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In vitro anti-cancer properties of natural *vs* synthetic conjugated linoleic acid*

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Anti-proliferative activity was investigated of $C_{18:2}$ 9*cis*,11*trans* (conjugated linoleic acid – CLA) isomer isolated from sheep milk fat. The research was conducted on sheep milk fatty acids in natural composition (FA-1) containing 2.0% of the $C_{18:2}$ 9*cis*,11*trans* CLA isomer and preparation enriched with CLA, containing up to 9.2% of the CLA isomer (FA-2). The process of enrichment of FA-1 was carried out in two steps. Step I was to remove mid-chain-length saturated fatty acids, primarily C_{16}, by forming adducts with urea (to obtain FA-U), while in step II the short-chain acids were removed by way of supercritical extraction with CO_2. The final preparation obtained (FA-2) contained 9.2% of the $C_{18:2}$ 9*cis*,11*trans* isomer.

A natural sheep milk fatty acid composition (FA-1), the CLA-enriched product (FA-2) and a commercial CLA preparation (CP) were tested for anti-proliferative activity against the cells of human cervical carcinoma (KB), and leukaemia (HL-60). The ID_{50} dose (resulting in a 50% inhibition of cell proliferation) of FA-2 was found lower than that of the CP preparation. The SRB test showed

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the proliferation of the KB cells to have been inhibited to a greater degree by FA-2 than by gravimetrically identical (100 μg/ml) doses of CP (85.3% *vs* 69.5%, respectively). Similarly, the MTT test showed the proliferation of the HL-60 cells to have been inhibited to a greater degree by FA-2 than by CP (99.0% *vs* 69.0%, respectively). Unmodified sheep milk fatty acids (FA-1) showed only a slight activity against the cells of both cancer lines (inhibition by about 10%).

KEY WORDS: carcinoma / conjugated linoleic acid (CLA) / leukaemia / sheep milk fat / supercritical extraction / urea

In recent years linoleic acid isomers, containing conjugated unsaturated bonds, became a subject of special interest. The term CLA (conjugated linoleic acid) indicates a mixture of linoleic acid isomers, of which the best known is the linoleic isomer 9*cis*,11*trans*. The isomer is formed in the rumen, in the presence of linoleic isomerase produced by *Butyrivibrio fibrisolvens*, as an intermediate in the biohydrogenation of linoleic acid. In the milk fat of ruminants CLA appears in small quantities (from 0.5 to about 2.0% of total fatty acids) and the $C_{18:2}$ 9*c*,11*t* isomer comprises about 90% of all the linoleic acid isomers with conjugated unsaturated bonds. The interest in those isomers increased considerably when, during studies on mutagenic and carcinogenic factors in grilled beef, such isomers were identified as compounds responsible for inhibited mutagenesis [Ha *et al*. 1987].

Studies on the effect of linoleic acid isomers, and principally of $C_{18:2}$ 9*c*,11*t*, on cancer cells (mammary gland, prostate or stomach cancer) are being conducted both *in vitro* and *in vivo*, mainly with synthetic preparations [Ha *et al*. 1990, Ip *et al*. 1994, Cesano *et al*. 1998]. Hitherto, in literature there are no descriptions of a practical use of the effect of natural sheep milk fat enriched with CLA isomers, on cancer cells.

The present paper is a continuation of studies by Walisiewicz-Niedbalska *et al*. [2001] aiming at increasing the share of isomer $C_{18:2}$ 9*c*,11*t* in sheep milk fat and an evaluation of the cytotoxic activity of the preparation obtained against the cancer cells, as compared to unchanged sheep milk fat or a synthetic, commercial CLA preparation. The antiproliferative activity of the preparations compared was tested *in vitro,* against the cells of two lines of human cancer: cervical carcinoma (KB) and leukaemia (HL-60).

Material and methods

Material

The free fatty acids, further referred to as FA-1 preparation, were separated from milk fat of Friesian sheep during their third lactation, summer season, using the method described by Folch *et al*. [1957], and next saponified with an alcoholic KOH solution. The resulting soaps were converted to free fatty acids by a HCl solution. Part of the FA-1, containing 2.0% of $C_{18:2}$ 9*c*,11*t*, was left without modifying, while the remaining fatty acids were used to enrich them with the isomer of linoleic acid $C_{18:2}$ 9*c*,11*t*, by way of crystallization from urea (FA-U fraction) and then extraction with supercritical CO_2.

During crystallization from urea the ability of saturated fatty acids to form permanent complexes with urea was used. The filtrate (FA-U) obtained after separating the saturated fatty acid complexes (principally C_{12} to C_{18}) with urea was fractioned by extraction with CO_2 under supercritical conditions [Kim and Lin 1999, Walisiewicz-Niedbalska *et al*. 2001a, Walisiewicz-Niedbalska *et al*. 2001b] to obtain a fraction further referred to as preparation FA-2 containing 9.2% of linoleic acid $C_{18:2}$ 9*c*,11*t* isomer.

Analytical evaluation

The composition of fatty acids was determined by gas chromatography. Methyl esters were obtained according to the Official Methods and Recommended Practices of the AOCS (Ce2-66) [1989]. The separation was performed on a gas chromatograph (HEWLETT-PACARD) with a FID detector, column CP Sil 88, 100 m long. The temperature of the column was 170°C, of the injector 200°C, and of the detector 250°C; helium was used as the carrier gas.

The qualitative analysis was carried out by comparing with standards the retention times of the components examined and confirming the identification with the gas chromatography-mass spectrometry (GC/MS) method. CLA isomers from SIGMA were used as standards. The identification of position isomers was performed using the specific fragmentation of fatty acid derivatives carried out with 2-amino-2-methyl-1-propanol.

The separation of fatty acid methyl esters into *cis*- (FA-*cis*) and *trans*- (FA-*trans*) isomer fractions was performed by way of thin-layer chromatography (TLC-Ag+). A 85:15 (v/v) toluene-hexane mixture was used as the developing system; 2.7-dichlorofluoroescein was used as the developer, while visualization was carried out under UV radiation. Bands containing FA-*cis* and FA-*trans* methyl esters were collected and used for obtaining the 4,4-dimethyloxazoline (DMOX) derivatives of fatty acids. The 2-amino-2-methyl-1-propanol was added to the fraction of methyl esters collected, the mixture was sealed in an ampoule and heated at 185±10°C for about three hours. The resulting derivatives were dissolved in dichloromethane, washed with distilled water and, after the solvent had been evaporated, analysed by the GC/MS method.

A SIGMA commercial CLA preparation (CP) containing about 80% $C_{18:2}$9*c*,11*t* was used for comparison.

The *in vitro* anti-proliferative activities of the three presented preparations – FA-1, FA-2 and CP – were examined against the cells of two lines of human cancer: cervical carcinoma (KB) and leukaemia (HL-60). The KB line cells were purchased at the American Type Culture Collection (Rockville, Maryland, USA), while the HL-60 cells were obtained from the European Type Culture Collection, by courtesy of Professor G. Spik and Dr. J. Mazurier (Laboratory of Biological Chemistry, USTL, Lille, France). Both cell lines are maintained in culture at the Cell Line Bank of the Polish Academy of Sciences Institute of Immunology and Experimental Therapy, Wrocław.

The cells used were cultured in an opti-MEM (KB) or RPMI 1640 (HL-60) medium, supplemented with 5% FCS (GIBCO, Grand Island, USA – media and serum), 50 mg/ml streptomycin, 50 U/ml penicillin (both antibiotics from POLFA, Tarchomin, Poland)

and 2 mM glutamine (GIBCO). The cells were maintained at 37°C in a humidified atmosphere saturated with 5% CO_2.

The initial solutions of the compounds tested, at a concentration of 1 mg/ml, were prepared *ex tempore* for each experiment, dissolving 1 mg of preparation in 100 µl DMSO (dimethylsulphoxide, POCh, Gliwice, Poland) or DMF (dimethylformamide, POCh, Gliwice, Poland) plus 900 µl of the culture media. The culture media were also used as a solvent for obtaining further solutions. The compounds were tested at final concentrations of 100, 10, 1 and 0.1 µg/ml. The analyses were preformed using SRB (for KB) and MTT (for HL-60) methods, which render it possible to determine the inhibition of cell proliferation during a 96 hours of *in vitro* culture.

SRB test. The results of cytotoxic tests were obtained colorimetrically in 96-well flat-bottom plates (SARSTEDT, Newton, USA). Cells were plated in 100 µl of culture medium – 10^4 cells per well. After 24 hours of incubation under culture conditions another 100 µl of medium was added (control of cell growth), or a medium containing the preparation tested. The plates were incubated under the same conditions for another 72 hours at 37°C.

Next, into each well 50 µl of cold 50% TCA (trichloroacetic acid, POCh, Gliwice, Poland) were added. Another incubation followed, 60 minutes long, at a temperature of 4°C after which the plates were emptied and 5 times rinsed with running water. After drying, 50 µl of a 0.4% SRB solution (sulphorhodamine B, SIGMA, St. Louis, USA) in a 1% acetic acid (POCh, Gliwice, Poland) were added in order to stain the protein remaining in the wells. Another 30 minutes of incubation followed at room temperature, after which the plates were emptied once more and rinsed 4 times with 1% acetic acid. After drying, 150 µl of 10 nM TRIS (hydroxymethyl) aminomethane, SIGMA, St. Louis, USA) were added, which dissolved the stain. The optical density of the fluid in the wells was read on a Multiscan RM photometer (LABSYSTEMS, Helsinki, Finland), at a weave length of 540 nm. The photometer was standardized on the basis of a fluid obtained from a well containing only the culture medium, without cells. In each experiment samples containing specified concentrations of the preparation were tested three times. Each experiment was repeated three times [Shekan *et al*. 1990].

MTT test. The inhibition of cell proliferation was measured in a 144 hour *in vitro* culture. The results were obtained colorimetrically from 96-well, flat-bottom plates (SARSTEDT, Newton, USA). Cells immersed in 100 µl of the culture medium were placed in the wells – 5×10^3 cells per well. After 24 hours of incubation under culture conditions another 100 µl of the medium were added to each well (control of cell growth), or a medium containing the preparation tested. The plates were incubated under the same conditions for another 120 hours at 37°C. Next, to each well 20 µl were added of the MTT solution (MTT – 3-(4,5-dimethylthiasol-2-yl)-2,5-diphenyl tetrazolium bromide, SIGMA, St. Louis, USA); basic solution – 5 mg/ml. After four hours of incubation at 37°C 80 µl of a lysing buffer (225 ml dimethylformamide, 67.5 g sodium dodecyl sulphate – both SIGMA, St. Louis, USA – and 225 ml distilled water) were added. After a further 24 hours of incubation at 37°C the optical density of the fluid

in the wells was read in a Multiscan RM photometer (LABSYSTEMS, Helsinki, Finland) at a weave length of 570 nm. Each experiment was repeated three times.

Results and discussion

In the process of removing saturated mid-chain fatty acids from sheep milk fat as complexes with urea the content of saturated fatty acids decreased – principally C_{14} to C_{18} (by about 84%) of which palmitic acid by about 95%. Short-chain acids were almost totally removed in the process of extraction with supercritical CO_2 (about 97%). Among the fatty acids of the final FA-2 preparation, as main components were identified: oleic acid and its isomers, principally vaccenic acid, linoleic acid and its isomers, including 9.2% of 9*cis*,11*trans*, and linolenic acid.

Table 1. Fatty acids of natural (FA-1) and natural modified (FA-2) sheep milk fatty acids preparations

Acids	FA-1 (%m/m)	FA-U (%m/m)	FA-2 (%m/m)
Saturated			
C_4, C_6, C_8	2.3	3.2	0.1
C_{10}	3.5	3.9	0.1
C_{12}	2.7	2.8	0.1
C_{13}, C_{15}	0.3	0.4	0.0
C_{14}	9.6	3.2	1.8
C_{16}	24.5	1.1	0.5
C_{17}	1.1	0.1	0.0
C_{18}	13.5	2.5	1.8
Unsaturated			
$C_{18:1}$	29.4	62.5	68.2
$C_{18:1}$ 11t	2.8	4.2	7.5
$C_{18:1}$ 10c	0.4	0.5	1.3
Diunsaturated			
$C_{18:2}$	2.4	5.6	4.8
$C_{18:2}$ *iso*	0.9	0.9	1.0
$C_{18:2}$ 9c,11t (CLA)	2.0	4.6	9.2
$C_{18:2}$ (remaining CLA)	0.2	0.3	0.3
Triunsaturated			
$C_{18:3}$	1.3	1.5	2.1
Unidentified	3.1	2.7	1.2

FA-1 – sheep milk fat fatty acids.
FA-U – FA-1 after crystallization with urea.
FA-2 – FA-U after extraction with supercritical CO_2.

Table 1 presents the composition of fatty acids of unmodified sheep milk fat (FA-1) and of the products obtained as result of their enrichment in $C_{18:2}$ 9*c*,11*t* by crystallization with urea (FA-U) and next extraction in supercritical conditions (FA-2).

The results for the cytotoxic activity tests are presented as ID_{50} values (preparation dose resulting in a 50% inhibition of cancer cell proliferation) and as proliferation inhibition per cent caused by the preparations tested at the highest concentration, *i.e*.

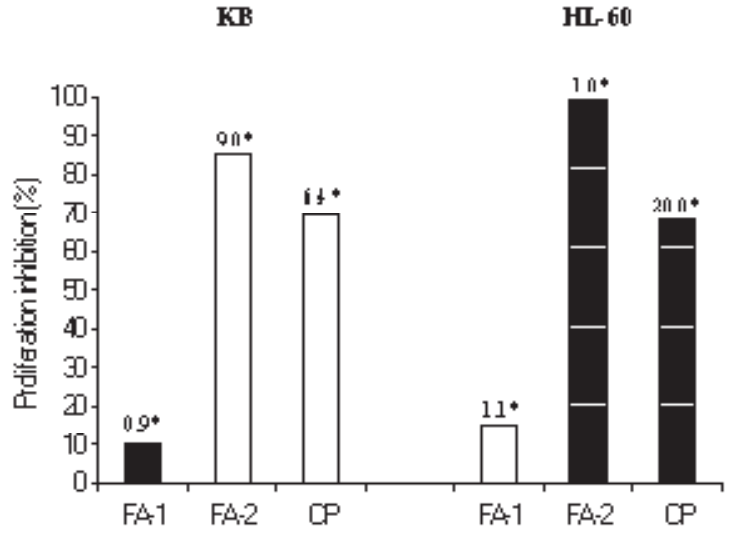

Fig. 1. Inhibition of cervical carcinoma (KB) and leukaemia (HL-60) cells growth by fatty acids preparations at a concentration of 100 μg/ml. FA-1 – sheep milk fat fatty acids; FA-2 – sheep milk fatty acids condensed after extraction with supercritical CO_2; CP – commercial preparation. Within cell line each mean differs significantly from remaining two. * Standard deviation.

Table 2. Anti-proliferative activity of fatty acids preparations against cancer cells of two lines

Cancer cells	Preparation	ID_{50} (μg/ml) mean	SD
Cervical cancer (KB)	FA-1	–	–
	FA-2	40.5	1.2
	CP	51.8	1.2
Leukaemia (HL-60)	FA-1	–	–
	FA-2	30.8	1.1
	CP	55.0	1.8

100 μg/ml. The anti-proliferative effect of the preparations used against cancer cells are presented in Figure 1 and Table 2.

The results presented here show the anti-proliferative activity of sheep milk fat preparation with the enriched level of CLA against both carcinoma cell types tested.

The synthetic commercial CLA preparation (CP), containing isomer $C_{18:2}$ 9*c*,11*t* demonstrated a lower anti-proliferative activity against the cancer cells tested than did the fraction of natural fatty acids isolated from sheep milk fat enriched with $C_{18:2}$ 9*c*,11*t* (FA-2). Also, the ID_{50} value for the synthetic CLA was higher than that observed for FA-2.

The ID_{50} value for the preparation FA-2 with an increased to 9.2% share of isomer $C_{18:2}$ 9*c*,11*t* against cells of the human line of cervical carcinoma (KB) proved to be 20% lower than that obtained for the CP (containing about 80% $C_{18:2}$ 9*c*,11*t*). In the case of leukaemia cells the effect of FA-2 was even more pronounced – ID_{50} for CP appeared as much as 40% lower than that found for FA-2. The proliferation inhibition of the cancer cells by 100 μg/ml of the natural enriched preparation of $C_{18:2}$ 9*c*,11*t* (FA-2) proved very high – 85.3 and 99%, against KB and HL-60, respectively. For the same dose of CP the proliferation inhibition of the cancer cells tested was lower, and amounted to 68 and 68%, respectively. These results confirm the data found in literature, pointing to the cytostatic activity of isomer $C_{18:2}$ 9*c*,11*t* against cancer of the mammary gland, prostate or stomach [Ip *et al*. 1994, Ip 1997, Cesano *et al*. 1998]. However, such significant differences in the anti-proliferative activity between the natural, enriched preparation FA-2 (9.2% of isomer $C_{18:2}$ 9*c*,11*t*) and the commercial standard preparation CP, indicate a specific character of the former, probably arising from its natural origin.

Compared to other literature data [Ip *et al*. 1991, Schultz *et al*. 1992, Ip 1994, Ip *et al*. 1994, Schonberg and Krokan 1995, Belury and Kempa-Steczko 1997, Ip 1997, Pariza *et al*. 2001] referring to the activity of synthetic preparations, the results presented here may indicate that the anti-cancer effect of sheep milk fatty acids includes also the effects of its other bio-active components.

The results of the study confirm the possibility of inhibiting the development of cancer by a preparation of natural origin, separated from sheep milk fat by traditional methods. The increased anti-proliferative activity of the fatty acids natural preparation containing 9.2% of isomer $C_{18:2}$ 9*c*,11*t* (CLA), when compared to the synthetic one containing 80% of the isomer, points to unique properties of the former. The importance of the results obtained should be evaluated further, also *in vivo*, on various lines of cancer cells.

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Badania *in vitro* właściwości przeciwnowotworowych naturalnego i syntetycznego preparatu CLA

Streszczenie

Do badań nad aktywnością antyproliferacyjną głównego izomeru kwasu linolowego $C_{18:2}$9*c*,11*t* (CLA), wybrano tłuszcz mleka owczego, który zawiera 1,8-3% izomeru. Porównywano efekt działania kwasów tłuszczowych mleka owczego, zawierających 2,0% izomeru $C_{18:2}$9*c*,11*t* (FA-1), kwasy po wzbogaceniu w izomery CLA przez zatężenie do 9,2% (FA-2) oraz handlowy preparat syntetyczny zawierający około 80% CLA. Aktywność antyproliferacyjną FA-1, FA-2 i preparatu handlowego (CP) badano wobec komórek dwóch linii ludzkich nowotworów: raka szyjki macicy (KB) oraz białaczki promielocytarnej (HL-60). Wartość ID_{50} (dawka powodująca zahamowanie proliferacji 50% populacji komórek nowotworowych) preparatu FA-2 była niższa niż ID_{50} preparatu handlowego. Wyniki testu SRB wykazały zahamowanie proliferacji komórek KB, dla wagowo identycznych dawek (100 μg/ml), wyższe dla preparatu FA-2 niż dla preparatu CP – odpowiednio 85,3% i 69,5%. Wyniki testu MTT wykazały zahamowanie proliferacji komórek HL-60, również wyższe dla preparatu FA-2 niż dla CP – odpowiednio 99,0% i 69,0%. Kwasy tłuszczowe mleka owczego bez modyfikacji (FA-1) wykazały niewielką aktywność wobec obu badanych linii komórek nowotworowych – około 10%.