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Antiproliferative effect of alkylglycerols as vehicles of butyric acid on colon cancer cells

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Abbreviations: AKG, 1-O-octadecyl glycerol; DMEM, Dulbecco´s modified Eagle's medium; DMSO, dimethyl sulfoxide; D-SCAKG, 1-O-octadecyl-2,3-dibutyroilglycerol; FBS, fetal bovine serum; GC, gas chromatography; M-SCAKG, 1-O-octadecyl-2-butyroilglycerol; MTT, thiazolyl blue tetrazolium bromide; PAF, platelet-activating factor

Abstract

The anticarcinogenic activity of synthetic 1-O-octadecyl-2,3-dibutyroilglycerol (D-SCAKG) in tumor-cell line of colonocytes (SW620) was performed. The effect of the previously digested D-SCAKG under *in vitro* intestinal conditions was compared to the bioactivity of non-digested D-SCAKG. Antiproliferative activity of each individual product from digestion (1-O-octadecyl-2-butyroilglycerol; 1-O-octadecyl glycerol; butyric acid) was also performed. The impact of solubilization of lipid products within micellar structures was also tested.

The 1-O-octadecyl glycerol was the most active compound, followed by 1-O-octadecyl-2-butyroilglycerol, D-SCAKG and butyric acid. The 1-O-octadecyl glycerol and butyric acid were the only molecules that showed antiproliferative effect in absence of micelles. Digested D-SCAKG was 4-fold more effective than non-digested D-SCAKG. A synergism between 1-O-octadecyl-2-butyroilglycerol and 1-O-octadecyl glycerol was evidenced.

As summary, the synthetic D-SCAKG seems to be an interesting antitumoral lipid against colonocytes, especially after previous intestinal digestion, and mainly due to the synergism of the major products, namely 1-O-octadecyl-2-butyroilglycerol and 1-O-octadecyl glycerol. At the same time, 1-O-octadecyl-2-butyroilglycerol would constitute a stable esterified form of butyric acid for its vehiculization.

Keywords: alkylglycerols, butyric acid, lipid digestion, lipid-delivery pro-drugs, antiproliferative compounds

1. Introduction

Alkyglycerols, alkylglycerophospholipids and their derivatives, namely ether lipids, have been the subject of much attention last years because of their special physiological functions (Magnusson and Haraldsson, 2011). These glycerides contain O-alkyl or O-alk-1-enyl groups at sn-1 position. Alkylmonoacylglycerols or alkyldiacylglycerols can be also found. Ether lipids are especially abundant as major components of total lipids in liver oil of marine invertebrates and vertebrates, especially shark liver oil. In the human body, they can be found in the cells of the immune system and in human breast milk as membrane components and cellular signaling molecules (Magnusson and Haraldsson, 2011; Torres et al., 2011). As anti-tumour agents, they have been related to inhibitory growth, antimetastatic activity, anti-neoangiogenic action, and induce differentiation and apoptosis in cancer cells (Brohult et al., 1978; Deniau et al., 2011; Krotkiewski et al., 2003; Pedrono et al., 2004; Pedrono et al., 2007). Immune stimulation properties have also been attributed to these substances (Braverman and Moser, 2012; Kantah et al., 2012; Mitre et al., 2005).

Within ether lipids, one of the most popular is the platelet-activating factor (PAF), a 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine, with diverse cell mediator effects in a variety of tissues and systems, including circulation, inflammation, development and reproduction (Hanahan, 1986; Prescott et al., 2000). Other important ether lipids are 1-O-alkyl-sn-glycerols, which have been also claimed to display various beneficial effects on human health and seems to amplify the production of PAF by incorporation within the PAF precursor, namely the 1-O-alkyl-2-acyl-sn-glycero-3-phosphocholine (Himachi et al., 1997; Kantah et al., 2012). On the other hand, 1-O-alkyl-2-acylglycerols are considered of interest as analogs of diacylglycerols, which would inhibit protein kinase C. The 1-O-alkyl-2-acylgycerols are produced following cell stimulation after alkylglycerol incorporation in membrane phospholipids. These products bind but do not activates protein kinase C-ε, and compete with

diacylglycerol, which could result in cell growth arrest (Deniau et al., 2011; Heymans et al., 1987; Houk et al., 2008; Marigny et al., 2002).

Taking into account the attractive properties of these lipids, synthetic ether lipids that do not occur in nature have been prepared to find therapeutic agents (Hartvigsen et al., 2006), such as PAF-like lipids, which retain a short-chain residue at the sn-2 position other than an acetyl group (Tokumura, 1995). In this sense, it has been shown that the shorter the sn-2 chain residue the more active the PAF-like lipid (Kern et al., 1998; Tanaka et al., 1995).

Recently, Torres et al. (2009 a) synthesized structured alkyldiacylglycerols containing residual short-chain fatty acids at sn-2 and sn-3 locations by enzymatic transesterification of 1-O-octadecyl glycerol and ethyl butyrate (1-O-octadecyl-2,3-dibutyroilglycerol; D-SCAKG). Ethyl butyrate was not only chosen by the nature of "short chain residue", but also on the bioactive importance of butyrate by itself. Besides having physiological role as the main metabolic fuel for colonocytes and control of colonic inflammation, butyrate seems to interfere with the pathogenesis of diverse cancers by inhibiting cell proliferation or inducing apoptosis, such as colorectal cancer, hepatocarcinoma, leukemia, breast and prostate cancer (Avivi-Green et al., 2001; Coradini et al., 1999; Kuefer et al., 2004; Kuroiwa-Trzmielina et al., 2009; Ooi et al., 2009). However, the potential application of butyrate as antitumor agent is limited by the problem to reach enough plasma concentrations required to exert its antiproliferative/differentiating actions. Moreover, it is rapidly metabolized, showing a short half-life (Kuroiwa-Trzmielina et al., 2009; Li et al., 2009). Therefore, there is a current interest on overcoming these drawbacks in order to allow its application as therapeutic agent. Pro-drugs, such tributyrin, have been proposed as alternative of natural butyrate (Clarke et al., 2008; Heidor et al., 2012; Kuroiwa-Trzmielina et al., 2009). Torres et al. (2009 a) suggested that the vehiculization of butyric acid by alkylglycerol backbone might be also proposed as interesting butyric pro-drug, due to the double advantage of alkylglycerols as potential lipid

vehicle of butyrate and the own bioactivity of each individual compound, alkylgycerol and butyrate, within the same molecule.

Preliminary studies have been performed in order to validate the synthesized D-SCAKG molecule. As first approach, the potential bioaccessibility was assayed by simulating the intestinal digestion of the molecule by pancreatic enzymes under *in vitro* conditions, since it would be the preliminary step in case of oral intake of this pro-drug (Martin et al., 2011). It was demonstrated that the major hydrolysis product after intestinal digestion of D-SCAKG was the molecule 1-O-octadecyl-2-butyroilglycerol, together with free butyric acid, 1-O-octadecyl glycerol and minor non-digested D-SCAKG. Therefore, regardless of the short-chain as butyrate, the major digestion product would be the potential bioactive structure under the form of 1-O-alkyl-2-acylglycerol. On the other hand, regardless of the alkylglycerol backbone, the major digestion product 1-O-alkyl-2-butyroilglycerol was a stable esterified form of butyrate. This was considered a positive result since it has been stated that efficient pro-drugs of butyrate should have a sufficiently stable bond between the carrier and butyrate residue to increase its *in vivo* half-life (Coradini et al., 1999). In fact, in the performed *in vitro* intestinal digestion study, it was shown that the traditional tributyrin proposed as pro-drug of butyrate was completely hydrolyzed to free butyric acid, which would not be the desired situation in case of butyrate pro-drugs. The potential of D-SCAKG as carrier of butyrate was concluded. Furthermore, the additional bioactive ether lipid, namely 1-O-octadecyl glycerol, commonly known as batyl alcohol, was also an interesting product of the *in vitro* intestinal digestion of the molecule D-SCAKG. Batyl alcohol has been previously tested as anti-tumour lipid (Ando et al., 1972) or antiinflamatory agent (Burford and Gowdey, 1968). Therefore, all the digestion products of the synthetic D-SCAKG after *in vitro* intestinal lipolisis might be considered attractive products for bioactive properties.

Taking into account all these preliminary data, the aim of the current study was to study a potential anticarcinogenic activity of the synthetic D-SCAKG in tumor-cell line of colonocytes. To perform this study, the digested D-SCAKG under *in vitro* intestinal conditions was tested, since this last would be the more probable situation in case of oral intake of this molecule, where the hydrolysis products would be the available compounds for intestinal cells. The bioactivities of hydrolysis products of digestion were also tested individually in order to explain the obtained results properly. Furthermore, the impact of solubilization of lipid products within micellar structures of bile salts and phospholipids on bioactivities was also tested, as it would be the most probable situation after *in vitro* intestinal digestion into the lumen gut environment (Martin et al., 2011).

2. Materials and methods

2.1. Reagents and Materials

The D-SCAKG was synthesized according to a methodology previously described by our group (Torres et al., 2009 a). Trizma, maleic acid, pancreatin, bile salts, phosphatidyl choline from egg yolk, butyric acid and 1-O-octadecyl glycerol were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Hydrochloric acid, sodium sulphate anhydrous, sodium chloride, calcium chloride were from Panreac (Barcelona, Spain). N-dodecane for synthesis was purchased from Merck (Darmstadt, Germany). All solvents used were of HPLC grade from Lab-Scan (Dublin, Ireland). Human colon cancer cells (SW620) were obtained from ATCC (Rockville, MD, USA). Dulbecco´s modified Eagle's medium (DMEM), fetal bovine serum (FBS), glutamine and antibiotic-antimycotic solution were from Gibco BRL (Grand Island, NY, USA).

2.2. In vitro lipid digestion of D-SCAKG

135 The *in vitro* lipid digestion model was performed according to Martin et al. (2011). Sample (1
136 g) of D-SCAKG was mixed with 0.5 g of bile salts, 0.2 g of lecithin, 5mM $CaCl_2$, 150mM
137 NaCl and 54 mL of 1 M Trizma-maleate buffer pH 7.5. The mixture was homogenized (Ultra-
138 Turrax IKA T18) for 20 min at 7000 rpm. The homogenized was placed in a thermostatically
139 controlled vessel (37ºC) under continuous stirring by magnetic stir bar at 1000 rpm.
140 Simulation of intestinal digestion was started by addition of fresh pancreatin extract (1 g of
141 pancreatin in 6 mL of Trizma-maleate buffer pH 7.5 stirred during 10 min and centrifuged at
142 1600 x g during 15 min) and continued up to 60 min. *In vitro* digestion of each sample was
143 performed in triplicate.
144 The total lipids from samples were extracted by hexane/methyl-tert-butyl ether (50:50, v/v).
145 Furthermore, this medium was acidified by hydrochloric acid (150 mM) in order to stop the
146 enzymatic reaction and to enhance the recovery of butyric acid. n-Dodecane (10 mg) was
147 added as internal standard. The mixture was vortexed for 1 min and centrifuged for 10 min at
148 15,000 rpm. Organic phase containing separated lipids was collected and anhydrous sodium
149 sulfate was added before further analysis.
150 Hydrolysis products were determined according to Torres et al. (2009 b) by gas
151 chromatography (GC) (Hewlett-Packard 5890 series II) with on-column injection using a 7 m
152 5% phenyl methyl silicone capillary column (Quadrex Corporation, New Haven, CT), 0.25
153 µm i.d. A deactivated column of 12 cm 530 µm i.d. was used as pre-column. Injector and
154 detector temperature was 43ºC and 360ºC, respectively. The temperature program was as
155 follows: starting at 40ºC and then heating to 250ºC at 42ºC min^{-1} with 10 min hold, followed
156 by heating from 250ºC to 325ºC at 7.5ºC min^{-1} with 30 min hold. Helium was used as carrier
157 gas at a pressure of 5.2 psi. The peaks were computed using GC chemstation software
158 (Agilent Technologies, Santa Clara, CA) and quantified according to the internal standard n-
159 dodecane.

160 The lipid composition of the digested D-SCAKG is shown in Table 1. This composition was
161 subsequently simulated by mixing the individual compounds at the proper proportions
162 according to Table 1 in order to perform the *in vitro* cell assays. Previously, the individual
163 hydrolysis product 1-O-octadecyl-2-butyroilglycerol (M-SCAKG) from *in vitro* digestion of
164 D-SCAKG was purified by semi-preparative HPLC, since it cannot be found as commercial
165 standard. The rest of hydrolysis products, namely butyric acid and 1-O-octadecyl glycerol
166 (AKG) were commercial standards.
167
168 *2.3. In vitro colonocytes assay*
169 2.3.1. Cell culture
170 Human colon cancer cells SW620 were cultured in DMEM supplemented with 10% FBS,
171 2mM glutamine and 1% of antibiotic-antimycotic solution (containing 10,000 units/mL of
172 penicillin base, 10,000 µg/mL of streptomycin base and 25,000 ng/mL of amphotericin B).
173 They were maintained under standard conditions of temperature (37ºC), humidity (95%) and
174 carbon dioxide (5%).
175
176 2.3.2. Cell viability assay
177 The antiproliferative activity of D-SCAKG and its derivates was measured by MTT (thiazolyl
178 blue tetrazolium bromide) assay. Cells in exponential growth phase were placed in 96-well
179 plates, using 6000 cells per well in a final volume of 200 µL. After 24h incubation, cells were
180 treated by replacing medium with new culture medium (blank wells), medium containing
181 different concentrations of the tested products, or micelles suspensions. Stocks of all
182 treatment compounds, both for mixing directly with DMEM as well as for preparing micelles,
183 were prepared in Ethanol.

Cell viability was determined at the same time of treatment and after 48h. To determine the number of viable cells, 20 µL of MTT solution (5 mg/mL in PBS) was added to each well and incubated during 3 h, subsequently the medium was removed and 200 µL of DMSO (dimethyl sulfoxide) was added in order to lyse the cells and resuspend the formazan (MTT metabolic product). Quantities of formazan product, which is directly related to the number of viable cells, were measured at 560 nm using a scanning spectrophometer microplate reader (UVM 340 Biochrom, Cambridge). Concentration values corresponding to the parameters IC50 (50% cell viability inhibition), GI50 (50% growth inhibition), TGI (total growth inhibition) and LC50 (50% cell death) were calculated according to the NIH definitions using a logistic regression.

2.3.3. Preparation of Mixed Micellar and Control Solution

Micelles were prepared with egg yolk lecithin and sodium taurocholate at final concentrations of 150µM and 0.5mM respectively. Micelle solutions were prepared by first adding appropriate amounts of each treatment compounds to a glass vial containing 0.5ml of hexane. For control solutions, a vial with 0.5ml of hexane was prepared. Vials content were dried using a nitrogen evaporator (N-Evap 111, Organomation Associates). Then 0.23 mg of lecithin dissolved in 0.575 mI of hexane was added to each vial and dried again in the same conditions; this was followed by the addition of 500ul of 2mM taurocholate prepared in ethanol and another evaporation step. Finally, dried content was resuspended in 1ml of DMEM without supplements and mixed with 1ml of complete DMEM, and 200ul of this mixture was added to each well.

3. Results and discussion

In order to analyze the potential anticarcinogenic activity of the synthetic D-SCAKG in tumoral colonocytes, the bioactivity of digested D-SCAKG under *in vitro* intestinal conditions was evaluated. As a first approach, the antiproliferative activity of each individual lipid product (D-SCAKG, M-SCAKG, AKG and butyric acid) was determined as reference. In addition, since the formation of micellar structures might constitute the most usual situation under intestinal digestion into the lumen gut environment (Martin et al., 2011), the impact of solubilization of lipid products within micellar structures of bile salts and phospholipids on the observed bioactivity was also tested, showing a noticeable different when determining the effect of the lipid product under free form (without micellization) or previously solubilized within mixed micelles.

As it can be observed in Figure 1, when compounds were added without previous inclusion in micellar structures of bile salts and phospholipids, the AKG and butyric acid were the only molecules that showed a bioactive effect at the assayed concentrations in tumor colonocytes, whereas lack of activity was detected for D-SCAKG and M-SCAKG. Under the free forms, the greatest activity was found for AKG (μM dose range) compared to butyric acid (mM dose range), accordingly with previous studies concerning other alkylglycerols or butyric prodrugs (Reynolds et al., 2000; Wächtershäuser and Stein, 2000). Concerning the specific case of AKG, the obtained bioactivity of this compound was not in agreement with the previous study of Deniau et al. (2011), because these authors reported a lack of antitumour effect of the same AKG (1-O-octadecyl glycerol) compared to other individual alkyglycerols. Nevertheless, such study was performed under *in vivo* conditions in solid grafted tumours in mice after oral administration, and studying the effect on tumour volume, spleen weight, or lung metastases, so the conditions would not be comparable to the present study. On the contrary, Ando et al. (1972) showed a positive anti-tumor activity of batyl alcohol (1-O-octadecyl glycerol) in Ehrlich carcinoma in mice, but such activity was worse than that for alkylglycerols with

shorter alkyl chains. The divergences between our and previous studies show the interest on go in depth on the bioactivities of individual alkylglycerols as potential antitumor agents, because such information is scarce.

When lipid compounds were added under a previous micellization with bile salts and phospholipids, bioactivities were also found for D-SCAKG and M-SCAKG. Again, the best antiproliferative effect was found for AKG, and the bioactivities for D-SCAKG and M-SCAKG were found at higher concentrations, being similar between them (Figure 1). Control assays of micelles without inclusion of the experimental compounds showed a lack of effect of such vehicles.

With the aim of further evaluating the bioactivity of the different conditions, growth inhibition and promotion of cell death were analyzed by IC50, GI50, TGI and LC50 calculation in those conditions in which a bioactive effect was initially observed. As it is shown in Table 2, AKG displayed greater bioactivity than D- and M-SCAKG even without including in micelles. Under micellization, AKG resulted 5-fold more active than the other two lipid compounds (48.3 in cell sensitivity to AKG with respect to 282.02 and 237.04 for D- and M-SCAKG respectively, and 65.38 in citostaticity with respect to 385.28 and 395.09). Regarding cell death, AKG was the only compound displaying an efficient cytotoxic effect at reached concentrations (85.9uM for LC50).

According to these results, it seems that the potential bioactivity of ether lipids in tumor colonocytes would be different depending on the esterification of the alkylglycerol backbone. Thus, either alkylmonoacylglycerols or alkyldiacylglycerols would be worse antiproliferative agents than non-esterified alkylglycerols. Nevertheless, it should be remarked that alkylmonoacylglycerols would be slightly better than alkyldiacylglycerols, since a value for LC50 was found for the former (M-SCAKG), whereas lack of LC50 dose was detected for alkyldiacylglycerols (D-SCAKG) when they were included under the form of mixed micellar

structures. The observed differences could be related to the own molecular size of the different structures of alkylglycerols. This reason could be also linked to the different solubility and/or polarity of the molecules. Thus, the presence of two hydroxyl groups in the case of AKG compared to the one or two acyl chains of butyrate in the case of M-SCAKG or D-SCAKG, respectively, might lead to better solubility of the AKG molecule in the aqueous media of the cell culture, allowing an easier access to colonocytes.

Regardless on the different bioactivities between compounds, the vehiculization of ether lipids within micellar structures enhanced their antiproliferative effects. Thus, whereas lack of activity was observed for D-SCAKG and M-SCAKG after direct addition to the media, a significant antiproliferative effect was observed under micellar solubilization conditions. Accordingly, AKG-mediated inhibition of tumoral cell growth and promotion of cell death were enhanced by micellization. These results would be in agreement with the expected situation at intestinal lumen, where most lipid compounds need the support of mixed micelles to be dispersed in the aqueous media to cross the unstirred water layer close to enterocytes previous to absorption (Christensen et al., 1995; Ramirez et al., 2001). At this respect, the non-esterified form of AKG might allow an easier formation of mixed micelles compared to D-SCAKG and M-SCAKG due to the availability of the two hydroxyl groups of the molecule, which confers emulgent-like properties, allowing its dispersion into mixed micelles and in turn the accessibility to colonocytes.

The obtained results contributed to the knowledge of the bioactivity of ether lipids, because to the best of our knowledge, previous studies showing differences between non-esterified and esterified alkyglycerols as antiproliferative agents by *in vitro* tumor-cell models have not been found. Furthermore, the effect of micellization on the efficacy of these compounds on intestinal cells has not been previously reported.

Nevertheless, it is important to remark that the results found for esterified forms of AKG might be considered an artifact in the particular case of an oral intake of D-SCAKG. This is because, as most esterified lipids, these are partially hydrolyzed at intestinal tract by digestive lipases. Subsequently, the hydrolysis products are those major included in mixed micelles of bile salts and phospholipids to allow their access to intestinal cells. In fact, according to Table 1 and in agreement to Martin et al (2011), the simulation of intestinal digestion of D-SCAKG produced a stable form of esterified butyric acid as 1-O-octadecyl-2-butyroilglycerol (M-SCAKG) as major hydrolysis product (> 50%). Free AKG (> 20%) and free butyric acid (20%) were also released as bioactive compounds of interest, together with minor residual D-SCAKG. Due to that, it was approached that the bioactivity of this mixture of compounds solubilized in micellar structures, namely M-SCAKG, AKG, butyric acid and residual D-SCAKG, should be tested for validate the bioactivity of the synthetic D-SCAKG in tumor colonocytes, in order to simulate the potential situation after oral intake of this lipid.

The bioactivity of digested D-SCAKG compared to that of non-digested D-SCAKG in shown in Figure 2. Furthermore, both compounds were also compared in terms of growth inhibition, citostaticity and citotoxicity in Table 3.a. In addition, data for each individual component of the mixture according to the proportions of Table 1 was estimated (Table 3.b). As it is shown in Figure 2 and Table 3.a, the digested D-SCAKG was around 4-fold more effective than the former non-digested D-SCAKG. Therefore, this result suggests that the digestion of D-SCAKG would improve efficiency on its antiproliferative activity of this synthetic lipid after oral intake. Of note, taking into account the dose of each individual compound in the mixture of hydrolysis products (Table 3.b), much lower effective concentrations were found for all compounds when they were mixed (Table 3.b) than when they were tested individually (Table 2). Especially attractive was the finding that the bioactivity of AKG, the most effective compound individually, was greater when this compound was added together with D-

SCAKG, M-SCAKG and butyric acid. Thus, 86 μM of AKG was necessary to reach the LC50 value when it was added individually to cells (Table 2), whereas such value was reduced by three times (26 μM LC50) when AKG was included in the micellized media together with the rest of hydrolysis compounds (Table 3.b). Similarly, IC50, GI50 and TGI reduced by twice. Taking into account that AKG was only present in the digested mixture at around 20% of total lipids (Table 1), whereas it was 100% when added individually, these results suggest a potential synergism between the hydrolysis products, promoting the efficacy of the individual compounds and in turn the total efficacy of the mixture.

In order to study the interaction of compounds further additional experiments were performed by the combination of the lipid products at the same proportion that obtained after *in vitro* digestion, except that butyric acid was not added to the media. This lead to a lipid mixture mainly formed by the major hydrolysis products M-SCAKG and AKG (68 and 29 g/100 g total lipids, respectively) . As shown in Figure 3, the combination of M-SCAKG and AKG showed almost the same results as found for the combination of all compounds, suggesting that the active compounds of the digested D-SCAKG were its major hydrolysis products, namely M-SCAKG and AKG. Furthermore, this result confirmed a synergism between M-SCAKG and AKG, since the antiproliferative dose of both compounds in the mixture was lower than the dose needed for each individual compounds separately (Table 3.b and Table 2). These results suggest two promising activities for the major hydrolysis product of D-SCAKG after *in vitro* intestinal digestion, namely M-SCAKG. On one hand, it would contribute together with AKG to a potential antitumoral activity against tumor colonocytes, and, on the other hand, M-SCAKG would constitute a stable esterified form of butyric acid for its vehiculization. This is because efficient pro-drugs of butyrate should have a sufficiently stable bond between the carrier and butyrate residue to increase its in vivo half-life (Coradini et al., 1999).

Additional studies might be conducted to further explain the mechanism of synergism between M-SCAKG and AKG. At this respect, concerning shark liver oil as the main natural source of alkylglycerols, it has been suggested that the bioactivity of such oil might be likely mediated by the presence of alkylglycerols (Pedrono et al., 2004), but the interaction with other lipid species in the oil (free alkylglycerols, mono- and di-esterified alkylglycerols with different fatty acids, and acylglycerols) could not be rejected. One explanation of the observed results of synergism might be related to the formation of micellar structures, both in the number or size of micelles. This is because in general, it has been shown that higher surface, extension and swelling of micelles with hydrolysis products enhance the inclusion of the rest of lipophilic compounds, and in turns their bioaccessibility (Porter and Charman, 2001). Similarly, it might be possible that the combination of M-SCAKG and AKG led to the formation of higher number and/or surface of micellar structures, compared to the sole presence of each individual component, allowing the access to colonocytes. According to this approach, further studies were performed by counting the number of micellar structures by light microscope from micelles preparations of AKG, M-SCAKG or their combination. However, no conclusive results were found (data not shown).

On the other hand, it would be also interesting to elucidate whether the observed responses might be also related to a synergistic action of M-SCAKG and AKG on the own colonocytes once the compounds were absorbed. In this regard is important to remark that the M-SCAKG molecule showed the typical structure of those analogues of diacylglycerols, namely 1-O-alkyl-2-acylglycerols, which have been pointed out as inhibitors of protein kinase C (Deniau et al., 2011; Marigny et al., 2002; Heymans et al., 1987). Additionally, once inside the enterocite the own forms of alkylglycerols would be derived to other diverse bioactive forms under the fast dynamic of lipid remodeling, leading to alternate fatty acids at sn-2 location

356 different to butyric acid, via the deacylation-reacylation pathway of the Lands's cycle (Perez
357 et al., 2006).
358 In summary, the synthetic D-SCAKG seems to be an interesting antitumoral lipid against
359 colonocytes, especially after previous intestinal digestion, after solubilization in mixed
360 micelles, and mainly due to the synergism of the major products, namely 1-O-octadecyl-2-
361 butyroilglycerol and 1-O-octadecyl glycerol. At the same time, 1-O-octadecyl-2-
362 butyroilglycerol would constitute a stable esterified form of butyric acid for its vehiculization.
363 The current study also showed the importance of considering the digestive hydrolysis process
364 of lipid compounds on the evaluation of bioactivities at intestinal level, as well as the
365 importance of vehiculize the lipid products as it would be at intestinal lumen, namely under
366 the form of mixed micelles. Nevertheless, further studies would be necessary in order to
367 explain the observed effects related to the intracellular metabolism once the lipid compounds
368 were absorbed.
369

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376

376 **References**

377 Ando, K., Kodama, K., Kato, A., Tamura, G., Arima, K., 1972. Antitumor activity of glyceryl
378 ethers. Cancer Res. 32, 125-129.

379 Avivi-Green, C., Polak-Charcon, S., Madar, Z., Schwartz, B., 2001. Apoptosis cascade
380 proteins are regulated in vivo by high intracolonic butyrate concentration: correlation with
381 colon cancer inhibition. Oncol. Res. Featuring Preclinical and Clinical Cancer Therapeutics
382 12, 83-95.

383 Braverman, N. E., Moser, A. B., 2012. Functions of plasmalogen lipids in health and disease.
384 BBA-Mol. Basis Dis. 1822, 1442-1452.

385 Brohult, A., Brohult, J., Brohult, S., 1978. Regression of tumour growth after administration
386 of alkoxyglycerols. Acta Obstet. Gynecol. Scand. 57, 79-83.

387 Burford, R. G., Gowdey, C. W., 1968. Anti-inflammatory activity of alkoxyglycerols in rats.
388 Arch. Int. Pharmacodyn. Ther. 173, 56-70.

389 Christensen, M. S., Høy, C. E., Becker, C. C., Redgrave, T. G., 1995. Intestinal absorption
390 and lymphatic transport of eicosapentaenoic (EPA), docosahexaenoic (DHA), and decanoic
391 acids: dependence on intramolecular triacylglycerol structure. Am. J. Clin. Nutr. 61, 56-61.

392 Clarke, J. M., Topping, D. L., Bird, A. R., Young, G. P., Cobiac, L., 2008. Effects of high-
393 amylose maize starch and butyrylated high-amylose maize starch on azoxymethane-induced
394 intestinal cancer in rats. Carcinogenesis 29, 2190-2194.

395 Coradini, D., Pellizzaro, C., Miglierini, G., Daidone, M. G., Perbellini, A., 1999. Hyaluronic
396 acid as drug delivery for sodium butyrate: Improvement of the anti-proliferative activity on a
397 breast-cancer cell line. Int. J. Cancer 81, 411-416.

398 Deniau, A. L., Mosset, P., Le Bot, D., Legrand, A. B., 2011. Which alkylglycerols from shark
399 liver oil have anti-tumour activities? Biochimie 93, 1-3.
400 Hartvigsen, K., Ravandi, A., Harkewicz, R., Kamido, H., Bukhave, K., Hølmer, G., Kuksis,
401 A., 2006. 1-O-alkyl-2-(ω-oxo) acyl-sn-glycerols from shark oil and human milk fat are
402 potential precursors of PAF mimics and GHB. Lipids 41, 679-693.
403 Hanahan, D. J. (1986). Platelet activating factor: a biologically active phosphoglyceride.
404 Annu. Rev. Biochem. 55, 483-509.
405 Heidor, R., Festa Ortega, J., de Conti, A., Prates Ong, T., Salvador Moreno, F., 2012.
406 Anticarcinogenic actions of tributyrin, a butyric acid prodrug. Curr. Drug Targets 13, 1720-
407 1729.
408 Heymans, F., Da Silva, C., Marrec, N., Godfroid, J. J., Castagna, M., 1987. Alkyl analogs of
409 diacylglycerol as activators of protein kinase C. FEBS letters 218, 35-40.

410 Hichami, A., Duroudier, V., Leblais, V., Vernhet, L., Le Goffic, F., Ninio, E., Legrand, A.,
411 1997. Modulation of Platelet-Activating-Factor Production by Incorporation of Naturally
412 Occurring 1-O-Alkylglycerols in Phospholipids of Human Leukemic Monocyte-Like THP-1
413 Cells. Eur. J. Biochem. 250, 242-248.
414 Houck, K. L., Fox, T. E., Sandirasegarane, L., Kester, M., 2008. Ether-linked diglycerides
415 inhibit vascular smooth muscle cell growth via decreased MAPK and PI3K/Akt signaling.
416 Am. J. Physiol.-Heart C. 295, H1657-H1668.
417 Kantah, M. K., Wakasugi, H., Kumari, A., Carrera-Bastos, P., Palmieri, B., Naito, Y.,
418 Catanzaro, R., Kobayashi, R., Marotta, F., 2012. Intestinal immune-potentiation by a purified
419 alkylglycerols compound. Acta bio-medica: Atenei Parmensis, 83, 36.
420 Kern, H., Volk, T., Knauer-Schiefer, S., Mieth, T., Rüstow, B., Kox, W. J., Schlame, M.,
421 1998. Stimulation of monocytes and platelets by short-chain phosphatidylcholines with and
422 without terminal carboxyl group. BBA-Lipid. Lipid Met. 1394, 33-42.
423 Krotkiewski, M., Przybyszewska, M., Janik, P., 2003. Cytostatic and cytotoxic effects of
424 alkylglycerols (Ecomer). Med. Sci. Mon. 9, 131-135.
425 Kuefer, R., Hofer, M. D., Altug, V., Zorn, C., Genze, F., Kunzi-Rapp, K., Gschwend, J. E.,
426 2004. Sodium butyrate and tributyrin induce in vivo growth inhibition and apoptosis in human
427 prostate cancer. Brit. J. Cancer 90, 535-541.

428 Kuroiwa-Trzmielina, J., de Conti, A., Scolastici, C., Pereira, D., Horst, M. A., Purgatto, E.,

429 Moreno, F. S., 2009. Chemoprevention of rat hepatocarcinogenesis with histone deacetylase
430 inhibitors: efficacy of tributyrin, a butyric acid prodrug. Int. J. Cancer 124, 2520-2527.
431 Li, Y., Le Maux, S., Xiao, H., McClements, D. J., 2009. Emulsion-based delivery systems for
432 tributyrin, a potential colon cancer preventative agent. J. Agric. Food Chem. 57, 9243-9249.
433 Magnusson, C. D., Haraldsson, G. G., 2011. Ether lipids. Chem. Phys. Lipids 164, 315-340.

434 Marigny, K., Pedrono, F., Martin-Chouly, C. A. E., Youmine, H., Saiag, B., Legrand, A. B.,

435 2002. Modulation of endothelial permeability by 1-O-alkylglycerols. Acta Physiol. Scand.

436 176, 263-268.
437 Martín, D., Morán-Valero, M. I., Señoráns, F. J., Reglero, G., Torres, C. F., 2011. In vitro
438 intestinal bioaccessibility of alkylglycerols versus triacylglycerols as vehicles of butyric acid.
439 Lipids 46, 277-285.
440 Mitre, R., Etienne, M., Martinais, S., Salmon, H., Allaume, P., Legrand, P., Legrand, A. B.,
441 2005. Humoral defence improvement and haematopoiesis stimulation in sows and offspring

by oral supply of shark-liver oil to mothers during gestation and lactation. Brit. J. Nutr. 94, 753-762.

Ooi, C. C., Good, N. M., Williams, D. B., Lewanowitsch, T., Cosgrove, L. J., Lockett, T. J., Head, R. J., 2009. Efficacy of butyrate analogues in HT-29 cancer cells. Clin. Exp. Pharmacol. P. 37, 482-489.

Pedrono, F., Martin, B., Leduc, C., Le Lan, J., Saiag, B., Legrand, P., Moulinoux, J., Legrand, A.B., 2004. Natural alkylglycerols restrain growth and metastasis of grafted tumors in mice. Nutr. Cancer 48, 64-69.

Pedrono, F., Moulinoux, J. P., Legrand, A. B., 2007. 1-O-Alkylglycerols reduce the stimulating effects of bFGF on endothelial cell proliferation in vitro. Cancer Letters 251, 317-322.

Perez, R., Matabosch, X., Llebaria, A., Balboa, M.A., Balsinde, J., 2006. Blockade of arachidonic acid incorporation into phospholipids induces apoptosis in U937 promonocytic cells. J. Lipid Res. 47, 484-491.

Porter, C. J., Charman, W. N., 2001. In vitro assessment of oral lipid based formulations. Adv. Drug Deliver. Rev. 50, S127-S147.

Prescott, S. M., Zimmerman, G. A., Stafforini, D. M., McIntyre, T. M., 2000. Platelet-activating factor and related lipid mediators. Annu. Rev. Biochem. 69, 419-445.

Ramirez, M., Amate, L., Gil, A., 2001. Absorption and distribution of dietary fatty acids from different sources. Early Hum. Dev. 65, S95-S101.

462 Reynolds, S., Cederberg, H., Chakrabarty, S., 2000. Inhibitory effect of 1-O (2 methoxy)
463 hexadecyl glycerol and phenylbutyrate on the malignant properties of human prostate cancer
464 cells. Clin. Exp. Metastasis 18, 309-312.
465 Tanaka, T., Tokumura, A., Tsukatani, H., 1995. Platelet-activating factor (PAF)-like
466 phospholipids formed during peroxidation of phosphatidylcholines from different foodstuffs.
467 Biosci. Biotech. Bioch. 59, 1389-1393.
468 Tokumura, A., 1995. A family of phospholipid autacoids: occurrence, metabolism and
469 bioactions. Prog. Lipid Res. 34, 151-184.
470 Torres, C., Martin, D., Torrelo, G., Casado, V., Fernandez, O., Tenllado, D., Vazquez, L.,
471 Moran-Valero, M.I., Reglero, G., 2011. Lipids as delivery systems to improve the biological
472 activity of bioactive ingredients. Curr. Nutr. Food Sci. 7, 160-169.
473 Torres, C. F., Vázquez, L., Señoráns, F. J., Reglero, G., 2009 a. Enzymatic synthesis of short-
474 chain diacylated alkylglycerols: A kinetic study. Process Biochem. 44, 1025-1031.
475 Torres, C. F., Tenllado, D., Senorans, F. J., Reglero, G., 2009 b. A versatile GC method for
476 the analysis of alkylglycerols and other neutral lipid classes. Chromatographia 69, 729-734.
477 Wächtershäuser, A., Stein, J., 2000. Rationale for the luminal provision of butyrate in
478 intestinal diseases. Eur. J. Nutr. 39, 164-171.
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Figure captions

Figure 1. Antiproliferative effect of free and micellized form of the different lipid compounds. Bioactivity was determined on sw620 human colon cancer cells by the MTT (thiazolyl blue tetrazolium bromide) assay. Butyric acid (d) was not included in the study of micellization since it is solubilized in the aqueous media. Data represent the mean +/- s.e.m.of at least 2 independent experiments, each performed in quadruplicate. AKG, 1-O-octadecyl glycerol; D-SCAKG, 1-octadecyl-2,3-dibutyroilglycerol; M-SCAKG, 1-O-octadecyl-2-butyroilglycerol.

Figure 2. Antiproliferative effect of digested D-SCAKG on sw620 human colon cancer cells. To test this compound, D-SCAKG, M-SCAKG, AKG and butyric acid were combined at the same proportion that obtained after in vitro digestion. AKG and their esterified forms were included in micelles, and butyric acid was added after micelle resuspension in culture medium. Data represents the mean +/- s.e.m.of at least 2 independent experiments each performed in quadruplicate. For comparative reasons, data are expressed at molar concentration of D-SCAKG equivalent. It the case of digested D-SCAKG equivalents, they were estimated from the weight of lipid mixture of hydrolysis products, which corresponded to a weight of non-digested D-SCAKG and, in turn to a specific molar concentration of D-SCAKG.

AKG, 1-O-octadecyl glycerol; D-SCAKG, 1-octadecyl-2,3-dibutyroilglycerol; M-SCAKG, 1-O-octadecyl-2-butyroilglycerol

Figure 3. Comparision of antiproliferative effect of digested D-SCAKG with a preparation in which butyric is excluded. M-SCAKG and AKG constitute the 97% of total composition. All hydrolisis products were combined at the same proportion that obtained after in vitro digestion. AKG and their esterified forms were included in micelles, while butyric acid (when used) was added after micelle resuspension in culture medium. Data represent the mean +/- s.e.m.of at least 2 independent experiments each performed in quadruplicate. For comparative reasons, data are expressed at molar concentration of D-SCAKG equivalent, as explained in figure 2.

AKG, 1-O-octadecyl glycerol; D-SCAKG, 1-octadecyl-2,3-dibutyroilglycerol; M-SCAKG, 1-O-octadecyl-2-butyroilglycerol

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Table 1. Lipid composition of digested D-SCAKG by *in vitro* intestinal model

	g/100 g total lipids
D-SCAKG[a]	2.2
M-SCAKG[b]	54.4
AKG[c]	23.4
Butyric acid	20.0

[a] D-SCAKG (1-octadecyl-2,3-dibutyroilglycerol); [b] M-SCAKG (1-O-octadecyl-2-butyroilglycerol); [c] AKG (1-O-octadecyl glycerol)

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Table 2. IC50, GI50, TGI and LC50 parameters of the esterified forms of AKG, as well as non-esterified AKG and butyric acid. These parameters were calculated from the results obtained in viability assays according to the NIH definitions using a logistic regression. Values are represented as the mean+/- s.e.m. of at least 2 independent experiments each performed in quadruplicate. NLO: No Lethality Observed.

	D-SCAKG			M-SCAKG			AKG						BUTYRIC		
	micelles			micelles			medium			micelles			medium		
	Conc. (µM)	±	s.e.m.	Conc. (µM)	±	s.e.m.	Conc. (µM)	±	s.e.m.	Conc. (µM)	±	s.e.m.	Conc. (µM)	±	s.e.m.
IC50	282,02	±	65,26	237,04	±	44,46	101,87	±	7,80	48,73	±	7,16	3791,95	±	1,13
GI50	150,52	±	28,89	178,79	±	31,58	64,13	±	1,84	26,94	±	6,94	1527,46	±	0,02
TGI	385,28	±	31,15	395,09	±	34,06	116,04	±	20,30	65,38	±	14,74	4551,65	±	1,33
LC50	NLO			732,76	±	20,93	130,56	±	25,57	85,90	±	23,14	11949,70	±	2,55

Table 3. IC50, GI50, TGI and LC50 parameters of digested AKG on sw620 human colon cancer cells. (a) Digested media versus non-digested media. Values were calculated as mean values +/- s.e.m. of at least 2 independent experiments each performed in quadruplicate. For comparative reasons, data are expressed at molar concentration of D-SCAKG equivalent. In the case of digested D-SCAKG equivalents, they were estimated from the weight of lipid mixture of hydrolysis products, which corresponded to a weight of non-digested D-SCAKG and, in turn to a specific molar concentration of D-SCAKG. (b) Calculated IC50, GI50, TGI and LC50 for each product of digestion taking into account their respective proportion in the mixture of digested D-SCAKG.

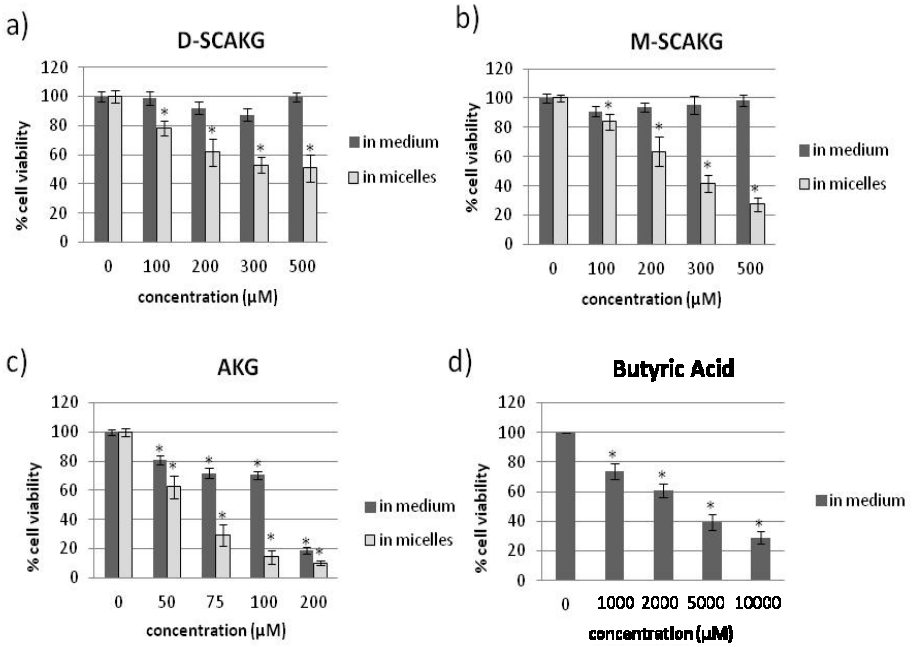
a)

	Digested D-SAKG (in micelles)			D-SCAKG (in micelles)		
	(uM D-AKG equivalent)		s.e.m.	Conc. (uM)		s.e.m.
IC50	74,0	±	13,7	282,02	±	65,26
GI50	50,8	±	12,0	150,52	±	28,89
TGI	79,8	±	15,4	385,28	±	31,15
LC50	85,2	±	9,7	no data	±	

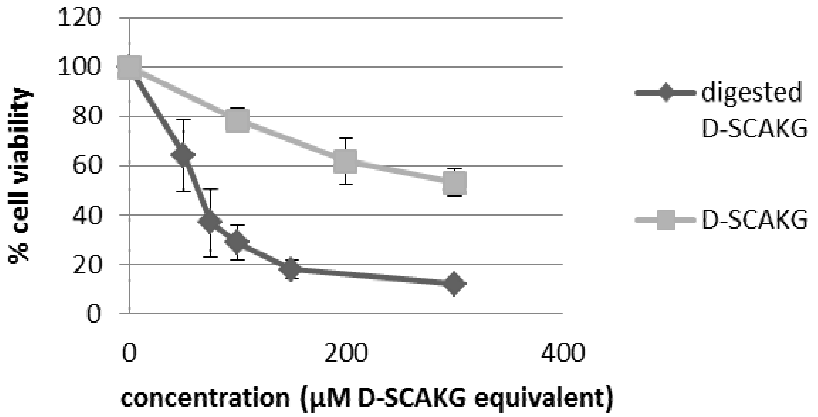
b)

Digested D-SAKG	D-SAKG	M-SAKG	AKG	BUTIRIC
	µM	µM	µM	µM
74µM (IC50)	1,3	44,3	22,7	78,32
50,8µM (GI50)	0,89	30,41	15,58	53,77
79,8µM (TGI)	1,4	47,77	24,48	84,46
85,2µM (LC50)	1,5	51	26,14	90,17

Figure 1



555 **Figure 2**



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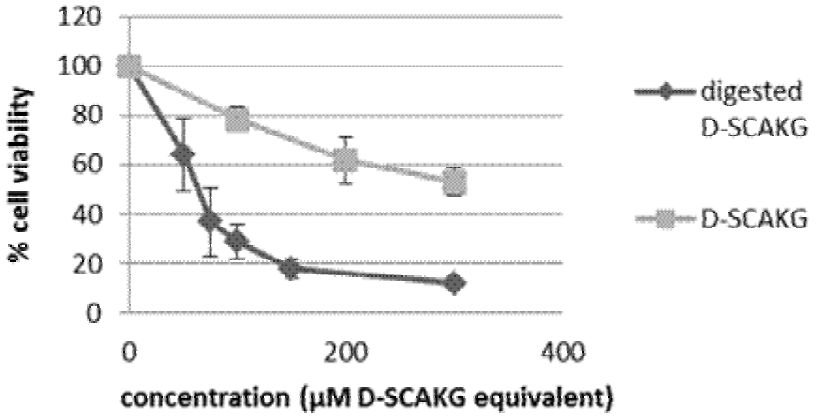

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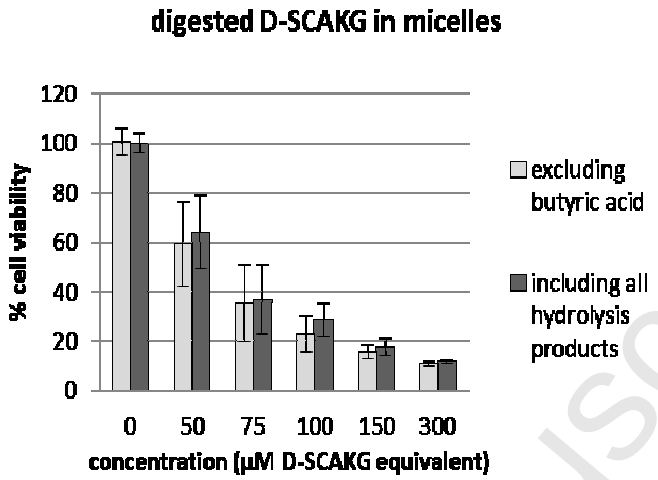
Figure 3



571 **Highlights**

572 Intestinal digestion of alkylglycerols diesterified with butyric acid was simulated.

573 The antitumor effect of digested alkylglycerol was performed in tumor colonocites.

574 Digested alkylglycerol was 4-fold more effective than the non-digested one.

575 Synergism between major digestion products was found.

576 Diesterified alkylglycerol would be a stable lipid for vehiculization of butyric acid.

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