CHARLES UNIVERSITY IN PRAGUE
FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

Department of Pharmacology and Toxicology

EFFECT OF COLCHICINE ON P-GLYCOPROTEIN EXPRESSION AND ACTIVITY IN CACO-2 CELLS

Diploma thesis

Thesis supervisor: PharmDr. Jana Pourová, Ph.D.

Hradec Králové 2011 Tereza Houdková

I declare I processed this thesis on my own. All bibliographic sources and other materials that I used for this work are listed in the references and cited properly.

Tereza Houdková

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ABSTRACT

Charles University in Prague

Faculty of Pharmacy in Hradec Králové

Department of Pharmacology and Toxicology

Candidate: Tereza Houdková

Supervisor: PharmDr. Jana Pourová, Ph.D.

Title of diploma thesis: Effect of colchicine on P-glycoprotein expression and activity in caco-2 cells

The caco-2 cell line is one of the most widely used human cell culture models. These cells derived from human colorectal adenocarcinoma and have been accepted as a reliable *in vitro* model for intestinal drug excretion mediated by P-glycoprotein studies. P-glycoprotein is an ATP-dependent efflux pump encoded by the MDR1 gene in humans, which is highly expressed in several cancer cells conferring a multidrug resistance phenotype.

P-glycoprotein is inducible by many drugs including dexamethasone, rifampicin, the herbal antidepressant St. John's wort (hyperforin and hypericin) and chemotherapeutic agents, namely doxorubicin, daunorubicin and vinblastine. The sensibility of P-glycoprotein from caco-2 cells to different inducing compounds is yet to be clearly established. Colchicine is a toxic natural product and secondary metabolite, originally extracted from plants of the genus Colchicum – Colchicum autumnale. This compound is used as an anticancer drug and was already reported as a P-glycoprotein inducer. Thus, the main objective of the present work was to evaluate the potential changes in P-glycoprotein expression and activity, when caco-2 cells are exposed to colchicine.

Caco-2 cells were exposed to a range of colchicine concentrations (0,1 μM – 100 μM), for a maximum period of 96 hours. Colchicine cytotoxicity was evaluated at different time points by the MTT assay. P-glycoprotein expression and transport activity were evaluated by flow cytometry, using a fluorescein isothiocyanate conjugated antibody (CD 243) and the P-glycoprotein fluorescent subtract rhodamine 123, respectively. The obtained results suggest that colchicine is cytotoxic for all the tested concentrations when caco-2 cells are exposed for more than 24 hours. For that reason, caco-2 P-glycoprotein expression and transport activity were evaluated only after 24 hours incubation with colchicine. Exposure of these cells to colchicine for 24 hours resulted in a small but significant increase in P-glycoprotein expression levels, although no significant changes were observed in P-glycoprotein transport activity. The observed results were important to characterize these cells in order to study the induction mechanism to protect cells from toxic compounds, including therapeutic drugs.

ABSTRAKT

Univerzita Karlova v Praze

Farmaceutická fakulta v Hradci Králové

Katedra farmakologie a toxikologie

Kandidát: Tereza Houdková

Školitel: PharmDr. Jana Pourová, Ph.D.

Název diplomové práce: Účinky kolchicinu na expresi a aktivitu P-glykoproteinu v caco-2 buňkách

Caco-2 buňky jsou jedním z nejpoužívanějších modelů lidských buněčných struktur. Tyto buňky pocházejí z lidských kolorektálních adenokarcinomů a jsou používány jako spolehlivý *in vitro* model pro střevní vylučování léků zprostředkované P-glykoproteinem. P-glykoprotein je ATP-dependentní efluxní pumpa kódovaná u člověka MDR1 genem a je vysoce vyjádřena v několika typech nádorových buněk.

P-glykoprotein je indukovatelný mnoha léky včetně dexametazonu, rifampicinu, třezalky - rostlinného antidepresiva (hyperforinu a hypericinu) a chemoterapeutik doxorubicinu, daunorubicinu a vinblastinu. Citlivost P-glykoproteinu caco-2 buněk k různým indukujícím sloučeninám zatím není ještě plně objasněna. Kolchicin je toxický, přírodní produkt a sekundární metabolit, původně získaný z rostlin rodu Colcichum – Colcichum autumnale. Tato látka se používá jako lék proti rakovině a je známa jako induktor P-glykoproteinu. Hlavním cílem této práce bylo zhodnotit potenciální změny v expresi a aktivitě P-glykoproteinu caco-2 buněk působením kolchicinu.

Caco-2 buňky byly vystaveny řadě koncentrací kolchicinu (0,1 μM – 100 μM) po dobu maximálně 96 hodin. Cytotoxicita kolchicinu byla hodnocena MTT testem v různých časových intervalech. Exprese a transportní aktivita P-glykoproteinu byla hodnocena průtokovou cytometrií, pomocí fluorescenčního isothiokyanátu a P-glykoproteinového fluorescenčního substrátu rhodaminu 123. Z výsledků vyplývá, že kolchicin je cytotoxický pro všechny zkoušené koncentrace, kterým jsou vystaveny caco-2 buňky více než 24 hodin. Z tohoto důvodu byla hodnocena exprese a transportní aktivita P-glykoproteinu po 24 hodinách inkubace s kolchicinem. Vystavení caco-2 buněk kolchicinu po dobu 24 hodin vyvolá malé, ale významné zvýšení exprese P-glykoproteinu. Naopak u transportní činnosti P-glykoproteinu nebyly pozorovány žádné významné změny. Pozorované výsledky umožnily charakteristiku těchto buněk za účelem studia mechanismu indukce.

1. INTRODUCTION

Pharmacy is a medical branch dealing scientifically and practically with the medication and drugs. The functions of pharmacy involve research, obtaining of scientific knowledge about drugs, evaluating and drug quality control among others. [21]

Toxicology is a multidisciplinary science, a branch of biology, medicine and chemistry concerned with the study of the adverse effects of chemicals on living organisms. It is the study of symptoms, mechanisms, treatments and detections of poisoning, especially the poisoning of people. [13]

The caco-2 cell line is one of the most widely used human cell culture models. These cells derived from human colorectal adenocarcinoma have been accepted as a reliable in vitro model for intestinal drug excretion mediated by P-gp (P-glycoprotein). P-gp is an ATP-dependent efflux pump encoded by the MDR1 gene in humans, which is highly expressed in several cancer cells conferring a multidrug resistance phenotype. P-gp is inducible by many kinds of drug. The sensibility of P-gp from caco-2 cells to different inducing compounds is yet to be clearly established. [2, 10]

2. THE AIM OF THE WORK

The aim of the work is to evaluate caco-2 cells P-gp expression and activity when exposed to colchicine, a known P-gp inducer.

3. THEORETICAL PART

3. 1. COLCHICINE

Colchicine is a mitotic poison that is a violently poisonous alkaloid, a toxic natural product and secondary metabolite, originally isolated from plants of the genus Colchicum – Colchicum autumnale. Colchicum autumnale is a perennial, usually 10 - 30 cm high poisonous herb. Naturally it is widespread in central, southern and western Europe. It occurs in damp meadows from lowlands to mountains. It is a sharp poisonous plant containing more than 20 alkaloids, of which colchicine and its derivatives are the most dangerous. Genus Colchicum includes nearly 200 species of plants. Colchicine can not be normally found in the human body. [18, 24, 23]

Fig. 1 Colchicum autumnale [22]

3. 1. 1. Chemistry of colchicine

Colchicine is an alkaloid of slightly alkaline nature and its salts are very unstable and strongly hydrolyzed in aqueous solution. It is a tricyclic derivative that binds to a specific site of tubulin. [24]

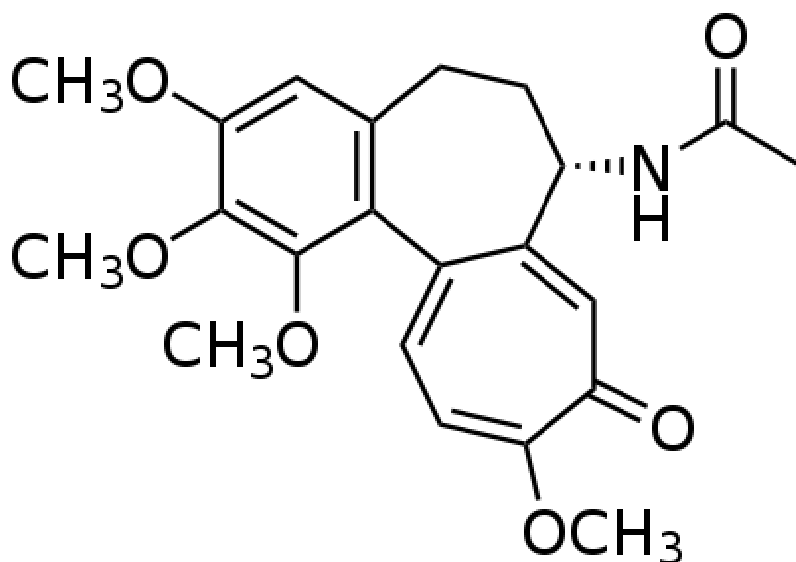

Fig. 2 Colchicine [25]

Systematic name: N-[(7S)-1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl] acetamid

Chemical formula: $C_{22}H_{25}NO_6$

3. 1. 2. Effects and application of colchicine

Colchicine has a cytostatic effect, and thus can prevent the uncontrolled cell division in the case of cancerous growths. It is a very powerful mitotic poison and one of the most effective cell poisons. It attacks the microtubules of spindle and thus interferes with the proper gauge of chromosomes in mitosis (mitosis is discontinued in metaphase). Therefore it is used in genetics for the artificial induction of genomic mutations. In medicine it is used to treat gout and chronic myeloid leukemia. Lethal dose for adult is 20 mg of colchicine. This compound is used as an anticancer drug and was already reported as a P-gp inducer. [18, 24, 23]

3. 2. CACO-2 CELLS

Caco-2 cells are a well-established human carcinoma cell line that closely mimics the enterocytes of the small intestine. [3]

These cells derived from human colorectal adenocarcinoma have been widely accepted as a reliable *in vitro* model for predicting drug intestinal absorption and excretion in humans. [10, 16]

Though derived from a human colon carcinoma these cells reconstitute as epithelial monolayers when grown on permeable supports and express many features (brush-border membrane hydrolases on the apical border and demonstrate absorptive transport of bile salts, cobalamin, glucose and dipeptides) of the normal human intestine, particularly those related to the ileum. [9]

Moreover, caco-2 cells exhibit spontaneous morphological and biochemical enterocytic differentiation after confluence in culture. [10]

Caco-2 cells have been reported to express P-gp, as well as other transporters and, except for breast cancer protein, the expression levels are in good agreement with those of normal human jejunum. [14]

Moreover, Hunter et al. stated the immunohistochemical localization of P-gp to the apical membrane of caco-2 cells, which demonstrates an additional membrane transport protein with polarized expression in this intestinal cell line in addition to those involved in glucose, bile salts and dipeptide transport. The polarized expression of P-gp in caco-2 cells was accompanied by net secretory (basal-to-apical) transport of vinblastine, a typical substrate, thus being in a good agreement with the morphological and functional measurements. The polarized nature of P-gp expression and functional activity in caco-2 cells provided a direct evidence for its role in the gastrointestinal epithelial barrier as a secretory detoxifying membrane protein. [9]

These results focused our research on the study of the mechanism underling P-gp induction adding enormous value to the development of an *in vitro* P-gp induction model in caco-2 cells in order to screen and select potent and safe P-gp inducers.

3. 3. P-GLYCOPROTEIN

P-gp is an ATP-dependent efflux pump encoded by the MDR1 gene in humans, which is highly expressed in several cancer cells conferring a multidrug resistance phenotype. P-gp MDR1 belongs to the ATP-binding cassette superfamily of transporters. [5]

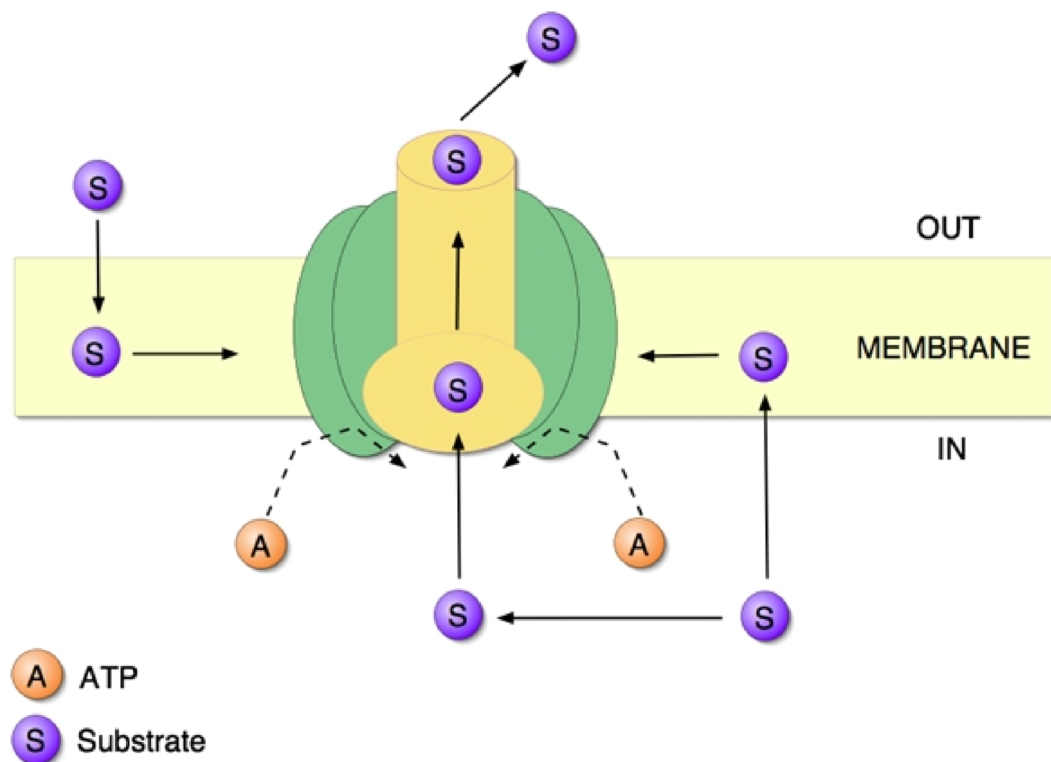

Fig. 3 Simplified cartoon of P-gp structure and function [4]

One of the most interesting characteristics of P-gp is its broad range of substrates that vary greatly in structure and activity, ranging from small molecules to macromolecules. [17]

P-gp confers resistance of cancer cells to chemotherapeutic drugs and has crucial impact on HIV protease inhibitors bioavailability. [7, 17]

Considerable efforts have been undertaken by the pharmaceutical industry to develop specific P-gp inhibitors for reducing multidrug resistance. [7]

In addition to the expression in tumor cells, P-gp is widely distributed in apical surfaces of normal human epithelial tissues like in gastrointestinal tract, kidney, placenta, testes. In normal tissues it plays an important role in the excretion of a variety of structurally and pharmacologically unrelated hydrophobic compounds and peptides thus significantly affecting the ADME (absorption – distribution – metabolism - excretion) of a number of clinically important drugs. [11]

After binding to P-gp the substrates are transported back to the apical surface of the tissue in an ATP-dependent manner preventing the accumulation of both exogenous and endogenous compounds. [3]

As the bioavailability of orally administered drugs is mainly affected by the absorption from the intestine, intestinal P-gp plays an important role in their pharmacokinetics. Because of those efflux properties, because of its polarized expression and as P-gp has a very broad substrate specificity with a relatively hydrophobic and amphophilic nature this protein may be suggested to be an intracellular detoxification mechanism against xenobiotics. The P-gp induction and inhibition seems to be a suitable tool to modulate the levels of xenobiotics in cells, organs or organisms. [16]

4. EXPERIMENTAL PART

4. 1. CACO-2 CELL CULTURE

Caco-2 cells were cultured in 75 cm^2 flasks which used DMEM (dulbecco's modified eagle medium) supplemented with 10% FBS (fetal bovine serum), 1% fungizone, 1% NEAA (non-essential amino acid), 1% antibiotic and transferrin (6 ug / ml). Cells were maintained in a 5% CO_2-95% air atmosphere the medium was changed every 2 days. Cultures were passaged by trypsinization (0.25% trypsin / 1 mM EDTA = ethylenediaminetetraacetic acid).

Practically:

- Use a 75cm^2 cell culture flask with caco-2 cells at confluence maintained in the incubator at 37º C
- Remove the cell culture media (DMEM)
- Wash the cells with 2 x 10 ml PBS (phosphate-buffered saline) 37° C and remove
- Add 1.5 ml trypsine and incubate at 37°C for 10 minutes to completely detach the cells
- Neutralize the trypsin by adding 10 ml of cell culture media as the FBS present in the cell culture medium will inactivate the enzyme
- Homogenate the cell suspension and count the cells with the newbauer chamber

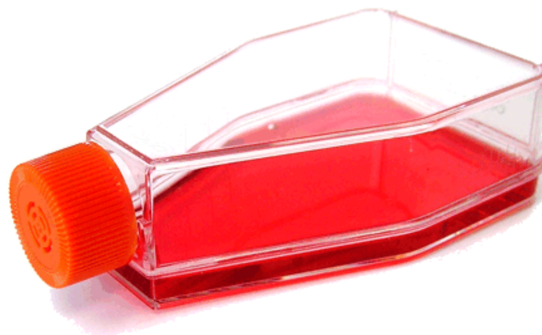

Fig. 4 Flask for culturing caco-2 cells [19]

4. 2. COLCHICINE CYTOTOXICITY ASSAY

Caco-2 cells were seeded into 96-well plates and treated, after reaching confluence, with 0 – 100 µM colchicine for 6 – 96 hours. Control cells (cells without colchicine) were maintained with DMEM medium during the time course of the experience. In the day of the experiment the cells were washed twice with PBS (pH 7.4) and exposed to colchicine. Positive control cells were maintained with DMEM with 1% triton X-100.

Practically:

• Plate the cells in a 96-well plate – 21 000 cells / well / 250 µl cell culture medium

• Tested compound concentration: 0; 0.1; 0.5; 1; 5; 10; 50 and 100 µM

• Different time of exposure: 6; 12; 24; 48; 72 and 96 hours

Tab. 1 Plating 96-well plate

PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
PBS	0	0.1	0.5	1	5	10	50	100	+	0	PBS
PBS	0	0.1	0.5	1	5	10	50	100	+	0	PBS
PBS	0	0.1	0.5	1	5	10	50	100	+	0	PBS
PBS	0	0.1	0.5	1	5	10	50	100	+	0	PBS
PBS	0	0.1	0.5	1	5	10	50	100	+	0	PBS
PBS	0	0.1	0.5	1	5	10	50	100	+	0	PBS
PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

0... Control cells (not exposed to colchicine); 0.1 – 100... Concentration of colchicine; PBS... Phosphate-buffered saline; +... Positive control

4. 2. 1. LDH assay

Cell damage caused by colchicine in the caco-2 cells cultures was assessed quantitatively by the measurement of LDH (lactate dehydrogenase) release into the medium (as a measure of cell membrane integrity and metabolic impairment) by means of a kinetic photometric assay. LDH is a cytoplasmic enzyme that is released into the cytoplasm upon cell lysis. The LDH assay, therefore, is a measure of membrane integrity.

Practically:

• After the selected time of exposure remove 50 µl of the cell culture media to a new 96-well plate

• Add 200 µl β-NADH (β-nicotinamide-adenine dinucleotide) (3 mg / 20 ml)

• Add 25 µl piruvate 22.7 mM

• Read the absorbance at 340 nm though a 4:30 minutes kinetic photometric assay (BioTek Instruments, Vermont, US)

4. 2. 2. MTT assay

The second assay for the *in vitro* evaluation of colchicine cytotoxicity was the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay which measures mitochondrial activity. MTT is a yellow, water-soluble tetrazolium dye that is reduced by live cells to a water-insoluble, purple formazan. The amount of formazan can be determined by solubilizing it in DMSO (dimethyl sulfoxide) and measuring it spectrophotometrically. Comparisons between the spectra of treated and untreated cells can give a relative estimation of cytotoxicity.

Practically:

• After the selected time of exposure remove the cell culture media

• Wash the cells with PBS (pH 7.4) 37° C

• Add 150 µl of the fresh cell culture media containing MTT (0.5 mg / l)

• Incubate for 30 minutes at 37° C in the incubator

• Remove the cell culture media

• Dissolve the formed crystals with 150 µl DMSO

• Leave to dissolve for 15 minutes in the dark

• Read the absorbance at 550 nm in a multiwell plate reader (BioTek Instruments, Vermont, US)

4. 3. P-GLYCOPROTEIN EXPRESSION AND ACTIVITY

Caco-2 cells were seeded into 24-well plates and treated, after reaching confluence, with 0 - 100 μM colchicine for 24 hours (P-gp expression and transport activity was only evaluated 24 hours after exposure). On the day of the experiment the cells were washed twice with PBS (pH 7.4) and exposed to colchicine.

Practically:

- Plate the cells in 24-well plates (4 plates) – 114 000 cells / well / 1ml
- Tested compound concentration: 0; 0.1; 0.5; 1; 5; 10; 50 and 100 μM
- Time of exposure: 24 hours

Tab. 2 Plating 24-well plate (plate 1)

PBS	PBS	PBS	PBS	PBS	PBS
PBS	0	0.1	0.5	1	PBS
PBS	0	0.1	0.5	1	PBS
PBS	0	0.1	0.5	1	PBS

0 - 1... Concentration of colchicine; PBS... Phosphate-buffered saline

Tab. 3 Plating 24-well plate (plate 2)

PBS	PBS	PBS	PBS	PBS	PBS
0	5	10	50	100	PBS
0	5	10	50	100	PBS
0	5	10	50	100	PBS

0 - 100... Concentration of colchicine; PBS... Phosphate-buffered saline

After the incubation period the cells were washed twice with PBS and trypsinized with 0. 25% trypsin / 1mM EDTA to obtain a cell suspension. The cells in each well were then divided in two aliquots of approximately 450 000 cells for the evaluation of P-gp activity and expression, respectively.

4 plates were plated altogether. Plates 1 (Table 2) and 3 are similar as well as plates 2 (Table 3) and 4. For plates 1 (Table 2) and 2 (Table 3) the cell suspension was divided into 2 aliquots for the evaluation of inhibited accumulation and inhibited accumulation + efflux. In the case of plates 3 (Table 2) and 4 (Table 3), the cells were divided into 2 aliquots for the evaluation of normal accumulation and P-gp expression.

4. 3. 1. P-glycoprotein expression

For the evaluation of P-gp expression the cells were centrifuged (300g / 10 minutes) and suspended in PBS buffer (pH 7.4) containing 10% FBS and P-gp antibody (UIC2) conjugated with FITC (fluorescein isothiocyanate). The antibody dilution used in this experiment was defined according to the manufacturer's instructions for flow cytometry. The cells were then incubated for 30 minutes at 37° C in the dark. After this incubation period the cells were washed twice with PBS (pH 7.4) containing 10% FBS, suspended in ice-cold PBS and kept on ice until analyses. Fluorescence measurements of isolated cells were performed with a fluorescent activated cell sorter (FACSCalibur, Becton -Dickinson Biosciences). Analysis was gated to include viable cells on the basis of their forward and side light scatters and the propidium iodide (4 µg / ml) incorporation based on the acquisition of data for 10 000 cells.

4. 3. 2. P-glycoprotein activity

P-gp activity was evaluated also by flow cytometry using 1 µM RHO 123 (rhodamine 123) as a P-gp fluorescent substrate. P-gp activity was evaluated by 2 different ways:

1- Analyzing the intracellular rhodamine fluorescence when the cells are submitted to normal accumulation conditions. In this case, cells with higher P-gp activity are expected to accumulate less RHO 123 as this substrate is being, at the same time, pumped out of the cell.

2- Comparing the inhibited accumulation + efflux (AiE) in control cells with treated cells, as the cells are left to accumulate RHO 123 without pumping it (inhibited accumulation) but then are submitted to an efflux phase (without inhibitors) and higher P-gp levels will result in a smaller RHO 123 accumulation.

For the inhibited accumulation phase, P-gp activity was blocked with 10 mM NaN_3 (sodium azide) (to inhibit energy production) and 10 μM cyclosporine (a known P-gp inhibitor) in order to accumulate the substrate inside the cells (designed as inhibitted accumulation). For the evaluation of inhibited accumulation followed by efflux, the cells were submitted to an accumulation phase similar to the described above, followed by an efflux phase where the energy-dependent P-gp function was re-established due to removal of the P-gp inhibitor (cyclosporine) and the addition of an energy source (DMEM with 4.5 g / mol glucose) (designed as inhibited accumulation followed by efflux).

P-gp activity was also evaluated through an accumulation phase in the absence of cyclosporine and NaN_3, known as normal accumulation. RHO 123 and cyclosporine cytotoxicity (evaluated after a 30 minutes incubation, the time necessary for RHO 123 accumulation) was previously determined by the propidium iodide incorporation assay (based on propidium iodide incorporation in nucleic acid helix of non-viable cells, with resultant increase in fluorescence intensity emission at 615 nm analyzed in the flow cytometer).

When P-gp activity increases, the amount of RHO 123 effluxed from the cells is higher and accompanied by a decrease in the fluorescence intensity due to the corresponding decrease in intracellular RHO 123. For the inhibited accumulation phase the cells were centrifuged (300 g / 10 minutes), suspended in PBS buffer (pH 7.4) containing 10 mM NaN_3, 10 μM cyclosporine and 1μM RHO 123 and incubated at 37º C for 30 minutes. After the accumulation of the fluorescent substrate, the cells were washed twice with ice-cold PBS with 10% FBS and centrifuged (300 g / 10 minutes) at 4° C. For the efflux phase the cell pellet submitted also to inhibited accumulation was suspended in DMEM medium containing 4.5 g / l glucose and incubated for 30 minutes at 37° C. After this efflux period, the cells were washed twice with ice-cold PBS with 10% FBS, suspended in ice-cold PBS and immediately analyzed as described above for the P-gp expression assay using the fluorescent activated cell sorter.

For the normal accumulation phase the cells were centrifuged (300 g / 10 minutes), suspended in PBS buffer (pH 7.4) containing 1µM RHO 123 and incubated at 37º C for 30 minutes. After the accumulation of the fluorescent substrate, the cells were washed twice with ice-cold PBS with 10% FBS and centrifuged (300 g /10 minutes) at 4° C.

5. RESULTS

5. 1. COLCHICINE CYTOTOXICITY

Colchicine cytotoxicity in caco-2 cells was evaluated by two different *in vitro* cytotoxicity assays:

- The MTT assay that measures mitochondrial activity.
- The LDH leakage assay that is a measure of membrane integrity, as the increase in extracellular LDH activity reflect the release of this cytoplasmatic enzyme to the extracellular media as a result of membrane damage.

5. 1. 1. MTT assay

Previous to the evaluation of the effect of colchicine on P-gp expression and activity, the cytotoxicity of this inducer was determined at different concentrations and time points. The conversion of MTT to formazan crystals by mitochondrial dehydrogenases was used as an index of mitochondrial viability and was evaluated up to a maximum period of 96 hours. Figure 5 presents the cytotoxic effects of 0 - 100 µM cholchicine in caco-2 cells. Colchicine exposure resulted in a concentration and time-dependent cytotoxic effect after 48 hours of incubation. The obtained results show that up to a period of 24 hours of incubation no significant effect on mitochondrial viability occurred at any of the tested colchicine concentrations. However, 48 hours after colchicine exposure significant changes in cell viability were observed for all tested concentrations. In fact, for exposure times higher than 48 hours, there was a significant and extremely pronounced reduction in mitochondrial activity, mainly at the 5 – 100 µM colchicine concentration range. At the end of the experiment (96 hours) mitochondrial viability decreased to values of 42 - 45% as compared to the control cells within this concentration range.

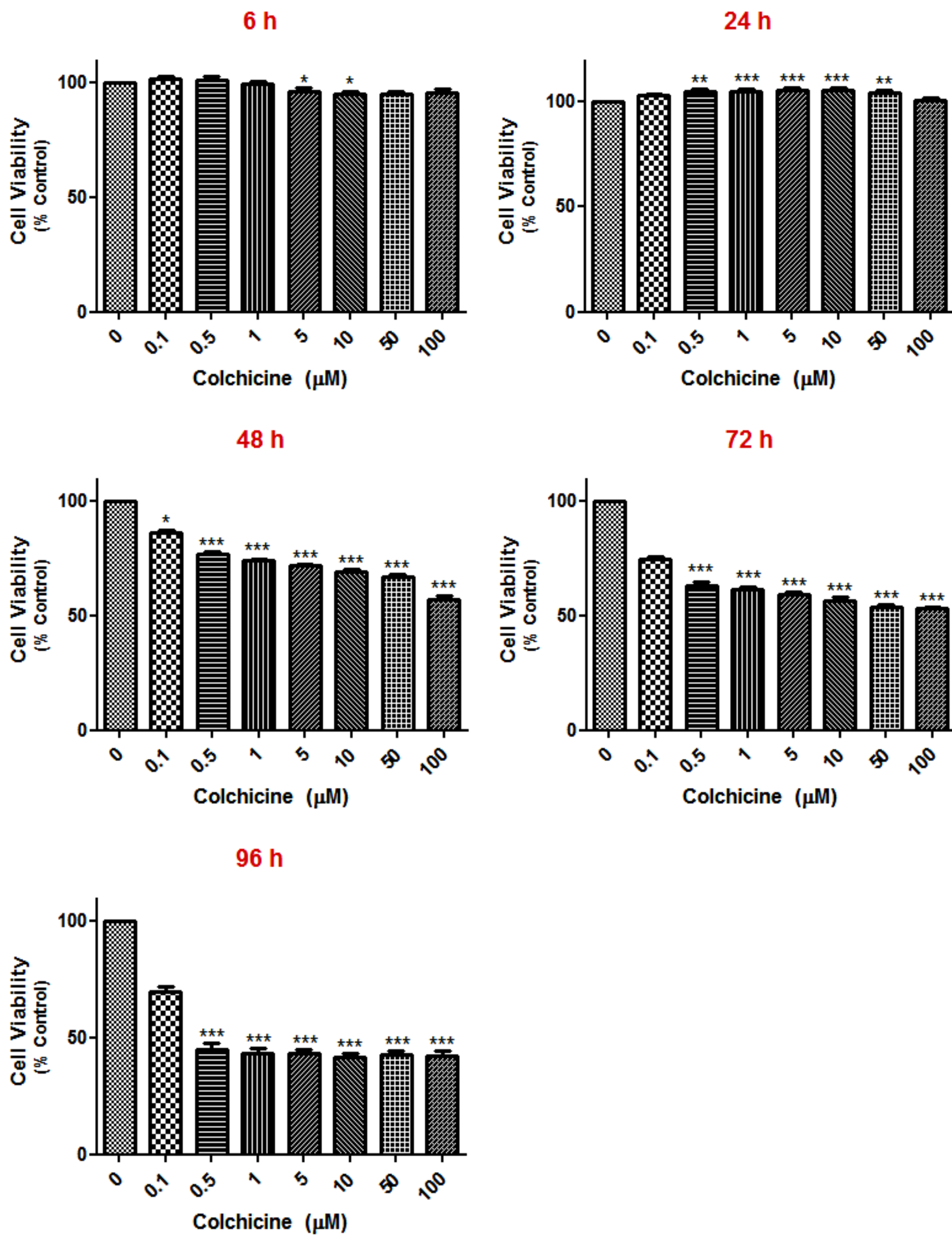

Fig. 5 The cytotoxic effects of 0 - 100 μM cholchicine in caco-2 cells

5. 1. 2. LDH leakage assay

LDH is a cytosolic enzyme which is released from damaged cells into the extracelular cell culture medium. Thus, cell damage caused by colchicine in the caco-2 cells cultures was assessed quantitatively by the measurement of LDH release into the medium by means of a kinetic photometric assay. The LDH assay, therefore, is a measure of membrane integrity and consists in the evaluation of the decrease in absorbance at 340 nm resulting from the oxidation of NADH (Figure 6).

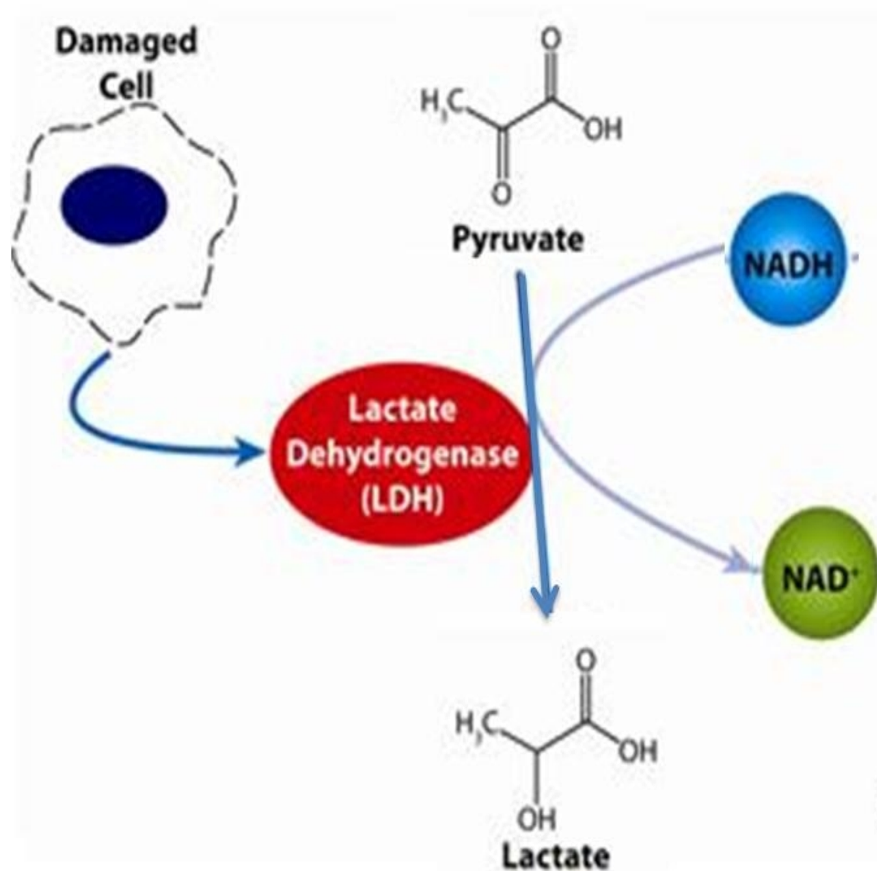

Fig. 6 Schematic representation of the LDH leakage assay [20]

Colchicine cytotoxicity was evaluated by the LDH leakage assay up to 72 hours after exposure (Figure 7).

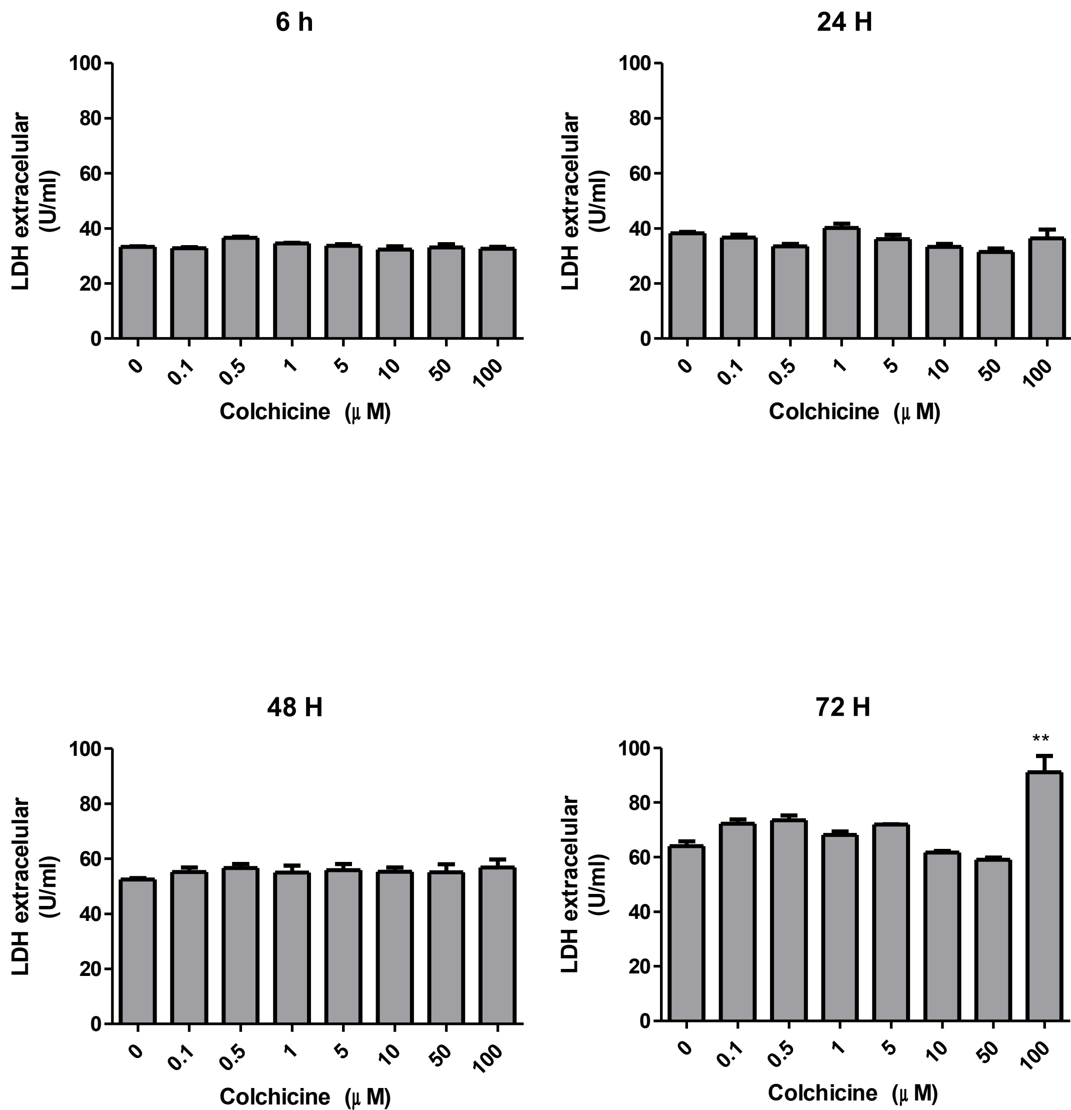

Fig. 7 Colchicine cytotoxicity by the LDH leakage assay up to 72 hours after exposure

Statistical comparisons were made using the Kruskal-Wallis test followed by the Dunn's Multiple Comparison post hoc test (*p<0.05; **p<0.01; ***p<0.001 vs control).

The obtained results show the absence of significant differences in LDH activity from all the tested concentrations when compared to control cells up to 48 hours of exposure. For longer exposure times (72 hours) it was possible to observe a significant increase in extracellular LDH activity when the cells are exposed to 100 μM colchicine.

These results suggest that the two *in vitro* cytotoxicity assays performed have different sensitivity with the MTT assay being more sensitive in detecting cytotoxic events compared to the LDH leakage.

Taking these results into consideration, the evaluation of colchicine effect on P-gp expression and activity was only performed for the incubation period of 24 hours, due to the lack of significant cytotoxicity demonstrated by the MTT assay.

5. 2. EVALUATION OF P-GLYCOPROTEIN EXPRESSION AND ACTIVITY

5. 2. 1. P-glycoprotein expression

The effect of colchicine on P-gp expression in caco-2 cells was evaluated by flow cytometry, using a P-gp monoclonal antibody (UIC2) conjugated with FITC. Non-specific binding of the FITC-labeled anti-P-gp antibody (UIC2) was not observed as estimated by the fluorescence obtained with the isotype-matched negative control. The results presented in Figure 8 show that colchicine significantly increased P-gp expression in a concentration-dependent manner.

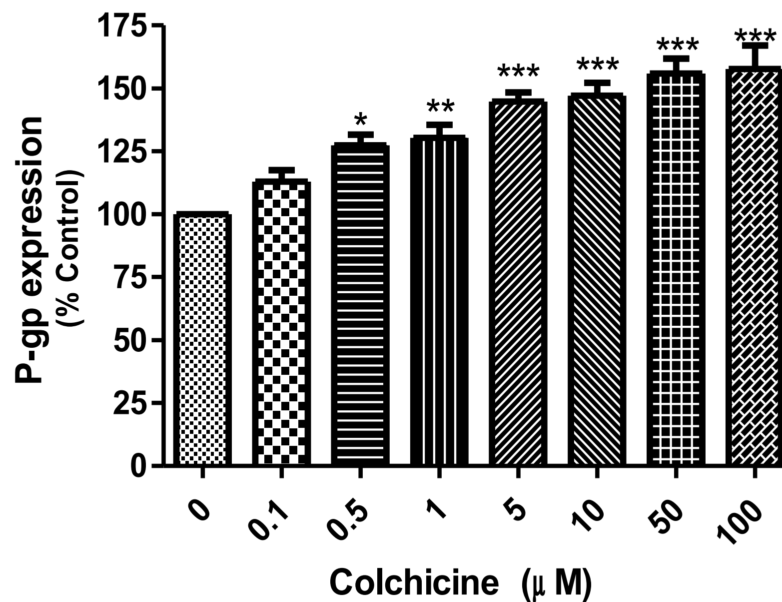

Fig. 8 P-gp expression in caco-2 cells exposed to colchicine (0 - 100 μM) for 24 hours

Results are presented as mean ± SEM from 4 independent experiments (triplicates were performed in each experiment). Statistical comparisons were made using One-way analysis of variance followed by the Bonferroni's Multiple Comparison post hoc test (*p<0.05; **p<0.01; ***p<0.001 vs control).

The obtained results show that the concentration range with optimal induction effect is between 5 - 100 μM. In fact, at 5, 10, 50 and 100 μM colchicine, a significant increase in P-gp expression was observed, achieving values of 145%, 147%, 156% and 157%, as compared to control, respectively. For the lower concentration (0.1 μM) no significant effect on P-gp expression was observed during the time course of the experiment. On the other hand, for the colchicine concentrations of 0.5 and 1 μM, it was observed a significant increase in P-gp expression levels, with values of 127 and 130% when compared to control, respectively.

5. 2. 2. P-glycoprotein activity

P-gp activity was evaluated by flow cytometry using 1 μM RHO 123 as a P-gp fluorescent substrate and was analyzed by 2 different ways: analyzing the intracellular rhodamine fluorescence when the cells are submitted to normal accumulation conditions. In this case, cells with higher P-gp activity are expected to accumulate less RHO 123 as this substrate is being, at the same time, pumped out of the cell. For this purpose, the measure of P-gp activity was 1/NA, where NA is the normal accumulation fluorescence. Cells with higher P-gp activity will have a smaller intracellular fluorescence as more RHO 123 is being pumped out the cells which results in a higher 1/NA value.

Comparing the inhibited accumulation fluorescence with the fluorescence when the cells are submitted to inhibited accumulation followed by efflux. In this case, the cells are left to accumulate RHO 123 without pumping it (inhibited accumulation) but then are submitted to an efflux phase (without inhibitors). The P-gp activity is evaluated by the ratio Ai/AiE where Ai is the cell fluorescence after inhibited accumulation and AiE is the fluorescence when the cells are submitted to inhibited accumulation followed by efflux. With this measure, higher P-gp levels will result in a smaller RHO 123 accumulation after AiE so will result in a higher Ai/Aie value.

The evaluation of cell fluorescence when they are submitted to normal accumulation is presented in Figure 9. It was possible to state that after exposure caco-2 cells to colchicine 0 - 100 µM no significant changes in the RHO 123 accumulation were observed.

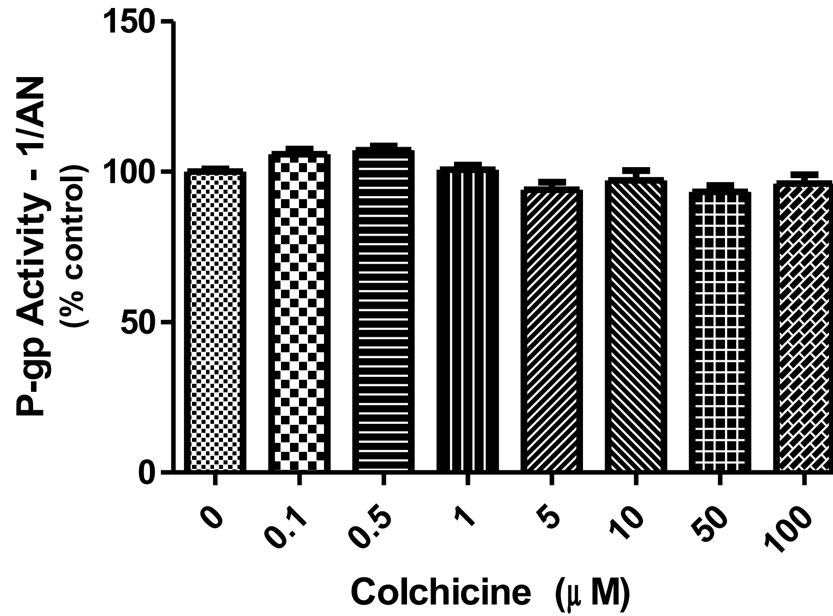

Fig. 9 P-gp activity in caco-2 cells exposed to colchicine (0 – 100 µM) for 24 hours evaluated by the normal accumulation emitted fluorescence

Results are presented as mean ± SEM from 4 independent experiments (triplicates were performed in each experiment). Statistical comparisons were made using One-way analysis of variance followed by the Bonferroni's Multiple Comparison post hoc test (*p < 0,05; **p < 0,01; ***p < 0,001 vs control).

P-gp activity was also evaluated by the ration Ai/Aie where the cells are accumulating the fluorescent substrate in inhibited P-gp conditions and then are submitted to an efflux phase where RHO 123 is being pumped out the cells. Cells with higher P-gp activity will accumulate less RHO 123 during the efflux phase resulting in a smaller fluorescence in the end of this efflux phase. Thus, higher P-gp levels result in a higher Ai/AiE ratio. The results are represented in Figure 10 and suggest that no significant changes in P-gp activity were observed for all the tested colchicine concentrations.

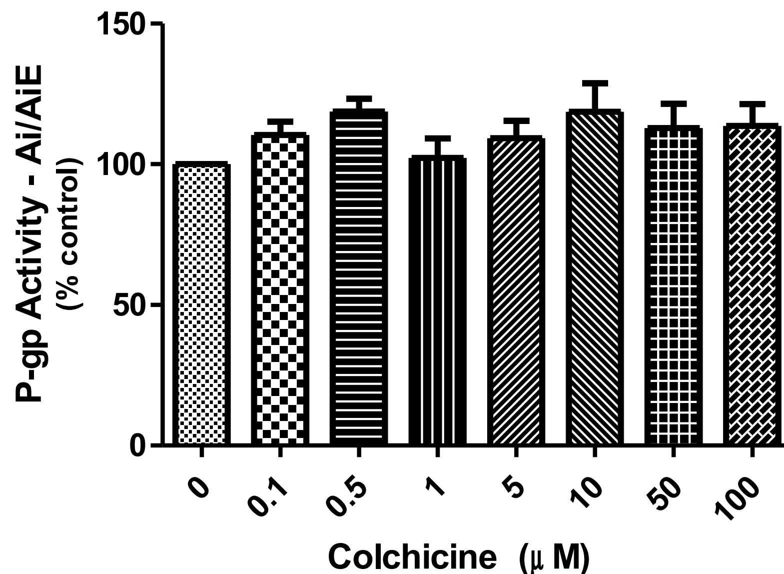

Fig. 10 P-gp activity in caco-2 cells exposed to colchicine (0 – 100 µM) for 24 hours, evaluated by the ratio between inhibited accumulation fluorescence (Ai) and inhibited accumulation + efflux fluorescence (AiE)

Results are presented as mean ± SEM from 4 independent experiments (triplicates were performed in each experiment). Statistical comparisons were made using One-way analysis of variance followed by the Bonferroni's Multiple Comparison post hoc test (*p < 0,05; **p < 0,01; ***p < 0,001 vs control).

The obtained results suggest that the two P-gp activity measures resulted in the same kind of final result and conclusions, and both are effective measure for the evaluation P-gp activity. Moreover, although colchicine is effective in increasing P-gp expression levels in a concentration dependent manner, no significant changes in P-gp activity were observed.

6. DISCUSSION

P-gp has been viewed as a therapeutic target for specific inhibition to overcome the well known problems of drug resistance in anticancer therapy. On the other hand, its polarized expression is consistent with the proposed role of P-gp as a secretory protective system contributing to the gastrointestinal epithelial barrier in limiting the bioavailability of its substrates. [9]

Thus, by using its efflux properties, a possible antidotal pathway against the damage induced by xenobiotics that are substrates of this transporter could be proposed. By increasing the expression and activity of this important transport protein, a consequent increase in the cellular efflux of such xenobiotics and a corresponding decrease in its accumulation could therefore culminate in an overall decrease in cytotoxicity.
Caco-2 cells have been reported to express P-gp and the expression levels are in good agreement with those of normal human jejunum. [6, 9, 11, 14, 16]

Therefore, this *in vitro* model could be used for the screening and selection of potent and safe P-gp inducers. To validate the caco-2 cells as a suitable *in vitro* model to study and select safe, potent, and specific P-gp inducers as a tool for cell protection against xenobiotics, colchicine, a known P-gp inducer was used to investigate changes in P-gp expression and activity.

Our results clearly indicated that colchicine is effective in increasing P-gp expression. In fact, in presence of this known P-gp inducer, P-gp expression increased in a concentration dependent manner. However, no significant changes in P-gp transport activity were found, suggesting that colchicine should not be the ideal inducer to be used as possible antidotal pathway against the damage induced by xenobiotics as the lack of increase in P-gp activity will not decrease the xenobiotics intracellular accumulation and will not culminate in a decrease in its cytotoxicity.

This suggests that although P-gp is being highly expressed and incorporated in the cell membrane (since the monoclonal antibody recognizes an external P-gp epitope) this

transport efflux pump is not yet fully functional. Noteworthy, our data suggest that, for the screening of P-gp inducers, both P-gp expression and activity should be investigated, since an increase in the first may not be reflected in an increase in the second parameter.

Similarly, Takara noted that P-gp transport function remained unchanged in Caco-2 cells exposed to several nonsteroidal anti-inflammatory drugs in spite of the observed increase in MDR1 mRNA. [15]

One possible explanation for the differences noted between P-gp expression and activity levels in these cells is that caco-2 full differentiation into enterocytes could be needed to obtain fully functional P-gp.

In fact, in a study performed by Hosoya et al., based on the directionality of cyclosporine and verapamil transport, it was observed that in spite of the observed early P-gp expression, the protein was only fully functional after the 17th day in culture. Moreover, in that study the rank order of P-gp expression levels was 4 weeks ≈ 1 week > 3 weeks > 2 weeks at equal loading of cell proteins. These authors also observed, at the late stage of culture (~ 27 days), an enhanced cyclosporine transport in the basal-to-apical direction, which was due not only to an increased level of P-gp expression in the apical cell membrane but also to the full development of cell polarity, which may be the most important factor in effecting efflux pump function. [8]

Other possible explanation is that colchicine, in spite not causing cell death evaluated by the MTT assay, could cause ATP depletion, and the new synthesized P-gp molecules are not able to work due to the lack of ATP.

7. CONCLUSION

Effective antidotal pathways can be achieved by promoting the cellular efflux of deleterious xenobiotics. As such, appropriate *in vitro* models addressing P-gp induction are needed. These results indicate that P-gp expression was increased by treatment with colchicine, although the transport function was unchanged. However, the presented *in vitro* model could be useful for the screening of potential P-gp inducers.

8. LIST OF ABBREVIATIONS

Ai	inhibited accumulation
AiE	inhibited accumulation + efflux
ADME	absorption – distribution – metabolism – excretion
ATP	adenosine triphosphate
PBS	phosphate-buffered saline
β-NADH	β-nicotinamide-adenine dinucleotide
CO_2	carbon dioxide
DMEM	dulbecco's modified eagle medium
DMSO	domethyl sulfoxide
EDTA	ethylene diamine tetraacetic acid
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
LDH	lactate dehydrogenase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA	normal accumulation
NaN_3	sodium azide
NEAA	non-essential amino acid
P-gp	P-glycoportein
RHO 123	rhodamine 123
UIC 2	P-glycoportein monoclonal antibody

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