New efflux pump inhibitors for Gram positive bacteria strains and cancer cells from an African medicinal plant

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INTRODUCTION

All living cells contain genes encoding multidrug transporters and some of them play an important role in conferring drug resistance in mammalian cancer cells and in microbial pathogens such as *Staphylococcus aureus*, *Enterococcus faecalis*, *Candida albicans*, *Plasmodium falciparum,* and *Leishmania donovani.* The over-expression of P- glycoprotein (P-gp) is one of the principal mechanisms of multidrug resistance (MDR) found in eukaryotic and prokaryotic cells (Fig.1). The inhibition of P-gp as a possible way of reversing MDR has been extensively studied. A large number of plant-derived compounds and synthetic molecules have been shown to block MDR pump efflux activity. Nevertheless, their pharmacokinetic interaction with chemotherapy and side effects have limited their clinical development.[1]

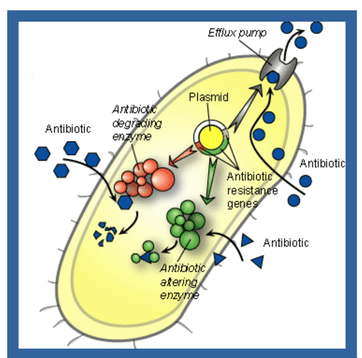

Fig. 1. Bacterial MDR mechanism.[2]

In our search for biologically active compounds from *Momordica balsamina* L. (Fig.2), we have isolated and characterized three new cucurbitane-type triterpenes, named balsaminagenin A and B, and balsaminoside A (**1-3**) and a known cucurbitacin (**4**). The isolated compounds (Fig.3) were evaluated for their efflux modulating effects of Gram positive and negative bacteria by a real-time fluorimetric method that utilizes the fluorochrome ethidium bromide (EB), a universal substrate of bacterial efflux pumps. Furthermore, the evaluation of the compounds as P-gp modulators of resistant cancer cells was also carried out by flow cytometry, using rhodamine 123, and by real-time fluorometry method, the latter method assessing accumulation of EB on a real-time basis.

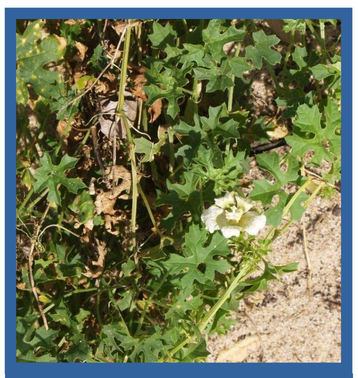

Fig. 2. *Momordica balsamina* L.

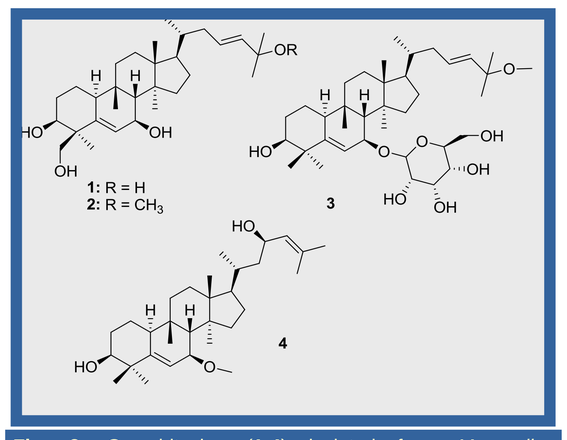

Fig. 3. Cucurbitacins (**1-4**) isolated from *Momordica balsamina.*

RESULTS

The structures of cucurbitacins **1-4** were established by means of spectroscopic techniques including 2D NMR experiments (COSY, HMQC, HMBC and NOESY), Fig. 4.

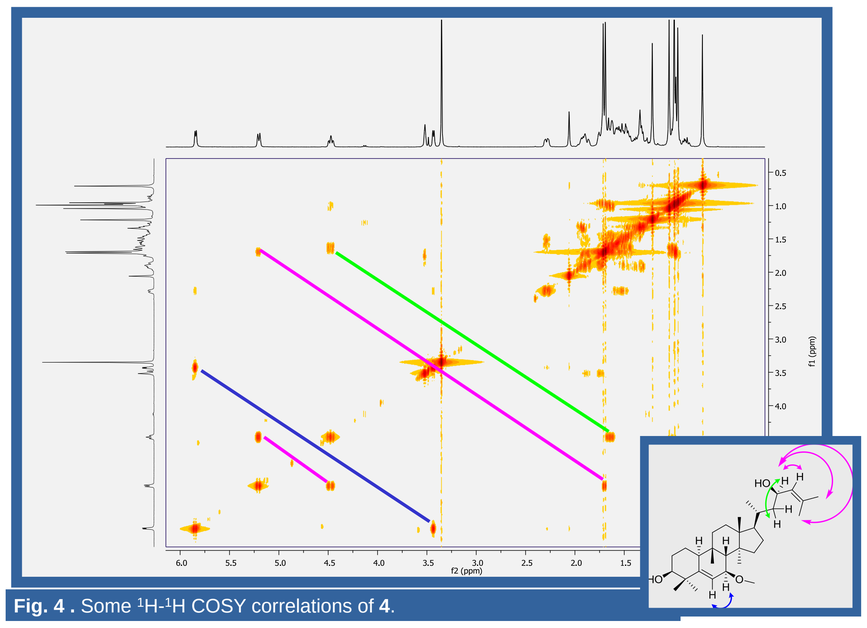

Fig. 4 . Some ^{1}H-^{1}H COSY correlations of **4**.

Cucurbitacins (**1-4**) were evaluated for their efflux modulating effects on resistant Gram positive (*Enteroccocus faecalis* and two methicillin-resistant *Staphylococcus aureus* strains) and Gram negative bacteria (two *Escherichia coli* and two *Salmonella enteridis* strains). Their ability as MDR modulators was also assessed in L15178 mouse T-lymphoma cell line transfected with the human *mdr 1* gene.

Whereas all the tested compounds increased the accumulation of ethidium bromide in Gram positive bacteria strains (*E. faecalis*, and methicillin-resistant *S. aureus* Col) in a dose-dependent manner, exhibiting compound **2** the highest activity in MRSA *S. aureus* Col (Fig. 5), no significant effect was observed in the Gram negative bacteria tested.

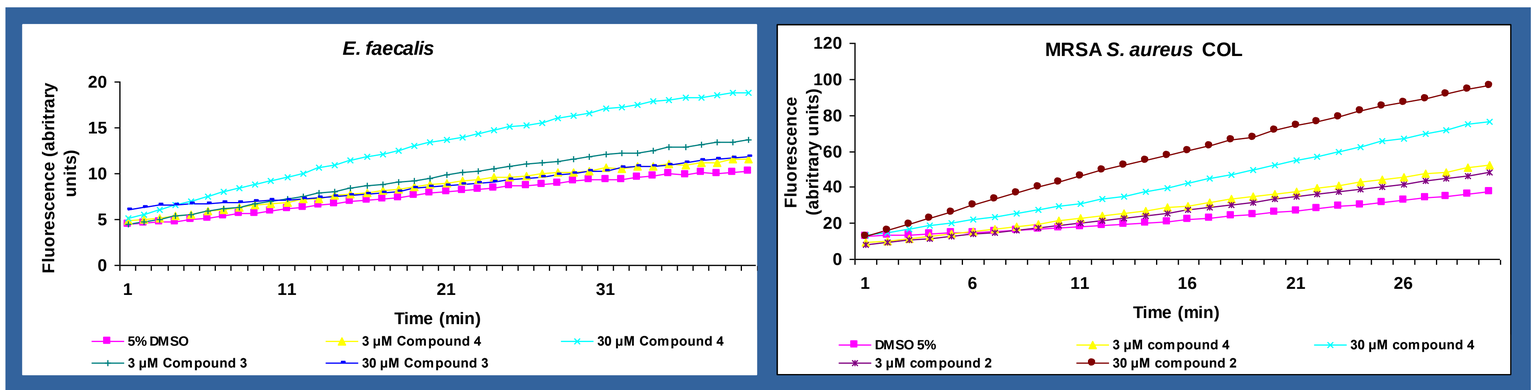

Fig. 5. Accumulation of EB (0.5 µg/ml, and 1 µg/ml), by *E. Faecalis* and MRSA *S. aureus* COL, respectively.

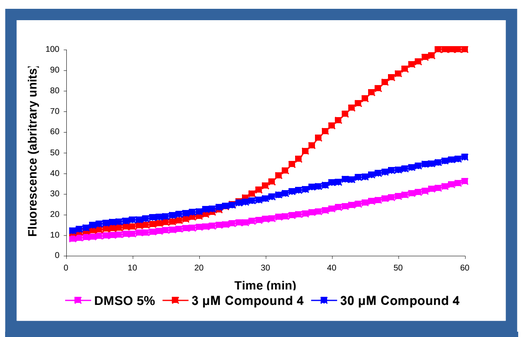

Fig. 6. Accumulation of EB (1 µg/ml) by MDR mouse lymphoma cells.

As can be observed in Table 1, all the compounds significantly increased the retention of rhodamine 123 in resistant mouse T-lymphoma cells, which over-express P- glycoprotein. Compound **4** was the most potent inhibitor. These data were corroborated by the results obtained from the semi-automated real-time ethidium bromide assay (Fig. 6 and Table 2).

Table 2. MDR reversal effects of compounds **1 – 4** on L 5178 resistant cell line (Real-time fluorometry).

Compound	Conc. (µM)	RFF
Verapamil	81.45	100
1	3.0	0
	30.0	64,02
2	3.0	3,60
	30.0	65,03
3	3.0	1,60
	30.0	86,70
4	3.0	64,02
	30.0	11,81
DMSO	10 µl	0.8

Table 1. MDR reversal effects of compounds **1 – 4** on L 5178 resistant cell line (Flow cytometry)

Compound	Conc. (µM)	FL-1	FAR
PAR+R123	—	956.7	
MDR+R123	—	18.1	
Verapamil	22.0	97.1	7.4
1	2.0	14.0	1.1
	20.0	579.8	44.3
2	2.0	78.4	6.0
	20.0	1365.1	104.2
3	2.0	19.0	1.5
4	0.5	12.7	1.5
	1.0	130.0	15.0
	2.0	365.7	42.1
	20.0	399.5	46.0
	20.0	1171.1	89.4
DMSO	10 µl	7.53	0.8

CONCLUSIONS

In conclusion, these results indicate that cucurbitane-type triterpenes can be considered as very promising lead compounds for the reversal of multidrug resistance in both cancer cells and Gram positive bacteria. This is especially important due to the increase of multidrug resistance in Gram positive pathogenic bacteria like *S. aureus*, *S. pneumoniae* and *Enterococcus spp*, and the lack of new anticancer drugs.

MATERIALS AND METHODS

Isolation of Compounds: The powdered aerial parts of *Momordica balsamina* were extracted at room temperature with methanol. The methanol extract was further fractionated, by liquid-liquid extraction. The resulting methanol:H2O extract was then fractionated by chromatographic methods, until the isolation of pure compounds **1-4**. All the structures were deduced from their physical and spectroscopic data.[3]

MDR Reversal Assay by flow cytometry: The assay was carried out in *MDR1* gene-transfected mouse lymphoma cells. An activity ratio (FAR) was calculated on the basis of the measured fluorescence values (FL-1) measured via the following equation: FAR = (FL-1MDR treated/FL-1MDR control)/(FL-1parental treated/ FL-1parental control).[3]

EB accumulation assay by real-time fluorometry: The assay was carried out in *MDR1* gene-transfected mouse lymphoma cells,[4] and Gram negative (*Escherichia coli* AG 100, *Escherichia coli* AG 100 TET 8, *Salmonella enteridis* 5408, and *Salmonella enteridis* 5408-cipro), and Gram positive [*Enteroccocus faecalis* ATCC 2921, *Staphylococcus aureus* (MRSA) COL, and *Staphylococcus aureus* (MRSA) COL 1600].

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References: 1. Szakács et al (2006) Nat Rev Drug Discov. 5: 219-234; 2. http://bioinfo.bact.wisc.edu/themicrobialworld/bactresanti.html; 3. Ramalhete et al. (2009) Bioorg. Med. Chem. 17, 6942-6951; 4. Spengler et al. (2009) Anticancer Res. in press.