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Biochimica et Biophysica Acta 1191 (1994) 384–388

BBA Biochimica et Biophysica Acta

Rapid Report

Calcein accumulation as a fluorometric functional assay of the multidrug transporter

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(Received 25 January 1994)

Abstract

Acetoxymethyl ester (AM) derivatives of various fluorescent indicators (fura-2, fluo-3, indo-1, BCECF, calcein) are actively extruded by the multidrug transporter (MDR1, P-glycoprotein – Homolya, L. et al. (1993) J. Biol. Chem. 268, 21493–21496). In the present paper we show that the measurement of the accumulation of a fluorescent cell viability marker, calcein, can be effectively used as a rapid and sensitive fluorometric and flow cytometric assay for studying P-glycoprotein function. The rate of calcein accumulation in human MDR1-expressing cells is significantly lower than in the control cells, while various drug-resistance reversing agents (verapamil, vinblastine, oligomycin, cyclosporin A and UIC2 monoclonal antibody) greatly increase calcein trapping only in the MDR1-expressing cells. Since calcein-AM is not fluorescent and free calcein is not a substrate of the multidrug transporter, the assay is readily applicable for rapid kinetic studies of the MDR1 function. Calcein has a high fluorescence intensity in the visible range, thus changes in calcein uptake can be easily visualised and MDR1-expressing and control cells separated by conventional flow cytometry.

Key words: MDR1; P-glycoprotein; Calcein; Fluorescent indicator; Flow cytometry; Fluorometry; Functional assay

Ineffectivity of tumor chemotherapy is often caused by the resistance of malignant cells to a wide range of hydrophobic cytostatic agents, including Vinca alkaloids, anthracyclines, epipodophyllotoxins, antibiotics like actinomycin D, and other chemically unrelated compounds of natural origin. The main characteristic of these multidrug-resistant cells is an energy dependent outward transport of drugs produced by a membrane glycoprotein, identified as P-170 or P-glycoprotein (MDR1, multidrug transporter). Another key feature is the potential reversibility of multidrug resistance by a great variety of agents, like verapamil, quinidine, calmodulin inhibitors, phenothiazines, reserpine, or cyclosporin A [2,3]

In order to assess the presence and functioning of the MDR1 protein in various normal and tumor cells, several methods have been established. The mdr1 mRNA is detected by RNA slot blot, RNAse protection assay, in situ hybridization, Northern blot, or reverse transcription-PCR. The expressed P-glycoprotein can be visualised with Western blotting, immunocytochemistry, or immunofluorescence labelling, by using various monoclonal and polyclonal antibodies. The functional demonstration of the presence of MDR1 requires in vitro cytotoxicity assays or, alternatively, the measuring of the extrusion of labelled or fluorescent drugs, or drug-mimicking molecules [4]. It has been shown that MDR1-expressing cells show a decreased uptake of certain fluorescent anthracyclines [5], as well as fluorescent dyes as Rhodamine 123 [6–8], or fluo-3 [9,10], and these compounds have been used to discriminate between drug-resistant and sensitive cells. A functional flow cytometric analysis is often required because of the non-uniform distribution of MDR1-expressing cells and different patterns of MDR1 expression depending on the type of tumor and stages of treatment regimen.

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Abbreviations: AM, acetoxymethyl ester; BCECF, 2′,7′-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; Mab, monoclonal antibody; MDR1, human multidrug-resistance protein; PBS, phosphate-buffered saline.

SSDI 0005-2736(94)00055-T

Various fluorescent dyes, like fura-2, fluo-3, indo-1, quin-2 and BCECF are widely used in biochemical studies focusing on changes in intracellular ionic composition and membrane characteristics during cell activation. Recently we have shown [1] that the hydrophobic, cell-permeant AM derivatives of these probes are actively extruded by the multidrug transporter, in contrast to the hydrophilic free acid forms produced by intracellular esterase cleavage. The significantly different rates of dye uptake by control and MDR1 cells and the excellent MDR1-ATPase activating properties of these probes suggested the possible development of a sensitive fluorescent approach for the functional characterisation and detection of P-glycoprotein in cellular systems. In the present report we demonstrate that calcein-AM, which is widely used in cell viability and proliferation assays, is a highly advantageous probe for a functional detection of the multidrug transporter.

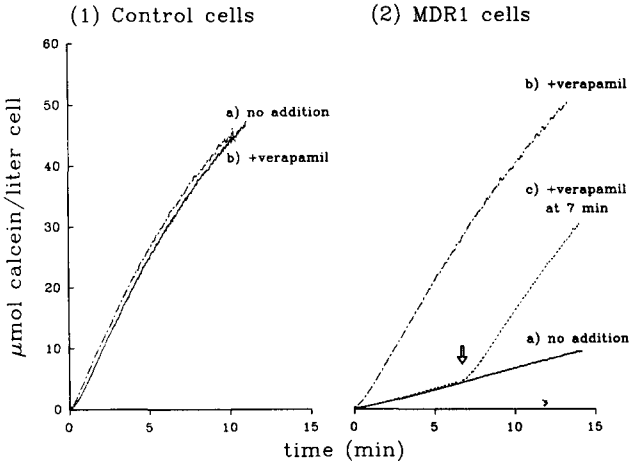

Fig. 1. Time-course of calcein accumulation in control and human MDR1-transfected NIH 3T3 fibroblasts. (Panel 1) Control NIH 3T3 cells were incubated with 0.25 μM calcein-AM in HPMI at 37°C. Curve a, no verapamil addition; Curve b, verapamil (40 μM) preincubation for 5 min before the loading period. (Panel 2) Curves a and b: MDR1-expressing cells incubated under identical conditions to curves a and b of Panel 1. Curve c, verapamil (40 μM) added at the time indicated by the arrow. NIH 3T3 fibroblasts were cultured as in Ref. [1]; human MDR1-transfected cells (NIH 3T3 MDR1 G185) were prepared and characterised for their drug-resistance as described previously [19]. Before each experiment the cells were trypsinised, then washed and stored in Dulbecco's modified Eagle's Medium (DMEM) at 25°C. Dye uptake was measured by incubating $2 \cdot 10^6$ cells/ml in HPMI medium [1] containing 0.25 μM calcein-AM (Molecular Probes, Eugene, OR, USA). Fluorescence was measured at 37°C with rapid stirring in a Hitachi F-4000 fluorescence spectrophotometer (excitation and emission wavelengths for calcein were 493 and 515 nm, respectively, with a band width of 5 nm). Calibration of dye concentration was based on the measurements of free calcein fluorescence in the same instrument under identical conditions. All experiments were repeated at least three times with different batches of cell preparations and representative data are shown. The mean volume of control and MDR1-expressing NIH 3T3 cells was estimated to be 1000 fl/cell, as determined by a Coulter Channeliser.

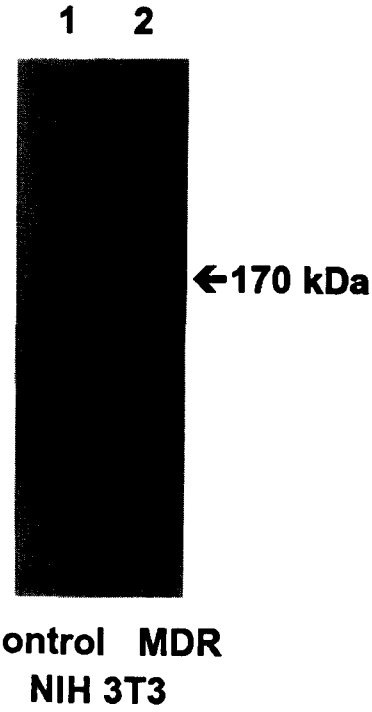

Fig. 2. Detection of P-glycoprotein by immunoblotting of the isolated NIH 3T3 cell membrane proteins with the 4077 polyclonal antibody specific to human MDR1. Lane 1, control cells; Lane 2, MDR1-transfected NIH 3T3 cells. Both lanes contained 10 μg membrane protein. Electrophoresis and immunoblotting with the 4077 polyclonal antibody [20] were carried out as described in [15].

Calcein acetoxymethyl ester (AM) is highly lipid soluble, rapidly penetrates the plasma membrane of cells, and is practically non-fluorescent. By cleavage of the ester bonds, intracellular esterases transform the dye to a hydrophilic and intensively fluorescent free acid form. We have shown previously [1] that calcein-AM (but not free calcein) is an excellent activator of the MDR1-ATPase in isolated membranes ($K_a \leq 1$ μM) and that calcein accumulation is prevented in MDR1-expressing cells. Calcein is a fluorescein derivative with a high molar emission coefficient (about 7.5-

Table 1
Effects of various inhibitors of *P*-glycoprotein on calcein accumulation in NIH 3T3 fibroblasts

Treatment	Calcein accumulation (μmol/min per liter of cells)	
	control cells	MDR1 cells
None	3.56 ± 0.51	0.63 ± 0.26
Verapamil (40 μM)	3.53 ± 0.70	3.8 ± 0.79
Vinblastine (50 μM)	3.6 ± 0.63	4.03 ± 1.10
Oligomycin (30 μM)	2.46 ± 0.46	2.83 ± 0.11
UIC2 (20 μg/ml)	3.55 ± 0.55	1.99 ± 0.35
Cyclosporin A (5 μM)	3.54 ± 0.42	3.84 ± 0.61

For experimental conditions see the legend to Fig. 1. Calcein accumulation rate was expressed as μmol free calcein/min per liter of cells, mean ± S.E. ($n = 3$).

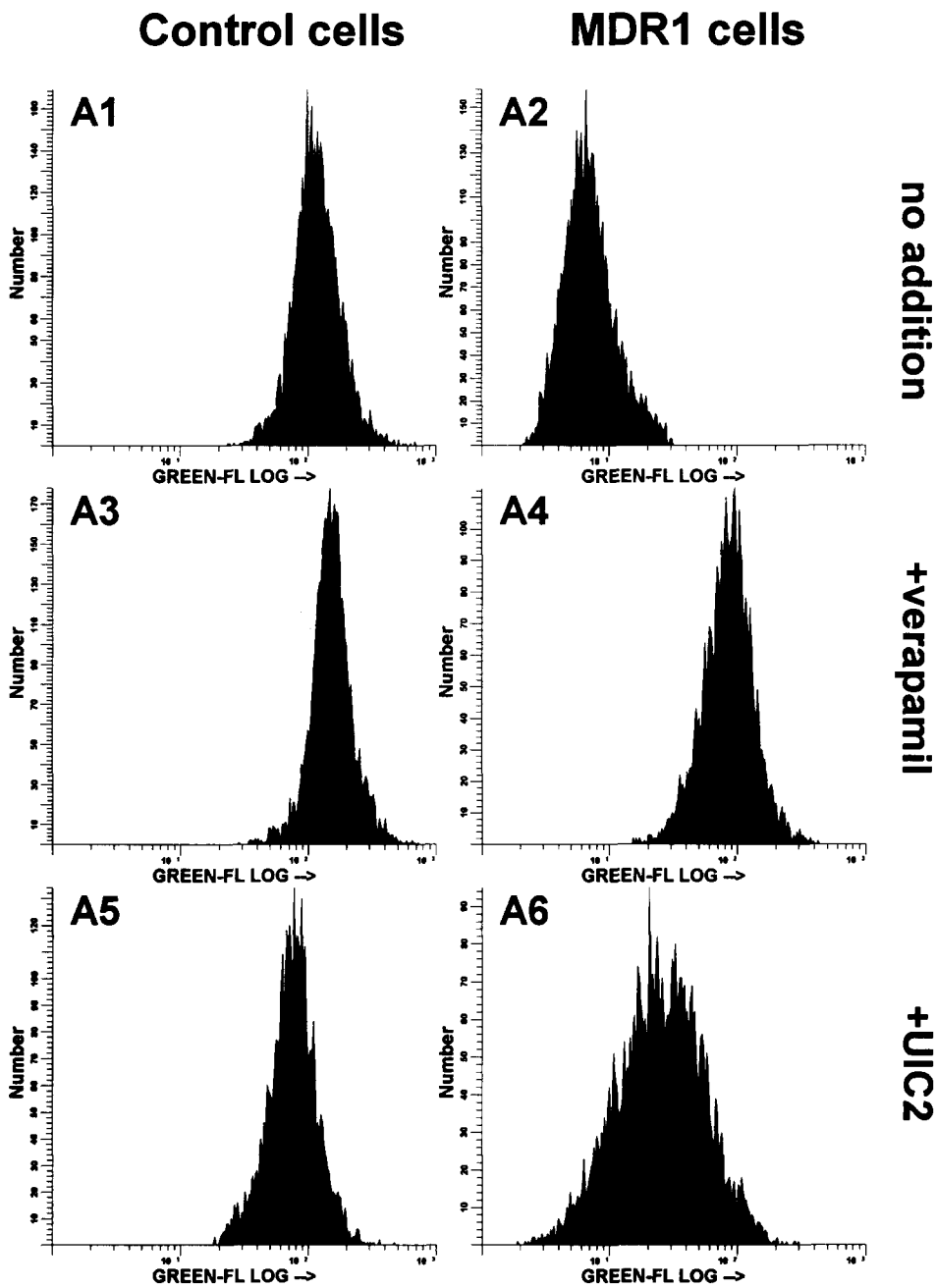

Fig. 3A.

fold higher than that of fura-2), with no apparent cytotoxicity. It is well retained by the cells ($T_{\frac{1}{2}}$ of calcein leakage is about 3 h at 37°C), with a fluorescence essentially insensitive to changes in pH, as well as Ca^{2+} or Mg^{2+} [11]. The fluorescence excitation and emission maxima of calcein are 496 and 517 nm, respectively, making this dye suitable for both conventional fluorescence/flow cytometric and laser-scanning microscopic applications.

In the experiments in Fig. 1 we measured calcein accumulation in a spectrofluorometer, by using control NIH 3T3 fibroblasts and the same cell line stably expressing the human MDR1. When the cells were incubated in the presence of calcein-AM, MDR1-expressing fibroblasts showed an about 6–8-times lower rate of calcein accumulation than the control cells. Preincubation of the cells with verapamil, an effective MDR1-reversing agent [12], restored the rate of dye accumulation in the MDR1-cells almost to the control level (Panel 2, Curve B), and the addition of verapamil during dye loading (Panel 2, Curve C), produced a similar increase in dye accumulation. In contrast, verapamil had no effect on calcein accumulation in the control NIH 3T3 cells (Panel 1, Curve B). After wash-

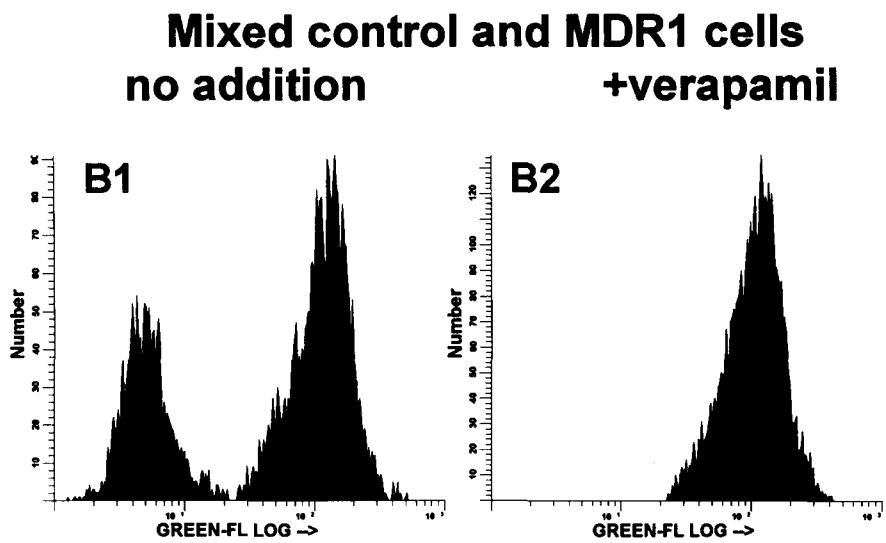

Fig. 3. Flow cytometric detection of P-glycoprotein function by calcein accumulation. (Panel A) Representative data of calcein loading experiments in control (A1, 3, 5) and MDR1-transfected (A2, 4, 6) cells. After a preincubation period (at 25°C) with the inhibitors (A1, A2: no addition; A3, A4: verapamil 40 μM for 6 min; A5, A6: UIC2 Mab, 10 μg/ml for 30 min) 10^5 cells/ml were further incubated in the calcein-AM (0.25 μM) containing medium at 37°C for 15 min. Green fluorescence intensity was measured and data are shown as number of cells plotted against log fluorescence intensity. (Panel B) Accumulation of calcein in a mixed population of control and MDR1-transfected cells in the absence (B1) and presence (B2) of verapamil. Loading conditions were identical as indicated at Panel A. Cellular fluorescence was measured with a Cytoronabsolute (Ortho, Ortho Diagnostics Systems, NJ, USA) flow cytometer. Non-living cells were detected and gated out by propidium iodide staining. Data were analysed by the Winlist software (Verity Software House).

ing and resuspending the cells in dye-free medium, no dye was observed to leak into the medium over a period of 180 min from either control or MDR1 cells. Addition of verapamil had no effect on dye retention of either cell type (data not shown). Calcein trapping properties are not influenced by changes in extracellular pH between 7.0 and 7.8 (data not shown). Taken together with our earlier data, indicating no interaction of free acid dyes with the multidrug transporter [1], these experiments suggest that free calcein is well retained by the cells and is not exported by the multidrug transporter.

Fig. 2 shows an immunoblot of the cell membrane proteins with the 4077 polyclonal antibody raised against human MDR1. These experiments clearly indicate the presence of large amounts of the 170 kDa P-glycoprotein in MDR1-transfected fibroblasts (lane 2), while no labelling can be observed in the control cells (lane 1).

In order to examine the effects of various inhibitors and substrates of the multidrug transporter, we compared the effects of verapamil, vinblastine, oligomycin, cyclosporin A, and an inhibitory monoclonal antibody, UIC2, on calcein accumulation in control and MDR1-expressing NIH 3T3 cells (Table I). As shown earlier (Fig. 1), verapamil increased calcein accumulation in the MDR1 fibroblasts without any effect on the control cells. Vinblastine, a well-known drug substrate of P-glycoprotein [13], increased dye trapping in the MDR1 fibroblasts, probably by competing on the MDR1 transporter, while no significant effect could be observed in the control cells. Cyclosporin A, an effective drug-resistance reversing agent [14] and oligomycin, a potent inhibitor of P-glycoprotein mediated drug transport [13] and MDR1-ATPase activity [15,16], also restored dye accumulation almost to the control level, although the latter slightly inhibited calcein accumulation in the control fibroblasts as well. The UIC2 monoclonal antibody, which recognises extracellular epitopes of P-glycoprotein and inhibits drug transport [17] increased calcein accumulation by almost 50% in the MDR1 fibroblasts, without any effect on the control cells.

All these data clearly indicate that measurement of calcein accumulation may be used to test for the functional presence of the multidrug transporter. The low environmental sensitivity, the intensive fluorescence of the intracellularly formed and trapped calcein and the continuous accumulation of this relatively inert dye make these investigations relatively simple and efficient. In addition, dye accumulation is suitable for studies of the kinetics of the multidrug transporter.

Previous fluorescence dye assays applied for studying MDR1 function had serious drawbacks. The mitochondrial dye, Rhodamine 123, which is exported by the multidrug transporter and which thereby has reduced uptake in MDR1-expressing cells [6–8], easily leaks out of the cells, accumulates in hydrophobic compartments and/or intracellular organelles [18], and shows a spectral shift and fluorescence intensity change inside cells due to its interaction with various cellular

components. The detection of a lower steady-state level of the fluorescent anthracycline, daunorubicin in drug-resistant cells [5] has similar problems of quantitation, because daunorubicin fluorescence is quenched upon binding to DNA [4]. The accumulation of fluo-3, a cytoplasmic calcium indicator, was found to be faster and an almost 7-times more sensitive measure of MDR1 function than the doxorubicin assay [9,10]. Although fluo-3 AM is indeed a good activator of MDR1-ATPase activity, and actively extruded by the multidrug transporter [1], the intracellularly formed free fluo-3 has a low fluorescence unless bound to calcium (K_d = 0.37–2.3 μM) and emission intensity increases more than 80-fold upon calcium binding [11]. Thus any change in cytosolic calcium levels (caused, e.g., by various calcium channel and calmodulin inhibitors, interacting also with MDR1) and free acid dye leakage, which may be significant in washed cells, greatly affect fluo-3 fluorescence in such in vitro studies.

In the following experiments flow cytometric measurements were carried out to determine whether the kinetic differences in calcein accumulation seen in the spectrofluorometric experiments allow a discrimination between MDR1 and control cell populations under quasi-steady-state loading conditions. Fig. 3, Panel A demonstrates that after a 15 min incubation of the cells with calcein-AM, P-glycoprotein expressing NIH 3T3 cells accumulated much smaller amounts of calcein than the control cells. Pretreatment with verapamil or the UIC2 inhibitory antibody resulted in an increased calcein accumulation in the MDR fibroblast population, without having any significant effect on the dye accumulation in the control cells (Fig. 3, Panel A). MDR1 expressing and drug-sensitive cell-mixing experiments showed a clear distinction between the two cell populations on the basis of their calcein fluorescence, while the addition of verapamil caused a significant fluorescence shift to high intensity levels, affecting only the MDR1 cell population (Fig. 3, Panel B).

These data clearly show that calcein accumulation is a fast and effective tool for measuring P-glycoprotein function both at single cell and cell population levels. In flow cytometry, immunofluorescence studies may be combined with kinetic data for calcein accumulation, which offers a unique possibility of simultaneous determination of P-glycoprotein expression and function.

This work was supported by research grants from OTKA (I/3-195) and OMFB (Mecenatura), Hungary. We would like to thank Dr. G. Gárdos (NIHBT, Budapest) and Dr. R.C. Boucher (UNC, Chapel Hill) for their valuable advice, R. Mihalik and Dr. M. Benczúr for their help and advice in flow cytometric measurements. We are grateful to M.M. Gottesman (NCI, NIH) for providing us the NIH 3T3 MDR1 G185 cell line. The UIC2 monoclonal antibody was a generous gift of Dr. I. Roninson and Dr. E.B. Mechetner. The technical help of Andrea Siposs is gratefully acknowledged.

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