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Elevated uptake of low density lipoprotein by drug resistant human leukemic cell lines

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Abstract

Overexpression of a 170 kD membrane glycoprotein, P-glycoprotein (Pgp), which acts as an energy dependent efflux pump for cytotoxic drugs is believed to be one of the factors that is responsible for clinical drug resistance. Recent studies suggest that Pgp is also responsible for the intracellular transport of cholesterol from the plasma membrane to the endoplasmic reticulum. Leukemic cells from patients with acute myelogenous leukemia have an elevated uptake of low density lipoprotein (LDL) when compared with white blood cells from healthy individuals. Since elevated LDL receptor expression and multidrug resistance are both common events in leukemic cells, we investigated LDL receptor expression in sensitive and drug resistant human leukemic cell lines. We found a 2- to 10-fold higher uptake of LDL in five out of five drug resistant K562 cell lines. All three drug resistant HL60 cell lines studied also had higher uptake than the parental cells. The LDL receptor expression in vincristine resistant Pgp positive K562 cells was less sensitive to downregulation by sterols than in parental cells. There was no selective effect of the Pgp inhibitor PSC-833 or other Pgp modulators on LDL receptor activity in Pgp positive cells. Since also resistant Pgp, multidrug resistance protein 1, and breast cancer resistance protein negative cells exhibited an elevated LDL receptor activity, we conclude that overexpression of these proteins is not the mechanism behind the elevated LDL uptake in the drug resistant leukemic cell lines. The findings are of interest for the concept of using lipoproteins as carriers of cytotoxic drugs in cancer treatment.

Keywords: Cholesterol; Leukemia; Low density lipoprotein; Multidrug resistance; P-glycoprotein

1. Introduction

Cancer chemotherapy with antineoplastic agents often fails to achieve complete and durable response. A major cause of treatment failure is drug resistance, primary or secondary. Overexpression of Pgp, a 170 kD membrane glycoprotein that is responsible for the efflux of naturally occurring cytotoxic compounds, causes the classical multidrug resistance (MDR) phenotype in cells [1]. Pgp belongs to the ATP-binding cassette (ABC) superfamily of transporters. Recent studies suggest that Pgp also transports cholesterol [2,3] indicating a relationship between cellular cholesterol disposition and drug resistance. That cholesterol may be involved in drug resistance is further supported by a study demonstrating that cellular cholesterol depletion resulted in an increased vincristine uptake and cytotoxicity in L5178Y murine leukemic lymphoblast cells [4]. It was also shown that an increment in the cholesterol content of resistant cells was directly proportional to the resistance to vincristine.

Cells have two ways to supply their cholesterol demands, either by uptake of LDL, which is the major cholesterol carrier in human plasma [5], or by endogenous cholesterol synthesis in which HMG-CoA reductase, is the rate-limiting enzyme [6]. Leukemic cells from patients with acute myelogenous leukemia (AML) have a higher uptake of LDL as compared to normal mononuclear blood cells, a phenomenon that has been found also in other human malignancies [7–10]. In addition to this, AML cells have an increased HMG-CoA reductase activity [11], which is the enzyme that catalyzes the rate-limiting step in the cholesterol synthesis. Since drug resistance and elevated uptake of LDL are common features of leukemic cells and since previous studies suggest that cellular cholesterol may be an important factor in drug resistance, we investigated whether drug resistant leukemic cells have an abnormal

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Abbreviations: LDL, low density lipoprotein; Pgp, P-glycoprotein; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; Calcein-AM, calcein-acetoxymethylester; MRP1, multidrug resistance protein 1; BCRP, breast cancer resistance protein.

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cholesterol metabolism using leukemic cell lines as a model. More specifically, we compared LDL receptor activities in drug sensitive and drug resistant leukemic cell lines with and without overexpression of Pgp or other MDR proteins like multidrug resistance protein 1 (MRP1) and breast cancer resistance protein (BCRP) [12,13].

2. Materials and methods

2.1. Materials

Na^{125}I (carrier free, pH 7–11) was from Bionuclear, uridine 5′-[*a*-^{35}S]thiotriphosphate (triethylammonium salt, >1000 Ci/mmol) were from Amersham. RNase A, RNase T1, proteinase K, transfer-RNA, trypan blue, and salmon sperm DNA were obtained from Sigma Chemical Co. Formamid (p.a.) was from Merck. The plasmid, pGEM4z, restriction endonucleases, and the *in vitro* transcription kit (Riboprobe Gemini II Core System) were from Promega Biotec. Sephadex G-50 columns (Nick columns) were from Pharmacia. 3-Hydroxy-3-methyl-[3-^{14}C]glutaryl coenzyme A (52 mCi/mmol) and DL-(2-^{3}H) mevalonic acid lactone (1.26 Ci/mmol) were from Amersham.

2.2. Cell lines

The human erythroleukemia cell line K562 and five drug resistant sublines (K562/Vcr30, K562/Vcr150, K562/Dauno75, K562/Mitox100, and K562/IDA20) and the human promyelocytic leukemia cell line HL60 and three drug resistant sublines (HL60/Mitox, HL60/AMSA, and HL60/AMSA/VP16) were kind gifts from Dr. Astrid Gruber (Department of Hematology and Infectious Disease, Karolinska Hospital, Sweden). Drug resistance was developed by continuous exposure to increasing concentrations of the drugs and cells were maintained in medium containing 30 and 150 nM vincristine (K562/Vcr30 and Vcr150; Eli Lilly and Company), 75 ng/mL daunorubicine (K562/Dauno75) (Rhone-Poulenc Rorer), 100 ng/mL mitoxantrone (K562/Mitox100; Wyeth Lederle), 20 ng/mL idarubicine (K562/IDA20) (Pharmacia & Upjohn), 100 ng/mL mitoxantrone (HL60/Mitox; Wyeth Lederle), 100 ng/mL amsacrine (HL60/AMSA; Warner Lambert/Pfizer), and initially 100 g/mL amsacrine which was later changed to 50 nM VP16 (HL60/AMSA/VP16).

2.3. Lipoproteins

LDL (density: 1.020–1.063 g/mL) and human lipoprotein deficient serum (LPDS; density > 1.215 g/mL) were isolated from serum of healthy blood donors by sequential ultracentrifugation. The purity of LDL and LPDS preparations was examined by agarose gel electrophoresis, and the absence of cholesterol in LPDS was confirmed by enzymatic cholesterol analysis (Merck). ^{125}I-Labeled LDL (sp. act. 150–410 cpm/ng protein) was prepared as described by Langer *et al.* [14]. Less than 1% of the radioactivity in the ^{125}I-labeled LDL preparation was present as free iodide. The concentration of LDL refers to protein and was determined by the method of Lowry *et al.* [15].

2.4. Cell culture

Cells were cultured at 37° in 25-cm^2 tissue culture flasks (Costar Corporation) in RPMI 1640 medium supplemented with 10% FCS and antibiotics (100 IU penicillin + 100 mg streptomycin/mL). The resistant cells were subcultured without drug prior to the experiments and the cells were subcultured once a week. Three days before each experiment the cells were subcultured in order to be subconfluent and in a growing stage at the time of the experiment (approximately 1×10^6 cells/mL).

2.5. Determination of LDL receptor activity

Before LDL receptor activity assay, cells were washed three times with phosphate buffered saline (PBS; 140 mmol/L NaCl, 2.7 mmol/L KCl, 9.5 mmol/L Na_2HPO_4, and 9.5 mmol/L KH_2PO_4, pH 7.4) by centrifugation. In some experiments, cell aliquots were removed and frozen at −70° for determination of HMG-CoA reductase activity and LDL receptor RNA levels. The remaining cells were dissolved in RPMI 1640 medium supplemented with 10% LPDS and antibiotics (100 IU penicillin + 100 mg streptomycin/mL). The high affinity degradation rate of ^{125}I-labeled LDL was used as a measure of LDL receptor activity as described previously [16,17]. In brief, 0.5×10^6 cells were incubated with 25 μg ^{125}I-LDL in 35 mm × 10 mm tissue culture dishes (Costar Corporation) at 37° in 1 mL of 10% LPDS in RPMI 1640 medium in the absence or presence of 500 μg unlabeled LDL. After 4 hr, the formation of noniodine trichloroacetic acid-soluble radioactivity in the medium was determined. The high affinity degradation rate of ^{125}I-LDL was calculated by subtracting the degradation of ^{125}I-LDL in the presence of excess unlabeled LDL (nonspecific degradation) from the degradation of ^{125}I-LDL in the absence of unlabeled LDL (total degradation) [5].

2.6. Sterol sensitivity and cellular uptake of sterols

In order to test end product repression of the LDL receptor and HMG-CoA reductase by sterols, the 10% LPDS medium was supplemented with a mixture of 25-hydroxycholesterol (Steraloids Inc.) and cholesterol (Sigma Chemical Co) in a ratio of 1:20 in ethanol (final ethanol concentration 0.2% v/v). Cells at a concentration of 0.5 × 106 mL were preincubated in 25-cm^2 tissue culture flasks (Costar Corporation) in 10% LPDS medium with sterols or in control medium for 17 hr and the high affinity ^{125}I-LDL degradation rate and HMG-CoA reductase activity was subsequently determined.

The cellular uptake of sterols was also determined. Cells, at a concentration of 1×10^6 cells/mL in 8 mL medium with 10% LPDS, were incubated in 25-cm^2 tissue culture flasks with a mixture of ^{3}H-25-hydroxycholesterol and ^{14}C-cholesterol in ethanol to which was added unlabeled cold sterols (final ethanol concentration was 0.2% v/v, and final concentration of sterols were 0.1 μg/mL 25-hydroxycholesterol and 2.0 μg/mL cholesterol) in 25-cm^2 tissue culture flasks for the indicated time points. After incubation at 37°, the cells were centrifuged and washed two times with PBS + BSA (1 mg/mL) and once with PBS. The cells were then resuspended in PBS, sonicated, and the radioactivity was determined in a scintillation counter (LKB/Wallac 1217 Rackbeta).

2.7. Effect of Pgp modulators on LDL receptor activity

The effect of the Pgp modulators verapamil (NM Pharma AB), tamoxifen (Sigma), and progesterone (Sigma), PSC-833 (Novartis), and the Pgp activator dexamethason on the high affinity degradation rate of ^{125}I-LDL was investigated in cells growing in 10% LPDS medium. Cells growing in 10% FCS were centrifuged and washed three times with PBS and resuspended in 10% LPDS medium at a concentration of 0.5×10^6 cells/mL. The drugs were then added at indicated concentrations and the cells were incubated at 37° in 1 mL of 10% LPDS medium in 35 mm × 10 mm tissue culture dishes. After 17 hr the high affinity degradation rate of ^{125}I-LDL was determined.

2.8. Determination of HMG-CoA reductase activity

HMG-CoA reductase activity in cell-free extracts from 1 to 2×10^6 cells was determined from the rate of conversion of 3-hydroxy-3-methyl-[3-^{14}C]glutaryl coenzyme A (25,000 dpm/nmol) to ^{14}C-mevalonate in detergent-solubilized extracts as previously described [11,18]. HMG-CoA reductase activity is expressed as picomoles of ^{14}C-mevalonate formed/min mg of detergent-solubilized protein. Protein concentration was determined by the method of Bradford [19].

2.9. Determination of RNA levels

LDL receptor RNA levels in cells were determined by a quantitative RNA–RNA solution hybridization method as previously described [11]. Briefly, total nucleic acids were prepared from 20 to 30×10^6 cells which were homogenized in 4 mL of 1 × SET (1% SDS; 20 mM Tris–HCl, pH 7.5; and 10 mM EDTA) with a Polytrone (Kinematica Type PT 16/35) for 10–15 s at setting 5–6 and DNA concentration was assayed by Hoechst fluorometry. Antisense and sense probes for LDL receptor RNA were prepared by *in vitro* transcription of a BamHI or HindIII cleaved plasmid, carrying a 265-bp fragment of cDNA encoding the human LDL receptor, with T7 (in the presence of ^{35}S-UTP) and SP6 (in the presence of unlabeled nucleotides only) RNA polymerases, respectively. The nucleic acid extracts or unlabeled sense RNA were hybridized with the antisense probes in solution. Following RNase treatment and precipitation with trichloroacetic acid, the RNase resistant precipitate was collected on 24-mm glass microfiber filters (Whatman International Ltd) and the radioactivity was determined in a liquid scintillation counter (Packard Instrument Company). RNA levels were quantified by comparing the hybridization signal with the linear part of the standard curve, which was generated by hybridization with different concentrations of sense RNA. All RNA values are based on three serial dilutions of each extract and presented as RNA copies per diploid genome (6 pg DNA).

2.10. Analysis of ABC membrane transporters

2.10.1. Calcein accumulation/extrusion

For detection of Pgp activity we measured the cellular uptake of the fluorogenic dye calcein-AM in the presence or absence of the Pgp drug-binding inhibitor cyclosporin A. The nonfluorescent hydrophobic calcein-AM enters the cells by diffusion and is de-esterified to fluorescent calcein in the cytoplasm [20]. The increase in fluorescence of cells incubated with calcein-AM represents calcein accumulation. Pgp in the membrane extrudes a portion of calcein-AM before it is de-esterified to fluorescent calcein, so that cell fluorescence increases more slowly. Cells at a concentration of 2.5×10^6 cells/mL were incubated at 37° in 200 μL 10% FCS medium for 30 min with 0.5 μM calcein-AM (Molecular Probes) in the presence or absence of cyclosporin A (10 μM; Novartis,) in 96-well microplates. The difference in fluoresence between cells incubated in the presence or absence of cyclosporin A represents Pgp function.

For detection of MRP1 we used a functional test by combining the MRP1-substrate calcein with the MRP1 inhibitor probenecid (Sigma) as described previously [21,22]. Two hundred microliters of cell suspension at 2.5×10^6 cells/mL were incubated in 96-well microplates at 37° for 30 min with 0.5 mM calcein-AM in presence or absence of 2.5 mM probenecid. At the end of the incubation, the cells were washed twice with ice-cold PBS, and thereafter incubated for 1 hr at 37° in the presence or absence of 2.5 mM probenecid in order to monitor the extrusion of calcein. Cells expressing MRP extrude calcein and this extrusion is inhibited by probenecid. The cellular fluorescence was measured on a FL600 microplate fluorescence reader at λ_{em} 494 nm and λ_{exe} 517 nm (Bio-tek Instruments).

2.10.2. Western blotting

Membrane proteins for detection of Pgp were separated on a polyacrylamide gel with 0.1% SDS and transferred to nitrocellulose membrane as described previously [23]. Membrane proteins for detection of BCRP and MRP1 were isolated as described previously [24,25]. Briefly, 40×10^6

cells were harvested by centrifugation (except for H69AR cells which were scraped at confluence in ice-cold PBS), washed twice with ice-cold PBS and homogenized for 10 s by sonication in an ice-cold hypotonic buffer consisting of 10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 7.4), and supplemented with protein inhibitors. The cell lysate was sequentially centrifuged at 1000 *g* for 10 min, at 3000 *g* for 15 min, and finally at 10,000 *g* for 15 min, respectively, and the supernatant was kept at −20°. Proteins were separated on 4–15% polyacrylamide gel and subsequently transferred to PVDF membrane (Gelman Laboratory). The membranes were incubated with the primary antibody mdr (ab-1, 1:500; Oncogene Science), BCRP (BXP-21, 1:200; Alexis Corporation) or the MRP1 (QCRL-1, 1:500; a kind gift from Dr. Susan Cole, Department of Pathology, Queen's University, Kingston, Ont., Canada) followed by a secondary HRP-labeled antibody (1:1000; Amersham). The breast carcinoma cell line MCF7 was used as a positive control for BCRP and the small cell lung cancer cell line H69AR was used as a positive control for MRP1. The bands were visualized on a Hyperfilm ECL film with the ECL kit (Amersham).

2.11. Determination of membrane fluidity

Fluorescence polarization values were measured with 1,6-diphenyl-1,3,5-hexatriene (DPH; Sigma) as a fluorescent probe in whole cells as described previously [26]. Labeling of cells with DPH was performed by injecting 8 μL of 2×10^{-3} M DPH in 95% ethanol into 2 mL 0.15 M KCl containing 5×10^6 cells. After 30 min of incubation at room temperature, fluorescence polarization was measured using a Perkin Elmer LS-50B fluorescence detector (Perkin Elmer, Inc.) with a polarization filters accessory. The excitation and the emission wavelengths were 340 and 426 nm, respectively. The emission intensity was measured parallel (I_{VV}) and perpendicular (I_{VH}) to the vertical plane of polarization of the excitation light and parallel (I_{HH}) and perpendicular (I_{HV}) to the horizontal plane of polarization of the excitation light. Polarization was calculated by the following equations: $P = (I_{VV} - GI_{VH})/(I_{VV} + GI_{VH})$ and $G = I_{HV}/I_{HH}$.

2.12. Determination of cholesterol in cells

For analysis of cellular cholesterol content, a combination of an enzymatic method and high pressure liquid chromatography was used with some modifications [27]. Approximately 5 to 10×10^6 cells were dissolved in 1–2 mL 0.5% (w/v) sodium cholate (Sigma) and sonicated for 30 s using a Vibra cell VC50T sonifier (Sonics & Materials, Inc.). Two 100-μL aliquots of the solution were taken for measurement of free and total cholesterol. The 100-μL aliquots were supplemented with 10 μL of a reaction mixture containing 500 mM MgCl2, 500 mM Tris–HCl (pH 7.4), 10 mM dithiothreitol, and 1% Triton X-100 (v/v). Thereafter, cholesterol oxidase (Boeringer Mannheim) or a mixture of cholesterol oxidase and cholesterol esterase (Sigma) were added in 10 μL of 0.5% sodium cholate (final concentration 0.4 unit/mL) for determination of free cholesterol and total cholesterol, respectively. The tubes were incubated at 37° for 30 min and then 380 μL methanol/ethanol (1:1) was added to stop the reaction. Samples were left for 30 min on ice and then centrifuged for 30 min at 1500 *g*. Fifty microliters of the supernatant was injected into the high pressure liquid chromatography system equipped with a Beckman Ultrasphere ODS 5 μ 4.6 mm × 25 mm C-18 column and a RCSS CN C-18 Guard-Pak precolumn insert (Waters Corporation). Methanol/acetic acid (99:1) were used as eluent at a flow rate of 2.0 mL/min, and absorbance was determined at 240 nm. The cellular cholesterol content was expressed as microgram per milligram protein which was determined by the method of Lowry *et al.* [15].

2.13. Statistics

Student's unpaired *t*-test was used for test of significance and $P < 0.05$ values were considered significant.

3. Results

3.1. LDL receptor and HMG-CoA reductase activities in K562/Vcr150 cells

The K562/Vcr150 cells have previously been shown to express the classical MDR phenotype with high levels of Pgp [28]. We, therefore, first investigated the high affinity ^{125}I-LDL degradation rate in continuously growing parental K562 and K562/Vcr150 cells at different time points after seeding. Trypan blue staining showed that cellular viability was 93–97% at all time points studied. The resistant cells showed up to four times higher ^{125}I-LDL degradation rates (Fig. 1A) and more than four times higher LDL receptor RNA levels (2.7 compared to 12.7 copies per cell) than the parental K562 cells. The HMG-CoA reductase activity was, on the contrary, lower in the resistant K562/Vcr150 cells than in the parental cells (Fig. 1B).

3.2. Sterol sensitivity of LDL receptor and HMG-CoA reductase activity

In order to know more in detail about the mechanism behind the altered cholesterol turnover in the K562/Vcr150 cells, we studied the feedback regulation of the LDL receptor and HMG-CoA reductase activities by sterols. The cells were exposed to 25-hydroxycholesterol + cholesterol (1:20) for 17 hr in medium with 10% LPDS before the determination of the high affinity ^{125}I-LDL degradation rate and HMG-CoA reductase activity. Both

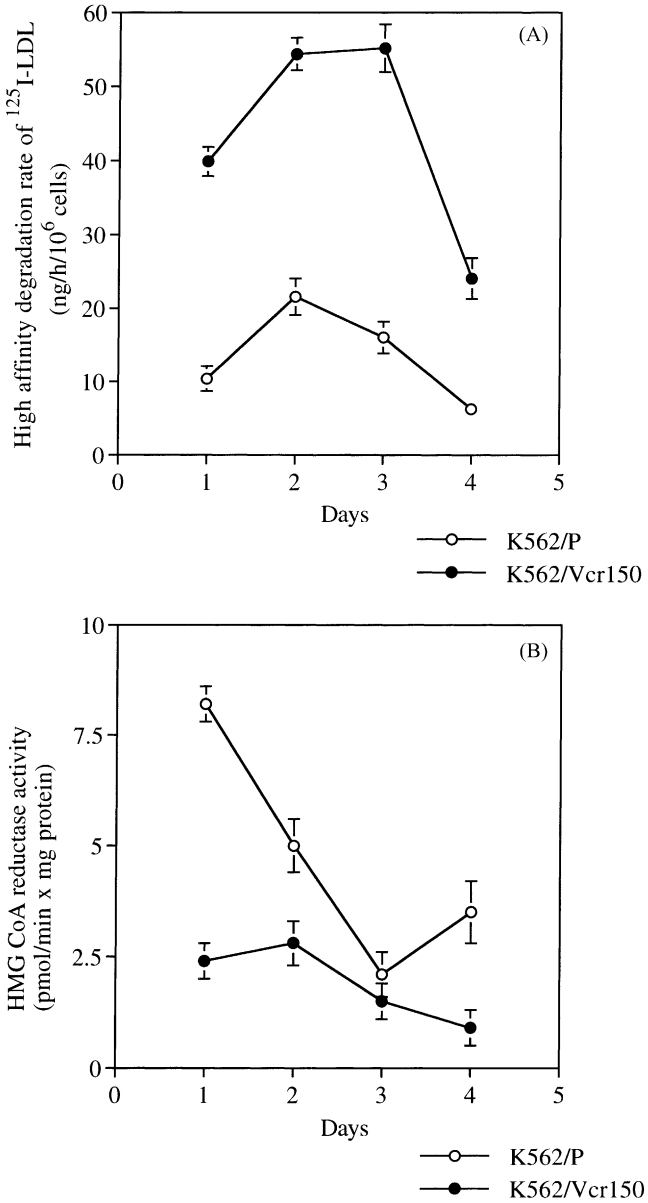

Fig. 1. (A) High affinity ^{125}I-LDL degradation rate and (B) HMG-CoA reductase activity in continuously growing parental and drug resistant K562/Vcr150 cells. Confluent cells were subcultured in 25-cm^2 tissue culture flasks in RPMI 1640 medium with 10% FCS at Day 0. After 1–4 days the cells were centrifuged and washed three times with PBS and resuspended in RPMI 1640 medium with 10% LPDS at a concentration of 0.5×10^6 cells/mL and the ^{125}I-LDL degradation rate was determined and aliquots were taken for determination of HMG-CoA reductase activity. Each data point shows the mean and standard deviation of triplicate incubations.

the LDL receptor and HMG-CoA reductase activities of K562/Vcr150 cells were considerably less sensitive, to the inhibitory effect of sterols, than the parental cells (Fig. 2A and B). The decreased sterol sensitivity was also found at the LDL receptor RNA level (Fig. 3A and B). In a separate experiment we studied the effect of five concentrations of the 25-hydroxycholesterol + cholesterol mix (0.025–0.4 μg/mL 25-hydroxycholesterol and 0.5-8.0 μg/mL cholesterol) on the LDL receptor activity in the parental K562 and the K562/Vcr150 cells. The median sterol concentration for 50% inhibition IC_{50} of LDL receptor activity was more than five times higher in the K562/Vcr150 cells than in the parental K562 cells (0.26 and 0.045 μg/mL 25-hydroxycholesterol, respectively) (not shown). In order to investigate if the decreased sensitivity to sterols could be explained by a reduced uptake of sterols, the uptake of ^{3}H-25-hydroxycholesterol and ^{14}C-cholesterol was determined in both cell lines. The uptake of ^{3}H-25-hydroxy-

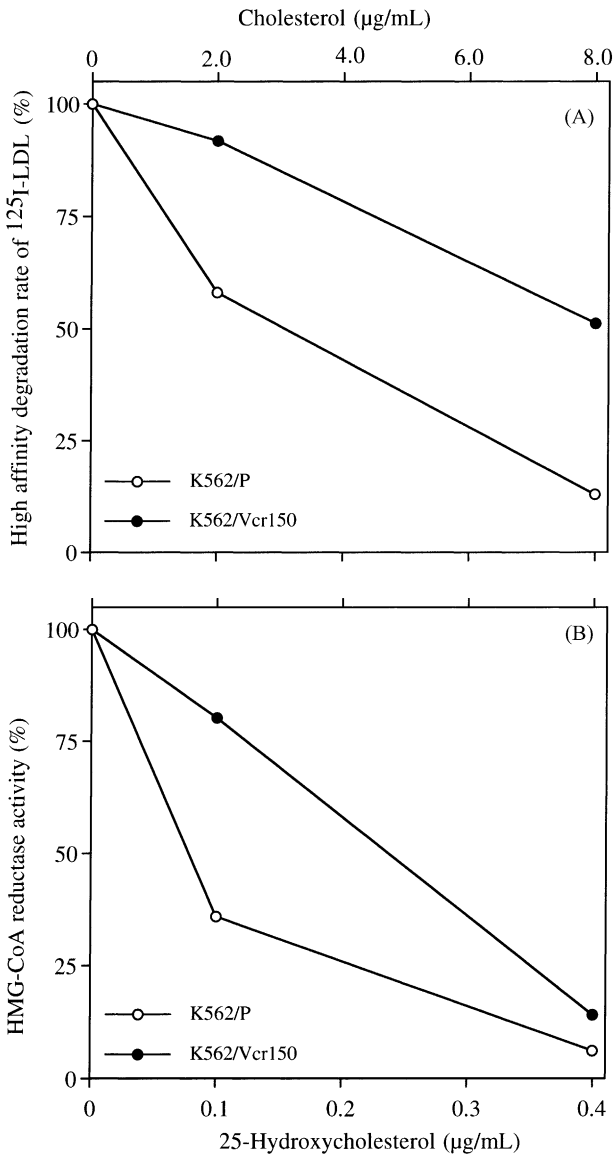

Fig. 2. Suppressive effect of sterols (25-hydroxycholesterol + cholesterol) on the (A) high affinity ^{125}I-LDL degradation rate and (B) HMG-CoA reductase activity in parental K562 and K562/Vcr150 cells. Cells were subjected to incubation in 25-cm^2 tissue culture flasks in 10 mL of RPMI 1640 medium with 10% LPDS at a concentration of 0.5×10^6 cells/mL with or without the presence of the indicated concentrations of sterols (added as a mixture in a ratio of 1:20). After 17 hr the high affinity ^{125}I-LDL degradation rate was determined and aliquots were taken for determination of HMG-CoA reductase activity the results are expressed as percent of cells incubated without sterols. Each data point shows the mean of duplicate incubations.

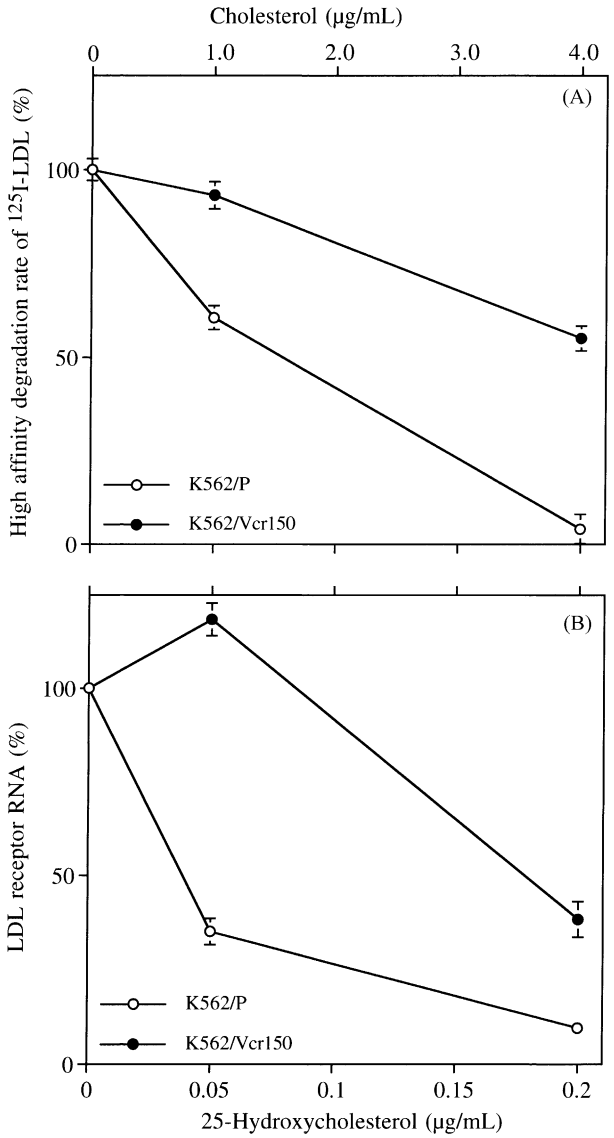

Fig. 3. Suppressive effect of sterols (25-hydroxycholesterol + cholesterol) on the (A) high affinity ^{125}I-LDL degradation rate and (B) LDL receptor RNA level in parental K562 and K562/Vcr150 cells. Cells at a concentration of 0.5×10^6 cells/mL were subjected to incubation in 35 mm × 10 mm tissue culture dishes in 1 mL of RPMI 1640 medium with 10% LPDS with or without the presence of the indicated concentrations of sterols. After 17 hr the high affinity ^{125}I-LDL degradation rate was determined and is expressed as percent of cells incubated without sterols. In parallel, incubations cells at a concentration of 0.5×10^6 cells/mL were subjected to incubation in 75-cm^2 flasks in 20 mL of RPMI 1640 medium with 10% LPDS with or without sterols. After 17 hr cells were washed three times with PBS and frozen for determination of LDL receptor RNA levels. Open circles represent parental K562 cells and closed circles represent K562/Vcr150 cells. Each data point shows the mean and standard deviation of triplicate incubations.

cholesterol was similar in both cell lines, while the uptake of ^{14}C-cholesterol was somewhat decreased (30%) in the K562/Vcr150 cells (Fig. 4A and B). LDL cholesterol isolated from human plasma or present in FCS was also less effective in downregulating the ^{125}I-LDL degradation rate in the K562/Vcr150 cells compared to the parental K562 cells (Fig. 5).

3.3. LDL receptor activity in other drug resistant cell lines

We also studied if an elevated LDL uptake occurred in other types of drug resistant cell lines. ^{125}I-LDL degradation rates were determined in the drug resistant K562 sublines (K562/Vcr30, K562/Dauno75, K562/Mitox100, and K562/IDA20) and parental HL60 and the drug resistant sublines (HL60/Mitox, HL60/AMSA, and HL60/AMSA/VP16). The resistant K562 sublines had up to 10 times elevated LDL receptor activities compared to the sensitive cells (Fig. 6A). The highest LDL receptor activity was found in the K562/Mitox100 cells, which do not express Pgp (described as follows). Also the resistant HL60 sublines had higher ^{125}I-LDL degradation rates than the parental cells, except for the first 2 days after subcultivation when ^{125}I-LDL degradation rates were similar (Fig. 6B).

3.4. Pgp activity and protein expression in drug resistant cell lines

In order to characterize Pgp expression in the cell lines, we used calcein-AM as a marker for functional Pgp activity and western blot to detect protein expression. The results showed an up to >50% reduced uptake of calcein-AM in the K562/Vcr30, K562/Vcr150, and K562/Dauno75 cell lines as compared to the parental K562 cells (Table 1). Moreover, the calcein-AM uptake was increased in the presence of cyclosporin A (10 μM) in these cell lines. In contrast, the calcein-AM uptake in K562/Mitox100 cells and K562/IDA20 and all the resistant HL60 cell lines, was higher than in the parental cells and the presence of cyclosporin A had little influence on calcein-AM uptake in these cell lines. Western blot analysis showed that the Pgp protein was present in membrane preparations from K562/Vcr30, K562/Vcr150, and K562/Dauno75 cell lines and absent in K562/Mitox100, K562/IDA20 cells, and all HL60 cell lines (Fig. 7A).

3.5. Other ABC transporters

The functional test for MRP1 was negative for all the resistant sublines since the uptake of calcein-AM by the cells during the loading experiment was not modified by the addition of probenecid, and the decline in fluorescence in the cells during the efflux experiment was not inhibited by the addition of probenecid (results not shown). Western blot analysis confirmed that none of the cell lines expressed MRP1 (Fig. 7B), while BCRP expression was found in the HL60/Mitox and the K562/Mitox100 cell lines (Fig. 7C). The highest BCRP expression was found in the HL60/Mitox cells.

3.6. Membrane fluidity and cellular cholesterol content

In order to investigate if the increased LDL uptake by the drug resistant cell lines would influence the cell membrane

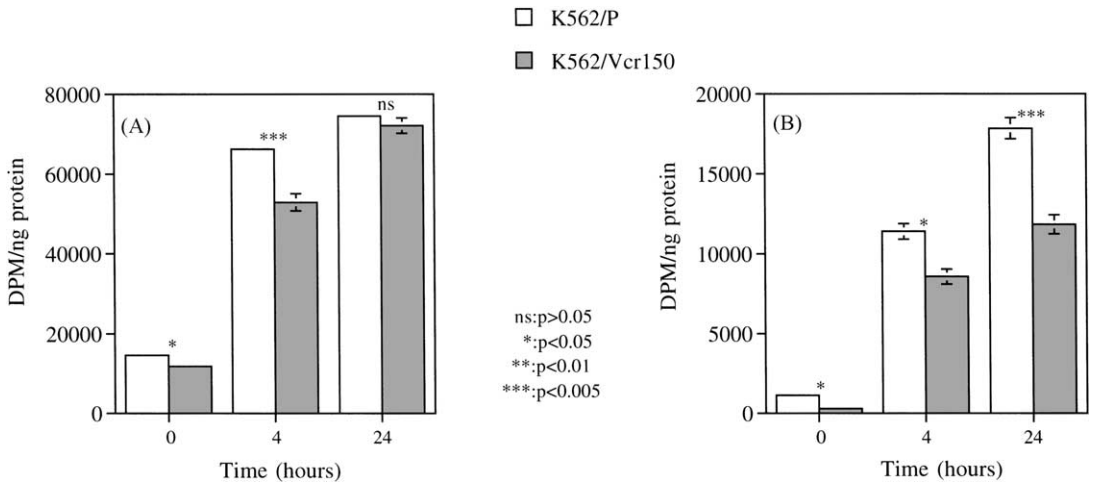

Fig. 4. Uptake of (A) ^{3}H-labeled 25-hydroxycholesterol and (B) ^{14}C-labeled cholesterol in parental K562 and K562/Vcr150 cells. Cells at a concentration of 1×10^{6} cells/mL were incubated at 37° in 25-cm^{2} tissue culture flasks with ^{3}H-labeled 25-hydroxycholesterol and ^{14}C-labeled cholesterol (final concentration of sterols were 0.1 μg/mL 25-hydroxycholesterol and 2.0 μg/mL cholesterol) in RPMI 1640 medium with 10% LPDS. After the indicated time periods the cells were washed three times and the radioactivity in relation to protein amount was determined. Each column shows the mean and standard deviation of triplicate incubations.

fluidity, we determined fluorescence polarization intensity as an estimate of the cell membrane fluidity (Table 2). In parallel, we also measured the cellular free and esterified cholesterol content in the cell lines. The results show that there were no significant differences in fluorescence polarization intensity between the parental and drug resistant cell lines, indicating that the membrane fluidity was not influenced by the increased LDL uptake. In accordance to this, the cellular free cholesterol levels were essentially the same in all cell lines (Table 2), while the cellular content of esterified cholesterol was 4–15 times higher in the drug resistant K562 cell lines.

3.7. Effect of Pgp modulators on LDL receptor activity

In order to investigate the mechanism behind the elevated uptake of LDL, we also studied the effects of the Pgp modulators verapamil, tamoxifen, progesterone, PSC-833, and dexamethason on LDL receptor activity in parental K562 and drug resistant K562 cells. Tamoxifen and

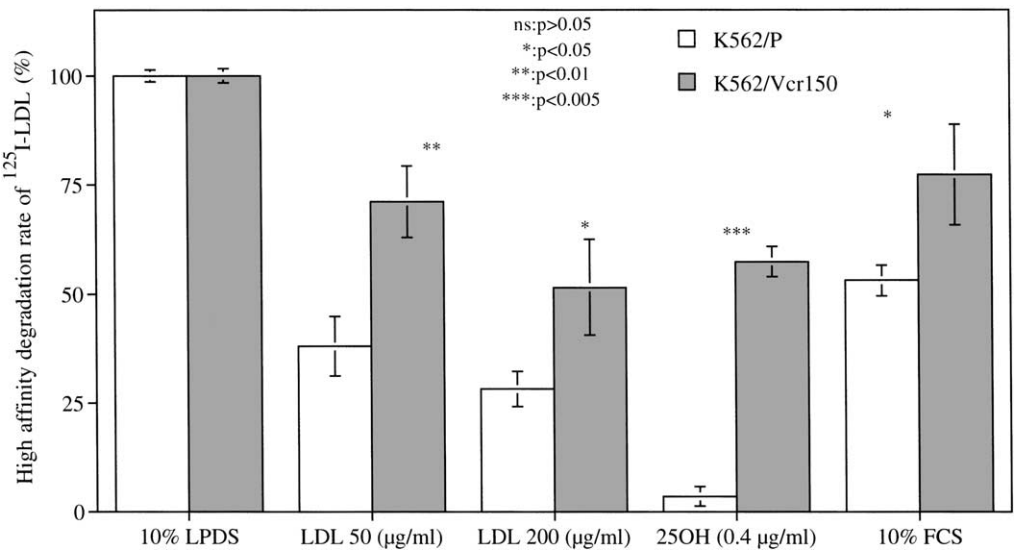

Fig. 5. Suppressive effects of lipoproteins, 25-hydroxycholesterol + cholesterol (1:20), or 10% FCS on the ^{125}I-LDL degradation rate in parental K562 and K562/Vcr150 cells. Confluent cells were washed three times with PBS and dissolved in RPMI 1640 medium with various additions. After 17 hr of incubation at 37° in 25-cm^{2} tissue culture flasks the cells were washed and dissolved in RPMI 1640 medium with 10% LPDS and the high affinity ^{125}I-LDL degradation rate was determined and is expressed as percent of cells incubated with 10% LPDS without any additions. Each column shows the mean and standard deviation of triplicate incubations.

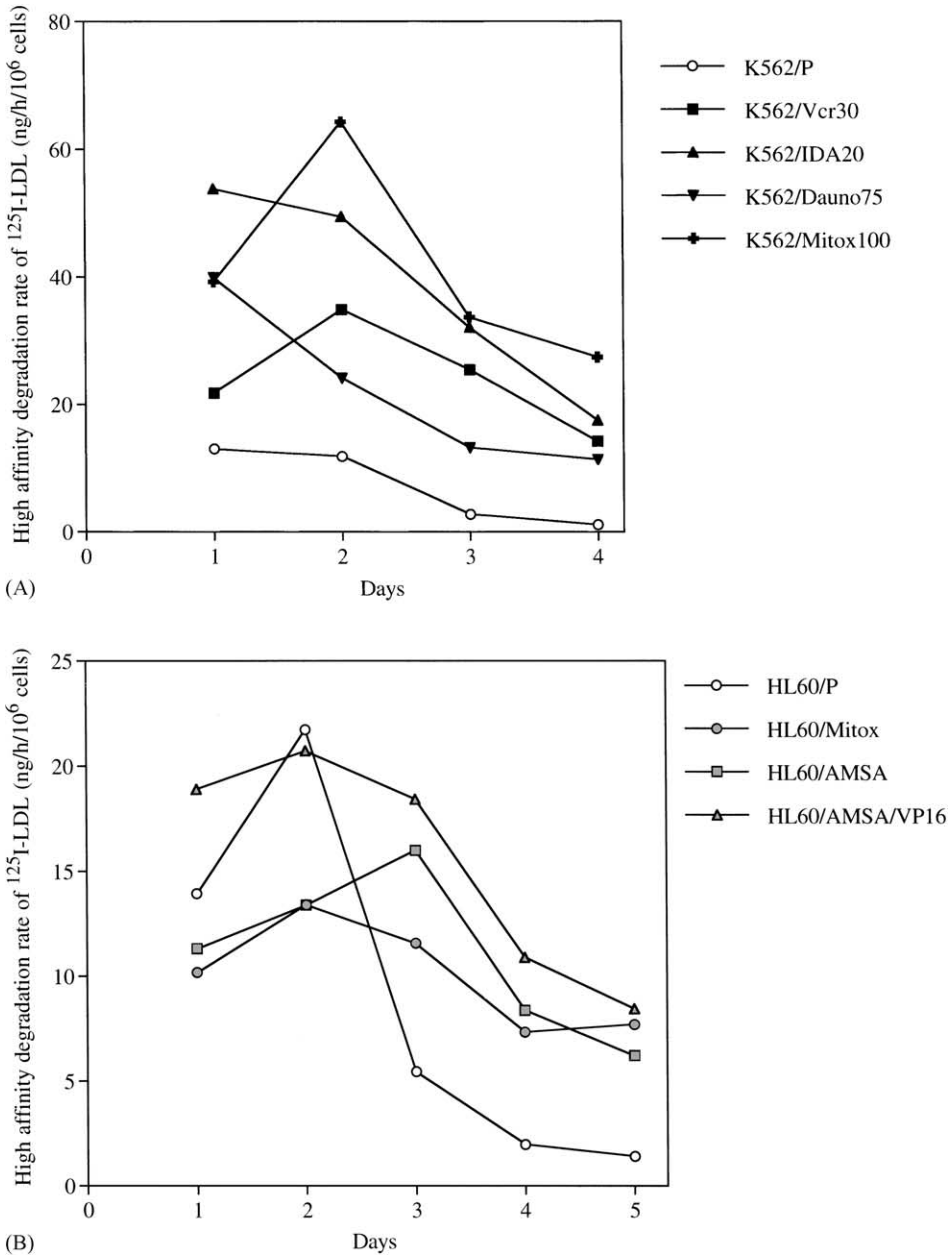

Fig. 6. High affinity ^{125}I-LDL degradation rate in continuously growing parental and drug resistant (A) K562 and (B) HL60 cell lines. Confluent cells were subcultured in 25-cm^2 tissue culture flasks in RPMI 1640 medium with 10% FCS at Day 0. After the indicated time the cells were centrifuged and washed three times with PBS and resuspended in RPMI 1640 medium with 10% LPDS at a concentration of 0.5×10^6 cells/mL. The ^{125}I-LDL degradation rate was then determined as described in Fig. 1. Each data point shows the mean of triplicate incubations.

progesterone (Fig. 8), and verapamil (Fig. 9) inhibited ^{125}I-LDL degradation rate in both the parental and the K562/Vcr150 cells. Verapamil produced a stronger inhibition of the high affinity ^{125}I-LDL degradation rate in the K562/Vcr150 cells compared to the parental cells (Fig. 9). Tamoxifen and progesterone, on the other hand, had a similar influence on the high affinity ^{125}I-LDL degradation rate in both cell lines (Fig. 8). The more specific Pgp inhibitor PSC-833 inhibited the ^{125}I-LDL degradation rate in K562/Vcr30, K562/Vcr150, K562/IDA20, and K562/Mitox100 cells, while the parental K562, K562/Dauno75, and all HL60 cells were not influenced by PSC-833 (Table 3). Incubation with the Pgp activator dexamethason (0.4–100 μM) produced a stimulation of the high affinity ^{125}I-LDL degradation rate in K562/Vcr150 and K562/Mitox100 cells by 25%, but the parental K562 cells were not influenced (results not shown).

4. Discussion

In this study, we found a markedly elevated LDL receptor activity in five drug resistant K562 and three HL60 cell lines as compared to their parental cells. The

Table 1
Calcein-AM uptake in the presence or absence of 10 μM cyclosporin A

Cell lines		Cyclosporin A (10.0 μM)
K562/P	100[a] (10.3)[b]	123.4 (5.2)
K562/Vcr30	65.8 (3.3)	161.9 (6.0)
K562/Vcr150	39.8 (2.3)	155.1 (23.0)
K562/IDA20	104.3 (6.9)	129.2 (22.4)
K562/Dauno75	30.3 (0.7)	130.6 (3.5)
K562/Mitox100	135.1 (13.8)	167.4 (30.0)
HL60/P	100 (7.3)	95.4 (0.8)
HL60/Mitox	120.4 (7.3)	104.2 (15.7)
HL60/AMSA	128.9 (4.2)	105.3 (4.9)
HL60/AMSA/VP16	110.1 (1.1)	102.0 (5.5)

[a] Calcein-AM uptake expressed as percent of parental cells.
[b] The numbers within parenthesis denote standard deviation.

Table 3
High affinity degradation rate of ^{125}I-LDL in cells exposed to 1 μM PSC-833 for 17 hr

Cell lines	PSC-833	Statistics[a]
K562/P	107.2 (5.9)	
K562/Vcr30	95.7 (2.0)	$P = 0.03$
K562/Vcr150	82.6 (9.1)	$P = 0.02$
K562/Dauno75	112.0 (9.0)	$P > 0.05$
K562/IDA20	88.4 (4.7)	$P = 0.01$
K562/Mitox100	81.0 (2.7)	$P = 0.002$
HL60/P	106.7 (5.4)	
HL60/AMSA	106.8 (7.0)	$P > 0.05$
HL60/AMSA/VP16	110.3 (7.3)	$P > 0.05$
HL60/Mitox	106.8 (5.3)	$P > 0.05$

Mean and SD in percent of cells treated with vehicle only (N = 3).
[a] Student's *t*-test vs.parental cells.

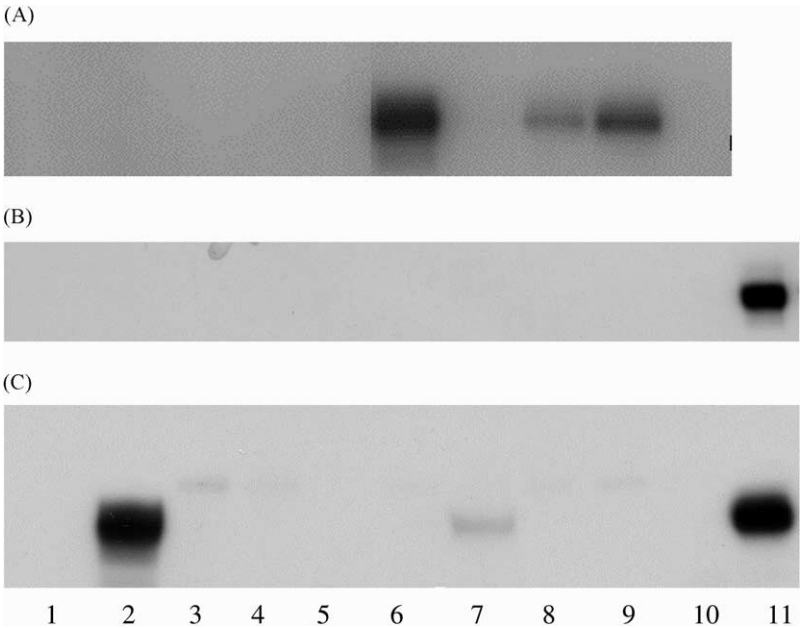

Fig. 7. Western blot analysis of (A) Pgp, (B) MRP1 and (C) BCRP in membrane preparations from HL60/P (lane 1), HL60/Mitox (lane 2), HL60/AMSA (lane 3), HL60/AMSA/VP16 (lane 4), K562/P (lane 5), K562/Dauno75 (lane 6), K562/Mitox100 (lane 7), K562/Vcr30 (lane 8), K562/Vcr150 (lane 9), and K562/IDA20 (lane 10) cells. Lane 11(B) contains membrane preparation from H69AR cells and lane 11(C) contains membrane preparation from MCF7 cells.

Table 2
Degree of fluorescence polarisation P obtained in cells labeled with DPH and cellular, total, free, and esterified cholesterol content

	P (N = 6)	Cholesterol (μg/mg protein)			
		Total (N = 3)	Free (N = 3)	Esterified (N = 3)	Statistics[a]
K562/P	0.257 (0.017)	35.1 (0.6)	33.4 (2.4)	1.6 (2.0)	
K562/Vcr30	0.249 (0.011)	43.4 (2.4)	35.2 (1.8)	8.2 (2.1)	$P = 0.011$
K562/Vcr150	0.252 (0.011)	43.6 (5.8)	33.7 (2.9)	10.0 (4.2)	$P = 0.033$
K562/Dauno75	0.273 (0.011)	53.7 (0.5)	46.3 (2.1)	7.4 (2.1)	$P = 0.02$
K562/IDA20	0.266 (0.010)	64.1 (5.2)	35.8 (4.9)	28.1 (2.6)	$P = 0.0001$
K562/Mitox100	0.241 (0.012)	45.5 (3.8)	25.1 (0.7)	20.5 (3.9)	$P = 0.001$
HL60/P	0.224 (0.010)	27.3 (1.3)	23.7 (3.8)	3.6 (1.4)	
HL60/AMSA	0.228 (0.006)	20.2 (0.6)	18.9 (0.8)	1.2 (0.9)	$P > 0.05$
HL60/AMSA/VP16	0.221 (0.001)	20.8 (1.6)	20.0 (1.4)	0.8 (0.5)	$P = 0.04$
HL60/Mitox	0.208 (0.007)	27.6 (0.6)	25.8 (0.9)	1.8 (0.8)	$P > 0.05$

The data are expressed as means and SD.
[a] Student's *t*-test, esterified cholesterol content vs. parental cells.

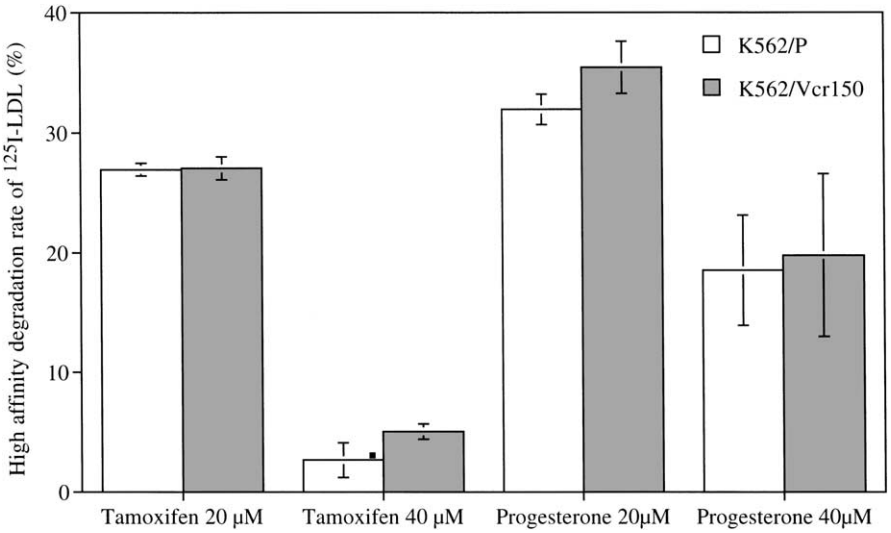

Fig. 8. Effect of tamoxifene and progesterone on high affinity ^{125}I-LDL degradation rate in parental K562 and K562/Vcr150 cells. Cells were subjected to incubation in 35 mm × 10 mm tissue culture dishes in 1 mL of RPMI 1640 medium with 10% LPDS at a concentration of 0.5×10^6 cells/mL in the presence of tamoxifen or progesterone at the indicated concentrations. After 17 hr ^{125}I-LDL degradation rate was determined and is expressed as percent of the ^{125}I-LDL degradation rate in cells incubated without tamoxifen or progesterone. Each data point shows the mean and standard deviation of triplicate incubations. Open columns represent parental K562 cells and filled columns represent K562/Vcr150 cells.

elevated LDL receptor activity was found irrespective of which drug was used for selection of drug resistance, and whether, if Pgp, MRP1 or BCRP was expressed or not. Specific LDL receptor overexpression was confirmed by RNA analysis. As the growth rate strongly affects the LDL uptake [5,29], the ^{125}I-LDL degradation rate was determined in continuously growing cells at several time points after seeding. The drug resistant K562 cells showed up to 10-fold elevated degradation rates of ^{125}I-LDL compared to the parental cells. The elevated LDL uptake by the drug resistant K562 cells was not caused by a higher growth rate since the parental cells had a higher growth rate (results not shown).

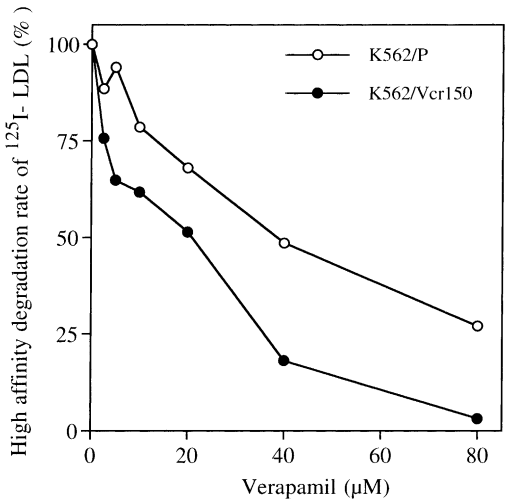

Fig. 9. The effect of verapamil on the high affinity ^{125}I-LDL degradation rate in sensitive K562 and K562/Vcr150 cells. Cells were subjected to incubation in 35 mm × 10 mm tissue culture dishes in 1 mL of RPMI 1640 medium with 10% LPDS at a concentration of 0.5×10^6 cells/mL in the presence of verapamil at the indicated concentrations. After 17 hr the ^{125}I-LDL degradation rate was determined and is expressed as percent of the ^{125}I-LDL degradation rate in cells incubated without verapamil. Each data point shows the mean of duplicate incubations. The variation between the samples was less than 5% of the mean. Open circles represent parental K562 cells and closed circles represent K562/Vcr150 cells.

We investigated the Pgp expressing K562/Vcr150 cells in more detail and found a lower HMG-CoA reductase activity in the K562/Vcr150 cells compared to the parental cells. It may be argued that the elevated uptake of LDL is a compensatory response to a decreased cholesterol synthesis. However, Ho *et al.* [16] showed that in normal mononuclear blood cells the synthesized cholesterol is quantitatively insignificant compared to the cholesterol derived by uptake of LDL. The HMG-CoA reductase activity in K562 cells (1–8 pmol/min mg protein) is similar to that in normal mononuclear blood cells (mean 6.8; range 2.6–14.3 pmol/min mg protein) [11]. On the contrary, the ^{125}I-LDL degradation rate was much higher in the K562/Vcr150 cells (20–55 ng/hr/10^6 cells) compared to normal mononuclear blood cells (mean 0.50; range 0.25–0.83 ng/hr/10^6 cells) [11]. We, therefore, consider it unlikely that a decrease in the supply of endogenously synthesized cholesterol in the K562/Vcr150 cells would cause such a strong compensatory response in LDL uptake. This is also supported by the measurements of cellular cholesterol content showing that all the drug resistant K562 cells had a higher total cholesterol content (20–80%) as compared to the parental K562 cells (Table 2).

We also investigated if overexpression of Pgp could be responsible for the elevated LDL uptake in the K562/Vcr150 cells by treating the cells with Pgp modulators. Although verapamil inhibited the ^{125}I-LDL degradation

rate to a higher extent in the K562/Vcr150 cells compared to the parental K562 cells, two other Pgp modulators, tamoxifen and progesterone, produced a similar inhibition of the ^{125}I-LDL degradation rate in both cell lines. Moreover, the more specific Pgp inhibitor, PSC-833, inhibited the ^{125}I-LDL degradation rate in all K562 cell lines except for the K562/Dauno75 cells and the parental K562 cells (Table 3). Thus, PSC-833 inhibited LDL receptor activity also in Pgp negative cells and the Pgp overexpressing K562/Dauno75 cells was not inhibited. Therefore, neither competitive inhibitors like verapamil, tamoxifene, or progesterone, nor the more specific inhibitor PSC-833 had a selective effect on LDL uptake in Ppg expressing cells. Treatment of Pgp positive K562/Vcr150 and Pgp negative K562/Mitox100 cells with dexamethason, a Pgp activator [30], gave a similar stimulation of LDL receptor activity in both cell lines. Together with the fact that also other non-Pgp overexpressing drug resistant cell lines, such as K562/Mitox100 and K562/IDA20 had an elevated LDL receptor activity, this demonstrates that the elevated LDL receptor activity is not caused by an overexpression of Pgp.

The expression of two other ABC transporters, MRP1 and BCRP (ABCG2), which are known to be selected by exposure to VP16 and mitoxantrone, were also investigated by western blot analysis. None of the cell lines expressed MRP1. Both the mitoxantrone resistant HL60 and K562 cell lines were found to overexpress BCRP. Although we cannot rule out that BCRP may influence the LDL uptake, it is unlikely that BCRP overexpression would be responsible for the increased LDL uptake in the resistant cells since BCRP was, beyond comparison, most abundant in the HL60/Mitox cells and they had the lowest LDL uptake among all the resistant cell lines (Fig. 7C). However, the results of the experiments with Pgp modulators and western blots could indicate that another ABC drug transporter is involved in the elevated LDL uptake in resistant cells.

Our results show that K562/Vcr150 cells had a decreased sensitivity to sterols (25-hydroxycholesterol + cholesterol) for both the LDL receptor and HMG-CoA reductase, compared with the parental cells. A decreased sensitivity to sterols at the LDL receptor RNA level was also demonstrated. It could be argued that the decreased sterol sensitivity in the K562/Vcr150 cells could be explained by a reduced uptake of sterols due to Pgp overexpression. Indeed, the uptake of cholesterol added in ethanol was approximately 30% lower in the K562/Vcr150 cells than in the parental cells (Fig. 4A). On the other hand, the uptake of the more potent and water soluble 25-hydroxycholesterol was similar in the two cell lines. Moreover, the results in Fig. 5 show that when the cells were given LDL cholesterol, the LDL receptor activity of K562/Vcr150 cells was less downregulated compared to the parental cells, in spite of the fact that the cholesterol supply was much higher in the K562/Vcr150 cells because of a higher LDL uptake. We, therefore, consider it unlikely that the minor reduction in cholesterol uptake explains the decreased sterol sensitivity in the K562/Vcr150 cells. The decreased sensitivity to both sterols and LDL cholesterol in the drug resistant K562/Vcr150 supports that a sterol-independent mechanism is responsible for the increased LDL receptor activity in the drug resistant cells. Measurements of the cellular cholesterol content revealed that the levels of esterified cholesterol were up to 15 times higher in the drug resistant K562 cells.

We have previously reported a decreased sensitivity to sterols in cells from patients with AML [11,31] and that the LDL receptor activity, directly after isolation from blood, correlated ($r = 0.53$, $P = 0.007$, $N = 27$) with IC_{50} values [31]. In addition, decreased downregulation of LDL receptor expression has been reported in Daudi Burkitt's lymphoma cells [32] and in a human prostate cancer cell line [33]. Interestingly, in the prostate cancer cell line it was demonstrated that both the LDL receptor RNA level and the level of the potent regulator of the *ldlr* promoter, SREBP2, were not downregulated by sterols. It is also noteworthy that incubation of cells with cytokines and mitogens has been shown to cause a sterol independent increase in LDL receptor gene expression both at the RNA and protein level [34–36].

The influence of an abnormal cholesterol turnover on the sensitivity to cytotoxic drugs in AML cells has not been studied. It can, for example, be speculated that an increased uptake of LDL may cause modifications of the cell membrane composition that may affect the passive diffusion of cytotoxic drugs across the cell membrane. Results by Mazzoni and Trave [37] showed that incubation of human ovarian cancer cells A2780 in LPDS medium caused a reduced uptake of doxorubicin. A reduced membrane cholesterol content, mainly esterified cholesterol, was held responsible for this phenomenon. In this study, we found elevated levels of esterified cholesterol in the drug resistant K562 cells but the membrane fluidity was, however, not different from that of parental K562 cells. More studies are needed to clarify if increased LDL uptake is a general phenomenon of drug resistant leukemia cells from AML patients and to investigate if AML cells with an increased LDL uptake are less sensitive to cytotoxic drugs compared to cells with a normal LDL uptake. The results of this study also support the concept of using LDL or synthetic LDL particles as carriers of cytotoxic compounds to increase the drug delivery to tumor cells with high LDL uptake [38,39].

Acknowledgments

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