

Betulinic acid does not modulate the activity of P-gp/ABCB1 or MRP1/ABCC1 in a non-tumoral renal cell line: Possible utility in multidrug resistance cancer chemotherapy

JOÃO MARCOS DE AZEVEDO DELOU, MÁRCIA ALVES MARQUES CAPELLA and CERLI ROCHA GATTASS

Instituto de Biofísica Carlos Chagas Filho, CCS, Universidade Federal do Rio de Janeiro, 21949-900 Rio de Janeiro, RJ, Brazil

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Abstract. Multidrug resistance (MDR) is a multifactorial phenomenon considered to be the main cause of failure in cancer chemotherapy. One of the underlying mechanisms of MDR is the overexpression of membrane transporter proteins, such as P-glycoprotein (P-gp/ABCB1) and multidrug resistance-associated protein 1 (MRP1/ABCC1). As these proteins are also expressed in normal tissues, considerable attention has been dedicated to the search for cytotoxic drugs that are not substrates for these proteins. This study investigated the effects of betulinic acid (BA) on the activity of ABCB1 and ABCC1 in Ma-104, a non-tumoral renal cell line constitutively expressing both proteins. The results indicated that concentrations of BA with low cytotoxicity to Ma-104 did not alter the activity of ABCB1 or ABCC1, nor did BA interfere with the accumulation of a classic chemotherapeutic, methotrexate. This suggests it would also be a good choice for use in drug cocktails. The lack of effect of BA on ABCB1 and ABCC1, as well as its antitumoral properties, suggest that this triterpene is a viable chemotherapeutic agent for MDR tumors.

Introduction

Multidrug resistance (MDR), the main cause of failure in cancer chemotherapy, is clinically defined as the cross-resistance of tumor cells to several structurally and mechanistically different chemotherapeutic drugs. Although MDR may result from several mechanisms, such as defects in the regulation of apoptosis control genes, an increase in drug detoxification mechanisms and alterations in the DNA-repair systems, its predominant cause is the overexpression of membrane transporter proteins of the ATP binding cassette (ABC) superfamily. These proteins, namely P-glycoprotein (P-gp/ABCB1) (1), multidrug resistance-associated protein 1

(MRP1/ABCC1) (2) and breast cancer resistance protein (BCRP/ABCG2) (3) act as active pumps, decreasing cellular drug accumulation and toxicity.

The expression of MDR proteins has been correlated with poor patient prognosis in several types of tumors (4-7). In the last decade, considerable attention has been dedicated to the search for new strategies to overcome the mechanisms of drug resistance. Therapeutic approaches to bypassing MDR include the use of inhibitors or modulators of the pumps as adjuvants in chemotherapy. However, as MDR proteins are also expressed in normal tissues, where they exhibit physiological functions, the use of such substances is generally accompanied by undesirable side effects (8). Over the years, more selective and less toxic inhibitors of these transporters have been developed, but the clinical results have generally been disappointing (9,10), indicating the need for new strategies. Among new approaches to the circumvention of MDR, one of the most promising is the use of drugs that are not substrates for transport, and that therefore do not lead to the undesirable side effects precipitated by the lack of function of the transporters.

Betulinic acid (BA), a triterpene of natural origin, was first described as an anti-neoplastic agent by Sheth *et al* in 1972 (11). Interest in this compound increased with the observation of its *in vitro* and *in vivo* activity against melanomas and tumors of neuro-ectodermal origin (12-14). Later, it was demonstrated that BA is also active in other types of tumors, including colon and prostate (15), lung, ovarian, cervical (16), leukemia (17), liver (18), and head and neck (19) carcinomas. Previous research by our group revealed that BA was cytotoxic to an MDR erythroleukemic cell line overexpressing P-glycoprotein, even in the absence of MDR modulators (20). This observation aroused interest in the further characterization of the cytotoxicity of this triterpene, particularly in non-tumoral cell lines.

Due to the growing interest in new compounds capable of overcoming the MDR phenotype for clinical praxis, the present study was performed to examine the effects of BA on the activity of ABCB1 and ABCC1 proteins in a non-tumoral renal cell line constitutively expressing both proteins (20). The results obtained indicate that BA had no effect on the activity or expression of these proteins in the cell line, and that it did not interact with a chemotherapeutic agent, methotrexate. This favors additional investigations directed towards the future use of BA in the treatment of MDR tumors.

Correspondence to: Dr Cerli Rocha Gattass, Laboratory of Cellular Immunology, Instituto de Biofísica Carlos Chagas Filho, CCS Bloco G, Universidade Federal do Rio de Janeiro, 21949-900 Rio de Janeiro, RJ, Brazil
E-mail: cerli@chagas.biof.ufrj.br

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Materials and methods

Cells and culture conditions. The renal epithelial cell line Ma-104 (monkey kidney embryo) was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) in disposable plastic bottles at 37°C. Cells were sub-cultured using trypsin-EDTA every 3-4 days.

Analysis of cellular viability. Cells were seeded in 96-microwell plates at a concentration of 2×10^4 cells/well. Experiments were performed 24 h after seeding to ensure the uniform attachment of the cells at the onset of the experiments. Cells were then incubated for 48 h with BA concentrations varying from 1 to 100 $\mu\text{g/ml}$. After incubation, cellular viability was measured by the thiazolyl blue tetrazolium (MTT) assay (21) on an ELISA reader (absorbance, 570 nm; reference filter, 655 nm). Each experiment was repeated at least three times using triplicate samples.

Intracellular accumulation of Rhodamine 123 and Fluo-3. Flow cytometry was used to measure intracellular fluorescence. Rhodamine 123 (Rho123, Sigma, USA) and Fluo-3 (Invitrogen, USA) were used for the measurement of ABCB1 and ABCC1 activities, respectively (21,22).

For each experiment, the cells were seeded in 24-well plates at a concentration of 1×10^5 cells/ml (1 ml/well). After 24 h, the cells were incubated for 30 min with 400 nM Rho123 or 1 μM Fluo-3 in the presence or absence of the desired BA concentrations. The classic ABCB1 and ABCC1 modulators cyclosporin A (1 μM) and MK571 (25 μM) were used as controls. The cells were then washed in PBS, harvested by trypsin-EDTA and kept on ice until evaluation by flow cytometry. Flow cytometry analysis was performed in a Becton-Dickinson cytometer (FACSCalibur) equipped with an air-cooled argon laser tuned to emit 15 mW at 488 nm. Fluorescence was measured through a 530 nm-long pass filter.

Western blot analysis. Expression of ABCB1 and ABCC1 was assessed by immunoblotting using specific antibodies (MRP6 and JSB-1, respectively). Cells were plated onto 6-well plates, and BA (10 $\mu\text{g/ml}$) was added the next day. After 24 h of incubation, the culture medium was aspirated and the cells were washed three times with PBS (pH 7.4) at room temperature, scraped, and centrifuged at $8,000 \times g$ for 90 sec. Whole cell extracts were prepared and diluted in sample buffer consisting of 1.5% sodium dodecyl sulfate (SDS), 10 mM Tris[hydroxymethyl]aminomethane (Tris-Cl), pH 6.8, 0.6% dithio-L-threitol, 0.5% β -mercaptoethanol and 6% glycerol. The proteins were then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA). The membranes were blocked with Western Breeze blocking solution (Invitrogen) and incubated with specific antibodies against ABCB1, ABCC1 or actin (Santa Cruz, USA). The phosphatase alkaline Western Breeze Kit (Invitrogen) was used to visualize the bands on the membranes.

Statistical analysis. Each experiment was repeated three to seven times. Data are expressed as the means \pm SEM and were analyzed by the paired one-tailed t-test (Figs. 2 and 4)

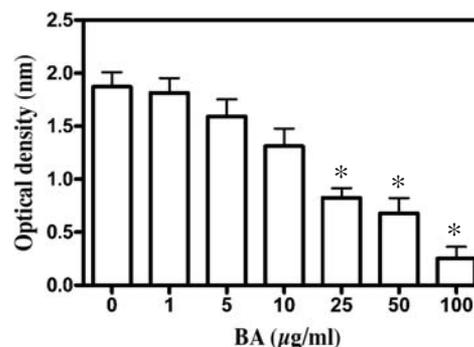


Figure 1. Effect of betulinic acid (BA) on Ma-104 cell viability. Cells were incubated with different BA concentrations for 48 h. Cell viability was measured by MTT. Results are derived from seven experiments performed in triplicate. * $p < 0.001$ in relation to the control.

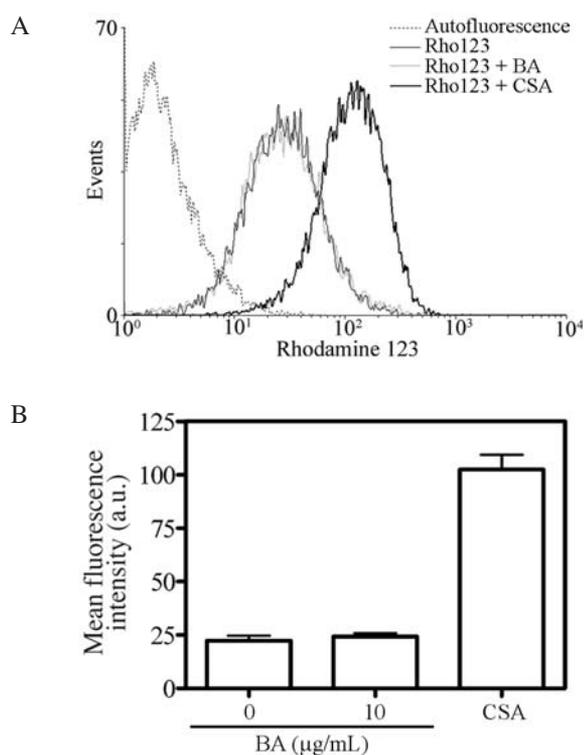


Figure 2. Effect of betulinic acid (BA) on Rhodamine 123 accumulation. Cells were incubated for 30 min with 400 nM Rho123 in the presence or absence of 10 $\mu\text{g/ml}$ BA. Cellular fluorescence was measured by flow cytometry. The accumulation of Rho123 in the presence of cyclosporin A was used as the control. (A) Representative histogram and (B) mean fluorescence intensity. Results are the mean of 3 experiments.

and one-way analysis of variance with Bonferroni's post test (Figs. 1 and 3). Values of $p < 0.05$ were considered statistically significant.

Results

The MTT assay was used to evaluate the effect of BA on Ma-104 cell viability. As shown in Fig. 1, BA decreased the viability of Ma-104 cells in a dose-dependent manner. High concentrations of BA were required to substantially decrease the viability of Ma-104 cells after 48 h of treatment; however, at 10 $\mu\text{g/ml}$, a concentration at which BA is highly cytotoxic

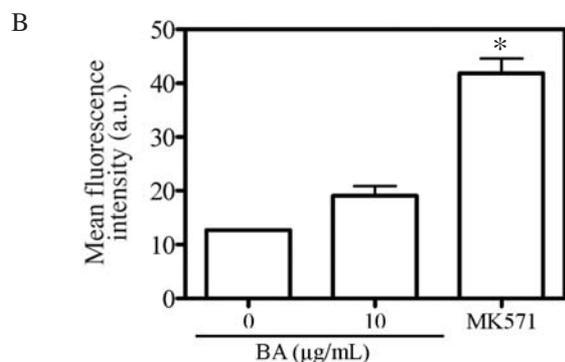
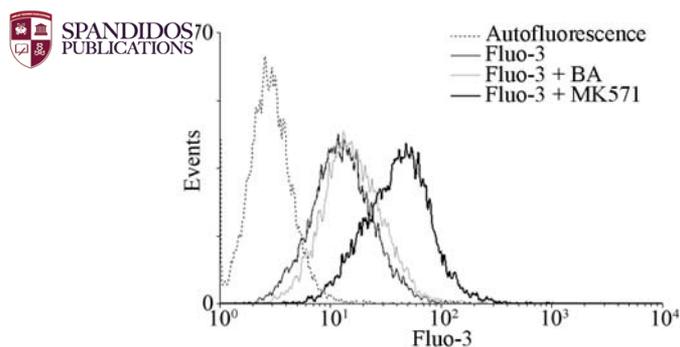


Figure 3. Lack of effect of betulinic acid (BA) on Fluo-3 accumulation. Cells were incubated for 30 min with 1 μ M Fluo-3 in the presence or absence of 10 μ M BA. Cellular fluorescence was measured by flow cytometry. Accumulation of Fluo-3 in the presence of MK571 (25 μ M) was used as the control. (A) Representative histogram and (B) mean fluorescence intensity. * $p < 0.01$ in relation to the control.

to several tumor cell lines (14-19), the effect of BA on Ma-104 was very low. Thus, to avoid the possible interference of cell death mechanisms on the measurement of ABCB1 and ABCC1 activities, subsequent experiments were performed using 10 μ g/ml of BA. At the incubation time used (30 min), this concentration had no effect on cell viability (results not shown).

We previously demonstrated that BA was cytotoxic to tumor cells overexpressing ABCB1 (24). However, there is no data regarding the effect of BA on ABCB1 and ABCC1 activity. To further analyze whether BA was able to inhibit ABCB1 or ABCC1 activity, Ma-104 cells were incubated for 30 min with Rho123 (ABCB1 substrate) or Fluo-3 (ABCC1 substrate) in the presence or absence of BA, and the intracellular fluorescence was measured.

Fig. 2 shows that no significant alteration was noted in the fluorescence of cells incubated with 400 nM Rho123 in the presence or absence of BA, while a significant increase in fluorescence was observed in the presence of the ABCB1 inhibitor cyclosporin A, indicating that BA has no effect on ABCB1 activity. Moreover, no significant alteration was noted in the fluorescence of cells incubated with 1 μ M Fluo-3 in the presence or absence of BA, while a significant increase in fluorescence was observed in the presence of the classical ABCC1 inhibitor MK571, indicating that BA does not have an effect on ABCC1 activity either (Fig. 3).

The above results suggest that BA does not modulate ABCB1 or ABCC1 activity. However, flow cytometry experiments were performed for 30 min, a time period which is too

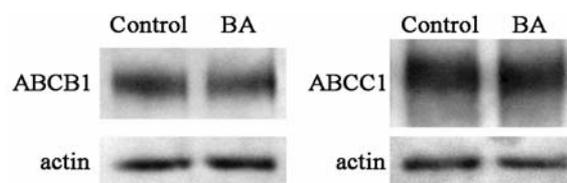


Figure 4. Betulinic acid (BA) does not alter ABCB1 or ABCC1 expression. Cells incubated with 10 μ M BA for 24 h were lysed and blotted against ABCB1 and ABCC1 antibodies. As a loading control, antibody against actin was used.

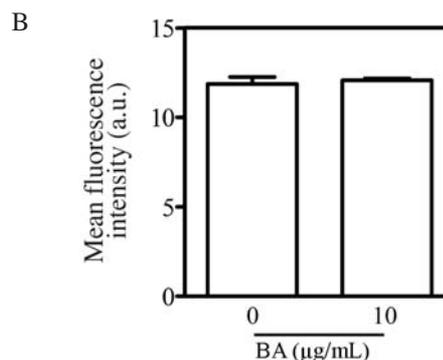
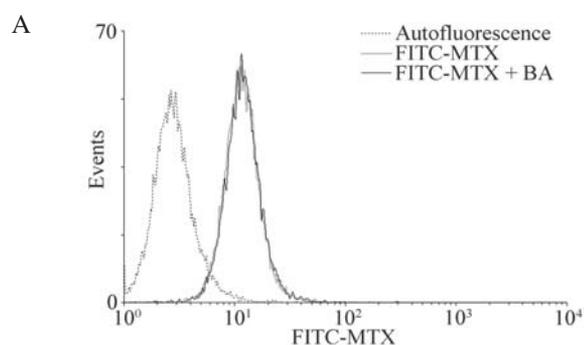


Figure 5. Betulinic acid does not affect methotrexate (MTX) accumulation. Cells were incubated for 30 min with 10 μ M FITC-MTX in the presence or absence of BA. Cellular fluorescence was measured by flow cytometry. (A) Representative histogram and (B) mean fluorescence intensity.

short for the observation of an alteration in protein expression. The remaining possibility that BA alters the expression of these proteins was therefore analyzed by immunoblotting. As shown in Fig. 4, BA did not alter the expression of ABCB1 or ABCC1 after 24 h of incubation, confirming that this triterpene has no effect on these proteins.

The fact that BA does not alter the expression or the activity of the two main proteins related to the MDR phenotype suggests that this triterpene does not alter the physiological functions of these transporters, nor the intracellular accumulation of the chemotherapeutics that are substrates for one (or both) proteins. Thus, BA could potentially be used in a chemotherapy cocktail without undesirable side effects, such as increased cytotoxicity. To test this hypothesis, we evaluated the intracellular accumulation of fluorescein methotrexate (FMTX) in the presence or absence of BA. As can be noted in Fig. 5, the results reveal that the intracellular accumulation of FMTX was indeed virtually the same in the presence or absence of BA.

Discussion

Multidrug resistance remains a major problem facing cancer chemotherapy. The complexity and versatility of the cellular resistance mechanisms present in MDR tumors hamper clinical chemotherapy, favoring a bad prognosis for patients with this type of tumor. The mechanisms underlying the development of MDR are considered multifactorial. However, of these mechanisms, the drug efflux mediated by several ATP binding cassette transporters (in particular ABCB1 and ABCC1) is one of the best studied. Since several anti-neoplastic drugs are substrates for the MDR proteins, clinical approaches to circumvent MDR mechanisms have included the use of inhibitors or modulators of these pumps (10). Nevertheless, although many MDR inhibitors have been identified, none have proven to be without side effects (8). The expression of MDR proteins on normal tissues is probably the main factor of the observed side effects (25). Therefore, the identification of new anti-neoplastic drugs that can bypass resistance and do not modulate the activity or alter the expression of the two main MDR proteins is of great importance.

Our group is studying the anti-tumoral activity of several triterpenes of natural origin, and we have shown that several are cytotoxic for MDR cell lines (20,24,26-28). Additionally, the effect of oleanolic acid on the activity of MDR proteins has been investigated. Although it did not interfere with ABCB1 activity, oleanolic acid did modulate ABCC1 activity (20).

Betulinic acid is already known as a promising anticancer drug (9,12,13,24), but its effects on MDR cells remain unclear. In a previous study, we demonstrated that BA was cytotoxic to an MDR erythroleukemia cell line that overexpresses ABCB1 (24). The antitumoral effect of BA was also observed in tumor cell lines with defective or mutated p53 (13,16), as well as in cell lines that acquire resistance to cytotoxic drugs (16,18). In this study, we investigated the effect of BA on Ma-104, a non-tumoral renal cell line that constitutively expresses P-gp/ABCB1 and MRP1/ABCC1 (20). The results show that, at lower concentrations ($\leq 10 \mu\text{g/ml}$), the cytotoxic effects of BA on Ma-104 cells are much smaller than those observed in tumor cell lines (14,16,19,29), including those resistant to cytotoxic drugs (16,30). Indeed, lack of BA activity at concentrations $\leq 20 \mu\text{g/ml}$ was reported in normal fibroblasts, peripheral blood lymphocytes, astrocytes and melanocytes (13,16,17,31). The present study also showed that BA had no effect on the accumulation of Rho123 and Fluo-3 in this cell line, nor did it alter the expression of ABCB1 or ABCC1 in a long-term incubation, confirming the complete absence of the effects of BA on these proteins. Corroborating this hypothesis, BA had no effect on methotrexate accumulation, a chemotherapeutic known to be a substrate for ABCC1 (32).

One of the problems confronting the chemotherapy of MDR tumors is the expression of MDR proteins in normal tissues, leading to altered pharmacokinetics and increased toxicity, which hamper the clinical development of chemosensitizers (33). In view of this, it was postulated that an ideal chemotherapeutic should not be a modulator of MDR proteins (34). BA has already been suggested as a chemotherapeutic candidate, and the kidneys are organs related to drug bioavailability. Thus, data in the literature demonstrating the

antitumoral properties of BA *in vitro* and *in vivo* and the results presented herein, indicating that BA does not modulate ABCB1 and ABCC1 activity and/or expression in a renal cell line, suggest that this triterpene is a promising drug for the treatment of tumors refractory to the currently available therapeutic agents. Therefore, BA is a viable option for the chemotherapy of MDR tumors.

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