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A Fluorescent Cell-Based Technique for Monitoring Efflux of MRP4

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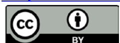
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Abstract

Background: Overexpression of efflux pumps is the drug resistance and adaptation mechanism employed by some eukaryotes and bacteria to transport endogenous and chemotherapeutic compounds from the intracellular to the extracellular environment. **Aim:** The study aimed at establishing a fluorescent cell-based assay to monitor the efflux activities of an ABC-transporter, multidrug resistance protein 4 (MRP4). **Methods:** DH5α competent *E. coli* cells were transformed with pcDNA-MRP4 by the heat-shock process. The presence of the MRP4 gene was analyzed by the digestion of plasmid using EcoRI and analyzed on a 1% agarose gel. HEK 293 cells were transfected with purified pcDNA-MRP4 under optimized conditions using a Polyethylenimine (PEI) protocol. The level of MRP4 in the HEK 293 cells was characterized by western blotting analysis using M_4I-10 anti-MRP4 and anti-Rat IgG (whole molecule)-Alkaline phosphatase antibodies. The fluorescent uptake study was performed by the incubation of 0.02 mM 8-[fluo-cAMP] with the MRP4-transfected and control HEK 293 cells for 1 h. The level of fluorescence was analyzed using fluorescence microscopy and spectrometer. **Results:** The agarose gel analysis showed a plasmid of 9.4 kb and restriction product of 5 kb, which correspond with the pcDNA and MRP4 sizes respectively. The western blot results of the transfection showed 4 µg pcDNA-MRP4 and the N/P ratio of 9 was the optimized condition to transfect our HEK 293 cells as it showed the broadest band. In the efflux studies, the fluorescence images of the MRP4-transfected HEK 293 cells were very low compared to the untransfected control. The level of fluorescence accumulation was significantly ($P \leq 0.0001$) higher 228.6 ± 13.1 RFU in the untransfected cells than the MRP4-transfected cells 8.6 ± 1.8 RFU. **Conclusion:** The higher levels of fluorescence detected in the control in both the fluorescent microscopy and spectrophotometer showed that MRP4-transfected cells had effluxed the

8-[fluo-cAMP] substrate out of the cell. This method could be employed in the detection of MRP4 functions in bacteria and cancer cells.

Keywords

Efflux Pump, Drug Resistance, ABC-Transporter, HEK 293, Fluorescence Assay

1. Introduction

The development of some novel drugs has been hampered due to the expression and upregulation of efflux pumps and secretory systems in some eukaryotic organisms and bacteria [1]. Efflux pumps are an energy-driven system used by both eukaryotes and some bacteria to transport molecules against the concentration gradient. This mechanism is both beneficial and deleterious. Some endogenous molecules in humans and microbes are translocated via this mechanism to sites that they are needed. During drug administration, cells that harbor these efflux pumps remove the drug from within the cell via these pumps rendering the drug inactive. In humans, a high level of expression of MRP4 mRNA has been found in kidney and prostate and low levels have been shown in blood cells, neurons, lungs, adrenal gland, testis, and ovary [2] [3] [4]. These efflux pumps have also been described in bacteria such as *Staphylococcus aureus* and *Escherichia coli* (MsbA) [5] [6].

Multidrug resistance protein 4 (MRP4) is an ATP-dependent transporter that pumps anionic biomolecules [2]. MRP4 translocates its substrates through the generation of energy via ATP hydrolysis. This energy causes the nucleotide-binding domain (NBD) to drive the conformational changes in the transmembrane domain (TMD) [7]. The molecules transported by MRP4 include cyclic adenosine monophosphate (cAMP), leukotrienes, folic acids, steroids, and antibiotics [3] [8] [9].

Cytotoxicity studies on MRP4-transfected cells showed broad substrate specificity as it can transport a range of endogenous and exogenous compounds [10]. The detection of the interactions between chemotherapeutic compounds and MRP4 is crucial for the development of the drugs to target cells or microorganisms that express this transmembrane transporter. These interactions provide information on bioavailability, drug-drug, and drug-biomolecular interactions. According to Lechner *et al.* [11], this will provide a more in-depth understanding of the pharmacokinetics of drugs in cells expressing MRP4.

This study aimed at developing a fluorescent cell-based assay to measure MRP-mediated efflux that could potentially be utilized for the screening of MRP4 inhibitors. 8-[Fluo-cAMP] was used as the substrate for the transporter because MRP4 is an active transporter of cyclic nucleotides monophosphates. 8-[fluo-cAMP] is a fluorescein-modified analog of the parent cAMP linked at the eighth position of the molecule via a six-atom spacer [12]. The study transfected human

embryonic kidney cells (HEK 293), a cancer cell line, known to express the MRP4 at extremely low levels. The essence of the transfection was to overexpress the transporter on the membrane of the HEK 293 cells. The MRP4-transfected cells and non-transfected control cells were treated with the fluorescent substrate, 8-[fluo-cAMP], and the level of fluorescence measured. This cell line has been previously used in the study of the MRP4 pump [10] [13]. The importance of this study is that there has been no previous record of a fluorescent cell-based investigation of MRP4. Previous researches detected MRP4 function by analyzing fluorescence in MRP4-containing vesicles and lysed cells [12] [14].

2. Methods

2.1. Materials

Dulbecco's modified eagle medium (DMEM) high glucose (4.5 gl^{-1}) with glutamine, Dulbecco's PBS (1×) without Ca and Mg and Trypsin-EDTA (1×) were purchased from PAA Laboratories GmbH, Austria. Anti-rat IgG (whole molecule)-Alkaline phosphatase antibody produced in rabbit and BCIP/NBT liquid substrate system were purchased from Sigma-Aldrich, USA. M_4I-10 anti-MRP4 antibody (Enzo Life Sciences); Penicillin-streptomycin, fetal calf serum (FCS), Polyethylenimine (PEI) (Sigma, USA); Bio-Rad D_c protein assay reagents A, B and S (Bio-rad, Hercules, CA); Immobilon-P (PVDF, pore size 0.45 um, Billerica, MA); 8-[fluo-cAMP] (BioLog Life Institute Germany) and Fluorescent spectrometer (Perkin Elmer, LS 55).

2.2. Transformation of *E. coli* with pcDNA-MRP4

The vector, pcDNA-MRP4, encodes the transporter gene MRP4 and ampicillin-resistance gene. DH5α competent *E. coli* cells were transformed with pcDNA-MRP4 by the heat-shock process. The heat-shocked *E. coli* cells were incubated in LB broth and plated on LB-ampicillin plates and incubated at 37˚C overnight. The transformed pcDNA-MRP4 *E. coli* cells were cultured by incubated individual colonies of the transformed cells in LB-ampicillin broth overnight.

2.3. Purification of the pcDNA-MRP4 Plasmid

The overnight culture of pcDNA-MRP4-transformed *E. coli* cells was purified according to GeneJET™ Plasmid Miniprep Kit (PureExtreme, Fermentas: Life Sciences). The concentration of the purified pcDNA-MRP4 was determined using a nanospectrometer at a wavelength of 230 nm. The purified pcDNA-MRP4 was stored at −20˚C. To show the presence of MRP4 in pcDNA-MRP4, the plasmid was digested with EcoRI. Both the undigested and digested pcDNA-MRP4 were analyzed on 1% agarose gel.

2.4. HEK 293 Cell Culture

HEK 293 cells were cultured in DMEM supplemented with 10% FCS (Invitrogen, Cergy Pontoise), and 1% Penicillin-Streptomycin, at 37˚C in a humidified

5% CO_2 atmosphere. Cell splitting of the HEK 293 cells are washed with PBS and detached from the flask using Trypsin-EDTA. Cell splitting was performed when cell confluency is up to 70%.

2.5. Seeding Cells into Six-Well Plates

The old medium was removed from the flask and cells were washed with PBS then cells were detached using trypsin-EDTA (at 37˚C). The old DMEM medium was replaced with a fresh medium and centrifuged at 10,000 rpm for 5 minutes at room temperature. The cell pellet was re-suspended in DMEM medium. The cell density was determined using hemocytometer and cells were seeded in a six-well plate at a concentration of 5×10^5 cells/ml and 2 ml per well. 25 kDa PEI was dissolved in distilled water to make the final concentrations of 10 mM while glucose was dissolved to make 5% of the distilled water. The dissolved PEI and glucose were filter sterilized. Because the efficiency of PEI transfection depends on the ratio of phosphate in the DNA to the nitrogen in the PEI (N/P ratio), the different transfection conditions were achieved by varying this ratio. The different amounts of PEI used were calculated as follows:

$$1\ \mu\text{g of DNA} = 3\ \text{nmol DNA phosphate}$$

$$1\ \mu\text{l of 10 mM PEI} = 10\ \text{nmol amine nitrogen}$$

$$\frac{(\mu\text{g of DNA} \times 3) \times \text{N : P ratio}}{10} = \mu\text{l 10 mM PEI}$$

The above equation was used to obtain the DNA amounts and PEI used. The transfection mix was 10% of the medium used 200 μl/well; 100 μl of DNA/glucose mix and 100 μl of PEI/glucose mix.

The sterile PEI was mixed with the 5% sterile glucose and pcDNA-MRP4 was also mixed with the 5% sterile glucose. The PEI/glucose complex was added to the pcDNA-MRP4/glucose mixture at different N/P ratios (*different amounts of DNA were used in some instances) (N/P = 9 [4 μg DNA]; N/P = 12 [*4 μg DNA]; N/P = 12 [*2 μg DNA]; N/P = 15 [*4 μg DNA]; and N/P = 15* [2 μg DNA]) and incubated at room temperature for 30 minutes. N/P ratios of PEI/DNA complexes were the ratios of moles of the nitrogen groups of PEI to those of the phosphate of DNA. The PEI/DNA/glucose mixtures were added to the DMEM medium. The old medium was discarded from the cells and PEI/DNA/glucose/medium was added to the cells and the N/P ratios of the different wells were as mentioned above. A control was set up by adding only DMEM medium and no transfection mixture. The cells were incubated at 37˚C in a humidified 5% CO_2 atmosphere for 48 h. The amount of protein of each fraction was estimated using a Bio-Rad protein assay (Bio-Rad, Hercules, CA).

2.6. Western Blot Analysis and Immunoblotting with Anti-MRP4 in Rabbit

The western blotting for MRP4 detection was carried out using a whole-cell lysate. The samples were loaded on a 7% SDS-PAGE and run at 100 volts for 1 h.

The proteins were transferred to a PVDF Immobilon transfer membrane (pore size 0.45 μm) at 100 volts for 1 h. Following protein transfer, the PVDF membranes were placed in blocking buffer (4% skimmed milk in 1 × TBS-T) for 1 h before the addition of the primary antibody. The M_4 1 - 10 anti-MRP4 antibody (primary antibody) produced in the rat was added to 4% skimmed milk in 1 × TBS-T in a ratio of 1:1000 and incubated with the PVDF membranes overnight at 4˚C. The membrane was washed three times with 5 minutes intervals in 1 × TBS-T. The PVDF membranes were transferred into 4% skimmed milk in 1 × TBS-T with anti-rat alkaline phosphate produced in rabbit (secondary antibody) in the ratio 1:5000. The PVDF membranes were incubated with the secondary antibody at room temperature for approximately 1 h and then washed five times in 1 × TBS-T with 5 minutes intervals of each washing. The membrane was developed by adding 2 ml of BCIP/NBT liquid substrate system and photographed using a camera.

2.7. 8-[Fluo-cAMP] Efflux Studies

HEK 293 cells were seeded in a six-well plate at 5×10^5 cells in 10 ml of DMEM medium. The cells were transfected with pcDNA-MRP4 (N/P = 9:1) and incubated for 48 h after which the western blot analysis was performed. The DMEM medium was removed and fresh medium containing 8-[fluo-cAMP] (0.02 mM) was added. Following 1 h incubation, the medium was removed and the wells were carefully rinsed with PBS. The PBS was discarded and a fresh PBS was added to the cells. The fluorescence of the cells was analyzed using a fluorescent microscope. The cells were harvested and centrifuged at 13,000 rpm for 2 minutes. The cell pellets were lysed and the amount of fluorescence in the cells was analyses using a fluorescence microscope and fluorescence spectrometer.

2.8. Data Analysis

The western blot paper was photographed with a camera. Where necessary data was analyzed on GraphPad Prism 8.2.1 (San Diego CA) using t-test and results were considered to be statistically significant at $P < 0.05$.

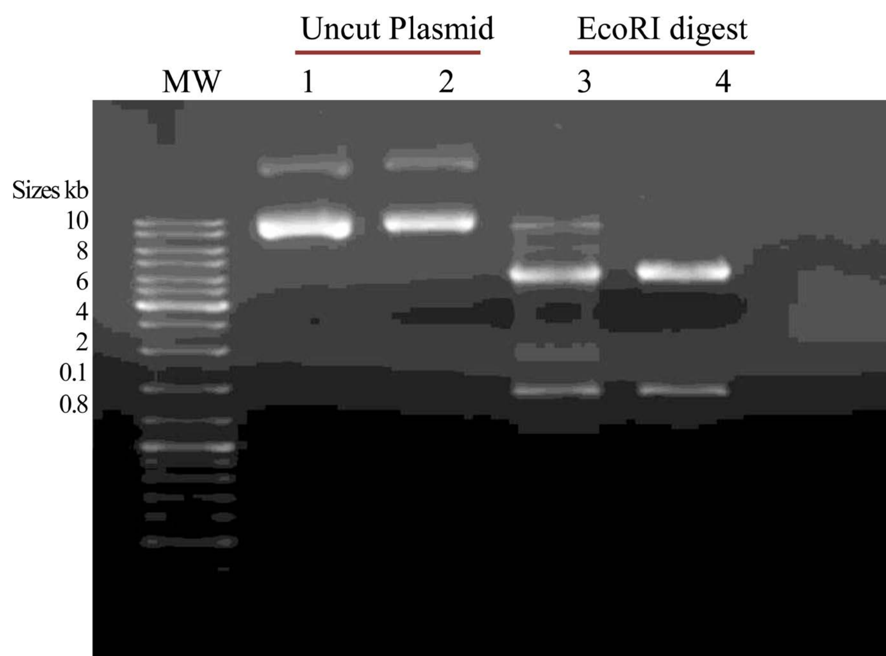
3. Results

3.1. pcDNA-MRP4 Products of EcoRI Digestion on Agarose Gel

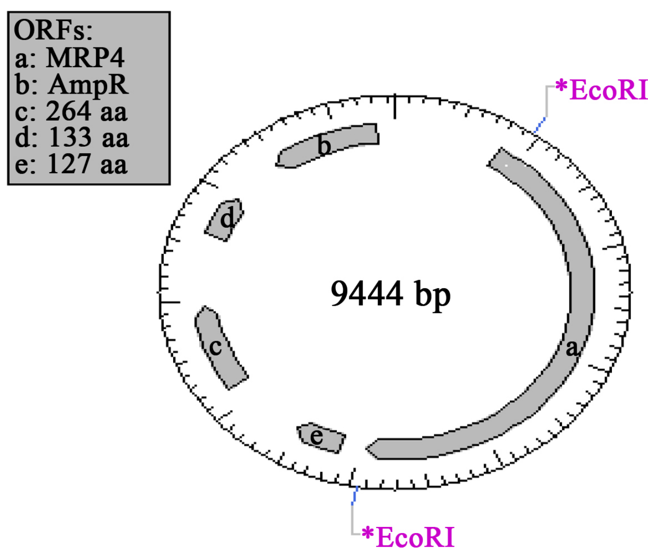
Figure 1 shows the products of the restriction digestion of the pcDNA-MRP4 plasmid on 1% agarose gel. The undigested plasmid (lanes 1 and 2) showed two bands; one at >10 kb and the other at 9.4 kb. Lanes 3 and 4 were loaded with the products of EcoRI digestion and produced 2 bands: one set of large bands of approximately 5 kb and another set of faint bands of about 0.8 kb.

3.2. MRP4 Expression in Transfected HEK 293

The levels of MRP4 in the transfected cell line during the optimization were



(a)



(b)

Figure 1. Agarose gel electrophoresis of the uncut and digested pcDNA-MRP4 used for HEK 293 cell transfection. (a) The lane M was loaded with a 1 kb standard DNA ladder. Both lanes 1 and 2 contained undigested pcDNA-MRP4 while lanes 3 and 4 contained EcoRI digested pcDNA-MRP4; (b) MRP4 plasmid size and the expected products of EcoRI digestion.

quantified using western blot analysis as shown in Figure 2(a). Each well was loaded with an equal amount (50 µg for Figure 2(a)) of the crude lysate. All the conditions in the optimization showed some bands (Lanes 3 - 7, Figure 2(a)) which traveled the same distance as the standard MRP4 control (Lane 2, Figure 2(a)) of 150 kDa. However, the treatment condition with the best transfection was HEK 293 treated with 4 µg of pcDNA-MRP4 with an N/P ratio of 9 shown

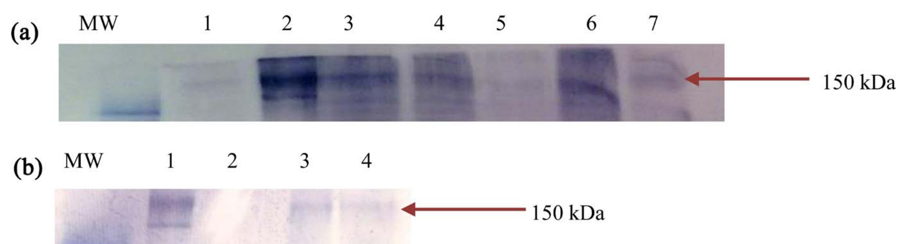


Figure 2. A western blot of the crude lysate of MRP4 in transfected HEK 293 cells. (a) Western blot for the six different transfection conditions loaded with 50 µg: MW (molecular weight) lane 1 HEK 293 control (no MRP4 DNA), lane 2 positive control (standard MRP4), lane 3 (4 µg DNA N/P = 9), lane 4 (4 µg DNA N/P = 12), lane 5 (2 µg DNA N/P = 12), lane 6 (4 µg DNA, N/P = 15) and lane 7 (2 µg DNA N/P = 15); (b) Western blot of the HEK 293 cells used for efflux studies, 30 µg of crude lysate was loaded per lane as follows: lane 1 positive control (standard MRP4), lane 2 HEK 293 control (no MRP4 DNA), lanes 3 and 4 were (4 µg DNA N/P 9). MRP4 was detected using M_4I-10 anti-MRP4 as primary antibody and anti-rat IgG (whole molecule)-Alkaline phosphatase antibody was the secondary antibody. The membrane was developed using BCIP/NBT liquid substrate system [N: nitrogen and P: phosphate].

in lane 3 (Figure 2(a)). This optimized condition was further analyzed with a lower amount of crude lysate of 30 µg (Figure 2(b)) using western blotting. The result showed the bands traveled at the distance as the corresponding positive control (Lane 2 Figure 2(b)). In both western blotting analysis, the untransfected control showed very faint bands (Lanes 1 for Figure 2(b) and Lane 2 Figure 2(b)).

3.3. 8-[Fluo-cAMP] Efflux Studies

The efflux studies analyzed the fluorescence generated both by qualitative and quantitative methods. Figure 3 shows the qualitative analysis of the fluorescence of HEK 293. This depicts the image of the fluorescent microscopic examination of untreated and MRP4-transfected HEK 293 cells. The MRP4-transfected cells showed a very low level of green fluorescence of the 8-[fluo-cAMP) while the control cells exhibited more visible fluorescence. Figure 4 demonstrates the fluorescent spectra of untransfected cells, MRP4-transfected cells, and 8-[fluo-cAMP] as well as the levels of 8-[fluo-cAMP] induced fluorescence in untransfected cells, MRP4-transfected cells. The fluorescent spectra (Figure 4(a)) show the peaks of the fluorescence in all cases occurred at approximately 517 nm. The fluorescein molecule shows the highest level of fluorescence of about 750 RFU followed by the untransfected control with about 230 RFU then the MRP4-transfected cells with about 9 RFU but no observable peak. The level of fluorescence accumulation was significantly ($P \leq 0.0001$) higher 228.6 ± 13.1 RFU in the untransfected cells than the MRP4-transfected cells 8.6 ± 1.8 RFU (Figure 4(b)).

4. Discussion

The movement of biological molecules across the membranes plays an indispensable role in the maintenance of normal physiological activities in many bacteria and

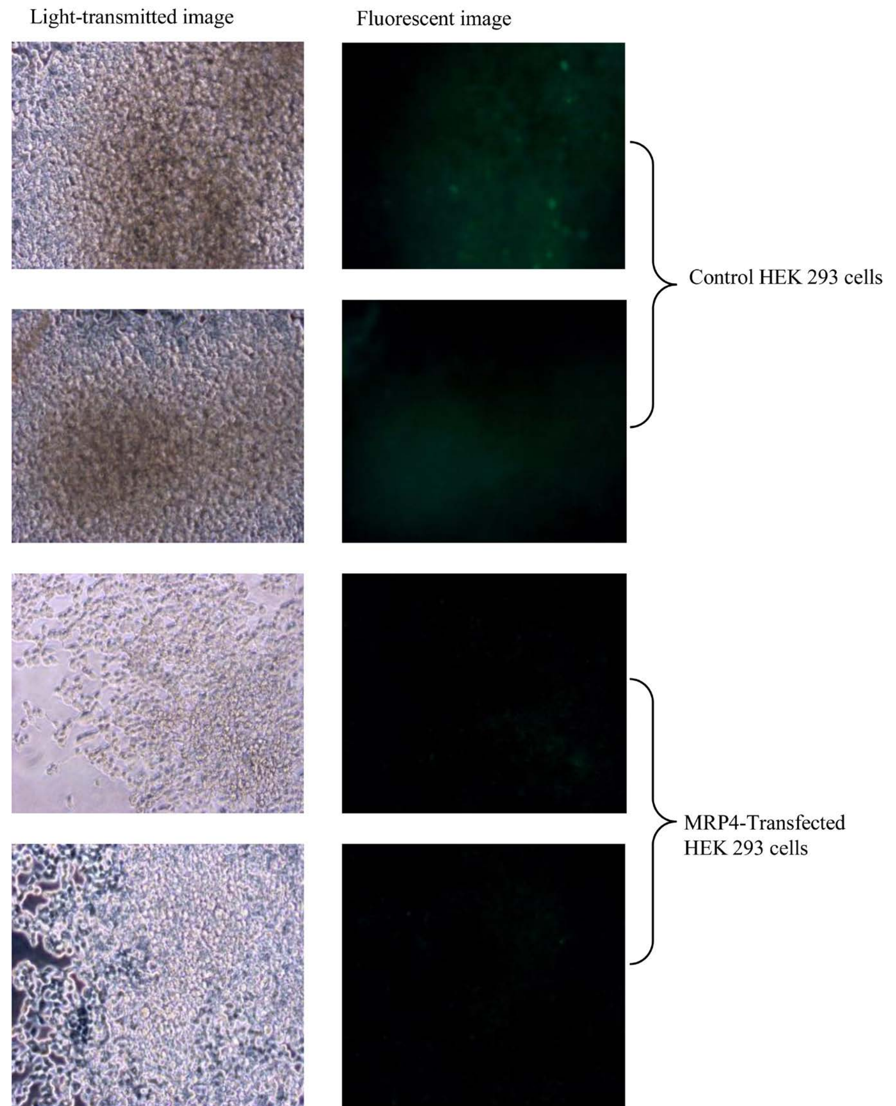


Figure 3. Fluorescent microscopic images of both control and MRP4-transfected HEK 293 cells treated with 8-[fluo-cAMP] used for the experiment. This was performed under the 40× objective of the fluorescent microscope.

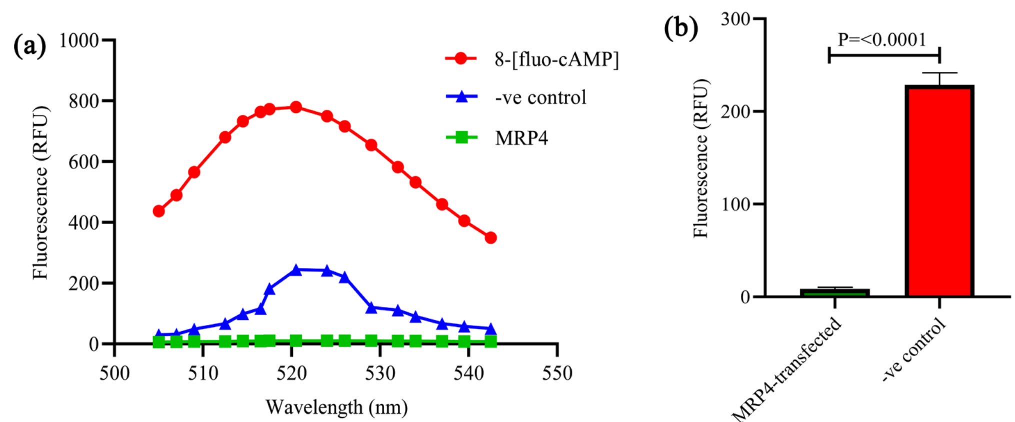


Figure 4. Fluorescence spectra and levels of (a) 8-[fluo-cAMP] and (b) both the control and MRP4-transfected HEK 293 cells.

cancer cells. This migration of biomolecules occurs mostly via the action of efflux pumps due to either the size or charge of these molecules [15] [16]. The current study investigated the efflux function of MRP4, a member of the ATP-binding cassette of efflux pumps that could be found in bacteria such as *E. coli*, *P. aeruginosa*, and *S. enterica* serotype *typhimurium* [1] [17] [18] [19].

A fluorescent cell-based approach offers a real-time, efficient, and rapid method of monitoring the efflux pump interactions. This method could further provide a suitable strategy for blocking its transport activities and potential drug-drug interactions indispensable in drug design [20].

The faint band seen in the undigested lane was more than 10 kb which could be another supercoiled plasmid present in the *E. coli* isolate while the 9.4 kb band represents the pcDNA-MRP4 plasmid (**Figure 1(a)** and **Figure 1(b)**). For lanes 3 and 4, the broadest bands represent the 5 kb which corresponds with the size reported by Barik *et al.* [21]. The other faint bands correspond to the ampicillin resistance gene and other associated regulatory genes. The purity of the digestion was high as there was no smear produced.

The western blot analysis showed the transfected MRP4 gene, using the PEI protocol, highly expressed the transporter in HEK 293 cells which could be confirmed by the presence of the bands at 150 kDa (**Figure 2**). The evidence to show that the optimization of the transfection protocol was effective is as a result of the extremely low level of expression noticed in the untransfected control cells. This level of expression in control condition is possible as HEK cells are kidney cells that have been reported to show some basal level of expression of the protein transporter molecule [22] [23] [24]. Some bacteria have been found to also overexpress the efflux pump either as a means of exporting endogenous substances or removing compounds that are cytotoxic to them [25] [26].

Other studies carried out to monitor the function of MRP4 used MRP4 in vesicles [11] [14] to detect the efflux activities of the transporter but not the transfections of the gene. The transfection protocol adopted in the current investigation used PEI which is a 25 kDa cationic polymeric molecule that condenses the DNA into positively charged particles enabling its endocytosis into the cell [27]. The determination of the appropriate N/P ratio and DNA concentration is crucial in influencing the transfection efficiency of the vector [27] [28].

The efflux studies utilized 0.02 nM of the fluorescein substrate incubation with the HEK 293 for 1 h. The fluorescent microscopic images show the untransfected control cells emitted higher fluorescence than the MRP4-transfected (**Figure 3**). The two top green images represent the control cells (untransfected) while the two bottom images represent the MRP4-transfected cells. A similar observation was noted for the quantitative evaluation of the fluorescence level (**Figure 4**). A lower level of fluorescence was expected in the MRP4-transfected cell as the efflux pump will remove the accumulated fluorescent substrate from the intracellular compartment of the HEK 293 cells to the surrounding thereby leading to the reduced substrate [14]. The higher the level of MRP4 expressed on the

membrane of the cells, the lower the expected fluorescence emission. However, as the level of fluorescence was much lower in the control cells than the 8-[fluo-cAMP] trace as shown in **Figure 4(a)**, it implies that more optimization techniques are necessary for the 8-[fluo-cAMP] uptake studies to increase the level of fluorescence. This could be achieved by altering the concentration of the fluorophore over different time intervals. Again, the level of fluorescence in the transfected cells was expected to be lower or completely non-existence as the expression of the pump will indicate the elimination of the fluorescent substrate. A better level of fluorescence can be achieved through more optimization of the transfection protocol.

5. Conclusion

The study has established a fluorescent cell-based method for MRP4 interactions using 8-[fluo-cAMP], evident in the lower level of fluorescent in MRP4-transfected cells compared to the control cells. It has also established the optimization of the transfection conditions for MRP4 using a PEI protocol.

Authors' Contribution

Monsi, T.P. contributed to the study design, performing the study, analysis of the data, writing the manuscript, literature search, and guarantor of the manuscript. Ben-Chioma, A.E. contributed to the analysis of the data, literature search, and reviewing of the manuscript. Onwuli, D.O. contributed to the integrity of the manuscript, analysis of the data, literature search, and reviewing of manuscripts.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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