Delivery Strategies to Improve In Vivo Stability of Immunogenic Peptide PADRE

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Abstract

PADRE, a peptide with potential in breast cancer immunotherapy exhibits low in vivo efficacy due to poor tumor uptake and significant enzymatic degradation. In the present work, novel delivery based approaches were utilized to improving the efficacy of PADRE. The utility of these approaches in the delivery of PADRE was tested on in vitro cell based models. In the in vitro systems, PADRE was not internalized in Caco-2 cells to a significant extent as observed from the concentrations in cell pellet, which were always lower than 5%. Also, 90% of PADRE externally associated with the Caco-2 cells was degraded within 4 hours, possibly due to the breakdown by ecto-peptidases associated with tumor cells. Enzyme inhibitors, antitrypsin reduced the PADRE degradation in Caco-2 cells (20%) as compared to untreated cells (55%). The lipoprotein based systems were formulated with and without 0.1% sodium lauryl sulfate that yielded 45 and 53% loading of PADRE, respectively. The efficacy of PADRE lipoprotein-based systems was determined by performing CD4 proliferation assay. The lipoprotein based systems with 20 µg/mL treatment with and without 0.1% sodium lauryl sulfate showed 3.2 and 2.8 times CD4 proliferation observed as compared to control. The CD4 proliferation on treatment with native PADRE was 4.3 times compared to a no treatment control, however this was not statistically significant (p>0.05) when compared to the PADRE activity from lipoprotein systems indicating that the lipoprotein-based approach was suitable delivering PADRE as it retained the immunogenic activity. The strategies studied to deliver PADRE were successful in vitro and showed potential for improving its immunogenic efficacy in vivo by improving its stability.

Keywords: PADRE delivery, peptidase inhibitors, lipoprotein delivery system

Introduction

Breast cancer is the most common cancer detected amongst women worldwide and also the second most leading cause of cancer related deaths amongst women in the world. According to statistics released by the National Cancer Institute, there were 2,30,480 cases of breast cancer in females in the United States alone reported in the year 2011 and an estimated 39,520 deaths. Breast cancer treatment involves radiation and surgery to locally treat and remove the primary tumor followed by adjuvant therapy to combat the metastasized tumor cells. Adjuvant therapy includes treatment with chemotherapy drugs, hormonal therapy and immunotherapy. Chemotherapy is effective but non-specific and therefore suffers from several adverse effects. Immunotherapy is gaining importance due to the selective recognition and destruction of cancer cells, thereby sparing the normal cells and minimizing the adverse effects.
Trastuzumab, a monoclonal antibody targeting the HER2 receptor, is a leading immunogenic therapy for breast cancers that exhibit HER2 overexpression. However, treatment with Trastuzumab is limited to around 25% of total breast cancer cases showing HER2 overexpression and the treatment also suffers from adverse effects like cardiotoxicity (1).

Breast cancer cells show expression of various MHC II antigens, which makes them suitable targets for treatment with immunogenic peptides. Several tumor associated antigens (TAA's) including HER2, p53, MUC1, BRCA2, survivin, IGFBP2 etc., have been identified in breast cancer patients and autoantibodies to these TAA's have been detected in the serum samples which gives further evidence of their immunogenic potential (2). This immunogenic response has been exploited for early diagnosis of these tumors and also for treatment of the tumors by targeted delivery of tumor specific immunogenic peptides. The immunogenic peptides mark the tumor cells for recognition by the immune system and eventually destroy them (3). Some immunogenic peptides exhibit strong immune response in vitro or when coated on to tumor surfaces before inoculation in vivo indicating that the peptides are recognized by the T lymphocytes. The physiological barriers and the metabolic enzymes may be responsible for the poor uptake and degradation of the peptides respectively, which prevents the same immune response to be observed when the peptides are injected systemically.

Pan DR Reactive Epitope (PADRE) is a 13 amino acid peptide sequence, aK(X)VAAWTLKAAa, which effectively binds to several MHC allelic variants and produces potent immunogenic responses in in vitro systems like T cell proliferation assays. PADRE is not effective on systemic administration, which could possibly be due to the enzymatic degradation of the peptide by the proteases and peptidases secreted by the tumor cells and also the physical and physiological barriers presented by the tumor cells. Wenning et al. developed a model for relating immunotoxin toxicity to cellular trafficking in a single cell and extrapolating with diffusive transport of immunotoxin in a solid tumor sphere. Immunotoxins were found to be less effective against multi-cell tumor spheroids (MTS) than monolayer cells under equivalent conditions. The poor efficacy was traced to either heterogeneous receptor distribution in MTS or significant barrier(s) to the penetration of the immunotoxin into the spheroid (4). In our previous publication, we reported the degradation of PADRE in biological matrices like intact tumor, tumor homogenates and plasma (5).

Improving the stability of PADRE and making them available for systemic and tumor uptake of PADRE using various delivery approaches is the objective of the current study. Two different approaches were investigated to improve the stability and in vivo immunogenic potential of PADRE, namely, use of enzyme inhibitors and use of lipoprotein based drug delivery systems. Based on previous studies, co-administration of peptidase inhibitors with proteins and peptides like insulin and nonapeptide PHPFHLFVF (a renin inhibitor) was found to improve their stability and absorption (6,7). We investigated the utility of this approach by selecting inhibitors of specific peptidases, which could possibly metabolize PADRE on the basis of its structure. The second approach investigated was the development of a lipoprotein based drug delivery system of PADRE to specifically target the low-density lipoprotein (LDL) receptors overexpressed on the tumors (8). The lipoprotein carriers have two major advantages over liposomes, mainly the smaller size and longer half lives of 3-5 days. Also, because lipoproteins are natural endogenous substances, they are non immunogenic and escape the reticuloendothelial system and will not interfere with PADRE immunogenicity. The LDL and HDL based drug delivery systems have been designed for the
delivery of some hydrophobic cytotoxic drugs to solid tumors. In a previous report, the LDL-drug complex was prepared for selective delivery of the cytostatic agent to tumors. However rapid dissociation of the complex in plasma resulted in stability issues preventing further development of these systems (9). In our study we explored lipoprotein approach for delivery of PADRE to tumors by physical entrapment of the peptide within the lipoprotein systems.

Materials and Methods

The peptide PADRE was synthesized by Genemed Synthesis Inc., San Francisco, USA. Low-density lipoproteins (LDL) and enzyme inhibitors were obtained from Sigma, USA. Caco-2 cells were obtained from ATCC (Manassas, VA, USA). The Caco-2 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen Co., Carlsbad, CA) containing 10% heat inactivated fetal bovine serum and 1% penicillin-streptomycin (5000 I.U./ml, Cellgro, Mediatech Inc, VA). The cells were incubated in an environment of 37 °C and 5% CO2. The cells were grown up to 80-90% confluency before further passage for experiments.

Effect of peptidase inhibitors on stability of PADRE: Caco-2 cells were cultured at a density of 1 x 10^6 cells. The cells were trypsinized and suspended in growth media. For the control studies, 500 lL of 100 ig/mL of PADRE was incubated in PBS with Caco-2 cells in culture tubes for 0.5, 1, 2, and 4 hours at 37°C. The cells were separated from the supernatant by centrifugation at 10,000 rpm for 5 minutes, digested, and the amount of peptide in supernatant and cell pellets was estimated by the HPLC method described previously (5). To determine effect of enzyme inhibitors on the stability, Caco-2 cells were pretreated with enzyme inhibitors (Anti-trypsin – 10 µM, Bestatin – 100 µM, Diprotinin A – 100 µM, Phospharidon –1 µM and Phenanthroline –100 µM) for one hour followed by treatment with PADRE (Table 1). At the end of 2 hour incubation, samples were deproteinated and the concentration of PADRE was analyzed by HPLC.

Preparation of lipoprotein delivery systems: Ten mg of LDL was triple washed with 5 mL of heptane to extract the lipids. The apoprotein B was suspended in 3 mL of PBS using a laboratory
vortex. To this, aqueous solution of peptide at 1 mg/mL was added, vortexed, and lyophilized at –50 °C for 12 hours to remove water. The heptane extract of lipids were then added to the powder mixture of apoprotein B and peptide and vortexed for 5 minutes. Heptane was slowly removed using rotoevaporator at 40 °C for 30 minutes under vacuum. Lipoprotein delivery systems were also prepared using exactly the same protocol except that 0.1% sodium lauryl sulphate was used during the suspension of the peptide in the apoprotein B.

Stability of PADRE-lipoprotein system in Caco-2 cells: Caco-2 cells were cultured at a density of 1 x 10^6 cells. The cells were trypsinized and suspended in growth media. PADRE-lipoprotein equivalent to 50 mcg/mL of peptide, was added to Caco-2 cells in culture tubes and incubated for 4 hours at 37 degrees. The cells were separated from the supernatant by centrifugation at 10,000 rpm for 5 minutes, digested, and the amount of peptide was estimated by the HPLC method. Native PADRE solution of 50 mcg/mL in water was used as control.

**CD4 proliferation assay to determine immunogenicity of PADRE-lipoprotein system:** CD4 positive T-lymphocytes were derived from immunized mice and subsequently selected via *in vitro* stimulation by peptide and dendritic cells. PADRE specific, CD4 cell proliferation assay was performed by incubating T-lymphocyte cells in triplicate with PADRE 10 and 20 µg/mL or equivalent amount of PADRE incorporated lipoprotein delivery systems, and dendritic cells in the presence of IL2 at 37°C for 48 hours. At the end of 48 hours, the cells were incubated with 3H-Thymidine for 4 hours and harvested. Thymidine incorporation was measured on a scintillation counter. CD4 proliferation was taken as a measure of immunogenicity of PADRE. Naïve lymphocytes served as a negative control.

![Graph](image.png)

**Fig. 2.** Improvement in PADRE stability upon pretreatment with peptidase inhibitors determined in Caco-2 cells.

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Results and Discussion

As reported in our previous manuscript, PADRE showed considerable degradation in all biological matrices like plasma, tumor and tumor homogenates, with most rapid degradation seen in plasma. PADRE was found to be stable in PBS (control) as shown in figure 1. PADRE stability in Caco-2 cultures was determined by incubating in the culture media. After Caco-2 cell digestion, the amount of PADRE present in the pellet represented the internalized fraction and amount present in the supernatant represented the PADRE associated with the cells. The amount of PADRE in the pellet at all times was less than 5%. PADRE present in the cell supernatant exhibited rapid degradation. The concentration of the peptide decreased to 50% of the original amount in 2 hours and up to 90% in 4 hours. This data suggested that PADRE is degraded by the ecto peptidases present on the tumor cell surface. The substrate specificity of these peptidases is found to be limited to small peptides (di-, tri-, and oligopeptides) up to a maximum of approximately 30 residues (10,11). Based on the possible degradation sites, peptidase inhibitors given in Table 1 were selected for co-delivery with PADRE. In a study by Takaori et al, the nonapeptide, PHPFLVF, was found to cross rabbit jejunum 90% intact in the presence of phosphoramidon, a metalloproteinase inhibitor (6). In the absence of protease inhibitors, 50% of the peptide was degraded in 5 min and 100% in 30 minutes. Insulin, another large peptide used to study the effect of protease inhibitors, was protected by diisopropyl fluorophosphate against serine proteinases (12), and aprotinin against RNAase (7). The unnatural D- amino acids at the N- and C- termini of the PADRE peptide and the unnatural amino acid cyclohexylalanine at position 3, rendered it more stable than other immunogenic peptides. The data from peptidase inhibitors shown in Figure 2 indicated that antitrypsin offered the maximum protection; only 20% of PADRE was degraded compared to 55% in untreated Caco-2 cells. Phosphoramidon and phenanthroline offered only marginal protection (45% and 49%, respectively).

To counter the problem of poor tumor barrier penetration, PADRE was formulated into a lipoprotein based drug delivery system. The LDL receptor overexpression on tumor surfaces improves the uptake of the lipoprotein based drug delivery systems (13). The PADRE lipoprotein based system exhibited a yield of 45%. The use of 0.1% sodium lauryl sulfate during the suspension of the PADRE in the apoprotein B, improved the yield to 53%. The stability of PADRE in Caco-2 cells was enhanced when incorporated into lipoprotein systems. The percent PADRE in Caco-2 cells remaining intact upon incubation for 4 hours increased from 3.5±0.4% to 23.1±2.4% when used in the lipoprotein system (Figure 3). The proliferation of CD4 cells when incubated with native peptide and lipoprotein delivery systems (without SLS and with SLS) was higher when compared with incubates without PADRE. As shown in Table 2, CD4 proliferation was increased by 4.3, 3.2, and 2.8 times at 20 µg/mL and 2.5, 2.0, and 2.3 times, at 10 µg/mL equivalents of PADRE for treatment with native peptide and lipoprotein delivery systems (without SLS and with SLS). The CD4 proliferation was not statistically significant amongst the PADRE treated groups (P>0.05). This data suggested that the immunogenicity of PADRE was preserved during the preparation of lipoprotein delivery systems and these systems were suitable in offering protection against loss of immunogenicity (Table 2). In previous studies, the lipoprotein based systems containing...
anticancer drug Daunorubicin (14), showed higher cytotoxicity in the LDL receptor positive Chinese hamster cells as compared to mutant cells (LDL negative). The lipoprotein-based approach has recently been utilized for siRNA delivery. The delivery of chol-siRNA via lipoproteins, improved the knock-down efficiency from 0% to 38% as compared to the delivery of the native siRNA at a 100 nM treatment in HepG2 cells (15). However due to receptor mediated endocytotic uptake, siRNA remained entrapped in the endo-lysosomal compartments. Disruption of the endosomes by “photochemical internalization” further increased the silencing efficiency to 78% (15). The results with the PADRE containing lipoprotein systems followed this trend, with significant CD4 proliferation observed for treatment with PADRE lipoprotein based systems. Thus, lipoprotein delivery systems have potential in the delivery of immunogenic peptides PADRE to tumor while maintaining their immunogenic potential.

**Conclusion**

Two approaches were studied for improving the stability and immunogenic potential of PADRE. The use of peptidase inhibitors provided protection against degradation of the PADRE in

<table>
<thead>
<tr>
<th>Peptidase Inhibitor</th>
<th>Enzyme/ Enzyme family/ inhibited</th>
<th>Recommended Concentration (micromolar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antitrypsin</td>
<td>Serine proteases</td>
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</tr>
<tr>
<td>Betastatin</td>
<td>Aminopeptidase N</td>
<td>100</td>
</tr>
<tr>
<td>Diprotinin A</td>
<td>Dipeptidyl peptidase IV</td>
<td>100</td>
</tr>
<tr>
<td>Phosphoramidon</td>
<td>Endopeptidase 24.11</td>
<td>1</td>
</tr>
<tr>
<td>Phenanthroline</td>
<td>Metallopeptidases</td>
<td>100</td>
</tr>
</tbody>
</table>

**Table 2.** Table 1. Different peptidase inhibitors selected for pretreatment with PADRE and test concentrations used.

<table>
<thead>
<tr>
<th>Control</th>
<th>Native PADRE</th>
<th>Treatment with PADRE-Lipoprotein without surfactant</th>
<th>Treatment with PADRE-Lipoprotein with surfactant</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>20 mcg/mL</td>
<td>10 mcg/mL</td>
<td>20 mcg/mL</td>
</tr>
<tr>
<td>X</td>
<td>3.5X</td>
<td>1.7X</td>
<td>3.4X</td>
</tr>
<tr>
<td></td>
<td>9.1X</td>
<td>3.2X</td>
<td>3.0X</td>
</tr>
<tr>
<td></td>
<td>2.7X</td>
<td>2.9X</td>
<td>3.5X</td>
</tr>
<tr>
<td></td>
<td>1.9X</td>
<td>2.3X</td>
<td>2.7X</td>
</tr>
<tr>
<td>AVEGARE</td>
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<td>2.5X</td>
<td>3.2X</td>
</tr>
<tr>
<td>SD</td>
<td>3.3X</td>
<td>0.7X</td>
<td>0.4X</td>
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the *in vitro* systems. Antitrypsin provided significant improvement in stability when compared to other inhibitors of ecto-peptidases. The lipoprotein based drug delivery approach offered better tumor barrier penetration due to the proposed endocytotic uptake and also helped in maintaining the immunogenic potential of the peptide. Thus, the *in vitro* results indicate that these delivery approaches have potential in improving *in vivo* efficacy of PADRE.

**References**