MDR-1 gene expression is a minor factor in determining the multidrug resistance phenotype of MCF7/ADR and KB-V1 cells

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Received 25 March 1997; revised version received 10 June 1997

Abstract The relevance of MDR-1 gene expression to the multidrug resistance phenotype was investigated. Drug-resistant cells, KB-V1 and MCF7/ADR, constantly expressed mRNA of the MDR-1 gene and were more resistant to vinblastine and adriamycin than drug-sensitive cells, KB-3-1 and MCF7. The drug efflux rate of KB-V1 was the same as KB-3-1 although the MDR-1 gene was expressed in only the resistant cell. The higher intracellular drug concentration of KB-3-1 than KB-V1 was due to the large drug influx. In the case of MCF7 and MCF7/ADR, the influx and efflux of the drug had nearly the same pattern and drug efflux was not affected by verapamil. The amount of ATP, cofactor of drug pumping activity of P-glycoprotein, was not changed by the resistance. These observations suggested that drug efflux mediated by MDR-1 gene expression was not a major determining factor of drug resistance in the present cell systems, and that the drug resistance could be derived from the change in drug uptake and other mechanisms.

Key words: MDR gene expression; KB-V1; KB-3-1; MCF7; MCF7/ADR; Influx; Efflux

1. Introduction

The majority of the acquired resistances of cancer cells to anticancer agents is considered to be related with MDR gene expression and the presence of P-glycoprotein (P-gp) which pumps out the intracellular drug [1]. However, recent studies have suggested that there are some exceptions. In the highly drug-resistant KB-A1 and KB-A10 cell lines, the level of drug resistance is not proportional to the expression of P-gp or to other common factors such as protein kinase C (PKC) activity, glutathione-*S*-transferase and topoisomerase II, which are thought to participate in drug insensitivity [2]. A multidrug-resistant HOB1/ADR lymphoma cell line showed a lack of reduced accumulation and of enhanced efflux of adriamycin [3]. Changes in subcellular drug distribution and cellular accumulation were observed in adriamycin-resistant sublines of SW-1573 lung cancer and MCF7 breast cancer cells [4]. It was found that multidrug resistance-associated protein (MRP) and MRP mRNA were expressed in the adriamycin-resistant KB cells but not in the parental KB-3-1 cells [5]. Accordingly, it could be assumed that drug resistances were mediated by many kinds of molecular mechanisms.

In the present study, we tried to find unique cases, which expressed MDR gene in the resistant variant but did not participate in the resistance. Two kinds of resistant and parental cells were selected and the cellular drug kinetics were examined. It was found that these cellular models could suggest the unique mechanisms of drug resistance.

2. Materials and methods

2.1. Chemicals

All chemicals, including adriamycin, vinblastine, and rhodamine 123, were purchased from Sigma (St. Louis, MO, USA). RPMI 1640 medium and Hanks' balanced salt solution were purchased from Gibco BRL (Grand Island, NY, USA).

2.2. Cell lines

Human cancer cells (MCF7, MCF7/ADR, KB-3-1 and KB-V1) were cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS, R10) at 37°C in a humidified atmosphere of 5% CO_2. For consistent MDR-1 gene expression, MCF7/ADR and KB-V1 cells were maintained in the presence of adriamycin (ADR, 0.1 μg/ml) and vinblastine (VBL, 0.1 mM), respectively.

2.3. Analysis of MDR-1 gene expression

The level of MDR-1 gene was determined by a modification of previous methods [6,7]. Total cellular RNA was isolated by using the Ultraspec-II RNA isolation system (Biotecx Lab. Inc., Houston, TX). cDNA was synthesized with 40 ng of total cellular RNA by using the GeneAmp RNA PCR kit (Perkin Elmer, Branchburg, NJ). Quantitative PCR was carried out with cDNA and 2.5 units of AmpliTaq DNA polymerase and human MDR-1 specific primer [8]. For the quantification of mRNA of MDR-1, mRNA of GAPDH was also amplified under the same conditions [9]. PCR products were separated on 3% agarose gel, followed by staining with ethidium bromide. MDR-1 mRNA levels for each cell line were evaluated by the densitometric scanning method using Molecular Analyst Software (Bio-Rad Lab., Hercules, CA) and were expressed by the relative ratio to mRNA levels for GAPDH.

2.4. Chemosensitivity

Chemosensitivity was performed by a modification of previous methods [10,11]. Cells were incubated in RPMI 1640 medium containing 5% FCS (R5) for 24 h and ADR (3–0.03 μg/ml) or VBL (10–0.1 μg/ml) was added to the medium. After further incubation for 48 h, cells were fixed with 50% (w/v) trichloroacetic acid (TCA) solution at 4°C for 1 h. After washing with tap water followed by drying in air, TCA-fixed cells were stained with 0.4% sulforhodamine B (SRB) dissolved in 1% acetic acid. After staining, the unbound dye was washed out with 1% acetic acid. To each well of the dried plate, 100 μl Tris base (10 mM, pH 10.5) was added. Optical density (OD) was measured with a microtiter plate reader (Molecular Devices, Model Emax, Menlo Park, CA) at 540 nm.

2.5. Determination of rhodamine 123 uptake and release

Cells (5×10^4 cells) were diluted in R10 and inoculated into each

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Abbreviations: MDR, multidrug resistance; R-123, rhodamine 123; ADR, adriamycin; VBL, vinblastine; VP, verapamil; SRB, sulforhodamine B

PII S0014-5793(97)00777-1

well of 24-well microplates. Cells were incubated overnight at 37°C in an atmosphere of 5% CO_2 and then washed two times with warm R5. Rhodamine 123 (R-123) uptake, the intracellular concentration of R-123, was determined at the designated time after addition of R-123 (1 μg/ml) and washed six times with cold R5. For R-123 release experiments, cells were incubated for the designated time in R5 containing R-123 and washed four times with cold R5. Then, the medium was changed to 37°C R5 without R-123 and incubated for the designated time and washed with cold R5. Intracellular R-123 was extracted twice with 0.2 ml *n*-butanol and the fluorescence intensity was measured with a Luminescence Spectrometer (Model LS50B, Perkin Elmer, UK) at an excitation wavelength of 518 nm and emission wavelength of 532 nm. The amount of R-123 was calculated by a standard curve of R-123 [12]. In the present experiment, R123 did not affect cell viability and morphology.

2.6. Effect of verapamil on R-123 and ADR release

Cells were incubated in Hanks' balanced salt solution (HBSS) containing either R-123 (1 μg/ml) [12] or ADR (10 μg/ml) for 15 min and washed four times with cold HBSS. The concentration of adriamycin was determined by the previous experiment on the dose-dependent uptake of adriamycin at a concentration which was non-cytotoxic. The medium was changed to 37°C HBSS with or without 5 μM verapamil and incubated for the designated time and washed with cold HBSS. Verapamil did not affect cell viability determined by trypan blue dye uptake. Intracellular R-123 was determined as described above. Intracellular ADR was extracted with 1 ml of 0.3 N HCl-50% ethanol [13] and the amount was determined by high-performance liquid chromatography (HPLC). The chromatographic separations were accomplished on a Waters Millipore model 510 liquid chromatograph (Millipore, Mariborough, MA) equipped with Lichrosorb RP-18 10 μm column of 10 μm particle size (Waters Assoc., Waltham, MA). An isocratic solvent system (mobile phase) was used consisting of 0.05 M NaH_2PO_4/methanol (70 : 30, v/v). The flow rate was 1.6 ml/min. Fluorescence was measured with a Model Waters 470 liquid chromatography fluorometer (Millipore). The excitation wavelength was 470 nm and the emission wavelength was 585 nm [13].

2.7. Determination of intracellular ATP concentration

After treatment of R-123 for 5 or 20 min, the amount of intracellular ATP was determined [14]. Intracellular ATP was directly extracted and quantified by using the ATP bioluminescence assay kit (Boehringer Mannheim, GmbH, Germany). ATP bioluminescence was measured with a Luminescence Spectrometer (Perkin Elmer) at an emission wavelength of 562 nm. The concentration of ATP was calculated by a standard curve of ATP (10^{-5} M–10^{-12} M).

3. Results

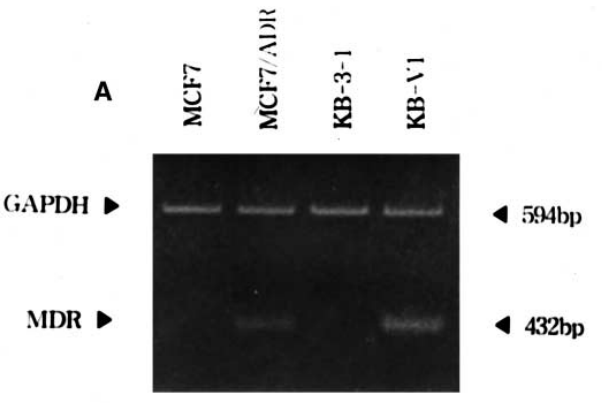
3.1. The expression of MDR-1 gene and chemosensitivity

Drug resistance phenotype was determined by MDR-1 gene expression and chemosensitivity. The RT-PCR analysis showed that the sensitive cells, MCF7 and KB-3-1, did not produce mRNA of the MDR-1 gene but the resistant variants, MCF7/ADR and KB-V1, expressed the MDR-1 gene (Fig. 1A). The ratios of the MDR-1 mRNA to GAPDH mRNA in MCF7/ADR and KB-V1 were calculated to 0.768 and 1.212, respectively (Fig. 1B).

The resistance of MCF7/ADR and KB-V1 to anticancer agents was also shown in the determination of chemosensitivity. Vinblastine suppressed the growth of KB-3-1 at a low concentration of 0.1 μg/ml but the same extent of cytotoxicity was observed only at concentrations higher than 3 μg/ml in KB-V1. In the case of MCF7 and MCF7/ADR, a significant difference of growth was observed at concentrations higher than 0.1 μg/ml of adriamycin (Fig. 2A). The calculated GI_{50} is shown in Fig. 2B. From the results, it was certain that the present resistant cells constantly expressed mRNA of MDR-1 and were more resistant than their parental cells.

3.2. Uptake and release of R-123

The rate of drug uptake and release was measured by using R-123. KB-3-1 had a higher uptake rate than KB-V1 for 30 min exposure, but the release rate of the two cell lines was the same (Fig. 3A). MCF7 had the same uptake rate of R-123

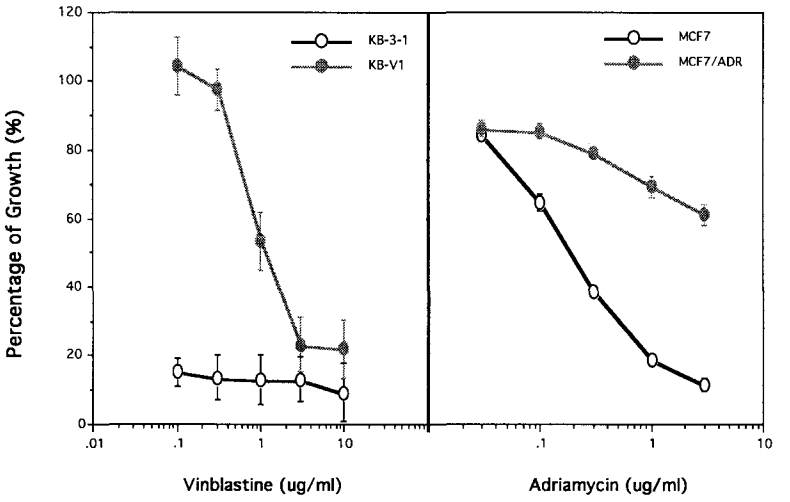


B

	MCF7	MCF7/ADR	KB-3-1	KB-V1
MDR-1 mRNA	0	0.768	0	1.212

Fig. 1. RT-PCR analysis of MDR-1 gene expression. Total RNA (40 ng) was amplified with the primers of MDR-1 (lower) and GAPDH (upper) (A). The product of RT-PCR was detected with agarose gel electrophoresis. Relative levels of MDR-1 and GAPDH mRNA were estimated as described in Section 2. The ratio of MDR-1 to GAPDH is listed in (B).

A



B

Cell Lines	GI_{50} (ug/ml)
KB-3-1	< 0.100
KB-V1	1.580
MCF7	0.120
MCF7/ADR	> 3.000

Fig. 2. Chemosensitivity of sensitive, KB-3-1 and MCF7, and resistant cells, KB-V1 and MCF7/ADR. Chemosensitivity of these cells was determined by sulforhodamine B (SRB) assay (A). Adriamycin was added from 0.3 to 3 μg/ml and vinblastine was added from 0.1 to 10 μg/ml. 50% growth inhibition (GI_{50}) was calculated and listed in (B). The data present the mean values ± standard deviation of three independent experiments which were done in six separate analyses.

with MCF7/ADR, and showed a slightly higher release rate of R-123 than MCF7/ADR (Fig. 3B).

Time-dependent uptake and release of R-123 were determined every 15 min. Fig. 4A shows that intracellular R-123 concentration in KB-3-1 steadily increases up to 60 min but that of KB-V1 slowly increases up to 30 min and then slowly decreases up to 60 min. Even though the intracellular concentrations of R-123 in KB-3-1 and KB-V1 were different at each time point, the release rate of R-123 was nearly the same. These results possibly reflect that MDR-1 gene expression was not related with the intracellular drug accumulation. The same experiment was done in MCF7 and MCF7/ADR and the result is depicted in Fig. 4B. These two cell lines had almost the same rate of uptake and release of R-123. Uptake and release of adriamycin in the resistant cells were also the same as in the sensitive cells (unpublished results). In these cells, MDR-1 gene expression did not match with the drug uptake and release.

3.3. *Effect of verapamil on drug release and quantification of ATP*

To confirm whether P-gp, product of MDR-1 gene, is involved in the determination of intracellular drug accumulation, verapamil (VP) was exposed to MCF7 and MCF7/ADR in the drug release experiment. Verapamil did not change the release rate of R-123 or ADR in either cell (Fig. 5). These results supported the previous results that P-gp was not involved in controlling the intracellular drug concentration of MCF7 and MCF7/ADR.

Next, the amount of ATP was determined to examine whether depletion of ATP affected the release and uptake of R-123. Fig. 6 shows that the amount of ATP, the cofactor of drug pumping activity of P-gp, was nearly the same between the resistant and sensitive cells, suggesting that P-gp was not functionally affected.

4. Discussion

KB-V1 cells showed the over-expression of MDR-1 gene, which is known as a major factor in changing intracellular drug concentration, and also showed a high resistance to vinblastine when compared with KB-3-1 cells. In spite of the difference in MDR-1 mRNA expression between sensitive and resistant cells, the decreased intracellular drug concentration in KB-V1 was mediated by the uptake rate but not the release rate. As shown in Fig. 4A, the intracellular R-123 concentration of KB-3-1 at 60 min after R-123 exposure was 592% of KB-V1, but the release rates of the two cells were almost the same. Accordingly, the low intracellular drug accumulation of KB-V1 might be mediated by a low drug influx but not a high drug efflux mediated by P-gp. In the present system, the drug accumulation was proportional to the extracellular concentration (data not shown). This might show that the influx of R-123 and other drugs was by

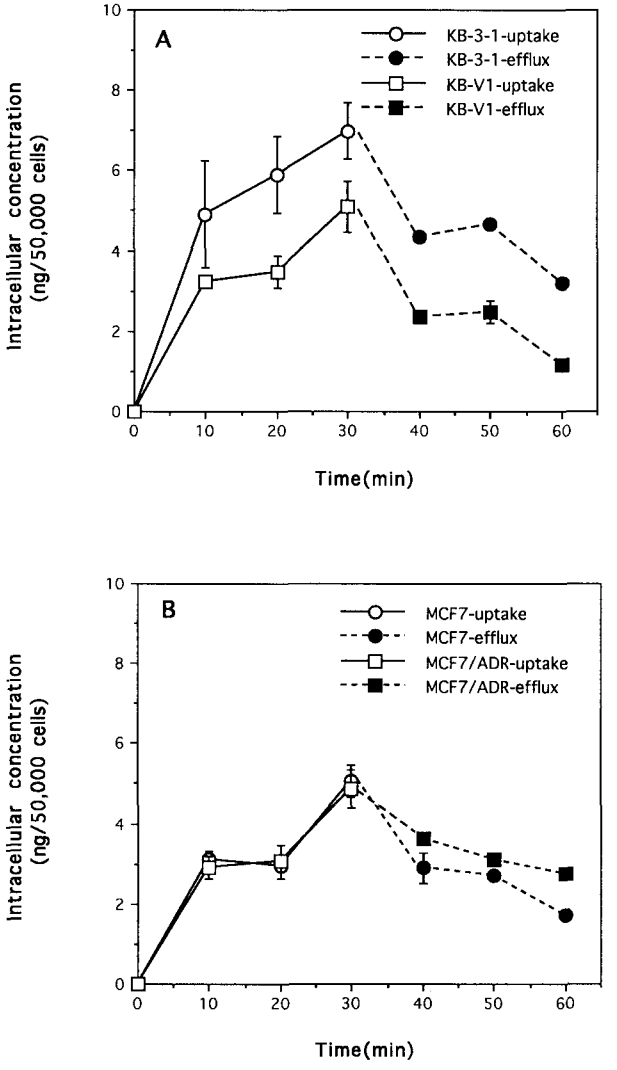


Fig. 3. R-123 uptake and efflux in KB-3-1 (A), KB-V1 (A), MCF7 (B), and MCF7/ADR cells (B). These cells were incubated with R-123 (1 μg/ml). To determine the uptake, intracellular drug concentration was determined every 10 min for 30 min. To determine the efflux, intracellular drug concentration was determined every 10 min after uptake R-123 for 30 min. The data here present the mean values ± standard deviation of three independent experiments which were done in six separate analyses.

diffusion through the cytoplasmic membrane. Then it could be assumed that the difference of drug influx between KB-V1 and KB-3-1 was caused by the changes of cytoplasmic membrane. These results were also observed in PC-9/VCR. The low intracellular drug concentration of resistant cells was mediated by a small drug uptake, which was reversed by ascorbic acid. PC-9/VCR cells have been suggested to have an ascorbic acid-sensitive drug uptake mechanism that differed from the P-gp-mediated MDR mechanism [15]. KB-V1 cells exhibited similar mechanisms with PC-9/VCR in that both cell types expressed multidrug resistance phenotype by reducing drug uptake.

Drug uptake and release rate of MCF7/ADR were the same as MCF7, suggesting that the intracellular drug concentration of both cells was the same. Also intracellular drug concentration was not changed by verapamil, a P-gp blocking agent. Although the intracellular drug concentration was not reduced in MCF7/ADR cells in comparison with parental cells, they constantly expressed the MDR-1 gene and showed a differential chemosensitivity to adriamycin. These observations demonstrated that, even though the MDR-1 gene was expressed in MCF7/ADR cells, it was not related to the resistant phenotype. Furthermore, the resistance of this cell was independent of the intracellular drug concentration. Further studies are needed to find the resistant mechanism not related with the intracellular drug concentrations and/or MDR-1 gene expression.

Although the multidrug resistance mechanism of the present cells was not clear, it might be suggested that the MDR-1 gene/P-gp did not properly work due to the changes of the modulating factors. Possible mechanisms seemed to be related to the changes of intracellular calcium and cAMP levels [16] and other common mediators such as PKC, which modulated the function of P-gp [17,18]. Alteration of the glu-

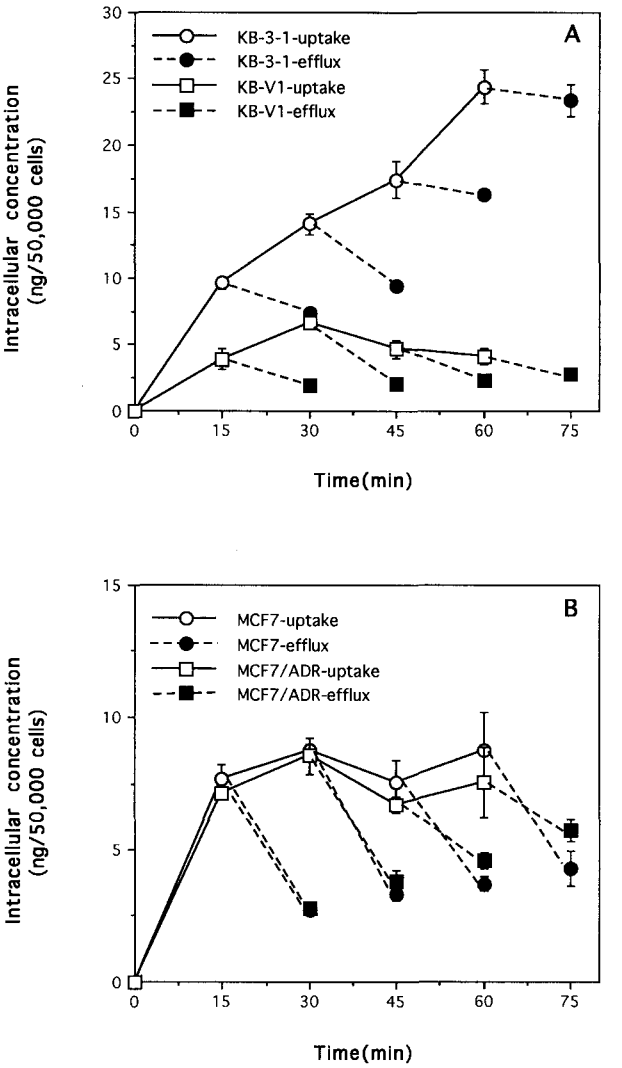


Fig. 4. R-123 uptake and efflux in KB-3-1 (A), KB-V1 (A), MCF7 (B), and MCF7/ADR cells (B). These cells were incubated with R-123 (1 μg/ml). To determine the uptake, intracellular drug concentration was determined every 15 min for 60 min. To determine the efflux, intracellular drug concentration was determined at 15 min after uptake of R-123 for indicated periods from 15 to 60 min. The data present the mean values ± standard deviation of three independent experiments which were done in six separate analyses.

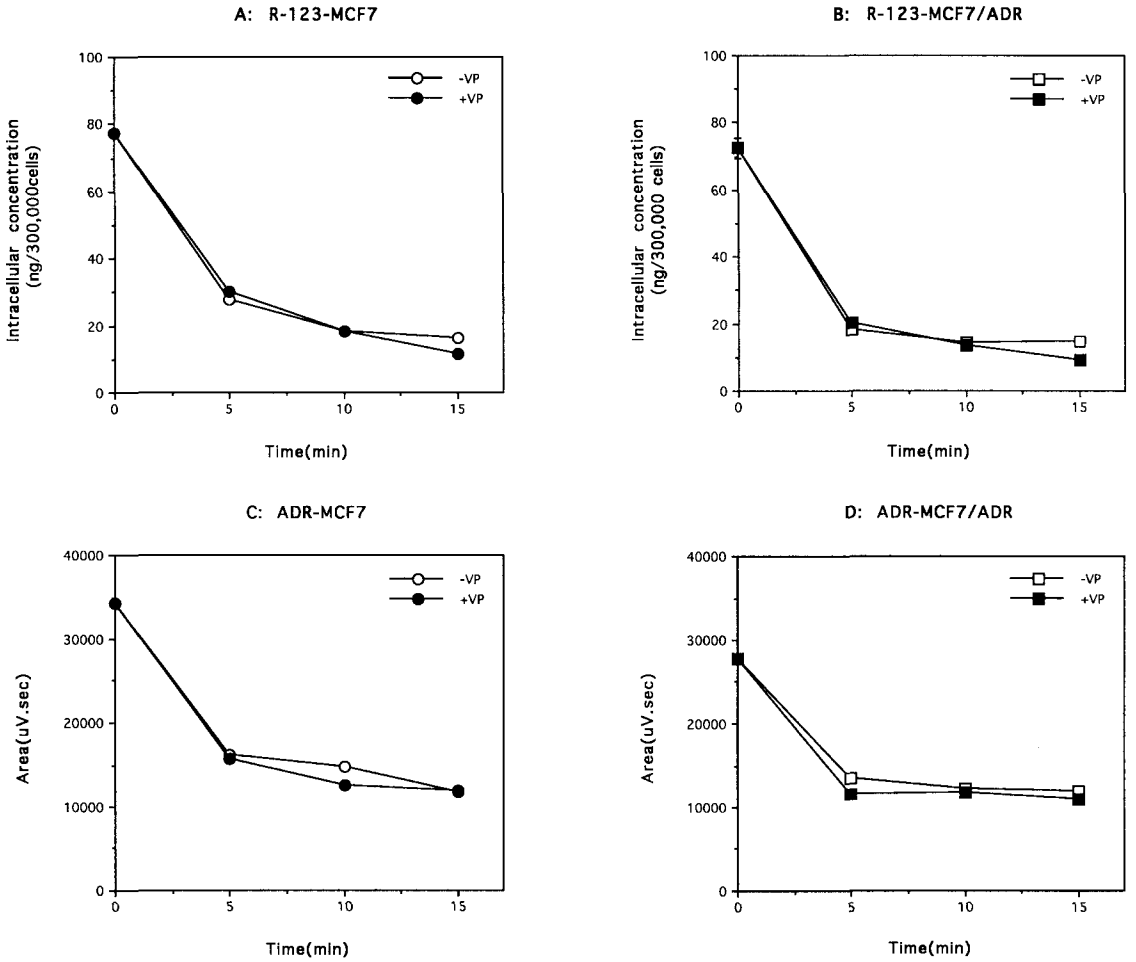


Fig. 5. Effects of verapamil on the efflux of R-123 (A, B) and ADR (C, D) in MCF7 (A, C) and MCF7/ADR cells (B, D). Either R-123 (1 μg/ml) or ADR (10 μg/ml) was administered to these cells. After washing verapamil was added at a final concentration of 5 μM for the indicated periods. To determine efflux, intracellular drug concentration was determined every 5 min. The data present the mean values of six separate analyses.

tathione redox cycle could modify the intracellular concentration of calcium and cAMP, affecting the function of P-gp [16]. The expression and activity of PKC isoenzymes in the human breast cancer cell line (MCF-7-WT) and MDR subline (MCF-7-MDR) were implicated in the regulation of the MDR phenotype through phosphorylation of P-gp [17]. The over-expression of PKC-α in an MCF-7 cell line already over-expressing P-gp has been shown to increase the MDR phenotype [18]. MRP might seem to contribute to the resistance phenotype, because MRP was known to reduce the intracellular drug concentration in the resistant cells (MCF7/VP) by MRP over-expression, in the absence of mdr1/P-glycoprotein expression and without any change in drug efflux [19]. The actual resistance mechanism of the present cells will be determined by examining these mechanisms. The relevance of drug uptake with intracellular calcium will be studied to find the resistant mechanism of KB-V1 and MCF7/ADR.

From these results, it was clear that MDR-1 gene expression did not affect the kinetics of drug influx and efflux in the present cell system, indicating that the transcription rate of the MDR gene was not a critical factor in the acquisition of multidrug resistance. Even though these two cell systems show different mechanisms of resistance, these are unique models in which the over-expressed MDR gene does not participate in drug resistance. It may be possible that the MDR gene expression is related to resistance mechanisms in some cancer cells but not others.

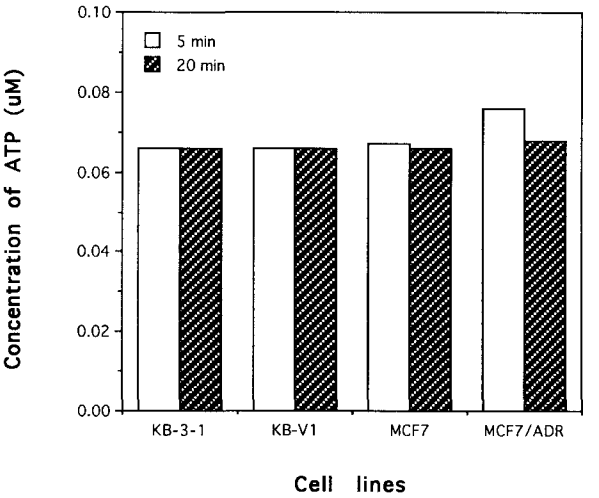


Fig. 6. Quantification of intracellular ATP concentration. The concentration of ATP was quantified in KB-V1, KB-3-1, MCF7, and MCF7/ADR cells up to 30 min by the bioluminescence detection method. The data present the mean values of six separate analyses.

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