Evaluation of Cucurbitane-type Triterpenoids from *Momordica balsamina* on P-Glycoprotein (ABCB1) by Flow Cytometry and Real-time Fluorometry

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Abstract. *Background: Cancer cells become refractory to chemotherapy as a consequence of their overexpressing ABC transporters that extrude not only the therapeutic agent but other unrelated compounds such as chemotoxins and biocides before they can reach their intended targets. A compound that can inhibit the activity of these transporters may find use as an adjunct to chemotherapy that had been rendered ineffective. Materials and Methods: Four curcubitane-type triterpenes isolated from Momordica balsamina Linn. (Cucurbitaceae), a plant from Mozambique were evaluated for their inhibition of the ABC transporter P-glycoprotein coded by the human ABCB1 gene transfected into mouse lymphoma cells. The evaluation was conducted by flow cytometry using rhodamine 123 and real-time fluorometry assessing accumulation of ethidium bromide (EB) on a real-time basis. Results: Among the compounds isolated, the most active was 7-methoxycucurbita-5,24-diene-3β,23(R)-diol, which inhibited the efflux of ethidium bromide (EB) and rhodamine 123 from the ABCB1-transfected mouse lymphoma cell. Conclusion: Real-time fluorometry replicated the flow cytometric results with significant advantages for the evaluation of efflux pump inhibitors. The substitution of side groups on the cucurbitane skeleton appears to be significant in the inhibition of ABCB1 activity.*

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Cancer becomes refractory to therapy by the initial chemotherapeutic agent as well as to other non-related anticancer drugs as a consequence of overexpression of distinct ATP-binding cassette (ABC) transporters that extrude the drug prior to it reaching its intended target (1). The ABCB1 (P-glycoprotein) drug efflux pump, encoded by the gene *ABCB1* (*MDR1*), is the most widely studied ABC transporter in multidrug resistance (MDR) (2). Efflux mediated by ABC drug transporters leads to decreased cellular accumulation of anticancer drugs, which is a main cause of the limited success of the chemotherapy. The ABCB1 transporter has an unusually broad specificity and can bind a large variety of hydrophobic natural-product drugs as they enter the plasma membrane. These drugs include many of the commonly used natural-product anticancer drugs such as doxorubicin, daunorubicin, vinblastine, vincristine and taxol (3). Also among these agents are compounds such as verapamil (4) and phenothiazines (5), which prevent the binding of Ca^{2+} to calcium-dependent ATPases.

During recent years, the search for compounds with the capacity to inhibit overexpressed transporters of cancer cells has been extended to plants (6). Although many such compounds have been isolated, very few have been evaluated for toxicity in the same study (7). Naturally occurring substances play an important role in drug discovery and development. In the herein report, we have evaluated a

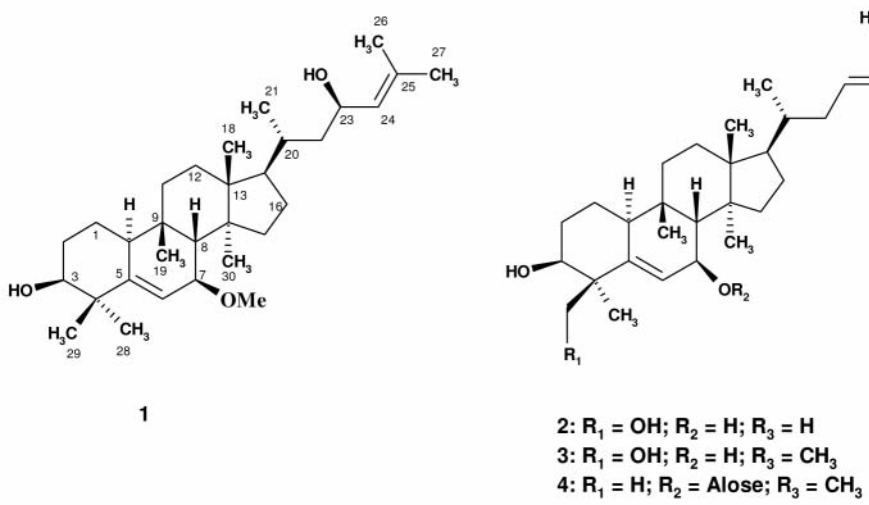

Figure 1. *Compounds isolated from Momordica balsamina L.*

Mozambican plant of the Cucurbitaceae family, *Momordica balsamina* L., for the presence of compounds that inhibit the activity of the ABCB1 transporter coded by human *ABCB1* gene transfected into mouse lymphoma cells.

The effect of an agent on the ABCB1 is normally conducted with the use of flow cytometry. This involves the employment of a fluorochrome substrate such as rhodamine 123 which is extruded by the ABCB1 transporter and which is increasingly retained if the transporter is inhibited (8). We previously developed an automated method that utilized the fluorochrome ethidium bromide (EB) which is considered to be a universal substrate of bacterial efflux pumps. Ethidium bromide has been shown to be particularly suitable for use as a probe because it emits weak fluorescence in aqueous solution (outside cells) and becomes strongly fluorescent in non-polar and hydrophobic environments. Based on our former results with bacterial (9) and eukaryotic (10) ABC-transporters, we employed this methodology, as well as flow cytometry (13), to detect and demonstrate the activity of the compounds isolated from *Momordica balsamina* on the eukaryotic efflux pump ABCB1.

Materials and Methods

Compounds tested. Four compounds with the cucurbitane skeleton, whose structures are presented in Figure 1, were tested for their anti-MDR activity: 7-methoxycucurbita-5,24-diene-3β,23(R)-diol (**1**), cucurbita-5,23(E)-diene-3β,7β,25,29-tetraol (**2**), 25-methoxycucurbita-5,23(E)-diene-3β,7β,29-triol (**3**) and 25-methoxycucurbita-5,23(E)-diene-3β-ol-7-*O*-β-D-allopyranoside (4). Compounds **1-4** were isolated and characterized previously by Ramalhete *et al*. (13) from the methanol extract of *Momordica balsamina*. All the compounds were dissolved in DMSO. The plant was collected in Gaza, Mozambique (August 2006). The plant was identified by Dr. Silva Mulhovo, and a voucher specimen (30 SM) has been deposited at the herbarium (LMA) of the Instituto de Investigação Agronómica of Mozambique.

Cell lines. L5178 mouse T-cell lymphoma cells 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, PAR) and the human *ABCB1*-gene transfected sub-line (MDR) were cultured in 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 (penicillin, streptomycin) at 37˚C and in a 5% CO_2 atmosphere.

Assay for antiproliferative effect. The effects of increasing concentrations of the drugs alone on cell growth were tested in 96-well flat-bottomed microtitre plates. The compounds were diluted in a volume of 50 μl medium and 1×10^4 cells in 0.1 ml of medium were added to each well, with the exception of the medium control wells. The culture plates were incubated at 37˚C for 72 h; at the end of the incubation period, 15 μl of dimethyl thiazolyl blue tetrazolium bromide (MTT; Sigma, St. Louis, MO, USA) solution (from a 5 mg/ml stock) were added to each well. After incubation at 37˚C for 4 h, 100 μl of sodium dodecyl sulfate (SDS) (Sigma) solution (10%) were measured into each well and the plates were further incubated at 37˚C overnight. The cell growth was determined by measuring the optical density (OD) at 550 nm (ref. 630 nm) with a Multiscan EX ELISA reader (Thermo Labsystems, Cheshire, WA, USA). Inhibition of the cell growth was determined according to the formula:

$$ID_{50} = 100 - \left[\frac{OD\,sample - OD\,medium\,control}{OD\,cell\,control - OD\,medium\,control}\right]\times 100$$

Where ID_{50} is defined as the inhibitory dose that reduces the growth of the compound- exposed cells by 50%. The ID_{50} values are expressed as means±SD from three experiments.

Table I. *Antiproliferative effects of compounds **1-4** on multidrug resistant mouse lymphoma cell line (L5178).*

Compound	ID_{50} (μM)
1	16.8±2.2
2	25.9±2.4
3	16.8±1.9
4	35.5±2.6

Values represent the mean±SD of three independent experiments.

EB accumulation assay. The cells were adjusted to a density of 2×10^6 cells/ml, centrifuged at 2,000×g for 2 minutes and re-suspended in phosphate-buffered saline (PBS) at pH 7.4. The cell suspension was distributed in 90 μl aliquots into 0.2 ml tubes. The tested compounds were individually added at different concentrations (3 and 30 μM) in 5 μl volumes of their stock solutions and the samples were then incubated for 10 minutes at 25˚C. Verapamil was used as a positive control (10). After this incubation, 5 μl (1 μg/ml final concentration) of EB (20 μg/ml stock solution) were added to the samples and the tubes were placed into a Rotor-Gene 3000™ thermocycler with real-time analysis software (Corbett Research, Sidney, Australia) 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 (9). The results were evaluated by Rotor-Gene Analysis Software 6.1 (Build 93) provided by Corbett Research. From the real-time data, the relative final fluorescence (RFF) of the last time point (minute 60) of the EB accumulation assay was calculated according to the formula given in Table III. The RFF value is the difference between the relative fluorescence (RF) at the last time point of the EB retention curve of cells in the presence of an inhibitor and the RF at the last time point of the EB retention curve of the untreated solvent control.

Flow cytometric assay for evaluation of a compound based on the retention of rhodamine 123. The cells were adjusted to a density of 2×10^6/ml, re-suspended in serum-free McCoy's 5A medium and distributed in 0.5 ml aliquots into Eppendorf centrifuge tubes. Test compounds were added at different concentrations in (10 μl of 0.025-1 mM), and the samples were incubated for 10 min at room temperature. Subsequently, 10 μl (5.2 mM final concentration) of the indicator rhodamine 123 were added to the samples and the cells were incubated for a further 20 min at 37˚C, washed twice and re-suspended in 0.5 ml PBS for analysis. The fluorescence uptake of the cell population was measured with FACS Star Plus flow cytometer (Beckton, Dickinson and Company, Franklin Lakes, NJ, USA). Verapamil was used as a positive control. The percentage mean fluorescence intensity was calculated for the treated MDR and PAR cell lines as compared to untreated cells. A fluorescence activity ratio (FAR) was calculated *via* the following equation, on the basis of the measured fluorescence values:

$$FAR = \frac{MDR\ treated\ /\ MDR\ control}{PAR\ treated\ /\ PAR\ control}$$

Table II. *Effect of compounds **1-4** on reversal of multidrug resistance (MDR) on human ABCB1 gene-transfected mouse lymphoma cells.*

Compound	Concentration (μM)	FSC	SSC	FL-1	FAR
PAR+R123	-	436.8	194.0	956.7	
MDR+R123	-	455.7	286.4	18.1	
Verapamil	22.0	451.4	277.0	97.1	7.4
1[a]	0.5	456.4	215.3	12.7	1.5
	1.0	460.7	217.8	130.0	15.0
	2.0	468.8	215.4	365.7	42.1
	20.0	551.8	206.8	399.5	46.0
2	2.0	441.8	261.2	14.0	1.1
	20.0	447.4	257.6	579.8	44.3
3	2.0	428.7	261.1	78.4	6.0
	20.0	454.4	238.6	1365.1	104.2
4	2.0	429.6	253.8	19.0	1.5
	20.0	441.6	253.8	1171.1	89.4
DMSO	10 μl	430.7	256.6	7.53	0.8

FSC: Forward scatter count of cells in the samples (cell size ratio); SSC: side scatter count of cells in the samples; FL-1: mean fluorescence intensity of the cells. FAR: fluorescence activity ratio, values were calculated by using the equation given in the experimental section. PAR control: a parental cell without *ABCB1* gene. MDR: a parental cell line transfected with human *ABCB1* gene. [a]The results of compound **1** (PAR + R123: FL-1=1034.3; MDR + R123: FL-1=9.5; verapamil: FL-1=73.7; FAR=8.5) were obtained from different assays.

The results presented were obtained from a representative flow cytometric experiment in which 10,000 individual cells of the population were evaluated for the amount of rhodamine 123 retained.

Results

The compounds isolated from *M. balsamina* L. were not toxic in the concentrations applied in this study and exerted just a slight antiproliferative effect (13) during a period of 72 hours (Table I).

The inhibition of ABCB1 is evident when FAR values are greater than 1 (8). As demonstrated by Table II (13) all the listed compounds significantly increased the retention of rhodamine 123. Compound **1** was the most potent inhibitor inasmuch as very low concentrations produced the greatest retention of the fluorescent substrate.

The flow cytometric data were confirmed using the semi-automated real-time ethidium bromide assay developed by Viveiros *et al*. (9) and modified by Spengler *et al*. (10). From the real-time data (examples presented by Figures 2 and 3), the RFF of the last time point (minute 60) of the assay was calculated (Table III). The data reveal that compound **1** displayed a significant inhibition of human ABCB1 transporter and in essence replicated the flow cytometric results.

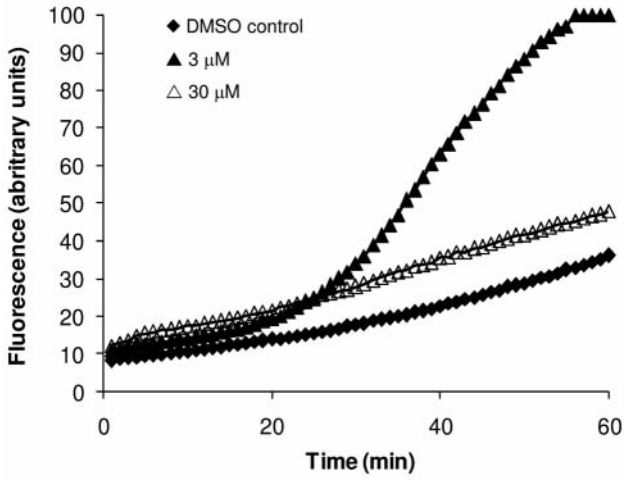

Figure 2. *Accumulation of ethidium bromide (1 μg/ml) by MDR mouse lymphoma cells in the presence of 3 and 30 μM of compound **1**.*

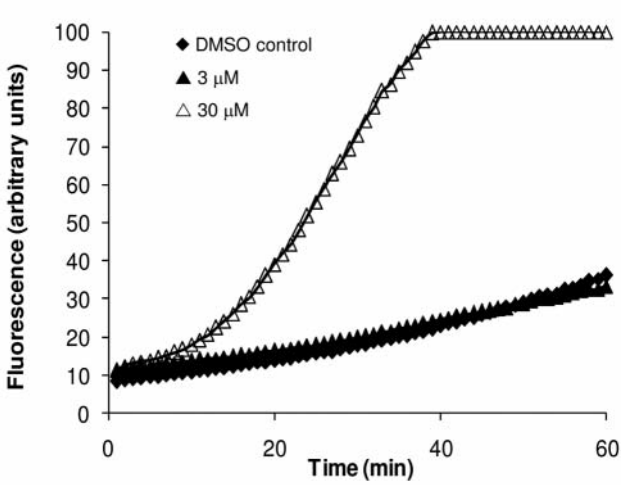

Figure 3. *Accumulation of ethidium bromide (1 μg/l) by MDR mouse lymphoma cells in the presence of 3 and 30 μM of compound **2**.*

Discussion

Cancer cells develop resistance to more than one agent as a consequence of being exposed to ineffective levels of the agent for a prolonged period of time (3). The resistance of these cells is mediated by overexpressed efflux pumps (10) that have the ability to extrude a large variety of unrelated chemicals (14). The search for plant-derived compounds, valuable for overcoming MDR in cancer has been intensified in recent years. Since inhibitors of ABC transporters can be used in combination with current drugs to increase the effective intracellular concentration of the latter, the possible impact of inhibitors of ABC transporters is of clinical interest (15).

It has been shown that *M. balsamina* fruit pulp extract contains a potent inhibitor of HIV-1 replication *in vitro* (16). The *in vitro* anti-plasmodial effect of methanolic extracts of *M. balsamina* has been demonstrated, suggesting possible clinical implications (17). In addition, extracts of *M. balsamina* also had low bactericidal activity against MDR *Shigella* species (18).

All of the compounds extracted from *M. balsamina* tested in this study as potential efflux pump inhibitors enhanced the intracellular accumulation of rhodamine 123 and EB. The most effective compound was compound **1** and, because of its low toxicity, it may have some clinical importance. It is important to note that the degree of retention of rhodamine 123 produced by 2 and 20 μM of compound **1** were similar (FAR values of 42.1 and 46.0, respectively), such that the latter concentration may exceed the saturation point of the transporter. All the compounds tested possess the same cucurbitane skeleton and we suppose that the side chain of compound **1** could be responsible for its greater potent inhibitory activity.

Table III. *Effect of compounds isolated from Momordica balsamina L. on the activity of MDR efflux pump of mouse lymphoma cells transfected with human ABCB1 gene by the real-time fluorometric method after 60 minutes.*

Relative final fluorescence (RFF)=(MDR treated) - (MDR untreated)$_{\text{solvent control}}$

Compound	Concentration (μM)	Relative final fluorescence (RFF)
Verapamil	81.45	100
1	3.0	64.0
	30.0	11.8
2	3.0	0
	30.0	64.0
3	3.0	3.6
	30.0	65.0
4	3.0	1.6
	30.0	86.7
DMSO	10 μl	0.8

The results presented in our study show that flow cytometry and the real-time fluorometry essentially provide the same information on the effects of agents on the activity of a eukaryotic transporter. Although the semi-automated real-time fluorometric method needs further standardization, it provides real-time, inexpensive and quick *in vitro* screening of candidate drugs compared to flow cytometry.

Although not evaluated in our present study, the inhibition of a transporter such as the ABCB1 by an agent is correlated with lower resistance of MDR cancer cells to cytotoxic agents commonly employed in the therapy of the respective

cancer (19). According to the results obtained by our study, cucurbitane-type triterpenoids do inhibit the major transporter of the mouse lymphoma cell that is responsible for its MDR phenotype. Besides their clinical potential, the understanding that the side chain of these agents is related to the degree of inhibitory activity affords a promising scaffold for the design of ABCB1 inhibitors.

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