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Modulation of MDR1 Gene Expression by a Chronic Treatment with Verapamil in Caco-2 Cells

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Abstract: The aim of this study was to investigate the effect of a chronic treatment with low doses of verapamil, as used in cardiovascular therapy, on the expression of mRNAs coding for transport proteins, in cultured Caco-2 cells. Caco-2 cells were selected *in vitro* through continuous exposure to stepwise increasing concentrations of verapamil. The Caco-2/ver cell line, a resistant sub line tolerating 50 µM verapamil, was obtained after a chronic treatment with this drug. Parental and resistant cells were grown for 20 days and then used for doxorubicin uptake and transport studies. The expression of P-gp and CYP3A4 mRNAs was examined by RT-PCR. Evaluation of doxorubicin uptake demonstrated a reduced drug accumulation in the resistant cell line compared to parental Caco-2 cells. Transport studies across cell monolayer showed an increased basal to apical transport rate in Caco-2/ver in comparison to Caco-2 cells. The relative expression of mRNA for MDR1 gene was greater in treated cells, whereas CYP3A mRNA levels were unaffected.

Key words: Caco-2 cells, induction, MDR1, CYP 3A4, verapamil

INTRODUCTION

A major problem in cancer chemotherapy is the development of multidrug resistance. This phenomenon is characterized by a cross-resistance between structurally and functionally unrelated drugs, such as anthracyclines, vinca alkaloids and taxans, by a decreased intracellular drug concentration and by an over expression of membrane energy-dependent transport proteins such as P-glycoprotein (P-gp) and Multidrug Resistance-related Proteins (MRPs)[1,2].

P-gp and MRPs belong to the super family of the ABC (ATP-binding cassette) membrane transport proteins and extrude substances from the cytoplasm. These proteins are present also in many normal tissues such as the liver, the kidney, the endothelia of the blood brain barrier and the intestine[3-5]. In the intestine, P-gp is located in the apical membrane of enterocytes and plays an important role in limiting the entry of xenobiotics from the gut lumen[6,7]. Proteins belonging to the MRP family are also expressed in the bowel, in particular MRP1 and MRP3 are located on the basolateral membrane of crypt cells[8-10], whereas MRP2 is located on the apical side of mature villus cells[11].

P-gp and MRP are expressed also in Caco-2 cells, a cell line that exhibits the functional characteristics of the lower small intestinal tract[12,13]. These cells are derived from a human colorectal carcinoma and in culture, spontaneously differentiate into a polarized cell monolayer with the characteristics of intestinal epithelial cells. Because of the morphological similarities between the Caco-2 cell monolayer and the intestinal epithelium, these cells are now widely used as an *in vitro* model to study intestinal absorption of drugs[6,14].

Several reports have shown that the expression of P-gp and MRPs can be modulated by chronic treatments with substances which are their substrates[15-20]. Verapamil is a substrate of P-gp and MRP; its administration at low dosages and for extended periods is extremely common in cardiovascular therapy as antiarrhythmic agent and in the treatment of hypertension, angina and certain cardiomyopathies[21]. The chronic use of the calcium antagonist in non neoplastic diseases could therefore determine an over expression of transport proteins in normal tissues and consequently could result in a modified pharmacokinetics and an altered efficacy and/or toxicity of verapamil as well as of other P-gp substrates. Whilst many studies have been conducted on the combined therapy with antineoplastic agents and verapamil used at high dosages as reverting agent[22], no data exist about the possible interaction between the calcium antagonist chronically employed in cardiac patients and antineoplastic drugs. Indeed, in clinical practice, it could happen that, among the patients who start an antineoplastic therapy, there are some who are currently taking verapamil for pre-existing cardiovascular pathologies.

The aim of the research was to investigate the effect of a chronic treatment with verapamil on cultured Caco-2 intestinal epithelial cells and to evaluate if such a

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treatment could affect the expression of mRNAs coding for transport proteins.

MATERIALS AND METHODS

Chemicals: Verapamil, doxorubicin, 3-(4,5-dimethylthiazol-2-yl)-2, 5 diphenyl-tetrazolium bromide (MTT), RedTaq^{TM} DNA polymerase and 100 bp DNA ladder were purchased from Sigma-Aldrich Srl, Milano, Italy. SV Total RNA Isolation System was from Promega, Milano, Italy; SuperScript^{TM} II RNase H$^-$ reverse transcriptase and synthetic oligonucleotide primers were from Invitrogen Life Technologies, San Giuliano Milanese, Italy. Cell culture media and supplements were obtained from Celbio, Pero, Italy. All other chemicals were of analytical grade.

Establishment of the verapamil-resistant clone: The human colorectal carcinoma cell line, Caco-2, was obtained from the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Brescia, Italy. Cells were used between passages 35–45 and cultured in Minimum Essential Medium Eagle containing 15% foetal bovine serum, 2 mM L-glutamine and 1 mM sodium pyruvate. Cells were fed twice weekly, grown to confluence and passaged using trypsin-EDTA. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air.

The Caco-2 drug resistant cell line was selected *in vitro*, through a continuous exposure to stepwise increasing concentrations of verapamil over an extended period of time. Cells were seeded at a cell density of 5×10^4 cells/mL in 35 mm dishes in 2 mL of complete medium and verapamil was added after 24 h. The initial concentration of verapamil was 1 μM, which was gradually increased to 50 μM, following the schedule: 1, 2, 4, 6, 8, 16, 25, 50 μM. Verapamil concentrations were increased when doubling time was similar to that of parental cells.

A resistant sub line tolerating 50 μM verapamil, CaCo-2/ver, was obtained and stored in liquid nitrogen.

In vitro **cytotoxicity assay:** The tetrazolium-dye assay of Mosmann[23] was used to assess cytotoxicity in the drug-sensitive and drug-resistant cell lines. The day before seeding, both cell lines were washed three times with ice cold D-PBS and incubated for 24 h in fresh medium without verapamil.

For the cytotoxicity test, 10^5 cells/well were seeded in 96-well plates in a volume of 200 μL of culture medium. Cells were exposed to predetermined concentrations of verapamil and allowed to grow for a further 3 days. At the end of the incubation, 20 μL of MTT solution (5 mg mL^{-1} in phosphate-buffered saline) were added and plates were maintained for 4 h at 37°C; after elimination of the medium, 200 μL of dimethylsulfoxide were added to dissolve the formazan crystals. Absorbance was measured on an Automated Microplate Reader EL311s (Bio-Tek® Instruments, Inc., Winooski, VT) with a reference wavelength of 630 nm and a test wavelength of 540 nm. Wells containing identical amounts of medium and MTT only were used as drug-free wells. Each experiment was performed using 8 replicate wells for each drug concentration and at least 3 separate experiments were carried out for each cell line. The IC_{50} was defined as the drug concentration required to reduce the optical density in each test to 50% of control. Relative resistance value was defined as the ratio between the IC_{50} of the resistant sub line and the IC_{50} of the parental control.

Doxorubicin accumulation: Caco-2 and Caco-2/ver (1×10^6 cells/well) cells were seeded in 35 mm dishes in 2 mL of complete medium or complete medium containing 50 μM verapamil, respectively. Doxorubicin uptake was measured on the 21st day after seeding. The day before the experiment, both cell lines were washed three times with ice cold D-PBS and incubated in fresh medium not containing verapamil. After 24 h, each dish was washed twice with 4 mL of PBS at 4°C; then 2 mL of complete medium containing doxorubicin (5, 25 and 50 μM) were added to each dish and the cells were incubated for a specified period. At the end of predetermined times, the medium was removed and cells were harvested by scraping. The final cell pellet was resuspended in 2 mL of 0.3 N HCl in 50% ethanol, mixed thoroughly in a vortex mixer and centrifuged at 700 xg. Doxorubicin content in the supernatant fraction was determined fluorimetrically by the method of Bachur *et al.*[24].

Drug transport: Transport of doxorubicin across Caco-2 monolayer was studied using 21 day aged cultured cells. Cells were plated at a density of 10^6 cells/well on tissue culture-treated inserts (24 mm diameter and 3.0 μm mean pore size polycarbonate membranes, Transwell^{TM}, Costar, Cambridge, MA). Two milliliter of medium were applied at both sides and medium was changed every 3 days. The quality of the cell monolayer was determined by measuring the transepithelial electrical resistance, using a Millicell-ERS Voltohmeter (Millipore, Vimodrone, Italy)[25]. After 20 days, both cell lines were washed three times with ice cold D-PBS and incubated for 24 h in fresh medium not containing verapamil. On day 21, culture medium was removed and replaced with transport medium (Hanks balanced salt solution containing 0.35 g L^{-1} sodium bicarbonate, 5.95 g L^{-1} HEPES, 4.5 g L^{-1} glucose, pH 7.4) or transport medium containing verapamil 100 μM.

After 1 h preincubation at 37°C, medium was replaced with fresh transport medium containing doxorubicin 100 μM in either apical or basolateral chamber. At the same time, in the opposite compartment, fresh drug-free medium was added. For time course studies, 100 μL of medium were removed from the opposite side with respect of the side of doxorubicin application at 60 min intervals and the volume replaced with drug free medium. Doxorubicin fluorescence was measured by the method of Bachur[24].

RT-PCR: Total cellular RNA from exponentially growing drug-treated or untreated cells was extracted using SV total RNA isolation system, according to the protocol provided by the manufacturer.

Five micrograms of total RNA were reverse transcribed using Superscript II RNAse H and the cDNA was used in PCR reaction in a final volume of 10 μL.

Synthetic oligonucleotide primers were used to investigate the presence of MDR1 and CYP 3A4 transcripts. For amplification of a 799 bp fragment of the MDR1 cDNA, encoding the P-gp, sense 5'-GATGGACAGGATATAGGACC-3' and antisense 5'-ATGGCACAAAATACACCAACA-3' primers were used; for amplification of a 245 bp fragment of the CYP 3A4 cDNA, sense 5'-GCTCTCATCCCAGACTTGGCCATGGAA-3' and antisense 5'-CAGCACAGGCTGTTGACCATCATA-3' primer pair was utilized. As a control for normalization of RNA quantity[26], a 500 bp fragment of the ß-actin gene was amplified using the following primers: sense 5'-CACCAAAGCTGAGAGGGAAATCGTCGTGA-3' and antisense 5'-ATTTGCGGTGCACGATGGAGGGGCCGGACT-3'.

The amplification was performed using a DNA thermal cycler Gene Amp® 9700 (PE Applied Biosystems, Foster City, CA). After denaturing at 94°C for 5 min, amplification proceeded through cycles (30 with MDR1 and CYP 3A4 primers and 20 with ß-actin primers) at 94°C for 1 min, 60°C for 1 min, 72°C for 1 min and final step at 72°C for 10 min. PCR products were separated by electrophoresis in 1% agarose and visualised by UV in the presence of ethidium bromide. Densitometric analysis of PCR product images was performed by the GelCompare 2 (Applied Math, Kortrijk, Belgium).

RESULTS

Establishment of verapamil-resistant clone and cytotoxicity assay: The human colorectal carcinoma cell line, Caco-2, was exposed to stepwise increasing concentrations of verapamil and a resistant sub line

tolerating 50 μM verapamil, Caco-2/ver, was obtained and stored in liquid nitrogen.

Drug-sensitive and drug-resistant cells were exposed to appropriate concentrations of verapamil and allowed to grow for 72 h, after this incubation time, verapamil cytotoxicity was evaluated by the MTT assay.

The 50% inhibitory concentration (IC_{50} value) was calculated for both cell lines (Table 1) Caco-2 and Caco-2/ver displayed an IC_{50} of 59.86±2.32 and 348.50±1.23 μM, respectively. Resistance Factor (RF) calculated as the two IC_{50} ratio, was 5.82.

Doxorubicin accumulation: To evaluate doxorubicin uptake, Caco-2 and Caco-2/ver cells, grown for 20 days in complete medium or complete medium containing 50 μM verapamil, respectively, were incubated with three different concentrations of doxorubicin. Compared to the parental line, the accumulation of doxorubicin was decreased in the resistant line after 1 and 4 h of exposure (Fig 1a) and the difference was evident with all the tested concentrations (Fig. 1b).

Doxorubicin transport: The time course of 100 μM doxorubicin transport across parental and resistant cell line monolayer was evaluated. The drug was added to the medium in the lower or upper compartment to measure basolateral to apical and apical to basolateral transport, respectively.

A direct comparison of the transport rate at a given concentration (Fig. 2a and 2b) showed that basolateral to apical permeation was higher than apical to basolateral permeation, suggesting the involvement of a transport system operating in the basolateral to apical direction. When sensitive and resistant cell lines were compared, an increased basolateral to apical transport rate was demonstrated in Caco-2/ver cells. Verapamil 100 μM had almost no effect in Caco-2 cells; on the contrary, the calcium antagonist significantly increased apical to basolateral and significantly reduced basolateral to apical transport, in Caco-2/ver cells.

RT-PCR: RT-PCR was performed to determine the relative expression of mRNA for MDR1 and CYP 3A4 genes in Caco-2 and Caco-2/ver cell lines. Figure 3 demonstrates an increase of MDR1 mRNA expression in verapamil treated

Table 1: Mean±SD of cytotoxicity assay

	IC_{50} (μM)		
	Caco-2	Caco-2/ver	RF*
Verapamil	59.86 ±2.32	348.50 ±1.23	5.82

Three or more experiments were performed to calculate IC_{50}.
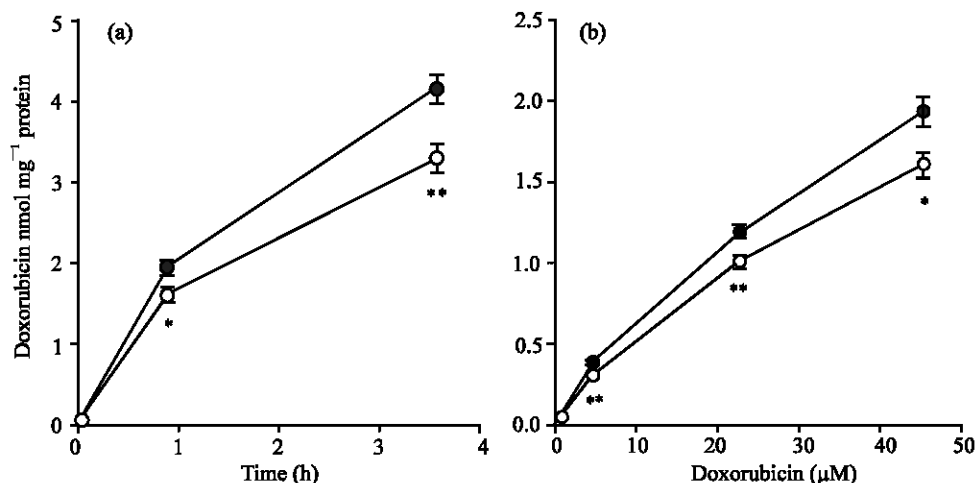*RF: resistance factor (IC_{50} resistant cell/IC_{50} sensitive cell)

Fig. 1a: Time dependent accumulation of 50 μM doxorubicin in Caco-2 (●) and Caco-2/ver (○) cells. b. Concentration dependent accumulation of doxorubicin in doxorubicin in Caco-2 (●) and Caco-2/ver (○) cells, incubated for 1 h. Results are the mean±SEM of three separate determinations.
Significantly different from Caco-2 cells at *: p<0.05; **: p<0.01, Student's t test for independent samples
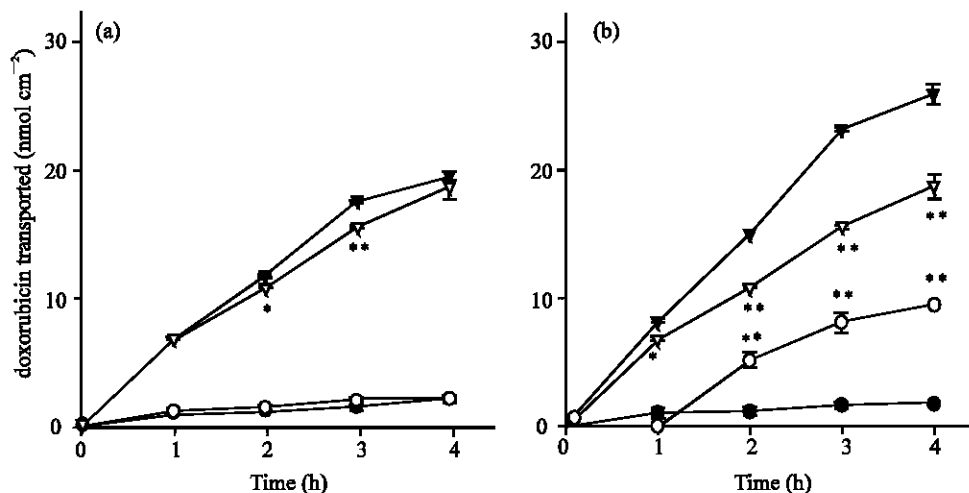

Fig. 2: Transepithelial transport of 100 μM doxorubicin by Caco-2 (a) and Caco-2/ver (b) monolayer. ●○: transport from the apical to the basolateral side; ▼▽: transport from the basolateral to the apical side. ○▽: monolayer was preincubated for 1 h in the presence of 100 μM verapamil. Results are the mean±SEM of three separate determinations.
Significantly different from Caco-2 cells at *: p<0.05; **: p<0.01, Student's t test for independent samples

compared to untreated cells. Densitometric analysis showed that the average increase was of 40%. In contrast, CYP 3A4 mRNA was almost undetectable in Caco-2 cells and verapamil treatment had no effect on the expression of this gene (data not shown).

DISCUSSION

In this study the modulation of the transport protein P-gp after verapamil treatment was demonstrated. This protein plays an important role in multidrug resistance in cancer cells[27], but is also physiologically expressed by a variety of normal tissues such as the liver, kidney and small bowel. In human intestine, P-gp is expressed on the apical membrane of enterocytes were it is assumed to pump xenobiotics from cells back to the gastrointestinal lumen and its expression increases from the proximal to the more distal parts[28].

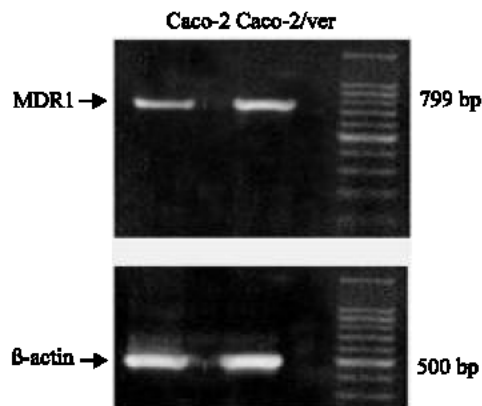In addition to P-gp, other transport proteins have been described in the normal gut. In particular, MRP1 was

Fig. 3:   Representative electrophoretic run of polymerase chain reaction products obtained with MDR1 and ß-actin primers

identified in the basolateral membrane of crypt cells, mainly Paneth cells[8], MRP2 mRNA was detected by Northern blots in duodenum and ileum[11] and MRP3 was detected in the basolateral membranes of small intestine and colon[9,10].

The calcium channel blocker verapamil is a well known substrate of P-gp and MRP and has been used as multidrug resistance modifying agent in tumours that express P-gp. The compound is widely used in cardiovascular therapy as antiarrhythmic agent and in the treatment of hypertension, angina and certain cardiomyopathies[21].

Since P-gp has been proposed to exert both protective mechanism against xenobiotics as well as endogenous substrates transport function, its expression could be regulated by several compounds transported, similarly to hepatic cytochrome system[29]. Among P-gp substrates, verapamil has been shown to influence the regulation of P-gp expression; Herzog *et al.*[15] have indeed shown that the calcium antagonist increases both the MDR1 mRNA levels and its product P-gp in the human colon carcinoma cell line LS180 and attributed this effect to posttranscriptional regulation occurring within the nucleus. More recently Anderle *et al.*[12] observed an induction of P-gp expression in Caco-2 cells cultured in medium containing verapamil, celiprolol or vinblastine. In contrast, Muller *et al.*[30] observed a decrease in P-gp expression at the mRNA level in multidrug resistant human leukemic cell lines and similar results were obtained by Hu *et al.*[31]. It appears therefore that the effect of modulators on transport protein gene expression is strictly dependent from the cell line employed, as well as from culture conditions.

The human colon carcinoma cell line Caco-2 functionally expresses the MDR1-gene product P-gp[32,33]; besides P-gp, MRPs are functionally expressed in Caco-2 cells and contribute to the active excretion of substrates in this cell line[13,34] and to reduction of the apical to basolateral permeability of MRP substrates[35]. On the contrary, these cells express insignificant levels of drug metabolizing enzymes of the cytochrome P450 class, which are found in high levels in the human intestine[36,37]. Thus this functional feature could permit the evaluation of the effect of inhibition or induction of transport proteins, without any interference of CYP 3A4 metabolizing enzymes.

Caco-2 cells spontaneously differentiate into polarized cell monolayer with the characteristics of intestinal epithelial cells and are a widely accepted model to investigate the contribution of P-gp to overall drug absorption and bioavailability of drugs. On the contrary, induction studies have been less successful with these cells; probably because the ideal point for induction is not clear.

In this study Caco-2 cells were grown in the presence of verapamil; the Caco-2/ver cells obtained has been developed through a continuous selection with increasing doses of the calcium antagonist over an extended period of time. The Caco-2/ver cell line obtained grows in the presence of a concentration of 50 µM of verapamil. Plasma concentrations of verapamil are usually lower than 0.5 µM, however, the concentrations reached in the intestine after administration of usual oral doses may reach much higher levels, comparable to or even higher than those used in this study[38]. Cells were cultured for 21 days before each experiment; it is indeed well known that the expression of transport proteins in Caco-2 cells varies with culture conditions[39-41].

The resistant cells obtained in this study exhibit a multidrug resistance profile, with reduced intracellular accumulation of doxorubicin and reversal of this response by verapamil, an increased transport of the antineoplastic agent from the basolateral to the apical side and moderately increased MDR1 mRNA levels estimated by RT-PCR. This technique is extremely sensitive and allows the detection of very low levels of expression, which may be however, of clinical importance; the precise quantification of gene expression with this technique may be difficult, because of the exponential increase of PCR products and several means have been used for solving this problem. In this study, the house keeping gene β-actin was used as an internal standard and the ratio between densitometric quantification obtained with

specific and with β-actin primers was calculated to normalize data[26]. Although this semi-quantitative technique cannot be considered ideal for evaluating the absolute amounts of specific mRNAs[42], it appears quite satisfactory for comparative evaluations of gene expression in a given cell line subjected to drug treatments[31].

In conclusion, chronic exposure of Caco-2 cells to verapamil can modify the phenotype of these cells by induction of P-gp expression and increases the efflux of doxorubicin. This effect of verapamil should be bore in mind when the drug is administered to patients for different pathologies. The drug could indeed increase the expression of transport proteins in the normal gut and consequently modify the pharmacokinetics of other substances that are P-gp or MRP substrates. In addition, the possibility that P-gp expression could be increased in colon carcinomas exposed to calcium blockers is of interest.

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