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ORIGINAL ARTICLE

Assessment of P-gp and MRP1 activities using MultiDrugQuant™ Assay Kit: a preliminary study of correlation between protein expressions and its functional activities in newly diagnosed acute leukaemia patients

Fazlina N*, Maha A***, Zarina AL**, Hamidah A**, Zulkifli SZ, Cheong SK*, Ainoon O*, Jamal R** and Hamidah NH*

*Haematology Unit, Departments of *Pathology and **Paediatrics, Faculty of Medicine, University Kebangsaan Malaysia, Kuala Lumpur, ***Division of Immunology, Clinical Laboratory of Sciences, Faculty of Medicine and Health Sciences, University Putra Malaysia, Kuala Lumpur*

Abstract

Multidrug resistance (MDR) is believed to be responsible for poor response of patients towards chemotherapy particularly patients with acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL). The best-characterized resistance mechanism is the one mediated by permeability-glycoprotein (P-gp) encoded by *MDR1* gene, which is responsible for drug efflux. We studied P-gp and multidrug resistance-associated protein 1 (MRP1) expression and functional activities in 43 newly diagnosed acute leukemia cases (19 paediatric ALL cases and 24 adult AML cases). The expression and functional activities were examined using flow cytometry and MultiDrugQuant™ assay kit (involving calcein AM uptake and efflux). P-gp and MRP1 expression and its functional activities were observed in 68.4% of paediatric ALL. In adult AML cases, all cases expressed MRP1 and its functional activities but only 58.3% were positive for P-gp and its functional activities. We were able to show a significant correlation between the expression of the multidrug resistant protein (P-gp and MRP1) and their functional activity in adult AML and paediatric ALL samples.

Key words: Multidrug resistance (MDR), permeability-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1), acute leukaemias.

INTRODUCTION

Studies on the resistance of tumour cells to cytotoxic drugs are necessary for understanding the mechanisms of the cells' defense against injury. The investigation of the insensitivity of malignant cells to chemotherapy is also needed for oncology practice because drug resistance is often considered to be a cause of tumour therapy failure. Ineffectiveness of the therapy may be provoked by many other causes besides tumour alterations; it may be connected with peculiarities of the pharmacokinetics of a drug, i.e., the inability of a drug to reach the target cells in adequate amounts and in active form.[1]

Recently, another potential factor causing poor response to chemotherapy particularly in acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) has been related to the presence of the multidrug resistance (MDR) phenomenon.[2,3] MDR was first described by Biedler & Riehm, who in 1970 obtained a cell line showing cross-resistance between unrelated anticancer drugs after exposure to one of these drugs; vinca alkoloids, epipodophyllotoxins, anthracyclines, dactinomycin and taxol, all having different cellular targets and mechanisms of action.[4]

The best-characterized resistance mechanism is the one mediated by the *MDR1* gene product permeability-glycoprotein (P-gp), which has been shown to be associated with poor treatment outcome in AML.[5,6] This gene maps on the long arm of chromosome 7.[2] P-gp belongs to the ATP-binding cassette (ABC) of the transporter gene superfamily, which is made up of homologous halves, each containing

Address for correspondence and reprint requests: Associate Professor Datin Dr. Noor Hamidah Hussin, Department of Pathology, Faculty of Medicine, University Kebangsaan Malaysia, Jalan Yaacob Latif, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia

six putative transmembrane domains and a nucleotide-binding sequence.[7] P-gp acts as an energy-dependent transmembrane pump that actively effluxes drugs and dyes.[2] Many authors have reported that acute leukemia is characterized by a positive correlation between P-gp expression and lower remission rates and a higher frequency of relapse.[2]

The functional capacity of P-gp in AML has been measured by the uptake/efflux of dye or drug using flow cytometry and shown to correlate with its protein expression. Several dyes (Rhodamine 123 (Rh123) and $DiOC_2$) and drugs (daunorubicin and doxorubicin) may be used to assess P-gp function. This functional test may then be used to correlate with treatment outcome.[8] However, in several studies, discrepant cases were reported, with increased efflux and no significant increase of MDR1 expression.[9] This suggests that alternative proteins, such as the recently recognized multidrug resistance-associated protein (MRP) or the lung resistance-related protein (LRP) may contribute to the MDR phenotype. However the role and functional capacity of these two proteins are still discussed and unclear in AML cases.[10] The functional test is important to determine the integrity of the MDR phenotype, but until now, only MDR1 functional test have been provided for AML.

Calcein AM (calcein acetoxymethyl ester) is a fluoregenic dye, which is a hydrophobic, non-fluorescent compound that readily penetrates the cell membrane. Cells exposed to calcein AM become fluorescent after the cleavage of calcein AM by endogenous esterases that produce a fluorescent derivative calcein. Calcein is a bright green-fluorescent dye, which does not affect most cell functions, including cell viability. P-gp, the product of multidrug transporter MDR1 gene, actively extrudes the calcein AM, but not the fluorescent calcein.[11] On the other hand, fluorescent calcein and calcein AM are extruded by the MRP.[11] Therefore, calcein AM uptake (with specific modulator of P-gp) can be used to assess whether MDR1 is functional and calcein efflux can explore MRP activity. Thus, with the calcein AM functional assay, the role and relative importance of P-gp and MRP can be clarified in acute leukaemia samples.

Most of the studies of these MDR proteins were done in AML cases. Amongst all the MDR proteins studied in AML cases, the role of P-gp expression has been shown to correlate with the clinical outcome.[12,13] However, the potential role of MRP and LRP expression in AML cases is still investigated. In this study, we examined the expression and functional activities of P-gp and MRP in newly diagnosed acute leukaemia cases in Hospital Universiti Kebangsaan Malaysia (HUKM).

MATERIALS AND METHODS

Patients

A total of 43 bone marrow (BM) and peripheral blood (PB) samples from newly diagnosed acute leukaemia cases (19 paediatric ALL cases and 24 adult AML cases) were analyzed. The diagnosis of acute leukemia was based on French-American-British (FAB) criteria. Immunophenotyping was performed by using flow cytometry. The age, sex and white blood cell count at diagnosis as well as response to induction chemotherapy was recorded (Table 1). Complete remission (CR) to induction chemotherapy was defined as normal marrow cellularity with less than 5% of blast cells and near normal peripheral blood counts after completion of the induction period.[11] Bone marrow and peripheral blood samples from patients who underwent bone marrow aspiration for diagnosis of non-malignant conditions were analyzed as negative controls. The samples were collected after consent was obtained from the patients.

Extraction of mononuclear (blast) cells

Bone marrow or peripheral blood samples that included more than 70% leukaemic cells were collected in EDTA anticoagulant after patient's informed consent. The mononuclear or blast cells were separated by density gradient centrifugation using Lymphoprep solution (PAA, Laboratories, USA) at 2400 rpm for 30 minutes at room temperature. After separation, the harvested blast cell fractions were collected and washed twice in RPMI 1640 with L-glutamine (PAA, Laboratories, Germany) supplemented with 1% heat-inactivated fetal calf serum (PAA, Laboratories, USA). Assessment of cells number and viability was performed by trypan-blue exclusion.

Detection of MDR proteins by flow cytometry

The expression of P-gp, LRP, MRPs and BCRP (breast cancer resistance protein) proteins was evaluated by flow cytometry (FACScan, Becton Dickinson (BD) Bioscience, USA). All monoclonal antibodies were employed using the indirect technique as previously described [2,14] with slightly modification. P-gp was detected by

TABLE 1: Clinical and biological characteristic of 43 patients with newly diagnosed acute leukaemia

	Pediatric ALL (n=19)	Adult AML (n=24)
Gender		
Male (%)	68.4%	45.8%
Female (%)	31.6%	54.2%
Age (years)		
Mean ± SD	5.47 ± 3.92	48.58 ± 21.02
Median	4.00	43.50
Blast (%)		
Mean ± SD	84.21 ± 12.18	78.39 ± 18.58
Median	90.00	85.00
WBC count ($x10^9$/L)		
Mean ± SD	120.79 ± 109.84	75.55 ± 79.90
Median	73.00	57.20
FAB classification (%)		
	L1= 57.9%	M0= 4.2%
	L2= 42.1%	M2= 8.3%
		M3= 12.5%
		M4= 50.0%
		M5a= 4.2%
		M5b= 4.2%
		M6= 4.2%
		UD= 12.5%

UD = undetermined FAB, n = number of cases/patients

the monoclonal antibodies 17F9 (extracellular epitope; Pharmigen, Becton Dickinson, USA), which is mouse IgG_{2b} antibody. In brief, after red cell lysis with 1X lysing buffer (eBioscience, USA), blast cells were incubated with the primary antibody for 30 min at room temperature. After two washes, staining was revealed by incubation with the fluorescein-isothiocyanate (FITC) goat anti-mouse antibody (GAM) (BD Pharmigen, USA) for 15 min in the dark, washed again and analyzed. The MRP1 expressions were detected by QCRL-3 (anti-MRP1; mouse IgG_{2a}) (Kamiya, Biomedical Company, USA). For intracellular staining, the cells were pretreated with a fixing and a permeabillizing buffer (eBioscience, USA). Cells (1×10^6) were fixed in a 1X fix buffer (eBioscience, USA) for 30 min in the dark at room temperature. After two washes in 1X PBS buffer (Sigma Diagnostic Kits; St. Louis, Missouri, USA), cells were incubated with 1X permeabillizing buffer containing MDR monoclonal antibodies for 30 min. Then, cells were washed twice in 1X permeabillizing buffer and re-incubated with either FITC-GAM or phycoerythrin (PE) GAM (BD Pharmingen, USA) for 15 min in the dark, washed again and analyzed. Negative controls were prepared in each experiment by replacing the specific primary antibody by irrelevant isotypic Ig (eBiosience, USA). Flow cytometric analysis was conducted using a (FACScan BD, USA) operated at 488 nm, which detects green (FITC) and red (PE) fluorescence. Data acquisition and analysis were performed with the CellQuest™ software (FACScan, BD, USA). Samples of peripheral blood from healthy donors (negative control) were investigated simultaneously. Results were expressed by the mean fluorescence index (MFI, i.e. by the ratio between the mean histogram fluorescence intensity generated by incubation with the MDR proteins, and MFI of negative isotypic controls).

Functional test using MultiDrugQuant™ Assay Kit (Chemicon, USA)

The assay consists of four well-separated stages: preparation of reagents and samples, reaction, measurement and calculations as provided in

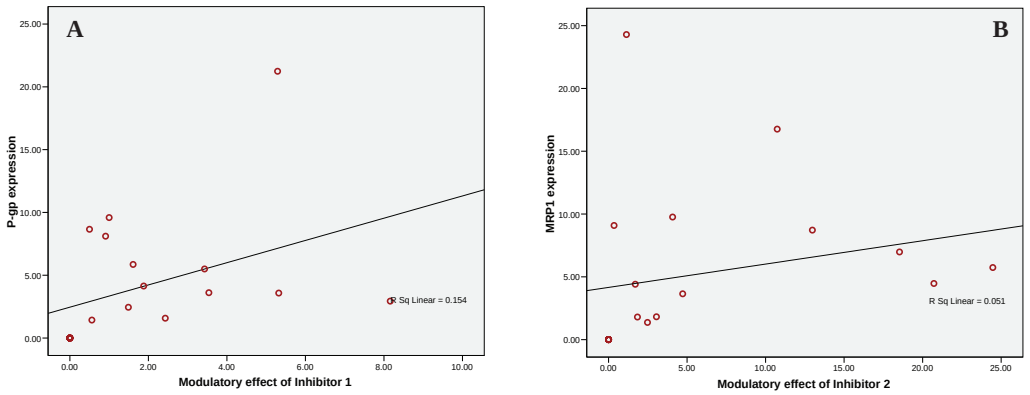

FIG. 1: Correlation between P-gp expression and the effect of Inhibitor 1 on calcein AM uptake (A) in leukaemic cells. Correlation between MRP1 expression and the effect of Inhibitor 2 on calcein efflux (B) in paediatric ALL cases.

manufacturer's protocol. Assaying one clinical sample involves three types of tubes measured in triplicate. In brief, a cell suspension containing 2-5 x 10^6 cells was prepared in 8mL of reaction buffer (1x). A total of 800 μL of cell suspension was aliquoted into tubes. Subsequently, 5μL of Inhibitor 1 was added into tubes labelled #1-3 and 5μL of Inhibitor 2 was added into tubes labelled #4-6. Samples were mixed thoroughly and incubated for 5 min at 37°C. Tubes labeled #7-9 served as negative controls. To begin the reaction, 200 μL of calcein AM solution were added into all nine tubes and incubated at 37°C for exactly 10 min. The reaction was stopped by rapid centrifugation and the cells were re-suspended in 0.5 mL of buffer containing propidium iodide (PI). Finally the samples were measured using flow cytometry within 24 hours. All data were calculated as the ratio of drug fluorescence with inhibitor divided by drug fluorescence without inhibitor after subtraction of the fluorescence of the control. Dead cells were gated out following scatter characteristics.

Statistical analysis

Test of normality using Shapiro-Wilk (W) for small samples size (<200) suggested the samples were not normally distributed (normality score W<1). Therefore correlation among levels of expression of continuous values was estimated using the Spearman rank coefficient.

RESULTS

Correlation of P-gp and MRP1 protein expression with functional activity in paediatric ALL cases

There is a significant correlation between P-gp expressions measured by flow cytometry and the modulatory effect of Inhibitor 1 on calcein-AM uptake ($r = 0.625$, $p<0.01$) (Figure 1A). We also found a significant correlation between MRP1 expressions and the modulatory effect of Inhibitor 2 on calcein efflux ($r = 0.676$, $p<0.01$) (Figure 1B). Two examples are shown in Figure 2. Thirteen (68.4%) of 19 cases showed P-gp expression and functional activity (Table 2).

TABLE 2: Median MFI* values for P-gp and MRP1 expressions and functional activities in paediatric ALL and adult AML cases (median ± interquartile)

	P-gp		MRP1	
	Protein expression (MFI)	Functional activity (MFI)	Protein expression (MFI)	Functional activity (MFI)
Paediatrics ALL (n=19)	4.14 ± 5.69 (n=13)	1.88 ± 3.46 (n=13)	5.74 ± 6.69 (n=13)	4.08 ± 13.49 (n=13)
Adults AML (n=24)	8.45 ± 14.86 (n=14)	4.48 ± 5.66 (n=14)	8.16 ± 7.28 (n=24)	12.09 ± 14.18 (n=24)

n = number of cases/patients; *MFI (median fluorescent intensity)

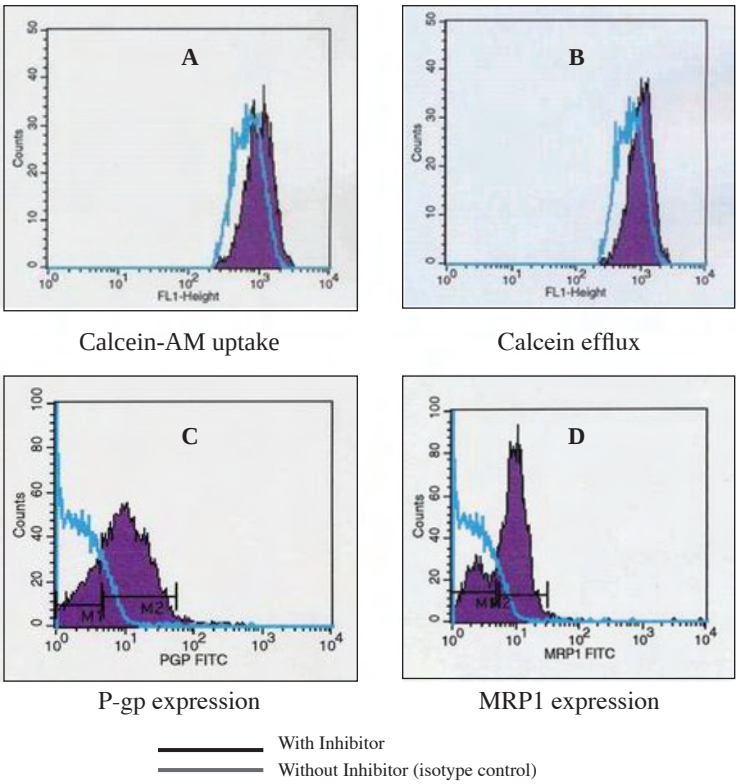

FIG. 2: Histogram profile of samples with or without P-gp and MRP1 expression and the modulatory effect of Inhibitor 1 and 2. The upper panel histogram presented samples with modulatory effect of Inhibitor 1 and 2. The lower histogram panel indicates the same samples with P-gp and MRP1 expression. The shifted histogram (purple) in A and B indicates population of cells with calcein-AM uptake and calcein efflux respectively. The histogram (purple) in C and D indicates the population of cells with high P-gp and MRP1 expression. The blue histogram line represents the isotype control (cells that were not exposed to any Inhibitor).

Thirteen (68.4%) cases also showed MRP1 expression and functional activitiy (Table 2). However, there were 6 cases (31.6%) which did not show P-gp or MRP1 expression and functional activity.

Correlation of P-gp and MRP1 Protein expressions with functional activity in adult AML cases

Figure 3A illustrates the significant correlation between P-gp expressions and the modulatory

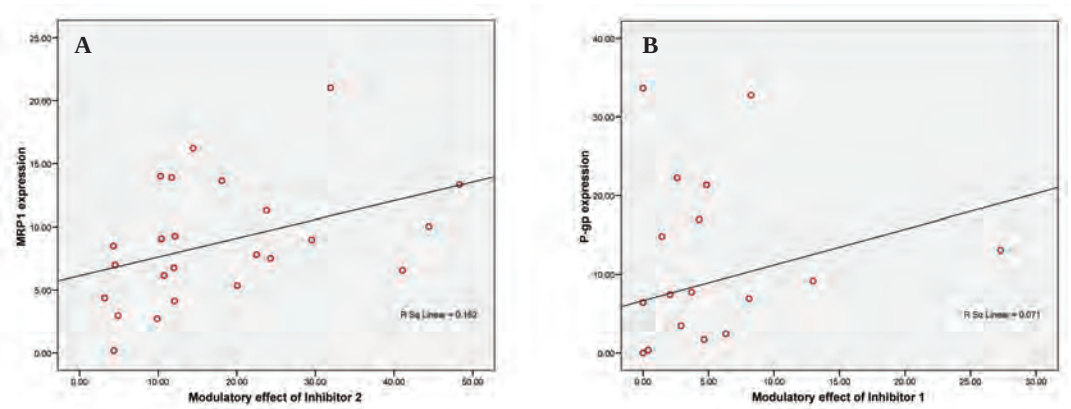

FIG. 3: Correlation between P-gp expression and the effect of Inhibitor 1 on calcein AM uptake (A) in leukaemic cells. Correlation between MRP1 expression and the effect of Inhibitor 2 on calcein efflux (B) in adult AML cases.

effect of Inhibitor 1 on calcein-AM uptake ($r = 0.625$, $p<0.01$). Figure 3B shows the significant correlation between MRP1 expression and the modulatory effect of Inhibitor 2 on calcein efflux ($r = 0.448$, $p<0.05$, $n = 24$). Fourteen (58.3%) of 24 cases showed P-gp expression and functional activity (Table 2). Eight (33.3%) cases showed no expression and functional activity of the P-gp protein. However, two (8.3%) cases showed P-gp expression but no functional activity. In addition, all these 24 cases showed MRP1 expression and functional activity (Table 2).

DISCUSSION

In this study, we evaluated the expression and function of two MDR-related proteins, P-gp and MRP1, in the leukaemic blast cells of 43 newly diagnosed acute leukemia cases. An *ex vivo* evaluation of the ability of the blast cells to accumulate calcein-AM or efflux calcein in either presence or absence of a specific inhibitor was used as a marker to determine functional activity of these proteins. Protein expression was evaluated by flow cytometry using specific monoclonal antibodies, and the results were expressed as mean fluorescence index (MFI = the ratio between the mean fluorescence intensity of cells incubated with specific monoclonal antibodies and the mean fluorescence intensity of respective isotypic controls) as according to previous studies on AML cases. [2, 11]

In previous studies, P-gp functional activity in AML cases were done by using Rh123 or daunorubicin with or without inhibitor and showed a good correlation between these MDR protein expressions and their functional activities.[15] However, recent studies have shown that calcein-AM was as specific and sensitive as Rh123 and daunorubicin.[11] In this study, we were able to show there is functional activity of P-gp and MRP1 proteins in newly diagnosed acute leukaemia cases by using the MultiDrugQuant™ Assay Kit. This functional test using calcein-AM correlated with P-gp and MRP1 protein expression in both paediatric ALL and adult AML cases. Therefore, calcein-AM might be used as a probe for both MRP1 and P-gp activities. An important consideration to note is that calcein efflux mediated by MRP1 might also be used by the other MRP1 homologues such as MRP2, MRP3, MRP4 and MRP5, which share similar drug transport mechanisms.

In a previous study on selected cell lines and fresh AML samples, the calcein-AM has been confirmed as a specific and sensitive probe for both P-gp and MRP functions.[11] The determination of the calcein-AM uptake (for P-gp analysis) and calcein efflux (for MRP analysis) of tested samples uses the same modulator (cyclosporine A) for both proteins. This method is time consuming as 90 minutes incubation time is required to determine calcein efflux for MRP functional analysis.[11] So far, only MultiDrugQuant™ Assay Kit has been developed to assess P-gp and MRP1 functional activity which is based on calcein-AM uptake or calcein efflux in leukaemic blast cells. There is very limited published information with regard to paediatric ALL cases, underscoring the need for further studies.

In conclusion, calcein-AM may be used in leukaemic blast cells to probe specifically both P-gp and MRP1 activities. The MultiDrugQuant™ assay kit is easy to use and may be recommended as a routine clinical diagnostic test and results can be obtained within one hour. This assay kit can be used as an essential tool to understand the MRP role in acute leukaemia cases.

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