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Evaluation of the Anti-Proliferative Activity of Natural Lipid Preparations Against Tumor Cell Lines

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Abstract: A few substances in the human diet have been identified as anticarcinogens but most of them are present in trace concentrations. Lipid preparations enriched in biologically active fatty acids like CLA, VA, EPA and DHA were used for Bioactive Lipid Complexes (BLCs) preparation. The compounds were examined for their antiproliferative activity *in vitro* against human lung (A549), melanoma (Hs294T), colon (HT-29), breast cancer (MCF-7) and promyelocytic leukemia (HL-60) cells. All compounds were also tested in combined treatment with several cytostatic agents. From all tested enriched lipid preparations and bioactive lipid complexes the compound BLC-2 containing 36.7% of CLA and 45.3% of EPA+DHA revealed the highest antiproliferative activity in tested human cancer models. Its activity was comparable to this of CLA-1 (98% c9, t11 C18:2 CLA) used alone as a reference. The results indicate that anti-proliferative activity of unsaturated fatty acid mixtures is associated with their composition.

Key words: Lipid preparations enriched in CLA, VA, EPA, DHA, bioactive lipid complexes, cancer, anti-proliferative activity, acid mixtures

INTRODUCTION

First information about an influence of food on cancer diseases have appeared at the beginning of 20th century (Willliams, 1993). It is currently believed that about 35-45% of cancer diseases incidents are connected to the diet (Bodkowski and Patkowska-Sokola, 2013a; Filip *et al*., 2010), especially fat and fatty acids constitute the main risk factors of breast, colon and prostate cancers (Woutersen *et al*., 1999). From the other hand, animal and plant origin products, contain biologically active fatty acids may be used in prevention and therapy of some diseases, e.g., in immunological system disorders, neurological disorders, circulatory system diseases or in cancer diseases (Adisakwattana *et al*., 2012; Bodkowski and Patkowska-Sokola, 2013b; Delas *et al*., 2008; Nunes *et al*., 2011).

The highest number of studies has been devoted so far to anticarcinogenic activity of CLA. It was demonstrated in *in vitro* experiments on human cancer cells and *in vivo* on mice and rats (Corl *et al*., 2013; Lipkowski *et al*., 2003) that conjugated dienes of linoleic acid, mainly cis-9, trans-11, decrease the development of skin, breast, colon and stomach cancers (Ip *et al*., 1991; Palombo *et al*., 2002). Antimutagenic and anticarcinogenic properties of CLA have been also confirmed in clinical studies, however in a lower degree (Aro *et al*., 2000; McCann *et al*., 2004).

The group of fatty acids characterized by profitable health promoting properties also includes n-3 family fatty acids, especially Eicosapentaenoic C20:5 (EPA) and Docosahexaenoic C22:6 (DHA) acid. Considerably less is known about anticarcinogenic activity of these acids (Begin *et al*., 1986; Boudreau *et al*., 2001; Calviello *et al*., 1998; Colquhoun and Schumacher, 2001; Connolly *et al*., 1999). Epidemiological studies and experiments on animals and tissue cultures proved that there is a relationship between consumption of polyunsaturated fatty acids

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and cancers prevalence (Caygill *et al*., 1996). The aim of this study was an enrichment of animal products (fish oil, sheep milk fat) and plant origin material (grapeseed oil) in biologically active fatty acids (CLA, VA, EPA, DHA) using methods elaborated or modified by the researcherss of this study. On this basis, the composition of Bioactive Lipid Complexes (BLCs) were elaborated and their influence on cancer cells proliferation *in vitro* was examined.

MATERIALS AND METHODS

Obtaining of lipid preparations enriched in biologically active fatty acids and elaboration based on them the composition of Bioactive Lipid Complexes (BLCs)
Synthesis of conjugated isomers of linoleic acid of c9, t11 and t10, c12 C18:2 configuration from linoleic acid c9, c12 C18:2 contained In Grapeseed Oil (IGO preparation): The enrichment process was conducted according to the methodology elaborated by Walisiewicz-Niedbalska *et al*. (2009). Grapeseed oil containing about 70% (m/m) of linoleic acid c9, c12 C18:2 was used as a raw material. Moreover, potassium hydroxide, ethylene glycol, concentrated hydrochloric acid and urea were used. Hexane was used as a solvent.

The synthesis of bioactive isomers of linoleic acid C18:2 of c9, t11 and t10, c12 configuration was conducted applying alkaline hydrolysis, free fatty acids acidification. The preparations were crystallized with urea, hydrochloric acid and hexane were removed (Fig. 1).

Enrichment of milk fat in linoleic 9c, 11t C18:2 and oleic acid isomer 11t C18:1 (MMF preparation): Sheep fat milk obtained from Polish mountain sheep in a process of double centrifugation (3000 rpm) of milk obtained, containing about 2% (m/m) of c9, t11 C18:2 isomer and 1.5% (m/m) of t11 C18:1 isomer was used as the raw material.

The enrichment process was conducted according to the methodology elaborated by Walisiewicz-Niedbalska *et al*. (2009). The process of increasing of the content of bioactive isomers c9, 11t C18:2 and t11 C18:1 in milk fat was conducted using the following processes: alkaline hydrolysis, crystallization with urea, freezing out and filtration but also using potassium hydroxide, hydrochloric acid and ethylene, methylene and hexane as solvents (Fig. 2). There are also other methods of plant oils isomerization reported in the literature (Walisiewicz-Niedbalska *et al*., 2011).

Analytical methods: Methyl esters of fatty acids of grapeseed oil and milk fat before and after the enrichment

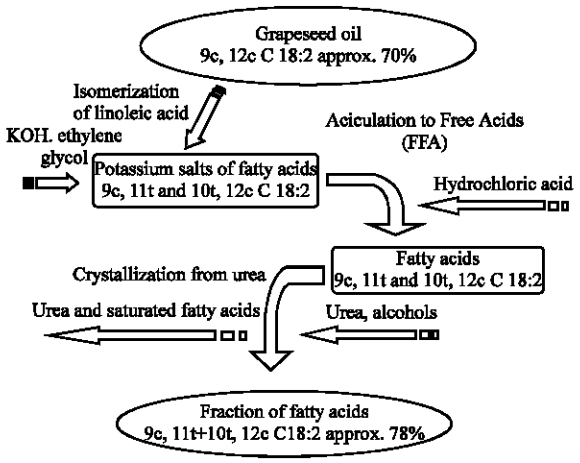

Fig. 1: Scheme of grapeseed oil enrichment in c9, t11 and t1t, c12 C18:2 acids

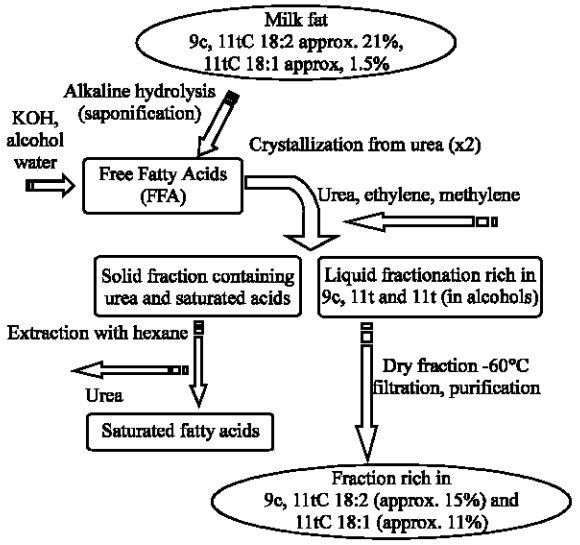

Fig. 2: Scheme of milk fat enrichment in c9, t11 C18:2 and t11 C18:1 acids

process were obtained according to AOCS Official Methods Ce2-66 while the analysis was conducted according to AOCS Official Methods Ce1f-96 (AOAC, 1995). For qualitative examinations the technique of capillary gas chromatography was used in a following conditions: Hawlett Pacard 5890 gas chromatograph with FID detector, SP-2560 column of Supelco company (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) of parameters LxI.D.100 m×0.25 mm, df 0.20 μm, furnace programme: 140°C for 1 min, accretion 1°C min^{-1} up to 180°C, 26 min isotherm, accretion 5°C min^{-1} up to 245°C, 25 min isotherm, FID 255°C detector, feeder-split/splitless 245°C, carrier gas-helium 0.98 mL min^{-1}.

The standards of Sigma (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) and Larodan (Larodan Fine Chemicals AB, Malmo, Sweden) companies and also standard oils: soybean, rapeseed, coconut as well as literature data were used for fatty acids identification (Roach *et al*., 2002). Heptadecanoic acid C17 of Fluka company (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) was used as an internal standard.

Enrichment of grapeseed oil and milk fat in biologically active fatty acids and also chromatographical examination of fatty acids profile were conducted in the Industrial Chemistry Research Institute in Warsaw, Poland.

Enrichment of fish oil in Eicosapentaenoic C20:5 (EPA) and Docosahexaenoic Acid C22:6 (DHA) (MFO preparation): Fat extracted from cod liver containing about 12.1% (m/m) of Eicosapentaenoic Acid (EPA) and about 19.5% (m/m) of Docosahexaenoic Acid (DHA) was used as a raw material. The enrichment process was conducted according to the modified methodology elaborated by Usydus *et al.* (2012) and with the use of potassium hydroxide, hydrochloric acid, dichloromethane and also ethylene and hexane as the solvents. The content of EPA and DHA fatty acids in fish oil was subject to an increase as a result of alkaline hydrolysis, acidification, extraction, dissolution and evaporation processes application (Fig. 3). The literature also provides the information of other methods concerning fish oil enrichment in omega-3 fatty acids (Bodkowski *et al*., 2011; Patkowska-Sokola *et al*., 2009).

Analytical methods: Methyl esters of fish oil fat acids before and after the enrichment process and their analysis were conducted according to AOAC (1995). Fatty acids profile was determined using gas chromatography method on Agilent Technologies 6890N chromatograph with an application of FID detector. Capillary column Restek

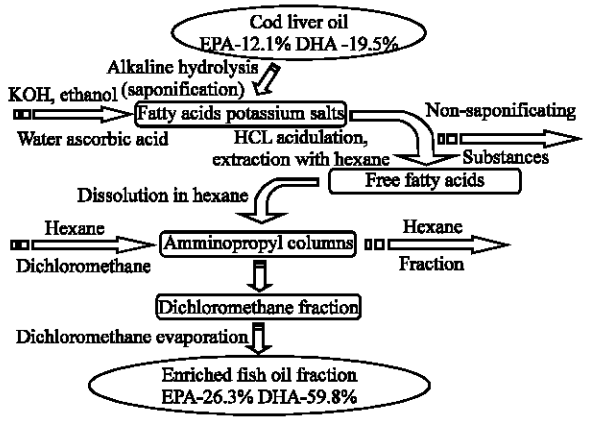

Fig. 3: Scheme of cod liver oil enrichment in EPA (C22:5) and DHA (C22:6) acids

RT-2560 of parameters L×I.D. 100 m× 0.25 mm, df 0.20 μm was used in the examinations. The samples of 2 μL were dosed in split mode 100:1. The temperature of the feeder was 250°C while that of detector was 260°C. Helium was used as a carried gas and nitrogen as masking one. The flow of a carrier gas by the column was 1.1 mL min^{-1}. Temperature program of the furnace was as follows: initial temperature 140°C (2 min) then temperature accretion of 2°C/min up to 225°C and 10 min isotherm, accretion 4°C/min up to 240°C and 10 min isotherm.

Identification and percentage content of particular fatty acids was determined based on retention times and peaks area for the standard mixture 37 FAME mix (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany). The +C18:4n3+C22:5n3 of Supelco company using Aligent Technologies software (Chemstation; 10 Rev A 10.02 (1757)). The reference material NIST 8415 (powdered eggs) was used for the confirmation method.

The processes of cod oil enrichment and chromatographic analyses were conducted in the laboratory of the Department of Food and Environmental Chemistry, National Marine Fisheries Research Institute in Gdynia, Poland.

Elaboration of the composition of Bioactive Lipid Complexes (BLCs): The composition of Bioactive Lipid Complexes (BLCs) was elaborated based on previously elaborated lipid preparations, i.e., Isomerised Grapeseed Oil (IGO), enriched sheep Milk Fat (MMF) and enriched cod liver oil (MFO). The preparations were mixed in a suitable ratios using magnetic stirrer (Ikamag EDA 9 of Electronic company) and then tocopherol dissolved in methanol was added in an amount of 200 mg/100 g of oil in order to protect unsaturated fatty acids against oxidation process.

The bioactive lipid complexes included: BLC 1-enriched sheep Milk Fat (MMF) and enriched Fish Oil (MFO), BLC 2-Isomerised Grapeseed Oil (IGO) and enriched Fish Oil (MFO), BLC 3-Isomerised Grapeseed Oil (IGO), enriched Fish Oil (MFO) and enriched sheep Milk Fat (MMF).

Methyl esters of fatty acids of the biopreparations elaborated (BLC1-BLC3) were obtained and analyzed according to analytical methods used also for milk fat and grapeeseed oil preparation. Chromatographic analyses of fatty acids profile were conducted in the Industrial Chemistry Research Institute in Warsaw, Poland.

Evaluation of lipid formulations influence on cancer cells proliferation: In the second part *in vitro* anti-proliferative assays were performed. The aim of this study was to evaluate the influence of obtained lipid formulations on

human cancer cell proliferation. The *in vitro* part was performed in the Laboratory of Experimental Anticancer Therapy, Department of Experimental Oncology, Institute of Immunology and Experimental Therapy PAS, Wroclaw (Poland).

Fatty acids, lipid formulations and bioactive lipid complexes tested *in vitro*: The antiproliferative activity of the following compounds was examined. Biologically active fatty acids obtained from Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany and Larodan Fine Chemicals AB, Malmo, Sweden:

- CLA1: the 98% of conjugated linoleic acid configuration c9, t11 C18:2
- CLA2: the 98% of conjugated linoleic acid configuration t10, c12 C18:2
- DHA: the 95% of docosahexaenoic acid C22:6
- EPA: the 95% of eicosapentaenoic acid C20:5
- VA: the 95% of vaccenic acid-isomer oleic acid t11 C18:1

Lipid preparations (fatty acid mixtures) enriched or modified using methods developed by the researcherss (Table 1):

- IGO: Isomerised Grapeseed Oil enriched with cis-9, trans-11 and trans-10, cis-12 C18:2
- MFO: Modified Fish Oil enriched with C20:5 (EPA) and C22:6 (DHA) acid
- MMF: Modified Milk Fat enriched with cis-9, trans-11 C18:2 and trans-11 C18:1

Bioactive Lipid Complexes (BLCs) prepared on the base on the obtained lipid formulations by the researchers (Table 1):

Table 1: Profile of lipids formulations enriched in bioactive fatty acids and prepared Bioactive Lipid Complexes (BLCn) (%) tested in *in vitro* experiment

	Tested compounds					
Fatty acids	IGO	MFO	MMF	BCL1	BLC2	BLC3
Saturated	1.5	0.3	5.6	3.0	1.1	2.6
Monounsaturated	20.6	2.5	60.1	31.3	10.2	23.1
Polyunsaturated, including:	78.7	97.2	33.5	64.6	88.4	73.9
Eicosapentaenoic C20:5 (EPA)	-	26.3	-	13.6	15.1	8.3
Docosahexaenoic C22:6 (DHA)	-	59.8	-	31.5	30.2	20.4
EPA+DHA	-	86.1	-	45.1	45.3	28.7
c9t11 C18:2	39.9	-	14.6	7.8	19.6	17.6
t10c12 C18:2	38.2	-	0.3	0.1	17.1	12.3
c9t11+t10c12 C18:2 (CLA)	78.2	-	14.9	7.9	36.7	29.9
11t C18:1 (VA)	-	-	11.3	5.8	-	2.3
CLA+VA	-	-	26.2	13.7	36.7	32.2

IGO: Isomerised Grapeseed Oil enriched with c9.t11 and t10.c12 C18:2; MFO: Modify Fish Oil enriched with EPA and DHA; MMF: Modify Milk Fat enriched with c9t11 C18:2 and t11 C18:1; BLC1: Composition of MFO and MMF; BLC2: Composition of IGO and MFO; BLC3: Composition of IGO, MFO and MMF

- BLC-1: composition of MFO and MMF
- BLC-2: composition of IGO and MFO
- BLC-3: composition of IGO, MFO and MMF

For the antiproliferative activity assay compounds were used alone or in combination with cytostatics. Pure fatty acids were also used as reference compounds for mixtures and biocomplexes. For every cancer model (as listed below) an adequate chemotherapeutic agent was chosen.

Prior to usage the compounds were dissolved in DMSO at the concentration of 10 mg mL^{-1} and subsequently diluted in culture medium to reach the required concentrations (ranging from 1-1000 μg mL^{-1}). IC50 values were calculated for all tested compounds.

Pilot studies on lipid formulation antiproliferative activity performed on human cancer cell lines have shown that the IC50 values were between 10 and 100 μg mL^{-1}. For combined anti-proliferative assay all compounds were used at the concentration of 50 μg mL^{-1} for fatty acids, fatty acid mixtures and bioactive lipid complexes to make the comparison of their activity more reliable.

Cytostatics were used at concentrations below their IC50 values: Cisplatin (Ebewe, Unterach, Austria), (CIS)-1 μg mL^{-1} for human non small lung cancer A549 and melanoma Hs294T cell lines. Doxorubicin (Institute of Biotechnology and Antibiotics, Warsaw, Poland) (DOX)-0.01 μg mL^{-1} for leukaemia HL-60 cell line. 5-fluorouracil (Ebewe, Austria) (5-FU)-5 μg mL^{-1} for colon cancer cells HT-29, tamoxifen (Ebewe, Austria) (TMX)-5 μg mL^{-1} for breast cancer MCF-7 cell line. All cytostatics were diluted in culture medium to reach the required concentrations.

Cell lines: Human, A-549 (lung), HL-60 (leukemia), Hs294T (melanoma), HT-29 (colon), MCF-7 (breast) cancer cell lines were obtained from the American Type Culture Collection (Rockville, Maryland, USA). All the cell lines are being maintained at the Institute of Immunology and Experimental Therapy, Wroclaw, Poland.

The leukemia cell line HL-60 was cultured in RPMI 1640 medium (Gibco, Scotland, UK) with 2 mM L^{-1} glutamine adjusted to contain 1.5 g L^{-1} sodium bicarbonate, 4.5 g L^{-1} glucose and 1.0 mM sodium pyruvate, 10% fetal bovine serum (all from Sigma-Aldrich Chemie GmbH, Steinheim, Germany). A-549 and HT-29 cells were cultured in RPMI 1640+Opti-MEM (1:1) (both from Gibco, Scotland, UK), MCF-7 cells maintained in Eagle medium (IIET, Wroclaw, Poland) and Hs294T in Dulbecco medium (IIET, Wroclaw, Poland) supplemented with 2 mM L-glutamine and 1.0 mM sodium pyruvate, 10% fetal bovine serum (all from Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

The culture of MCF-7 cells was supplemented with 0.8 mg L^{-1} of insulin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). All culture media were supplemented with 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (both from Polfa Tarchomin S.A., Warsaw, Poland). All cell lines were grown at 37°C, 5% CO_2 in humidified atmosphere.

Anti-proliferative assay *in vitro*: The 24 h before addition of the tested compounds, the cells were plated in 96 well plates (Sarstedt, Germany) at a density of 1×10^4 cells per well for all cell lines. An SRB for solid tumour cell lines or MTT for HL-60 leukaemia cell line assay was performed after 72 h of exposure to varying concentrations of the tested agents.

The results were calculated as a percentage (%) of proliferation inhibition of the cancer cell population. Mean values were calculated for each experiment separately and mean values±SD are presented in Table 2 and 3. Each lipid formulation used at a concentration of 50 μg mL^{-1} was tested in six replicates (6 wells per 1 lipid compound and 6 wells for combined assay: lipid compound+cytostatic) in a single experiment. Chemotherapeutic agents were used at concentrations below their IC50 values. Every single experiment (anti-proliferative assay) was repeated 3-5 times.

Cytotoxic tests

SRB: The cells were attached to the bottom of plastic wells by fixing them with cold 50% TCA (trichloroacetic acid, Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The plates were incubated at 4°C for 1 h and then washed five times with tap water. The cellular material once fixed with TCA was stained with 0.4% sulphorhodamine B (SRB, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) dissolved in 1% acetic acid (POCH, Gliwice, Poland) for 30 min. Unbound dye was removed by rinsing (4x) in 1% acetic acid. The protein-bound dye was extracted with 10 mM Tris base

Table 2: The antiproliferative effect expressed as proliferation inhibition of fatty acid formulations used alone or applied with drug against human lung cancer A549 and melanoma Hs294T cells

		A549			Hs294T		
		Proliferation inhibition of (%)			Proliferation nhibitiion of (%)		
Parameters	Composition	Compound	Compound+drug	H (%)	Compound	Compound+drug	H (%)
Drug		CIS	-	-	CIS	-	-
		-23.5±3.1			31.6±1.5		
Pure fatty acids	CLA-1	8.3±7.4	23.6±7.30	30^{n}	64.4±0.8	73.1±19.9	76^{n}
	CLA-2	-0.3±3.0	10.6±6.70	23^{an}	45.5±22.8	57.3±14.5	63^{sub}
	DHA	-1.0±1.3	12.5±4.90	23^{an}	0.8±1.0	34.2±0.80	32^{n}
	EPA	0.1±1.9	22.5±0.70	24^{n}	0.4±0.5	30.4±2.50	32^{n}
	VA	-0.8±2.2	13.3±8.20	23^{an}	20.8±18.6	33.7±6.10	46^{sub}
Fatty acid mixtures	IGO	4.2±0.7	22.5±6.70	27^{n}	18.6±2.8	39.3±8.40	44^{sub}
	MFO	1.0±2.1	15.9±8.60	24^{an}	56.1±5.7	48.9±19.6	70^{n}
	MMF	14.7±10.7	36.4±11.3*	35^{ad}	13.8±1.3	34.1±1.40	41^{sub}
Bioactive Lipid Complex (BLC)	BLC-1	2.2±3.0	22.7±6.70	25^{n}	7.8±7.7	32.7±4.40	37^{n}
	BLC-2	83.7±21.5	85.4±15.7	88^{n}	90.7±3.8	89.0±4.50	94^{n}
	BLC-3	2.9±1.5	28.7±7.20	26^{n}	29.8±5.1	42.0±2.40	52^{sub}

*p<0.05 when compared to cisplatin. Kruskal-Wallis ANOVA and Mann-Whitney; **p<0.05 when compared to doxorubicin. Kruskal-Wallis ANOVA and Mann-Whitney; n: no effect; an-antagonism; ad-additive effect; sub-subadditive effect

Table 3: The antiproliferative effect expressed as proliferation inhibition of fatty acid formulations used alone or applied with drug against leukemia HL-60, colon cancer HT-29 and breast cancer MCF-7 cells

		HL-60			HT-29			MCF-7		
		Proliferation inhibition of (%)			Proliferation inhibition of (%)			Proliferation inhibition of (%)		
Parameters	Composition	Compound	Compound+drug	H (%)	Compound	Compound+drug	H (%)	Compound	Compound+dtug	H (%)
Drug		DOX			5-FU			TMX		
		46.8 ± 2.9	-	-	48.5±7.9	-	-	18.2±2.7	-	-
Pure fatty acids	CLA-1	81.3±1.20	92.6±6.10**	90^{ad}	37.2±0.6	67.5±8.50	68^{ad}	82.6±21.4	59.2±7.70	86^{n}
	CLA-2	9.2±2.40	49.7±4.60	52^{ad}	1.2±1.0	54.2±7.30	49^{ad}	2.6±11.7	23.1±2.70	20^{ad}
	DHA	5.3±7.50	44.7±28.4	50^{an}	-3.0±3.6	48.5±8.00	47^{n}	19.3±2.7	19.9±1.90	34^{n}
	EPA	18.1±10.3	11.5±5.10	56^{an}	-2.1±2	49.7±6.20	47^{n}	11.3±4.8	32.9±10.3	27^{ad}
	VA	1.6±1.50	8.8±3.60	48^{an}	-0.8±0.8	46.2±5.00	48^{n}	10.7±5.6	25.0±5.90	27^{ad}
Fatty acid mixtures	IGO	59.4±2.50	76.8±3.10**	78^{ad}	22.1±11.6	48.1±8.80	60^{n}	4.2±2.6	23.1±6.90	22^{ad}
	MFO	28.2±6.90	55.3±8.70	62^{sub}	-1.0±0.3	44.4±5.10	48^{an}	-0.9±0.9	21.6±5.60	17^{ad}
	MMF	24.8±19.4	63.7±6.00	60^{ad}	0.6±1.6	44.6±5.60	50^{an}	1.2±3.3	27.8±9.80	19^{ad}
Bioactive Lipid	BLC-1	5.8±8.70	-6.5±3.00	50^{an}	1.1±1.1	50.2±7.20	49^{n}	-3.4±3.3	37.6±13.5	15^{ad}
Complex (BLC)	BLC-2	90.0±6.70	93.5±3.80	95n	26.88±0.1	56.3±13.8	73^{n}	24.7±11.5	37.2±0.30	38^{ad}
	BLC-3	83.5±15.3	89.1±2.80	91^{n}	4.3±2	50.3±10.7	51^{n}	4.4±2.7	22.2±8.70	22^{ad}

(POCH, Gliwice, Poland) for determination of optical density (λ = 540 nm) in a Multiskan RC computer-interfaced, 96-well microtiter plate reader photometer (Labsystems, Helsinki, Finland).

MTT (for HL-60 cell line): The 20 μL of MTT solution (MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (Sigma-Aldrich Chemie GmbH, Steinheim, Germany); stock solution: 5 mg mL^{-1} was added to each well and incubated at 37°C for 4 h. After the incubation time was complete, 80 μL of the lysing mixture was added to each well (lysing mixture: 225 mL Dimethylformamide, 67.5 g Sodium Dodecyl Sulphate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and 275 mL of distilled water). The optical densities of the samples were read after 24 h on a Multiskan RC photometer (Labsystems, Helsinki, Finland) at 570 nm.

Evaluation of the influence of lipid formulations on the anti-proliferative activity of cytostatics: The results are presented as percentages (%) of proliferation inhibition of cancer cells caused by the compound used alone or in combination with chemotherapeutic agents (Table 2 and 3). For anti-proliferative activity evaluation of the tested formulations in combined *in vitro* treatment a comparison was performed of the Hypothetic (H%) value for proliferation inhibition of two combined compounds on the basis of current experimental data. The expected percent of inhibition used to estimate the effect of combination of two compounds was evaluated using the equation:

$$H(\%) = \frac{100-\left[(100-\text{Cytostatic}\%)\times(100-\text{Comp.}\%)\right]}{100}$$

Where:

H (%) = Hypothetical percentage of proliferation inhibition caused by combined treatment (two compounds)

Cytostatic (%) = Experimentally determined percentage of proliferation inhibition caused by chemotherapeutic agent

Comp. (%) = Experimentally achieved percentage of proliferation inhibition caused by the tested compound

The observed effects in proliferation inhibition caused by a combination of two compounds:

- Synergism-the experimental percentage of proliferation inhibition achieved for two compounds in combined treatment is higher than the H (%) value
- Additive effect-the experimental percentage of proliferation inhibition achieved for two compounds and H (%) value are the same
- Sub-additive effect the experimental percentage of proliferation inhibition achieved for two compounds in combined treatment is lower than the % H value but higher than the percentage of proliferation inhibition for cytostatic used alone
- Antagonism the experimental percentage of proliferation inhibition achieved for two compounds in combined treatment is lower than the percentage of proliferation inhibition for cytostatic used alone

Statistical evaluation: Statistical analysis was performed using Statistica Version 7.1 (StatSoft Inc., USA). The data were analyzed by Kruskal-Wallis ANOVA. Multiple Comparisons p values (2-tailed) and Mann-Whitney test were performed for further analysis. The $p>0.05$ were considered significant.

RESULTS

Enrichment of lipid preparations in biologically active fatty acids and elaboration of Bioactive Lipid Complexes (BLCs) composition based on them: An application of alkaline isomerisation process and crystallization with urea (Walisiewicz-Niedbalska *et al*., 2009) allowed to synthesize conjugated dienes (CLA) of linoleic acid cis-9, cis-12 C18:2 contained in grapeseed oil. As a result of alkaline isomerisation process the change in an arrangement of double bonds in chains of unsaturated fatty acids (positional) and change in radicals arrangement with respect to the axis of double bond (geometric) was noted. Finally, two conjugated dienes of linoleic acid of cis-9, trans-11 and trans-10, cis-12 configuration not observed naturally in grapeseed oil were formed. As a result of the process of crystallization with urea in turn most of the saturated fatty acids was removed in a form of an adduct what allowed an increase of CLA concentration in a final product (IGO) up to 78% of all fatty acids pool (Table 1).

An application of double complexing with urea in turn and dry fractionation (Walisiewicz-Niedbalska *et al*., 2009) allowed to increase the content of vaccenic acid t11C18:1 and linoleic acid isomer of cis-9, trans-11 from 1.5 and 2.1-11.3 and 15.8%, respectively (Table 1). The modified method of aminopropyl column extraction (Roach *et al*., 2002) allowed in turn to increased concentration of omega-3 acids in cod liver oil from 34 up to 91% and EPA+DHA acids from 31.6-86.1% (Table 1).

The composition of Bioactive Lipid Complexes (BLCs) was elaborated based on the above lipid preparations. BLC-1 including enriched sheep Milk Fat (MMF) and enriched Fish Oil (MFO) contained 95.9% of Unsaturated Fatty Acids (UFA) including 64.6% of

Polyunsaturated (PUFA) and 31.3% of Monounsaturated ones (MUFA) and 3% of Saturated Fatty Acids (SFA). The content of biologically active fatty acids EPA+DHA and CLA+VA was 45.1 and 13.7%, respectively (Table 1). BLC-2 including Isomerised Grapeseed Oil (IGO) and enriched Fish Oil (MFO) contained in turn 98.6% UFA (including 10.2% MUFA and 88.4% PUFA) and 1.1% SFA. The contribution of EPA+DHA and CLA was on a level of 45.3 and 36.7%, respectively (Table 1). Biopreparation BLC-3 included Isomerised Grapeseed Oil (IGO), enriched Fish Oil (MFO) and enriched sheep Milk Fat (MMF). That preparation contained 97% UFA including 73.9% PUFA and 23.1% MUFA. The content of biologically active fatty acids EPA+DHA and CLA+VA were 28.7 and 32.2%, respectively (Table 1).

The influence of biologically active fatty acids and developed lipid formulations on cancer cell proliferation: Table 2 and 3 present the percentage of proliferation inhibition by the tested compounds at a dosage of 50 μg mL^{-1} used alone or in combination with chemotherapeutic agents. Cisplatin, tamoxifen and 5-fluorouracil were used in concentrations that inhibit proliferation of <50% of cells population. The cells were exposed to all compounds and subsequently to cytostatics during 72 h.

Anti-proliferative activity of lipid formulations (pure fatty acids, fatty acid mixtures and bioactive lipid complexes) towards several human cancer cell lines: All compounds were examined for their anti-proliferative activity *in vitro* against the human lung A549, breast MCF-7, colon HT-29, leukaemia HL-60 and melanoma Hs294T cancer cell lines (Table 2 and 3).

In the pure fatty acids group, the lowest activity was observed against lung A549 and colon HT-29 cancer cell lines. Only compound 1 (CLA-isomer) revealed proliferation inhibition of about 8.3% for A549 and 37.2% for HT-29 cell ine.

The leukaemia, melanoma and breast cancer cell lines were more sensitive to pure fatty acids, especially to CLA-1. Researchers observed that CLA-1, DHA, EPA, VA decreased the proliferation of MCF-7 cells by 82.6, 19.3, 11.3 and 10.7%, respectively. For the HL-60 cell line compounds revealed the following percentage of cell growth inhibition: 81.30% for CLA-1, 18.1%-EPA, 9.2%-CLA-2 and 5.3% for DHA. The results for Hs294T were little different than those for breast and leukaemia cell lines. The observed proliferation inhibition was caused by both CLA isomers; 64.4% for CLA-1, 45.5% for CLA-2 and by VA-20.8%.

Comparing the anti-proliferative activity of the second group of lipid formulations (fatty acid mixtures) against all tested cancer cell lines, the best results were observed on HL-60 and Hs294T. IGO, MFO and MMF inhibited the growth of 59.4, 28.2 and 24.8% of growth inhibition of leukaemia cells and 18.6, 56.1 and 13.8% of melanoma. In the case of HT-29 colon cells only IGO decreased cell proliferation by 22.1%. The growth of A549 cells was inhibited in 14.7% only by MMF. This effect could be dependent on the composition of these compounds; IGO contains 39.9% of CLA-1 (78.2% of both: CLA-1 and CLA-2) while MMF only 14.9%. However, the MFO-fish oil is enriched with 26.3% EPA and 59.8% DHA and contains no CLA. The last group of tested lipid preparations (BCL) was thought to reveal the highest anticancer activity *in vitro*. It was observed that the activity of BCLs strongly correlates with their composition, the CLA percentage is positively correlated with anti-proliferative activity. Elevated DHA and EPA content combined with the absence of CLA does not provide high activity.

BCL-1 with a DHA+EPA content of 45.1% (DHA-31.5% of composition) and a low level of CLA (7.9%) showed the lowest activity of all the tested bioactive lipid complexes against all cancer cell lines. All results obtained for BCL-1 used alone were <10% of proliferation inhibition.

BCL-2 which is a mixture of CLA-1+CLA-2 (36.7%) and DHA+EPA (45.3%), revealed higher anti-proliferative activity than pure CLA-1 against all cancer cell lines tested, apart from MCF-7. In this case, the anti-proliferative activity was dramatically reduced when compared to CLA-1 alone. The proliferation inhibition for the MCF-7 cell line was 24.7% for BLC-2 whereas for CLA-1-82.6%. In the case of the other cancer models the proliferation was decreased by 90.7% in Hs294T, 90.0% in HL-60, 83.7% in A549 and 48.5% in HT-29 cells.

The last preparation, BCL-3, containing 29.9% CLA (CLA-1+CLA-2) showed high antiproliferative activity only against leukaemia (83.5% of proliferation inhibition) and melanoma cells (29.8%).

The influence of fatty acid formulations on the anti-proliferative activity of chemotherapeutic agents against different human cancer cell lines: Comparing the results for both CLA-1 and BLC-2 (containing 36.7% CLA) used alone and in combination with cytostatic agents revealed high antiproliferative activity against human cancer cells.

In case of A549 cell line, the proliferation inhibition revealed by compounds used alone were much lower when compared to other cancer models. Only BCL-2 inhibited the cell growth in 83.7% when used alone or in 85.4% when combined with cisplatin. Researchers observed additive effect when MMF was used with

cisplatin (statistically significant). MMF+CIS achieved 36.4% proliferation inhibition where MMF alone received 14.7% and CIS alone 23.5%. It could be important to mention that MMF contains only 14.9% of CLA.

The results obtained on the HL-60 cell line show that polyunsaturated fatty acids did not improve the antiproliferative activity of doxorubicin excluding CLA-1 and IGO. Additive effects were observed for these two formulations and DOX combined treatment. BLC-2 and BLC-3 revealed no effect on doxorubicin activity when compared to the data received for BCL-2 and BCL-3 used alone. The inhibition of HL-60 cell growth received by BCL-2 and BCL-3 alone was 90.0 and 83.5%, respectively and in combined treatment with doxorubicin 93.5% (BCL-2+DOX) and 89.1% (BCL-3+DOX).

Antiproliferative activity results achieved for all compounds used with cisplatin showed subadditive of no effects on melanoma Hs294T cells. Most of these compounds: CLA, VA, IGO, MFO, MMF, BLC-2 and BLC-3 used alone revealed high proliferation inhibition. The percentage of the inhibition was 64.4, 45.5, 20.8, 18.6, 56.1, 90.7 and 29.8% for CLA-1, CLA-2, VA, IGO, MFO, MMF, BLC-2 and BLC-3, respectively.

When analyzing the results of combined treatment for 5-Fu with all tested compounds on HT-29 cells, only in case of CLA-1 and CLA-2 an additive effect was observed. CLA-1, IGO and BCL-2 used alone revealed 37.2, 22.1 and 26.5% of proliferation inhibition, respectively when 5-Fu used alone inhibited the growth of HT-29 cells by 48.5%.

Noteworthy are the results for combined treatment obtained on the MCF-7 cell line: CLA-1 used alone revealed 82.6% of proliferation inhibition and only 59.2% when combined with Tamoxifen (TMX). Fatty acid mixtures and Bioactive Lipid Complexes (BLC-s) combined with TMX revealed additive effects. For IGO, MFO, MMF combined with TMX the proliferation inhibition achieved 23.1, 21.6 and 27.8%, respectively where the compounds alone achieved only 4.2, 0.9 and 1.2% of proliferation inhibition. For BLC-1, BCL-2 and BLC-3 combined with TMX the anti-proliferative activity revealed 37.6, 37.2 and 22.2%, respectively. BLC1-3 compounds used alone achieved-3.4, 24.7 and 4.4%. Tamoxifen alone at a dose of 5 $\mu g\ mL^{-1}$ revealed 18.2% proliferation inhibition.

DISCUSSION

The aim of the research was to examine the anti-cancer activity of new obtained bioactive lipid formulations *in vitro*. Of all the tested bioactive lipid complexes and fatty acid mixtures BLC-2 containing 36.7% CLA revealed the highest anti-proliferative activity in all tested human cancer models. Its activity was comparable to that of CLA-1 (98% 9c, 11t C18:2 CLA) used alone as reference. Many *in vitro* studies on human cancer cell lines (Lipkowski *et al*., 2003) and *in vivo* studies on mice and rat models revealed anticancer activity of CLA. Mainly treatment with cis-9, trans-11 conjugated linoleic acid acts on retardation of tumor growth or reduce tumor development in melanoma, breast, colon and gastric cancer models (Corl *et al*., 2013; Ip *et al*., 1991, 1994, 1999; Ip, 1997). Clinical trials also reveal the antimutagenic and anticancer properties of CLA (Aro *et al*., 2000; Kritchevsky, 2000; McCann *et al*., 2004). Conjugated dienes of linoleic acid are so far, the best known and most studied fatty acids. The research confirms the ability of CLA to inhibit the proliferation of lung, leukemia, melanoma, colon and breast cancer cells.

Much less is known in the literature about the anticancer activity of omega-3 acids but studies on DHA, EPA, ALA and GLA revealed that these fatty acids can increase the cytotoxic activity of anticancer drugs. Taking into consideration the results for proliferation inhibition of all tested fatty acid compounds, formulations and bioactive lipid complexesresearcherscan recommend some of them for further *in vitro* investigations or for *in vivo* studies. Compounds chosen for every tested human cancer model used in these *in vitro* studies are listed below:

- Lung cancer A549-BLC-2
- Leukaemia HL-60-CLA-1, IGO, BLC-2 and BLC-3
- Melanoma Hs249T-CLA-1, CLA-2, MFO and BLC-2
- Colon HT-29-CLA-1 and BLC-2
- Breast MCF-7-CLA-1 and BLC-2

Analysing the results for combined *in vitro* treatment, researchers can recommend for further investigations the formulations which caused an additive effect when used with chemotherapeutic agents. Pairs cytostatic+compound chosen for each cancer cell line are listed below:

- A-549-Cisplatin+MMF
- HL-60-Doxorubicin+CLA-1, MMF
- HT-29-5-fluorouracil+CLA-2
- MCF-7-Tamoxifen+CLA-2, EPA, IGO, MFO, MMF, BLC-1

CONCLUSION

Results received in the studies indicate that the anti-proliferative activity of polyunsaturated fatty acid

mixtures is associated with their composition. Moreover, the ability to reduce cancer cell growth is dependent on the percentage of CLA in the formulation composition.

Nevertheless, despite all the research done by many research teams towards understanding the anticancer activity of CLA, the mechanisms mediating these effects are still not fully understood. Taking into consideration the safety of a high dose CLA anticancer therapy or the probability that some toxic or side effects in these therapies could occur, further investigations on *in vivo* treatment needs to be done.

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REFERENCES

AOAC, 1995. Official Methods of Analysis. 16th Edn., Association of Official Analytical Chemists, Washington, DC., USA.

Adisakwattana, S., J. Intrawangso, A. Hemrid, B. Chanathong and K. Makynen, 2012. Extracts of edible plants inhibit pancreatic lipase, cholesterol esterase and cholesterol micellization and bind bile acids. Food Technol. Biotechnol., 50: 11-16.

Aro, A., S. Mannisto, I. Salminen, M.L. Ovaskainen, V. Kataja and M. Uusitupa, 2000. Inverse association between dietary and serum conjugated linoleic acid and risk of breast cancer in postmenopausal women. Nutr. Cancer, 38: 151-157.

Begin, M.E., G. Ells, U.N. Das and D.F. Horrobin, 1986. Differential killing of human carcinoma cells supplemented with n-3 and n-6 polyunsaturated fatty acids. J. Natl. Cancer Inst., 77: 1053-1062.

Bodkowski, R. and B. Patkowska-Sokola, 2013a. Reduction of body fatness and meat fat content in lambs by supplementation of isomerised grapeseed oil. Anim. Sci. Pap. Rep., 31: 219-238.

Bodkowski, R. and B. Patkowska-Sokola, 2013b. Modification of fatty acids composition of lambs' fat by supplementation isomerised grapeseed oil. Anim. Sci. Pap. Rep., 31: 147-158.

Bodkowski, R., J. Szlinder-Richert, Z. Usydus and B. Patkowska-Sokola, 2011. An attempt of an optimization of fish oils low-temperature crystallization process. Przem. Chem., 90: 703-706.

Boudreau, M.D., K.H. Sohn, S.H. Rhee, S.W. Lee, J.D. Hunt and D.H. Hwang, 2001. Suppression of tumor cell growth both in nude mice and in culture by n-3 polyunsaturated fatty acids: Mediation through Cyclooxygenase-independent pathways. Cancer Res., 61: 1386-1391.

Calviello, G., P. Palozza, E. Piccioni, N. Maggiano, A. Frattucci, P. Franceschelli and G.M. Bartoli, 1998. Dietary supplementation with eicosapentaenoic and docosahexaenoic acid inhibits growth of Morris hepatocarcinoma 3924A in rats: Effects on proliferation and apoptosis. Int. J. Cancer, 75: 699-705.

Caygill, C.P., A. Charlett and M.J. Hill, 1996. Fat, fish, fish oil and cancer. Br. J. Cancer, 74: 159-164.

Colquhoun, A. and R.I. Schumacher, 2001. γ-Linolenic acid and eicosapentaenoic acid induce modifications in mitochondrial metabolism, reactive oxygen species generation, lipid peroxidation and apoptosis in Walker 256 rat carcinosarcoma cells. Biochim. Biophys. Acta Mol. Cell Biol. Lipids, 1533: 207-219.

Connolly, J.M., E.M. Gilhooly and D.P. Rose, 1999. Effects of reduced dietary linoleic acid intake, alone or combined with an algal source of docosahexaenoic acid, on MDA-MB-231 breast cancer cell growth and apoptosis in nude mice. Nutr. Cancer, 35: 44-49.

Corl, B.A., D.M. Barbano, D.E. Bauman and C. Ip, 2013. *Cis*-9, *trans*-11 CLA derived endogenously from trans-11 18: 1 reduces cancer risk in rats. J. Nutr., 133: 2893-2900.

Delas, I., M. Popovic, T. Petrovic, F. Dels and D. Ivankovic, 2008. Changes in the fatty acid composition of Brain and liver phospholipids from rats fed fat-free diet. Food Technol. Biotechnol., 46: 278-285.

Filip, S., R. Fink, J. Hribar and R. Vidrih, 2010. Trans fatty acids in food and their influence on human health. Food Technol. Biotechnol., 48: 135-142.

Ip, C., 1997. Review of the effects of trans fatty acids, oleic acid, n-3 polyunsaturated fatty acids and conjugated linoleic acid on mammary carcinogenesis in animals. Am. J. Clin. Nutr., 66: 1523S-1529S.

Ip, C., M. Singh, H.J. Thompson and J.A. Scimeca, 1994. Conjugated linoleic acid suppresses mammary carcinogenesis and proliferative activity of the mammary gland in the rat. Cancer Res., 54: 1212-1215.

Ip, C., S. Banni, E. Angioni, G. Carta and J. McGinley *et al*., 1999. Conjugated linoleic acid-enriched butter fat alters mammary gland morphogenesis and reduces cancer risk in rats. J. Nutr., 129: 2135-2142.

Ip, C., S.F. Chin, J.A. Scimeca and M.W. Pariza, 1991. Mammary cancer prevention by conjugated dienoic derivative of linoleic acid. Cancer Res., 51: 6118-6124.

Kritchevsky, D., 2000. Antimutagenic and some other effects of conjugated linoleic acid. Br. J. Nutr., 83: 459-465.

Lipkowski, A.W., W. Walisiewicz-Niedbalska, B. Patkowska-Sokola, A. Opolski and R. Bodkowski <>., 2003. *In vitro* anti-cancer properties of natural vs synthetic conjugated linoleic acid. Anim. Sci. Pap. Rep., 21: 47-55.

McCann, S.E., C. Ip, M.M. Ip, M.K. McGuire and P. Muti *et al*., 2004. Dietary intake of conjugated linoleic acids and risk of premenopausal and postmenopausal breast cancer, Western New York exposures and breast cancer study (WEB study). Cancer Epidemiol. Biomarkers Prev., 13: 1480-1484.

Nunes, G.F.M., A.V. de Paula, H.F. de Castro and J.C. dos Santos, 2011. Optimization of the enzymatic interesterification of milk fat and canola oil blends using immobilized *Rhizopus oryzae* lipase by response surface methodology. Food Technol. Biotechnol., 49: 385-390.

Palombo, J.D., A. Ganguly, B.R. Bistrian and M.P. Menard, 2002. The antiproliferative effects of biologically active isomers of conjugated linoleic acid on human colorectal and prostatic cancer cells. Cancer Lett., 177: 163-172.

Patkowska-Sokola, B., Z. Usydus, J. Szlinder-Richert and R. Bodkowski, 2009. Technology of omega-3 fatty acids obtaining from fish oils and their protection against oxidative changes. Przem. Chem., 88: 548-552.

Roach, J.A.G., M.M. Mossoba, M.P. Yurawecz and J.K.G. Kramer, 2002. Chromatographic separation and identification of conjugated linoleic acid isomers. Analitica Chimica Acta, 465: 207-226.

Usydus, Z., R. Bodkowski, J. Szlinder-Richert, B. Patkowska-Sokola and Z. Dobrzanski, 2012. Use of aminopropyl column extraction for fish oil enrichment in omega-3 acids. Przem. Chem., 91: 1043-1048.

Walisiewicz-Niedbalska, W., B. Patkowska-Sokola, A.W. Lipkowski, R. Bodkowski, Z. Dobrzanski, H. Gwardiak and K. Rozycki, 2009. Linoleic acid with conjugated double bonds in modified vegetable oils and milk fat. Przem. Chem., 88: 579-582.

Walisiewicz-Niedbalska, W., B. Patkowska-Sokola, K. Rozycki, H. Gwardiak and R. Bodkowski, 2011. Synthesis of biologically active linoleic acid isomers using oxide catalyst. Przem. Chem., 90: 1058-1060.

Willliams, G.M., 1993. Food: Its Role in the Etiology of Cancer. In: Food and Cancer Prevention: Chemical and Biological Aspects, Waldron, K.W., I.T. Johnson and G.R. Fenwick (Eds.). The Royal Society of Chemistry, USA., ISBN: 9780851864556, pp: 3-11.

Woutersen, R.A., M.J. Appel, A. van Garderen-Hoetmer and M.V. Wijnands, 1999. Dietary fat and carcinogenesis. Mutat. Res./Genet. Toxicol. Environ. Mutag., 443: 111-127.