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Vincristine transcriptional regulation of efflux drug transporters in carcinoma cell lines

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Abbreviations:
ABCB1, multidrug resistance protein 1 (mdr1) or P-glycoprotein (P-gp)
ABCC1, multidrug resistance associated protein 1 (mrp1)
ABCC2, multidrug resistance associated protein 2 (mrp2) or canalicular multispecfic organic anion transporter (cMOAT)
ABCC3, multidrug resistance associated protein 3 (mrp3)
CAR, constitutive androstane receptor
CYP3A4, Cytochrome P450 3A4

ABSTRACT

The increased expression of drug transporters following cancer chemotherapy contributes to resistance. This may reflect transcriptional up-regulation and/or clonal selection. We quantified the expression of mRNA for *ABCB1* (mdr1), *ABCC1* (mrp1), *ABCC2* (mrp2) and *ABCC3* (mrp3) to evaluate the potential contribution of induction. *ABCB1*, *ABCC1–3* mRNAs were quantified by real time RT-PCR and normalized to *GAPDH*. We used intestinal cells that express high pregnane X receptor (LS174T), low pregnane X receptor (Caco-2) and lung cells (A549) that express glucocorticoid receptor and low pregnane X receptor. Rifampin (10 μM) caused significant induction of *ABCB1* (595 ± 263%, $p < 0.05$) in LS174T cells but induction was absent in Caco-2 or A549 cells. *ABCC1* was not induced in any cell at 24, 48 and 72 h following rifampin treatment. In contrast, vincristine (10 nM and 100 nM), a ligand for *ABCB1* and *ABCC1–3* and a potential PXR/CAR ligand, induced *ABCC2* and *ABCC3* expression in LS174T cells at 48 h (372 ± 87% and 303 ± 42%, respectively, $p < 0.05$). A similar induction of *ABCC2* and *ABCC3* genes was also seen with 10 nM VCR in A549 cells following 48 h treatment. In summary, there may be a significant contribution of transcriptional activation to multi-drug resistance. However, this is cell selective and is not necessarily dependent on PXR mediated effects.

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GR, glucocorticoid receptor
PXR, hPXR, SXR, human pregnane X receptor
RIF, rifampin
RT-PCR, reverse transcriptase polymerase chain reaction
VCR, vincristine

1. Introduction

Chemotherapy is the most effective treatment for metastatic tumors, but success is limited by the ability of cancer cells to become simultaneously resistant to several different drugs, a trait known as multidrug resistance. Three decades of multidrug resistance research have identified a myriad of ways in which cancer cells can elude chemotherapy, and it has become apparent that resistance exists against every effective drug. Therefore, the ability to predict and circumvent drug resistance is likely to improve chemotherapy [1]. One of the most important reasons for cancer chemotherapy resistance is the increased expression of drug transporters in cancer cells that mediate the efflux of anticancer agents out of cells [1]. The ATP-binding cassette (ABC) transporters, which include ABCB1 (P-glycoprotein [P-gp, MDR1]), ABCC1 (multidrug resistance associated protein 1, [MRP1]), ABCC2 (multidrug resistance associated protein 2, [MRP2] or canalicular multispecfic organic anion transporter [cMOAT]) and ABCC3 (multidrug resistance associated protein 3, [MRP3]), are the most prominent drug efflux transporters, and their functional expression has been associated with tumor response and outcome for a number of different cancers [2–4].

Vincristine (VCR) is a vinca alkaloid that is widely used in the treatment of acute leukemias and solid tumors. Development of resistance to VCR can be an important barrier to successful cancer treatment. VCR appears to be transported by ABCB1, ABCC1, ABCC2 and ABCC3 [4–8]. Importantly, the expression of many drug efflux transporters may be induced by exposure to compounds they transport; in turn, this may result in the development of resistance to anticancer drugs during therapy, and ultimately, treatment failure. Currently, the potential for VCR to increase the expression of its associated efflux transporters is not known. Furthermore, the role of nuclear factors, such as pregnane X receptor (PXR) in the xenobiotic-mediated induction of drug resistance transporters is not clear. Recently, however, Giessmann et al. demonstrated that the drug rifampin (RIF) could induce *ABCB1* and *ABCC2* gene expression through the PXR mediated mechanisms in human intestine, suggesting a significant role for this nuclear factor in the induction of transporters [9]. The objective of this study was to examine the propensity for VCR to cause induction of the transporters *ABCB1*, *ABCC1*, *ABCC2* and *ABCC3* in three carcinoma cell lines. Specifically, we hypothesized that VCR could induce the gene expression of the transporters responsible for its own transport in a PXR dependent manner.

2. Materials and methods

2.1. Materials

LS174T cells (a human intestine carcinoma cell line), Caco2 cells (a human intestine adrenal carcinoma cell line) and A549 cells (a human lung carcinoma cell line), were obtained from American Type Culture Collection (ATCC, Manassas, VA). Dulbecco's modified eagle's medium, containing 4.5 g/L glucose and L-glutamine (DMEM), non-essential amino acid, HEPES, penicillin-G/streptomycin, sodium pyruvate, fetal bovine serum (FBS), PBS, Trypsin-EDTA and F-12K Nutrient Mixture (Kaighn's modification) were purchased from GIBCO® Invitrogen (Carlsbad, CA). [^{3}H] vincristine sulphate was obtained from Amersham Bioscience (Piscataway, NJ). VCR and RIF were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and reagents were of the highest grade.

2.2. Cell culture

LS174T cells were grown in DMEM supplemented with 10% FBS. Caco2 cells were grown with DMEM supplemented with 1% non-essential amino acid, 1% HEPES, 100 U/ml penicillin-G/streptomycin, 1% sodium pyruvate and 10% FBS. A549 cells were grown with F-12K Nutrient Mixture (Kaighn's modification) supplemented with 10% FBS. All cells were grown at 37 °C in a humidified 5% CO_2 atmosphere. Media was changed every 48 h and cells were passaged once weekly at approximately 80% confluence by trypsinization. Passages 130–140, 20–30, and 90–100 were used for LS174T, Caco2 and A549 experiments, respectively.

2.3. Cell viability tests

Cell viability was assessed using CellTiter 96 AQueous One solution (Promega, Madison, WI). Briefly, at the time of passage, cell suspensions (100 μL, 1×10^5 cells/ml) were added into the wells of Microtest 96 tissue culture plates (Becton Dickinson and Company, Franklin Lakes, NJ). After 24 h stabilization, treatment was started by aspirating the media and adding 100 μL of different concentrations of VCR solutions. VCR concentrations used in these experiments were 0, 0.1% MeOH vehicle control, 5, 10, 50, 100, 200, 500 and 1000 nM. At the end of each treatment, CellTiter 96 AQueous One solution (20 μL) was added to each well. Plates were incubated at 37 °C for 2 h. Absorbance at 490 nm was recorded using a 96-well plate reader (MKII Titertek Multi Skan Plus,

Labsystem, Finland). The percentage of viable cells was calculated by equation:

$$\%\,\text{viability} = \frac{(\text{OD}_{\text{experiment}} - \text{OD}_{\text{background}})}{(\text{OD}_{\text{vehicle control}} - \text{OD}_{\text{background}})} \times 100\%.$$

2.4. Drug induction treatment

Concentrations of VCR ranging from 0 to 2000 nM were tested in Caco2 cells for mRNA induction experiments. At the time of passage, cell suspension (1 ml 1×10^5 cells/ml) was added to each well of Multiwell 6-well tissue culture plates (Becton Dickinson and Company, Franklin Lakes, NJ). Three milliliters of different concentrations of VCR or 10 μM RIF were added to corresponding treatment wells in triplicate. The plates were then incubated for 48 h. Fresh prepared drug containing media was replaced every 24 h.

Induction experiments were also conducted in LS174T, Caco2 and A549 cells. At the time of passage, 1×10^5 cells/ml cell suspensions were added to the wells of 6-well plates. After 24 h, cells were treated with either 3 ml of 10 μM RIF, or 3 ml of VCR [10 or 100 nM] for 24 or 48 h.

2.5. RNA extraction and real-time RT-PCR

mRNA expression of *ABCB1* and *ABCC1–3* before and after VCR and RIF treatment was determined by real-time RT-PCR. Briefly, RNA was extracted using RNeasy Mini kits (QIAGEN Inc., Valencia, CA) and DNA was removed with DNA-free™ (Ambion Inc., Austin, TX). RNA quality assessment and quantification were conducted using the RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA). Subsequently, mRNA was reverse transcribed to cDNA (Promega reverse transcription system, Madison, WI). Real-time RT-PCR was performed for *ABCB1*, *ABCC1*, *ABCC2*, *ABCC3*, *PXR*, *CAR*, *CYP3A4* and *GAPDH* with the Bio-Rad iCycler system. All gene expression assays were developed and optimized in our laboratory and conducted using iQ SYBR Green supermix (Bio-Rad Laboratories, Richmond, CA). All primers were purchased from Integrated DNA Technologies (Coralville, IA). Primer sequences and RT-PCR conditions are shown in Table 1.

2.6. VCR uptake experiments

VCR uptake experiments were conducted in LS174T, Caco2 and A549 cells. Cell suspension (1 ml, 1×10^6 cells/ml) was added to each well of 12-well tissue culture plates. Following an incubation period of 24 h, VCR (10 nM) in media or blank control media was added to corresponding treatment wells in triplicate and plates were incubated for 24 or 48 h. After VCR pretreatment, cells were washed twice with blank media and incubated with blank media. Twenty-four hours later, 100 nM [^{3}H] VCR was added to each well for period of 2 h. Subsequently, supernatants were aspirated and cells were thrice washed with ice cold PBS. Cells were incubated with a lysis buffer [1% SDS, 1.0mM sodium *ortho*-vanadate, 10 mM Tris pH 7.4] for 3 h. Protein content was quantified using a Bradford assay (Sigma Chemical Co. St. Louis, MO), and intracellular [^{3}H] VCR was analyzed using a liquid scintillation counter (Packard Tri-carb 2100 TR, Boston, MA).

2.7. Data analysis

Each cycle threshold (C_t) value obtained from real-time RT-PCR was normalized to the housekeeping gene GAPDH. The mRNA expression fold change in compared to vehicle control was calculated by $2^{-\Delta\Delta C_t}$ method, of which ΔC_t is the C_t value difference between gene of interest and housekeeping gene, and $\Delta\Delta C_t$ is the ΔC_t difference between treatment and control experiment [10]. Two assumptions, the equal amplification efficiency of transporter genes and *GAPDH* and the consistency of *GAPDH* gene expression over time, were further

Table 1 – Real time RT-PCR primers and running conditions for *ABCB1*, *ABCC1*, *ABCC2*, *ABCC3*, *PXR*, *CAR*, *GAPDH* and *CYP3A4*

Genes	Primer sets	RT-PCR conditions
ABCB1	F: 5′-TGCTCAGACAGGATGTGAGTTG-3′ R: 5′-TAGCCCCTTTAACTTGAGCAGC-3′	1 cycle of 50 °C/2 min, 95 °C/3 min, 40 cycles of 95 °C/30s, 64.2 °C/2 min
ABCC1	F: 5′-TACCTCCTGTGGCTGAATCTGG-3′ R: 5′-CCGATTGTCTTTGCTCTTCATG-3′	1 cycle of 50 °C/2 min, 95 °C/3 min, 40 cycles of 95 °C/30s, 64.2 °C/2 min
ABCC2	F: 5′-CAAACTCTATCTTGCTAAGCAGG-3′ R: 5′-TGAGTACAAGGGCCAGCTCTA-3′	1 cycle of 50 °C/2 min, 95 °C/3 min, 40 cycles of 95 °C/30s, 64.2 °C/2 min
ABCC3	F: 5′-CTTAAGACTTCCCCTCAACATGC-3′ R: 5′-GGTCAAGTTCCTCTTGGCTC-3′	1 cycle of 50 °C/2 min, 95 °C/3 min, 40 cycles of 95 °C/30s, 64.2 °C/2 min
PXR	F: 5′-ACCTTTGACACTACCTTCTCCCAT-3′ R: 5′-CGCAGCCACTGCTAAGCA-3′	1 cycle of 50 °C/2 min, 95 °C/3 min, 40 cycles of 95 °C/15s, 60 °C/1 min
CAR	F: 5′-CCAGCTCATCTGTTCATCCA-3′ R: 5′-GGTAACTCCAGGTCGGTCAG-3′	1 cycle of 50 °C/2 min, 95 °C/3 min, 42 cycles of 95 °C/15s, 61.4 °C/1 min
GAPDH	F: 5′-GAAGGTGAAGGTCGGAGTC-3′ R: 5′-GAAGATGGTGATGGGATTTC-3′	1 cycle of 50 °C/2 min, 95 °C/3 min, 42 cycles of 95 °C/15s, 60 °C/1 min
CYP3A4	F:5′-CATTCCTCATCCCAATTCTTGAAG T-3′ R: 5′-CCACTCGGTGCTTTTGTGTATCT-3′	1 cycle of 50 °C/2 min, 95 °C/3 min, 42 cycles of 95 °C/15s, 60 °C/1 min

"F" denotes for forward primer; "R" reverse primer; "min" minute(s) and "s" represents seconds.

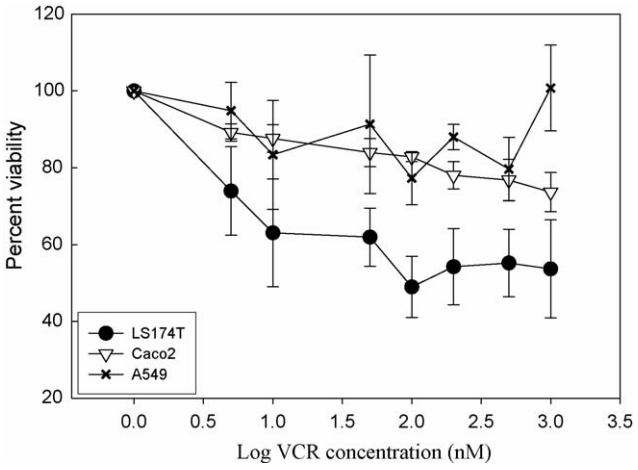

Fig. 1 – Cell growth inhibition after 48 h VCR treatment in LS174T, Caco2 and A549 cells. Data are presented as mean ± S.D. from three repeats.

evaluated. To assess the equal amplification efficiency of transporter genes and *GAPDH*, we examined how ΔC_t varies with template dilution. cDNA samples were diluted to 0.5, 0.2, 0.1, 0.05, 0.02 and 0.01 times. Real-time amplifications were performed for both *GAPDH* and target genes at their optimal thermal conditions. The average C_t was calculated for each gene and the ΔC_t ($C_{t,\ transporter} - C_{t,\ GAPDH}$) was determined. A plot of the log cDNA dilution versus ΔC_t was made for each transporter gene. The consistency of *GAPDH* gene expression over time was validated through the $\Delta C_t'$ method by plotting the $\Delta C_t'$ ($C_{t,\ time\ x} - C_{t,\ time\ 0}$) versus time. Data are presented as mean ± standard deviation. Two tailed Student's *t*-tests with Bonferroni correction were performed between each treatment and vehicle control. Corrected *p*-values <0.05 were considered statistically significant.

3. Results

3.1. Cell viability after VCR treatment

When treated with VCR (5–1000 nM, 48 h), both LS174T and Caco2 cells displayed concentration dependent declines in viability. However, A549 cells were relatively insensitive to VCR treatment, and elevated VCR dose did not correspond to decreased cell viability (Fig. 1). Based on these data, 10 and 100 nM VCR concentrations were chosen for further experiments, because the viability difference between 24 and 48 h was greater than that between 48 and 72 h in 10 and 100 nM VCR treated LS174T cells, which suggests development of resistance in LS174T cells [this difference was statistically significant for 10 nM VCR treatment ($p < 0.05$)] (data not shown). Additionally the viability of different cell lines after these intermediate concentrations VCR treatment was suitable for further mRNA collection at the end of the study period.

3.2. RIF and VCR effects on the expression of transporter genes

Because we chose to use $2^{-\Delta\Delta C_t}$ method to calculate the relative gene expression change, the two assumptions, equal amplification efficiencies of transporter genes and *GAPDH* and consistent *GAPDH* expression during VCR treatment, were confirmed. The absolute values of the slopes for log cDNA dilution versus ΔC_t ($C_{t,\ treatment} - C_{t,\ GAPDH}$) were close to zero at the given conditions (data not shown). Therefore, the efficiencies of the transporter genes and the *GAPDH* were similar, which confirmed that it was valid to use $\Delta\Delta C_t$ calculation for the relative quantification of transporter gene expression.

The $\Delta C_t'$ method, which plotted the $\Delta C_t'$ ($C_{t,\ time\ x} - C_{t,\ time\ 0}$) versus time, indicated no effect of VCR treatment on *GAPDH* C_t level (data not shown). This confirmed that *GAPDH* was a suitable internal control to quantitate the effects of VCR on transporter expression.

At baseline, our real-time RT PCR assay was able to detect all four transporter genes, which displayed variable expression in different cell lines. LS174T cells have low level of baseline *ABCC2* mRNA expression. A549 cells have low level of baseline *ABCB1* gene mRNA expression (Table 2).

3.2.1. LS174T cells

In LS174T cells, RIF (10 μM) significantly increased *ABCB1* expression at 24 and 48 h (595 ± 263% and 408 ± 167%, respectively, $p < 0.05$, Fig. 2A). With VCR treatment, 10 nM and 100 nM VCR significantly induced *ABCC2* mRNA expression at 48 h (372 ± 87% and 149 ± 55%, respectively, $p < 0.05$, Fig. 4A and B). 10 nM VCR significantly increased *ABCC3* expression 303 ± 42% at 48 h. One hundred nanomolar VCR induced *ABCC3* expression 84 ± 51% and 222 ± 57% at 24 and 48 h, respectively ($p < 0.05$).

3.2.2. Caco2 cells

In Caco2 cells, RIF (10 μM, 24 h) treatment significantly down-regulated *ABCB1* expression (28 ± 9%, $p < 0.05$, Fig. 2B). However, RIF (10 μM, 48 h) treatment appeared to have no effect on

Table 2 – *ABCB1*, *ABCC1*, *ABCC2*, *ABCC3* and *PXR* gene mRNA expression in LS174T, Caco2 and A549 cells

Origin		Transporter gene expression				
		ABCB1	*ABCC1*	*ABCC2*	*ABCC3*	*PXR*
LS174T	Human intestinal carcinoma	22.7	23.9	31.4	28.5	25.2
Caco2	Human intestinal carcinoma	23.8	25.6	24.3	28.6	34.2
A549	Human lung carcinoma	33.6	20.6	21.1	25.0	35.0

Gene expression is shown as the absolute C_t value obtained from real-time RT-PCR ($n = 6$/group).

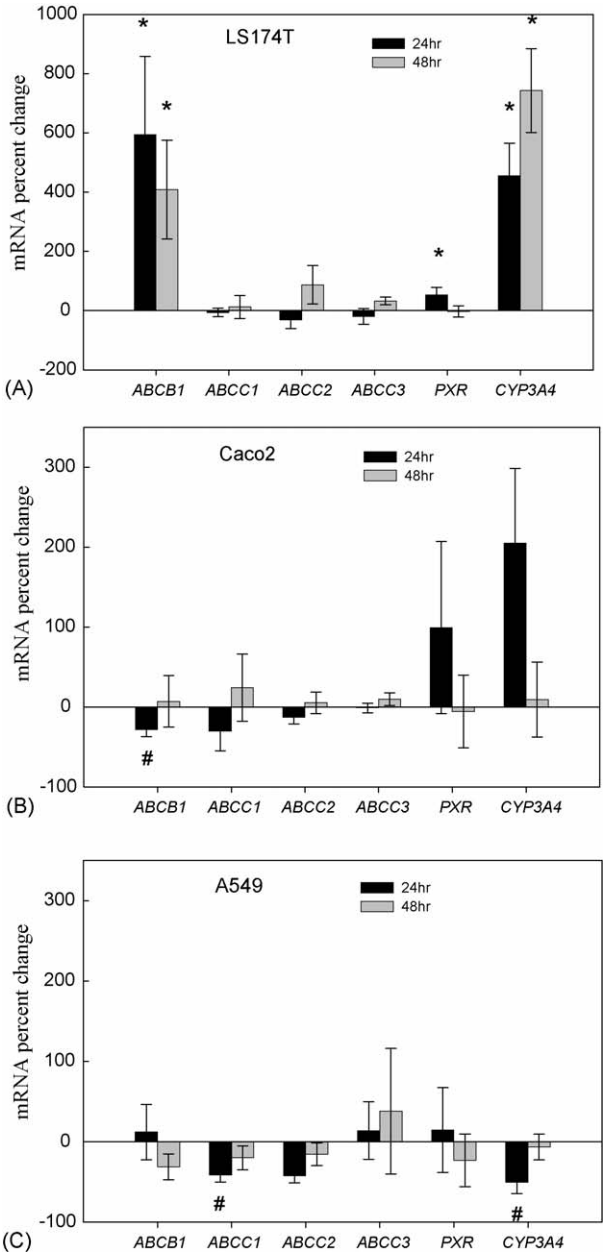

Fig. 2 – Effect of 10 μM RIF 24 h and 48 h treatment on *ABCB1*, *ABCC1*, *ABCC2*, *ABCC3*, *PXR* and *CYP3A4* mRNA expression when compared to 0.1% MeOH treatment in: (A) LS174T; (B) Caco2; (C) A549 cells. Data are presented as mean ± S.D. ($n = 6$). *Statistically significant (adjusted p-value <0.05) increase. #Statistically significant (adjusted p-value <0.05) decrease.

ABCB1, *ABCC1*, *ABCC2*, and *ABCC3* gene expression in Caco2 cells (Fig. 3). After VCR treatment (48 h, 5–2000 nM), more than 60% of the cells were viable. Increasing VCR concentration resulted in increasing total RNA level within the treatment group, suggestive of general induction. Our real-time RT-PCR assay showed differential effects of 5–2000 nM concentrations of VCR on transporter gene expression (Fig. 3). Five and ten nanomolar VCR 48 h treatment significantly decreased *ABCB1* expression (59 ± 7% and 43 ± 4%, respectively, $p < 0.05$). However, 1000 and 2000 nM VCR 48 h treatment significantly increased *ABCB1* expression 74 ± 7% and 35 ± 6%, respectively ($p < 0.05$, Fig. 3A). At 5–2000 nM range, VCR 48 h treatment had no effect on *ABCC1* gene expression. However, it induced *ABCC2* and *ABCC3* gene expression significantly. VCR treatment (5, 10, 50, 100, 500 and 2000 nM, 48 h) significantly induced *ABCC2* expression by 42–159% and VCR treatment (5, 10, 50, 100, 500, 1000 nM, 48 h) significantly induced *ABCC3* expression by 23–330% ($p < 0.05$, Fig. 3C and D). VCR treatment (10 nM, 48 h) significantly increased *ABCC2* expression 43 ± 19% ($p < 0.05$, Fig. 4C). One hundred nanomolar VCR increased *ABCC2* expression at 24 and 48 h (42 ± 18% and 63 ± 32%, respectively, $p < 0.05$, Fig. 4D). One hundred nanomolar VCR treatment also significantly down-regulated *ABCB1* expression 51 ± 10% at 24 h ($p < 0.05$, Fig. 4D).

3.2.3. A549 cells

In A549 cells, RIF (10 μM, 24 and 48 h) treatment had no induction of *ABCB1*, *ABCC1*, *ABCC2* or *ABCC3* (Fig. 2C). In fact, RIF (10 μM) significantly down-regulated *ABCC1* by 41 ± 9% at 24 h ($p < 0.05$, Fig. 2C). 10 nM VCR significantly induced *ABCC2* and *ABCC3* expression at 48 h (57 ± 7% and 134 ± 59%, respectively, $p < 0.05$, Fig. 4E). In contrast, 100 nM VCR showed no induction effect on *ABCB1*, *ABCC1*, *ABCC2* and *ABCC3* expression, with statistically significant down-regulation of *ABCC1* and *ABCC2* expression (Fig. 4F).

3.3. RIF and VCR effect on the expression of PXR gene

The three human carcinoma cell lines selected demonstrated a differential expression of PXR. Specifically, LS174T cells express much higher baseline *PXR* mRNA than Caco2 and A549 cells (Table 2).

After RIF treatment (10 μM, 24 h), *PXR* mRNA increased 53 ± 26% in LS174T cells ($p < 0.05$, Fig. 2A). RIF treatment did not induce *PXR* expression in Caco2 and A549 cells (Fig. 2B and C). VCR treatment (10 nM, 48 h) significantly down-regulated *PXR* 42 ± 10% in LS174T cells, and induced *PXR* by 221 ± 117% in A549 cells ($p < 0.05$, Fig. 5A). VCR (10 nM, 48 h) had no induction on PXR expression in Caco2 cells (Fig. 5A). One hundred nanomolar VCR led to 119 ± 48% induction of *PXR* at 24 h and 65 ± 11% down-regulation at 48 h in LS174T cells ($p < 0.05$, Fig. 5B). At this concentration, VCR had no effect on PXR expression in Caco2 and A549 cells (Fig. 5B).

3.4. RIF and VCR effects on the expression of other genes

We also quantified *CYP3A4* and *CAR* gene expression before and after RIF or VCR treatment in the three cell lines. At baseline, the mRNA expression level of *CYP3A4* was low, with rank order of LS174T > A549 > Caco2. *CAR* gene expression in all three cell lines was very low (C_t value is at the detection limit of 39).

RIF (10 μM, 24 and 48 h) treatment significantly induced *CYP3A4* expression in LS174T cells (455 ± 110% and 743 ± 142%, respectively, $p < 0.05$, Fig. 2A). In A549 cells, RIF (10 μM) significantly down-regulated *CYP3A4* 50 ± 14% at 24 h ($p < 0.05$, Fig. 2C).

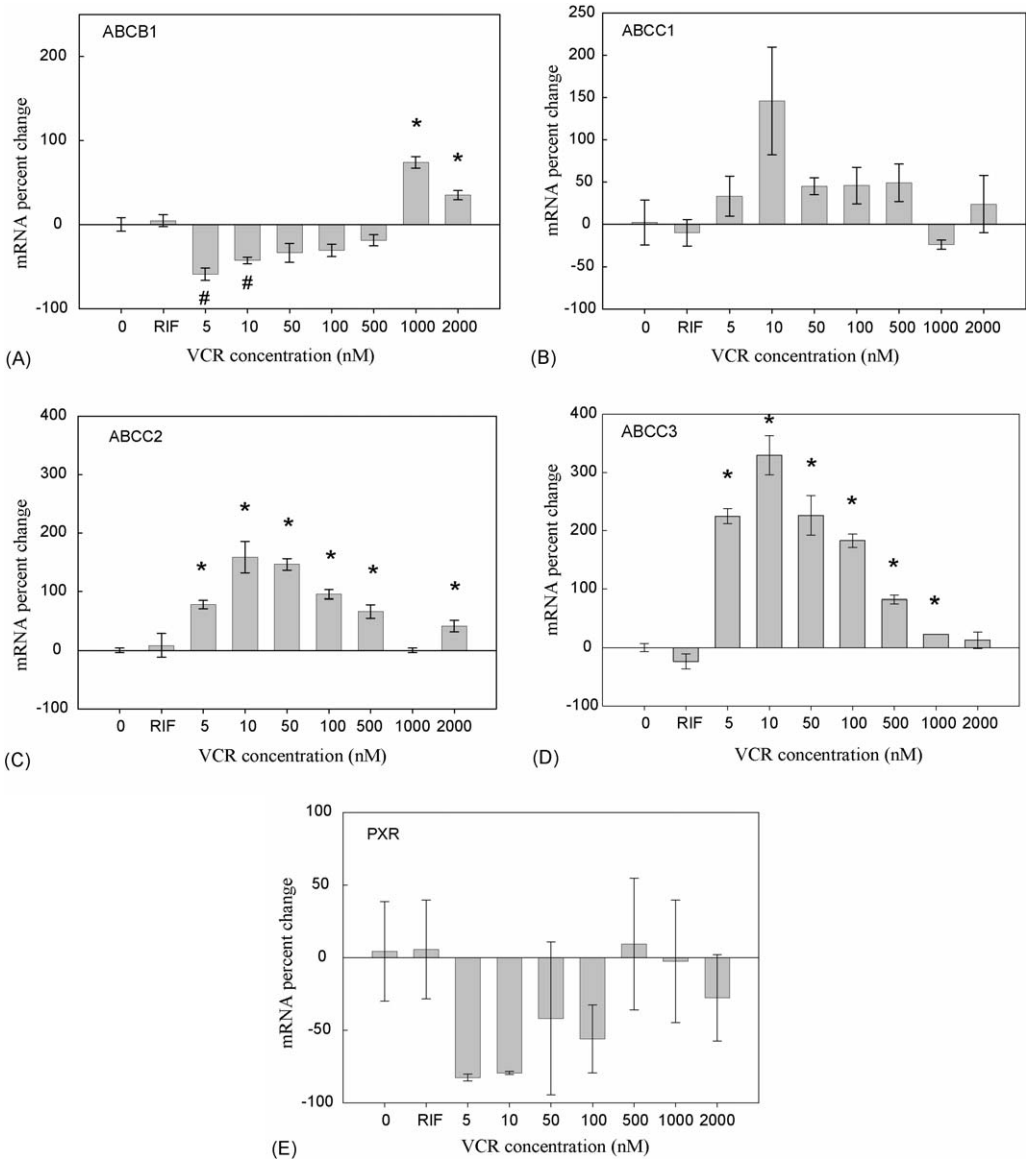

Fig. 3 – Effect of 0, 5, 10, 100, 500, 1000 and 2000 nM VCR or 10 μM RIF 48 h treatment on *ABCB1* (A), *ABCC1* (B), *ABCC2* (C), *ABCC3* (D) and *PXR* (E) mRNA expression in Caco2 cells. Data are presented as mean ± S.D. ($n = 6$). *Statistically significant (adjusted p-value <0.05) increase. #Statistically significant (adjusted p value <0.05) decrease.

VCR (10 nM, 24 and 48 h) treatment significantly induced *CYP3A4* expression in LS174T cells (93 ± 38% and 75 ± 29%, respectively, Fig. 5C). Ten nanomolar VCR also induced 154 ± 29% *CYP3A4* expression in A549 cells at 48 h ($p < 0.05$, Fig. 5C). VCR (100 nM, 24 and 48 h) treatment significantly down-regulated *CYP3A4* expression in LS174T cells (52 ± 22% and 79 ± 12%, respectively, $p < 0.05$, Fig. 5D). One hundred nanomolar VCR also down-regulated *CYP3A4* in A549 cells at 24 h (34 ± 21%, $p < 0.05$, Fig. 5D).

3.5. *VCR treatment effects on transporter activity*

We examined [^{3}H]VCR uptake following VCR pretreatment (10 nM for 24 or 48 h) in the three cell lines. VCR pretreatment for 24 h decreased [^{3}H]VCR uptake in Caco2 cells ($p < 0.05$, Fig. 6A), and a similar trend was observed in LS174T cells. In contrast, treatment for 48 h significantly decreased VCR uptake in A549 cells ($p < 0.05$, Fig. 6B).

4. Discussion

To improve the success of cancer chemotherapy, a better understanding of the mechanistic basis of cancer resistance to chemotherapy is necessary. Our study has demonstrated significant induction of drug transporter gene expression by VCR. The functional consequences of these changes (i.e., increased VCR efflux) were confirmed by VCR uptake experiments. This suggests transcriptional activation may be an important mechanism in the development of transporter mediated multi-drug resistance to chemotherapy. Of interest, the induction of *ABCB1*, *ABCC1*, *ABCC2* and *ABCC3* gene

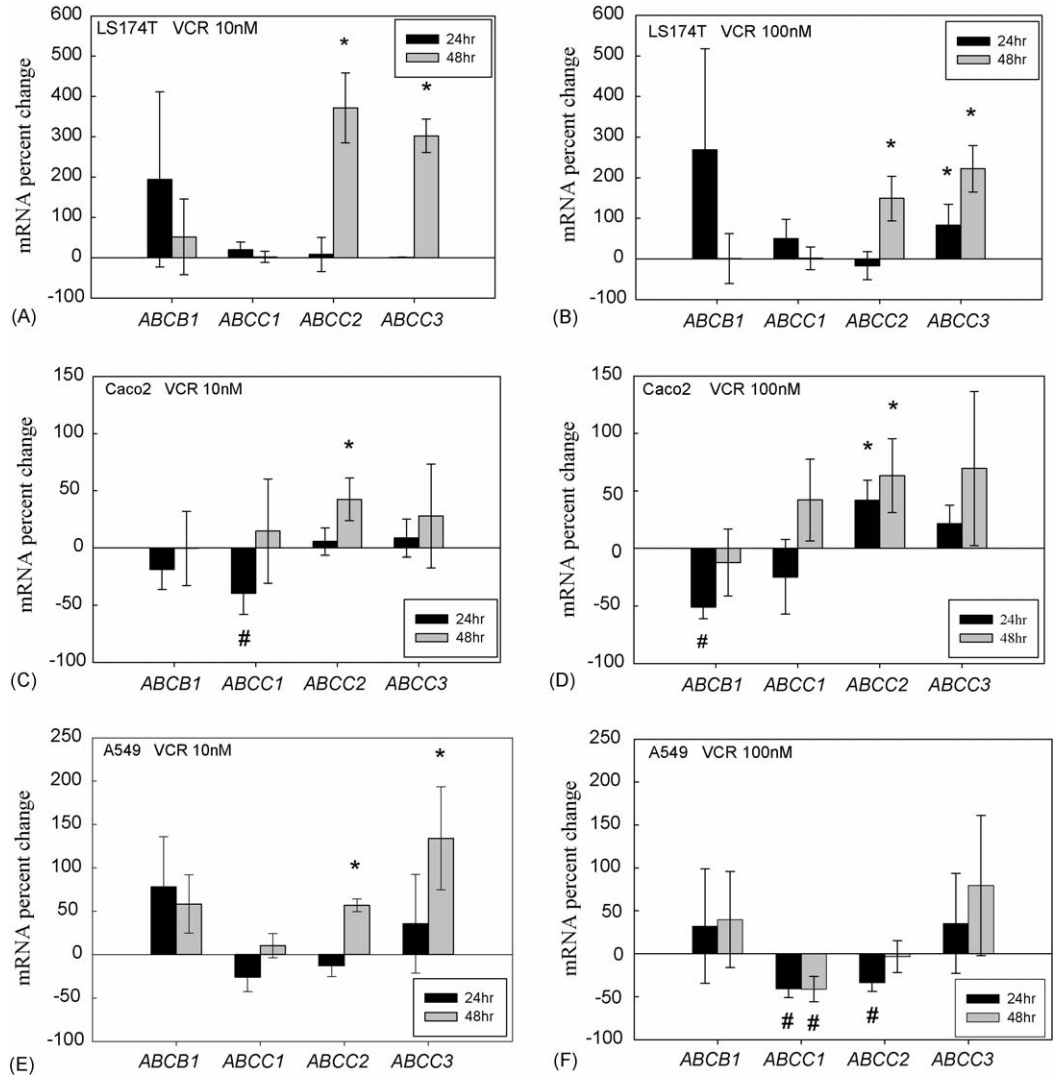

Fig. 4 – Effect of 10 nM (A, C, E) or 100 nM (B, D, F) VCR 24 h and 48 h treatment on *ABCB1*, *ABCC1*, *ABCC2* and *ABCC3* mRNA expression when compared to 0.1% MeOH treatment in LS174Tcells (A and B), Caco2 cells (C and D) and A549 cells (E and F). Data are presented as mean ± S.D. ($n = 9$). *Statistically significant (adjusted p-value <0.05) increase. #Statistically significant (adjusted p-value <0.05) decrease.

expression appears to depend on cell type, exposure time and drug concentration.

VCR concentrations ranging from 5–1000 nM were chosen for the cytotoxicity experiments based on human pharmacokinetic data [11]. At this concentration range, the growth of LS174T and Caco2 (ABCB1 expressing cells) is VCR dependant. LS174T cells had the lowest viability. This was consistent with the results of uptake experiments of which the highest VCR uptake were seen. Our cytotoxicity experiments demonstrate a decrease in cytotoxicity between 48 and 72 h when compared to that between 24 and 48 h, suggesting a potential role of transporter induction in the development of VCR resistance. Again, this is supported by significant decreases in VCR uptake after VCR treatment. It should be noted however, that a direct link between transporter induction and viability is difficult to establish, and that multiple factors may influence cell viability.

When examining mRNA expression changes in Caco2 cells after treatment with 5–2000 nM concentrations of VCR for 48 h, differential effects were seen. Specifically, 10 nM VCR showed the most profound effects on transporter genes, with approximately 160 and 330% induction of *ABCC2* and *ABCC3* genes respectively after 48 h and 43% down-regulation of *ABCB1* gene. Interestingly, the induction of transporter gene expression was not a simple function of VCR dose. The *ABCC2* and *ABCC3* induction reached the maximum at 10 nM VCR treatment and decreased afterwards. These data suggest that higher concentrations of VCR cause less induction of transporter gene expression and a corresponding increase in cytotoxicity. Both features are preferred for anti-cancer treatment.

Many studies have shown that RIF can induce *ABCB1* and *CYP3A4* expression through a mechanism that is at least partly dependent on PXR activation. Geick et al. have demonstrated a

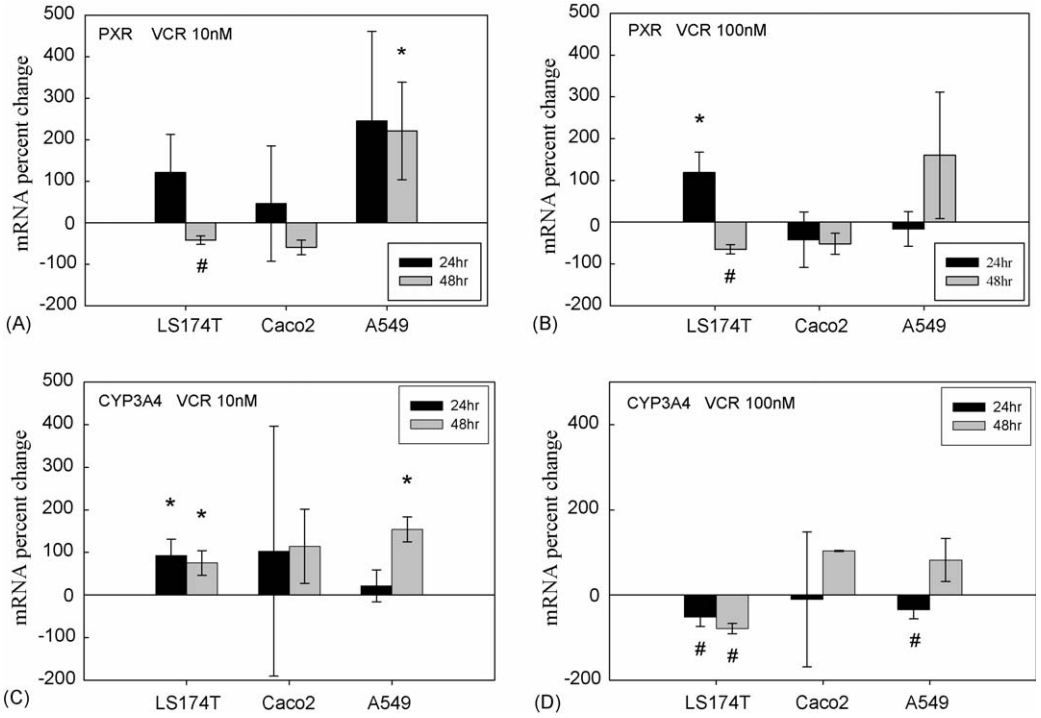

Fig. 5 – Effect of 10 nM (A and C) or 100 nM (B and D) VCR 24 h and 48 h treatment on *PXR* (A and B) and *CYP3A4* (C and D) mRNA expression when compared to 0.1% MeOH treatment in three immortal cell lines. Data are presented as mean ± S.D. ($n = 9$). *Statistically significant (adjusted p-value <0.05) increase. #Statistically significant (adjusted p-value <0.05) decrease.

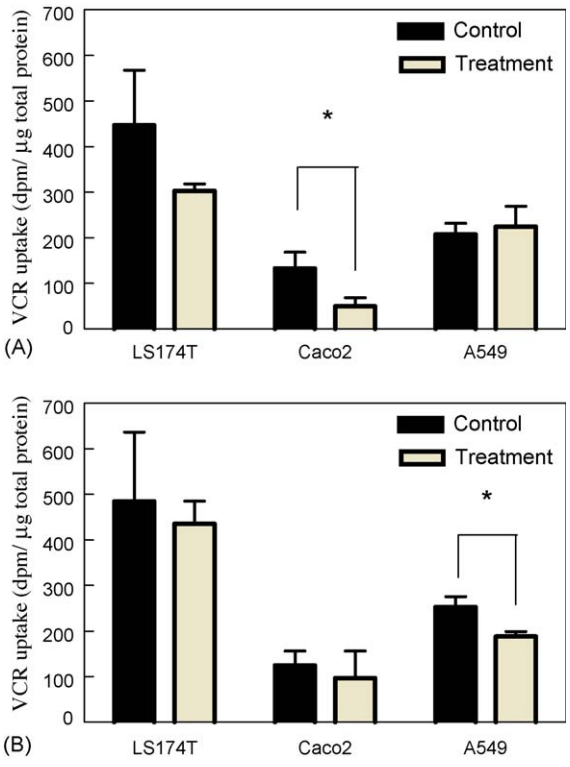

Fig. 6 – Effect of 10 nM VCR on 100 nM [^{3}H]VCR uptake in LS174T, Caco2 and A549 cells after (A) 24 h and (B) 48 h treatment. Data are presented as mean ± S.D. ($n = 3$). *Statistically significant (p-value <0.05).

time- and concentration-dependent induction of *ABCB1* by RIF in LS174T cells [12]. RIF induction of *ABCB1* mRNA expression has also been observed in human lymphocytes [13]. Therefore, we used 10 μM RIF treatment as a PXR dependent positive control. Our data demonstrate that 10 μM RIF induced *ABCB1* as well as *CYP3A4* gene expression in LS174T cells after treatment for 24 and 48 h. This *ABCB1* induction was not seen in Caco2 or in A549 cells that express low levels of *PXR* (Table 2). This is consistent with the differential expression of PXR in these three cell lines.

Previously, Fromm et al. have shown RIF induced *ABCC2* mRNA and protein expression in duodenal tissue [14]. Additionally, Giessmann et al. have shown the induction of *ABCB1* and *ABCC2* at both mRNA and protein level in human intestine after treatment with RIF 600 mg/day for 9 days [9]. In our study, we observed an 88% induction of *ABCC2* mRNA in LS174T cells after 10 μM RIF treatment for 48 h, however, this was not statistically significant.

We examined the effect of VCR (10 nM and 100 nM, 24 and 48 h) treatment on *ABCB1*, *ABCC1*, *ABCC2*, *ABCC3*, *PXR*, *CAR* and *CYP3A4* gene expression in three cancer cell lines. Ten nanomolar VCR 48 h treatment significantly induced *ABCC2* gene expression in all three cell lines. VCR (10 nM, 48 h) treatment also induced *ABCC3* gene expression in both LS174T and A549 cells. One hundred nanomolar VCR induced *ABCC2* and *ABCC3* gene expression in LS174T cells. VCR (100 nM) only induced *ABCC2* gene expression in Caco2 cells and had no inductive effect on *ABCC2* gene in A549 cells. Among the four efflux transporters of interest, *ABCC1* was relatively resistant to 10 μM RIF or VCR induction in LS174T, A549 or Caco2 cells.

We further explored the potential mechanisms of transporter transcriptional activation. Synold et al. demonstrated that PXR activates *ABCB1* expression in human primary hepatocytes and intestinal (LS180) cells and this activation results in enhanced drug efflux [15]. Geick et al. had identified a DR4 motif in the upstream enhancer region of *ABCB1* gene, where PXR can bind to induce *ABCB1* gene expression after RIF treatment [12]. Although not statistically significant, we did observe a trend of increasing *ABCB1* expression in LS174T cells after 24 h VCR treatment together with significant induction of *PXR*. This increase of *ABCB1* expression disappeared at 48 h with down-regulation of *PXR*. Therefore, VCR induced *ABCB1* gene expression may occur as a result of PXR mediated mechanisms, so that when VCR down-regulates the expression of PXR gene, the inductive effect of VCR on *ABCB1* gene disappears.

The fact that *ABCC2* and *ABCC3* genes were induced by VCR in all three cell lines suggests *PXR* may not be responsible for this induction. This was further supported by lack of *PXR* induction after VCR treatment (10 or 100 nM, 24 or 48 h) in Caco2 and A549 cells. Although we observed a 3-fold induction of *PXR* in A549 cells at 48 h after 10 nM VCR treatment, the induced *PXR* expression level was far below the *PXR* expression level in LS174T cells.

Multiple studies indicate a role for PXR and CAR in the co-regulation of genes that are important in drug metabolism and drug transport [16–18]. Because we observed significant induction of *ABCC2* and *ABCC3* gene expression by VCR, after examining the expression of *PXR*, we also quantified *CAR* expression. *CAR* expression is low in all three cell lines. After 10 μM RIF or VCR treatments, we did not find a significant increase in the amount of CAR. This suggested the induction effect on *ABCC2* and *ABCC3* genes were unlikely to be a result of CAR activation.

Because CYP3A4 has been suggested to be the major metabolism enzyme in VCR metabolism, and may share common regulation pathways with *ABCB1*, we also examined *CYP3A4* expression in the three cell lines. The baseline expression level of CYP3A4 in all three cell lines was low. Without sufficient information on VCR metabolism, it is possible that the effect of VCR on transporter gene expression was caused by VCR and/or its metabolite(s) by CYP3A4 or other enzymes. Finally, the experimental conditions selected for our experiments were based on the characteristics of our model and previewed physiologic relevance; it is possible that different conditions could yield different results.

In summary, our study suggests VCR could significantly induce the drug efflux transporter gene expression, and that this phenomenon may influence VCR cellular uptake. We also demonstrated that VCR mediated *ABCC2* and *ABCC3* induction could occur in the absence of *PXR* and *CAR*. Further efforts exploring the role(s) of other nuclear receptors in transporter induction in order to identify molecular targets in overcoming chemotherapy resistance are warranted.

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