (cells remaining in culture) is a more reliable measure of the effectiveness of the DHA and sodium butyrate combination treatment because dead cells are rapidly eliminated from the cultures.

Data analysis. The data (total cell count) were analyzed by one-way ANOVA and the difference between two treatment points by the Newman-Keuls test. A $p \leq 0.05$ was considered statistically significant.

Results

Figures 1 A-D show the effects of different concentrations of sodium butyrate in combination with 0, 5, 10 and 20 μM of DHA on Molt-4 cells. Figures 2 A-D show the effects of similar treatments on human lymphocytes. Both DHA and sodium butyrate had significant cytotoxic effects on Molt-4 cells, whereas their effects on lymphocytes were minimal. To illustrate the effects and interaction between the two compounds, the effects of control (no drug added), DHA alone (20 μM), sodium butyrate alone (1 mM) and DHA (20 μM) + sodium butyrate (1 mM) on Molt-4 and lymphocytes at the 24-hour time-point are plotted in Figures 3 and 4, respectively. One-way ANOVA of the Molt-4 data showed a significant treatment effect ($F[3, 8]=1534$, $p<0.001$). The Newman-Keuls test showed that DHA ($p<0.001$) and sodium butyrate ($p<0.001$) alone significantly decreased the cell counts compared to the control. Furthermore, a combination of DHA and sodium butyrate significantly further enhanced
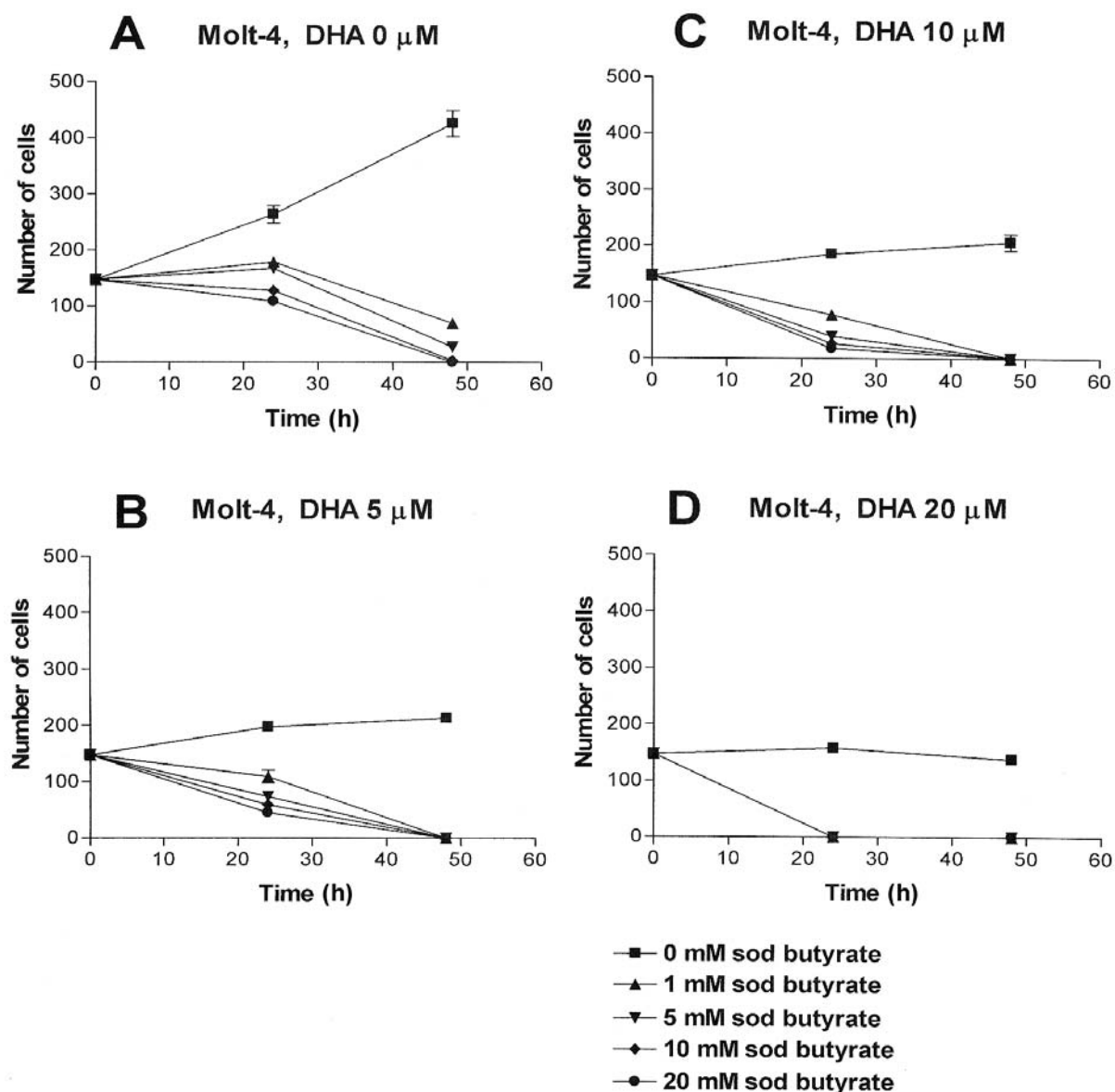
Figure 1. *Graphs showing the number of Molt-4 cells (Mean±SD, N=3) at 0-, 24- and 48-hour time-points with different treatment combinations of DHA and sodium butyrate. Graphs show the effects of 0, 1, 5, 10 and 20 mM sodium butyrate added to cultures containing 0, 5, 10 and 20 μM DHA (Figures 1 A-D, respectively).*

the effect of either drug alone (*i.e.*, the cell count of the drug-combination treatment was significantly lower than either drug alone, $p < 0.001$ for both comparisons). This suggests that the effects of DHA and sodium butyrate are synergistic. One-way ANOVA of the lymphocyte data showed no significant treatment effect ($F[3,8]=1.39$, $p > 0.05$). The Newman-Keuls test also did not reveal any significant difference between treatments. These results indicate that both DHA and sodium butyrate have selective toxicity towards Molt-4 cells.

Discussion

From Figure 1, it is clear that a minimum concentration of 20 μM of DHA is needed for a decrease in Molt-4 cell numbers from 0 to 48 hours. Other concentrations of DHA (5 and 10 μM) were unable to decrease the cell numbers significantly in Molt-4 cultures at 48 hours. On the other hand, all concentrations of sodium butyrate (1, 5, 10, 20 mM) decreased the Molt-4 cell numbers at all time-points.
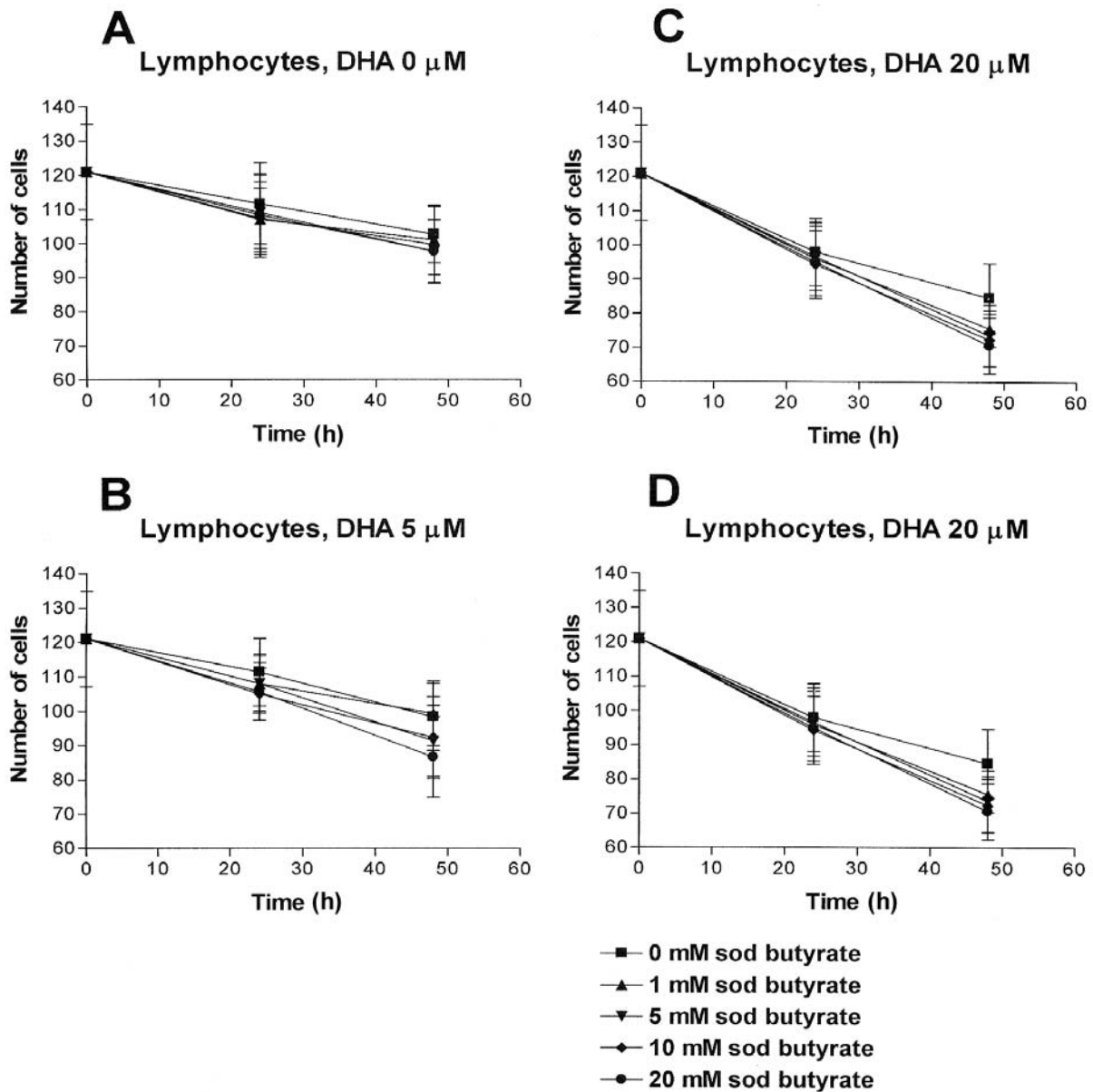
Figure 2. *Graphs showing the number of human lymphocytes (Mean±SD, N=3) at 0-, 24- and 48-hour time-points with different treatment combinations of DHA and sodium butyrate. Graphs show the effects of 0, 1, 5, 10 and 20 mM sodium butyrate added to cultures containing 0, 5, 10 and 20 μM DHA (Figures 2 A-D, respectively).*

In a previous study (4), we found that 10 μM DHA was more effective in killing Molt-4 cells within 8 hours than in the present study. In the present study, 20 μM of DHA alone was not as effective even at the 24- and 48-hour time-points. One of the reasons for this discrepancy could be the use of DMSO to dissolve DHA and its presence in all cultures at 1% concentration, compared to the culture medium used to dissolve DHA in our previous study. DMSO is an antioxidant and probably decreases the effectiveness of DHA.

Several studies on different cancer cell types have shown butyrate exerting its cytotoxic effects *via* apoptosis (25, 26, 30-33), although the mechanism by which butyric acid induces apoptosis is not well understood (26). Vidali *et al.* (30) observed H3 and H4 histones hyperacetylation due to inhibition of nuclear deacetylase(s) by butyrate. Hyperacetylation of histones leads to decondensation of chromatin, making it vulnerable to endonuclease-mediated apoptosis (31). However, butyric acid is known to be weak
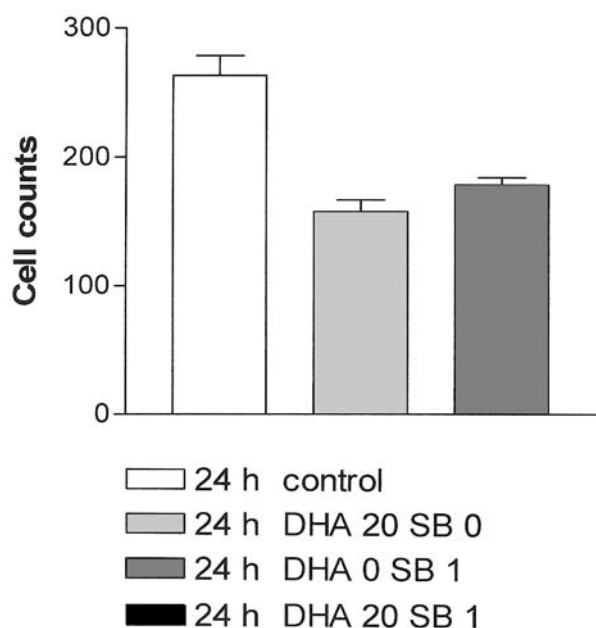
Figure 3. *Bar graph of mean cell numbers (±SD) from three experiments showing the effects of control (no treatment), 20 μM DHA, 1 mM sodium butyrate and a combination of 20 μM DHA and 1 mM sodium butyrate on Molt-4 cells at the 24-hour time-point. (The drug combination reduced the cell count to zero).*

Figure 4. *Bar graph of mean cell numbers (±SD) from three experiments showing the effects of control (no treatment), 20 μM DHA, 1 mM sodium butyrate and a combination of 20 μM DHA and 1 mM sodium butyrate on human lymphocytes at the 24-hour time-point.*

compared to natural and synthetic deacetylase inhibitors used as anticancer agents (34). On the contrary, Rozental *et al.* (25) showed that apoptosis induced by sodium butyrate in the MSN neuroblastoma cell line was independent of deacetylase inhibition. These authors also observed that sodium butyrate did not raise the intracellular calcium levels needed for activation of endonucleases.

Short chain fatty acid enemas have been used in inflammatory bowel disease in rats (35) and in humans (36, 37). Venkatraman *et al.* (38) found sodium butyrate enema in rats to be cytoprotective in dextran sulfate-induced colitis and showed that the effect was partly due to suppression of heat shock protein 70 and transcription factor NF-kappaB, both of which play roles in inflammation.

Two major mechanisms may possibly overlap to enhance the apoptotic effect of the two agents: i) butyric acid causes chromatin decondensation allowing free radicals formed by artemisinin to trigger apoptotic pathways, and ii) butyric acid redistributes intracellular iron in such a way that it is more readily available for interaction with artemisinin to generate free radicals. Several studies, exploring the role of iron in differentiation (39-41), have indicated that butyric acid is involved in intracellular iron redistribution. Our results, showing a synergistic effect of artemisinin and butyric acid in killing Molt-4 cells, may be due to these mechanisms.
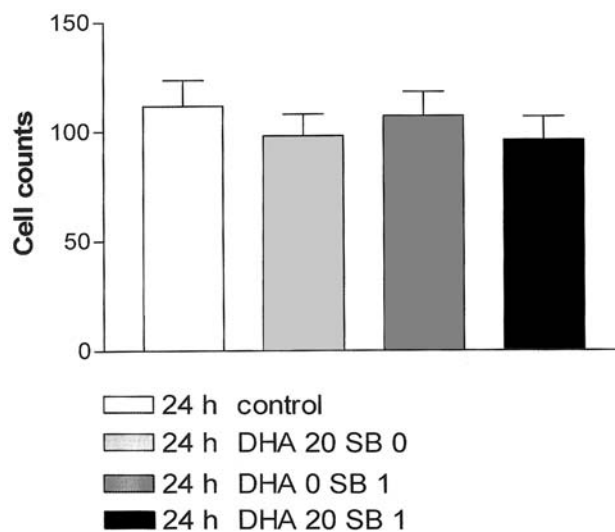
Edelman *et al.* (42) treated 20 terminal cancer patients with tributyrin, a butyric acid prodrug. Two patients with small cell lung carcinoma improved after treatment with tributyrin and a conventional chemotherapeutic agent, indicating the effectiveness of the combined therapy. Low concentrations of either DHA or sodium butyrate alone are unable to totally eliminate Molt-4 cells. However, the combination of low concentrations of sodium butyrate (1 mM) and DHA (20 μM) kills all leukemia cells in culture within 24 hours, suggesting that the combination of short chain fatty acids and artemisinin or its analogs may be an effective treatment for cancers. One primary advantage of the butyric acid and artemisinin combined therapy could be the ability of both compounds to induce apoptosis exclusively in cancer cells. We did not observe any necrosis by butyric acid alone, DHA alone, or a combination of the two. Thus, the combination could be developed into a safe, effective and inexpensive cancer therapy.

The oral intake of artemisinin and its analogs (7, 43) and butyric acid (44) is safe. Oral administration of the artemisinin analogs, artesunate (100 mg in a healthy adult) resulted in micromolar plasma concentration of DHA (45). The basal plasma concentration of butyric acid is normally >1 μM (42). Oral or intravenous administration of sodium butyrate (50 mg/kg) has been shown to increase the plasma butyrate concentration to the millimolar range in rats (46). The artemisinin-butyrate combination may work well, particularly for colon cancer where butyrate concentrations can be raised by simple oral probiotic intake of butyric acid-producing Lactobacilli.

Acknowledgements

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