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Artículo Original

Verapamil modulates p-glycoprotein expression in resistant colon cancer cells

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Abstract

The multidrug resistance (MDR) phenotype is a great problem in the chemotherapy of cancer patients, being responsible of many cases of therapeutic failure. One of these MDR mechanisms are the overexpression of p-glycoprotein (P-GP), an ATP-binding cassette (ABC) protein which expel the chemotherapeutic drugs from the cells. Some molecules blocking the activity of P-GP and inhibit the expulsion of drugs from the cell, such as verapamil. However, the expected results of this activity are not observed in clinical trials and could be related with the modulation of P-GP expression. Tumor cells with overexpression of P-GP (HCT15) and no-overexpressing P-GP cells (T84 and HT29) were exposed to verapamil and/or doxorubicin (DOX) to study their proliferation by sulforhodamine B assay and the P-GP expression by qPCR and flow cytometry. The results showed a clear blocking activity of verapamil in HCT15 with a reduction in proliferation percentages (up to 50.3 % higher in 1μM) compared to cells treated only with DOX. Furthermore, verapamil induced an increase of the P-GP expression in HCT15 resistant cell line at the RNA level (almost twice) and protein level (1.2 times) compared to HCT15 no treated with verapamil. This alteration of P-GP expression by verapamil could be due to the cell's own resistance mechanism as a response to a stressful situation and could be related with the therapeutic failure in its clinical application.

Keywords: verapamil, p-glycoprotein, colon cancer, MRD, HCT15 cell line.

Introduction

The cell population in a tumor is heterogeneous. Inside this heterogeneity, many tumor cells may have some cellular mechanisms which make them resistant (inherent or acquired) to a broad range of chemotherapeutic drugs. This is the multidrug resistance (MDR) phenotype; a problem in chemotherapy treatment in patients as many drugs are ineffective (1). One of these mechanisms of MDR is the overexpression of ATP-binding cassette

(ABC) transporters (2). This MDR pump family of proteins include p-glycoprotein (P-GP) which is usually overexpressed in chemotherapy resistant tumor cells that expel out the drugs from cells (3).

There are some substances that are able to block the activity of P-GP such as verapamil or cyclosporine A; retaining the drug inside cells and therefore enhancing their cytotoxic effect (4) (Figure 1). However, the use of this blocking-agents in some clinical trials have not shown clearly satisfactory results for reasons still unclear (5). Some molecules have been shown to be able to modify the expression and/or activity of P-GP such as drugs, hormones or physiological factors (6-8) and verapamil could be involved in this modification (6,9,10).

The aim of the present study was to investigate if verapamil may enhance de expression of P-GP, and therefore could be one of the possible reasons of the clinical therapeutic failure of this compound in combination with antitumor drugs.

Material and methods

Cell culture

Human colon cancer cell lines T84, HT29 and HCT15 were obtained from the European Collection of Cell Culture and the Scientific Instrumentation Center (University of Granada, Spain) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) with a supplementation of 10% fetal bovine serum (FBS) and 1% of a mixture of antibiotics (Penicillin-Streptomycin) (Sigma-Aldrich). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO2. HCT15 is a tumor cell line which overexpress P-GP, whereas T84 and HT29 not overexpress this protein, and therefore they were used as negative controls.

Verapamil-blocking activity on P-glycoprotein

The cell lines T84 and HCT15, were seeded in 24-well plates with a cell density of 8x103 to 15x103 cells/well respectively. The next day, cells were pre-incubated 1 hour with verapamil (Sigma-Aldrich) at a non-toxic dose of 7 ug/ml. The selection of the verapamil dose and its time of pretreatment were done based on previous experiments (data not shown). After this incubation time, cells were treated with increasing doses of doxorubicin; a chemotherapeutic drug that is P-GP substrate. After 48 hours of treatment, cell proliferation were evaluated by sulforhodamine B assay (11).

P-glycoprotein expression analysis by qPCR

The colon cancer cell lines HCT15 and HT29 were seeded in culture flasks (25cm2) to a

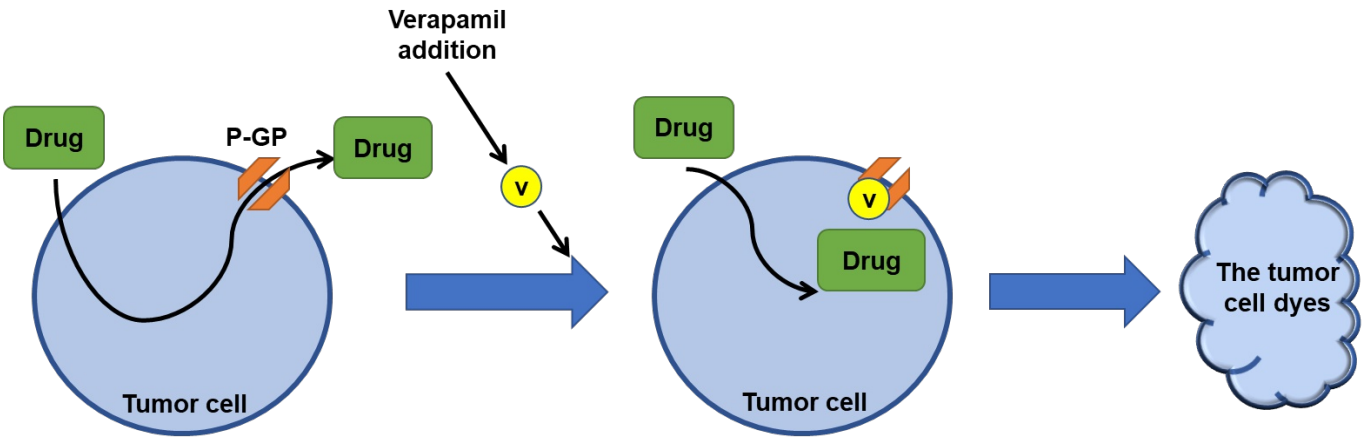

Figure 1. Tumor cells that overexpress P-GP expel the chemotherapeutic drug out during treatment. When a P-GP blocker such as verapamil is used, the drug cannot leave the cell and therefore tumor cells are more sensitive to treatment and die.

confluence of 80%. After 24 hours, cells were treated with verapamil at a dose of 7 ug/ml for 1 and 24 hours of exposition. One culture flask of each cell line remained untreated to maintain the basal expression of P-GP. Once exposure times were elapsed, quantitative-PCR was performed to study the P-GP expression on colon cancer cells. For this, 1.5 µg of RNA (TRI Reagent® from Sigma-Aldrich and RNeasy Mini Kit from Qiagen) was reversed transcribed with AMV Reverse Transcriptase (Reverse Transcription System, Promega). SYBR Green-based amplification (Applied Biosystems) was performed with the CFX96 Real-Time PCR Detection System (Bio-Rad). The primer used was for the amplification of ABCB1 (Hs00184500_m1, TaqMan® Gene Expression, Thermo Fisher Scientific). The qPCR cycling program was 50°C (2min), 95°C (2 min), 40 cycles of denaturation at 95°C (30 s), annealing at 60°C (30s), and extension at 72°C (40 s), followed by a melting curve analysis (range 56–95°C) with increments of 0.5°C/5 s to assess the primer specificity. The housekeeping gene used was GADPH, and the RNA of the untreated cells was used as control with a basal expression. The results were expressed as relative quantification (RQ) of gene expression.

P-glycoprotein expression analysis by flow cytometry

Colon cancer cells were seeded in 6-well plates and after 24 hours were treated with 7 ug/ml of verapamil for 1 hour. Then cells were detached with a PBS-EDTA solution and washed in cold PBS. After that, cells were directly immunostained with FITC-conjugated anti-P GP antibody (Cat. ab66250, Abcam). An isotype control was made to determine the level of background fluorescence of untreated cells. Afterwards, P-GP expression were analized by flow cytometry with a FACSCanto II flow cytometer (BD Bioscience, San Diego, CA, USA) in order to determine the mean fluorescence intensity and the percentage of positively labelled cell.

Results and discussion

Verapamil-blocking activity on P-glycoprotein

A pre-treatment with verapamil administered on a cell line that overexpressed P-GP (HCT15) produced a blocking effect on the protein that could allow a greater accumulation of drug within the cell leading to higher cell death compared to the proliferation percentage of cells treated with free DOX (up to 50.3 % higher in 1µM) (Figure 2). This effect was not observed in the cell line that not overexpress P-GP with similar percentage of proliferation between cells with pre-treatment and without pre-treatment with verapamil. This blocking of P-PG by verapamil has been extensively described in previous studies for a long time. Verapamil enhances the retention of the drugs inside cells by a competition for binding sites on P-GP (12-14). On this way, we demonstrate in vitro this activity in our cell lines to corroborate its biological activity.

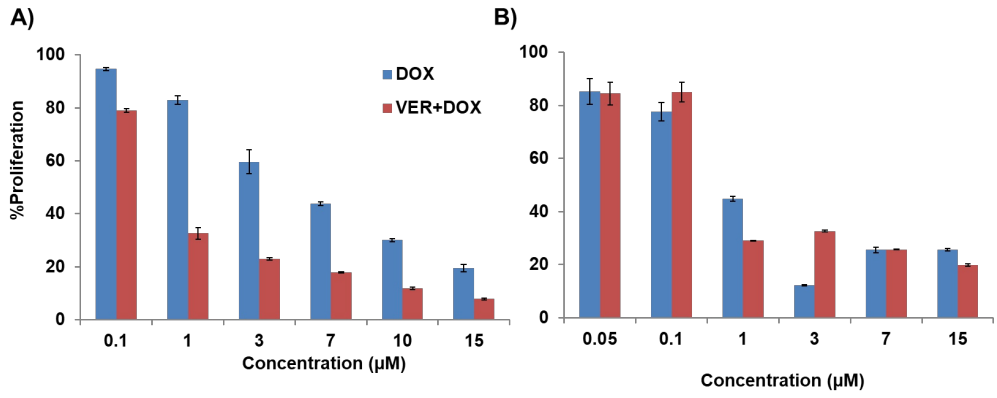

Figure 2. Proliferation assay to show blocking activity of verapamil on P-GP. The cell lines HCT15 (A) and T84 (B) were exposed to verapamil and doxorubicin for 49 and 48 hours respectively and then were analized by sulforhodamine B assay.

P-glycoprotein expression analysis by qPCR and flow cytometry

The expression assays revealed an increased P-GP expression in HCT15 cells exposed to verapamil. By qPCR it can be observed increases in RNA expression almost twice with a verapamil exposure for 24 hours, while in the HT29 cell line, RNA expression remains unchanged (Figure 3). In addition, an increase in concentration-dependent expression can be observed. At the protein level, an increased expression of P-GP was also observed in the HCT15 cell line exposed to verapamil (1.2 times), which was not observed in control cells (Figure 4). Furthermore, a percentage of cell labelling around double was observed in HCT15 in relation to control cell line. These results could mean that the exposure to verapamil could alter the expression P-GP in both, at the RNA and the protein level. It has been previously described that chemotherapeutic drugs may enhance the expression and/or activity of P-GP in tumor cells (15). However, the effect of verapamil in the P-GP expression of human colon cancer cells has not been described. Some authors have shown the modulating effect of verapamil on the expression of P-GP in other cells. Overexpression of P-GP has been demonstrated in human monocyte-derived dendritic cells exposed to verapamil, what could be an answer to the fundamental role that P-GP has in activated myeloid derived antigen presenting cells (10). This increased expression has also been observed in Ehrlich ascites carcinoma cells inoculated intraperitoneally in CD-1 mice treated with verapamil orally. Concretely, the enhanced expression was observed at the protein level (6). In the cell line L1210 with P-GP overexpression achieved by transfection, the combined treatment with verapamil and all-trans retinoic acid induced small increases in P-GP expression without any changes in its efflux activity (9). On the contrary, some authors have shown a decrease in P-GP expression. Rats with hepatocellular carcinoma induced were treated with verapamil and showed lower P-GP expression at RNA level compared to those untreated mice (16). These results could be due to the no-presence of previous resistance with an overexpression of P-GP as we have observed in our tumor cell lines T84 and HT29, since the tumors were not induced by cell inoculation, but by Nitrosodiethylamine (NDEA) exposition.

Finally, our results could indicate that the alteration of P-GP expression by verapamil could be due to the cell's own resistance mechanism (MDR), since it does not appear in non-resistant cells. By subjecting the cell to a stressful situation, such as blocking proteins that expel out toxic substances, the cells could respond by over-expressing the protein to survive to the adverse conditions (17).

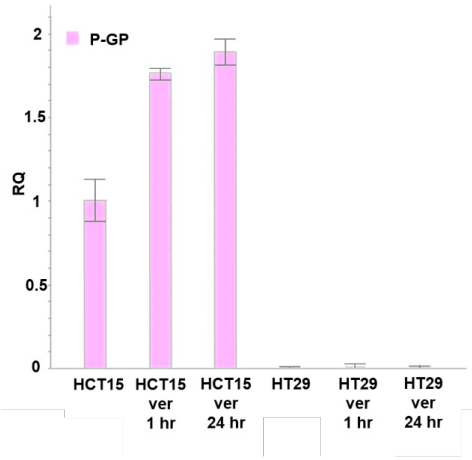

Figure 3. mRNA expression of P-GP in colon cancer cell lines exposed to verapamil for 1 and 24 hours by qPCR respect to a basal cell line HCT15.

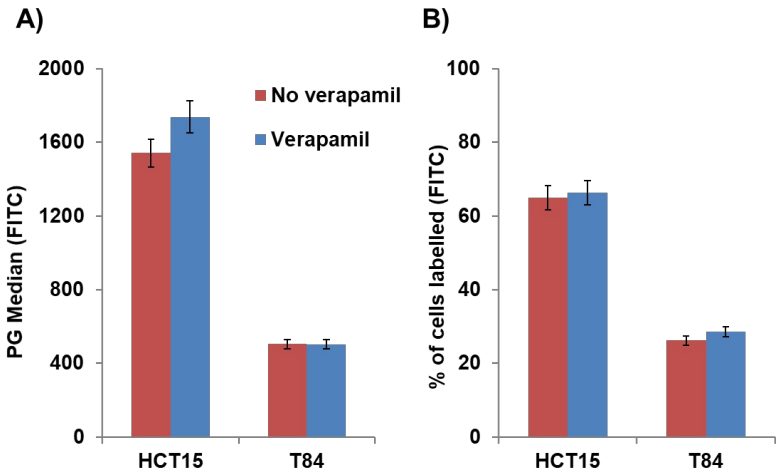

Figure 4. Protein expression of P-GP in colon cancer cell lines exposed to verapamil by flow cytometry. Fluorescence intensity of labelled cells (A). Percentage of labelled cells (B).

Conclusion

P-glycoprotein expression seems to be modulated by a treatment with verapamil. Concretely, verapamil increases the expression of P-GP both at the RNA level and at the protein level. The increase in expression was much more evident at the RNA level than at the protein level, perhaps because not all mRNA translates to protein or perhaps due to insufficient exposure time to verapamil. Finally, the increased expression of P-GP induced by verapamil, could be involved in therapeutic failure in its clinical application. However, further studies are necessary to demonstrate this premise.

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