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Antiproliferative activity of buttermilk lipid fractions isolated using food grade and non-food grade solvents on human cancer cell lines

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ABSTRACT

Buttermilk is a dairy by-product with a high content of milk fat globule membranes (MFGMs), whose protein constituents are reported to be antiproliferative. Lipids represent about half of the composition of MFGM. The aim of this study was to isolate buttermilk lipid fractions and evaluate their potential antiproliferative effect. Selective extraction with food grade or non-food grade solvents was performed. Antiproliferative effectiveness of lipid extracts and their neutral and polar fractions was evaluated on nine human cancer cell lines. Fractions obtained using food grade ethanol gave a higher yield than those obtained using non-food grade solvents, and they effectively inhibited cell viability of the cancer cell lines investigated. These fractions, rich in phospho- and sphingolipids, were strongly antiproliferative against human ovary and colon cancer cells. This observation allowed us to hypothesize further analyses aimed at promoting the use of buttermilk polar lipid fractions as functional food additives.

1. Introduction

Fat in milk occurs as an oil-in-water emulsion with a unique stabilizing lipoprotein membrane, referred to as the milk fat globule membrane (MFGM). The globule core is mainly composed of triacylglycerols (TAGs) (98%), including a large number of esterified fatty acids. Milk fat is protected against both chemical attack and physical destabilization by the MFGM (Danthine, Blecker, Paquot, Innocente, & Deroanne, 2000), that is mostly composed of proteins associated with membrane as well as polar lipids (PLs), mainly phospho- and sphingolipids. Although the MFGM has a unique trilayer structure formed by an inner layer from endoplasmic reticulum and covered by an outer bilayer from the mammary cell (Mather, 2000), it is noteworthy that PLs account for only 0.5–1% of total milk lipids (Rombaut, Camp, & Dewettinck, 2006). Despite this low amount, this fraction is important because the structure acquired from cellular membranes provides MFGM with a unique PL composition in comparison with other commercial PL sources (Kuchta, Kelly, Stanton, & Devery, 2012). However, some milk by-products such as buttermilk (BM) and, especially, its MFGM fraction contain up to 40% (in weight) of PLs, of which around 30% is phosphatidylethanolamine (PE), 7% is phosphatidylinositol (PI), 5% is phosphatidylserine (PS), 31% is phosphatidylcholine (PC) and 20% is sphingomyelin (SM) (Rodríguez-Alcalá & Fontecha, 2010).

Studies carried out with PLs from different sources are being actively investigated for their role in human physiology and health. There is a wide range of diseases whose outcome could potentially be influenced by intake of PLs. Most of these research studies have tended to focus on some types of cancers, neurological pathologies and liver diseases, as has been reviewed by Kullenberg, Taylor, Schneider, and Massing (2012).

However, the anticarcinogenic effect of MFGM has not been comprehensively studied and it has been attributed to both, membrane proteins and PLs (Spitsberg, 2005). Previous studies, carried out in our laboratory, suggested that the use of some organic solvents extensively employed for lipid extraction and/or fractionation procedures could lead to the loss of bioactive compounds (Castro-Gómez et al., 2013). Moreover, these solvents

Abbreviations: MFGM, milk fat globule membrane; TAG, triacylglycerols; DAG, diacylglycerols; CHOL, cholesterol; PLs, polar lipids; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; BM, buttermilk; FG, food grade method; NFG, non-food grade method; FBS, fetal bovine serum; FC, flash chromatography; ELSD, evaporative light scattering detector; TGI, total growth inhibition; GI50, 50% growth inhibition.

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are unsuitable for animal or human consumption, which hampers the practical use of PL isolates as functional ingredients.

In this study, the antiproliferative effect of different BM lipid fractions isolated using either food grade (FG) or non-food grade (NFG) solvents, have screened against 9 human cancer cell lines. We also investigated the composition of each isolated lipid fraction and its relation to the inhibition of cell proliferation.

2. Materials and methods

2.1. Samples and reagents

BM powder samples were kindly donated from Reny-Picot (Asturias, Spain). All solvents were HPLC grade, and MS grade when available. Chloroform, dichloromethane, hexane, methanol, isooctane, isopropanol, acetone, diethyl ether, and ethanol (95%) were purchased from Labscan (Dublin, Ireland). Methanol (F.C.C.) Aditio for industrial food use, sea sand, potassium hydroxide and sodium carbonate were from Panreac (Barcelona, Spain). Formic acid (98%), triethylamine (99.5%), tritridecanoin, pelargonic acid (C9), cholesterol (CHOL), CHOL ester, monostearin, diolein and the phospholipids PI, PS, PE, PC and SM were purchased from Sigma-Aldrich (Bellefonte, PA, USA). The fetal bovine serum (FBS) was from Gibco (Gibco BRL, Gaithersburg, MD, USA).

2.2. Total fat extraction using a pressurized liquid system

A pressurized liquid extraction (PLE) procedure was carried out to isolate total lipids from BM powder following the optimized conditions described by Castro-Gómez et al. (2014) with a PLE (Dionex Corp., Sunnyvale, CA), using either food grade (FG) or non-food grade (NFG) solvents. Briefly, 2 g of BM powder was mixed with 2 g of sea sand and loaded into a stainless steel extraction cell covered with filters on both sides. For the FG lipid extraction procedure, ethanol was used as the only organic solvent during 4 static cycles of 5 min at a pressure of 10.3 MPa and 60 °C. For the NFG lipid extraction procedure, dichloromethane-methanol solution (2:1) as in Castro-Gómez et al. (2014) was used. The BM lipid extracts obtained were concentrated by removing the organic solvent in a rotary vacuum evaporator (Strike 202 model; Steroglass S.R.L., Perugia, Italy) and dried under a gentle stream of nitrogen. Then, the lipid extracts were weighed and stored in amber vials, exposed to a stream of nitrogen and frozen at −40 °C until analysis. Each extraction was performed in triplicate.

2.3. Lipid fractionation by flash-chromatography

The BM lipid extracts obtained were fractionated by using a preparative Reveleris® Flash Chromatography (FC) system (Grace, Deerfield, IL, USA) equipped with an evaporative light scattering detector (ELSD). The lipid fractionation that was carried out depended on whether FG or NFG solvents were used. For the FG fractionation procedure, in which the FC system was equipped with a C18 silica cartridge, BM fat was dissolved in 4 mL of methanol for food industry use (50 mg/mL) and loaded onto a preconditioned 4 g C18 silica cartridge (Reveleris; Grace, Deerfield, IL, USA). The elution solvent program consisted first of the methanol and then ethanol. Each step was maintained for 5 min at a flow rate of 7 mL/min. Pressure during fractionation was below 100 psi. The two lipid fractions collected (F1, F2) were evaporated under a nitrogen stream, weighed, and kept at −40 °C until further analysis. The NFG fractionation was carried out as in the procedure described by Castro-Gómez, Holgado, Rodríguez-Alcalá, Montero, and Fontecha (2015), using a normal silica cartridge. Briefly, the BM fat was dissolved in 4 mL of hexane (50 mg/mL) and loaded onto a normal preconditioned 4 g silica cartridge. The elution solvent program consisted of hexane/diethyl ether (98:2), hexane/diethyl ether (95:5), and finally methanol. Two lipid fractions were also collected (F1, F2) as above, and were evaporated and kept frozen until further analysis. In all cases the assays were carried out in triplicate.

2.4. Lipid class composition by HPLC-ELSD

Separation of lipid classes was accomplished with an Agilent Technologies HPLC, model 1200 (Palo Alto, CA, USA) coupled to an ELSD detector (SEDERE, SEDEX 85 model, Alfortville Cedex, France) using prefiltered compressed air as the nebulizing gas at a pressure of 3.5 bar and temperature of 60 °C, and with the gain set at 3. Two Zorbax Rx-SIL columns (Agilent Technologies, Palo Alto, CA, USA) of 250 mm × 4.5 mm and 5 μm particle size were used in series together with a precolumn with the same packing. Samples of BM fat, as well as the isolated BM fat fractions, were prepared at 5 mg/mL in dichloromethane, and the injection volume was 50 μL. The solvent gradient was as detailed in Castro-Gómez et al. (2014). Analyses were carried out with freshly prepared solvents. Lipid standards were analysed under the same conditions and used for further identification. Analyses were carried out in triplicate.

2.5. Antiproliferative activity assay

The *in vitro* antiproliferative assay was performed according to Monks et al. (1991). The human tumour cell lines were kindly donated by Frederick Cancer Research and Development Center, National Cancer Institute (NCI) and the HaCat cell lines by Prof. Dr. Ricardo Della Coletta (FOP/UNICAMP) (Table 1). All cell lines were maintained in 25 cm^3 (Costar/Corning) bottles with 5 mL of RPMI 1640 culture medium supplemented with 5% FBS, and incubated at 37 °C in a humid atmosphere with 5% CO_2. All cells were maintained below passage 20, after which they were replaced by new cells of the same strain. The exponential growth of all cell lines was checked in a Neubauer chamber after 48 h of incubation. A volume of 100 μL per well of each cellular suspension, at the concentration shown in Table 1, was applied in 96-well culture plates in RPMI/5% FBS, and penicillin:streptomycin (1000 U/mL:1000 μg/mL, 1%) medium. They were incubated at 37 °C in a humid atmosphere with 5% CO_2 for 24 h. 5 mg of each sample was dissolved in 50 μL of dimethyl sulfoxide followed by 950 μL of RPMI/5% FBS medium. These solutions were diluted in culture medium to reach 0.25, 2.5, 25 and 250 μg/mL and applied on cell lines (100 μL per well). Doxorubicin (0.025–25 μg/mL) was used as a positive control. The dimethyl sulfoxide final concentration (up to 0.25%) did not affect cell viability. After 48 h, cells were

Table 1
Human cancer cell lines used to assess the antiproliferative activity.

Human cell lines	Organ/ disease	Embryonic origin	Inoculation density (10^4 cells/mL)
U251	Skin/melanoma	Ectoderm	4.0
MCF7	Breast/adenocarcinoma	Ectoderm	6.0
NCI/ADR-RES	Ovary/adenocarcinoma	Ectoderm	5.0
786-0	Kidney/adenocarcinoma	Mesoderm	5.0
NCI-H460	Lung/carcinoma	Endoderm	4.0
OVCAR-3	Ovary/adenocarcinoma	Mesoderm	7.0
HT29	Colon/adenocarcinoma	Endoderm	5.0
K-562	Bone marrow/Myeloid leukaemia	Mesenchyme	6.0
HaCaT	Skin (keratinocyte)/ no tumour	Ectoderm	4.0