

# Induction of Apoptosis by the Medium-Chain Length Fatty Acid Lauric Acid in Colon Cancer Cells due to Induction of Oxidative Stress

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## Key Words

Medium-chain fatty acid · Short-chain fatty acid · Colorectal cancer · Caco-2 · IEC-6 · Apoptosis · Redox · Cell cycle · Glutathione · Reactive oxygen species

## Abstract

**Background:** Fatty acids are classified as short chain (SCFA), medium chain (MCFA) or long chain and hold promise as adjunctive chemotherapeutic agents for the treatment of colorectal cancer. The antineoplastic potential of MCFA remains underexplored; accordingly, we compared the MCFA lauric acid (C12:0) to the SCFA butyrate (C4:0) in terms of their capacity to induce apoptosis, modify glutathione (GSH) levels, generate reactive oxygen species (ROS), and modify phases of the cell cycle in Caco-2 and IEC-6 intestinal cell lines. **Methods:** Caco-2 and IEC-6 cells were treated with lauric acid, butyrate, or vehicle controls. Apoptosis, ROS, and cell cycle analysis were determined by flow cytometry. GSH availability was assessed by enzymology. **Results:** Lauric acid induced apoptosis in Caco-2 ( $p < 0.05$ ) and IEC-6 cells ( $p < 0.05$ ) compared to butyrate. In Caco-2 cells, lauric acid reduced GSH availability and generated ROS compared to butyrate ( $p < 0.05$ ). Lauric acid reduced Caco-2 and IEC-6 cells in G0/G1 and arrested cells in the S and G2/M phases. Lauric

acid induced apoptosis in IEC-6 cells compared to butyrate ( $p < 0.05$ ). Butyrate protected IEC-6 cells from ROS-induced damage, whereas lauric acid induced high levels of ROS compared to butyrate. **Conclusion:** Compared to butyrate, lauric acid displayed preferential antineoplastic properties, including induction of apoptosis in a CRC cell line.

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## Introduction

Scientific evidence describing the potential preventative and chemotherapeutic properties of fatty acids is increasing for the treatment of intestinal cancers, especially colorectal cancer (CRC) [1, 2]. Fatty acids such as butyrate, docosahexaenoic acid (DHA), and conjugated linoleic acid (CLA) have demonstrated the capacity to induce apoptosis in Caco-2, HT-29, and SW620 CRC cell lines [1, 3]. Fatty acids, therefore, could hold promise as potential adjunctive chemotherapeutic agents.

Fatty acids are bioactive molecules classified by their carbon atom chain length as short chain (SCFA;  $<C8:0$ ), medium chain (MCFA;  $C8:0-14:0$ ), or long chain (LCFA;  $>C16:0-9$ ) [4–7]. The differing carbon atom chain length classifications (short, medium, and long) are be-

lieved to be a critical factor influencing their apoptotic capacity [1, 2, 8]. The SCFA butyrate has demonstrated the capacity to induce apoptosis by: inhibiting histone deacetylase activity, inducing cell cycle arrest, promoting differentiation, activating NF- $\kappa$ B, downregulating  $\alpha\beta_1$ , modifying glucose availability, and inducing caspase activation in CRC cell lines [1, 2, 9–12]. LCFA have also demonstrated apoptotic properties, with palmitic acid,  $\alpha$ -linoleic acid, CLA, and DHA inducing apoptosis in a range of CRC cell lines, including Caco-2, HTC-116, and COLO 205 cell lines. This is believed to be a consequence of lipid peroxidation, modification of cell cycle phases, induction of p21, an increase in the proapoptotic gene NAG-1, and altered P13 kinase and p38 MAPK pathways [13–17].

Cancer chemotherapy can induce apoptotic cell death and increased oxidative stress in transformed cells [18]. This is often due to reduced levels of the major antioxidant glutathione (reduced form: GSH) or increased levels of reactive oxygen species (ROS) [19]. Manipulation of this system holds promise for cancer treatment [20, 21]. The cellular redox state of a cell is a tightly regulated coupled system and a key modulator of intestinal cell homeostasis, regulating the balance between cell proliferation and apoptosis [19]. The major regulators of redox balance in a cell are the redox couplet GSH/GSSH (reduced and oxidized GSH, respectively) [19, 22]. Imbalance in this system can result in oxidative stress, leading not only to the initiation of diseases including diabetes, cardiovascular disease, and cancers of the skin, breast, and stomach but also, paradoxically, to the induction of apoptosis in neoplastic cells [19, 23, 24].

Exogenous manipulation of intracellular GSH levels has been demonstrated to increase the sensitivity of neoplastic cells to chemotherapy and radiotherapy [25]. However, few studies have investigated the potential role of fatty acids in modulation of the intracellular redox system and regulation of apoptosis in both transformed and nontransformed intestinal epithelial cells [19, 23, 26]. While several studies on the role of SCFA and LCFA in GSH modulation and regulation of apoptosis have been undertaken, no such studies have been reported on MCFA. MCFA could potentially combine the apoptotic properties of SCFA and LCFA.

We investigated the MCFA lauric acid (C12:0) for its capacity to modify the redox state of CRC (Caco-2) and nontransformed (IEC-6) intestinal cell lines by evaluating the bioavailability of the antioxidant GSH, the downstream effects on phases of the cell cycle, generation of ROS, and the resultant induction of apoptosis.

## Materials and Methods

### Materials

Caco-2 and IEC-6 cell lines were obtained from the American Type Culture Collection (ATCC®) (HTB-37™) (CRL-1592™) (Manassas, Va., USA). Dulbecco's modified Eagle's medium (DMEM), Glutamax™, penicillin/streptomycin (10,000 units of penicillin and 10,000  $\mu$ g of streptomycin), and  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free phosphate-buffered saline (PBS) were purchased from Gibco®, Invitrogen (Australia). Heat-inactivated fetal bovine serum (HI-FBS), sodium lauric acid (NaLa), sodium butyrate (NaB), fatty acid-free bovine serum albumin (FAF-BSA), propidium iodide, and RNase (DNase free) were all purchased from Sigma-Aldrich (Castle Hill, N.S.W., Australia). Annexin-V fluorescein isothiocyanate (FITC) was obtained from BioSource, Invitrogen (Australia) and 2',7'-dichlorodihydrofluorescein diacetate (H2-DCF-DA) was obtained from Molecular Probes™ (Invitrogen, Australia). Glutathione reductase and L-glutathione (GSH) were obtained from Sigma-Aldrich. All tissue culture hardware was CELLSTAR products purchased from Greiner Bio-One (Frickenhausen, Germany).

### Cell Culture

The Caco-2 and IEC-6 cell lines were determined to be mycoplasma negative via polymerase chain reaction undertaken at the Institute of Medical and Veterinary Science, Australia. Both cell lines were cultured in complete DMEM containing 2 mM Glutamax with 10% HI-FBS and penicillin/streptomycin (73.5 units/ml, 73.5  $\mu$ g/ml) in 75-cm<sup>2</sup> tissue culture flasks and maintained in a humidified atmosphere at 37°C in 5% CO<sub>2</sub>.

### Experimental Protocol

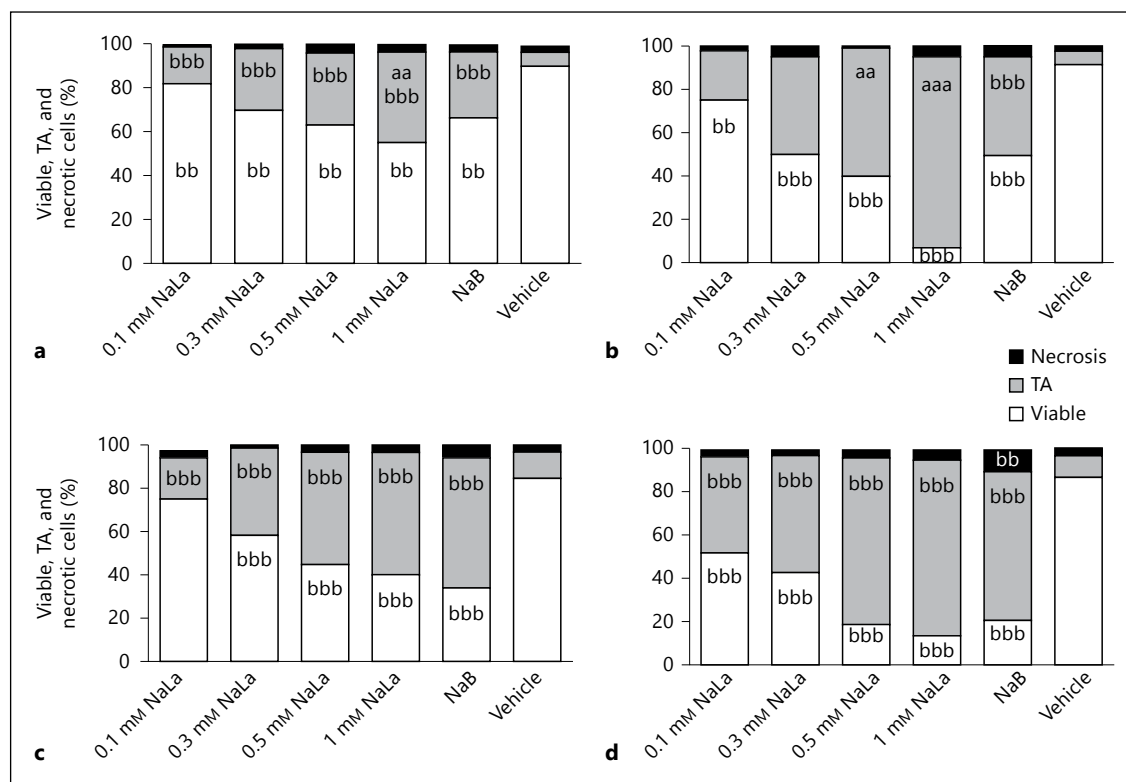
For the cytotoxic dose response, the apoptotic index, cell cycle analysis, and GSH availability, both cell lines were harvested by enzymatic dissociation and seeded into 24-well tissue culture plates at  $2.5 \times 10^5$  and  $1.5 \times 10^5$  cells/ml, respectively. After 24 h of incubation, the medium was replaced with 1 ml of complete DMEM containing freshly prepared (50 mM) NaLa, conjugated to 0.4% FAF-BSA [27] to final concentrations of 0.1, 0.3, 0.5, and 1 mM and compared to NaB (5 mM) with 50  $\mu$ l of 0.4% FAF-BSA as a vehicle control and incubated in a humidified atmosphere at 37°C in 5% CO<sub>2</sub> and, after enzymatic dissociation, assayed at 24, 48, 72, and 96 h. All experimental studies were undertaken in triplicate and measured in duplicate.

### Flow Cytometric Analysis of Apoptosis Induced by Lauric Acid

Apoptosis was measured using a standard fluorescence-activated cell sorting (FACS) assay by annexin V FITC and propidium iodide staining as per Matthews et al. [26]. The Caco-2 and IEC-6 cell lines were incubated with NaLa, NaB, and vehicle controls as per the experimental design to assess the apoptotic index. Ten thousand events were collected for all cytometric analyses on a FACScan™ flow cytometer (Becton Dickinson Biosciences, San Jose, Calif., USA) and analyzed using BD CellQuest Pro™ software. Cells were gated into viable, early/late apoptosis, and necrotic cells against standard controls.

### Cell Cycle Analysis

Cell cycle analysis was undertaken as previously described by Matthews et al. [26]. Caco-2 and IEC-6 cells were cultured in 5% FCS for 24 h prior to NaLa, NaB, and vehicle control incubation as



**Fig. 1.** Caco-2 cells treated with increasing concentrations of NaLa and cell viability, TA, and necrosis compared to NaB (5 mM) and the vehicle control over 24 (a), 48 (b), 72 (c), and 96 h (d). Data are presented as means  $\pm$  SEM (n = 3). Statistical significance be-

tween treatment groups is denoted as: <sup>aa</sup> p < 0.01 (NaLa vs. NaB), <sup>aaa</sup> p < 0.0001 (NaLa vs. NaB), <sup>bb</sup> p < 0.01 (NaLa or NaB vs. vehicle control), and <sup>bbb</sup> p < 0.0001 (NaLa or NaB vs. vehicle control).

per the experimental protocol. Twenty thousand events were collected on a FACScan (BD). The phases of the cell cycle were categorized into G0/G1, S, and G2/M and analyzed using BD CellQuest Pro software.

#### Determination of GSH Availability and ROS Detection

##### GSH Availability

Assessment of GSH availability was undertaken as previously described by Matthews et al. [26]. Caco-2 and IEC-6 cell lines were incubated with NaLa, NaB, and vehicle controls as per the experimental design.

##### ROS Detection

A modified assay from Alexandre et al. [28] and Traver et al. [29] was employed to detect ROS. Caco-2 and IEC-6 cells were incubated with NaLa, NaB, and vehicle controls as per the experimental design. A final concentration of 5  $\mu$ M H<sub>2</sub>-DCF-DA was used. Twenty thousand events were collected on a FACScan (BD). The resulting difference in fluorescence intensity was compared to vehicle controls and cell counts adjusted to  $5 \times 10^5$  cells/ml.

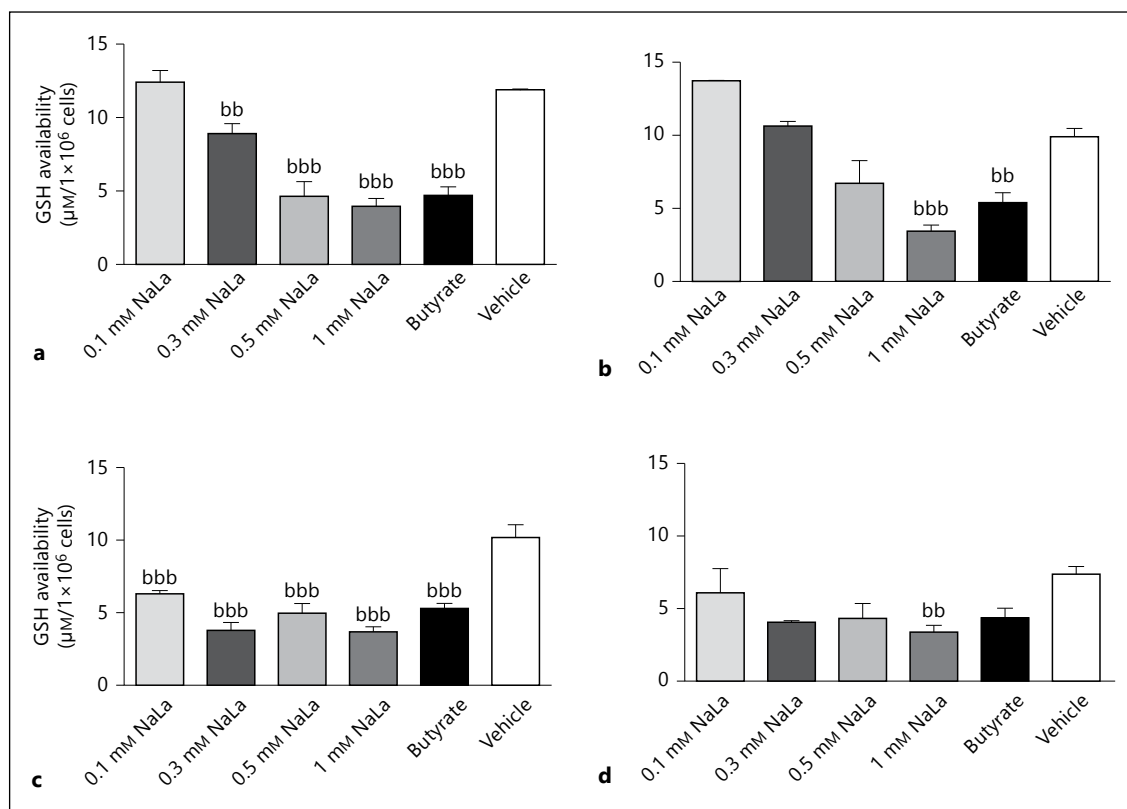
##### Statistics

All results are expressed as means  $\pm$  SEM of groups and were compared using Student's t test or one-way ANOVA with the Bonferroni post hoc test. p < 0.05 was considered statistically significant.

## Results

### A Cytotoxic Dose of Lauric Acid Significantly Reduced Caco-2 Cell Viability Compared to Butyrate

After 24 h of treatment with NaLa, Caco-2 cell viability was not significantly reduced compared to NaB; however, NaLa (0.3, 0.5, and 1 mM) ( $70.8 \pm 7.4\%$ ,  $63.4 \pm 5.9\%$ , and  $55.1 \pm 7.5\%$ , respectively) significantly reduced cell viability when compared to vehicle controls ( $90.1 \pm 5.6\%$ ) (p < 0.01). NaB ( $66.5 \pm 8.0\%$ ) also significantly reduced cell viability compared to vehicle controls (p < 0.01) (fig. 1a). A dose-dependent reduction in cell viability was demonstrated after 48 h of treatment with NaLa (0.5 and 1 mM) ( $40.8 \pm 6.4\%$  and  $7.0 \pm 4.3\%$ ) compared to NaB (5 mM) ( $44.5 \pm 2.4\%$ ) (p < 0.01 and p < 0.0001) and vehicle controls ( $91.4 \pm 3.0\%$ ). NaLa (0.1 and 0.3 mM) ( $76.5 \pm 3.5\%$  and  $50.0 \pm 8.8\%$ ) and NaB ( $44.5 \pm 2.4\%$ ) reduced cell viability compared to vehicle controls ( $91.4 \pm 3.0\%$ ) (p < 0.01, p < 0.0001, and p < 0.0001) (fig. 1b). NaLa continued to reduce cell viability at 72 and 96 h postincubation compared to NaB and ve-



**Fig. 2.** GSH availability in Caco-2 cells treated with increasing concentrations of NaLa (0.1, 0.3, 0.5, and 1 mM) and NaB (5 mM) and compared to the vehicle control over 24 (a), 48 (b), 72 (c) and 96 h (d). Data are presented as means  $\pm$  SEM (n = 3). Statistical signifi-

cance between treatment groups is denoted as: <sup>bb</sup> p < 0.01 (NaLa/NaB vs. vehicle control) and <sup>bbb</sup> p < 0.0001 (NaLa/NaB vs. vehicle control).

hicle controls [(0.5 and 1 mM) ( $44.8 \pm 9.8\%$  and  $40.1 \pm 8.8\%$ ) and ( $18.6 \pm 3.9\%$  and  $13.6 \pm 5.3\%$ ) ( $p < 0.0001$ )] (fig. 1c, d).

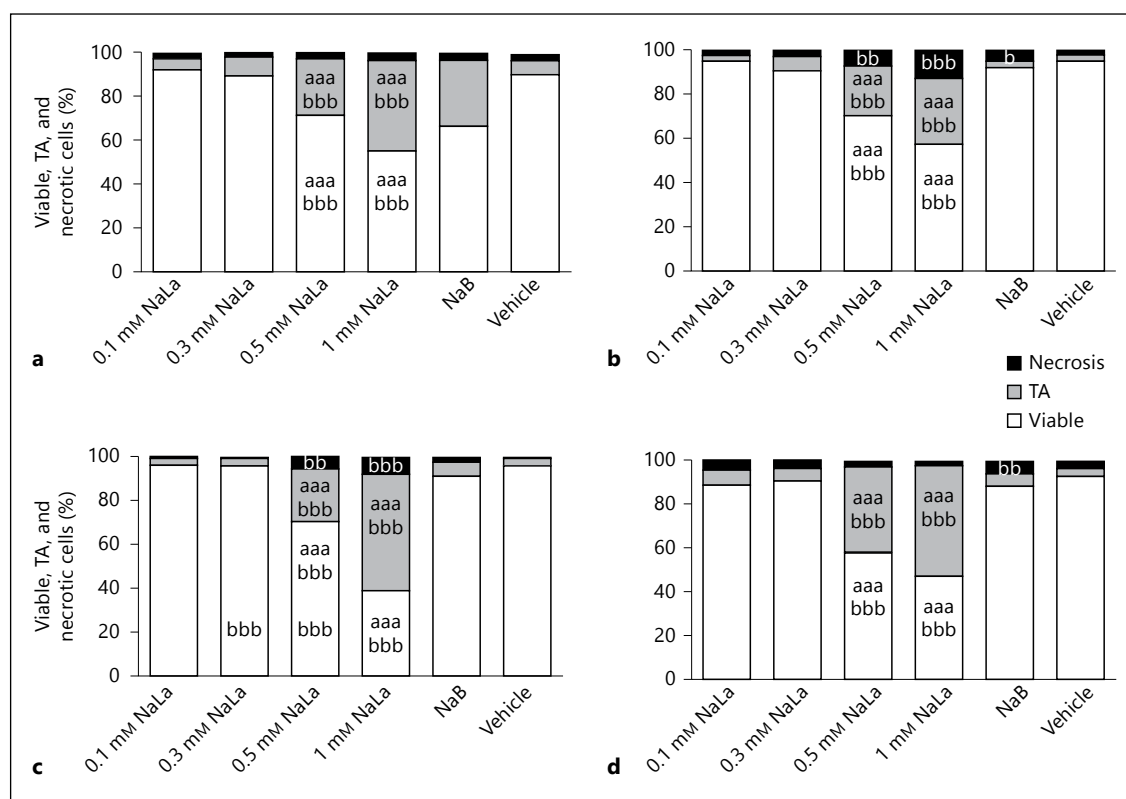
#### *Lauric Acid Induced Apoptosis in the Caco-2 Cell Line*

At 24 h post-NaLa treatment (1 mM) ( $41.5 \pm 3.5\%$ ) the proportion of Caco-2 cells in total apoptosis (TA) (TA = early + late apoptosis) was significantly increased compared to NaB ( $66.5 \pm 5.7\%$ ) ( $p < 0.01$ ). All concentrations of NaLa (0.1, 0.3, 0.5, and 1 mM) ( $16.9 \pm 3.3\%$ ,  $28.1 \pm 2.5\%$ ,  $32.8 \pm 3.0\%$ , and  $41.5 \pm 3.5\%$ ) and NaB ( $66.5 \pm 5.7\%$ ) induced a significantly higher percentage of cells undergoing TA compared to vehicle controls ( $p < 0.01$ ,  $p < 0.0001$ ,  $p < 0.0001$ ,  $p < 0.0001$ , respectively) (fig. 1a). After a further 48 h of treatment, NaLa (1 mM) ( $88.0 \pm 5.6\%$ ) induced a higher percentage of cells in TA compared to NaB ( $45.4 \pm 3.2\%$ ) ( $p < 0.0001$ ). NaLa, at concentrations of 0.3 mM and 0.5 mM ( $45.0 \pm 5.8\%$  and  $59.1 \pm 6.7\%$ ), and NaB ( $45.4 \pm 3.2\%$ ) also significantly increased the TA compared to vehicle controls ( $p < 0.0001$ )

(fig. 1b). From 72 to 96 h posttreatment, the rate of TA induced in Caco-2 cells remained constant and was not significantly different between NaLa (0.1, 0.3, 0.5, and 1 mM) and NaB. However, all NaLa and NaB induced significant TA compared to vehicle controls ( $p < 0.0001$ ; fig. 2c, d). Over the 96-hour assay period, NaLa (1 mM) induced an increase in Caco-2 cells ( $67.6 \pm 10.0\%$ ) in TA compared to NaB-treated Caco-2 cells ( $49.3 \pm 7.0\%$ ) ( $p < 0.05$ ). A significant difference in the induction of necrosis was noted at 96 h postincubation with 5 mM NaB compared to the vehicle control ( $p < 0.01$ ).

#### *Lauric Acid Significantly Reduced IEC-6 Cell Viability*

To ascertain whether NaLa or NaB altered the viability of normal intestinal epithelial cells, IEC-6 cells were treated with NaLa (0.1, 0.3, 0.5, and 1 mM) and NaB and compared to the vehicle control. After 24 h, NaLa (0.5 and 1 mM) ( $71.5 \pm 4.1\%$  and  $60.4 \pm 5.2\%$ ) reduced IEC-6 cell viability significantly compared to NaB ( $87.8 \pm 6.7\%$ ) ( $p < 0.01$ ) and vehicle controls ( $91.4 \pm 5.5\%$ ) ( $p < 0.05$ ) (fig. 3a).



**Fig. 3.** IEC-6 cells treated with increasing concentrations of NaLa and cell viability, TA, and necrosis compared to NaB (5 mM) and the vehicle control over 24 (a), 48 (b), 72 (c), and 96 h (d). Data are presented as means  $\pm$  SEM (n = 3). Statistical significance be-

tween treatment groups is denoted as: <sup>aaa</sup>  $p < 0.0001$  (NaLa vs. NaB), <sup>b</sup>  $p < 0.05$  (NaLa or NaB vs. vehicle control), <sup>bb</sup>  $p < 0.01$  (NaLa or NaB vs. vehicle control), and <sup>bbb</sup>  $p < 0.0001$  (NaLa or NaB vs. vehicle control).

At 48 h posttreatment, IEC-6 cell viability was significantly reduced compared to NaLa (0.5 and 1 mM) ( $70.3 \pm 7.1\%$  and  $57.4 \pm 6.3\%$ ), NaB ( $92.0 \pm 4.3\%$ ) ( $p < 0.05$  and  $p < 0.0001$ , respectively), and vehicle controls ( $95.0 \pm 5.5\%$ ) ( $p < 0.05$  and  $p < 0.0001$ , respectively) (fig. 3b). At 72 h posttreatment NaLa (0.5 and 1 mM) reduced cell viability significantly compared to NaB ( $p < 0.0001$ ). NaLa (0.1, 0.3, 0.5, and 1 mM) and NaB reduced cell viability significantly compared to vehicle controls ( $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.0001$ ,  $p < 0.0001$ , and  $p < 0.0001$ , respectively) (fig. 3c). At 96 h posttreatment, only NaLa (1 mM) significantly reduced cell viability compared to NaB and vehicle controls ( $p < 0.0001$ ) (fig. 3d).

#### Lauric Acid Induced Apoptotic Cell Death in IEC-6 Cells

To ascertain whether treatment with NaLa or NaB induced apoptosis or necrosis on normal gut epithelia, the experimental conditions were reproduced in the IEC-6 cell line evaluating induction of cell death. At 24–96 h post-

treatment with NaLa (0.5 and 1 mM), IEC-6 cell viability was significantly reduced compared to NaB and vehicle controls due to the preferential induction of apoptosis ( $p < 0.0001$ ). NaB did not induce apoptosis at any assay time point compared to vehicle controls (fig. 3a–d). A significantly higher level of necrosis was noted at 48 h posttreatment with NaLa (0.5 and 1 mM) and NaB compared to the vehicle control ( $p < 0.001$ ,  $p < 0.0001$ , and  $p < 0.05$ , respectively) and once again at 72 h posttreatment with NaLa (0.5 and 1 mM) compared to the vehicle control ( $p < 0.01$  and  $p < 0.0001$ ). In the final assay period of 96 h, NaB induced a significant higher number of cells undergoing necrosis compared to the vehicle control ( $p < 0.01$ ) (fig. 3b–d).

#### In Caco-2 Cells, Lauric Acid Reduced G0/G1 and Arrested Cells in the S and M Phases

Over the 72-hour assay period, NaLa at the higher concentrations of 0.5 and 1 mM tended to reduce the mean percentage of Caco-2 cells ( $36.3 \pm 1.7\%$  and  $37.9 \pm 5.6\%$ ) in the G0/G1 phase compared to NaB ( $42.7 \pm 0.9\%$ ); how-



**Table 1.** Cell cycle results for Caco-2 cells following treatment with NaLa compared to NaB

Cell cycle phase	Fatty acid (mM)	Duration of treatment (h)		
		24	48	72
G0/G1	0.1 NaLa	48.7±0.1 <sup>a,e</sup>	41.7±0.1 <sup>e</sup>	40.1±0.5
	0.3 NaLa	37.6±0.8 <sup>d</sup>	48.0±0.4 <sup>d</sup>	48.1±0.4
	0.5 NaLa	30.0±0.4 <sup>b,f</sup>	40.3±0.1 <sup>e</sup>	38.2±0.2 <sup>b</sup>
	1 NaLa	32.0±0.6 <sup>a,e</sup>	49.3±0.4 <sup>d</sup>	32.6±0.4 <sup>c</sup>
	5.0 NaB	40.9±0.2 <sup>h</sup>	43.9±1.0 <sup>h</sup>	42.4±0.1 <sup>i</sup>
	Vehicle control	62.1±0.3	63.7±0.3	64.1±0.1
S	0.1 NaLa	21.0±0.1 <sup>d</sup>	23.7±0.3 <sup>d</sup>	20.8±0.1 <sup>a,f</sup>
	0.3 NaLa	24.4±0.1 <sup>d</sup>	23.6±0.3 <sup>d</sup>	22.8±0.2 <sup>a,e</sup>
	0.5 NaLa	29.0±0.2 <sup>e</sup>	24.4±0.1 <sup>d</sup>	25.0±0.1 <sup>c,e</sup>
	1 NaLa	35.9±0.4 <sup>a,i</sup>	21.9±0.1 <sup>d</sup>	32.6±0.4 <sup>c,e</sup>
	5.0 NaB	24.1±0.3	21.8±0.9 <sup>g</sup>	18.6±1.0 <sup>h</sup>
	Vehicle control	15.6±0.2	12.7±0.9	13.8±0.4
M	0.1 NaLa	32.3±0.1 <sup>e</sup>	32.9±0.1 <sup>a</sup>	24.2±0.1 <sup>b</sup>
	0.3 NaLa	34.1±0.1 <sup>b,e</sup>	22.4±0.8	29.5±0.5 <sup>d</sup>
	0.5 NaLa	31.1±0.1 <sup>b,e</sup>	26.2±0.3 <sup>b</sup>	34.2±0.2 <sup>b,e</sup>
	1 NaLa	32.2±0.1 <sup>d</sup>	24.7±0.3	43.0±0.9 <sup>b,e</sup>
	5.0 NaB	28.4±0.5 <sup>g</sup>	26.2±0.2 <sup>g</sup>	30.1±0.3 <sup>g</sup>
	Vehicle control	18.3±0.4	15.7±0.2	19.6±0.1

Data are presented as mean percentages ± SEM (n = 3). Cell cycle phases are compared between NaLa, NaB, and the vehicle control. Statistical significance between treatment groups is denoted as: <sup>a</sup> p < 0.05 (NaLa vs. NaB), <sup>b</sup> p < 0.01 (NaLa vs. NaB), <sup>c</sup> p < 0.0001 (NaLa vs. NaB), <sup>d</sup> p < 0.05 (NaLa vs. vehicle control), <sup>e</sup> p < 0.01 (NaLa vs. vehicle control), <sup>f</sup> p < 0.0001 (NaLa vs. vehicle control), <sup>g</sup> p < 0.05 (NaB vs. vehicle control), <sup>h</sup> p < 0.01 (NaB vs. vehicle control), and <sup>i</sup> p < 0.0001 (NaB vs. vehicle control).

ever, this was not significant (p > 0.05). Caco-2 cells were arrested in the S phase following NaLa treatment. Once again, higher doses of NaLa (0.5 and 1 mM) arrested a significantly higher percentage of Caco-2 cells (26.2 ± 0.7% and 26.8 ± 1.6%) compared to NaB (21.5 ± 0.8%) (p < 0.05). Cells were also arrested in the M phase after treatment with NaLa (1 mM) (39.4 ± 2.3%) compared to NaB (29.6 ± 0.7%) (p < 0.0001). All fatty acids reduced Caco-2 cells in G0/G1 and arrested cells in the S and M phases compared to vehicle controls (G0/G1, 62.7 ± 0.6; S, 14.9 ± 0.7, and M, 18.7 ± 1.3%), NaLa (0.3, 0.5, and 1 mM), and NaB for all phases (p < 0.0001).

#### Lauric Acid Reduced G0/G1 and Arrested the S and M Phases in IEC-6 Cells

IEC-6 cells treated with NaLa had a reduced mean percentage of cells in the G0/G1 phase with treatment of 0.5 and 1 mM (60.4 ± 0.06% and 53.6 ± 1.8%) compared to NaB (75.4 ± 0.2%; p < 0.001 and p < 0.0001) and the ve-

**Table 2.** Cell cycle results for IEC-6 cells following treatment with NaLa compared to NaB

Cell cycle phase	Fatty acid (mM)	Duration of treatment (h)		
		24	48	72
G0/G1	0.1 NaLa	68.0±0.7	80.9±0.1 <sup>b</sup>	83.2±0.3
	0.3 NaLa	59.0±0.6 <sup>e</sup>	71.8±0.1	64.7±0.4 <sup>f</sup>
	0.5 NaLa	56.4±0.1 <sup>a,e</sup>	68.6±0.05	56.3±0.1 <sup>c,f</sup>
	1 NaLa	56.9±0.1 <sup>a,e</sup>	53.4±0.1 <sup>c,e</sup>	50.5±0.1 <sup>c,f</sup>
	5.0 NaB	68.0±0.3	72.1±0.01	86.0±0.3
	Vehicle control	78.6±0.4	78.3±0.3	87.5±0.05
S	0.1 NaLa	9.5±0.7	6.2±0.03	6.7±0.1
	0.3 NaLa	14.5±0.2 <sup>b,e</sup>	5.8±0.1	6.1±0.2 <sup>a</sup>
	0.5 NaLa	14.2±0.1 <sup>b,e</sup>	7.5±0.5 <sup>a,d</sup>	12.7±0.2 <sup>b,e</sup>
	1 NaLa	13.5±0.4 <sup>a,d</sup>	8.8±0.1 <sup>b,e</sup>	13.5±0.3 <sup>b,e</sup>
	5.0 NaB	8.1±0.2	5.2±0.1	8.6±0.3
	Vehicle control	6.4±0.2	5.4±0.2	7.4±0.5
M	0.1 NaLa	13.3±1.3	13.6±0.06	8.4±0.03 <sup>d</sup>
	0.3 NaLa	18.2±0.5 <sup>b</sup>	19.5±0.2	17.8±0.4 <sup>b,d</sup>
	0.5 NaLa	20.7±0.1 <sup>c,d</sup>	21.3±0.4 <sup>b,d</sup>	31.7±0.1 <sup>c,f</sup>
	1 NaLa	22.3±0.1 <sup>c,e</sup>	31.1±0.1 <sup>c,e</sup>	36.2±0.1 <sup>c,f</sup>
	5.0 NaB	14.5±0.2	18.2±0.2	13.0±0.3 <sup>g</sup>
	Vehicle control	15.6±0.5	16.5±0.3	10.0±0.06

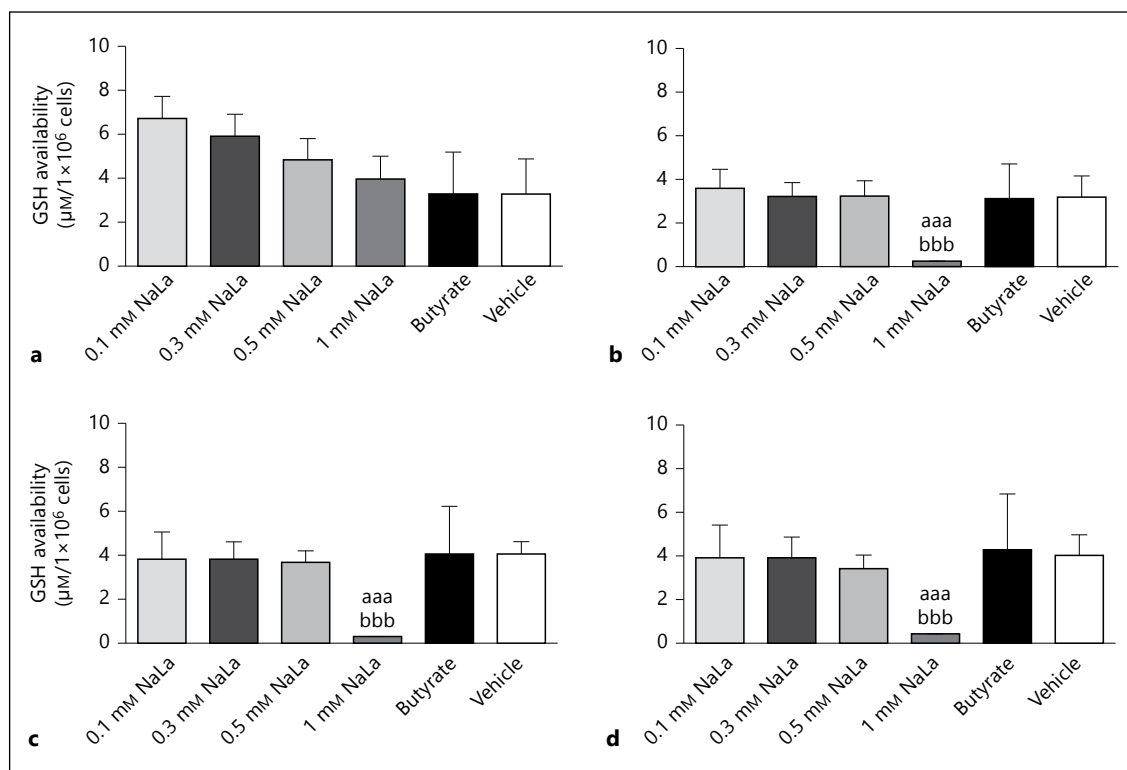
Data are presented as mean percentages ± SEM (n = 3). Cell cycle phases are compared between NaLa, NaB, and the vehicle control. Statistical significance between treatment groups is denoted as: <sup>a</sup> p < 0.05 (NaLa vs. NaB), <sup>b</sup> p < 0.01 (NaLa vs. NaB), <sup>c</sup> p < 0.0001 (NaLa vs. NaB), <sup>d</sup> p < 0.05 (NaLa vs. vehicle control), <sup>e</sup> p < 0.01 (NaLa vs. vehicle control), <sup>f</sup> p < 0.0001 (NaLa vs. vehicle control), <sup>g</sup> p < 0.05 (NaB vs. vehicle control).

hicle control (81.5 ± 0.3%) (p < 0.0001) over the 72-hour assay period. NaLa at doses of 0.3, 0.5, and 1 mM (8.8 ± 0.2%, 11.5 ± 0.2%, and 11.9 ± 0.3%) arrested IEC-6 cells in the S phase compared to NaB (7.3 ± 0.1%; p < 0.05). The same NaLa doses arrested a mean percentage of cells compared to vehicle controls (6.4 ± 0.3%; p < 0.05). Once again, NaLa at does of 0.5 and 1 mM arrested a mean percentage of IEC-6 cells (24.5 ± 0.4% and 29.8 ± 0.1%) in the M phase compared to NaB (15.2 ± 0.2%) (p < 0.05) and vehicle controls (14.0 ± 0.2%) (p < 0.05). NaB did not significantly reduce the G0/G1, S, or M phase at any time point in IEC-6 cells (table 2).

#### Reduction of GSH Availability and Generation of ROS

##### GSH Availability

After 24 h, the GSH concentration was significantly reduced in NaLa-treated cells (0.3, 0.5, and 1 mM) (p < 0.01, p < 0.0001, and p < 0.0001) and NaB-treated Caco-2 cells (p < 0.0001) compared to the vehicle control. At 48 h



**Fig. 4.** GSH availability in IEC-6 cells treated with increasing concentrations of NaLa (0.1, 0.3, 0.5, and 1 mM) and NaB (5 mM) and compared to the vehicle control over 24 (a), 48 (b), 72 (c), and

96 h (d). Data are presented as means  $\pm$  SEM ( $n = 3$ ). Statistical significance between treatment groups is denoted as: <sup>aaa</sup>  $p < 0.0001$  (NaLa vs. NaB) and <sup>bbb</sup>  $p < 0.0001$  (NaLa/NaB vs. vehicle control).

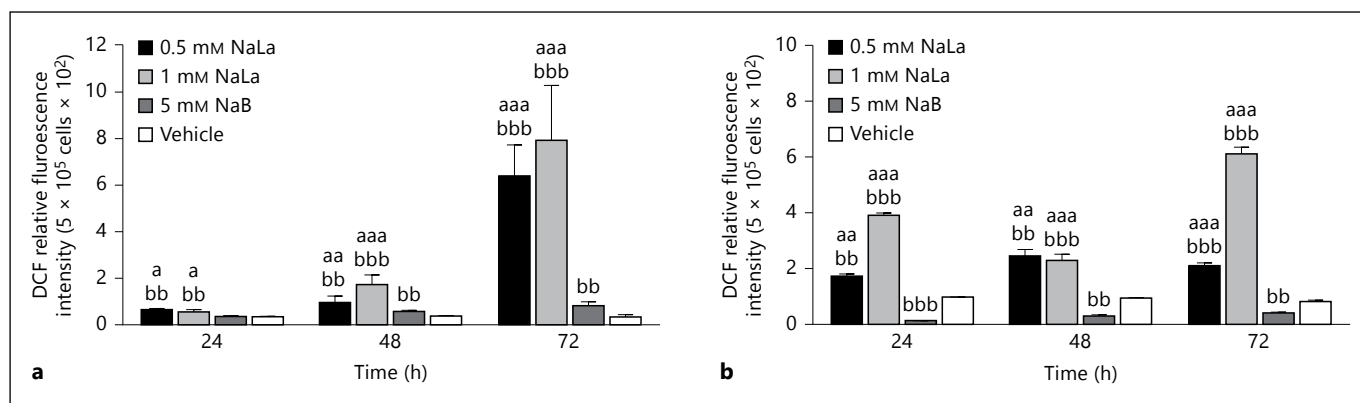
posttreatment, only 1 mM NaLa ( $p < 0.0001$ ) and NaB ( $p < 0.001$ ) significantly reduced GSH levels compared to the vehicle control. Interestingly, after 72 h of incubation with NaLa (0.1, 0.3, 0.5, and 1 mM) (all  $p < 0.0001$ ) and NaB ( $p < 0.0001$ ) compared to the vehicle control. At the last time point of measurement, only NaLa at 1 mM ( $p < 0.001$ ) led to significantly reduced levels of GSH compared to the vehicle control. GSH levels of the vehicle control at 96 h ( $7.4 \pm 0.6 \mu\text{M}/1 \times 10^6$  cells) were less than those of vehicle controls at 72 h ( $10.2 \pm 0.9 \mu\text{M}/1 \times 10^6$  cells), which may have had an impact on the significance of the results at 96 h (fig. 2a–d). GSH available in the IEC-6 cell line did not significantly change following treatment with either NaLa or NaB at 24 h postincubation. However, at the time points of 48–96 h the NaLa dose of 1 mM significantly reduced GSH levels compared to 0.5 mM NaLa and NaB ( $p < 0.0001$ ; fig. 4a–d).

#### ROS Generation

In Caco-2 cells, at 24 h posttreatment NaLa (0.5 and 1 mM) induced a significant increase in ROS compared to NaB ( $p < 0.05$  and  $p < 0.05$ ) and vehicle controls ( $p < 0.01$

and  $p < 0.01$ ). However, no significant increase was detected between NaB and vehicle controls ( $p > 0.05$ ). ROS generation at 48 h was significantly increased for NaLa (0.5 and 1 mM) compared to NaB ( $p < 0.01$  and  $p < 0.0001$ ) and vehicle controls ( $p < 0.01$  and  $p < 0.0001$ ). NaB induced a significant increase in ROS compared to vehicle controls ( $p < 0.01$ ). In the final 72 h of analysis, the increase in ROS generation continued significantly between NaLa (0.5 and 1 mM) and NaB and vehicle controls ( $p < 0.0001$ ,  $p < 0.0001$ , and  $p < 0.0001$ ). NaB also induced a significant difference in ROS generation compared to the vehicle control ( $p < 0.01$ ) (fig. 5a).

In IEC-6 cells, at 24 h posttreatment NaLa (0.5 and 1 mM) induced a significant increase in ROS ( $p < 0.0001$  and  $p < 0.01$ , respectively) compared to NaB and vehicle controls ( $p < 0.05$ ). Interestingly, NaB generated lower levels of ROS than the vehicle controls at all time points ( $p < 0.0001$ ,  $p < 0.001$ , and  $p < 0.05$ ). ROS levels at 48 h after treatment with NaLa (0.5 and 1 mM) were significantly increased compared to NaB and vehicle controls ( $p < 0.0001$ ,  $p < 0.0001$ , and  $p < 0.0001$ ). At the final assay point of 72 h, once again, NaLa (0.5 and 1 mM) increased



**Fig. 5. a, b** ROS detected in Caco-2 cells (**a**) and IEC-6 cells (**b**) treated with increasing concentrations of NaLa (0.1, 0.3, 0.5, and 1 mM) as detected by flow cytometric analysis of oxidation of the probe dichlorodihydrofluorescein diacetate to DCF compared to NaB (5 mM) and the vehicle control over 24, 48, and 72 h. Data are

presented as means  $\pm$  SEM ( $n = 3$ ). Statistical significance between treatment groups is denoted as: <sup>a</sup>  $p < 0.05$  (NaLa vs. NaB), <sup>aa</sup>  $p < 0.01$  (NaLa vs. NaB), <sup>aaa</sup>  $p < 0.0001$  (NaLa vs. NaB), <sup>bb</sup>  $p < 0.01$  (NaLa/NaB vs. vehicle control), and <sup>bbb</sup>  $p < 0.0001$  (NaLa/NaB vs. vehicle control).

ROS compared to NaB and vehicle controls ( $p < 0.0001$ ,  $p < 0.0001$ , and  $p < 0.0001$ ) (fig. 5b). The reduced levels of ROS induced by NaB compared to vehicle controls suggest a protective effect of this SCFA.

## Discussion

The current study primarily investigated the antineoplastic potential of the MCFA lauric acid on a CRC cell line in vitro. It was hypothesized that this (C12:0) MCFA acid, with a carbon atom chain length positioned between the SCFA (<C8:0) and the LCFA (>C16:ω3–9) would augment its antineoplastic properties. It was shown that lauric acid induced apoptosis in the Caco-2 cell line, proposed to be due to a reduction in the levels of antioxidant glutathione (reduced) (GSH), concomitant with a reduction of G0/G1 and arrest of the S and M cell cycle phases, together with increased generation of ROS.

In the evaluation of new chemotherapeutic bioactive agents, few in vitro investigations have evaluated the deleterious side effects of the target agents in nontransformed cell lines. This study used the rat small intestinal cell line IEC-6 as a model of normal intestinal epithelial cells. It was demonstrated that lauric acid at concentrations of 0.5 and 1 mM induced considerable cell death due to apoptosis in IEC-6 cells. This was associated with a reduction of GSH, a reduction of cells in the G0/G1 phase, an increase in cells in the S and M phases, and an increase in generation of ROS. Once again, in this cell line higher doses of LA induced significant cell death which did not

correspond with a reduced availability of GSH. Therefore, this high level of apoptosis may have been associated with reductions in phases of the cell cycle and increased generation of ROS independent of GSH availability. Butyrate did not induce apoptosis in the IEC-6 cell line, nor did it decrease GSH availability, although cells accumulated in the G0/G1 phase at the initial time points. Interestingly, butyrate generated lower levels of ROS than PBS control values, which is consistent with previous studies [44].

Previous studies have alluded to the cytotoxic capabilities of lauric acid over other MCFA [8, 30]. Little is known about the cytotoxic actions of lauric acid on any in vitro neoplastic cell type. However, Lima et al. [8] treated the leukemic cell lines Jurkat and Raji with increasing concentrations of lauric acid (0.005–1 mM) and determined that the optimal cytotoxic dose was greater than 0.2, although the underlying mechanism of cell death was not determined [8]. Differential rates of cell death induced by fatty acids have been proposed to be influenced by cell lineage, and the study of Lima et al. [8] did not detect differences in fatty acid-induced cytotoxicity between T and B lymphocytes. However, it has been suggested that blood-borne cancers are more sensitive to cytotoxic agents than are solid malignancies [8, 26, 31].

In the current study, levels of apoptosis induced by the cytotoxic doses of lauric acid (0.5 and 1 mM) were evaluated against the SCFA butyrate, and rates of apoptosis comparable to those published previously were induced in butyrate-treated Caco-2 cells [12, 32]. Litvak et al. [32] employed flow cytometric methods similar to those of the



current study and reported a significant increase in apoptosis in butyrate-treated Caco-2 cells [32]. This is further supported by the findings of Roy et al. [33], who demonstrated that Caco-2 cells treated with butyrate induced a 65.7% increase in apoptosis compared to negative controls, as detected by flow cytometry and further confirmed by ELISA [32, 33]. In the current study, lower concentrations of lauric acid (0.3 mM) also induced cell death compared to controls but not to butyrate. Moreover, lower lauric acid concentrations ( $\leq 0.1$  mM) did not display any cytotoxic activity.

In the current study, lauric acid induced a significantly higher mean rate of cell death (TA) compared to butyrate and, more importantly, the mechanism of cell death was determined to be due to apoptosis as opposed to necrosis. This result was surprising considering that lauric acid has previously demonstrated inflammatory responses in vivo and in vitro [34]. To date, few studies have investigated the apoptotic properties of lauric acid. However, published literature has reported that lauric acid exerts antimicrobial effects by inhibiting growth or reducing cell viability in bacterial and viral species [35–37].

Little is known about the mechanisms promoting these growth inhibitory properties and there are even fewer reports of apoptotic effects, either in vivo or in vitro, in any prokaryote or eukaryote cell type. However, two separate studies demonstrated that lauric acid treatment of Jurkat and Raji cells induced morphological changes characteristic of apoptosis which included cell shrinkage, loss of membrane integrity, increased granularity and reduction of cell viability [8]. Isolated rat colonic cells treated with LA (10 mM) induced activated cytochrome c, reduced expression of Bcl-2, activated caspases 9 and 3, and induced DNA fragmentation, indicative of apoptosis [34]. These few studies support the postulate that lauric acid can induce apoptosis both in vitro and in vivo. Due to increasing carbon atom chain lengths, this action could be comparable to the apoptotic actions of LCFA including palmitic acid (C14:0) and linoleic acid (18:2), which have previously demonstrated morphological changes characteristic of apoptosis in Caco-2 cells, although definitive rates of apoptosis were not measured [13, 14].

Modulation of the redox system underpins the induction of apoptosis, and previous studies have reported that butyrate-treated colorectal (HT-29) and gastric cancer cells (Kato III) reduced GSH levels corresponding to an increase in alkaline phosphatase activity and increased levels of apoptosis [26, 38]. Longer-chain fatty acids which may be closer in carbon atom chain length to

MCFA also reduce GSH levels in CRC cell lines [13]. To date, there are no cited reports of the effects of lauric acid on GSH levels, either in vivo or in vitro. To this end, the current study demonstrated that lauric acid (1 mM) reduced GSH levels in Caco-2 cells. However, GSH availability to the cell was not significantly reduced compared to butyrate. This is an interesting finding, as lauric acid induced a higher percentage of cells in TA compared to butyrate. Furthermore, the lower concentrations of lauric acid also reduced GSH to levels comparable to the cytotoxic dose of lauric acid without inducing high levels of apoptosis. This suggests that reduction of the intracellular availability of GSH in lauric acid-treated Caco-2 cells may not be the only contributing factor in the induction of apoptosis. GSH availability was reduced in IEC-6 cells treated with 1 mM lauric acid at 48–96 h posttreatment. This corresponds with increased levels of apoptosis. Interestingly, low doses of lauric acid initially elevated GSH levels, which then returned to control levels over the study period. Lima et al. [8] demonstrated that the fatty acid concentration influenced cytotoxicity in leukemic cell lines treated with a wide range of carbon chain atom length fatty acids which may have influenced the GSH levels in IEC-6 cells [8]. However, the redox couplet of GSH/GSSH was not determined; thus it could be argued that this was not a true measure of the redox potential. Nevertheless, in the current study, the generation of ROS was indicative of an increased oxidative state of the cell [39].

In further defining the underlying mechanism driving lauric acid-induced apoptosis, the current study demonstrated that both lauric acid and butyrate reduced G0/G1 and arrested the S and M phases. Previous studies have determined that apoptosis can also be induced in Caco-2 cells due to modification of phases of the cell cycle by redox modulation, specifically G1 to the S phase [40]. In a study by Xiang et al. [41], butyrate increased the number of cells in the M phase and decreased cells in the S phase [41]. This inconsistency with the current study was possibly due to a lower dose of butyrate (1 mM) and a shorter assay period [41]. However, Matthews et al. [1] demonstrated that butyrate (5 mM) treatment of Caco-2 cells reduced cells in G0/G1 and arrested cells in the S and G2/M phases [1]. Treatment of Caco-2 cells with longer-chain fatty acids (fish oil-based emulsions) demonstrated G2/M cycle arrest and, interestingly, cotreatment with fish oil and 5-fluorouracil further arrested cells in the S phase [42].

Increased generation of ROS is presumed to be a final consequence of imbalance of the redox system and a shift

from the reductive to the oxidative state in a cell [19,43]. In the current study, lauric acid treatment of Caco-2 cells generated ROS over the entire time course of the assay, whereas butyrate only increased during the final 96-hour assay period. In the evaluation of new chemotherapeutic bioactive agents, few in vitro investigations have evaluated deleterious side effects on nonneoplastic cell lines. The current study determined, not unexceptionally, that lauric acid at concentrations of 0.5 and 1 mM induced considerable apoptosis in IEC-6 cells. This was associated with a reduction in GSH, a reduction in the number of cells in G0/G1 and arrested cells in the S and M phases, and an increase in ROS generation. Once again, in this cell line, higher doses of lauric acid induced significant cell death which did not correspond with increased GSH availability. Therefore, this high level of apoptosis may have been associated with modification of phases of the cell cycle and increased generation of ROS independent of GSH availability. Butyrate did not induce apoptosis in the IEC-6 cell line, nor did it decrease GSH availability or modify any phase of the cell cycle. Interestingly, butyrate generated lower levels of ROS than vehicle control values, consistent with previous studies [44].

While lauric acid at higher concentrations reduced viability in normal intestinal epithelial cells, this study defined the apoptotic impact of this MCFA and its potential antineoplastic properties in a CRC cell line. Advances in targeted drug therapy, for example using microencapsulation or nanoparticles, may hold promise in the delivery of lauric acid directly to neoplastic cells, thereby reducing the cytotoxicity in normal epithelia and potentially preventing conditions such as chemotherapy-induced mucositis [45]. This initial study into the potential antineoplastic effects of MCFA represents an important first step for more intensive investigations into the specific apoptotic mechanisms of fatty acid action together with the possible augmentative and protective effects of MCFA and SCFA.

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