Bull Environ Contam Toxicol (2011) 86:105–109
DOI 10.1007/s00128-010-0158-y

Multi Drug Resistance-1 (MDR1) Expression in Response to Chronic Diazinon Exposure: An In vitro Study on Caco-2 Cells

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Received: 7 February 2009 / Accepted: 11 November 2010 / Published online: 4 December 2010
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Abstract To evaluate the effect of chronic exposure to diazinon in the gastrointestinal tract, Caco-2 cells were made resistant by growing in low concentrations of diazinon (0.02 μM) that was gradually increased to 20 μM within 4.5 months. Resistant cells showed significant higher growth in the presence of 15, 45 and 135 μM of diazinon (96.08, 81.80 and 65.16% of control) compared to parent cells (79.71, 71.76 and 29.50% of control, respectively; $p < 0.05$). P-glycoprotein (P-gp) expression increased significantly in resistant cells (P-gp to beta-actin ratio 0.586 for parent and 1.255 for resistant cells, respectively; $p < 0.05$) without any alteration in MDR-1 mRNA level ($p > 0.05$).

Keywords P-glycoprotein · Multi drug resistance 1 · Caco-2 · Diazinon

The organophosphate insecticide, diazinon [*O*, *O*-(6-methyl-2(1-methylethyl)-4-pyrimidinyl) phosphorothioate], is widely used in agriculture as well as homes and gardens (WHO 1998). Frequent contacts in animals and people living in farming regions have been documented in several studies (Bailey et al. 2000; Andrew Clayton et al. 2003; McCauley et al. 2003). Although, chronic exposure to diazinon is a recognized phenomenon, only a few studies has been performed about the effects of chronic exposure to this pesticide. In the previous study, we reported immunotoxic effects of diazinon on C57b1/6 mice which was prominent in doses above 2 mg/kg (Neishabouri et al. 2004). Additionally, genotoxicity and adverse effects on reproductive system are discussed further in other studies (Ibrahim and El-Gamal 2003; Sanchez-Pena et al. 2004).

P glycoprotein (P-gp) expression results in multidrug resistance by augmenting the dynamic efflux of chemotherapeutic drugs from the cells (Gottesman and Pastan 1993), and has an identified role in the transport of diverse drugs according to the structure, such as vinca alkaloids, digoxin, cyclosporin A, anthracyclines, and glucocorticoids (Tanigawara et al. 1992; Ueda et al. 1992; Wacher et al. 1995; Tanaka et al. 1997). According to the higher rate of diazinon toxicity in spontaneous contact compared to chronic exposure, it has been suggested that chronic exposure may induce P-gp expression as a resistance mediator.

To the best of our knowledge, only a few reports exist about increased expression of P-gp in chronic exposure to diazinon in Rat intestine (Lecoeur et al. 2006) and none has confirmed increased mRNA transcription and translation in controlled in vitro models for epithelial cells. We performed the following study to find out whether Caco-2 cells can be made resistant to low concentrations of diazinon and if these cells may demonstrate higher endurance in higher concentrations of diazinon compared to non-resistant cells. Additionally, P-gp as the possible mediator, have been investigated to confirm its expression and activity with the use of RT-PCR and western blotting methods.

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Materials and Methods

The human colon adenocarcinoma cell line, Caco-2, obtained from Iranian Pasteur institute at passage number 21, was grown in the form of monolayer in Dulbecco's modified Eagles' medium (DMEM), 10% fetal bovine serum (FBS) and 1% antibiotic. The cultures were kept in an incubator with an atmosphere of 5% CO_2 and 90% relative humidity, at 37.8°C.

Caco-2 cells were cultivated in the presence of DMEM containing 20% FBS which was inactivated with heat, 1% nonessential amino acid (L-glutamine, 20 mM), penicillin (100 U/ml) and streptomycin (100 mg/ml) for 72 h in the same temperature and humidity setting. Afterwards, the cells were transferred to the same medium containing different concentrations of diazinon. Initial diazinon concentration was 0.02 μM that was increased gradually in the period of 4.5 months to 20 μM. During the period, culture medium was changed three times a week and the diazinon concentration was increased gradually.

For determining growth curve and viability, 5×10^4 cells were cultured in each well of a 24 well plates at different concentrations of diazinon. Cell proliferation in both parent and resistant cells were evaluated using MTT hydrogenase assay.

For MTT test, cultivated cells at appropriate timing were withdrawn from the culture medium. Afterwards, 100 μl of 5 mg/ml Thiazolil blue solution in phosphate buffer solution was added to each well. Acidified formazan crystals formation was quantified with a reference wavelength of 690 nm at 570 nm.

For P-gp expression, 10^6 cells were grown in 35 mm dish. After treatment period, cells were collected using trypsin followed by centrifuging at 1,500*g* for 15 min at 4°C. The pellet was lyzed using RIPA-L solution, incubated on ice for 30 min and centrifuged at 14,000*g* for 15 min at 4°C (Ghahremani et al. 1999). The supernatant was transferred to a micro-tube and kept at −80°C for further analysis.

The protein samples were subjected to SDS–PAGE (10%) and blotted on PVDF membrane. The blots were blocked in 1% casein in TBS for 2–3 h followed by overnight incubation at 4°C with P-gp monoclonal antibody (Abcam, UK) at 1/1,000 dilution. The bands were detected using HRP-conjugated secondary antibody and visualized using luminol on Biomax film (Kodak, UK). Beta-actin polyclonal antibody (Santa Cruz, USA) at 1/1,500 dilution was used as internal control. The bands were digitized using Scion image software (Scion corporation, USA), normalized to beta-actin bands and reported.

Total RNA was extracted from Caco-2 cells by means of TRIZOL (Roche, Germany) method for extracting pure total RNA. Briefly, after cell sample lysis using TRIZOL, 0.5 ml chloroform was added to the lysate followed by shaking vigorously. After being centrifuged for 30 min at 11,000 rpm at 4°C, the supernatant phase was transferred to new clean tube and mixed with 0.6 ml isopropyl alcohol and centrifuged again in order to separate the RNA precipitate. After washing the RNA with 70% ethanol and partial drying, RNA precipitate was dissolved in RNase free water (DEPC water). Total RNA concentrations and quality were evaluated by spectrophotometer at 260 and 280 nm. The 260/280 ratios were above 1.7 for all prepared samples.

Then cDNA was made using 2 μg of Total RNA with reverse transcriptase enzyme for 1 h at 42°C. PCR was performed using two sets of primers specifically designed for MDR1 gene. Beta-actin mRNA was used as internal control (Table 1).

Beta-actin mRNA was detected as a 201 bp PCR product on agarose gel electrophoresis. In addition, two sets of primers that were used to detect expression of-MDR1 mRNA showed two PCR products one at 157 and another one at 280 bp on agarose gel. Results of RT-PCR were visualized and photographed using photodoc (Bio Doc It, UK) followed by band analysis in a semi-quantitative manner using Lab work software.

Results and Discussion

The viability curve of parent and diazinon resistant cells in the presence of different concentrations of diazinon is shown in Fig. 1. It should be noted that resistant cells were the same according to nutritional requirements and morphology compared to parent cells although culture medium of resistant cells contained 20 μM diazinon with the purpose of maintaining the cells in the resistant condition.

Table 1 Specific PCR primers for MDR1 and beta-actin mRNA

mRNA	Forward	Reverse	Product size (bp)
MDR1	5′-GTT-CAA-ACT-TT-GCT-CCT-GA-3′	5′-CCC-ATC-ATT-GCA-ATA-GCA-GG-3′	157
MDR1	5′-TC-TGC-AGT-TAA-AGG-G-5′	5′-GTT-CAA-ACT-TCT-GCT-CCT-GA-3′	280
Beta-actin	5′-GTC-CTG-TGG-CAT-CCA-CGA-AAC-T-5′	5′-TAC-TTG-CGC-TCA-GGA-GGA-GCA-A-3′	201
Beta-actin	5′-ACG-GGG-TCA-CCC-ACA-CTG-TGC-3′	5′-CTA-GAA-GCA-TTT-GCG-GTG-GAC-GAT-G-3′	660

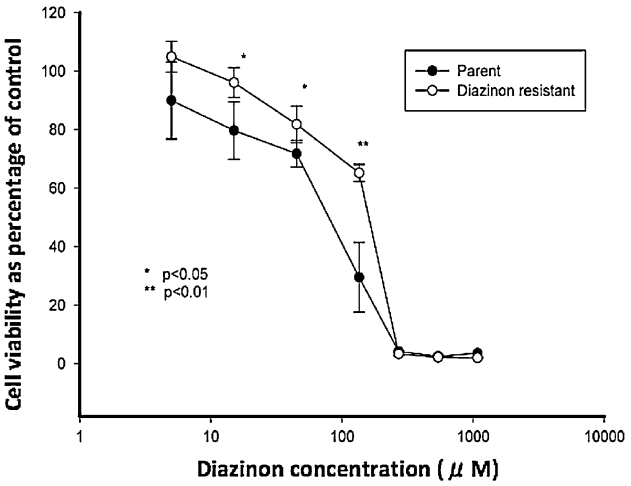

Fig. 1 The effect of different concentration of diazinon in parent and diazinonresistani Caco2 cells after 96 h (MTT; n = 9, ±SD)

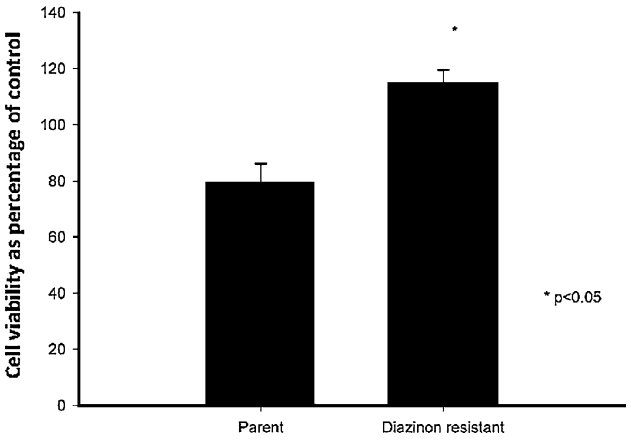

Fig. 2 The effect of 20 μM diazinon on the parent and resistant Caco2 cells after 5 days (MTT assay ± SD, n = 15)

As shown in Fig. 1, resistant cells were significantly different compared to the parent cells in the existence of diazinon up to the 135 μM concentrations. Crude results of MTT are also shown in Table 2 for clarification. The difference between parent and resistant cells viability, after 5 days of confrontation with 20 μM diazinon, is shown in Fig. 2.

Caco-2 cells expressed low levels of P-gp in normal condition. However, in resistant Caco-2 cells, the level of P-gp has been increased significantly (Fig. 3). The ratio of P-gp expression to beta-actin was 0.586 and 1.255 in parent and resistant cells, respectively (Fig. 3).

Band densitometry of MDR1 and beta-actin results using Lab work software revealed that MDR1 mRNA is of the same level in both parent and resistant cells. Figure 4 shows an example of our results with both sets of primers.

In the current study, Caco-2 cells were confronted with low doses of diazinon for a considerable period, in order to simulate chronic exposure to the toxin. 20 μM concentration of diazinon, which is equivalent to the amount of 6 mg toxin per kilogram of soil is used in this study. This amount

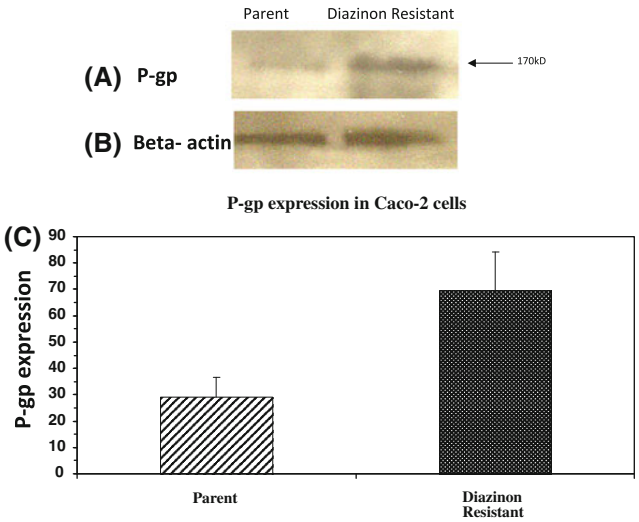

Fig. 3 P-gp expression in parent and diazinon resistant cells. Pannel **a** and **b** Western blot results for P-gp and beta-actin, respectively. **c** Comparison of P-gp expression in Diazinon resistant cells with Parent cells (Normalized ratios to beta-actin as percentages). The results were expressed as mean ± SD

Table 2 MTT results for parent and diazinon resistant cells in the presence of different diazinon concentrations

Diazinon concentration	Parent				Diazinon resistant			
	Crude value	% of control	Standard deviation	Negative control	Crude value	% of control	Standard deviation	Negative control
5	0.278876	89.96	13.18	0.31	0.136357	104.89	5.28	0.13
15	0.247101	79.71	9.83	0.31	0.124904	96.08	5.09	0.13
45	0.222456	71.76	4.6	0.31	0.10634	81.8	6.26	0.13
135	0.09145	29.5	11.92	0.31	0.084708	65.16	2.94	0.13
270	0.012958	4.18	0.42	0.31	0.004329	3.33	0.587	0.13
540	0.007781	2.51	0.63	0.31	0.002795	2.15	0	0.13
1,080	0.011036	3.56	0	0.31	0.002548	1.96	0.196	0.13

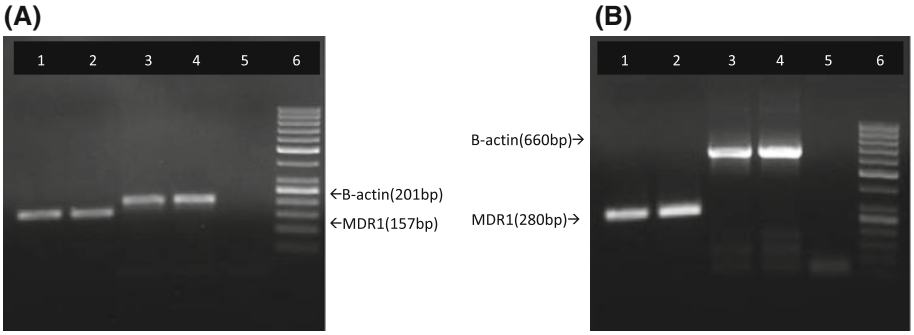

Fig. 4 RT-PCR results for MDR1 mRNA with both sets of primers normalized to beta-actin results. *Lanes 1* and *2* shows parent and resistant Caco-2 cells results for mdr1. Beta-actin results for parent and resistant Caco-2 cells are shown in *lanes 3* and *4*, respectively. *Lane 6* contains DNA ladder and *lane 5* has been used for running negative control

has been reported in polluted industrial areas (Krapac et al. 1995) and houses in which the pesticide has been used (Simcox et al. 1995). Minnesota Children's Pesticide Exposure Study (MNCPES) revealed that the ingestion is the principal root of pesticide exposure (Andrew Clayton et al. 2003) and it has been shown that during the precipitation season the amount of pesticides are higher in urban waterways which might cause exposure through ingestion (Bailey et al. 2000). According to these findings, diazinon was used in this study to model the chronic contact in the gastrointestinal cells before being metabolized to diazinon-oxon.

It has been shown that monolayer cultures of Caco-2 cells have the capability of expressing P-gp in the presence of various drugs and chemicals. Recent studies have also shown that MDR1 mRNA expression decreases in differentiated cells and the expression is at the maximum level within the first 3 days, then declines (Goto et al. 2003) but the process of MDR1 mRNA reduction in vivo differs from in vitro experiments and remains elevated up to 12 days (Lecoeur et al. 2006). According to the fact that our cells were in contact with low concentrations of diazinon, we predicted that MDR1 mRNA might decrease in these cells compared to the control, which might be confirmed by these results. Previous studies reported inhibitory effect of diazinon on digoxin transport through binding to P-gp (Taipalensuu et al. 2004). P-gp inducers usually do not transfer via P-gp related pomp but diazinon transportation in Caco-2 cells is through this system. Though, diazinon's role on its transportation is very important.

Initially, cellular toxicity in resistant and parent cells was compared. Obviously, IC50 was significantly higher in the resistant cells. It has been defined that cellular resistance decrease noticeably in the presence of specific inhibitors of diazinon (Cavret et al. 2005), consequently, it's obvious that cellular resistance is related to P-gp expression. The current study states that P-gp increased expression is not at the level of mRNA transcription. This points toward a steady state in which protein level has reached to constant levels and there is no need for increasing transcription. In this state, via increasing protein stability and decreasing its destruction, resistant cells might maintains protein levels at the cell surface as this is correlated to the inhibition of toxin collection in the cell.

Briefly, increased expression P-gp is observed in resistant Caco-2 cells to low doses of diazinon and these cells had higher tolerance to the toxin compared to controls but we did not notice any change in mRNA amount. Although this is probably due to adequate levels of protein in the cell, special subtype selection in heterogeneous population of Caco-2 cells in low concentrations of diazinone cannot be ruled out by this study and other studies have to be performed under controlled conditions to examine this hypothesis.

In conclusion, our results in addition to other studies, define P-gp role in cellular tolerance to diazinon and proves the role of low concentrations of the pesticide in preventing toxicity of higher doses in vitro.

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