

Evaluation of the Expression of P-Glycoprotein in Propoxur-Resistant Caco-2 Cells

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Abstract- There is a great concern about the effect of propoxur, as one of the more common N-methyl carbamate pesticides, on human health due to its extensive use in agricultural and non-agricultural applications. Caco-2 cells became resistant to propoxur, and the resistance was confirmed through MTT assay. Then the cell membrane integrity and P-glycoprotein expression were measured by LDH assay and western blot analysis, respectively and compared to the parent cells. Contrary to what was expected, the expression of P-glycoprotein in propoxur resistant cells was lower than parent cells. Conclusion: This study indicates that the resistance to propoxur may not be related to P-glycoprotein expression directly, since P-glycoprotein expression has decreased in these cells.

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Introduction

N-methylcarbamate pesticides have become increasingly popular in recent years, as alternatives of organochlorine insecticides, due to their short-term environmental persistence and low mammalian toxicity (1). The great success in agricultural applications has led to an increase in the use of this group of pesticides (2).

Propoxur [2-(1-methylethoxy) phenyl methylcarbamate, Baygon], an important N-methylcarbamate shows both systemic and contact activity against insects, and it is used as a broad spectrum pesticide on a variety of pests in either agricultural or non-agricultural applications, so it is a popular choice for pest control (3) and due to its toxic potential effects, there is a great concern about human health (4).

In physiological conditions, detoxification systems have thus to minimize the potential of toxic xenobiotics damage within intestinal cells (5). Transportation of

drug substances across the intestinal membrane is a dynamic process includes the passage of compounds through various functional pathways in parallel. P-glycoprotein (P-gp) is a member of the ATP-binding cassette (ABC) super family of transport proteins that transport a wide variety of compounds directly out of the membrane (6).

P-gp is highly expressed in tissues with excretory and elimination functions like apical surface of mucosal cells in small and large intestine, kidneys, liver, placenta and blood-brain barrier (7). P-gp seems to play a role in the development of resistance to various cytotoxic drugs, a phenomenon called multidrug resistance (MDR) (6) and it has also been implicated in development of some carbamate pesticide resistance (8). As drug efflux proteins are functionally expressed in their apical membrane, Caco-2 cell line is a suitable model of P-gp dependent jejunal efflux of xenobiotics (9) and is widely used to assess P-gp function and drug transportation (10).

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Despite the existing information based on in vivo and in vitro studies, it has not been determined that how chronic exposure to pesticides will effect on human health and whether P-gp transporter is involved in the mechanism of resistance to propoxur. The object of this study was to characterize the effects of exposure to propoxur in resistant and parent Caco-2 cells, as an intestinal cell model, and evaluation P-gp expression in the obtained resistant cells compared to the parent cells.

Materials and Methods

Cell culture

Caco-2 cells were obtained from National Cell Bank Pasteur Institute (Iran, Tehran). The cells were maintained in 50% Dulbecco's Modified Eagle's Medium-Ham F12 (DMEM-F12), 39% RPMI-1640, 10% heat activated Fetal Bovine Serum, 1% Penicillin (100 U/ml) and Streptomycin (100 µg/ml) and were kept in humidified 5% CO₂ incubator at 37°C. Subculture numbers 20-25 were used for the experiment.

For preparation propoxur resistant Caco-2 cells, the cells were kept in a medium with sequential increasing concentration of propoxur up to 20 µM for 50 days according to results of MTT assay.

Trypan blue dye exclusion assay

Viable cells exclude trypan blue while the dye stains dead cells. In this method, trypan blue (20µl; 0.4% w/v) was added to the cell suspension (20 µl) and the cells not stained by the dye were counted following microscopic examination. Briefly, cells were treated with different concentration of propoxur for 5 days and viability of cells were measured as mentioned in the parent cells compared to the resistant cells and expressed as a percentage of a parallel, untreated control. The 50% of maximal effective concentration (EC₅₀) of propoxur was determined.

MTT assay

Cells were seeded at a density of 4×10⁴ cells per well in 24-well plate. Cells were then treated by different concentrations of propoxur (10- 400 mM) and incubated at 37°C. Reduction of the tetrazolium salt (11) was measured in parent and resistant cells after 5 days.

Briefly, in this method, medium was discarded, and MTT (5 mg/ml in PBS) was added to the cells. Cells were incubated for 3 hours at 37°C and formazan crystals dissolved in DMSO and aliquots (200µl) of the resulting solutions were transferred in 96-well plates and the absorbance was measured at 570 nm with a

reference wavelength of 690 nm using the microplate reader (Biochrom Anthos 2020 Microplate Reader). Each experiment was carried out in triplicate. The amount of dye produced is proportional to the number of metabolically active live cells. Dose-response curves were computer plotted after converting the mean data values to percentages of the control response.

Lactate dehydrogenase release test

Test was performed according to the manufacturer's instructions and were on the basis of the rate of reduction of pyruvate to lactate in the presence of LDH with a parallel reduction of NAD. Cells were seeded on 24-well plates at a density of 4×10⁴ cells per well and were treated by different concentrations of propoxur (10- 400 mM) and incubated for 5 days. Then aliquots of media and warm reagent were mixed in a 96-well plate and absorbance NADH formation was indirectly measured at 490 nm, by using stoichiometric conversion of a tetrazolium dye. This test measures membrane integrity as a function of cytoplasmic LDH released into the medium. Results were analyzed and presented as expressed units/2 x 10⁶ cells.

Western blot analysis

Cells of both normal and propoxur resistant Caco-2 cells were harvested after 14 days post-seeding in Phosphate Buffer Saline (PBS). Cells were then centrifuged at 1400 g for 5 minutes and resuspended in RIPA-L lysis solution [Tris 1M, MgCl₂ 1M, KCl 1M, dithiothreitol 0.1M, phenyl methyl sulfonyl fluoride 0.1 M, Nonidet P-40 0.1%, Sodium dodecyl sulfate 10%, Na-deoxycholate, Leupeptine 500 µg/ml] (12). The suspension was passed through 25G needle twice to sheer the DNA and transferred to precooled microfuge tube. The suspension was incubated on ice for 30 minutes and centrifuged at 10000g for 10 minutes at 4°C. The supernatant was transferred to a clean precooled tube and stored at -80°C for further analysis.

The protein extracted was determined by the Bradford method using Bovine Serum Albumin (BSA) as a standard. Equal amount of samples (20- 30 µL equal to 15-20 µg) of proteins was loaded onto a 12% SDS polyacrylamide gel (13). The proteins were then transferred to PVDF membrane in a semidry apparatus using transfer buffer (Tris 25 mM, Glycine 192 mM, Methanol 20% v/v) at 1.5 mA/cm² for 75 minutes. Blots were then blocked in Casein 1%, Tween-20 0.1% in TBS (Tris Buffer Saline, pH=8) for 4 hours and incubated with the mouse anti P-gp primary antibody (1:500 in Casein 1%) overnight at 4°C. Blots were

Evaluation of the expression of p-glycoprotein

washed (4×10min) with TBS and incubated with the secondary antibody (anti mouse HRP-conjugated, 1:1000) in Casein 1% blocking solution for an hour at room temperature. Then blots were washed again, and P-gp bands were detected using the Chemiluminescence's System (Roche, Germany) and quantified by densitometric analysis.

Statistical analysis

Statistical comparison was carried out by unpaired t test for the obtained data from propoxur resistant and parent cells. The results are presented as percent of control ± SD.

Results

The growth curve of parent and propoxur resistant Caco-2 cells

Figure 1 shows a significant decrease in proliferation rate of propoxur resistant Caco-2 cells (P-value < 0.02) at the sample time. Subsequently the doubling time has increased up to 100 hours, in comparison to wild-type parent cells with that of 62 hours.

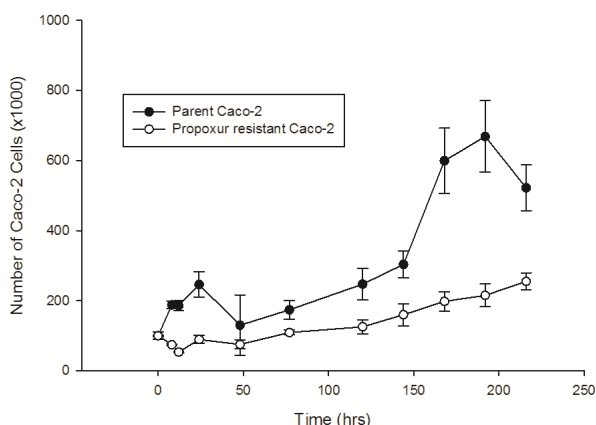


Figure 1. The growth curve of normal and propoxur resistant Caco-2 cell using trypan blue method

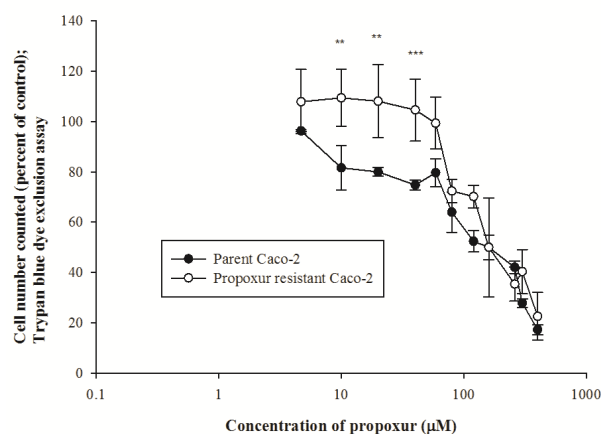
Cells were cultured in 24 well plates in triplicate at the density of 5×10^4 cells per well, and growth was evaluated for 9 days. The data presented as Mean ± SD (n=6). According to the growth curve, the doubling time has increased up to 100 hours, in comparison to wild-type parent cells with that of 62 hours

Viability assays

Propoxur cytotoxicity was evaluated in concentrations from (10 to 400 μM) using trypan blue method (Figure 2A) and MTT test (Figure 2B) in both parent and resistant cells. As it is seen in Figure 2, the toxicity of propoxur was significantly decreased in the resistant cells compared

to the parent cells at the concentration from 10 μM to 40 μM using trypan blue assay (Figure 2A) as well as MTT assay (Figure 2B). In this regard, the EC₅₀ of propoxur was obtained 173 ± 12 μM in normal cells, consistent with the previous report (14) and 191 ± 14.5 μM in propoxur resistant Caco-2 cells.

A



B

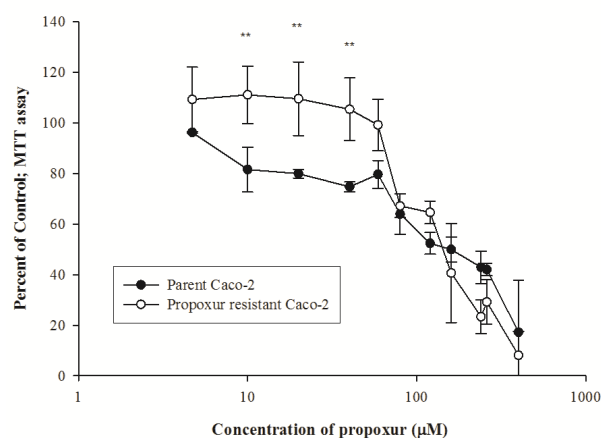


Figure 2. The effect of Propoxur on the viability of Caco-2

Normal and resistant cells were plated in 24 well plates (trypan blue exclusion) or 96 well plates (MTT assay) in the presence of various concentrations of propoxur for 5 days. Viability measured using trypan blue method (A), MTT assay (B) and data presented as Mean ± SD (n=6). The static analysis shows significant difference between concentrations of 10 to 40 μM (P-value < 0.01).

LDH membrane integrity assay

To indicate the cell membrane integrity as a declaration of the cytotoxic effects of propoxur, LDH assay was performed. Different concentrations of propoxur were tested over 5 days exposure to both

parent and resistant cells, and the results showed the membrane damage in a dose dependent manner in both cell types. However, there was no significant difference between the amount of lactate dehydrogenase leakage from parent and resistant cells (Figure 3).

The effect of long-term exposure of low dose propoxur on P-gp expression in wild-type and propoxur resistant Caco-2 cells was measured by western blot analysis. Western blot analysis revealed that the expression of P-glycoprotein in resistant cells was decreased compared to the normal (wild type) Caco-2 cells.

As shown in Fig. 4, the 170 kDa band correspond to P-gp was detected in both cell lines. The long-term exposure to propoxur decreased the expression of P-gp significantly in resistant Caco-2 cells compared to the parent cells (*P*-value < 0.01) (Figure 4).

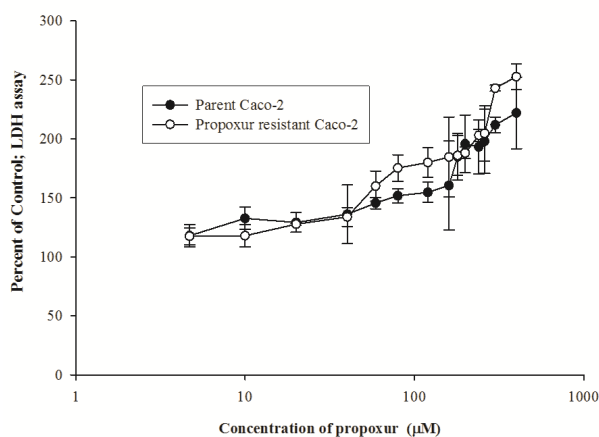
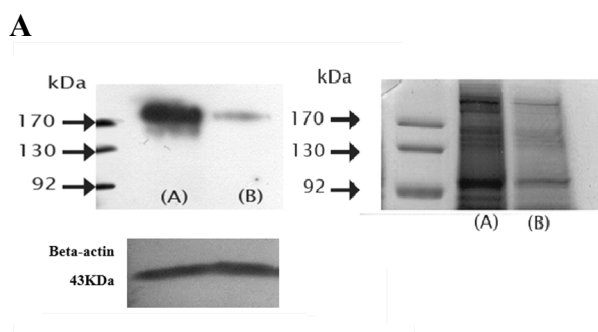


Figure 3. The effect of Propoxur on the viability of Caco-2 cells using LDH assay

Normal and resistant cells were plated in the presence of various concentrations of propoxur (10- 400 µM) for 5 days. Viability measured using LDH assay and data presented as Mean ± SD (n=6).

There is no significant difference between parent and propoxur resistant Caco-2 cells



B

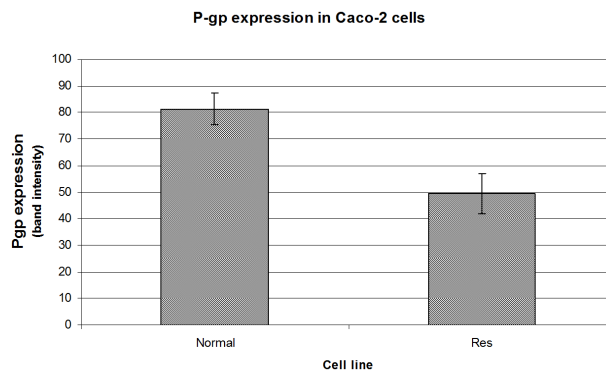


Figure 4. Effect of long-term exposure to low dose propoxur on P-glycoprotein expression in Caco-2

Cells were grown in the presence of 20µM propoxur for 50 days. Protein was extracted from both resistant and wild type parent cells and measured by Western Blotting and on Coomassie blue-stained polyacrylamide gel. (A) Immunoblotting was performed with P-gp antibody, using control parent (normal) cells (lane A), propoxur resistant cells (lane B). Each lane contains 30µL protein from cell lysates. (B) The bands were quantified by densitometric analysis and results presented as Mean ± SD for three independent experiments (n=3)

Discussion

Propoxur is one of the N-methylcarbamate esters that is used as an important insecticide due to its low acute toxicity to mammals (15).

We have developed propoxur resistant Caco-2 cells by means of continuously exposure to repeated low dose of propoxur (20 µM), in order to simulate the effect of the contaminated environment on gut epithelial cells. This concentration is not lethal for the Caco-2 cells in a long time exposure (3-6 months) (16). This method of developing resistant cells has been confirmed and used previously (17,18). In our study, as figure 1 shows, the rate of cell proliferation has been decreased significantly in the treated cultures, and the doubling time has been increased from 62 hours to 100 hours. This may be due to the cell cycle delay and may represent slow regeneration of epithelial cells following propoxur exposure. Based upon the previous data, carbofuran, a carbamate pesticide, could induce significant concentration-dependent delay in cell-cycle progression as well as a significant decrease in the proliferation (19). The cytotoxicity studies in parent and propoxur resistant Caco-2 cells indicates that the tolerance to propoxur increases by continuous exposure to the pesticide, as IC50 has been altered from 173 ± 12 µM in parent cells to 191 ± 14.5 µM in resistant cells. Both results of MTT and trypan blue assays confirms each other and show a

Evaluation of the expression of p-glycoprotein

significant decrease in cell proliferation inhibitory effect of propoxur on the second group which was under the method of developing resistant cells. These data confirm our method.

On the other hand, according to the LDH results there is no difference between the cell integrity of resistant and parent cells. Furthermore, the involvement of the mitochondrial pathway for propoxur-induced cell death has been suggested in the past (4). This result could demonstrate the independence of resistance to propoxur on cell membrane damage, although further investigations are needed to clarify the mechanism of resistance to propoxur.

One possible mechanism in resistant to the toxin is the presence of P-glycoprotein (20). In consequence, we have measured P-gp expression in normal and propoxur resistant cell types by western blot analysis. In this study, propoxur resistant Caco-2 cells showed a decrease in P-gp expression (ratio of P-gp expression in resistant cells to parent cells is 0.6 ± 0.07) (Figure 4). It has been investigated that flavonols like quercetin and kaempferol could significantly decrease P-gp expression and function resulting in inhibition of P-gp activity in KB-V1 cells, while isoflavones were able to inhibit P-gp function with no effect on P-gp expression (21). Also, it has been reported that P-gp expression in Caco-2 cells is not constant and is influenced by culture conditions and drug exposure (22). But in one investigation an increase was observed in P-gp expression in Caco-2 cells treated with repeated low doses of diazinon (9). It was reported that diazinon possess a P-gp mediated efflux in Diaz/Caco-2 cells (20), and Lecoeur has suggested a role for P-gp in the pesticide transfer (9). Further studies are needed to find the involved mechanisms in propoxur resistant.

This study indicates that the prolonged exposure to propoxur decreased both proliferation and P-gp expression in Caco-2 cells. Moreover, resistance to propoxur may not be related to P-gp expression directly, since P-gp expression level has been decreased to about 40% compared to the parent cells and further investigation is required to clarify the precise mechanism of resistance to propoxur in these cells.

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