

Original Article

Multidrug resistance P-glycoprotein dampens SR-BI cholesteryl ester uptake from high density lipoproteins in human leukemia cells

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Abstract: Tumor cells are characterised by a high content of cholesterol esters (CEs), while tumor-bearing patients show low levels of high-density lipoproteins (HDLs). The origin and significance of high CE levels in cancer cell biology has not been completely clarified. Recent evidence that lymphoblastic cells selectively acquire exogenous CE from HDL via the scavenger receptor SR-BI has drawn attention to the additional membrane proteins involved in this pathway. P-glycoprotein-MDR1 (P-gp) is a product of the *MDR1* gene and confers resistance to antitumor drugs. Its possible role in plasma membrane cholesterol trafficking and CE metabolism has been suggested. In the present study this aspect was investigated in a lymphoblastic cell line selected for MDR1 resistance. CEM were made resistant by stepwise exposure to low (LR) and high (HR) doses of vincristine (VCR). P-gp activity (³H-vinblastine), CE content, CE and triglycerides (TG) synthesis (¹⁴C-oleate), neutral lipids and DiI-HDL uptake (fluorescence), SR-BI, ABCA1 and P-gp protein expression (western blotting) were determined. To better evaluate the relationship between CE metabolism and P-gp activity, the ACAT inhibitor Sandoz-58035 and the P-gp inhibitors progesterone, cyclosporine and verapamil were used. CE content and synthesis were similar in the parental and resistant cells. However, in the latter population, SR-BI protein expression increased, whereas CE-HDL uptake decreased. These changes correlated with the degree of VCR-resistance. As well as reverting MDR1-resistance, the inhibitors of P-gp activity induced the CE-HDL/SR-BI pathway by reactivating membrane cholesterol trafficking. Indeed, CE-HDL uptake, SR-BI expression and CE content increased, whereas there was a decrease in cholesterol esterification. These results demonstrated that P-gp overexpression impairs anticancer drug uptake as well as the SR-BI mediated selective CE-HDL uptake. This suggests that these membrane proteins act in an opposite manner on the same transport mechanism. Therefore, the dampening activity of P-gp in this pathway and its reversal by P-gp inhibitors open new strategies for antitumor therapy in drug-resistant tumors.

Keywords: Tumor biology, multidrug resistance, cell membrane, cholesterol traffic, neutral lipids, P-glycoprotein-MDR1 inhibitors

Introduction

A high rate of cholesterol synthesis, uptake and esterification in proliferating tissues, accompanied by a reduction in plasma cholesterol-HDL, during tumor growth, has been extensively described [1-6]. Free cholesterol (FC) greater requirement has been attributed to its structural and functional properties, whilst the elevated content of its esterified form has mainly been ascribed to the preferential shift of membrane cholesterol to ER, where it is esterified by the Acyl-coenzyme A: cholesterol acyltransfer-

ases (ACAT), rather than being delivered to HDLs. Furthermore, cholesteryl esters (CEs) are mostly considered an additional store of sterol that ensures continuous membrane biogenesis [1, 2, 6], even if the CE levels seem to be well over what is required by the proliferating cancer cell. High rates of cholesterol esterification have been associated with higher growth rates in several lymphoblastic cell lines [7]. The inhibition of ACAT, the enzyme esterifying cholesterol in the ER, partially blocks cell proliferation in non-malignant cell types [8, 9]. However, no such inhibitory effects were observed in the

investigated tumor cell lines, even when the drug doses used were remarkably higher than those utilised to completely inhibit cholesterol esterification [10]. It has recently been demonstrated that HDLs deliver the whole CE molecule directly to cancer cells, probably via the plasma membrane protein SR-BI [10, 11]. Furthermore, CE-HDL uptake increases during endogenous CE deprivation, indicating that this pathway may supply CE when other routes (LDL uptake or CE synthesis) are insufficient to ensure an optimal lipid pool for cellular functions. Although it is unknown whether and how the maintenance of high CE content affects tumor proliferation, these results definitely demonstrate that low plasma levels of HDL, along with the reduction of cholesterol efflux, are a consequence of the active supply of CE-HDL to cancer cells [10, 11]. However, low levels of CE-HDL have also been described in several chronic diseases such as sepsis and atherosclerosis [12]. It has been observed that, as compared to normal cells, malignant ones continuously demand exogenous CEs from these lipoproteins via a pathway that is typically utilised only by specialised cells (i.e. the suprarenal glands and liver). Even if all pathways for obtaining CEs are enhanced in tumor cells, the need for HDL-derived CEs makes these lipoproteins a possible trojan horse for delivering drugs with the same lipophilic profile as that of CEs [13, 14]. The potential for using this pathway as a new anticancer strategy warrants investigation into the underlying proteins and mechanisms. P-gp is a plasma membrane protein and a product of the *MDR1* gene that confers resistance to antitumor and anti-HIV drugs [15-18]. A possible physiological function for this protein (beyond that in multi-drug resistance) has still not been identified, but its involvement in membrane cholesterol trafficking has been suggested [16]. Such a hypothesis derives from the fact that several blockers of P-gp activity also inhibit cholesterol esterification [19] by preventing membrane cholesterol transport to the ER [20]. However, the potential involvement of P-gp in CE metabolism has yet to be completely understood and accepted by all researchers [21, 22]. To determine whether P-gp is an additional player in the control of CE metabolism in cancer cells, the acquisition of CE was investigated in a lymphoblastic CCRF-CEM cell line selected for MDR1-based VCR resistance.

Materials and methods

Cells

ATCC® CRM-CCL-119™ (CCRF-CEM, wild type, WT) cells, isolated from the peripheral blood of a 4 year old Caucasian female with acute lymphoblastoid leukemia, were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). This cell line was chosen because it easily develops MDR1 resistance in the presence of several drugs. MDR1-resistant cells were selected via exposure to VCR at a starting concentration of 1 nM [23]. The VCR concentration was then increased to induce 50% mortality and further increased when the treated cells reached at least 95% viability. VCR increments produced MDR1 subclones resistant to 50 nM, 100 nM, 200 nM, 300 nM, 500 nM and 1 µM VCR. After a preliminary characterisation of the different subclones for experimental purposes, 50 nM CEM-VCR (low resistance, LR) and 500 nM CEM-VCR (high resistance, HR) were used for further studies. Although resistant cells were always cultured in the presence of the drug, they were routinely tested for P-gp expression. Cultures were maintained in the exponential growth phase (between 2.0×10^5 cells/ml and 1.5×10^6 cells/ml) in T-25 cm² tissue culture flasks (Falcon, M19, Milan, Italy) containing RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich, Milan, Italy) and 1% penicillin-streptomycin at 37°C in a humidified incubator with 5% CO₂. The cells were diluted and cultured in new media twice a week, and viability was determined using trypan blue dye exclusion. In order to evaluate what role is played by the different cellular pathways in obtaining CEs, in terms of cell growth, CE metabolism and P-gp activity, cholesterol esterification was inhibited in some experiments by directly blocking the ACAT with Sandoz-58035 solubilised in dimethylsulfoxide (SZ, 4 µM). In another series of experiments, we used drugs known to inhibit both membrane cholesterol movement to the ER and also P-gp activity [24-26], namely, progesterone (PG, final concentration 20 µM in WT and LR, 10 µM in HR), cyclosporine (CLS, final concentration 2.5 µM), verapamil (VPM, final concentration 10 µM) (Sigma-Aldrich, Milan, Italy) all solubilised in ethanol (97%). The drug doses used were those with the highest inhibitory efficacy on cell growth (>40%) but the low-

MDR1 resistance influences high density lipoprotein metabolism

est toxicity (cell mortality < 5%) (data not shown) in both the WT and resistant cells. To evaluate the short- and long-term effects of ACAT inhibition, the cells were treated with SZ for 72 h (acute treatment, ACS) or cultured for at least 16 passages (approximately 50 cell cycles) with the drug (chronic treatment, CRS).

Determination of drug resistance

[³H]-vinblastine cell accumulation was measured using a modified method from Pani et al. [22]. To maintain the same specific activity and correctly evaluate the degree of resistance 50, 100, 500 and 1000 nM of CEM-VCR cells (1.0×10^6 cells/ml) received 3.7, 7.4, 22.0 and 37.0 GBq of [³H]-vinblastine (Perkin Elmer, MA, USA), respectively. Following 1 h of incubation, the cells were rapidly washed three times with ice-cold PBS, solubilised in 1 M NaOH, and analysed for protein [27] and [³H]-vinblastine content. The amount of [³H]-vinblastine accumulated is reported as dpm per μg of total cellular protein.

Cell proliferation and viability

Cell number and viability were measured in all experiments, using a Burker Chamber and the trypan blue dye exclusion test, respectively. An aliquot of cells was hydrolysed and processed to evaluate protein content.

Determination of cholesterol content

FC and CE content were measured in all experiments. The Cholesterol Quantification Kit (MAK043-1KT, Sigma Aldrich, Milan, Italy) was used according to the manufacturer's instructions.

Determination of cholesterol synthesis

To determine the rate of cholesterol synthesis, 10^6 cells/ml were incubated for 3 h with 185 kBq/ml sodium [¹⁴C]-acetate followed by separation via centrifugation and collection. Lipids were extracted with ice-cold acetone, and neutral lipids were separated via thin layer chromatography (TLC) Kieselgel plates using a solvent system containing eptane/isopropylether/formic acid (60:40:2 v/v/v). FC and CE bands were identified via comparison with simultaneous standards and visualised using iodine vapour [28]. For radioactive 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 reported as the mean values (variation between triplicates was less than 10%). All data are expressed as the rate of [¹⁴C]-acetate incorporation into cholesterol per μg of protein.

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

Cells were incubated for 4 h in medium containing [¹⁴C]-oleate bound to bovine serum albumin (BSA). To prepare the oleate-BSA complex, 3.7 MBq of [¹⁴C]-oleic acid in ethanol (specific activity 2.04 GBq/mmol) was mixed with 1.4 mg KOH, and the ethanol evaporated. PBS (1.5 ml) without Ca^{2+} or Mg^{2+} and containing 4.24 mg BSA (fatty acid-free) was added to the mixture, which was then shaken vigorously. This solution was added to each well at a final concentration of 74 KBq/ml. After 4 h of incubation, the cells were washed with ice-cold PBS and the lipids were extracted with acetone. Neutral lipids were separated via TLC, and the incorporation of [¹⁴C]-oleate into TGs and CEs was measured. An aliquot of cell lysate was processed for protein content.

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, the 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. The cell suspension was then placed in the wells of a 35-mm glass-bottom dish (MatTek, Ashland, MA, USA) for microscopic observation. Nile Red is a fluorescent dye that differentially stains polar lipids (i.e. phospholipids) and neutral lipids (i.e. CEs and TGs). Polar lipids display a red emission, while neutral lipids display a yellow emission [29, 30]. A red emission was observed with 540 ± 12.5 nm excitation and 590 LP emission filters. A yellow emission was observed with 460 ± 25 nm excitation and 535 \pm 20 LP emission filters. The cells were observed using an Olympus 1 \times 71 inverted microscope (Olympus, Tokyo, Japan) fitted with a 20 \times /0.7 planapochromatic objective. Twelve bit-images were captured using a

MDR1 resistance influences high density lipoprotein metabolism

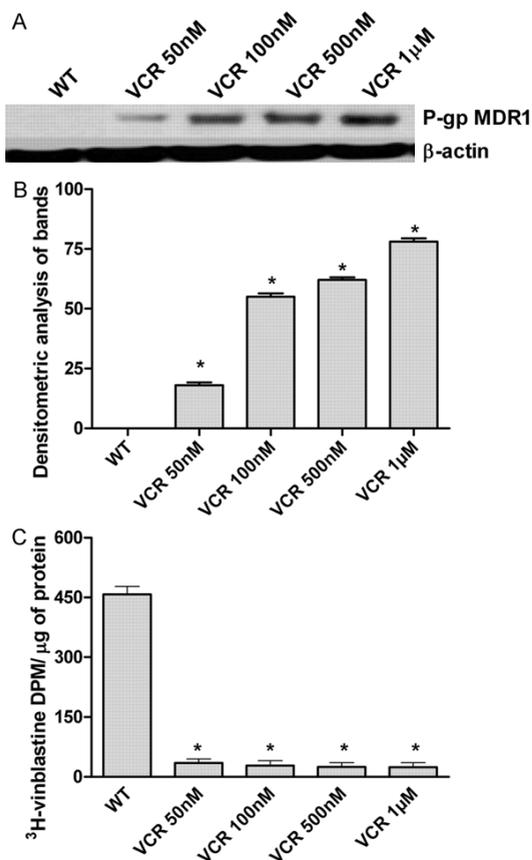


Figure 1. Clonal selection of CEM-VCR-resistant cells. Parental CCRF-CEM (WT) cells were exposed to VCR at the initial concentration of 1 nM (>50% mortality). When the cells reached 95% viability, the VCR doses were progressively increased to select subclones with higher resistance to the drug. At a concentration of 1.0×10^6 cells/ml, the cells were harvested and processed for western blot analysis, as described in the Materials and methods section. A. P-gp-MDR1 protein expression progressively increased at higher doses of VCR. B. Quantification of the protein bands was accomplished via densitometric analysis. C. To evaluate the degree of VCR resistance, the cells were incubated with [³H]-vinblastine; accumulation was evaluated (see Materials and methods). Data are expressed as the means \pm SEM of triplicate samples. All experiments were performed at least twice. * $p < 0.05$ vs. WT, WT: wild type; VCR: vincristine.

cooled CCD camera (Sensicam PCO, Kelheim, Germany) electronically coupled to a mechanical shutter placed between the 100 W Hg lamp and the microscope to limit photobleaching. The excitation source was attenuated with a 6% neutral density filter. The nominal resolution of images was $0.3 \mu\text{m}/\text{pixel}$. A quantitative analysis of the images was performed with the ImagePro Plus package (Media Cybernetics, Silver Springs, MD, USA). At least 200 individual

Table 1. Time course of cell proliferation in WT and resistant cells

	WT (cell number $\times 10^5$)	LR (cell number $\times 10^5$)	HR (cell number $\times 10^5$)
0 h	3.03 \pm 0.16	3.33 \pm 0.90	3.07 \pm 0.67
24 h	5.42 \pm 0.55	6.13 \pm 0.01	4.80 \pm 0.73
48 h	12.10 \pm 0.10	14.80 \pm 0.80*	7.31 \pm 0.69**
72 h	19.30 \pm 3.20	31.00 \pm 1.00*	16.20 \pm 0.20**

Exponentially growing cells (3.0×10^5 cells/ml) were cultured, and cell number and viability were determined at 0, 24, 48 and 72 h, using a Burker Chamber and the trypan blue dye exclusion test, respectively. WT: wild type; LR: low resistance; HR: high resistance; Data are expressed as the means \pm SEM. * $p < 0.05$ statistically significant vs WT. ** $p < 0.05$ statistically significant vs WT and LR. Cholesterol content in WT and resistant cells.

cells from each experimental group were measured.

Dil-HDL uptake

After HDL binding to the SR-BI protein, only the CE component passes through the membrane [31], whereas the HDL holoparticle is not internalized by this protein [32]. To determine the selective uptake of CE-HDL we utilised lipoproteins bound to the fluorescent dye Dil (1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate) (Bioquote, London). Dil is a fluorescent probe bound to the inner lipid component (mainly CEs) which, when excited with wavelengths around 514 nm, features an emission peak in the range of 550 nm, suitable for viewing with a common filter for Rhodamine. We have previously demonstrated that the selective CE uptake from HDL is already present at 4°C and that, differently from LDL, the Dil-CE component does not go to the endolysosomal pathway [11]. Unless otherwise indicated, cells were seeded at a concentration of 2.0×10^5 cells/ml and incubated at 4°C for 2 h with 10 $\mu\text{g}/\text{ml}$ Dil-HDL [33, 34]. Afterwards, the samples were processed as previously described [11]. Fixed cells were observed using an inverted Olympus 1 \times 71 microscope (Olympus, Tokyo, Japan) equipped with a 20 \times /0.7 planapochromatic objective. The nominal resolution of images was $0.3 \mu\text{m}/\text{pixel}$. Quantitative analysis of images was performed with the ImagePro Plus package (Media Cybernetics, Silver Springs, MD). At least 200 individual cells from each experimental group were measured.

MDR1 resistance influences high density lipoprotein metabolism

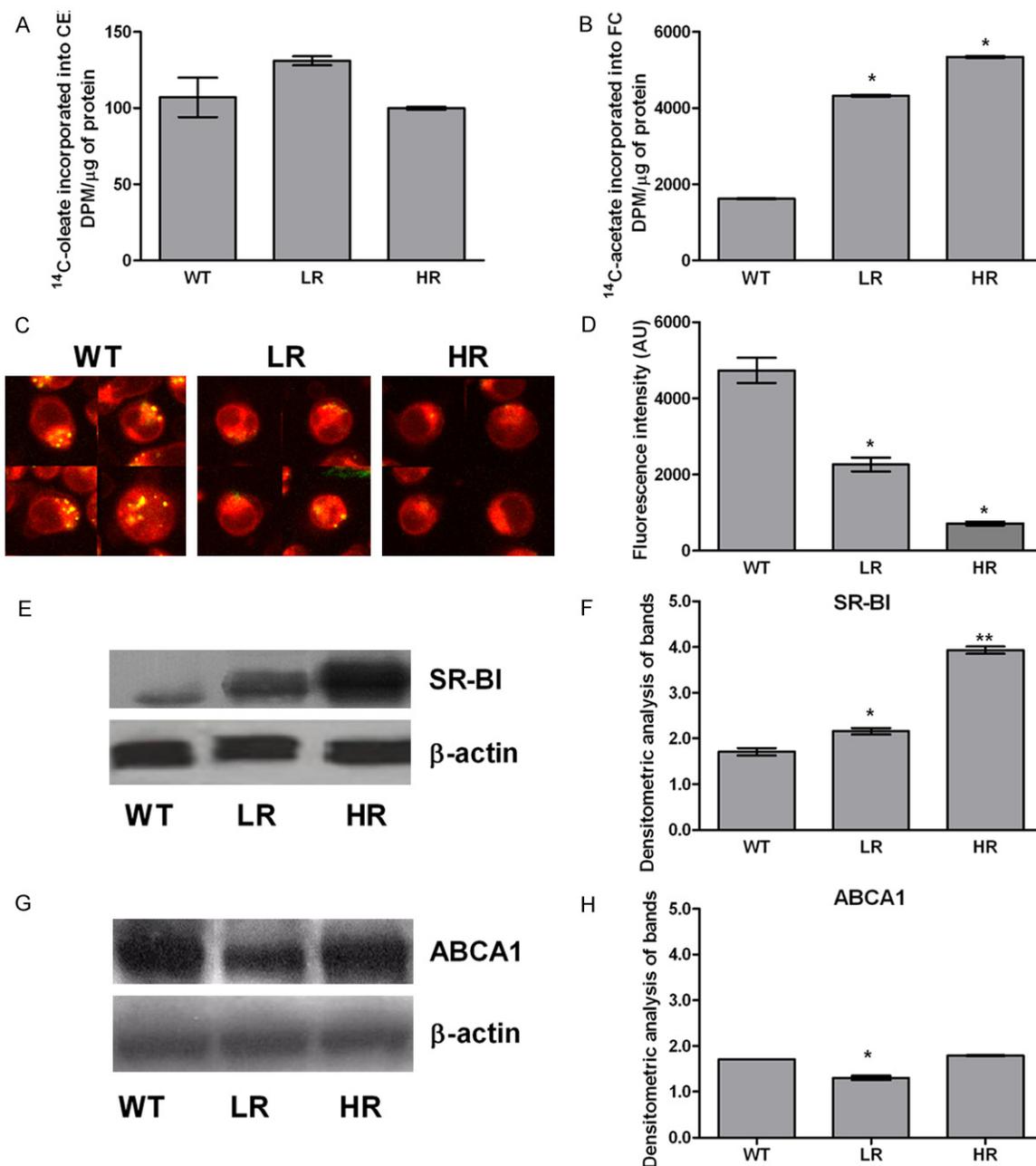


Figure 2. CE metabolism in WT and resistant cells. (A) Cells at a concentration of 1.0×10^6 cells/ml were incubated for 4 h in the presence of 74 KBq/ml [14 C]-oleate bound to bovine serum albumin (BSA). After incubation, the cells were processed as described in the Materials and methods section. (B) Cells at a concentration of 1.0×10^6 cells/ml were incubated for 3 h with 185 KBq/ml sodium [14 C]-acetate and processed as described. (C) Neutral lipids *in situ* were stained with Nile Red, and fluorescence was visualised with a Sensicam PCO (see Materials and methods). (D) Quantitative analysis was performed with the ImagePro Plus package, and at least 400 individual cells were measured for each experimental group. (E) Western blot images of SR-BI expression and (F) densitometric analysis of the bands. (G) Western blot images of ABCA1 expression and (H) densitometric analysis of the bands. Data are expressed as the means \pm SEM of triplicate samples. All experiments were performed at least twice. * $p < 0.05$ vs WT, ** $p < 0.05$ for HR vs LR. WT: wild type; LR: low resistance; HR: high resistance; SR-BI: scavenger receptor BI; ABCA1: ATP-binding cassette transporter A1.

Western blot analysis

The cells were lysed at 4°C in PBS buffer containing 10% SDS, 50 μg TRIS, 1 μM EDTA, pH

7.5, 50 μM DTT and a protease/phosphatase inhibitor cocktail. The cells were homogenised using a UP100H Compact Ultrasonic Laboratory Device (Hielscher Ultrasonic GmbH, Teltow,

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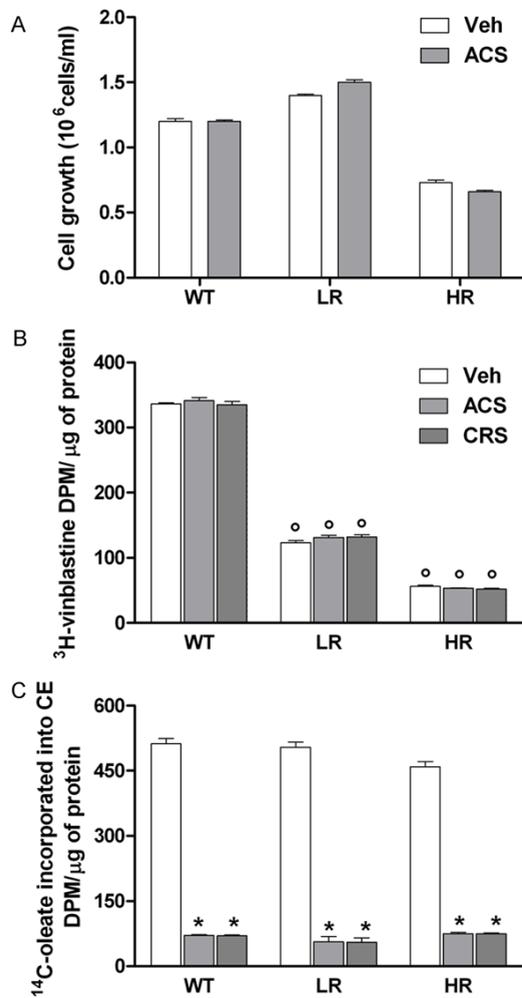


Figure 3. Sandoz inhibits cholesterol esterification but not cell proliferation or P-gp activity in WT, LR and HR cells. A. Cells were cultured at 2.0×10^5 cells/ml and treated for 72 h with 4 μ M SZ. The cells were then harvested and counted. B. Cells at a concentration of 1.0×10^6 cells/ml were incubated for 4 h in the presence of 74 KBq/ml [3 H]-vinblastine bound to bovine serum albumin (BSA). After incubation, the cells were processed as described in the Materials and methods section. C. The cells at a concentration of 1.0×10^6 cells/ml were incubated for 1 h in the presence of 3.7 KBq/ml [3 H]-vinblastine. After incubation, the cells were processed as described in the Materials and methods section. Data are expressed as the means \pm SEM of triplicate samples. All experiments were performed at least twice. * $p < 0.05$ vs WT, * $p < 0.05$ vs Veh. WT: wild type; LR: low resistance; HR: high resistance; Veh: vehicle, corresponding untreated cells; ACS: Sandoz 72 h; CRS: Sandoz treatment for two months.

Germany). The protein content of each sample was determined using a BCA (Sigma-Aldrich, Milan, Italy) assay and processed as previously described [11, 13]. Briefly, protein samples (30 μ g/lane) were separated via electrophoresis

(acrylamide precast gel; Bio-Rad, Laboratories Inc., Milan, Italy) and transferred to 0.45- μ m-pore nitrocellulose (Millipore, Milan, Italy) using a standard electro-blotting procedure. The blots were pre-treated with a solution containing 5% non-fat dried milk at 4°C in TBST (50 μ M TRIS-HCl, pH 7.6, 0.15 M NaCl and 0.05% Tween-20) for at least 1 h prior to the addition of the primary antibodies (diluted 1:200 to 1:5000) for SR-BI and ABCA1 (anti-rabbit polyclonal antibody) and β -actin (anti-goat polyclonal antibody). After overnight incubation at 4°C, the primary antibodies were removed, and the appropriate horseradish peroxidase-conjugated secondary antibodies were added in a dilution range of 1:2500 to 1:10000 for at least 1 h at room temperature. All antibodies were purchased from Abcam (Cambridge, UK). Proteins were detected using enhanced chemiluminescence (Millipore, Milan, Italy) and exposure to X-ray film (Sigma-Aldrich, Milan, Italy) for the required exposition time. Quantification of the protein bands was then accomplished using a densitometric analyser (ImageJ software, NIH Bethesda, MA, USA).

Statistical analysis

All data were expressed as the means \pm SEM of triplicate experiments and were analysed using Student's *t*-test and one or two-way ANOVA; post-hoc tests (Tukey or Bonferroni tests) were completed when required. Statistical significance was set at $p < 0.05$.

Results

Clonal selection of CEM-VCR-resistant cells

Parental CCRF-CEM (WT) cells were exposed to VCR at the initial concentration of 1 nM (>50% mortality). When the cells reached 95% viability, the VCR doses were progressively increased, and subclones with a higher resistance to the drug and enhanced P-gp-MDR1 protein expression were selected (Figure 1A). These data were confirmed via densitometric analysis of the bands (Figure 1B). Cell resistance (as evaluated according to vinblastine accumulation) was completed in all MDR1-resistant cells (Figure 1C) and was observed in more than 95% of those tested. Preliminary experiments evaluated the differences in cholesterol metabolism between the subclones, and most studies were carried out with low resistance (LR, VCR 50 nM) and high resistance (HR, VCR 500 nM) cells.

MDR1 resistance influences high density lipoprotein metabolism

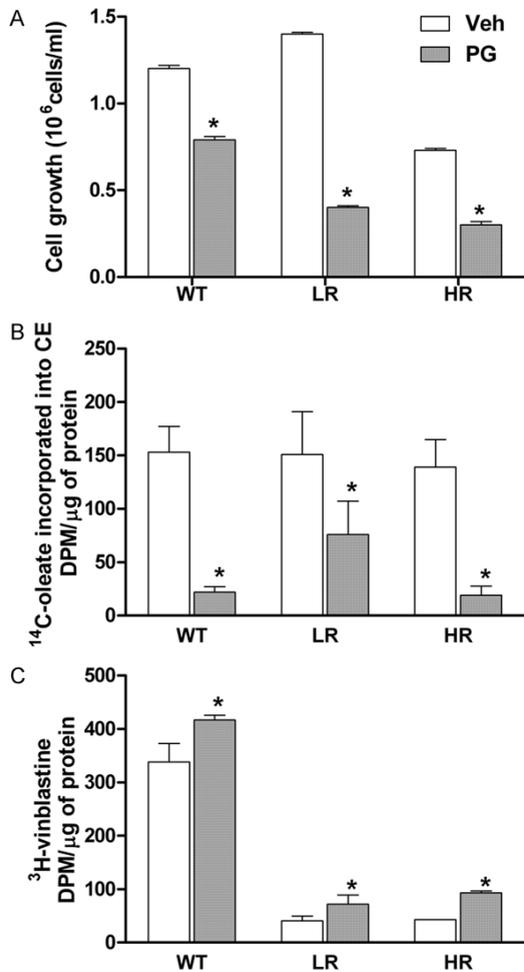


Figure 4. Progesterone inhibits cell proliferation, CE synthesis and P-gp activity in WT, LR and HR cells. A. WT and LR cells (2.0×10^5 cells/ml) were cultured for 48 h in the presence of 20 μ M of PG, while HR cells received 10 μ M PG (see Materials and methods). The cells were subsequently counted and evaluated for viability. The results show the percentage of viable cells compared with the respective control. B. Cells at a concentration of 1.0×10^6 cells/ml were incubated for 4 h in the presence of 74 KBq/ml [14 C]-oleate. After incubation, the cells were processed as described above. C. Cells at a concentration of 1.0×10^6 cells/ml were incubated for 1 h in the presence of 3.7 KBq/ml [3 H]-vinblastine. After incubation, the cells were processed as described in the Materials and methods section. Data are expressed as the means \pm SEM of triplicate samples. All experiments were performed at least twice. * $p < 0.05$ vs Veh. WT: wild type; LR: low resistance; HR: high resistance; Veh: vehicle, corresponding untreated cells; PG: progesterone.

Cell proliferation and CE metabolism in WT, LR and HR cells

The curves of all groups showed similar growth rates in the first 24 h. Thereafter, the LR cells

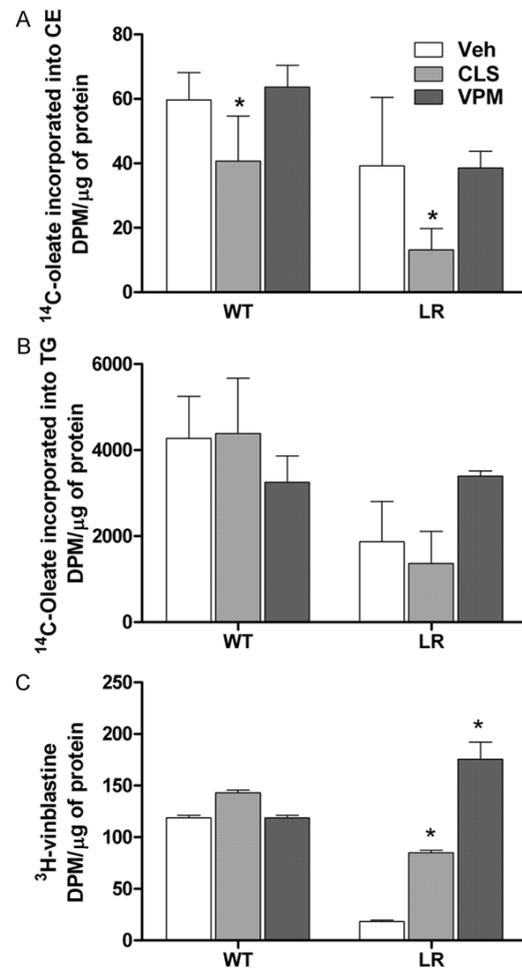


Figure 5. Cell proliferation, CE synthesis and P-gp activity in WT, LR and HR cells cultured in the presence of CLS and VPM. WT and LR cells (2.0×10^5 cells/ml) were cultured for 48 h in the presence of CLS (2.5 μ M) or VPM (10 μ M). A. Cells at a concentration of 1.0×10^6 cells/ml were incubated for 4 h in the presence of 74 KBq/ml [14 C]-oleate. After incubation, the cells were processed as described above for CE synthesis. B. Cells at a concentration of 1.0×10^6 cells/ml were incubated for 4 h in the presence of 74 KBq/ml [14 C]-oleate. After incubation, the cells were processed as described above for TG synthesis. C. Cells at a concentration of 1.0×10^5 cells/ml were incubated for 1 h in the presence of 3.7 KBq/ml [3 H]-vinblastine. After incubation, the cells were processed as described in the Materials and methods section. Data are expressed as the means \pm SEM of triplicate samples. All experiments were performed at least twice. * $p < 0.05$ vs Veh. WT: wild type; LR: low resistance; HR: high resistance; Veh: vehicle, corresponding untreated cells; CLS: cyclosporine; VPM: verapamil.

showed a significantly higher proliferation rate, whereas HR slowed down (Table 1). As demonstrated in Figure 2, CE synthesis did not significantly change in the LR or HR cells compared

MDR1 resistance influences high density lipoprotein metabolism

Table 2. FC, CE, TC and ratio CE/CT in WT and resistant cells

		FC $\mu\text{g}/10^6$ cells	CE $\mu\text{g}/10^6$ cells	TC $\mu\text{g}/10^6$ cells	CE/TC %
WT	Veh	12.26 \pm 1.04	1.79 \pm 0.44	14.05 \pm 1.48	13
	PG	9.93 \pm 0.2	1.64 \pm 0.47	11.57 \pm 0.52	14
	CLS	10.96 \pm 1.9	2.13 \pm 0.33	13.09 \pm 1.57	16
	VPM	11.62 \pm 1.86	2.58 \pm 0.45	13.33 \pm 0.72	19
LR	Veh	11.26 \pm 0.98	1.59 \pm 0.45	14.92 \pm 0.35	12
	PG	8.70 \pm 1.85	2.87 \pm 0.68*	11.57 \pm 2.47	25
	CLS	10.23 \pm 0.93	3.46 \pm 1.45*	13.69 \pm 1.30	25
	VPM	11.53 \pm 2.61	4.60 \pm 1.05*	16.14 \pm 2.42	29
HR	Veh	13.46 \pm 0.34	2.06 \pm 0.15	14.81 \pm 1.01	14
	PG	10.56 \pm 0.04	4.53 \pm 1.88*	13.73 \pm 2.12	33

Free cholesterol (FC), cholesteryl ester (CE), total cholesterol (TC) content and CE/TC (%) were measured in exponentially growing WT and resistant CEM cells. For details see Materials and methods section. WT: wild type; LR: low resistance; HR: high resistance; PG: progesterone; CLS: cyclosporine; VPM: verapamil; Veh: vehicle, corresponding untreated cells. Data are expressed as the mean \pm SEM. * p <0.05 statistically significant vs WT and LR.

with the WT cells (**Figure 2A**), whereas FC synthesis (**Figure 2B**) was significantly higher in all resistant cells. Nile Red emission was significantly lower in the resistant cells compared with the WT cells (**Figure 2C, 2D**), whereas SR-BI protein expression (**Figure 2E, 2F**) was progressively higher as cell resistance increased. ABCA1 protein expression was slightly reduced in LR cells, compared with the other groups.

Cell proliferation, CE metabolism and P-gp activity in the presence of SZ

As shown in **Figure 3**, treatment with SZ did not significantly change the cell growth rate (**Figure 3A**) or vinblastine accumulation (**Figure 3C**). CE synthesis was almost absent in both the acute (ACS) and chronically (CRS) ACAT-inhibited cells (**Figure 3B**) and in both the parental and resistant populations.

Cell proliferation, CE metabolism and P-gp activity in WT and resistant cells cultured in the presence of inhibitors

As shown in **Figure 4**, PG treatment significantly decreased cell growth (**Figure 4A**) in all cells (66, 30 and 42% for WT, LR and HR, respectively vs. their respective controls). As shown in **Figure 4B**, cholesterol esterification was inhibited in all cells cultured with PG. Vinblastine accumulation was significantly affected by the

drug (**Figure 4C**). CLS inhibited cell proliferation in both the WT and LR cells (61% and 48% when compared to the respective control, p <0.05). VPM exerted a weaker but significant inhibitory effect on cell proliferation (28 and 31% vs WT and LR respectively, p <0.05). However, VPM inhibited WT cell proliferation but reduced LR cell proliferation to a lesser extent. As reported in the Material and Methods section, low doses of CLS were toxic to HR cells; therefore, the effects of CLS and VPM were only evaluated in the LR cells. As demonstrated, both drugs only slightly affected CE synthesis in the WT cells; this effect was more pronounced in the LR cells, with CLS being more effective than VPM (**Figure 5A**). As shown in **Figure 5B**, P-gp inhibitors did not significantly modify TGs synthesis, when compared with the respective control (Veh). Accordingly, as shown in **Figure 5C**, [^3H]-vinblastine accumulation increased in resistant cells in the presence of CLS or VPM, and this effect was more consistent in the presence of VPM. The two drugs did not modify [^3H]-vinblastine accumulation in parental cells.

CE content, neutral lipids and SR-BI protein expression in resistant cells cultured in the presence of inhibitors

CE content increased in resistant cells treated with PG (**Table 2**), while did not exert any difference in WT cells. On the contrary the drug increased Nile Red fluorescence in all cells (**Figure 6A, 6B**). SR-BI protein expression significantly increased in both the WT and resistant cells cultured with the drug (**Figure 6C, 6D**). CE content increased in LR cells cultured in the presence of the P-gp inhibitors, as compared to the corresponding control (**Table 2**). As shown in **Figure 6E** Nile Red fluorescence increased in presence of P-gp inhibitors only in resistant cells. These results were confirmed by the analytical analysis of the images (**Figure 6F**). As depicted in these images, SR-BI protein expression (**Figure 6G, 6H**) was higher in the presence of CLS and VPM in both the WT and LR cells.

CE-HDL uptake in WT, LR and HR cells cultured in the presence of inhibitors

As shown in **Figure 7**, CE-HDL uptake increased in resistant cells in the presence of PG com-

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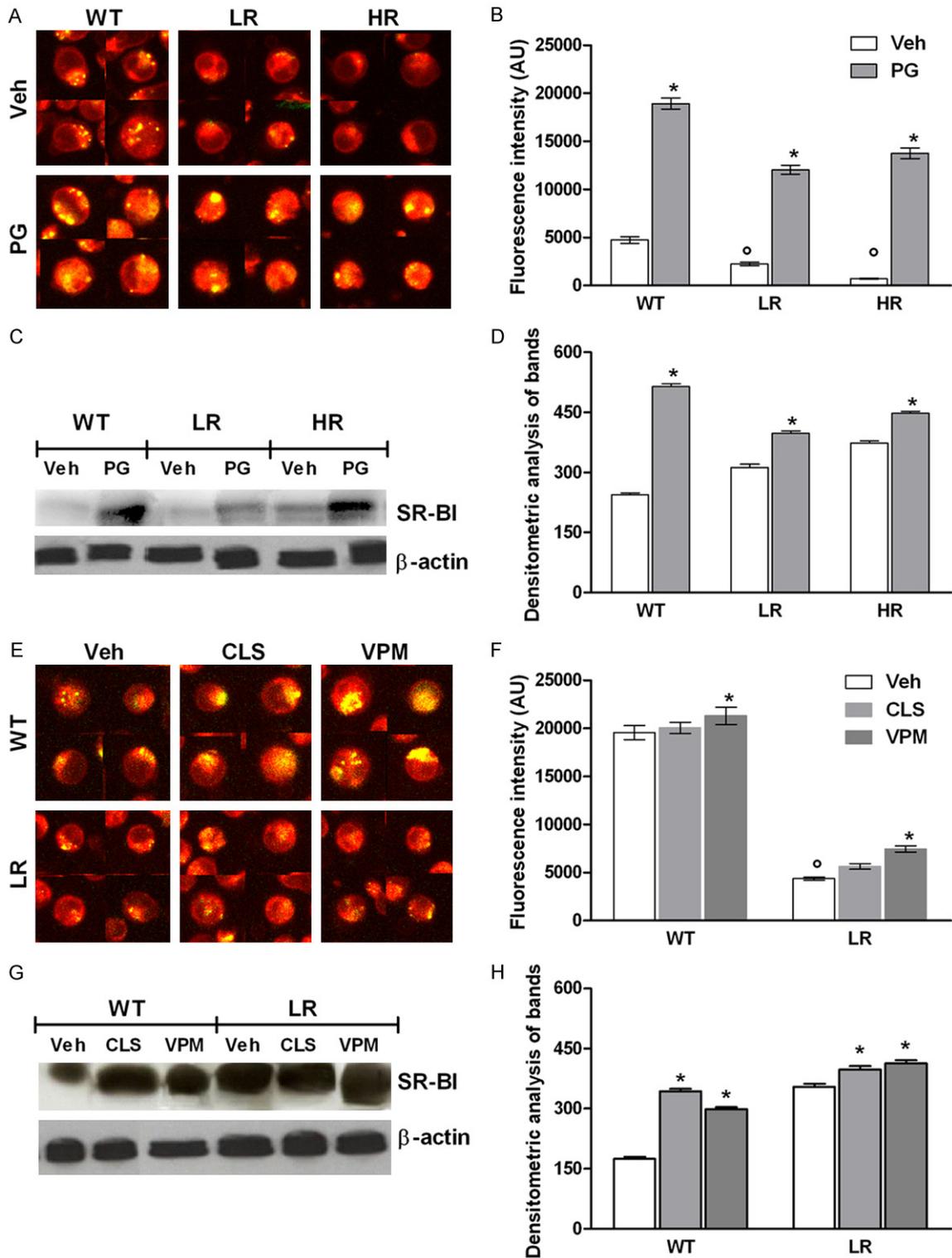


Figure 6. Neutral lipids and SR-BI protein expression increase in WT and resistant cells cultured in the presence of PG, CLS and VPM. WT and resistant cells (2.0×10^5 cells/ml) were cultured for 48 h in the presence of: PG, CLS or VPM (see Material and methods). Neutral lipids *in situ* were stained with Nile Red (A and E) and fluorescence visualised with a SencicamPCO and measured by imaging analysis (B and F) performed with the ImagePro Plus package (see Materials and methods). At the indicated time, the cells were harvested and processed for western blotting (C and G) and bands were measured by densitometric analysis (D and H). Data are expressed as the means \pm SEM of triplicate samples. All experiments were performed at least twice. * $p < 0.05$ vs Veh, ° $p < 0.05$ vs WT. WT: wild type; LR: low resistance; HR: high resistance; Veh: vehicle, corresponding untreated cells; PG: progesterone, CLS: cyclosporine; VPM: verapamil.

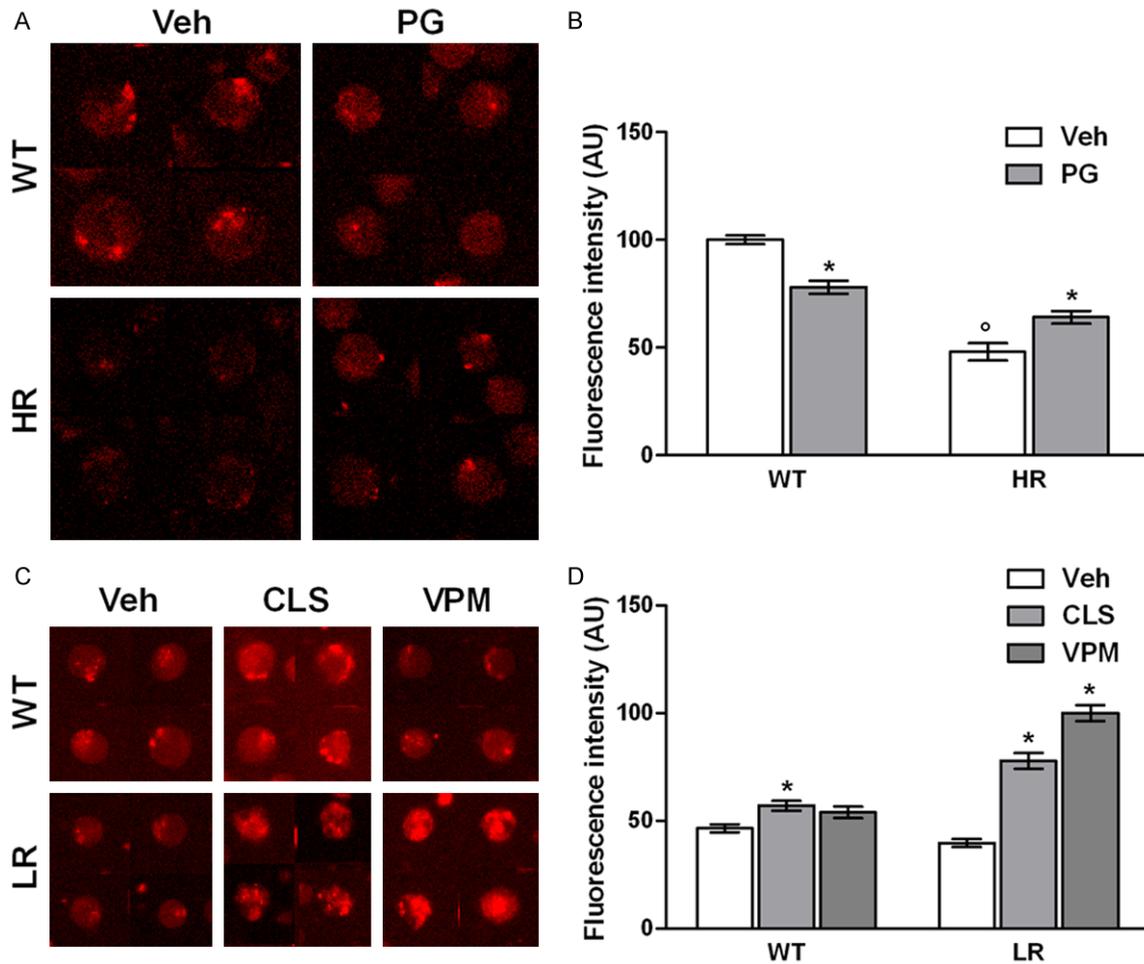


Figure 7. CE-HDL uptake in WT and resistant cells in the presence of PG, CLS or VPM. Cells were seeded at a concentration of 2.0×10^5 cells/ml and incubated at 4°C for 2 h with $10 \mu\text{g/ml}$ Dil-HDL. The cells were then fixed and observed using an inverted microscope (A and C) fitted with a $20\times/0.7$ planapochromatic objective (see Materials and methods). At least 200 individual cells from each experimental group were measured by imaging analysis (B and D) performed with the ImagePro Plus package (see Materials and methods). Data are expressed as the means \pm SEM of triplicate samples. All experiments were performed at least twice. $^{\circ}p < 0.05$ vs WT and $*p < 0.05$ vs Veh. WT: wild type; LR: low resistance; HR: high resistance; Veh: vehicle, corresponding untreated cells; PG: progesterone; CLS: cyclosporine; VPM: verapamil.

pared with the relative control (Figure 7A), as confirmed by the quantitative analysis of the appropriate images (Figure 7B). CE-HDL uptake increased in WT and LR cells in the presence of VPM and CLS compared with the relative control (Figure 7C), as confirmed via quantitative image analysis (Figure 7D).

Discussion

High CE content accompanied by a reduction in plasma HDL is a consistent finding in tumor-bearing patients [1-6]. A high rate of cholesterol synthesis and esterification as well as reduced cell cholesterol efflux to HDL typically act in combination to maintain a high CE content in

tumor tissues [2, 6]. However, in recent years, growing evidence has demonstrated that HDL contributes to the maintenance of high CEs by directly releasing the neutral lipids to tumor cells [33, 34]. SR-BI is the plasma membrane protein involved in this selective process. Indeed after its binding to HDL, it allows the passage of the inner lipophilic component of the lipoprotein, mainly through a selective CE uptake, peculiar to specialised cells. The biological significance of this route in cancer cells is unclear, and it is an open question whether high CE levels are related to cancer cell proliferation and biology or simply an epiphenomenon. HDL has occasionally been described as functioning as a growth factor for some cancer

MDR1 resistance influences high density lipoprotein metabolism

cells [35, 36], whereas SR-BI is considered as facilitating the extra-lysosomal hydrolysis of CE-HDL through the hormone-sensitive lipase, providing additional 'free' cholesterol for the new membranes and the intracellular signalling pathways involved in the regulation of cell proliferation [37]. It has been demonstrated in previous cell models that whole CE is removed from HDL and subsequently stored in lipid droplets. Recent evidence indicates that CEs from HDLs are neither hydrolysed nor re-esterified but, rather, directly increase the cellular pool of CEs as preformed molecules stored in lipid droplets. For this reason, some authors have suggested that HDLs may be involved in cancer cell biology by playing an active role in tumor CE metabolism [10, 11]. The data presented in this article indicate that proteins resident in the plasma membrane are involved in regulating CE content in cancer cells, with SR-BI as one of the main regulators of this process [37-38]. In addition to SR-BI, the P-gp protein has also been implicated in the regulation of cholesterol esterification [16, 24]. This protein is overexpressed in some types of cancer cells and confers multi-drug resistance to these populations. The correlation of P-gp overexpression with CE metabolism is primarily because some of the drugs that block its activity also inhibit cholesterol esterification [16]; however, this function is not widely accepted [19, 21]. CE esterification does not always differ between cells selected for resistance and parental lines. Notably, the only method used to evaluate CE metabolism in the cited articles is the rate of CE synthesis [16, 19, 21, 24], which may account for the debated roles ascribed to P-gp MDR1 in CE metabolism. Several articles have reported that P-gp regulates plasma membrane cholesterol in some way [39] or, conversely, that cholesterol may regulate P-gp activity [40]. Evidence is however often indirect and performed by artificially replenishing and depleting membrane cholesterol [39]. The present study demonstrates that CE metabolism significantly differs between parental and resistant CCRF-CEM cells and that these differences are related to a dampening effect exerted by P-gp on CE-HDL selective uptake despite the high SR-BI protein expression. We have hypothesised that the observed SR-BI overexpression may be considered an attempt to compensate for the interference exerted by P-gp on CE-HDL uptake. This hypothesis is supported by the fact that when P-gp activity is

inhibited, there is a concomitant recovery of CE-HDL selective uptake, and an increase in SR-BI expression, and CE content. Moreover, the fact that P-gp inhibitors reduce cholesterol esterification, strongly suggests that CE increase is due to CE-HDL SR-BI-mediated uptake. Although CE reside in lipid droplets and Nile Red fluorescence follow the same direction, the fact that CE and TG are co-localized in lipid droplets has been taken into account for the discrepancy between Nile Red fluorescence and CE content among the different experimental groups. The involvement of P-gp is further reinforced by the fact that these effects were not present in parental cells. PG almost completely inhibited CE synthesis, although the inhibitory effect on P-gp activity was weaker than that exerted by the CLS and VPM. It must be considered that, apart from its effect on P-gp-mediated membrane cholesterol traffic [24], PG inhibits cholesterol biosynthesis in the latter steps, which results in the accumulation of a number of sterol precursors [20]. Overall, these data suggest that P-gp activity modifies the CE-HDL/SR-BI pathway by raising the possibility that CE-HDL selective uptake and P-gp MDR1 drug resistance act in an opposite manner on the same transport mechanism. Although the molecular mechanisms underlying this pathway need further investigation, two main aspects must be taken into account: i) the reduced CE-HDL uptake as well as the decreased amount of CE content are related to resistance; ii) the effect of CE synthesis inhibitors on P-gp activity (and, in turn, that of P-gp inhibitors on CE metabolism) acts at the plasma membrane level. This pathway warrants further research on other cancer cell lines. Furthermore, similar studies need to be conducted in cells made resistant to different drugs or after insertion of the *MDR1* gene. In conclusion, these data further support the hypothesis that (1) cancer cells possess a preferential way of obtaining CEs from HDL via SR-BI; (2) HDLs represent a promising carrier for targeting lipophilic drugs to cancer cells; (3) the dampening activity of P-gp in this pathway and its reversal by P-gp inhibitors open new strategies for anti-cancer therapy in drug-resistant tumors.

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MDR1 resistance influences high density lipoprotein metabolism

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Abbreviations

ACS, acute treatment; BSA, bovine serum albumin; CE, cholesterol esters; CLS, cyclosporine; CRS, chronic treatment; Dil, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; FC, free cholesterol; FCS, fetal calf serum; HDL, high-density lipoproteins; HR, high resistance; LDL, low-density lipoproteins; LR, low resistance; Nile Red, 9-diethylamino-5H-benzo[α]phenoxazine-5-one; PFA, paraformaldehyde; PG, progesterone; P-gp, P-glycoprotein-MDR1; SR-BI, scavenger receptor class B type I; SZ, sandoz-58035; TG, triglycerides; TLC, thin layer chromatography; VCR, vincristine; VPM, verapamil; WT, wild type.

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