ORIGINAL ARTICLE

Effects of peptides selected from artificial peptide libraries to p-glycoprotein activity

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Abstract

Peptides selected from artificial peptide libraries have special interest in cell targeting, drug discovery, molecular diagnosis and multidrug reversal. Peptides selected from artificial peptide libraries against doxorubicin resistant K562 cells have the capacity in the reversal of the doxorubicin resistance. Our aim is to determine the characteristics of these peptides on recombinant human P-glycoprotein membrane fractions to be able to understand their interactions. Peptides were selected against doxorubicin resistant K562 cells. The effects of synthesized peptides on these cells viability was done by XTT viability assay. The interactions of peptides with P-glycoprotein were done by Pgp-Glo™ Assay System by measuring the ATPase activity of P-glycoprotein. According to our results; four selected peptides effected on doxorubicin resistant K562 cells viability and stimulated the P-glycoprotein activity in the presence of doxorubicin at different levels. If the P-glycoprotein stimulation occurs on living doxorubicin resistant K562 cells, cell viability will not be affected due to multidrug resistance effect of P-glycoprotein. Peptides selected from artificial peptide libraries are useful tools in the reversal of multidrug resistance and for understanding the membrane structure-function relationships.

Keywords: Recombinant peptides, multidrug resistance, Pp-glycoprotein, membrane biophysics

Introduction

The development of drug resistance in cancer cells is an important problem in the treatment of cancer. Failure of antitumor drugs in cancer therapy is associated with over expression of structures on the cell membrane. Thus, intracellular concentrations of the drugs used do not reach effective levels and loss their activity. Cell surface proteins are responsible for multidrug resistance and cell function changes in drug resistant cells [1]. In recent years, studies have accelerated in the cancer cells to be able to overcome the p-glycoprotein (P-gp) mediated multidrug resistance. P-gp which is one of the energy (ATP) dependent drug efflux pumps is a member of a large ABC super family of the transporter proteins. These proteins are over expressed in cancer cells which play an important role in the resistance of various cancer cells in chemical therapeutic approaches [2-4-7]. Recent studies have shown that it is possible to reverse the resistance of drugs that can attract P-gp in these cells, to prevent P-gp functions and to develop new generation molecules [3,8,9].

The use of phage containing artificial peptide libraries has prompted researches on cellular functions by targeting proteins on the cell membrane [10-14]. Peptides selected from artificial peptide libraries are used in researches as well as cell targeting, diagnostic or therapeutic purposes in model cell systems such as cancer cells [14-17]. On the other hand; the use of artificial phage libraries has also increased significantly in areas such as molecular imaging and drug development [18,19].

Human erythroleukemia cells from chronic myeloid leukemia patients are described as K562 cells [20]. Well characterized doxorubicin-resistant K562 cell lines (K562-dox) were over expressed drug resistance proteins, especially P-gp molecules more than wild K562 cells [2,21-24]. Doxorubicin (adriamycin) is a P-gp substrate which stimulates the P-gp activity and used in the treatment of various types of cancer [3,25]. The K562-dox cells are resistant to a certain concentration of doxorubicin. Therefore, the number of P-gp in these cells increased and they became resistant to doxorubicin. K562 cell types, which are capable of obtaining resistance to certain drugs, are used as model cells in drug resistance studies [3,8,26]. In our previous study, based on the phage display technology and the properties of the resistant K562 cells, we have identified peptides that were carried by phages that could affect drug resistance in these cells [14].

In the first step of this study we aim to investigate the effect of the identified peptides on K562-dox cells directly and in the second step the effect of artificial peptides on P-gp activity.

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Materials and Methods

Cell Lines and Cell Culture

K562-dox cell lines were obtained from Prof. Dr. Hakan Akça (Pamukkale University Faculty of Medicine, Denizli, Turkey). The cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 Units/ml penicillin and 100 µg/ml streptomycin. Cells were incubated at 37 °C in 5% CO2, 95% air atmosphere. K562-dox cells were periodically cultured at 1 mM doxorubicin concentration in RPMI 1640 medium.

Peptides

In our previous study, selected phages against to K562-dox cells by phage display technology were named membrane penetrated phages as KPP; membrane bound phages as KPB [14]. We selected peptides that were negatively affected K562-dox cell viability and peptides sequences were synthesized as KPB7 (SLNTTFTGPSRP), KPB10 (TSHHDSHGLHRV), KPB25 (WPTYLNPSSLKA) and KPP8 (FMPKSHKSRLPS) (Invitrogen, *USA*).

Cell Viability Assay

To investigate the effects of artificial peptides in the presence and absence of 1 mM doxorubicin in the first step of our study, K-562-dox cells were seeded onto 96-well plates as 1x105 cells per well. Peptide concentrations were arranged as 0.04 µmol, 0.08 µmol and 0.15 µmol and assay was repeated three times for each peptide. After incubation at 37 °C with 5% CO2 for 24 hours, XTT (*Biological Industries, Israel Beit Haemek Ltd.*) solution was added to each well at a volume of 50 µl. The cells were further incubated for 24 hours and 72 hours to allow XTT production. Cell viability was calculated according to the manufacturer's instructions (*Biological Industries, Israel Beit Haemek Ltd.*).

P-gp ATPase Activity Assay

In the second stage of our study, changes in ATP use due to P-gp ATPase activity were detected with the P-gp Glo assay system (*The Pgp-GloTM Assay Systems, Promega*) when the P-gp molecule developed interaction with other molecules. Possible interactions with P-gp molecules were investigated at different concentrations of artificial peptides (0.04 µmol, 0.06 µmol, 0.08 µmol, 0.1 µmol and 0.12 µmol) and 1 mM doxorubicin in the absence and presence. These studies were performed in the Pgp-Glo ™ Assay System manufacturer's instructions and the luminescence measurements were performed with the Glomax Multi Detection System (*Promega*) to determine the results.

In this study, the control results obtained in experiments firstly with no addition to the medium as basal control, secondly by adding the specific inhibitor of the P-gp molecule, Na3VO4 and the activator of the P-gp, verapamil were used as activity controls. These control results were compared with the results obtained from the synthesized peptide and P-gp interactions. In the next step, P-gp ATPase activity stimulated by synthetic peptides was calculated as nmol ATP consumed/µg P-gp/minute and the changes in P-gp activity that occurred at different concentrations of peptides in the presence and absence of doxorubicin were calculated.

Statistical Analysis

Statistical analyses were performed with Student's t-test. Statistically, $p < 0.05$ was considered as significant. Standard deviations (SD) were shown on each figure.

Results

Our initial findings indicate that the peptides we identified as negatively affected for K562-dox cell viability in our previous study and identified as amino acid sequences KPB7 (SLNTTFTGPSRP), KPB10 (TSHHDSHGLHRV), KPB25 (WPTYLNPSSLKA) and KPP8 (FMPKSHKSRLPS). All peptides were used in different concentrations (0,04 µmol, 0.08 µmol and 0.15 µmol) and the viability of K562-dox cells studied in the presence/absence of 1 mM doxorubicin.

The results of cell viability assay of control K562-dox cells are shown in Figure 1. According to these findings, the viability of these cells, cultured under optimum conditions, observed as (170% ± 5,6) at 24 hours, (256% ± 52) at 48 hours and (300% ± 20) at 72 hours. The viability assay results obtained at different concentrations (0.04 µmol, 0.08 µmol and 0.15 µmol) of peptides in the presence/absence of doxorubicin are shown in Figure 2. With these findings, cell viability remained within the range of 58-68% for each peptide at different concentrations without doxorubicin. In the presence of doxorubicin, these rates remained similar for KPB7, KPB10 and KPB25, but decreased to 47% for KPP8 at 0.15 µmol concentration (Figure 2). It was observed that synthetic peptides at different concentrations had negative effects on cell viability (p <0,05) when compared with the control K562-dox cells. It has been shown that the rate of cell survival of synthetic peptides in the presence or absence of doxorubicin is similar to peptides on the phage surface [14].
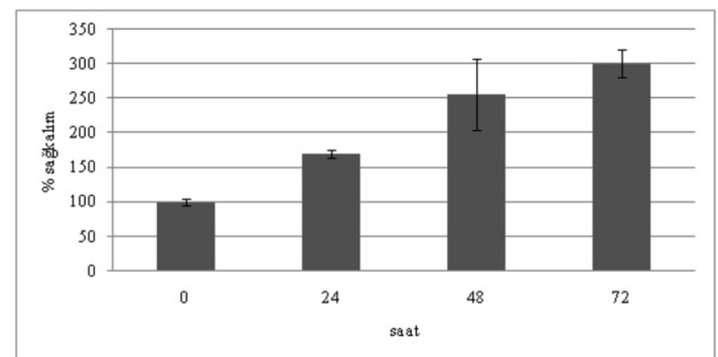

Figure 1. Cell viability of control K562-dox cells (p<0.05)
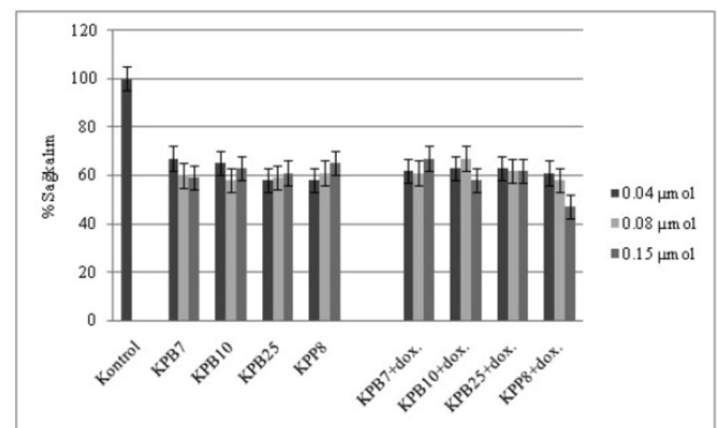

Figure 2. XTT viability assay results of K562-dox cells with peptides (p<0.05)

In the next step, the findings of possible interactions between P-gp molecules and synthetic peptides KPB7, KPB10, KPB25 and KPP8 were obtained with a human P-gp membrane model in the Pgp-Glo ™ Assay. In this system, peptide concentrations were used as 0.04 µmol, 0.06 µmol, 0.08 µmol, 0.1 µmol and 0.12 µmol based on the concentration range used for XTT viability analysis. The results obtained in these experiments with Na3VO4 and verapamil were used as controls and compared with those obtained from synthetic peptides and P-gp interactions. As shown in Figure 3, basal P-gp ATPase activity was calculated as 0.03 ± 0.01 nmol/µg P-gp/min. P-gp stimulation with verapamil as a positive control, was found to be 1.7 times greater than basal control, and there was no ATPase activity in the presence of Na_3VO_4. It was determined that, the P-gp ATPase activity of all peptides decreased at a concentration of 0.12 µmol, based on the interaction results of synthetic peptides with P-gp at different concentrations in the absence of doxorubicin. KPB7 peptide alone was found to cause lower activity at all concentrations than the control results with verapamil. In contrast, KPB10, KPB25 and KPP8 peptides were found to cause more P-gp ATPase activity in this system than verapamil activity. The highest P-gp ATPase activity was found to be 3.25-fold in the presence of 0.04 µmol KPP8 peptide compared to basal control.

According to our results, it was observed that P-gp activity did not have large changes due to synthetic peptide concentration differences (Figure 3). Synthetic peptides in the presence of doxorubicin caused an increase in the activity of P-gp ATPase only according to the results they used. The results between the presence and absence of doxorubicin were compared; the highest P-gp ATPase activity (4.1 fold) was obtained at a concentration of 0.08 µmol of peptide KPB7. In contrast, it was found that the peptide KPB10 and doxorubicin together increased the activity of P-gp ATPase approximately 3.5-fold compared to the basal control. In the presence of doxorubicin and peptide KPB25, 4.9-fold (0.04 µmol), 5.48 fold (0.06 µmol), 4.75 fold (0.08 µmol), 4.43 fold (0.1 µmol) and 5-fold (0.12 µmol) P-gp ATPase activity was found to be the highest activity. Peptide KPP8 at 0.1 µmol concentration has the lowest activity with doxorubicin than the P-gp ATPase activity in the absence of doxorubicin (Figure 3).
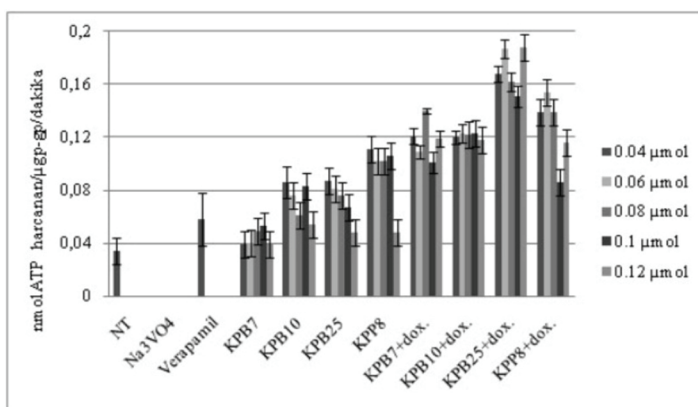
Figure 3. ATPase activity results of P-gp with peptides (p<0.05)

Discussion

The development of drug resistance in cancer cells constitutes a major problem in cancer therapy. In recent years, studies have accelerated in the cancer cells to be able to overcome the P-glycoprotein (P-gp) mediated multidrug resistance. K562-dox cells are also used as model cancer cells in cell viability assays [2,21-24]. Artificial peptide-phage display technology has become a common method to target cell membrane structures, to redirect drugs to target cells and to investigate their contribution to the treatment of the diseases [10-14]. In addition, researchers have shown that multidrug resistance of cells can be reversed by following the effects of different molecules on P-gp activity in various cancer types [8,27].

In our previous study, we reported that the peptides displayed on the phage surface caused a decrease in the viability of K562-dox cells. The use of doxorubicin with phage displayed peptides has been shown to further enhance the effect of doxorubicin on resistant cells [14]. We tried to find an answer to the question of how the peptides synthesized separately from the phage interact with the cell surface and how it can reverse doxorubicin resistance. One of the membrane proteins that cause drug resistance in cells is the P-gp molecule, which is a member of the ABC super family. The artificial membranes with P-gp protein were established as a model system for investigating the effects of molecules by following the activity of P-gp.

Possible effects of artificial peptides on both cell viability and the ATPase activity of P-gp on human artificial P-gp membrane in the presence and absence of doxorubicin were studied because of the contribution of P-gp as a cell membrane protein to multiple drug resistance. According to the results of the cell viability assay, we observed that the control K562-dox cells survived because they were resistant to doxorubicin (Figure 1). In the presence of doxorubicin, artificial peptides were found to similarly reduce K562-dox cell survival in our previous study. KPP8 peptide at 0.15 µmol concentration and together with doxorubicin was found the most effective peptide on cell viability (47%) as compared with others (Figure 2). In the presence of the peptide alone, cell death was still observed, although less than in the presence of peptide/ doxorubicin combination. Therefore, they are thought to cause cell death by acting on cell resistance mechanism. However, we studied the effects of these peptides on p-glycoprotein functions primarily in order to elucidate what changes in the membrane are caused by peptides properties, what structures they interact with or which molecules functions they increase/decrease on the cell membrane. However, the effect of these peptides on p-glycoprotein functions was investigated when we tried to elucidate what kind of changes in the membrane caused by the peptides themselves, their effect on the membrane with which structures or which molecules disrupted the function.

Obtained results show that KPB7, KPB10, KPB25 and KPP8 peptides increase the activity of P-gp. On the other hand, the increase in P-gp ATPase activity was shown to be in the range of 2.52-5.5-fold with the use of peptides at different concentrations with doxorubicin (Figure 3).

Peptide and peptide/doxorubicin combinations have been shown to reverse K562-dox cell viability by reversing doxorubicin resistance. However, these peptides have an effect on the activity of artificial P-gp. At this point we think that the pathway to cell viability is through different molecular pathways such as P-gp. If the peptides were interacting with P-gp on the cell membrane, the viability of K562-dox cells would not be affected. So/therefore,

the effect of these peptides has many interactions not only with the P-gp activity. To be able to identify the exact mechanisms of interactions between peptides, doxorubicin and P-gp or mechanisms of different membrane proteins on cell membrane, it should be further investigated by different biophysical techniques such as atomic force microscopy (AFM), X-ray crystallography and patch clamp. The use of target-specific peptides from artificial peptide libraries is thought to be useful tools in cell targeting, multidrug resistance reversal and elucidating the biophysical phenomena on living cell membranes.

Acknowledgement

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