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Original Paper

Flow Cytometric Functional Analysis of Multidrug Resistance by Fluo-3: a Comparison With Rhodamine-123

S. Koizumi, M. Konishi, T. Ichihara, H. Wada, H. Matsukawa, K. Goi and S. Mizutani

Using four cell lines including drug-sensitive K562/Parent cells, P-glycoprotein (Pgp)-mediated multidrug resistant (MDR) K562/VCR, K562/ADR and revertant K562/ADR-R cells, two fluorescent agents, Fluo-3 and rhodamine-123 (Rh-123), were compared as indicators in a functional assay of MDR. Cells were incubated with 4 μM Fluo-3 or 1 μM Rh-123 for 45 min and then the intracellular accumulation of the agent was measured using a flow cytometer. Verapamil (20 μM) or cepharanthine (biscoclaurine alkaloid, 10 μM) was added just before the fluorescent agents. Efflux patterns were also studied 60 min after incubation with or without verapamil and cepharanthine. Increased intracellular accumulation and a delayed efflux pattern of Fluo-3 by verapamil and cepharanthine were demonstrated in multidrug resistant K562/VCR and K562/ADR cells, indicating that Fluo-3 is another good indicator of MDR. However, a similar, but lower, increase in uptake and a delayed efflux pattern of Fluo-3 by verapamil and cepharanthine were also demonstrated even in Pgp-non-overexpressed K562/Parent cells. In contrast, accumulation of Rh-123 was not affected by verapamil and cepharanthine. To further study the Pgp dependency of Fluo-3, another cell line, K562/NC16 expressing minimum *MDR1* mRNA, was cloned. Increased uptake and a delayed efflux pattern of Fluo-3, but not Rh-123, with verapamil or cepharanthine were again demonstrated in K562/NC16 cells, indicating that intracellular accumulation of Fluo-3 may be non-specifically influenced by verapamil and cepharanthine at very low levels of Pgp-related MDR, while the influx and efflux patterns of Rh-123 may be specifically affected by Pgp overexpression.

Key words: multidrug resistance, P-glycoprotein, rhodamine-123, Fluo-3, K562 cells
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INTRODUCTION

CYTOTOXIC DRUG RESISTANCE is a common cause of failure of chemotherapy in various malignancies. The phenomenon of multidrug resistance (MDR), in which drug resistance to a variety of apparently unrelated cytotoxic agents can be induced *in vitro* following prolonged exposure to a single agent, readily develops in experimental tumours and there is evidence to indicate its role in certain human neoplasms including acute leukaemia, lymphoma and neuroblastoma [1, 2]. MDR is associated with overexpression of a cellular membrane 170 kDa glycoprotein termed P-glycoprotein (Pgp) which is the product of the human *MDR1* gene [3–6]. Pgp is believed to act as an energy-dependent drug efflux pump which causes a decrease in cytotoxic drug accumulation [3, 4, 7, 8]. Based on this functional capacity of Pgp, intracellular accumulation of fluorescent probes, including doxorubicin [9, 10] and Hoechst 33342 [11], has been studied as an indicator of MDR. Rhodamine 123 (Rh-123) has been observed to accumulate in the mitochondria of cells and has been investigated as a standard functional indicator of MDR [12, 13]. Furthermore, several new fluorescent indicators have been synthesised [14] and, among them, Fluo-3 has been reported to accumulate rapidly in drug-sensitive cell lines but not in MDR cells [15].

MDR mediated by Pgp can be reversed by verapamil, a calcium channel blocker [16, 17]. Cepharanthine, a bisbenzylisoquinoline (biscoclaurine) alkaloid, which is extracted from a menispermaceous plant, *Stephania cepharantha* Hayata, has been reported to reverse MDR by the same mechanism as that of

Correspondence to S. Koizumi at the Department of Paediatrics, Kanazawa University School of Medicine, 13-1 Takaramachi, Kanazawa 920, Japan.
S. Koizumi, T. Ichihara, M. Konishi, H. Wada and H. Matsukawa are at the Department of Paediatrics, Kanazawa University School of Medicine, 13–1 Takaramachi, Kanazawa 920; K. Goi and S. Mizutani are at the Department of Virology, The National Children's Medical Research Center, 3-35-31 Taishido, Setagaya, Tokyo 154, Japan.
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verapamil [18–20]. In this paper we investigated the ability of a flow cytometric technique using a new fluorescent agent, Fluo-3, combined with verapamil and cepharanthine to detect MDR, and compared the results with those for Rh-123. The results showed that there were significant differences in Fluo-3 accumulation beween the drug-sensitive K562 cell lines and the drug-resistant variants, and that intracellular accumulation of Fluo-3 at low levels of MDR may be non-specifically affected by MDR-reverting agents, while uptake and efflux patterns of Rh-123 may be strongly dependent on Pgp overexpression.

MATERIALS AND METHODS

Reagents

Vincristine (VCR) and doxorubicin (ADR) were obtained from Shionogi Pharmaceutical Co., Ltd (Osaka, Japan) and Kyowa Hakko Co., Ltd (Tokyo, Japan), respectively. Fluo-3 acetoxymethyl ester dissolved in dimethyl sulphoxide (Fluo-3/AM) was purchased from Dojin Biochemical Co., Ltd (Tokyo, Japan). Verapamil was provided by Eisai Pharmaceutical Co., Ltd (Tokyo, Japan) and cepharanthine, a bisbenzylisoquinoline (biscoclaurine) alkaloid, was purchased from Wako Biochemical Co., Ltd (Tokyo, Japan). A monoclonal antibody, MRK16 (IgG2a), which recognises an epitope of the extracellular domain of Pgp, was a gift from Dr. T. Tsuruo (University of Tokyo, Japan).

Cells

The drug-sensitive human erythroleukaemic K562 cell line was purchased from the ATCC (American *Type Culture* Collection; Rockville, Maryland, U.S.A.). The multidrug-resistant K562/VCR cell line was established in our laboratory by exposure of the parent cells to gradually increasing concentrations of VCR. The multidrug-resistant K562/ADR cell line, which was originally established by exposure of cells to ADR, was provided by T. Tsuruo (University of Tokyo, Japan). K562/NC16 cells expressing minimum *MDR1* mRNA were cloned from the K562 parent cells using a limiting dilution method. These cells were maintained in RPMI 1640 medium (Gibco, Grand Island Biological Co., New York, U.S.A.) supplemented with 5% fetal calf serum (FCS, Flow, McLean, Virginia, U.S.A.), 2 mM glutamine, 100 units/ml penicillin, 5 μg/ml gentamicin and the drugs at appropriate concentrations at 37°C in a humidified atmosphere of 5% CO_2 in air.

Clonogenic assay and cytotoxicity study

After cells were incubated in complete medium without drugs for 1 week, the parent K562 cells and the multidrug-resistant K562/VCR and K562/ADR cells (10^3 cells) were plated in 1 ml of RPMI 1640 with 5% FCS containing increasing concentrations of VCR (10^{-10} to 10^{-5} M) or ADR (10^{-10} to 10^{-5} M). After overnight (approximately 18 h) incubation in a humidified atmosphere of 5% CO_2 in air at 37°C, cells were centrifuged and the supernatant was discarded. Cells were then replated in 0.8% methylcellulose (Fisher, Fair Lawn, New Jersey, U.S.A.) to prepare a clonogenic assay as described previously [21]. After additional incubation for 6 days, colonies were counted using an inverted microscope (Olympus, Tokyo, Japan). To study the effects of MDR-reversing agents, verapamil (2 and 10 μM) or cepharanthine (1 and 2 μM), cells were pre-incubated overnight with each agent and then prepared for the clonogenic assay without removing each agent. The agents alone at these concentrations had neither cytotoxic nor growth inhibitory actions against cells.

Measurement of intracellular Fluo-3 and Rh-123 accumulation by flow cytometry

Prewarmed cells (10^6/0.5 ml) were incubated with 4 μM Fluo-3/AM or 1 μM Rh-123 for 45 min. Fluorescence was measured using a Cytoron Absolute flow cytometer (Ortho Diagnostic Systems, Raritan, New Jersey, U.S.A.) using a green filter. From our preliminary studies, saturated levels of concentrations of these fluorescent probes were used in this study. For a quick examination of the effects of MDR-reversing agents, higher concentrations of verapamil (20 μM) or cepharanthine (10 μM) were added just before the addition of fluorescent agents. For the study of efflux patterns of Fluo-3 and Rh-123, cells were incubated with Fluo-3 or Rh-123 combined with verapamil or cepharanthine for 45 min, washed with cold RPMI 1640 medium, and incubated for an additional 60 min with or without verapamil and cepharanthine. The MDR-reversing agents alone under these concentrations showed no cytotoxicity nor growth inhibition of cells. Effects of verapamil and cepharanthine on intracellular accumulation of Fluo-3 and Rh-123 were expressed as an "index" which was calculated as follows:

$$\text{Index} = \frac{\text{The mean channel of fluorescence intensity of the cells incubated with verapamil or cepharanthine}}{\text{The mean channel of fluorescence intensity of the cells incubated without verapamil or cepharanthine}}$$

Immunofluorescence assay of Pgp expression

To determine whether Fluo-3 accumulation was associated with Pgp expression, cell surface expression of Pgp was analysed with a monoclonal antibody, MRK16, using a standard indirect immunofluorescence assay. Briefly, 10^6 cells were incubated with MRK16 (5 μg/ml) at 4°C for 30 min. After washing twice with phosphate-buffered saline (PBS, pH 7.4) containing 1% FCS, cells were incubated with fluorescein-conjugated goat anti-mouse immunoglobulin (Becton Dickinson, Mountain View, California, U.S.A.) for another 30 min at 4°C, washed again, and analysed on a flow cytometer (Cytoron Absolute). A non-immune mouse IgG2a fraction (Becton Dickinson) was substituted for the primary antibody as a negative control.

Expression of MDR1 mRNA by reverse transcriptase–polymerase chain reaction (RT–PCR)

Total cellular RNA was prepared from each cell line by the acid guanidinium thiocyanate–phenol–chloroform extraction method [22]. cDNA was synthesised with 5 μg of total cellular RNA and 100 ng of random hexadeoxynucleotide primer (Pharmacia, Uppsala, Sweden) and RAV-2 reverse transcriptase (Takara, Tokyo, Japan) at 43°C for 1 h. PCR was carried out with cDNA derived from 1 μg of RNA, 1 unit of Amplitaq polymerase and reaction kits (Takara) in a final volume of 25 μl. Each PCR cycle comprised 30 s of denaturation at 94°C, 1 min of primer annealing at 55°C and 2 min of extension/synthesis at 72°C. *MDR1*-specific sequences were amplified using the sense strand primer 5′ CCCATCATTGCAATAGCAGG 3′, (residues 2596–2615) and the antisense-strand primer 5′ GTTTCAAACTTCTGCTCCTGA 3′ (residues 2733–2752) which yielded a 167 bp product [23]. As an internal control, the primers for the phosphoglycerate kinase (PGK) -specific sequences were 5′ CAGGGGTCCTAGGCTTGGAG 3′ (sense strand, residues 708–727) and 5′ GGATCCATCCCCG AAGGTGA 3′ (antisense strand, residues 793–812) which

Table 1. Multidrug resistance of the K562 cell subclones

Cell lines	IC_{50} VCR (μM)	IC_{50} ADR (μM)
K562/Parent	0.0014 (1)±0.0005	0.028 (1)±0.011
K562/VCR	0.84 (600)±0.25	1.47 (53)±0.65
K562/ADR	0.54 (380)±0.15	1.85 (66)±0.65
K562/ADR-R*	0.0013 (0.9)±0.0005	0.025 (0.9)±0.010
K562/NC16	0.0015 (1.1)±0.0005	0.034 (1.2)±0.010

Vincristine (VCR); doxorubicin (ADR). Each value represents mean ± S.D. of at least three separate experiments. Values in parentheses indicate the relative resistance index compared with K562 parent cells. *ADR-R, revertant cell line.

yielded a 105 bp product. PCR was carried out in a DNA thermal cycler (Perkin-Elmer/Cetus, Norwalk, Connecticut, U.S.A.) for 25 cycles. PCR products were separated on 2% agarose gels and were transferred to a nylon membrane for hybridisation. The specific bands of PCR products were hybridised with ^{32}P-labelled internal probes, 5′ GGAAGATCGCTACTGAAGCA 3′ for *MDR1* and 5′ GGAATTTCTAGCCGCATTTTCCC for PGK oligonucleotide, and visualised on autoradiograms.

RESULTS

Establishment of MDR cell lines of K562 cells

Table 1 shows the characteristics of the MDR K562/VCR and K562/ADR cell lines. Based on the IC_{50} values from our clonogenic assays, the K562/VCR cells showed approximately 600-fold resistance to VCR and the K562/ADR cells demonstrated 66-fold resistance to ADR compared with the parent cells. The degrees of resistance of the K562/VCR cells to ADR and the K562/ADR cells to VCR were also high (53-fold and 380-fold, respectively).

Reversal effects of verapamil and cepharanthine on MDR

As shown in Table 2, Pgp inhibitors, verapamil and cepharanthine, clearly reversed the drug resistance of these two MDR K562/VCR and K562/ADR cell lines in a dose-dependent manner as demonstrated in *in vitro* growth experiments.

Pgp expression and Fluo-3 accumulation

Figure 1 shows the correlation between Pgp expression and intracellular accumulation of Fluo-3 with or without verapamil. Overexpression of Pgp using the MRK16 monoclonal antibody in the drug-resistant K562/VCR and K562/ADR cells was demonstrated. Overexpression of Pgp in the K562/VCR and K562/ADR cells, but not in other cells, was confirmed by Western blotting (data not shown). An obvious increase in Fluo-3 accumulation was observed in the drug-sensitive K562 parent cells (Figure 1). Fluo-3 accumulation in both the drug-resistant K562/ADR and K562/VCR cell lines was markedly lower than that in the parent cell line. Revertant cells from K562/ADR after incubation without ADR showed almost complete reversal in Fluo-3 accumulation which strongly correlated with a reduction of Pgp expression. Verapamil caused a remarkable increase in Fluo-3 accumulation in the drug-resistant K562/VCR and K562/ADR cells. The drug-sensitive K562 parent and revertant cells also showed a small, but not significant, effect of verapamil on Fluo-3 accumulation. These results were essentially the same as those for Rh-123 and for cepharanthine (data not shown), suggesting that Fluo-3 may provide another sensitive and functional way to monitor MDR.

Expression of MDR1 mRNA

Figure 2 shows that *MDR1* mRNA was overexpressed in the MDR K562/ADR and K562/VCR cells and that, even in the

Table 2. Reversal effects of verapamil and cepharanthine on in vitro growth of multidrug-resistant K562/VCR and K562/ADR cells

Agents	IC_{50} for vincristine (VCR)			IC_{50} for doxorubicin (ADR)		
	K562/Parent (μM)	K562/VCR (μM)	K562/ADR (μM)	K562/Parent (μM)	K562/ADR (μM)	K562/VCR (μM)
None	0.0014 (1)±0.0005	0.84 (600)±0.25	0.54 (380)±0.15	0.028 (1)±0.011	1.85 (66)±0.65	1.47 (53)±0.07
Verapamil						
2 μM	0.00054 (1)±0.00036	0.12 (220)±0.03	0.056 (100)±0.020	0.017 (1)±0.002	0.28 (16)±0.06	0.18 (11)±0.05
10 μM	0.00033 (1)±0.00032	0.013 (39)±0.001	0.0088 (27)±0.0029	0.012 (1)±0.005	0.11 (9.2)±0.04	0.43 (3.6)±0.031
Cepharanthine						
1 μM	0.0013 (1)±0.0001	0.054 (42)±0.035	0.053 (41)±0.008	0.021 (1)±0.002	0.18 (8.6)±0.03	0.12 (5.7)±0.04
2 μM	0.0012 (1)±0.0001	0.020 (17)±0.007	0.021 (18)±0.004	0.022 (1)±0.011	0.12 (5.5)±0.06	0.069 (3.1)±0.035

Vincristine (VCR); doxorubicin (ADR). Each value represents mean ± S.D. of at least three separate experiments. Values in parentheses indicate the relative resistance index compared with K562 parent cells.

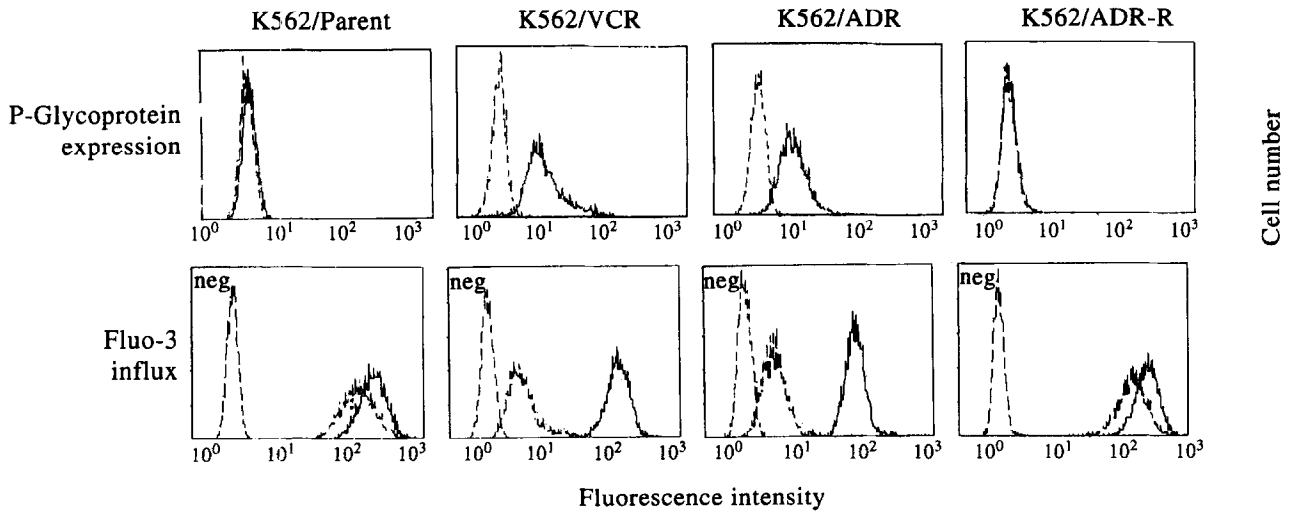

Figure 1. P-glycoprotein (P-gp) expression and influx patterns of Fluo-3 of drug-sensitive K562/Parent cells, multidrug-resistant K562/ADR and K562/VCR cells, and revertant K562/ADR-R cells. (a) P-gp expression was examined in a standard indirect immunofluorescence assay using the MRK16 monoclonal antibody (solid line). A non-immune mouse IgG2a fraction was used as a negative control (broken line). (b) Influx patterns of Fluo-3 were obtained after the cells were incubated with Fluo-3 combined with (solid line) or without verapamil (broken line). neg, negative controls without Fluo-3.

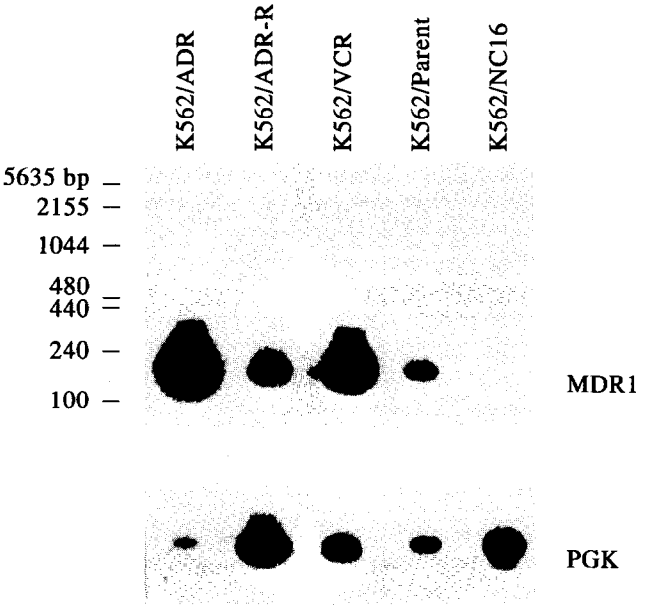

Figure 2. Expression of *MDR1* mRNA in drug sensitive K562/Parent, K562/ADR-R and K562/NC16 cells, and multidrug-resistant K562/ADR and K562/VCR cells. PM_2/*Hind*III was used as a size marker.

K562/Parent and the revertant K562/ADR-R cells, *MDR1* mRNA was expressed clearly under our experimental conditions.

Comparison of Fluo-3 with Rh-123 in the efflux pattern combined with verapamil

Figure 3 shows a comparison of the efflux pattern of Fluo-3 with that of Rh-123 in the K562/Parent cells and their drug-resistant variants. In the MDR K562/VCR and K562/ADR cells, verapamil showed marked effects on the retention of the Fluo-3 and Rh-123 fluorescent agents. In contrast, in the non-Pgp overexpressing cells, including K562/Parent and K562/ADR-R cells, no significantly delayed efflux patterns of Rh-123 after addition of verapamil were observed, while the intracellular retention of Fluo-3 was significantly delayed after the addition of verapamil in the efflux phases similar to those for K562/VCR and K562/ADR cells. Cepharanthine also showed almost the same patterns as those for verapamil (data not shown). These results suggested that the efflux pattern of Fluo-3 might demonstrate very low levels of Pgp expression, since the *MDR1* mRNA was detected even in K562/Parent and K562/ADR-R cells as shown in Figure 2, or that Fluo-3 might be non-specifically affected by verapamil and cepharanthine.

Establishment of a K562/NC16 cell line and accumulation of Fluo-3 and Rh-123 in the cells

To further study the Pgp dependency of Fluo-3, a new cell line, referred to as K562/NC16, was established from the K562 parent cell line using the limiting dilution technique. The degree of drug sensitivity was almost the same as that for K562/Parent cells (Table 1). As shown in Figure 2, the K562/NC16 cells expressed no *MDR1* mRNA using our RT–PCR method. Figure 4 shows the influx and efflux patterns of Fluo-3 compared with those of Rh-123 in the K562/NC16 cells. Intracellular accumulation of Fluo-3, even with verapamil or cepharanthine, decreased in the efflux phase, and once again, significantly increased influx and delayed efflux patterns of Fluo-3, but not Rh-123, were observed in the minimum Pgp-expressing K562/NC16 cells after addition of verapamil or cepharanthine. Table 3 shows the "indices" of the effects of verapamil and cepharanthine on the influx and efflux patterns of Fluo-3 and Rh-123 in K562/NC16 cells. Intracellular Fluo-3 accumulation was significantly influenced by these Pgp inhibitors both in the influx and efflux patterns as compared with those for Rh-123. These results indicated that intracellular accumulation of Fluo-3 might be non-specifically influenced by MDR-reverting agents at minimum levels of Pgp-related MDR, while the influx and efflux patterns of Rh-123 might be strongly affected by Pgp overexpression.

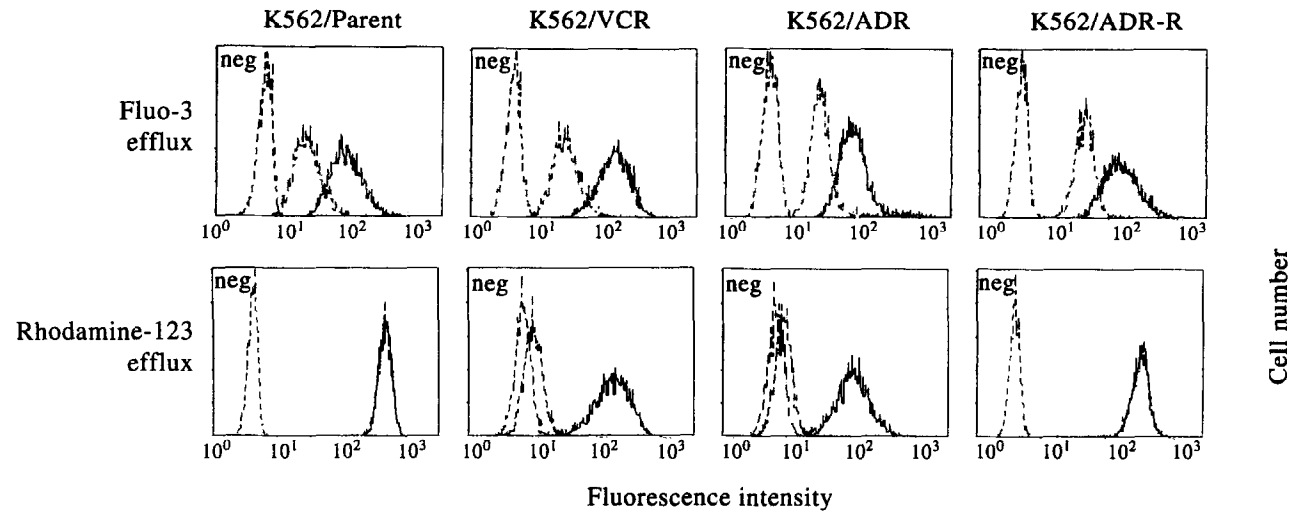

Figure 3. Comparison of the efflux patterns of Fluo-3 and rhodamine-123 (Rh-123) combined with verapamil. neg, negative control; solid line, additional verapamil incubation; broken line, no additional verapamil incubation.

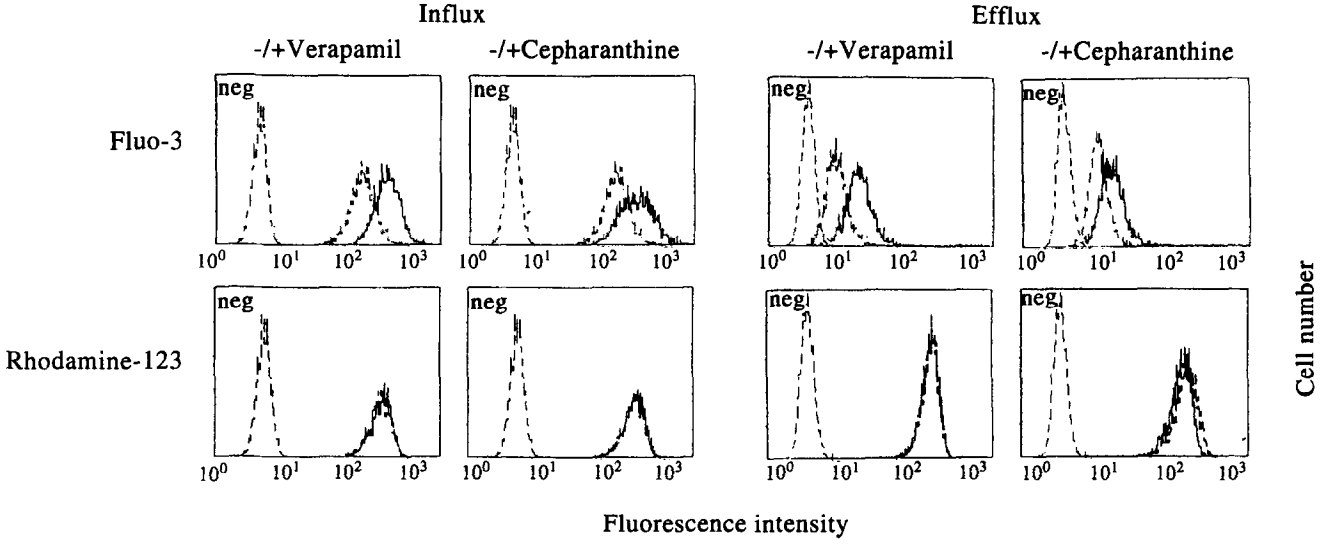

Figure 4. Comparison of the influx and efflux patterns of Fluo-3 and rhodamine-123 (Rh-123) in K562/NC16 cells expressing minimum *MDR1* mRNA. neg, negative control; solid line, additional verapamil or cepharanthine incubation; broken line, no additional verapumil or cepharanthine incubation.

Table 3. Comparison of the effects of verapamil and cepharanthine on the influx and efflux patterns of Fluo-3 and rhodamine-123 in K562/NC16 cells

	Fluo-3	Rh-123	*P* value*
Influx pattern (n = 4)			
Index of verapamil effect	2.65 ± 0.20	0.99 ± 0.07	<0.01
Index of cepharanthine effect	2.93 ± 0.89	1.01 ± 0.06	<0.05
Efflux pattern (n = 4)			
Index of verapamil effect	2.18 ± 0.22	1.20 ± 0.23	<0.01
Index of cepharanthine effect	2.97 ± 0.65	1.04 ± 0.20	<0.01

* *t*-test.

DISCUSSION

The usefulness of Fluo-3 as an indicator of intracellular calcium metabolism has been studied by Minta and associates [14] and Kao and colleagues [24]. Its practical adaptation in a functional assay of MDR was initially reported by Wall and associates [15] who showed that Fluo-3 was a more rapid and sensitive fluorescent indicator than doxorubicin which had previously been used as a drug-accumulation indicator for MDR cells. Rh-123 has been noted to accumulate in the mitochondria of cells, and its retention has been found to be inversely correlated with Pgp expression of MDR cell lines [12, 13]. Thus, we compared these two fluorescent agents for their abilities to functionally detect MDR using established K562 leukaemic cell lines.

Significant differences in Fluo-3 accumulation between the drug-sensitive K562 cells and the two drug-resistant variant cells, K562/ADR and K562/VCR, were shown to be similar to those with Rh-123. However, in contrast to Rh-123, differential accumulation of Fluo-3 in the presence and absence of verapamil or cepharanthine was demonstrated even in the non-*MDR1* mRNA-expressing K562/NC16 cells, suggesting that Fluo-3 might show non-specific and false positive intracellular accumulation since verapamil is well known to be a specific inhibitor of Pgp. Intracellular accumulation of Fluo-3 by verapamil may not be because of minimum levels of Pgp expression, but may be due to changes of intracellular calcium content after addition of calcium channel-blocking agent. Cepharanthine has also been shown to inhibit the release of intracellular calcium to the extracellular medium [25]. At very high levels of MDR such as in the experimentally established, MDR cell lines such as K562/ADR and K562/VCR cells, the intracellular accumulation of Fluo-3 clearly correlated with the levels of MDR, but the flow cytometric technique using Fluo-3 might not be a good method to monitor low levels of MDR. Although Wall and associates [26] recently showed that, for the detection of MDR in clinical samples of chronic lymphocytic leukaemia, a flow cytometric assay using Fluo-3 was more sensitive than other assay systems, further careful examination may be needed because the levels of Pgp expression during the multiple courses of chemotherapy in the clinical setting have been reported to be relatively low [1]. A clear picture of the influx and efflux pathways of Fluo-3 has not been shown. This may be another reason why it is difficult to advocate use of this fluorescent agent as a test for Pgp transport.

Cepharanthine (biscoclaurine) is known to decrease the fluidity of various biological membranes and to reverse MDR through the same mechanism as that of verapamil, which blocks the labelling of Pgp with a photoaffinity analogue of cytotoxic drugs such as vinblastine [19]. In this study cepharanthine also showed a significant reversal of MDR and Rh-123 accumulation by drug-resistant cells in a dose-dependent manner similar to verapamil, suggesting that cepharanthine, like verapamil, may be useful for the assessment of MDR mediated by Pgp.

In conclusion, both Fluo-3 and Rh-123 may be good indicators for the functional assay of MDR at high levels, while for the detection of relatively low levels of MDR, measurement of intracellular uptake and efflux of Fluo-3 with MDR-reversing agents such as verapamil and cepharanthine may not be useful because of the non-specific appearance of Fluo-3 accumulation.

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