RESEARCH ARTICLE

A lipoprotein source of cholesteryl esters is essential for proliferation of CEM-CCRF lymphoblastic cell line

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Abstract Tumour are characterised by a high content of cholesteryl esters (CEs) stored in lipid droplets purported to be due to a high rate of intracellular esterification of cholesterol. To verify whether and which pathways involved in CE accumulation are essential in tumour proliferation, the effect of CE deprivation, from both exogenous and endogenous sources, on CEM-CCRF cells was investigated. Cholesterol synthesis, esterification and content, low-density lipoprotein (LDL) binding and high-density lipoprotein (HDL)-CE uptake were evaluated in cultured in both conventional and delipidated bovine serum with or without oleic or linoleic acids, cholesteryl oleate, LDL and HDL. High content of CEs in lipid droplets in this cell line was due to esterification of both newly synthesised cholesterol and that obtained from hydrolysis of LDL; moreover, a significant amount of CE was derived from HDL-CE uptake. Cell proliferation was slightly affected by either acute or chronic treatment up to 400 μM with Sz-58035, an acyl-cholesteryl cholesterol esterification inhibitor (ACAT); although when the enzyme activity was continuously inhibited, CE content in lipid droplets was significantly higher than those in control cells. In these cells, analysis of intracellular and medium CEs revealed a profile reflecting the characteristics of bovine serum, suggesting a plasma origin of CE molecules. Cell proliferation arrest in delipidated medium was almost completely prevented in the first 72 h by LDL or HDL, although in subsequent cultures with LDL, it manifested an increasing mortality rate. This study suggests that high content of CEs in CEM-CCRF is mainly derived from plasma lipoproteins and that part of CEs stored in lipid droplets are obtained after being taken up from HDL. This route appears to be up-regulated according to cell requirements and involved in low levels of c-HDL during cancer. Moreover, the dependence of tumour cells on a source of lipoprotein provides a novel impetus in developing therapeutic strategies for use in the treatment of some tumours.

Keywords Cholesteryl esters · Tumour cell proliferation · Lipoproteins

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Introduction

The cholesterol metabolism changes dramatically during tumour growth, being directed towards the uptake and storage of cholesterol in tumour tissues [1–3]. Moreover low levels of cholesterol-low-density lipoprotein (LDL) and cholesterol-high-density lipoprotein (HDL) have frequently been reported in cancer patients [4–8]. The authors previously hypothesised that during proliferative burst, the excess of membrane cholesterol is preferentially diverted to ER for esterification rather than delivered to HDL, this shift consequently increases intracellular CE and decreases it in the plasma compartment [1, 2].

CEs synthesis by acyl-CoA–cholesterol acyl transferase (ACAT) is a homeostatic mechanism that concurs towards preventing cellular accumulation of free cholesterol, whilst acting, in specialised organs, as a reservoir for bile acid production and steroid hormone biosynthesis [9, 10]. Intracellular CEs are confined to metabolically active droplets, hydrolyzed and synthesised according to cell requirements [11]. Gebhard [12] first reported that clear renal carcinoma in the rat contains 35-fold more CEs than the corresponding normal cells. Similar findings were also observed in the corresponding human renal cancer, as well as several experimental [13, 14] and human tumours [1, 2, 15, 16]. High levels of CEs are considered to be related to the large amounts of cholesterol needed by malignant cells to sustain membrane biogenesis. Indeed, to meet the increased demand, an increase in cell cholesterol synthesis and uptake is manifest, whilst, at the same time its release to HDL is remarkably reduced. As the major function attributed to HDL is the maintenance of cholesterol homeostasis in normal cells by removing excess cholesterol, the authors suggest that during tumour growth, the observed decrease registered in HDL-cholesterol and concomitant accumulation of CEs may be due in part to a reduction in cholesterol efflux from proliferating cells [17]. This phenomenon has been described in normal proliferating fibroblasts [18]. In a previous study, our group focused on cholesterol esterification, efflux and the expression of correlated genes during exponential and stationary growth of two different cell lines, CEM and MOLT4, isolated from patients with lymphoblastic T cell leukaemia. The results obtained revealed how a greater capacity to esterify and accumulate cholesterol in CEM-CCRF cells was associated with a higher growth rate than MOLT-4 [17, 19]. We moreover demonstrated that cholesterol esterification in smooth muscle cells is a key event in vascular proliferative diseases [20]. Furthermore, inhibition of CEs synthesis partially blocked cell proliferation, although the extent of the latter effect was dependent on the cell type [20–23], suggesting the potential involvement of cholesterol esterification as a new pathway implicated in cell growth. On the other hand, several studies have demonstrated how proliferation of tumour cells is arrested in a delipidated medium, with inhibition being almost completely prevented following addition of LDL and/or HDL [24, 25]. Thus it can be hypothesised that plasma lipoproteins are also implicated in regulation of cholesterol metabolism in tumour cells.

Despite the abundance of literature published on this topic, it is still a matter of debate whether maintaining high content of CEs is a requisite for tumour cell proliferation. The question therefore arises as to whether and to what extent endogenous synthesis and exogenous source of cholesteryl esters provided by lipoproteins contribute towards this process. Accordingly, in the present study, the effect of CEs deprivation on lymphoblastic line CEM-CCRF growth was investigated.

Materials and methods

Cell culture

The cell line CCRF-CEM, isolated from a child with acute human T cell leukaemia, a highly proliferative and malignant tumour, was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cultures were maintained in exponential growth (between 2×10^5 cells/ml and 1.5×10^6 cells/ml) at 37°C in T-25-cm^2 tissue culture flasks (Falcon, M19, Milan, Italy) containing RPMI 1640 medium supplemented with 10% foetal calf serum (FCS; Sigma-Aldrich, Milan, Italy) or 10% bovine calf serum (BCS; Bioquote Ltd, York, UK), streptomycin (100 μg/ml) and penicillin (100 U/ml; growth medium). Stock cultures were diluted and seeded at a density of ≈2×10^5cell/ml in growth medium; in some experiments, cells were treated with 4 μM Sz-58035 (Sigma-Aldrich, Milan, Italy) for 72 h (acute treatment). To investigate the effect of chronic ACAT inhibition, cells were cultured up to 6 months with the drug. In some experiments, cells were diluted and seeded in medium containing RPMI 1640 supplemented with 10% lipoprotein delipidated bovine calf serum (LDBCS). In these cells, growth and cholesteryl ester metabolism were evaluated in presence of oleic (1.5 μM) and linoleic acids (1.5 μM), cholesteryl oleate (1 mg/ml), 100 μg/ml LDL and 250 μg/ml HDL (Biochemical Technology, MA, USA). Lipid content in culture media and cells was evaluated in all experiments.

Determination of cholesterol synthesis

To determine the rate of cholesterol synthesis 10^6/ml cells were incubated for 3 h with 185 kBq/ml of sodium [^{14}C]-acetate. After incubation, they were separated by centrifugation and collected. Lipids were extracted with cold acetone and neutral lipids separated by thin layer chromatography (TLC) Kiesegel plates using a solvent system containing eptane/isopropylether/formic acid (60:40:2 $v/v/v$). Free cholesterol and cholesterol ester bands were identified by comparison with standards running simultaneously with samples and visualised using iodine vapour [17]. For counting, the bands were excised and added directly to counting vials containing 10 ml of Ultima Gold. All incubations were carried out in triplicate and the results of individual experiments are given as mean values, variation between triplicates was

less than 10%. All data are expressed as the rate of [^{14}C] acetate incorporation into cholesterol for microgrammes of protein.

Determination of cholesterol esterification and triglyceride synthesis from [^{14}C]-oleate

Cells were incubated for 4 h in medium containing [^{14}C]-oleate bound to bovine serum albumin (BSA). To prepare the oleate–BSA complex, 3.7 MBq of [^{14}C]-oleic acid in ethanol (specific activity 2.035 GBq/mmol) was mixed with 1.4 mg KOH and the ethanol evaporated. PBS (1.5 ml) without Ca^{2+} and Mg^+ containing 4.24 mg BSA (fatty acid-free) was added and the mixture shaken vigorously. This solution was added to each well at a final concentration of 74 KBq/ml. After incubation, cells were washed with ice-cold PBS and lipids extracted with acetone. Neutral lipids were separated by TLC [17] and incorporation of [^{14}C] oleate into cholesterol esters was measured. An aliquot of cell lysate was processed for protein content [26].

Cholesterol esters and triglyceride synthesis from [^{14}C]-cholesteryl oleate

To determine the rate of cholesterol and triglyceride synthesis from an exogenous source of cholesteryl oleate, 10^6/ml cells were incubated for 6 h with 185 kBq/ml of [^{14}C]-cholesteryl oleate. After incubation cells, were washed with ice-cold PBS and lipids extracted with acetone and proceeded as above.

Analysis of free cholesterol and cholesteryl esters in media and cells

Total lipids were extracted from the cells and media using the method described by Folch et al [27]. Aliquots from chloroform phase were dried down under vacuum and dissolved in methanol for HPLC analysis. Separation of cholesteryl esters was carried out as described in [28] with an Agilent 1100 HPLC system (Agilent, Palo Alto, CA) equipped with a diode array detector and mass spectrometer in line. UV and mass spectra were recorded to confirm the identification of the HPLC peaks [29]. Cholesterol esterified with conjugated linoleic acid (18:2 CLA), a fatty acid biosynthesized exclusively in the rumen and characteristic of the tissues and milk of ruminants [30] was likewise identified using the above method. Indeed, human CEM-CCRF cells are unable to produce CLA, thus all intracellular CLA derive from medium culture which is added with 10% bovine serum. We utilised CLA as a marker of exogenous uptake of CE when endogenous synthesis was inhibited.

Lipid droplet imaging and measurements

To visualise neutral lipids in situ, cells were fixed by adding a proportional amount of paraformaldehyde (PFA) from a 16% stock solution to the growth medium to obtain a final concentration of 4% PFA. After 30 min, cells were washed in PBS and resuspended in 300 nM Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one; Fluka, Buchs, SG, Switzerland) in PBS [31]. The cell suspension was then placed in the well of a 35-mm glass-bottomed dish (MatTek, Ashland, MA) for microscope observation. Nile Red is a fluorescent dye that stains differentially polar lipids (i.e. phospholipids) and neutral lipids (i.e. CEs and triglycerides) [32]. Polar lipids display a red emission, while neutral lipids display a yellow emission. Red emission was observed with 540±12.5 excitation and 590 LP emission filters. Yellow emission was observed with 460±25 excitation and 535±20 emission filters. Cells were observed using an Olympus IX 71 inverted microscope (Olympus, Tokyo, Japan) fitted with a ×20/0.7 planapochromatic objective. Twelve-bit images were captured using a cooled CCD camera (Sensicam PCO, Kelheim, Germany) electronically coupled to a mechanical shutter interposed between the 100-W Hg lamp and the microscope to limit photobleaching. Excitation light was attenuated with a 6% neutral density filter. The nominal resolution of images was 0.3 μm/pixel. Quantitative analysis of images was performed with the ImagePro Plus package (Media Cybernetics, Silver Springs, MD). At least 200 cells were individually measured for each experimental group.

Dil-LDL binding and Dil-HDL uptake

To evaluate binding of LDL and uptake of CE-HDL, we utilised lipoproteins bound with the fluorescent dye Dil (Bioquote, London). Cells were seeded at a concentration of $2.0×10^5$/ml and incubated at 4°C for 2 h with 10 μg/ml of Dil-LDL or Dil-HDL. Afterwards, samples were processed as described by Stephan et al [33]. Fixed cells were observed by using an inverted microscope, Olympus IX71 (Olympus, Tokyo, Japan), equipped with a ×20/0.7 objective planapochromatic. The nominal resolution of images was 0.3 μm/pixel. The quantitative analysis of images was performed with the ImagePro Plus package (Media Cybernetics, Silver Springs, MD). At least 200 cells were measured individually for each experimental group.

Cell proliferation

Cell number and viability, determined by a Burker Chamber and by the trypan blue dye exclusion test,

Table 1 FC, CE and CLA content in media utilised for experimental purposes (nanomoles per millilitre)

10% Serum in culture media	FC	CE total	CE/FC	CLA in CE
FCS	54.72	202.85	3.71	0.74
BCS	77.44	338.52	4.37	0.40
LDBCS	n.d	n.d	n.d.	n.d
LDBCS+LDL 100 μg/ml	109.75	275.76	2.51	0.88
LDBCS+HDL250μg/ml	49.37	221.55	4.49	0.92

FCS foetal calf serum, *BCS* bovine calf serum, *LDBCS* lipoprotein-deprived bovine calf serum, *LDL* low-density lipoprotein, *HDL* high-density lipoprotein

respectively, were measured in all experiments. To evaluate DNA synthesis in some experiments cells were incubated

for 2 h with 185 kBq/ml of [³H]-thymidine. At the end of the incubation period, cells were rinsed with ice-cold phosphate-buffered saline (PBS), washed with 5% cold trichloroacetic acid at 4°C and hydrolyzed with 1 M NaOH at room temperature. Radioactivity was measured by a Beckman β counter (Palo Alto, CA) using Ultima Gold as scintillation fluid. An aliquot of cell hydrolysate was processed for protein content [26].

Statistical analysis

All data were expressed as the mean±SD of experiments in triplicate and analysed by Student't test and one- or two-way ANOVA, post hoc tests (Tukey or Bonferroni test) applied when required. Significance was set at $p<0.05$.

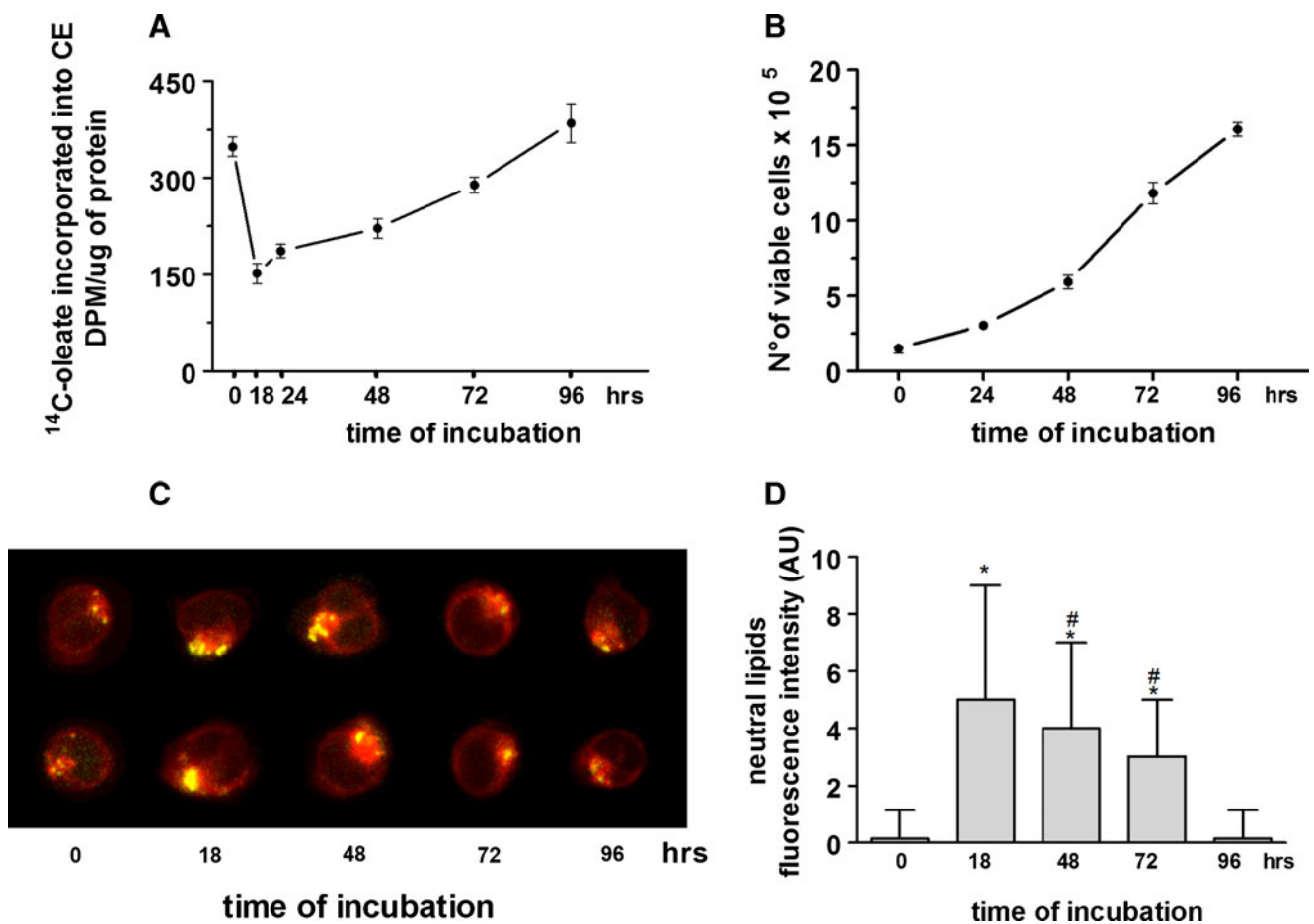**Fig. 1** Cholesterol ester (*CE*) synthesis and content during the growth of CEM-CCRF The cells were seeded at a density of 2×10^5 cells/ml in tissue culture plates and grown in RPMI 1640 medium supplemented with 10% FCS. All determinations were evaluated at the indicated times. **a** At 4 h before the indicated times cells were labelled with 3.7 MBq of [¹⁴C]-oleic acid in ethanol (specific activity 2.035 GBq/mmol) and the amount of [¹⁴C]oleate incorporated into CEs was determined as described in the materials and methods section. **b** Cell number were determined with a Coulter Counter and were corrected for viability by Trypan Blue dye exclusion. **c** Neutral lipids were visualised by Nile Red fluorescence (yellow spots observed with 460±25 excitation and 535±20 emission filters). **d** Quantitative analysis of Nile Red yellow emission was performed with the ImagePro Plus package. At least 200 cells were individually measured for each experimental group (for details see materials and methods). Data represent means±SD for triplicate determinations of a representative experiment. *$p<0.001$ vs time 0 and 96 h; #$p<0.01$ vs 18 h (Newman–Keuls multiple comparison test)

Results

Lipid content of culture media

Table 1 shows the content in free cholesterol (FC), CE and CLA-CE in culture media with different sera and treatments utilised for experiments.

Cholesterol esterification and content during the growth of CEM-CCRF

Figure 1 shows cholesterol esterification (panel a) during the growth of CEM-CCRF (panel b). Cholesterol ester synthesis dropped over the first 18 h of culture at a lower cell density, and subsequently increased progressively together with cell number. However both lipid droplets (panels c and d) and CE featuring extremely low content at the time of dilution (time 0) were significantly ($p < 0.001$) increased following 18 h incubation (203.85 ± 18.31 and 431.19 ± 35.74 pmol/10^7 confluent vs. exponentially growing cells; see also Table 2), with both values decrease at cell density saturation ($\sim 1.5^6$ cell/ml) at 96 h.

Fluorescence imaging of Dil-LDL and Dil-HDL in CEM-CCRF

Fluorescence imaging of Dil-LDL and Dil-HDL in CEM-CCRF showed an efficient LDL binding and a consistent amount of CE deriving from HDL (Fig. 2). Although all incubations were carried out at 4°C, the effect of temperature affected differently LDL and HDL. Indeed at this temperature, LDL binds to its receptor but internationalisation is avoided; on the contrary, HDL does not bind to any receptor and only the CE component is internalised. Previous experiments showed that CE uptake was independent from the culture temperature.
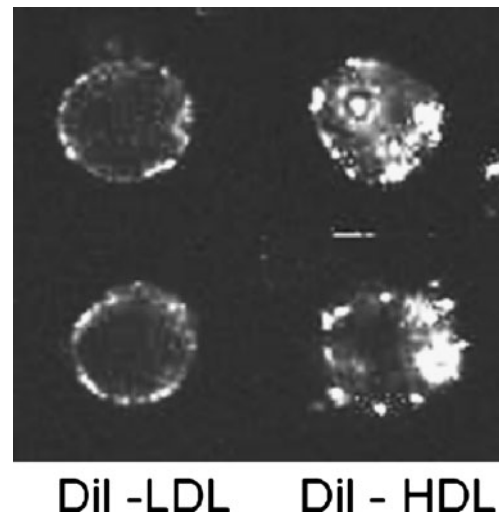

Dil -LDL   Dil - HDL

Fig. 2 Fluorescence imaging of *Dil-LDL* and *Dil-HDL* in CEM-CCRF cells were seeded at a concentration of 2.0×105/ml and incubated at 4°C for 2 h with 10 μg/ml of Dil-LDL or Dil-HDL. Afterwards, samples were processed as described by Stephan et al [33]. Fixed cells were observed by using an inverted microscope, Olympus IX71 (Olympus, Tokyo, Japan), equipped with a ×20/0.7 objective planapochromatic. The nominal resolution of images was 0.3 μm/pixel. The quantitative analysis of images was performed with the ImagePro Plus package (Media Cybernetics, Silver Springs, MD). At least 200 cells were measured individually for each experimental group

Effect of ACAT activity inhibition on cell growth and CE synthesis and content

As shown in Fig. 3 (panel a), acute and chronic treatment with Sz-58035 (4 μM) was accompanied by a marked and significant reduction of cholesterol esterification in both groups (311.65 ± 10 vs. 77.22 ± 8 and 72.35 ± 7, control vs. ACS and CRS respectively, $p < 0.001$). CRS treatment determined a reduction of cholesterol synthesis (panel b),

Table 2 FC, CE and CLA content in cholesteryl esters (picomole per 10^7 cells)

	FC	CE tot	CE/FC	CLA in CE
CEM-EG	89.13±17.21	431.19±35.74	4.84±0.93	0.75±0.15
CEM-SG	74.97±6.32	203.80±18.31*	2.72±0.95	0.47±0.12
CEM-ACS EG	69.95±12.47	485.62±52.86	6.94±1.52	0.74±0.22
CEM-ACS SG	70.19±18.22	221.04±29.31*	3.15±1.76	1.52±0.41***
CEM-CRS SG	70.56±21.66	367.11±31.48**	5.20±1.21	1.30±0.33***

Analysed by Newman-Keuls multiple comparison test

FC free cholesterol, *ACS* acute Sandoz-58035, *EG* exponentially growing, *SG* stationary growing, *CRS* chronic Sandoz-58035

* $p < 0.01$ CEM-SG vs CEM-EG and CEM-ACS EG

** $p < 0.05$ CEM-CRS SG vs CEM-ACS SG and CEM-SG

*** $p < 0.01$ CEM-SG vs CEM-ACS SG and CEM-CRS SG, CEM-ACS EG vs CEM-ACS SG, CEM-EG vs CEM-ACS SG
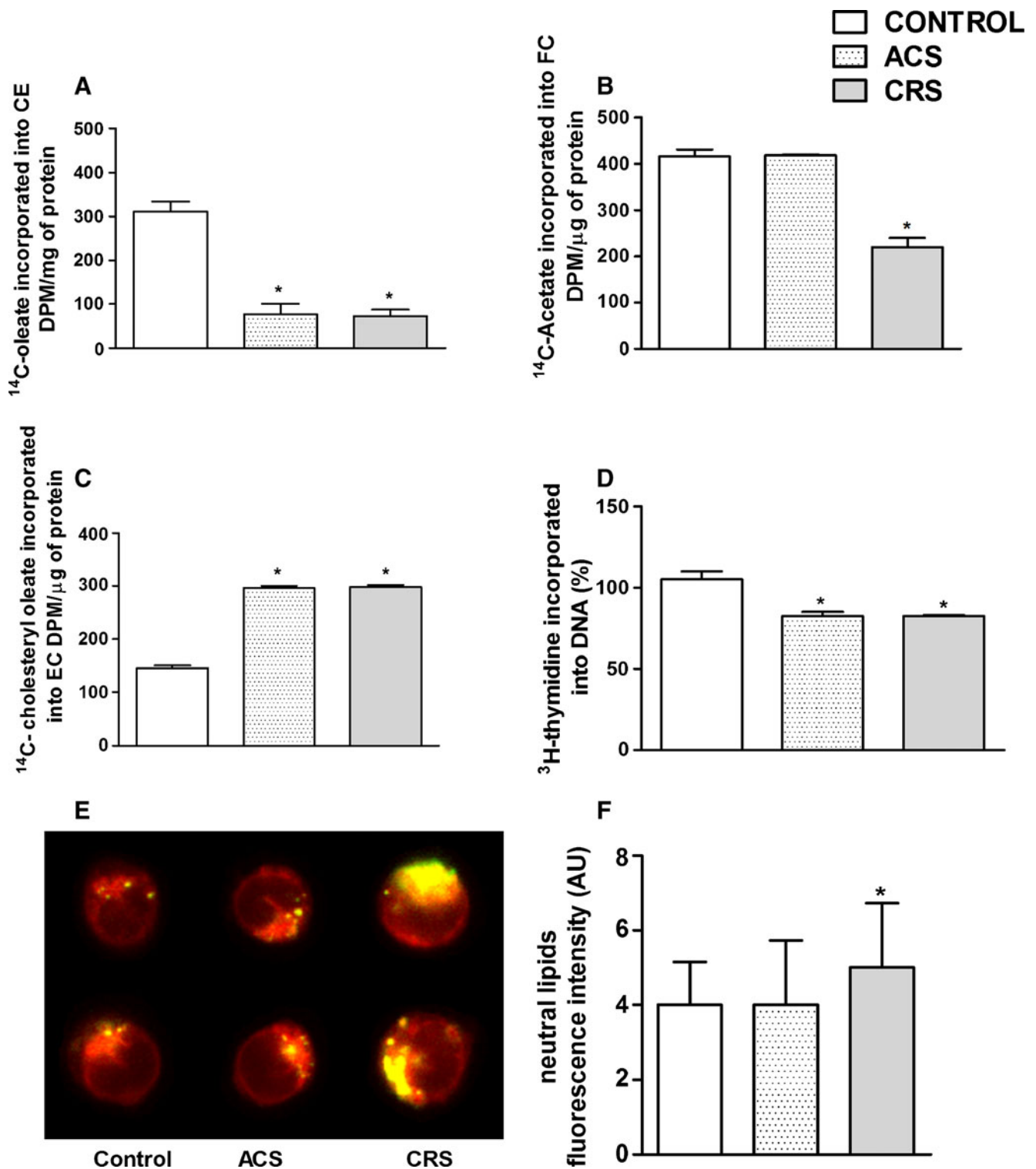
Fig. 3 Effect of acute (ACS) and chronic (CRS) ACAT activity inhibition on cell growth, free cholesterol (FC) and CE synthesis and content. Control cells, ACS (4 µM Sz-58035 for 72 h) and CRS (4 µM Sz-58035 for 6 months) were plated at a density of ≈2× 10^5 cell/ml and cell analyses were performed after 72-h culture. a CE synthesis in cells labelled with [^{14}C]-oleate (see Fig. 1). b FC synthesis in cells labelled for 3 h with [^{14}C]-acetate and processed as for CE. c Cholesteryl oleate uptake in cells labelled 6 h with 18.5 kBq/ml [^{14}C]-cholesteryl oleate. d To evaluate DNA synthesis, cells were incubated for 2 h with 185 kBq/ml of [^{3}H]-thymidine. e Neutral lipids were visualised by Nile Red fluorescence (yellow spots) and f quantitative analysis of Nile Red yellow emission was performed as described in Fig. 1. Data represent means±SD for triplicate determinations of a representative experiment. *$p<0.005$ CRS vs control, #$p<0.25$ CRS vs control and ACS (Newman–Keuls multiple comparison test)

whereas ^{14}C-cholesteryl oleate uptake significantly increased in both (panel c). Cell growth (panel d) was only slightly but significantly inhibited (about 10–20%) in both groups. Higher doses of the drug (up to 400 μM) did not further affect cell growth or viability (data not shown). Moreover, lipid droplet content was significantly increased in CRS when compared to control and ACS groups (panel e and f; $p < 0.05$). Table 2 shows cholesterol and CE contents, and CLA in CE of cells. An early significant increase ($p < 0.01$) in CE content was found in exponentially growing cells, either control or Sz-58035-treated cells (221.04±29.31 SG vs. 485.62±52.86 EG). A significant difference ($p < 0.05$) in CE content was found in chronically treated SG cells when compared to other corresponding groups. These results were confirmed by lipid droplet evaluation (Fig. 3, panel e, f). As expected cholesteryl CLA was detected in FCS and BCS (see Table 1). Accordingly, a substantial increase of cholesteryl CLA in cells cultured with BCS in the presence of Sandoz is strongly suggestive of an exogenous uptake of CE (Table 2).

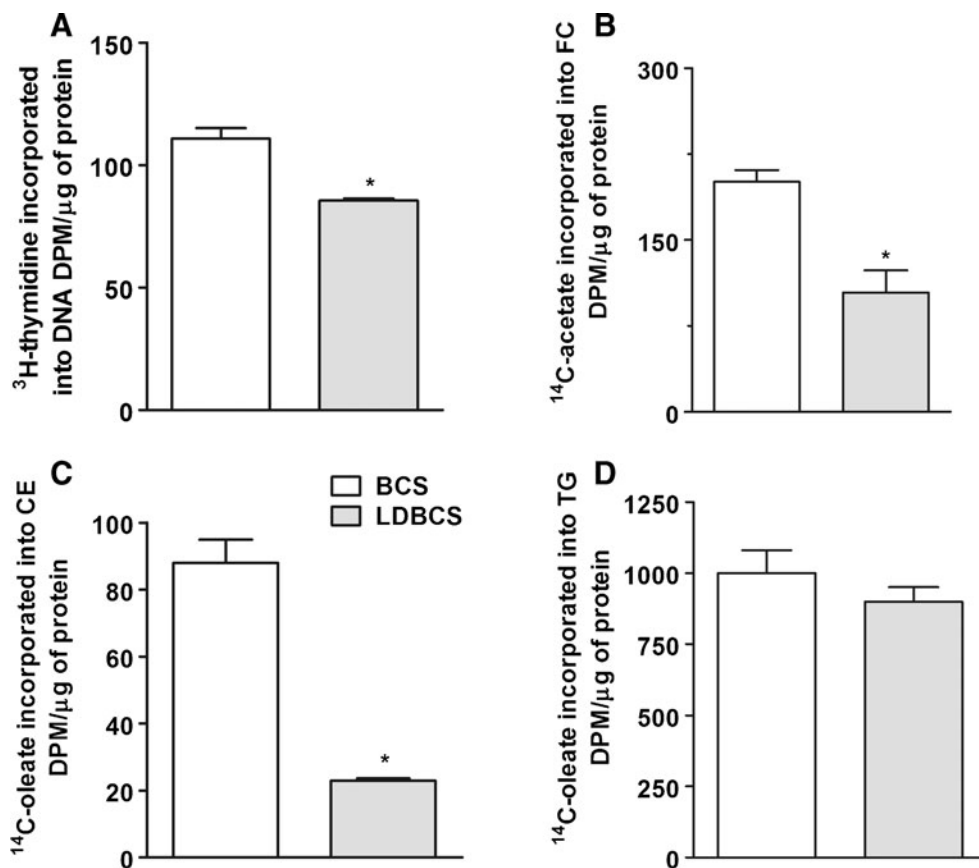## Effect of delipidated serum on cell growth and lipid synthesis

The effect of 72-h culture with LDBCS on cell growth and lipid synthesis is shown in Fig. 4. In these experiments, control cells were cultured with the corresponding whole serum (BCS). LDBCS caused a significant reduction of cell growth (panel a). Free (panel b) and esterified cholesterol (panel c) and triglyceride synthesis (panel d) were all markedly and significantly reduced. It is interesting to note how cholesterol synthesis increased over the first 24–36 h following culture in LDBCS and was subsequently markedly reduced (data no shown).

Effect of lipids and lipoproteins on cell cultured in LDBCS

As shown in Fig. 5 panel a, oleic acid (OA) and linoleic acid (LA) did not significantly modify CE synthesis in control cells. On the contrary, in the presence of cholesteryl oleate (CO), cholesteryl esterification was significantly reduced in BCS cells; whereas when added to LDBCS, cell lipid alterations (panel b) and growth inhibition (Table 3) were not prevented. As shown in Fig. 6, HDL (250 μg) and LDL (100 μg) showed a proven ability to revert growth inhibition in LDBCS throughout the first 72 h of incubation. Moreover, Sz-58035 prevented the effect of LDL on cell growth whilst proving ineffective in HDL-treated cells (panel a). Lipoproteins (panel b) however exerted an opposite effect on cholesterol esterification that was significantly reduced by HDL and induced by LDL,

Fig. 4 Effect of bovine calf serum (*BCS*) and lipoprotein delipidated bovine calf serum (*LDBCS*) on cell growth and *FC*, *CE* and triglyceride (*TG*) synthesis. The cells were cultured, at a density of ≈2×10^5 cell/ml, in medium containing RPMI 1640 supplemented with 10% bovine calf serum (*BCS*) or 10% lipoprotein delipidated bovine calf serum (LDBCS). Cell analyses were performed after 72-h culture. **a** [3H]-thymidine incorporation. **b** FC synthesis; **c** CE and **d** TG synthesis. Data represent means±SD for triplicate determinations of a representative experiment. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs BCS (Student *t* test)
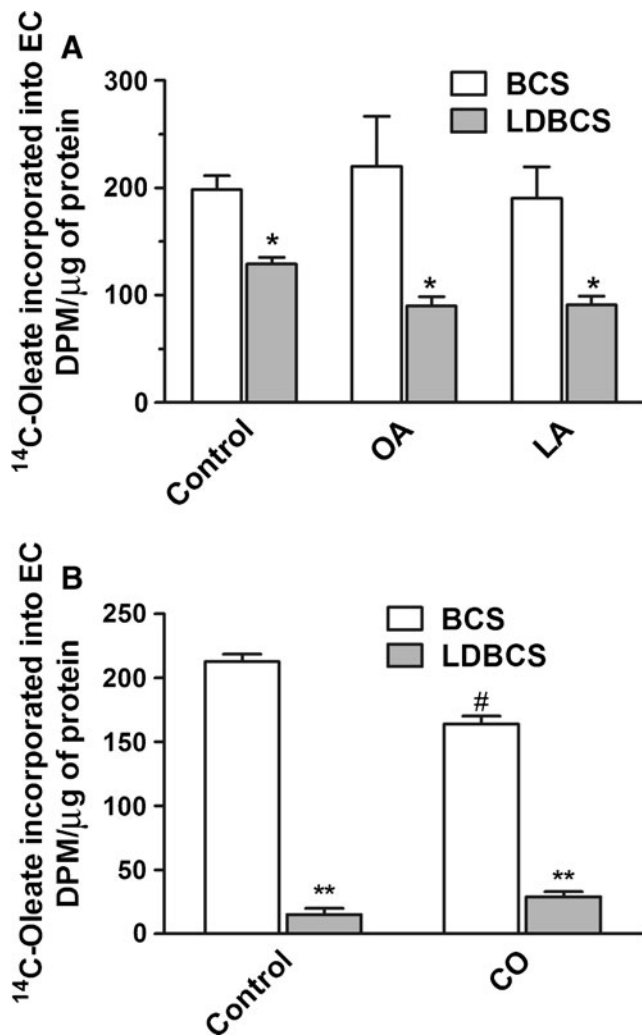
Table 3 CEM-CCRF proliferation in presence of BCS, LDBCS, CO; OA, LA, CLA

Culture conditions	Number of viable cells×10^5	
	0 h	72 h
BCS	2.0	15.0±0.30
LDBCS	2.0	4.0±0.08[*]
LDBCS+CO	2.0	4.2±0.10[*]
LDBCS+OA	2.0	4.3±0.07[*]
LDBCS+LA	2.0	4.1±0.10[*]
LDBCS+CLA	2.0	4.0±0.07[*]

BCS bovine calf serum, *LDBCS* lipoprotein-deprived bovine calf serum, *CO* cholesteryl oleate, *OA* oleic acid, *LA* linoleic acid, *CLA* conjugated linoleic acid

[*] $p<0.001$ vs BCS (Bonferroni post-test)

Fig. 5 Effect of lipid addition on CE synthesis and proliferation in cells cultured in LDBCS. The cells were cultured, at a density of ≈2× 10^5 cell/ml, in medium containing RPMI 1640 supplemented with 10% bovine calf serum (*BCS*) or 10% lipoprotein delipidated bovine calf serum (*LDBCS*). Cell analyses were performed after 72-h culture. CE synthesis in presence of oleic acid (*OA*, 1.5 μM) and linoleic acid (*LA*, 1.5 μM) (**a**) and in presence of cholesteryl oleate (*CO*, 1 mg/ml) (**b**). Data represent means±SD for triplicate determinations of a representative experiment. *$p<0.05$ vs the corresponding control group (**a**); **$p<0.0001$ vs the corresponding controls and #$p<0.05$ BCS-CO vs BCS (Bonferroni post test)

this induction significantly prevented by Sz-58035. Additionally, after 72-h culture in LDBCS only cells to which HDL had been added were still alive and proliferated in the subsequent cell cycles, whereas those grown in LDL progressively died (data not shown).

Discussion

The present study demonstrates how high contents of CE are required for proliferation of lymphoblastic cell line

CEM-CCRF. To ensure the storage of high levels of CEs in lipid droplets, cells provide them through both endogenous synthesis and exogenous uptake. The data obtained in this study strongly suggest the involvement of exogenous sources of esterified sterols in the regulation of cell growth. To this aim, it was observed that: (1) an almost complete inhibition of endogenous CE synthesis by Sz-58035 only slightly affected cell proliferation even at exceedingly high doses or when chronically administered; (2) under experimental conditions of chronic ACAT inhibition, cells continue to display very high concentrations of CE stored in lipid droplets; (3) during the initial proliferative burst observed when shifting cells from elevated to lower density, CE content is remarkably increased, although endogenous synthesis is reduced. CLA-CE, deriving mainly from bovine serum [30], also increases considerably in CEM with complete inhibition of ACAT activity, thereby suggesting that CE in lipid droplets have also an exogenous origin. According by other authors [24, 34–37], by culturing cells in delipidated serum only the addition of LDL or HDL was able to prevent inhibition of cell proliferation. As early as in the 1980s, reports were published referring how when culturing cells in delipidated serum, following an increase in cholesterol synthesis in the first 24–36 h, cell proliferation was progressively arrested [24]; these results however were not given the attention they deserved. Delipidated serum contains nutrients, hormones and all factors required for growth, and cells possess an intact capacity to synthesise and esterify cholesterol. In the present study, the addition of fatty acids failed to prevent growth inhibition, although an elevated triglyceride synthesis confirmed uptake of these compounds. Culture of LDBCS cells with either HDL or LDL prevented inhibition of proliferation and CE increased markedly in both conditions. The failure of LDL to prevent growth inhibition in the
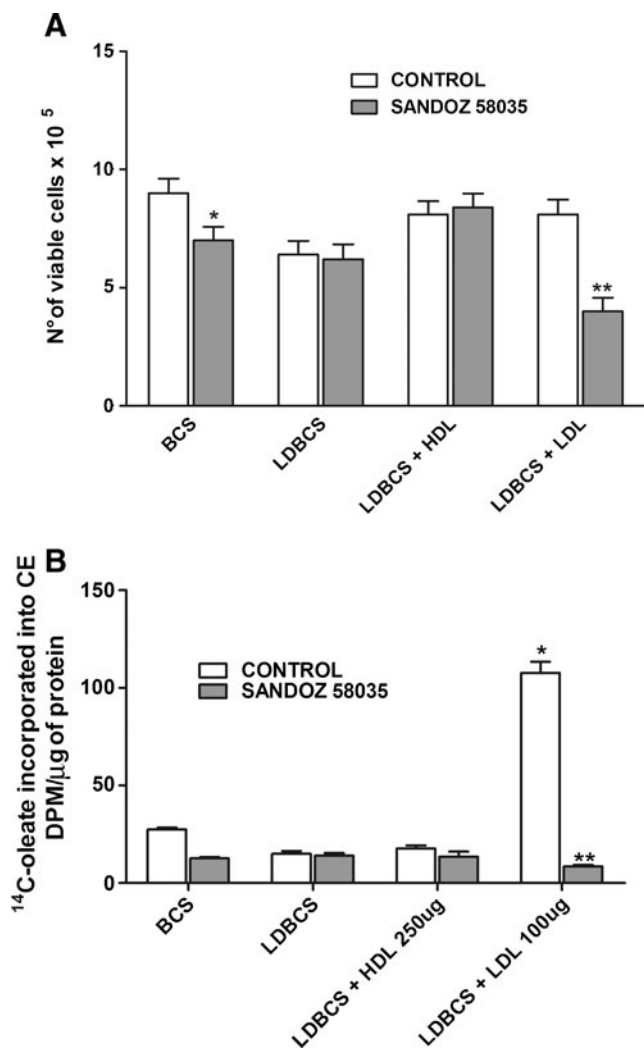
Fig. 6 Effect of lipoprotein addition on CE synthesis and proliferation in LDBCS cells with or without ACAT inhibition. BCS and LDBCS cells were seeded at a density of $\approx 2 \times 10^5$ cell/ml and harvested after 72 h. Some of LDBCS cells were treated either with 100 μg LDL/ml or 250 μg/ml HDL in presence or absence of 4 μM Sz-58035. **a** CE synthesis and **b** cell proliferation (for details see Figs. 3 and 4, as well as Materials and methods). Data represent means±SD for triplicate determinations of a representative experiment. *$p < 0.01$ and **$p < 0.0001$ vs the corresponding controls (Bonferroni post test)

presence of an inactive ACAT suggests that LDL exerts its effect also by inducing CE production. Intracellular CE synthesis was diversely affected by the two classes of lipoproteins, being markedly induced by LDL in absence of HDL. On the other hand, the lack of CE esterification in cells growing with only HDL shows that, in such experimental condition, very low amounts of free cholesterol deriving from exogenous CE are available for esterification. It was subsequently observed in LDBCS cultures that only cells to which HDL had been added continued to survive and proliferate, whereas those growing in LDL progressively died. Accordingly, these results seem

to suggest a short-lived efficacy in endogenously formed CEs, whilst at the same time suggesting the need for availability of HDL-CE for cell proliferation. Up to now, however, our data are not sufficient to clearly establish the crucial role of CE-HDL for tumour growth.

As established elsewhere, cells obtain exogenous cholesterol either via LDL receptor-mediated endocytosis, in which free cholesterol is made available only following complete lysis of lipoproteins and re-esterification by ACAT [38], or when CE is taken up directly from HDL [25] by a plasma-membrane scavenger receptor protein (SR-B1) in rodents [39], and CLA-1 in humans [40]. Further to its presence in specialised organs, such as the adrenal cortex and liver, a selective uptake of HDL-CE by SR-B1 has been described in rapidly proliferating tumour cells [41–43]. This protein is considered to facilitate extra-lysosomal hydrolysis of HDL-CE by the hormone-sensitive lipase, providing additional 'free' cholesterol for new membranes, and for intracellular signalling pathways involved in the regulation of cell proliferation [40]. Although lipoproteins have long been considered a source of free cholesterol for increased membrane biogenesis, the results obtained here demonstrate that also CEs, both exogenous and newly synthesised, stored in lipid droplets are essential for cell proliferation. Moreover, CE content seems to be strictly controlled since ACAT inhibition is accompanied by an increased uptake of CE from medium, whereas in absence of HDL-CE synthesis remarkably increases. These results suggest that having tumour cells, different pathways to increase intracellular CE, ACAT inhibition is not sufficient to consistently affect cell growth in our cell lines. The present results therefore could explain, at least in part, the mitogenic effect of both LDL [24] and HDL [44–46] described in the literature, although it would appear that only HDL is crucial in providing CEs to growing cells [23]. A physiological role for this pathway could be suggested particularly as the uptake or synthesis of CE by routes other than HDL does not suffice in ensuring an optimal CE pool for cell proliferation. On the other hand, this route has been reported to be essential for several functions, such as an optimal adrenal activity [47]. This novel mechanism, fundamental in maintaining high CE concentrations in tumour cells could account for the reduction of cholesterolemia, mainly c-HDL, described in experimental and human tumours [1–3]. Such a singular modification may be further supported by the reduction of cholesterol efflux observed mainly in cultured proliferating cells [18].

In conclusion, the present study demonstrates how high levels of CE stored in lipid droplets in CEM-CCRF are dependent on both cholesterol esterification synthesis and CE deriving from HDL suggesting that a source of lipoprotein is essential for their growth. This finding may

justify the low plasma lipid levels found in individuals affected by large tumours. Future studies moreover may lead to the establishing of promising therapeutic options for use in the treatment of cancer.

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