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Research Article

MDR1, cholesterol esterification and cell growth: a comparative study in normal and multidrug-resistant KB cell lines

A. Pani[a,*], B. Batetta[b], M. Putzolu[b], F. Sanna[b], O. Spano[b], S. Piras[b], M. F. Mulas[b], R. R. Bonatesta[b], C. Amat di S. Filippo[a], L. Vargiu[a], T. Marceddu[a], L. Sanna[a], P. La Colla[a] and S. Dessì[b]

[a]Dipartimento di Biologia Sperimentale, Sezione di Microbiologia, Cittadella Universitaria, SS 554, Km 4,500, I-09042 Monserrato (Italy), Fax + 39 70 675 4210, e-mail: pania@vaxca1.unica.it
[b]Istituto di Patologia Sperimentale, Università degli Studi di Cagliari (Italy)

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Abstract. The product of the *MDR1* gene (P-gp) has been implicated in the transport of cholesterol from plasma membrane to endoplasmic reticulum for esterification. In previous studies on leukemia cell lines, we suggested that cholesterol esterification may regulate the rate of cell growth and that the *MDR1* gene might be involved in this process by modulating intracellular cholesterol esters levels. To further investigate this matter, the rate of cell growth, cholesterol metabolism, expression of the *MDR1* gene, and P-gp activity were compared in KB cell lines displaying differences in expression and function of P-gp (drug-sensitive phenotype versus MDR phenotype). The rate of cell growth correlated with cholesterol esterification in all KB cell lines, whereas the over-expression of *MDR1* observed in the MDR cell lines was not always associated with an increased capacity of cells to esterify cholesterol. Two known inhibitors of P-gp activity, progesterone and verapamil, strongly inhibited both cholesterol esterification and cell proliferation in all KB cell lines, but they affected intracellular accumulation of labeled vinblastine only in MDR cell lines. These results further support a role for cholesterol esters in the regulation of cell growth and suggest that the P-gp expressed in MDR KB cells is not involved in the general process leading to cholesterol esterification.

Key words. MDR; P-gp; cell growth; cholesterol esterification.

The product of the *MDR1* gene, or class 1 P-gp, has been identified as an energy-dependent multidrug efflux pump localized at the cell surface that prevents the accumulation of certain anticancer drugs within cells, and it is responsible for at least one of the mechanisms that confers the multidrug resistance (MDR) phenotype on tumor cells. Substrates of P-gp are a wide variety of structurally and functionally unrelated drugs, such as doxorubicin, the vinca alkaloids vincristine and vinblastine, actinomycin D and taxol, to which MDR cells show a particular pattern of cross-resistance [1, 2].
Although P-gp is over-expressed in tumors following relapse after chemotherapy [3], it is also highly expressed in some tumors not previously treated with drugs [2–4]. The expression of the *MDR1* gene is in this case limited to tumors developed from tissues that constitutively express substantial levels of P-gp (i.e., colon, liver, and adrenal). Nevertheless, the physiological role and function played by P-gp in the normal cell remain to be unequivocally established.

* Corresponding author.

The ability of P-gp to catalyze the efflux of synthetic compounds led to the hypothesis that *MDR1* normally functions in detoxification [5, 6], whereas other studies have suggested that the physiologic role of *MDR1* is to catalyze the transport of cellular lipids across membranes [7, 8]. A number of more recent lines of evidence indicated that *MDR1* activity is required for cholesterol esterification and that amphiphilic agents known to modulate *MDR1* activity block transport of the cholesterol substrate from the plasma membrane to the endoplasmic reticulum, the site of cholesterol esterification by acyl-CoA:cholesterol acyltransferase (ACAT) [9–13]. Using verapamil, progesterone and other steroid hormones, which are known to physically interact with P-gp to inhibit its activity, Debry et al. [13] found a correlation between *MDR1* activity and cholesterol esterification in a number of cultured human cell lines.

In our studies on the role of cholesterol esterification during cell proliferation, we have shown that proliferative processes, either normal or pathologic, are characterized by a marked esterification of cholesterol [14–19]. More recently, we have reported that cell growth rate correlates with cholesterol esterification and with the expression of both *ACAT* and *MDR1* genes [20, 21]. These results were obtained in three lymphoblastoid cell lines, MOLT4, CEM, and L1210, which show different doubling times. In these studies, absolute levels of cholesterol synthesis and cholesterol uptake did not appear to be directly correlated with the differences in growth rate.

On the basis of these results, we hypothesized that a larger store of cholesteryl esters within the cell would allow for more rapid mobilization and utilization of cholesterol for new membrane biogenesis, and that the *MDR1* gene might contribute to regulate the rate of cell growth by modulating intracellular cholesteryl esters levels.

In the present study, to better define the involvement of the *MDR1* gene in cholesterol trafficking and cell proliferation, we compared cell lines differing in expression and possibly function of the *MDR1* gene product P-gp (drug-sensitive versus MDR phenotype). To this end, cell growth rate, cholesterol metabolism, *MDR1* gene expression, P-gp activity, and drug resistance pattern were compared in the following human nasopharyngeal carcinoma KB cell lines: parental KB (drug-sensitive phenotype); KB^{V20C} (MDR phenotype: KB selected in the presence of increasing drug concentrations); KB^{MDR} (MDR phenotype: KB engineered with the human *MDR1* gene).

Materials and methods

Materials. Doxorubicin, vincristine, etoposide, cis-platinum, camptothecin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma (Milan, Italy).

Cell lines. The human nasopharyngeal carcinoma KB cell line and the MDR subclones KB^{V20C} and KB^{MDR} were a generous gift of Prof. Y. C. Cheng, Yale University, USA. KB^{V20C} cells over-express *MDR1* P-gp [22] following stepwise selection for resistance with increasing concentration of vincristine. KB^{MDR} are KB cells infected with a retroviral vector containing a full-length cDNA for the human *MDR1* gene [23] that conferred the full MDR phenotype on cells. All cell lines were grown in RPMI-1640 medium supplemented with 10% FCS, 100 units/ml penicillin and 100 μg/ml streptomycin and incubated at 37 °C in a humidified, 5% CO_2 atmosphere. The absence of mycoplasma contamination was checked periodically by the Hoechst staining method. The KB^{V20C} and KB^{MDR} sublines were maintained under the same conditions as outlined above with media supplemented with 20 nM vincristine and 37 nM doxorubicin, respectively, until 8 days before the initiation of the individual experiments described below. All experiments reported in this study were performed during the exponential growth phase of the cells.

Antiproliferative assays. Antiproliferative activity was evaluated in exponentially growing cultures seeded at 5×10^4 cells/ml and allowed to adhere overnight to culture plates before addition of the drugs. Cell viability was determined by the MTT method as previously described [24]. Cell viability at each drug concentration was expressed as a percentage of untreated controls and the concentration resulting in 50% viability (IC_{50}) was determined by linear regression analysis.

Vinblastine accumulation assay. Vinblastine accumulation was measured according to the method described by Metherall et al. [12], with the following modifications. Cells were seeded at 1×10^5 cells/ml. After 48 h of incubation, a total of 5×10^5 cells were washed twice with PBS and incubated at 37 °C in half final volume of RPMI-1640 medium supplemented with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. One hour later, cells were refed an equal volume of identical medium containing 0.1 μCi/ml [G-^{3}H]vinblastine sulfate (Amersham Life Science 15.5 Ci/mM). Inhibitors of P-gp were added as indicated. Following a 1-h incubation, cells were washed rapidly three times with ice-cold PBS, solubilized in 0.1 N NaOH, and analyzed for protein and ^{3}H-vinblastine content. The amount of ^{3}H-vinblastine accumulated is reported as cpm per microgram of total cellular protein.

Biosynthesis, uptake, and esterification of cholesterol. Since the main differences in cholesterol metabolism of CEM and MOLT4 cell lines [20] were observed during exponential growth (48–72 h), cholesterol synthesis, uptake, and esterification were determined after 48 h of incubation of cells seeded at

1×10^5/ml. For cholesterol biosynthesis, cells were pulse labeled with 5 μCi/ml of sodium [^{14}C]acetate (DuPont NEN 50 mCi/mmol). After 3-h incubation, cells were thoroughly washed with PBS. Cell lipids were then extracted with acetone and neutral lipids were separated by thin layer chromatography (TLC) on kiesegel plates (Merck, Darmstadt, Germany) using a solvent system containing n-heptane/isopropyl ether/formic acid (60:40:2, v/v/v). Free cholesterol and cholesteryl ester bands were identified by comparison with standards that were run simultaneously on one side of the plate and visualized using iodine vapor. For counting, the silica was added directly to the counting vials containing 10 ml Econofluor (DuPont NEN) liquid scintillation fluid.

The uptake of exogenous cholesterol was measured in cells pulse labeled with 3 μCi/ml [1,2-^{3}H(N)]cholesterol (DuPont NEN 45 Ci/mmol) in medium without FCS for 30 min. After incubation, cells were washed with PBS, extracted with acetone and processed for intracellular free and esterified cholesterol as above.

Cholesterol esterification was also evaluated by incubating cells for 4 h in medium containing [1-^{14}C]oleic acid (DuPont NEN 55 mCi/mmol), bound to bovine serum albumin (BSA). To prepare the [^{14}C]oleate-BSA complex, 100 μCi of [1-^{14}C]oleic acid in ethanol was mixed with 1.4 mg KOH and the ethanol evaporated. PBS (1.5 ml) without Ca^{2+} and Mg^{2+} containing 4.24 mg BSA (essentially fatty acid free, Sigma) was added and the mixture shaken vigorously. This solution was added to each well at a final concentration of 2 μCi/ml. After incubation, cells were washed with PBS and extracted with acetone. Lipid subclasses were separated by TLC as described above and incorporation of [^{14}C]oleate into cholesterol esters was measured.

RNA isolation and reverse transcriptase-polymerase chain reaction. Expression levels of the *MDR1* gene were determined in cells seeded at 1×10^5/ml and incubated for 48 h. Total RNA was isolated from cells by the guanidine isothiocyanate phenol-chloroform extraction method. The RNA concentration was measured spectrophotometrically at 260 nm, and the 260/280 ratio was evaluated to check for protein contamination. Equal amounts of total RNA (1 μg) were reverse transcribed into cDNA and subsequently amplified by the polymerase chain reaction (PCR) in the presence of the specific MDR1 primer according to the instructions provided by the manufacturer (GeneAmp RNA PCR Kit, Perkin-Elmer Cetus). PCR products separated on agarose and stained with ethidium bromide showed a major band of the predicted size (167 bp). During the PCR reaction, the nonradioactive label digoxigenin-11-dUTP (DIG) (Boehringer, Mannheim) was incorporated, and the DNA fragments separated by electrophoresis in agarose were blotted onto a nylon membrane for 16 h in 10 × SSC. The blot was exposed to X-ray film (Kodak X-OMAT) for 2–10 min in an X-ray cassette at room temperature. A Kodak Digital Science BandScanner Image Analysis System containing HP ScanJet, ID Image Analysis Software assessed the intensity of the bands in the autoradiographs. β-Actin cDNA was amplified as an internal control. Since a low yield of PCR products was often obtained when cDNA segments were coamplified with the internal standard gene in the same tube, the relative levels of gene expression have therefore been determined by comparing the PCR products of the target cDNA and β-actin gene in separate tubes. The target gene/β-actin ratio was taken to represent the relative expression of the genes studied.

Immunoblotting of P-gp protein. To directly compare amounts of P-gp in the various cell lines, Western transfer analysis of plasma membrane protein from the two MDR KB cell lines and their respective control KB line with C219 antibody (Signet Laboratories, Mass.) was carried out. Techniques for Western transfer [25, 26] and immunoprecipitation [27] were as previously described. Western blots were probed using the enhanced chemiluminescence (ECL) detection system (Amersham International, Little Chalfont, UK), and horseradish peroxidase-linked (HRP anti-mouse immunoglobulin; Amersham) was used as second antibody. Nitrocellulose sheets were exposed to X-ray film for up to 2 min.

Peripheral blood lymphocytes. Peripheral blood lymphocytes (PBLs) from healthy donors were obtained by separation on Ficoll-Hypaque gradients. After extensive washings, cells were resuspended (1×10^6 cells/ml) in RPMI-1640 with 10% FCS and incubated overnight. For evaluations in resting PBLs, 1×10^6 cells/ml nonadherent cells were incubated in RPMI-1640 10% FCS in the absence or presence of the inhibitors at the indicated concentrations, and labeled with the radioactive precursors. For experiments with proliferating PBLs, after washings and overnight incubation, 1×10^6 cells/ml nonadherent cells were incubated at 37 °C in RPMI-1640 10% FCS supplemented with PHA (2.5 μg/ml) for 48 h. PHA-stimulated PBLs were then labeled in the absence and presence of the inhibitors. Radioactive precursors and drugs, times and labeling conditions, and procedures for radioactivity measurements in resting and proliferating PBLs were as described above for KB cell lines.

Statistical analysis. Means $\pm$ SE for triplicate determinations are presented. Statistical significance of differences was determined by a nonparametric Mann-Whitney test. $P < 0.05$ was the criterion for significance. All experiments were repeated at least twice.

Chemical analysis. Proteins were determined by the method of Lowry et al. [28], and bovine albumin was used as a standard.

Results

Drug resistance pattern of KB cell lines. The drug-sensitive phenotype of parental KB cells, and the MDR phenotype of KB^{MDR} and KB^{V20C} cell lines were assessed by testing their comparative sensitivities to a number of cytotoxic agents (table 1). Parental KB cells were susceptible to the inhibitory effects of a number of P-gp drugs, such as vincristine, doxorubicin, and etoposide, and also to drugs not subjected to the efflux pump P-gp, such as cis-platinum and camptothecin (IC_{50} in the range 1 nM–83 μM). In contrast, KB^{MDR} and KB^{V20C} cell lines showed patterns of cross-resistance expected for cells with an MDR phenotype: KB^{MDR} cells were 40-fold, 500-fold, and 90-fold more resistant to doxorubicin, vincristine, and etoposide, respectively, relative to parental KB cells; KB^{V20C} cells were approximately 100-fold more resistant to vincristine relative to the parental KB cell line and 20-fold and 40-fold more resistant to etoposide and doxorubicin, respectively. As expected, cis-platinum and camptothecin maintained in the two MDR cell lines the same degree of cytotoxicity shown against parental KB cells.

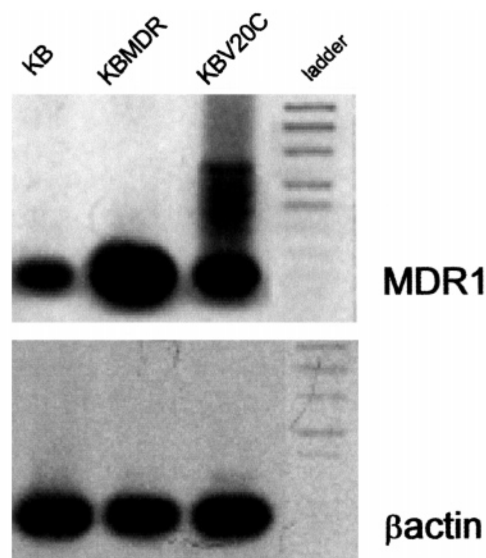
Growth characteristics of KB cell lines. The parental KB cell line had a doubling time of about 24 h and grew as homogeneous surface-adherent monolayers with few floating viable cells. KB^{MDR} cells had a doubling time of about 20 h and grew as parental KB cells but with increased acidification of the medium. KB^{V20C} had a doubling time of approximately 30 h and grew as islands of surface-adherent cells with higher numbers of floating cells than parental KB cells (data not shown).

Characterization of P-gp in KB cell lines. Relative to parental KB cells, KB^{MDR} and KB^{V20C} over-express P-gp as assessed by both RT-PCR of *MDR1* mRNA (fig. 1) and immunoblotting with antibodies against P-gp (fig. 2). The C219 antibody recognizes the broad Mr 170,000 P-gp and the narrow band of Mr 180,000 P-gp [29]. As can be seen, in KB^{MDR} and KB^{V20C}, both bands were visualized, Mr 170,000 being prominent. In the parental KB cells, the bands were too weak to be visualized on the photograph.

Table 1. Drug resistance pattern of MDR KB cell lines compared to the drug sensitivity of parental KB cells.

Drug	IC^*_{50} (μM) ± SE		
	KB	KB^{MDR}	KB^{V20C}
Vincristine	0.001 ± 0.00015	0.52 ± 0.09	0.11 ± 0.038
Doxorubicin	0.017 ± 0.007	0.73 ± 0.078	0.82 ± 0.042
Etoposide	0.23 ± 0.031	22.4 ± 3.8	8.76 ± 0.72
Cis-platinum	83.5 ± 5.2	89.3 ± 6.2	78.7 ± 5.6
Camptothecin	0.03 ± 0.002	0.07 ± 0.005	0.04 ± 0.007

*IC_{50} (inhibitory concentration 50): values represent the drug concentration ± SE required to reduce cell growth by 50% with respect to untreated controls as measured by the MTT methods.



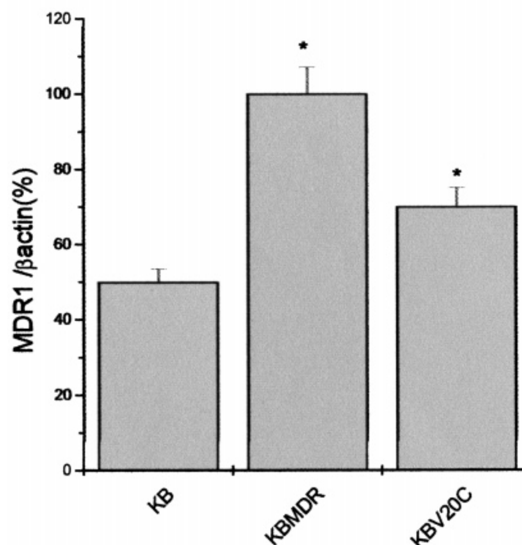


Figure 1. MDR1 mRNA levels in KB cell lines. Bars represent MDR1 mRNA levels relative to β-actin mRNA. Values are the mean ± SE of three independent experiments (*$P < 0.05$ vs KB). Representative autoradiographs are shown.

However, because the expression and function of P-gp are not always directly related [30], we measured the activity of the drug efflux pump based on the influx of ^{3}H-vinblastine, a known substrate for P-gp: the net cell content of ^{3}H-vinblastine is inversely proportional to expression of functional P-gp. As shown in figure 3, the

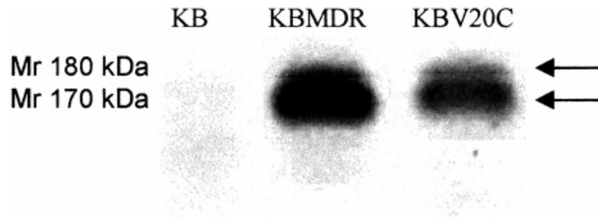


Figure 2. Immunoblotting of P-gp protein in KB cell lines. Autoradiogram of Western blots from cell lysate proteins with C219 antibody detected by chemiluminescence. Fifty micrograms per lane of proteins were subjected to gel electrophoresis (7.5% SDS-PAGE) and to immunoblotting. The position of prestained molecular size markers is shown.

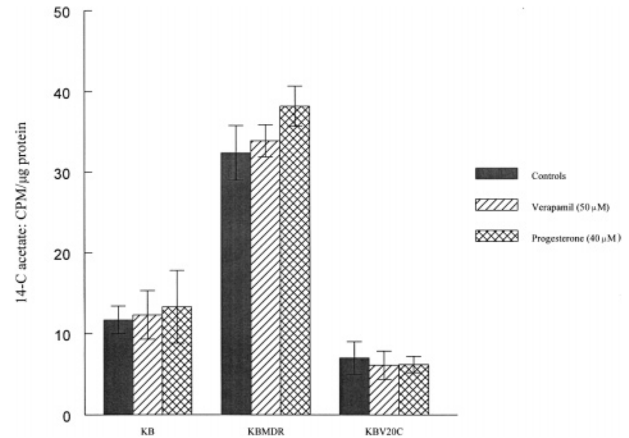


Figure 4. ^{14}C-acetate incorporated into free cholesterol in KB cell lines. Cells were seeded at 1×10^5/ml and incubated in growth medium. After 48 h, cultures were pulse labeled with 5 μCi/ml of [^{14}C]acetate for 3 h. Cell lipids were extracted and processed as described in Materials and methods. Data represent means ± SE for triplicate determinations of a representative experiment.

total amount of functional P-gp in KB^{MDR} and KB^{V20C} cells greatly increased with respect to the parental KB cells.

Synthesis, uptake, and esterification of cholesterol in KB cell lines. To investigate the possible relationship among cholesterol trafficking, P-gp activity, and growth rate, we next compared synthesis (fig. 4), uptake (fig. 5) and esterification (figs 6–8) of cholesterol in the above KB cell lines that express different amounts of functional P-gp as well as different rates of cell growth.

Relative to parental KB cells, the amount of ^{14}C-acetate incorporation into free cholesterol was higher in KB^{MDR} and lower in KB^{V20C} (fig. 4). In contrast, cholesterol uptake was higher (twofold) in KB^{V20C} cells and lower in KB^{MDR} compared to parental cells (fig. 5). Under basal conditions, the esterification of ^{3}H-cholesterol was again greater in KB^{MDR} than in parental cells, whereas, despite the high levels of ^{3}H-cholesterol up-

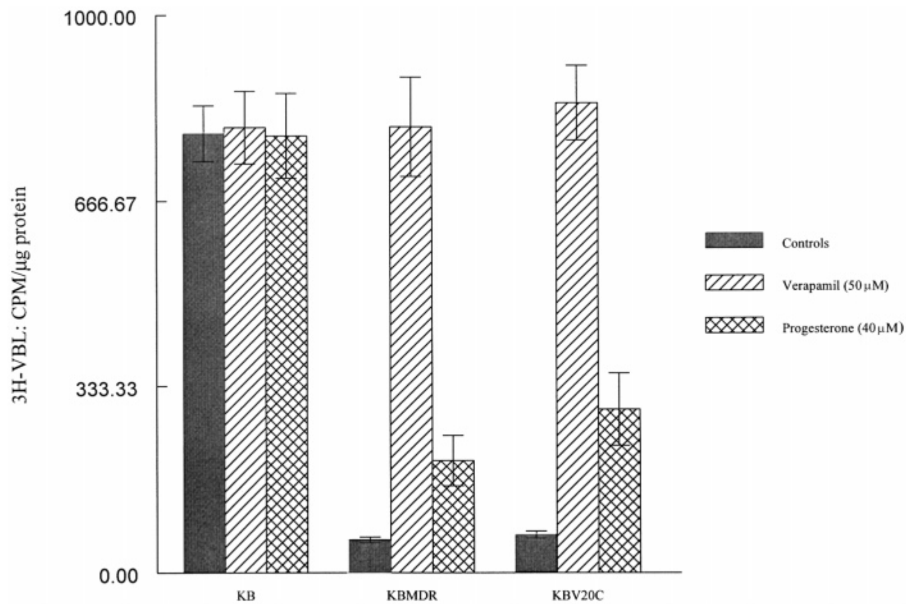


Figure 3. ^{3}H-vinblastine accumulation in KB cell lines. Cells were seeded at 1×10^5/ml and incubated in growth medium. After 48 h, cultures were incubated in fresh medium containing 0.1 μCi/ml of ^{3}H-vinblastine, with 40 μM progesterone or 50 μM verapamil as indicated. After 1 h, the cultures were processed to determine both intracellular radioactivity and protein content. Values represent cpm of ^{3}H-vinblastine per microgram of protein ± SE.

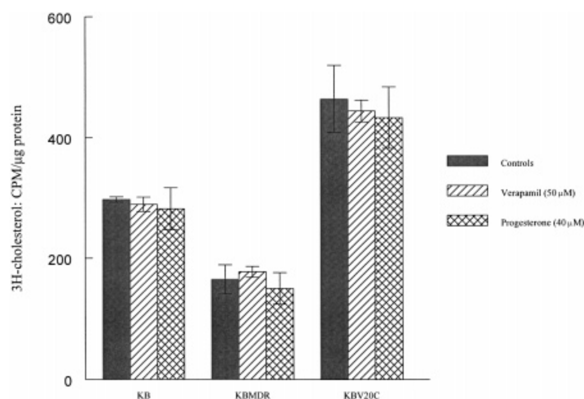


Figure 5. Uptake of ^{3}H-cholesterol in KB cell lines. Cells were seeded at 1×10^5/ml and incubated in growth medium. After 48 h, cultures were pulse labeled for 30 min with 3 μCi/ml of [^{3}H]cholesterol in medium without FCS. Cell lipids were extracted and processed as described in Materials and methods. Data represent means $\pm$ SE for triplicate determinations of a representative experiment.

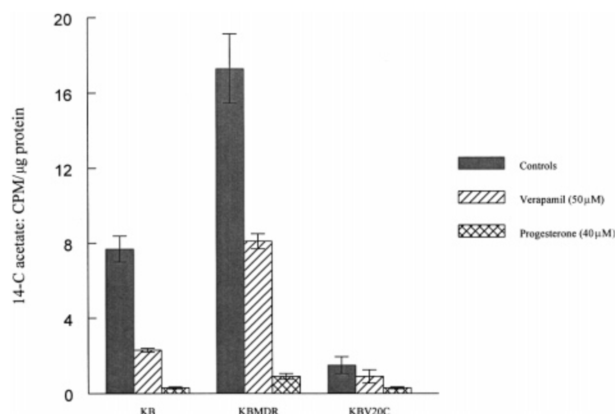


Figure 7. ^{14}C-acetate incorporated into cholesterol esters in KB cell lines. Cells were seeded at 1×10^5/ml and incubated in growth medium. After 48 h, cultures were pulse labeled with 5 μCi/ml of [^{14}C]acetate for 3 h. Cell lipids were extracted and the amount of [^{14}C]acetate cholesterol esters was determined as described in Materials and methods. Data represent means $\pm$ SE for triplicate determinations of a representative experiment.

take, KB^{V20C} exhibited very low ^{3}H-cholesterol ester incorporation (fig. 6). Similar results were obtained when cholesterol esterification was measured as ^{14}C-acetate and ^{14}C-oleate incorporated into cholesterol esters (figs 7, 8).

Overall, these data indicate that cholesterol esterification does not correlate with *MDR1* gene expression and P-gp activity in all the KB cell lines tested. Relative to parental KB cells, both KB^{MDR} and KB^{V20C} show increased levels of *MDR1* gene and functional P-gp expression, but only KB^{MDR} also showed an increased capacity to esterify cholesterol. Conversely, the strong relationship observed in all KB cell lines between the capacity of cells to esterify cholesterol and the length of their doubling time supports the idea that cholesterol esterification might be an effective modulator of cell growth and division.

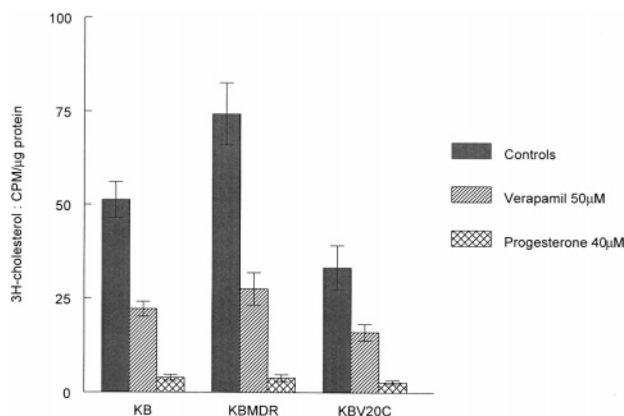


Figure 6. ^{3}H-cholesterol incorporated into cholesterol esters in KB cell lines. Cells were seeded at 1×10^5/ml and incubated in growth medium. After 48 h, cultures were pulse labeled with 3 μCi/ml of [^{3}H]cholesterol in medium without FCS for 3 h. Cell lipids were extracted and the amount of [^{3}H]cholesterol esters was determined as described in Materials and methods. Data represent means $\pm$ SE for triplicate determinations of a representative experiment.

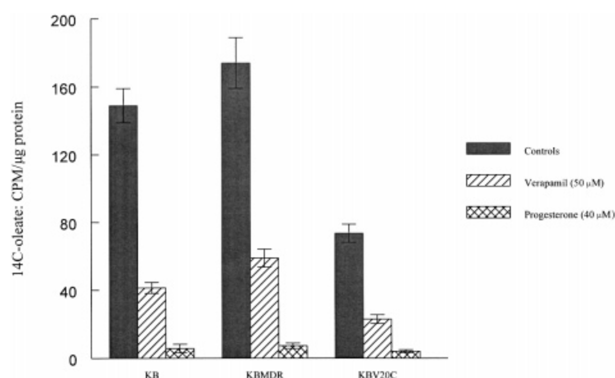


Figure 8. ^{14}C-oleate incorporated into cholesterol esters in KB cell lines. Cells were seeded at 1×10^5/ml and incubated in growth medium. After 48 h, cultures were pulse labeled for 4 h with 2 μCi/ml of [^{14}C]oleate-BSA complex. The amount of [^{14}C]oleate incorporated into cholesterol esters was determined as described in Materials and methods. Data represent means $\pm$ SE for triplicate determinations of a representative experiment.

Effects of P-gp inhibitors in KB cell lines. To further substantiate this hypothesis, we then investigated the effects of verapamil and progesterone (two nonspecific inhibitors of both ACAT and P-gp activity) on ^{3}H-vinblastine accumulation (fig. 3), cholesterol metabolism (figs 4–8) and cell growth (table 2), in the different KB cell lines.

Verapamil completely inhibited P-gp function by increasing the net content of intracellular labeled vinblastine of both KB^{MDR} and KB^{V20C} cell lines up to the level found in parental KB cells (fig. 3). Although to a lesser extent, progesterone also increased the intracellular levels of labeled vinblastine in both MDR cell lines. In contrast, despite detectable levels of *MDR1* mRNA, in the parental KB cells, neither progesterone nor the more potent verapamil affected the net intracellular content of ^{3}H-vinblastine (fig. 3).

In parental as well as MDR KB cell lines, both verapamil and progesterone inhibited the incorporation of ^{3}H-cholesterol (fig. 6), ^{14}C-acetate (fig. 7), and ^{14}C-oleate (fig. 8) into cholesteryl esters. Contrary to the effects shown on P-gp activity, however, progesterone inhibited cholesterol esterification more effectively than verapamil. Similar results were obtained by Luker et al. [30] in other MDR cell lines.

It has also to be noted that, in our experimental conditions, neither progesterone nor verapamil affected the biosynthesis or the uptake of cholesterol (see figs 4, 5). In agreement with earlier work conducted in several MDR cell lines [31, 32 and references cited therein], in KB cell over-expression of P-gp was also associated with hypersensitivity (collateral sensitivity) to steroids and calcium antagonists, which are known chemosensitizing compounds in MDR cells. The antiproliferative activities of both progesterone and verapamil were more potent against KB^{MDR} and KB^{V20C} than against parental KB cells (table 2). Although both drugs showed a selective cytotoxicity against MDR cells, the inhibitory concentrations of progesterone against KB^{MDR} and KB^{V20C} were tenfold lower than those active against parental KB cells. The corresponding concentrations of verapamil were only twofold lower, suggesting that MDR cells are more susceptible to the growth inhibitory effect of progesterone than to that of verapamil.

In conclusion, while verapamil was a stronger inhibitor than progesterone of P-gp activity, progesterone was a more potent inhibitor than verapamil of both cholesterol esterification and cell proliferation.

Table 2. Effect of P-gp inhibitors on growth of parental and MDR KB cell lines.

Cell line	IC_{50}^{*} (μM) ± SE	
	Progesterone	Verapamil
KB	33.5 ± 2.65	56.5 ± 3.8
KB^{MDR}	2.4 ± 0.53	23.6 ± 2.3
KB^{V20C}	2.7 ± 0.47	27.8 ± 1.9

*IC_{50} (inhibitory concentration 50): values represent the drug concentration ± SE required to reduce cell growth by 50% with respect to untreated controls, as measured by the MTT methods.

Table 3. P-gp activity and cholesterol esterification in resting versus mitogen-induced PBLs.

PBLs*	cpm/μg protein ± SE		
	^{3}H-vinblastine	^{14}C-oleate	^{3}H-thymidine
Resting	73.3 ± 4.3	21.1 ± 4.4	<3
PHA stimulated	75.1 ± 6.7	63.7 ± 14.9	114.9 ± 15
PHA stimulated + progesterone	79.3 ± 4.7	5.4 ± 1.2	34.8 ± 1.9
PHA stimulated + verapamil	72.8 ± 5.2	10.9 ± 5.6	not detected

*PBLs: resting and PHA-stimulated PBL cultures were obtained, labeled and processed as described in Materials and methods.

P-gp activity and cholesterol esterification during growth of PBLs. Although negative for P-gp protein (fig. 2) and P-gp activity (fig. 3), our control cells (parental KB cell line) did express detectable levels of *MDR1* mRNA (fig. 1). Therefore, we deemed it useful to investigate the correlation between P-gp and cholesterol esterification in cells that are P-gp negative. To this end, PBLs from healthy donors were used and the above parameters were evaluated in resting compared to mitogen-induced PBLs. Both resting and PHA-stimulated PBLs did not show detectable levels of *MDR1* mRNA as determined by RT-PCR (data not shown). However, PHA stimulation of PBLs, which resulted in increased amounts of ^{3}H-thymidine incorporated into DNA, caused a marked increase in ^{14}C-oleate incorporated into cholesterol esters. As can be seen, cholesterol esterification was strongly inhibited by progesterone and to a lesser extent by verapamil (table 3). In contrast, intracellular levels of labeled vinblastine did not significantly change in PHA-stimulated PBLs with respect to resting cells. Moreover, neither progesterone nor verapamil affected intracellular vinblastine accumulation, suggesting that their inhibitory effect on cholesterol esterification is independent on P-gp expression levels.

Discussion

Cell growth rate correlates with cholesterol esterification in normal and MDR KB cells. In agreement with our previous findings on CEM and MOLT4 cell lines [20, 21], the rate of cell growth correlates with the rate of cholesterol esterification also in KB cell lines, irrespective of their drug phenotype. We suggest that the rate-

limiting factor for cell growth is cholesterol esterification rather than cholesterol biosynthesis for the following reasons. KB^{MDR} cells, which have a doubling time shorter than parental KB cells (20 vs 24 h), show higher rates of cholesterol esterification as measured by the amount of cholesterol esters derived from (i) membrane-incorporated cholesterol (^{3}H-cholesterol), (ii) neosynthesized cholesterol (^{14}C-acetate), (iii) total cholesterol (^{14}C-oleate). In contrast, KB^{V20C} cells that have a consistently longer doubling time (30 h) than the other two cell lines, show the lowest cholesterol esterification capacity following exposure to any of the above labeled precursors. Moreover, KB^{V20C} cells that take up twice as much ^{3}H-cholesterol into the plasma membrane as KB^{MDR} cells do, incorporated into cholesterol esters only 6% of that ^{3}H radioactivity compared to 29% and 16% in KB^{MDR} and parental KB cells, respectively. Finally, progesterone and verapamil inhibit cholesterol esterification and cell growth, but have no effect on cholesterol biosynthesis.

Lack of correlation between *MDR1* gene, P-gp activity, and cholesterol esterification in MDR cells. Our previous findings [21] suggested that the *MDR1* gene might be involved in regulation of the rate of cell growth by modulating intracellular levels of cholesterol esters. Thus, cell lines displaying documented differences in the expression and function of P-gp, i.e., cells with a MDR phenotype versus drug-sensitive cells, appeared suitable to investigate this matter further. Several lines of evidence indicate that the drug-resistant phenotype of the two MDR KB cell lines used in this study is mediated by increased levels of functional P-gp: (i) relative to parental KB cells, both KB^{MDR} and KB^{V20C} show increased resistance to vincristine, doxorubicin, and etoposide, but not to cis-platinum and camptothecin; (ii) they have tenfold lower levels of intracellular labeled vinblastine that can be increased up to parental KB cell levels by the P-gp inhibitors verapamil and progesterone, and (iii) both MDR cell lines over-express *MDR1* mRNA and P-gp protein relative to parental KB cells.

Contrary to the P-gp present in the drug-sensitive CEM and MOLT4 cells [21], the P-gp functioning in MDR KB cells does not seem to be involved in the process leading to transport and esterification of cholesterol. In fact, the increased levels of both *MDR1* gene expression and functional P-gp in the two MDR cell lines paralleled an increased capacity to esterify cholesterol only in the KB^{MDR} cells. In KB^{V20C} cells, instead, over-expression of both the *MDR1* gene and its product was associated with a reduced cholesterol esterification capacity.

Moreover, in both parental and MDR cell lines, the process of cholesterol transport and esterification seems independent of the activity of the expressed P-gp. In fact, the inhibitory effect of P-gp inhibitors against cholesterol esterification does not always correlate with their effect on P-gp activity. For example, while inhibiting cholesterol esterification in all KB cell lines, neither verapamil nor progesterone caused an increase in intracellular ^{3}H-vinblastine in the parental KB cells, despite detectable levels of *MDR1* mRNA in these cells. In addition, although both compounds are effective inhibitors of cholesterol esterification and P-gp activity, verapamil proved to be a more potent inhibitor of the drug efflux pump P-gp, whereas progesterone more effectively inhibited cholesterol esterification.

That the observed effects of progesterone and verapamil on cholesterol esterification are independent of P-gp expression levels appears also supported by the data obtained in PBLs. These cells, which remain P-gp negative after mitogen stimulation and are not affected by progesterone and verapamil in their ability to accumulate labeled vinblastine, are strongly inhibited in cholesterol esterification capacity by these P-gp inhibitors. In PBLs also, progesterone proved to be a more potent inhibitor of cholesterol esterification than verapamil.

However, that the P-gp expressed in MDR cells is not involved in the transport/esterification process of cholesterol remains to be unequivocally demonstrated. KB^{MDR} cells might represent a better model than KB^{V20C} cells to study the role of *MDR1* and its product in the processes of cholesterol esterification and cell growth rate. KB^{MDR} cells were obtained following infection with a retroviral vector carrying the human *MDR1* gene and likely differ from the parental KB cell line only at the level of expression of this gene. In contrast, KB^{V20C} cells were obtained by stepwise drug selection of resistant cells in the sensitive population. Therefore, their drug-resistant phenotype might be associated to genotype modifications additional to that of the *MDR1* gene, which may in turn influence the overall biochemical behavior and cycle of the cell.

Possible implications in cancer therapy. P-gp inhibitors, verapamil and progesterone among others, are potentially beneficial to chemosensitize MDR tumor cells to cytotoxic agents [33]. In agreement with earlier work [31, 32 and references therein], the results reported in the present study indicate that inhibitors of P-gp activity might prove clinically useful in the therapy of MDR tumors not only through this mechanism. The strong inhibition exerted by verapamil and progesterone on the cholesterol esterification process is also associated with a selective cytotoxicity of these inhibitors against MDR cells relative to normal cells. Progesterone, in particular, shows a cytotoxic activity tenfold more potent on MDR cell lines than against normal cells, and its inhibitory effect leads to cell death of MDR but not normal cells. Although the mechanism of the selective cytotoxicity of verapamil and progesterone it is not yet known, it will

be of interest to test whether other steroid hormones that inhibit P-gp activity have the same selective cytocide effect on MDR cells and whether this also holds true in other MDR cell models.

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