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Functional Fluo-3/AM Assay on P-Glycoprotein Transport Activity in L1210/VCR Cells by Confocal Microscopy

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Abstract. Multidrug resistance (MDR) phenotype of L1210/VCR cell line, acquired by selection for vincristine (VCR), is predominantly mediated by P-glycoprotein (Pgp). Calcein/AM (Cal) was recently described as a fluorescent substrate for Pgp and may be used for measuring of transport activity of Pgp. Expression of Pgp in the cells prevents them to be loaded with the fluorescent marker. To detect the activity of Pgp, verapamil (Ver) or cyclosporine A (CsA) has to be used as Pgp inhibitors. Multidrug resistance protein (MRP), another drug efflux pump, may be inhibited by probenecid (Pro), i.e, the inhibitor of a wide variety of anion transporters. Ver, but not Pro, is able to induce the loading of L1210/CR cells by Cal that is measurable by fluorescence-activated cell sorter (FACS). Another dye, fluo-3/AM (F-3), has a similar behaviour like Cal. Using confocal microscopy we have proved that L1210/VCR cells, in contrast to parental sensitive cells, are not loaded with F-3. Marking of cells with the dye can be achieved using inhibitors of Pgp like Ver or CsA but not by Pro. These results indicate that F-3 is usable for detection of Pgp function in various MDR tissue cells.

Key words: P-glycoprotein — Multidrug resistance — Fluo-3/AM — Confocal microscopy — L1210 cells

Abbreviations: CsA, cyslosporine A; Cal, calcein/AM; DMSO, dimethyl sulfoxide; F-3, fluo-3/AM; FACS, fluorescence-activated cell sorter; MDR, multidrug resitance; MRP, multidrug resistance protein; Pgp, P-glycoprotein; Pro, probenecid; VCR, vincristine; Ver, verapamil

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Introduction

Overexpression of Pgp (plasma membrane glycoprotein that functions as an ATP-driven drug efflux pump) in neoplastic cells is generally known to induce the MDR that represents the real obstacle in chemotherapy of neoplastic diseases. Pgp is a member of superfamily of ABC membrane transporters with large specificity for diverse spectrum of different lipophilic substances (for a review see Kvačkajová-Kišucká et al. 2001). Function of Pgp in MDR cells could be studied using several fluorescent substrates of Pgp like doxorubicin (Weaver et al. 1991; Breier et al. 1998; Drobná et al. 2002; Fu et al. 2002), rhodamine 123 (Tapiero et al. 1984; Weaver et al. 1991), fura-2/AM (Fu et al. 1997, 2002), calcein/AM (Eneroth et al. 2001; Karaszi et al. 2001). Last two substances represent esterified analogues of fura-2 and calcein that were originally developed for measurement of intracellular free concentration of calcium in animal cells. Another intracellular calcium indicator, F-3, was also used for the measurement of transport activity of organic anion transporter in human colon carcinoma cells HT29 clone 19A (Abrahamse and Rechkemmer 2001) or transport activity of multidrug resistance protein (MRP1, MRP2 and MRP4, i.e., another drug efflux transport pump with ABC consensus) in several cell models (Cantz et al. 2000; Prechtl et al. 2000; Cui et al. 2001; Masereeuw et al. 2003).

L1210/VCR cell line represents a Pgp positive (Fiala et al. 2003) MDR cell model selected from parental cells by repeated cultivation in sublethal concentration of VCR (Polekova et al. 1992). These cells exert crossresistance to actinomycin D, mitomycin C, doxorubicin, cyclophosphamide, dexamethasone (Boháčová et al. 2000; Breier et al. 2000) and resistance may be reversed by several known chemosensitisers like Ver (Barančík et al. 1994) but also by blockers of mitogen activated protein kinases cascades (Barančík et al. 2001; Kišucká et al. 2001) or derivatives of pentoxifylline (Drobná et al. 2002; Kupsaková et al. 2002, 2004). Several differences in ultrastructure between L1210 and L1210/VCR cells under normal and hypoxic conditions have recently been described (El-Saggan and Uhrík 2002; El-Saggan et al. 2003; Fiala et al. 2003)

The aim of the present study is to test if F-3 may be used for the measurement of Pgp transport activity in L1210/VCR cells using confocal microscopy.

Materials and Methods

Reagents

Components of cell cultivation RPMI 1640 medium, L-glutamine, gentamycin and foetal bovine serum were purchased from Life Technologies (Scotland). VCR was obtained from Gedeon Richter Co., F3 – 4-(6-acetoxymethoxy-2,7-dichloro-3-oxo-9-xanthenyl)-4′-methyl-2,2′(ethylene dioxy)dianiline-N,N,N′,N′-tetra-acetic acid tetrakis(acetoxymethyl) ester and Cal – calcein O,O′-diacetate tetrakis(acetoxymethyl) ester were purchased by Sigma-Aldrich Co., Ltd. (USA). All other chemicals were supplied from Sigma-Aldrich Co.

Cell culture

L1210 and L1210/VCR cells were cultivated 3 days in RPMI 1640 medium supplemented by L-glutamine (1 mg/ml), 4% foetal bovine serum and gentamycin (1 μg/ml) in the humidified atmosphere by CO_2 to 5% at 37 ℃. Resistance of cells was tested by cultivation of cells in the presence of VCR or other cytostatics or by estimation of content of mRNA encoding Pgp (Fiala et al. 2003). After cultivation period cells were counted in haematocytometer and used for experiments with F-3 or Cal.

Calcein/AM assay

Cells (5×10^5) were washed two times in PBS containing 0.2% of bovine serum albumin adjusted on 200 μl in the same buffer and were incubated with 10 μmol/l Ver or 5 mmol/l Pro (disolved using dimethyl sulfoxide (DMSO) at final concentration of 0.5%) for 30 min at 37 ℃. Cal (200 pmol *per* sample) was added directly to the incubation medium after incubation period. After next 20 min lasting incubation at 37 ℃, cells were washed two times in PBS containing 0.2% BSA cooled to 4 ℃. Fluorescent measurements were made in the FACS Calibur fluorescent flow cytometer (Becton-Dickinson). The samples were measured using excitation at 488 nm. Emission at 520 nm was detected on FL1 channel for each 10^4 cells.

Fluo-3/AM assay

Cells (5×10^5) were incubated in 0.5 ml RPMI medium (without bovine foetal serum) in the humidified atmosphere with 5% CO_2 at 37 ℃ in the absence or presence of Ver (10 μmol/l), CsA (0.83 μmol/l) or Pro (2.5 mmol/l disolved using DMSO at a final concentration of 0.5%). Directly to this medium, 1.8 μmol/l F-3 and 0.02% pluronic acid F127 (to dissolve the F-3 and to prevent against aggregation; Drummond et al. 1987) were added and the mixtures were incubated for additional 30 min of the same condition. Samples were washed two times with isotonic MOPS saline medium at pH 7.0. Finally, samples were evaluated in the confocal microscope Leica TCS SP-2 AOBS using excitation at 488 nm. Emission at the range 493–620 nm was detected. Cells incubated without F-3 but with Ver, CsA or Pro were used in control experiments.

Results

Pgp was proved to be dominating in MDR phenotype of L1210/VCR cells (Boháčová et al. 2000). This drug pump prevents cells to be loaded by Cal (Fig. 1). In contrast, sensitive L1210 cells were extensively loaded by this fluorescent dye. Presence of Ver but not Pro induced loading of L1210/VCR cells by Cal to the similar extent as it was observed for sensitive L1210 cells (Fig. 1).

Sensitive cells were extensively stained also by fluo-3 (Fig. 2). Presence of CsA and Pro showed a trend for an increased staining of L1210 cells by fluo-3. Resistant L1210/VCR cells were unstained by this fluorescent dye (Fig. 3). Presence of Pro

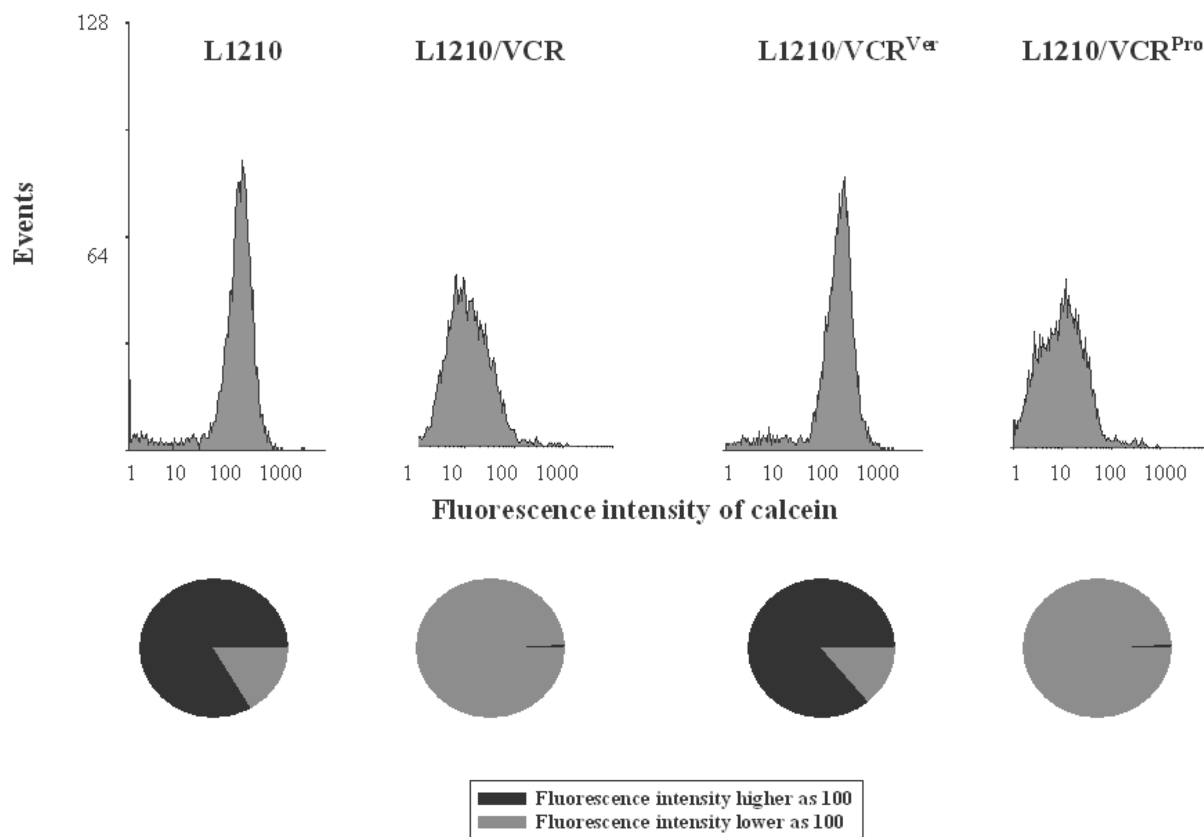

Figure 1. Representative FACS histograms of L1210 and L1210/VCR cells after loading with Cal superimposed according increased calcein fluorescence. Exponents Ver or Pro indicates that cells were loaded with Cal in the presence of Ver or Pro (for details see chapters Materials and Methods). The proportion of cells with fluorescence exceeding and non exceeding fluorescence relative intensity 100 were summarised on pie diagrams for the respective experimental groups. Similar results were obtained for cells from three independent cultivations.

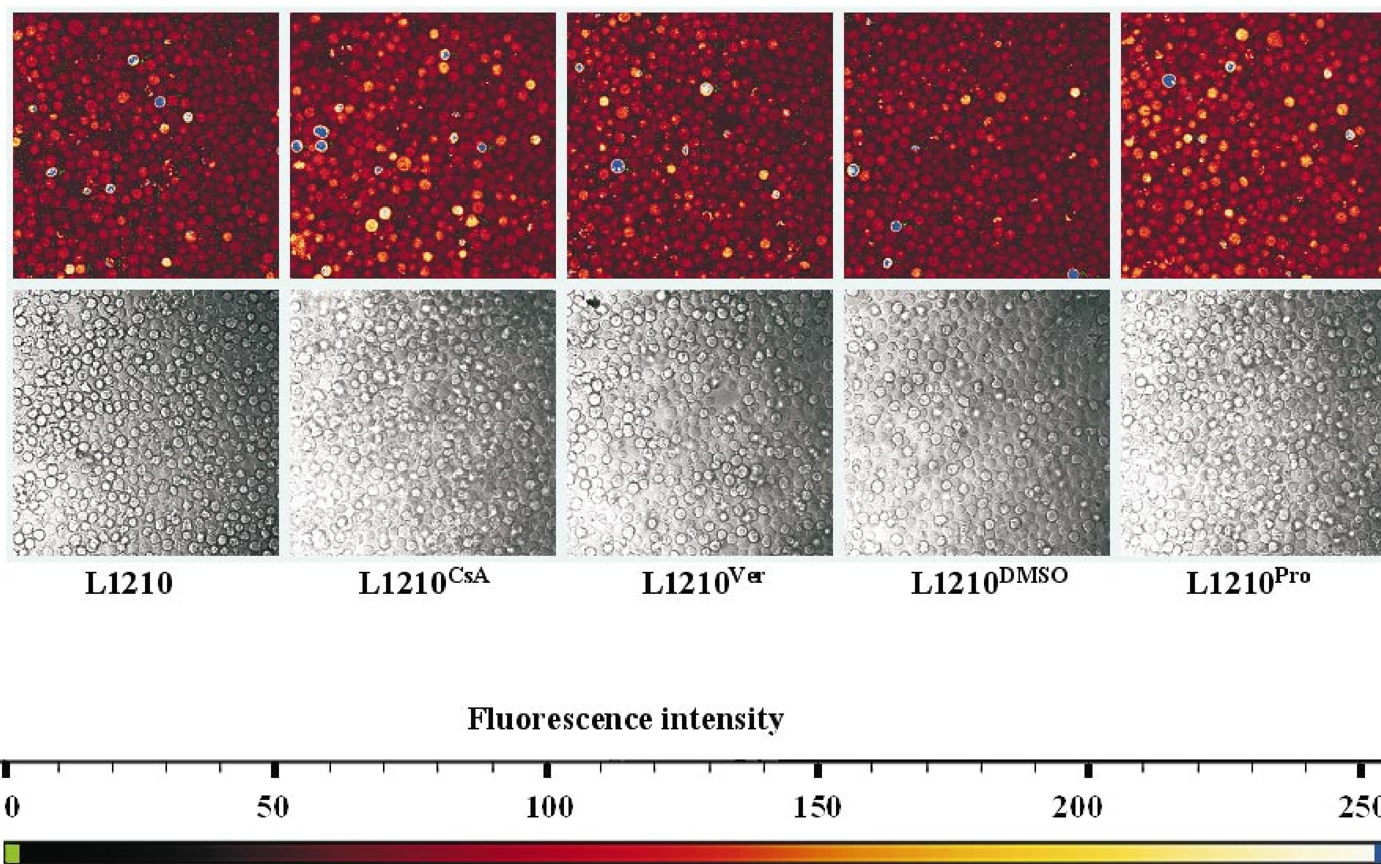

Figure 2. Confocal microscopy of L1210 cells after loading with F-3. Micrographs were obtained by registration of fluo-3 fluorescence (upper panel), the same samples registered using transmitted light (lower panel). Exponents CsA, Ver, DMSO or Pro indicates that cells were loaded with F-3 in the presence of CsA, Ver, DMSO or Pro (for details see chapters Materials and Methods).

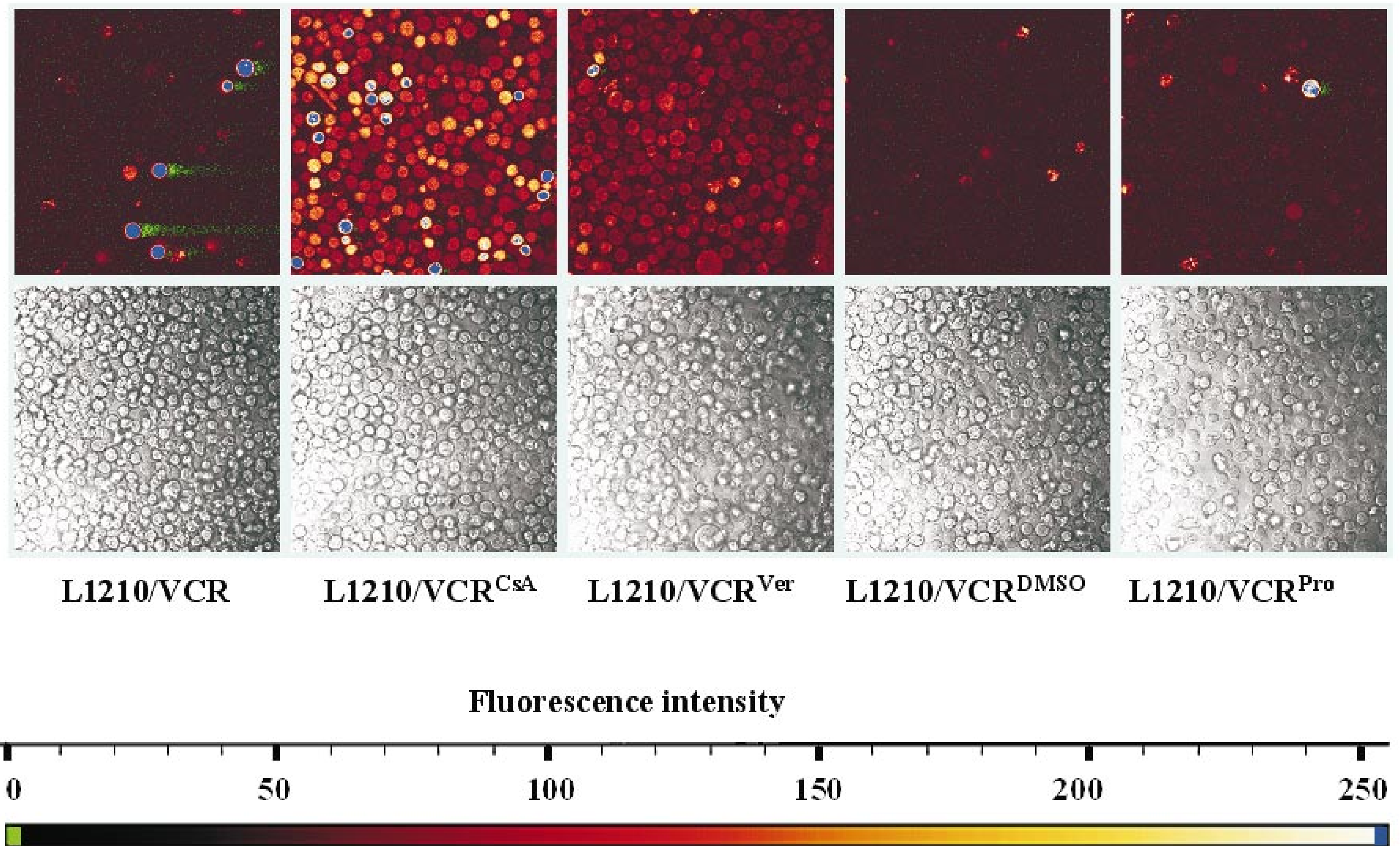

Figure 3. Confocal microscopy of L1210/VCR cells after loading with F-3. Description of figure is same as in Fig. 2.

was not effective and resistant cells remained unstained. In contrast, presence of Pgp antagonizing agents like CsA or Ver caused an extensive staining of L1210/VCR cells. In the presence of CsA, staining of L1210/VCR cells was more pronounced as in the presence of Ver (Fig 3).

Discussion

L1210/VCR cells represent the Pgp-mediated MDR cell model exerting high and stable expression of Pgp (Fiala et al. 2003). Pgp was described to be dominating system that secures MDR phenotype of the above cells (Boháčová et al. 2000). These facts agree with elevating effects of Pgp inhibitors (Ver and CsA; Silbermann et al. 1989; Lincke et al. 1990) on staining of resistant L1210/VCR cells by Cal or F-3. Ineffectivity of Pro, i.e., inhibitor of diverse group of anion transporters (Cunningham et al. 1981; Di Virgilio et al. 1988), on staining of cells by F-3 and Cal, indicates that these trasporters do not play a role in prevention of L1210/VCR cells to be stained by both fluorescent dyes. Cal as fluorescent substrate of Pgp has already been described (Eneroth et al. 2001; Karaszi et al. 2001). On the other hand, fluo-3 is known as a substrate of several anion transporters (Cantz et al. 2000; Prechtl et al. 2000; Abrahamse and Rechkemmer 2001; Cui et al. 2001). Demethylated stain, i.e., fluo-3, has to be a substrate for these anion transporters. In contrast, Pgp probably prefers methylated analogue F-3 because it is known as a transporter for hydrophobic neutral substances very often without charge (Sharom 1997; Ferté 2000). Demethylated fluo-3 is retained in cells also after wash out of Pgp inhibitors Ver and CsA, i.e., it is not effluxed by Pgp from the cells.

Fluo-3 fluorescence depends on concentration of free Ca^{2+} (Kao et al. 1989; Minta et al. 1989). Calcium entry blocker – Ver may induce a decrease of intracellular Ca^{2+} content in L1210/VCR cells that probably explains why lower level of cell staining was observed in the presence of Ver than in the presence of CsA.

Our data clearly show, that F-3 represents a substrate of Pgp in L1210/VCR cells. Thus, this substance, similarly as Cal, may be used for measurement of Pgp transport activity.

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