

Demonstration of the Activity of P-glycoprotein by a Semi-automated Fluorometric Method

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Abstract. *Background:* We have developed a semi-automated fluorometric method that utilizes ethidium bromide (EB), a common substrate of bacterial efflux pumps. The method is sufficiently sensitive to characterize the efflux pump systems of bacteria. Because EB is also recognized and extruded by ATP-binding cassette (ABC) transporters and these have similarity to P-glycoprotein (P-gp), the method has been extended for the evaluation of agents that can inhibit the extrusion of EB on a real-time basis by mouse lymphoma cells containing the human ABCB1 (*mdr1*) gene. *Materials and Methods:* Monitoring of uptake and extrusion of EB was assessed using the Rotor-Gene™ 3000 (Corbett Research) under different conditions. *Results:* Whereas extrusion of EB took place readily, the addition of known inhibitors of efflux pumps (verapamil, reserpine) caused retention of EB. *Conclusion:* This method is inexpensive and allows the detection of neoplastic cells with increased efflux activity as well as the screening of large numbers of compounds for inhibition of the P-gp.

The major reason for the failure of chemotherapy of cancer is the refractory nature of the cell that results from prior exposure to the same or other chemotherapeutic agents. Since

chemotherapy is the treatment of choice for ~50% of all types of cancer, acquired resistance during the course of the therapy has also become commonplace (1). The major mechanism of multi-drug resistance (MDR) is the overexpression of drug transporters such as *ABCB1* [also known as P-glycoprotein (P-gp)], *ABCC1* [also known as multi-drug resistance-associated protein (MRP1)], *ABCG2* [also known as breast cancer-resistance protein (BCRP) and mitoxantrone-resistance protein (MXR)], all of which belong to the ATP-binding cassette (ABC) superfamily (1, 2). MDR mediated by the overexpression of efflux pumps, such as P-gp, makes therapy problematic in a large number of patients (2). Although the overexpression of drug transporters in MDR cancer cells is mainly a consequence of chemotherapy, P-gp coded by the *ABCB1* gene (previously known as *MDR1*) is often overexpressed in untreated cancer. Such tumours include those derived from the colon, kidney, adrenal gland, liver and pancreas. Intrinsic MDR is also found in carcinoid tumours, chronic myelogenous leukaemia in blast crisis, cell lines of non-small cell lung carcinoma (NSCLC) with neuroendocrine properties, acute lymphocytic leukaemia (ALL) and acute non-lymphocytic leukaemia (ANLL) in adults, indolent non-Hodgkin's lymphoma and neuroblastoma, for example (3). *ABCB1* gene expression may be considered an unfavourable prognostic factor for these and other malignancies (4, 5).

To overcome the MDR due to the efflux activity of the ABC transporters, various chemosensitizers have been developed (2). Agents which inhibit extrusion of chemotherapeutic agents render the cancer cell susceptible to the agent to which it had become resistant. Obviously, targeting these transporters is of interest if cancer chemotherapy is to be viable.

Flow cytometry is the standard method for the screening of P-gp modulators. These modulators are assessed for their ability to cause the retention of the fluorochrome substrate

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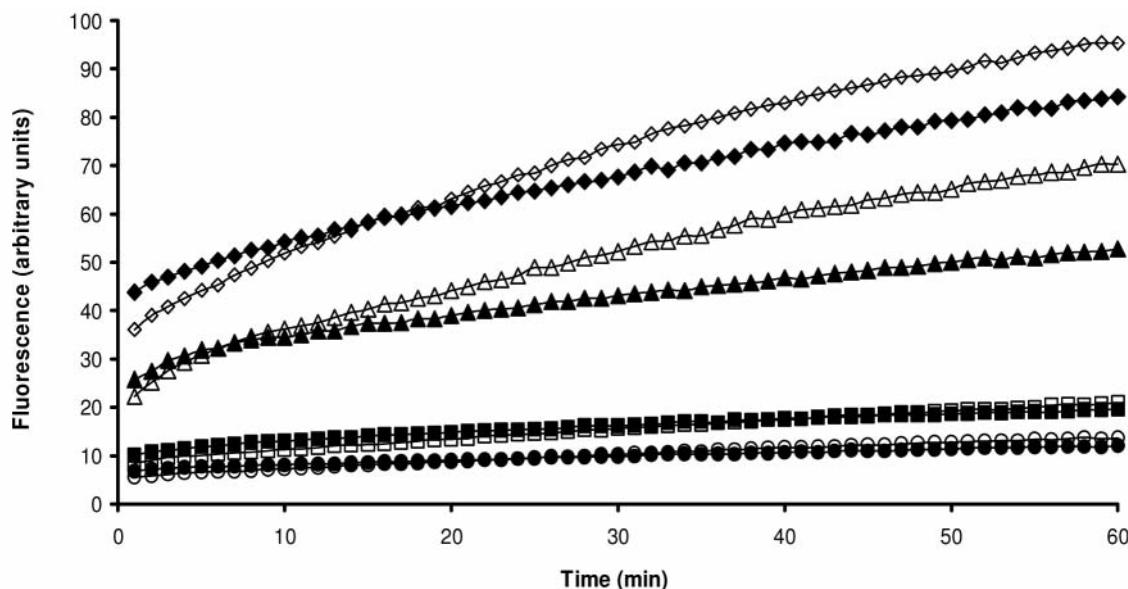


Figure 1. Determination of the lowest ethidium bromide (EB) concentration that is barely accumulated by the parental (closed symbols) and the multidrug-resistant (open symbols) mouse lymphoma cells. ●, ○ EB 0.5 $\mu\text{g}/\text{ml}$; ■, □ EB 1 $\mu\text{g}/\text{ml}$; ▲, △ EB 2 $\mu\text{g}/\text{ml}$; ◆, ◇ EB 3 $\mu\text{g}/\text{ml}$.

rhodamine 123 by cancer cells (6, 7). Although the flow cytometric method is sensitive, it does not provide a real-time assessment of the activity of the modulator. Moreover, the method requires a flow cytometer which is a very expensive instrument and a technician with demonstrated experience in this area.

We developed a simple method that assesses the real-time accumulation and extrusion of the fluorochrome ethidium bromide (EB), which is a common substrate of bacterial efflux pumps (8). This assay employs a new application of the semi-automated system, Rotor-GeneTM 3000 (Corbett Research, Sidney, Australia), which provides, on a real-time basis, the assessment of transport kinetics, reflecting the balance between accumulation of EB *via* passive diffusion (through the membrane permeability) and extrusion *via* efflux. Since the system can handle as many as 36 tubes that lack or contain the agents that are to be screened for activity against efflux pumps (*i.e.* efflux pump inhibitors, EPIs), employs an inexpensive fluorochrome, and is user-friendly, we have applied this methodology for the assessment of the P-gp activity of mouse lymphoma cells transfected with the human ABCB1 gene.

Materials and Methods

Cell lines. L5178 mouse T-cell lymphoma cells (9, 10) were transfected with pHa MDR1/A retrovirus, as described elsewhere (11, 12). The ABCB1-expressing cell lines were selected by culturing the infected cells with 60 ng/ml of colchicine to maintain the MDR phenotype. L5178 mouse T-cell lymphoma cells

(parental) and the human ABCB1-gene transfected sub-line were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, L-glutamine and antibiotics (penicillin, streptomycin) at 37°C and in a 5% CO₂ atmosphere.

Medium. McCoy's 5A medium (Lonza BioWhittaker, Verviers, Belgium) supplemented with 10% heat-inactivated horse serum (Sigma-Aldrich Química SA, Madrid, Spain), L-glutamine (Lonza BioWhittaker) and antibiotics (Lonza BioWhittaker).

Compounds. EB, verapamil and reserpine were purchased from Sigma-Aldrich Química SA.

Viability assay. The cytotoxicity of EB and the EPIs, verapamil and reserpine was determined by the trypan blue exclusion method, previously described in detail (13).

EB accumulation assay. The cells were adjusted to a density of 2×10⁶ cells/ml, centrifuged at 2000×g for 2 minutes and resuspended in phosphate-buffered saline (PBS) solutions of different pH (5, 7.4 and 8). The cell suspension was distributed in 90 μl aliquots into 0.2 ml tubes. The tested compounds were individually added at different concentrations in 5 μl volumes of their stock solutions and the samples incubated for 10 minutes at 25°C. After this incubation, 5 μl (1 $\mu\text{g}/\text{ml}$ final concentration) of EB (20 $\mu\text{g}/\text{ml}$ stock solution) were added to the samples and the tubes were placed into a Rotor-GeneTM 3000 and the fluorescence monitored on a real-time basis. Prior to the assay, the instrument was programmed for temperature (37°C), the appropriate excitation and emission wavelengths of EB (530 nm bandpass and 585 nm highpass, respectively), and the time and number of cycles for the recording of the fluorescence (8). The results were evaluated by Rotor-Gene Analysis Software 6.1 (Build 93) provided by Corbett Research.

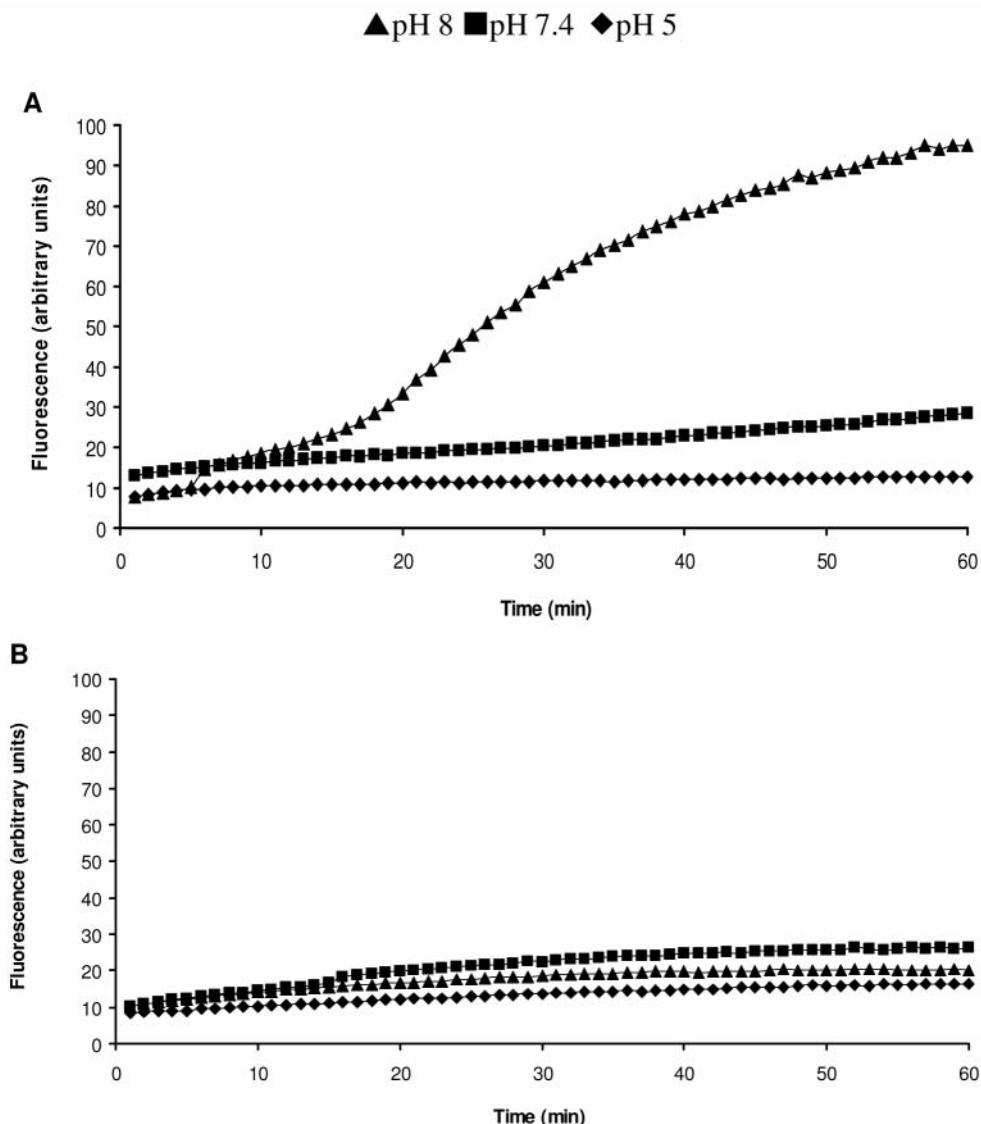


Figure 2. Accumulation of EB (1 µg/ml) at different pH by the parental (A) and the multidrug resistant (B) mouse lymphoma cells.

Results

In order to confirm whether EB can be used to monitor the accumulation without affecting the viability of the cells, the effect of different concentrations of EB was tested in combination with reserpine and verapamil on mouse lymphoma cells. Viability was monitored over a period of 180 minutes. Concentrations of EB below 2 µg/ml did not affect the cell viability over this time period (data not shown).

Selection of the EB concentration. This new approach provides the detection of transport kinetics reflecting the

balance between accumulation and efflux. The desired aim is to easily and accurately detect and quantify the transport of EB through the cell membrane at working concentrations that will not affect cell viability nor perturb cellular functions, using a methodology that allows the distinction between the direction of transport across the cell membrane. If conditions that affect the accumulation of EB are to be studied, it is important to determine the lowest concentration of EB accumulated. We concluded that the optimum concentration of EB for studying accumulation was 1 µg/ml; at a lower concentration of 0.5 µg/ml the signal was very low and hence was below the sensitivity of the instrument (Figure 1). Employing this concentration (1 µg/ml) under

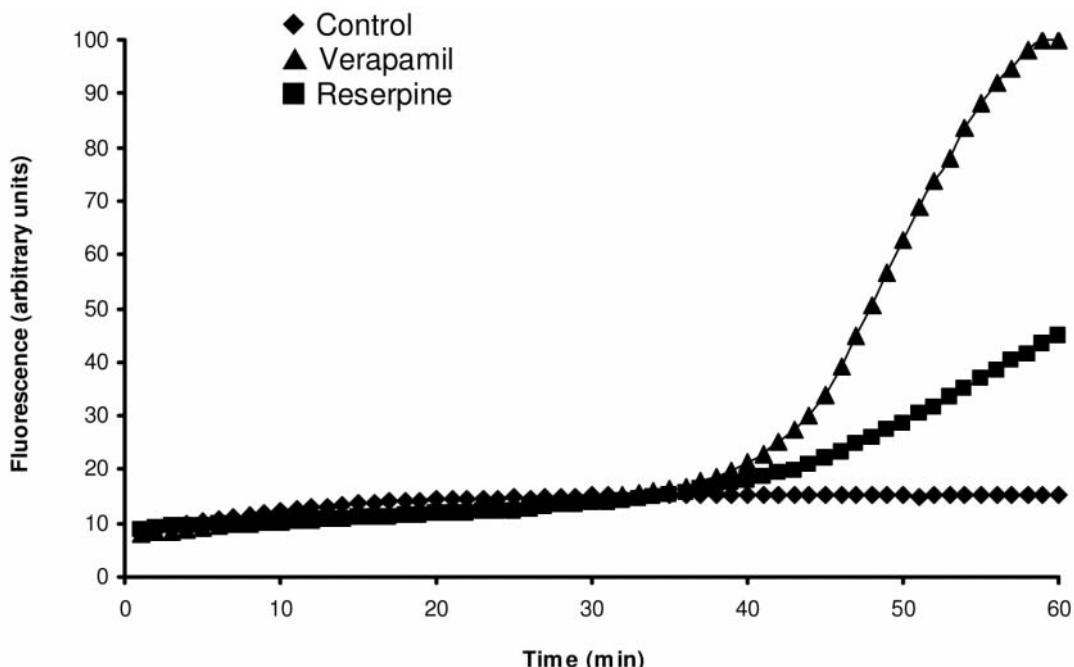


Figure 3. Accumulation of EB (1 µg/ml) at pH 7.4 by MDR mouse lymphoma cells in the presence of 40 µg/ml of efflux pump inhibitors verapamil and reserpine.

different conditions of the medium, the accumulation and efflux processes can be studied under different conditions, as well as in the presence of EPIs. These assays were repeated five times and the results with the parental and the MDR cells depicted by Figure 1 have a standard deviation (SD) of 2.67 and 3.82 arbitrary units, respectively.

Effect of pH on the accumulation of EB. The accumulation of EB was affected by pH, as shown by Figure 2A and B. There was a difference between the accumulation properties of the parental and the *ABCB1* gene-transfected cell line. Whereas the parental cells accumulated more EB as the pH increased (Figure 2A), the *ABCB1*-transfected cells were not affected by the increase of pH. These assays were repeated three times and gave consistent results.

Effect of the EPIs, verapamil and reserpine. The inhibition of the P-gp of the *ABCB1*-transfected mouse lymphoma cells can be demonstrated using this method. As shown by Figure 3, the presence of verapamil and reserpine in the medium at pH 7.4 promoted accumulation of EB, as compared to the control where these EPIs were not present. In our study, pH 7.4 was selected since this pH was used in other flow cytometry studies reported (5). These assays were repeated three times and the quantitative data presented in Figure 3 is representative of the consistent data.

Discussion

There are various methods to detect the effect of drugs on P-gp activity (14). The interaction of drugs with P-gp is complex, however, and because each method has advantages and disadvantages, quantitative comparison of data is difficult. The results obtained in this study show that accumulation of EB is a pH-dependent process only observable in the parental cells. Because efflux pumps can derive their energy from the proton gradient, and those that are part of the ABC superfamily contribute to this gradient from the hydrolysis of ATP (15), at pH 8, the parental cells are unable to maintain the extrusion of EB over time due to the weaker proton gradient. In contrast to this, the *ABCB1* transfected sub-line maintains extrusion of EB at pH 8 throughout the assay. These results suggest that the activity of P-gp is not dependent upon the proton motive force. Furthermore, because energy is needed for the functioning of P-gp, the question of the source of energy used by this transporter is intriguing. That this energy may come from the hydrolysis of ATP by the *ABCB1*-transfected cell line is suggested by the following: at pH 8, the membrane potential of the cell is quite low and the protons that are generated by the F1Fo ATP synthase of the mitochondria favor ATP hydrolysis (ATPase activity). Our results confirm those obtained by others that demonstrated that P-gp-mediated

drug efflux does not require external acidification and that acute changes in the intracellular pH do not alter the P-gp-mediated rhodamine 123 efflux (16). Marbeuf-Gueye *et al.* had similar results, namely that changes of extra- and/or intracellular pH yielded no modification of the ABCC1-mediated efflux of hydroxyrubicin in GLC4/ADR adriamycin-resistant human small cell lung carcinoma cell line (17). These results, when coupled to ours, suggest a major difference between the transporter of the parental mouse lymphoma cell and that of the human P-gp subline, a difference that may be exploited when studying the transfection of mouse lymphoma cells with genes that code for other human transporters.

The desired aim of the method described is to easily and accurately detect and quantify the transport of EB through the cell membrane, at concentrations that will not affect cell viability nor perturb the cellular function, in order to readily assess efflux activity in neoplastic cells or to screen for new EPIs against cancer cells by a high-throughput process. Although the method needs further standardization, it provides real-time, inexpensive and quick *in vitro* screening of candidate drugs that may result in the development of new MDR modulating agents that can be applied in the chemotherapy of cancer.

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