 doi:10.20944/preprints202009.0734.v1

Research article

AE36 HER2/neu-derived peptide linked to positively charged liposomes with CpG-ODN as an effective therapeutic and prophylactic vaccine for breast cancer

Nastaran Barati [a], Amin Reza Nikpoor [b], Fatemeh Mosaffa[c], Atefeh Razazan [d], Ali Badiee [c,e], Mousa Motavallihaghi[f], Javad Behravan*[c,h] , Mahmoud Reza Jaafari* [c,e]

[a] Vice chancellor for Research and technology, Hamadan University of Medical Sciences, Hamadan, Iran

[b] Molecular Medicine Research Center, Hormozgan Health Institute, Hormozgan University of Medical Sciences, Bandar Abbas, Iran.

[c] School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran

[d] Department of Molecular Medicine, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran Iran

[e] Nanotechnology Research Center, Pharmaceutical Technology Institute, Mashhad University of Medical Sciences, Mashhad, Iran

[f] School of medicine, Hamadan University of Medical Sciences, Hamadan, Iran

[g] Mediphage Bioceuticals, Inc., MaRS Centre,Toronto, ON, Canada

[h] Biotechnology Research Center, Pharmaceutical Technology Institute, Mashhad University of Medical Sciences, Mashhad, Iran

*Javad Behravan, Pharm.D., Ph.D.

School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran

Mediphage Bioceuticals, Inc., MaRS Centre,Toronto, ON, Canada

Mashhad, Iran, P.O. Box: 91775-1365, Tel: 0513 7112472; Fax: 0513 8823251

Email:behravanj@mums.ac.ir

*Mahmoud Reza Jaafari, Pharm.D., Ph.D.

School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran

Nanotechnology Research Center, Pharmaceutical Technology Institute, Mashhad University of Medical Sciences, Mashhad, Iran

Mashhad, Iran, P.O. Box: 91775-1365, Tel: 0513 7112472; Fax: 0513 8823251

E-mail: jafarimr@mums.ac.ir

* **Corresponding authors**

 doi:10.20944/preprints202009.0734.v1

Abstract

In the present day and age, cancer is still a life-limiting factor whose one of the therapeutic strategies is immunotherapy with vaccines. This research aims to prepare and characterize nanoliposomal vaccine formulation attached to HER2/neu-derived peptide (AE36) with or without CpG-ODN, and to evaluate its immunological responses to the therapy using BALB/c mice with HER2 overexpressing breast cancer. Methods: AE36 was conjugated to the liposomes containing DOTAP, DOPE and Cholesterol. Such formulations are able to produce CD8+ and CD4+ responses and induce synthesis of cytokines detectable via Enzyme-linked immunosorbent assay kits, cytotoxicity testing and intracellular cytokine staining combined with flow cytometry. Therapeutic and prophylactic effectiveness were evaluated through the formulation in samples. The highest effectiveness was found in DDC-peptide + CpG-ODN in both prophylactic and therapeutic studies, which decreased the size of tumors significantly and increased time of survival. These nanoliposomes linked to AE36 could be regarded as an appropriate candidate for the treatment and also for prophylaxis of $HER2^{+}$ breast cancer; however further studies are needed.

Keywords: AE36 HER2/neu-derived peptide, Nanoliposomes, CpG-ODN, Vaccine, Cancer

1. Introduction

During 20 past years, cancer immunotherapy has drawn a great deal of scientific attention to itself due to ineffectiveness of other ways of treatment, such as chemotherapy, radiotherapy and surgery [[1]]. In cancer immunotherapy, cellular immunity could be induced when cytotoxic T lymphocytes (CTLs), particularly CD8+ T cells (Cytotoxic T cells with CD8 surface protein), are activated [[2, 3]]. The immune cells can recognize the cancer cells following active production of immune responses. Epidermal growth factor receptor proteins in human (HER2 or HER2/neu), are overexpressed in metastatic and non-metastatic cancer cells. This overexpression causes unrestricted cell division, which ultimately results in tumors. The immune system is stimulated via sequences of this protein and the sequences (AE36) are possibly applied in prophylaxis and treatment of HER2+ cancers. AE36 is taken into account as one of those sequences [[4-6]]. Immunity against HER2/neu sequences could reduce tumor progression and improve prognosis [[7, 8]]. Liposomes are carriers for proteins or peptide antigens. Materials may attach to their outer surface, be encapsulated in the internal aqueous spaces, or be reconstituted with the liposomes lipid bilayers. These carriers prompt immune reactions by interactions with antigen presenting cells. Moreover, it has been demonstrated that antigens could be presented by liposomes to helper T lymphocytes. [[9, 10]]. Contents of liposomes can be reserved from environmental hazards before releasing at the target area [[11]]. In the present research, AE36 (Ac-GVGSPYVSRLLGICL-NH2), as a peptide derived from HER2 intracellular domain containing 15 amino acids attached to the liposomes, was employed as a vaccine [[12-14]]. AE36 is attached to the MHC II molecules producing T cell responses to peptides in mice and also synthesizes T lymphocytes in patients in vitro [[15, 16]]. Additionally, plasmacytoid dendritic cells and B cells are activated by CpG motifs in order to express Toll-like receptor 9 for generating an innate immune response. Generation of this response is attainable with production of T helper 1 (Th1) together with pro inflammatory cytokines synthesis, which is currently applied as an effective adjuvant in cancer immunotherapy studies [[17-19]]. In the previous study, the peptide was encapsulated in liposome and here, the peptide was linked to liposome in order to get better effects [20]. In the present study, the effectiveness of AE36 peptide conjugated to liposomal formulation composed of DOTAP: DOPE: CHOLESTROL for the commencement of CTL response was evaluated in BALB/c mice model with TUBO breast cancer tumor, which shows the overexpression of HER2/neu oncogene.

2. Results

2.1. Synthesis of AE36 peptide-PEG2000-DSPE

Thin layer chromatography was done to determine binding of Maleimide-PEG2000-DSPE to AE36 peptide. Disappearing the PEG2000-DSPE spot in mixture, revealed the end of the reaction (Fig.1.B).

 doi:10.20944/preprints202009.0734.v1

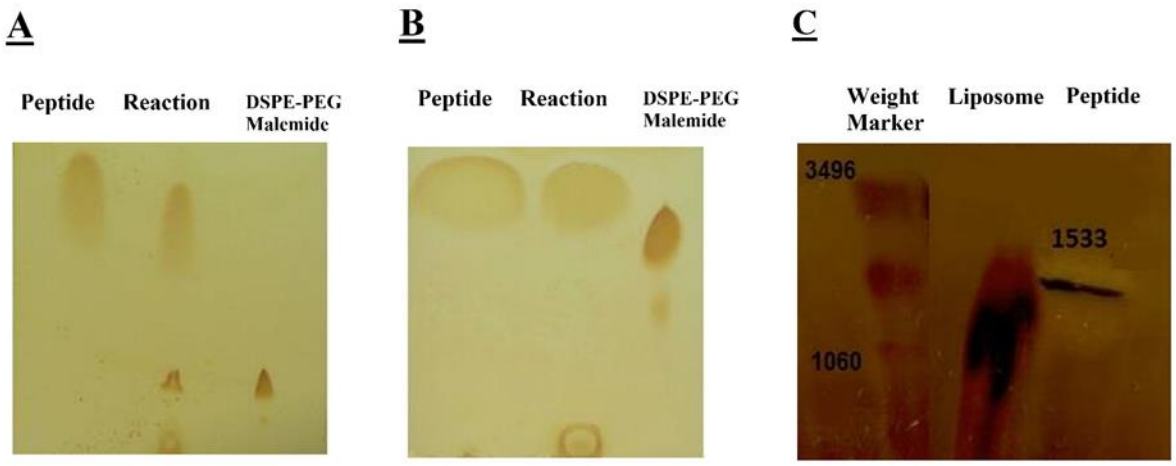

Fig 1.A represents B TLC of the conjugation of AE36 peptide to. Picture a shows the phase before peptide linked to DSPE-PEG Maleimide, and B is after the ultimate step of the reaction. There is no spot of PEG2000-DSPE observed in the mixture in picture b which shows the final step of the reaction. Fig 1.C represents Gel electrophoresis: Column 1) pure peptide, AE36, with molecular weight of 1533.84 g/mol. Column 2) Molecular weight marker ranged between 1060 and 26600 Dalton. Marker, Column 3) liposomal sample. The peptide amount was 5µg in liposomal and pure peptide samples.

2.2. Liposomes characteristics

Table 1 demonstrates the physical characteristics of liposomal formulations. Based on table 1, particle volume was in the range of 185.6± 3 nm and 200± 0.6nm prior to adding CpG-ODN and there were no significant differences in the particle volume in the prepared formulations (P.value>0.05). Liposomes volume enhanced by adding CpG; however, zeta potential reduced. Moreover, particles .revealed the polydispersity index (PdI) of approximately 0.2.

Table 1: Particle size, zeta potential and poly disparity of formulations. (n =3; mean±SD).

Formulation	**Phospholipids molar ratio**	**Size, nm (mean ±SD)**	**Zeta potential, mV (mean±SD)**	**Poly disparity index (mean±SD)**
DOTAP/DOPE/Chol/AE36	1:1:1	200±0.6	43±0.5	0.24±0.01
DOTAP/DOPE/Chol/AE36 + CpG	1:1:1	303±10	30±0.7	0.22±0.02

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 30 September 2020 doi:10.20944/preprints202009.0734.v1

DOTAP/DOPE/Chol	1:1:1	186±3	45±0.5	0.22±0.006
DOTAP/DOPE/Chol + CpG	1:1:1	290±9	33 ± 0.6	0.22 0.005

2.3. Gel electrophoresis

For determination of the band of peptide, polyacrylamide gel electrophoresis was performed. We assessed samples for, 1) pure peptide (molecular mass: 1533.84 g/mol), 2) marker (molecular mass: 1060-26600 D), and 3) liposomal formulation. 5 µg was calculated as the total extent of peptide in liposomes as well as pure peptide specimens. Phospholipids in liposomal formulation caused the smear in liposomal sample (fig.1.C).

2.4. Serum IL-4 and IFN-γ cytokines assay by ELISA

Serum of mice (three mice of each group) were isolated two weeks after the last vaccination to evaluate the immune responses of T cells. According to the standard curves, the amounts of IL-4 and IFN-γ were calculated for each group. DDC-P+CpG group was the most stimulator of IL-4 production (fig. a). (P.value<0.05). The differences were not significant between groups for INF-γ production (fig. 2). (P.value>0.0

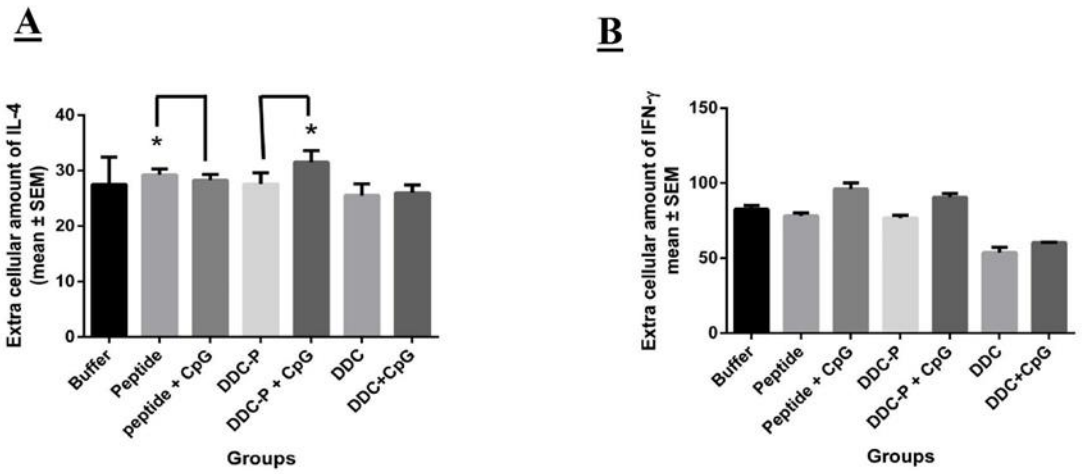

Fig. 2 shows Cytokine production in serum of mice immunized with three booster doses of 10 µg AE36 in different liposomal vaccines. Fourteen days after the last booster, serums of mice (three per group) were isolated for the evaluation of the amount of IL-4 (a) and IFN-γ (b), by ELISA in which DDC-P+CpG caused the most secretion of IL-4, but the differences were not significant for IFN-γ (P.value > 0.05). n=3 (mean ± SEM).

2.5. Intracellular cytokine assay by flowcytometry on splenocytes

Flow cytometry was utilized for intracellular cytokine assay for IL-4 and IFN-γ on CD4+ and CD8+ T cell splenocytes. As shown in Figure 3, which is rather similar to result of ELISA assay, Peptide + CpG group was produced the highest amount of IFN-γ from CD8+ T-cells, yet it was not significant.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 30 September 2020 doi:10.20944/preprints202009.0734.v1

Peptide+CpG group stimulated a higher amount of IL-4 against control group (DDC+CpG). (P.value < 0.05). Moreover, the Treg percent was not significant between studied groups (fig.3 (A,B,C,D)).

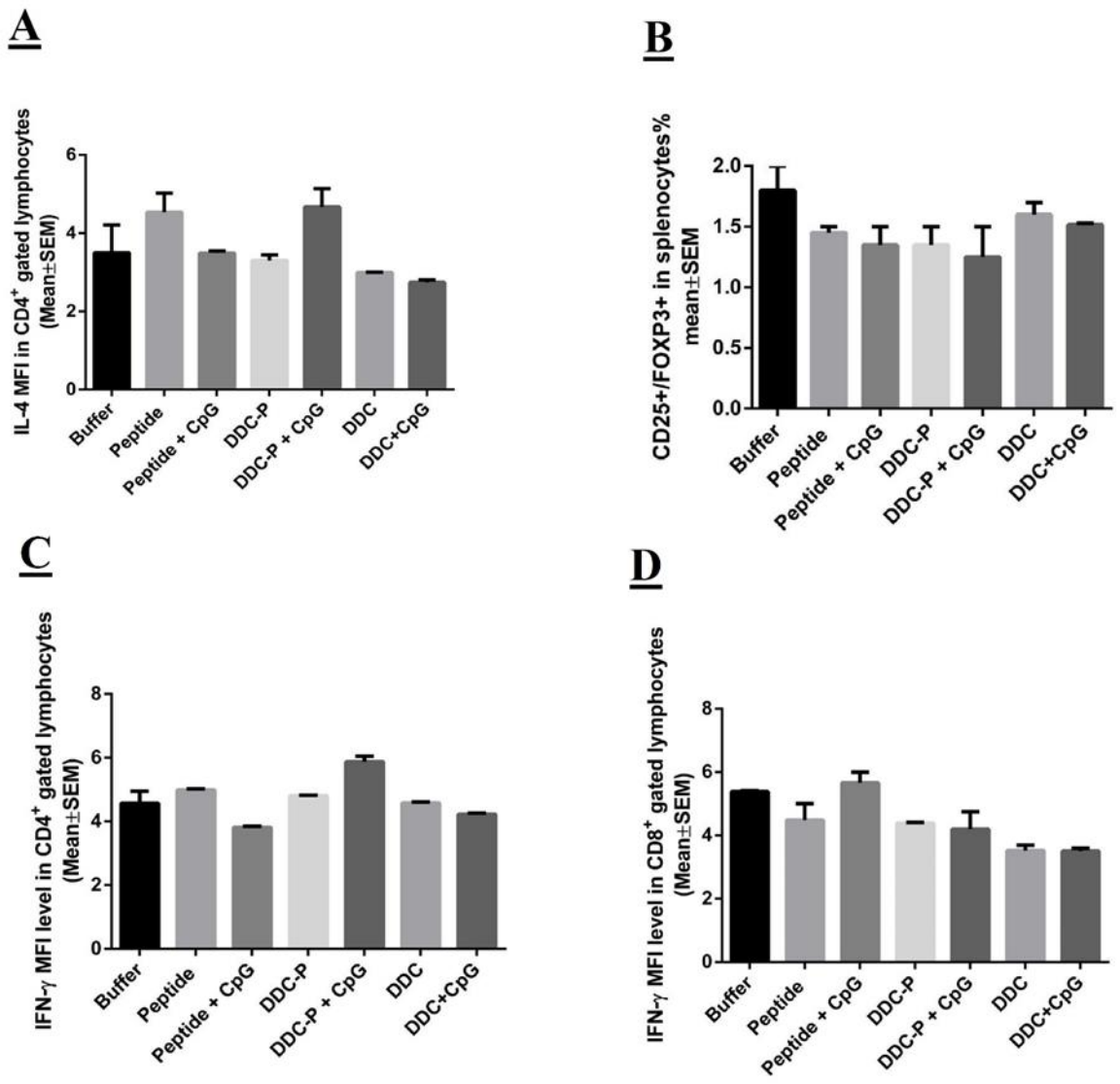

Fig 3 demonstrates Intracellular cytokine production in splenocytes of mice immunized with three booster doses of 10 μg AE36 in different liposomal vaccines. Fourteen days after the last booster, spleens of mice (three per group) were isolated and stained with PE labeled CD8 or CD4 surface markers, and then stained with anti-IFN-γ-FITC or anti-IL-4-PE antibodies, respectively. The frequency of IFN-γ producing cells within the CD8+ or CD4+ population (c & d) and IL-4 producing cells within the CD4+ population (a) were subsequently determined by FACSCalibur Cell analyzer. The data represent mean ± SEM (n = 3). (P.value<0.05)

2.6. Cytotoxicity assay

Cell cytotoxicity assay was performed to assess how vaccination affected generation of CTL response in spleen cells. Splenocytes of the vaccinated animals did not affect CT26 cells (control) indicating their HER2-specific toxicity. In three effectors to target cells ratios, DDC-P+CpG was 3.25-fold

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 30 September 2020 doi:10.20944/preprints202009.0734.v1

compared to the buffer group (P.value < 0.0001) (fig.4).

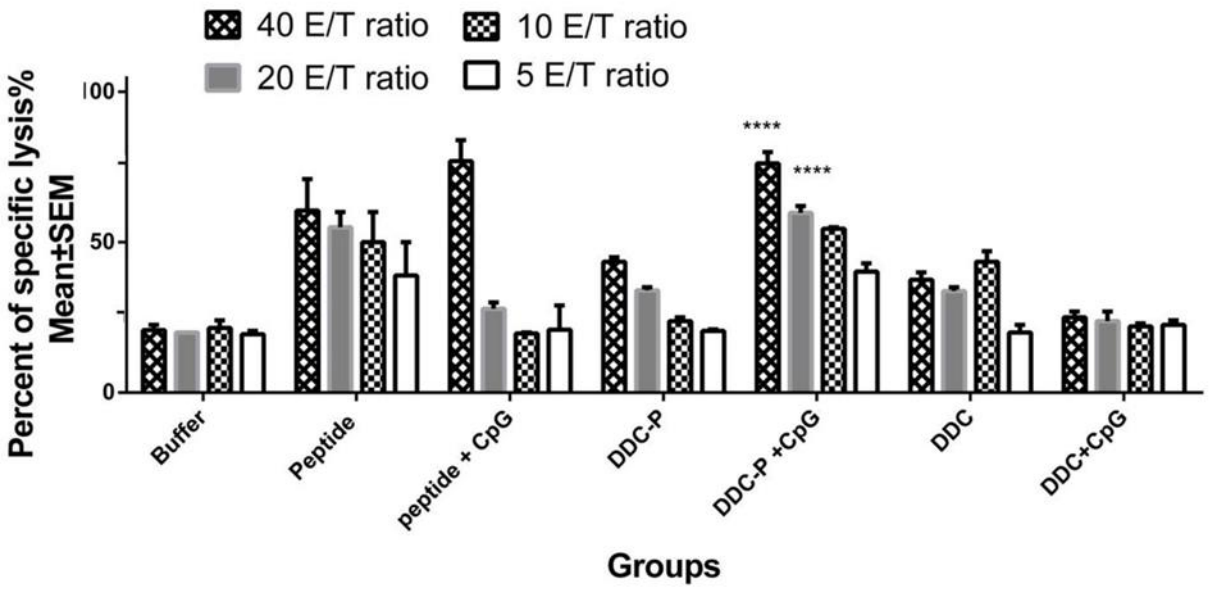

Fig. 4 illustrates Cell cytotoxicity assay which shows how vaccination affected generation of CTL response in spleen cells. Splenocytes of the vaccinated animals did not affect CT26 cells (control) indicating their HER2-specific toxicity. CTL response between splenocytes and TUBO cells was induced by various formulations. Splenocytes from mice (3 in each group) were incubated with Calcein AM-loaded rHER2/neu-expressing TUBO tumor cells and rHER2/neu-expressing negative CT26 cells (as control). The results represent mean ± SEM (n = 3). DDC-P + CpG group shows a higher percent of lysis versus buffer among all of the effectors to target cells ratios (P.value < 0.0001) E/T ratio: Effector to target cells ratio.

2.7. Prophylactic model of mice

The model was initiated by vaccination of the studied formulations for three times and a two-week interval. Two weeks after the last vaccination, the groups were exposed to TUBO cells. The animals were monitored in order to make sure whether there are mice with a touchable tumor at the injection (right flank) area. Weights and sizes were calculated until 3 mentioned situations occurred. As it is shown in fig.5 and table 2, DDC-P + CpG group decreased the size of tumor significantly. (P.value < 0.01). One of the mice in this group was cured and completely became tumor free. However, without any remarkable statistical significance, the survival time increased in the mentioned group compared to other groups.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 30 September 2020 doi:10.20944/preprints202009.0734.v1

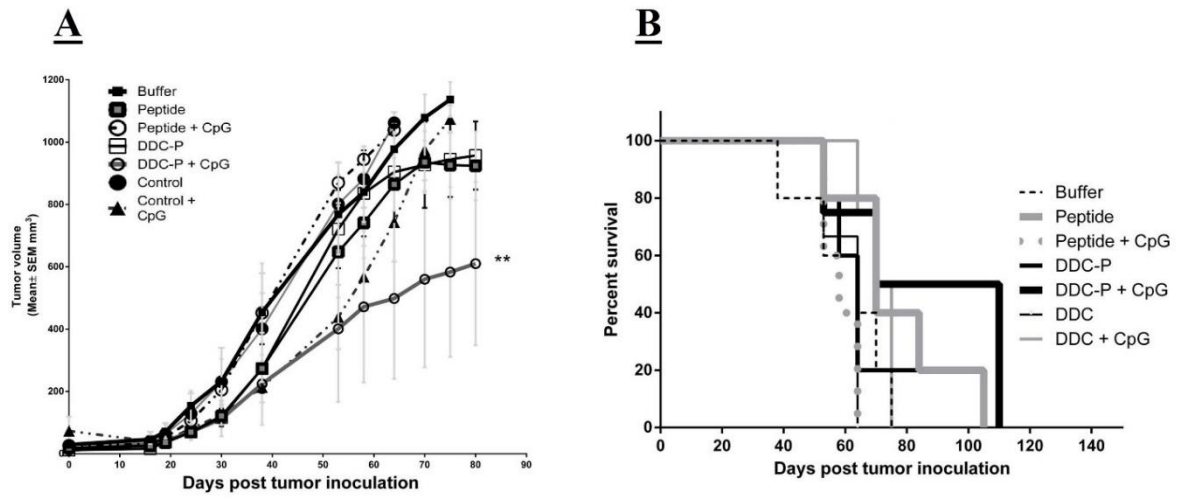

Fig. 5.A shows Protective effects of vaccination with different formulations in BALB/c mice against a TUBO tumor model. 14 days after the last vaccination, mice were challenged with injection of 5 × 105 TUBO cells. Sizes of tumors were calculated. The values are expressed as the means of tumor size ± SEM. (n = 5). DDC-P + CpG group decreased the rate of tumor growth significantly. P.value < 0.01.

Fig. 5.B represents Survival analysis of vaccinated groups. Effects of immunization on survival time were monitored for a period of 110 days among BALB/c mice. The majority of mice in DDC-P + CpG group survived compared to other groups, yet the difference was not significant. (n =5).

Table 2: Protective efficacy data of different liposomal vaccine formulations in TUBO tumor mice model

(n = 5)

Formulation	MST[a](Day)	TTE[b](Day)± SD	TGD[c] (%)
Peptide	70	76±20	31
Peptide + CpG	58	57±5	0
DDC-P	64	66±20	15
DDC-P + CpG	90	82±27	43
DDC	64	59±8	2
DDC+ CpG	72.5	70±6	21
Buffer	64	58±13	0

[a] Median survival time.

[b] Time to reach end point.

[c] Tumor growth delay.

2.8. Therapeutic model of mice

In the therapeutic model, subcutaneous injection of TUBO cells was done. Following finding palpable tumor, the vaccination schedule started and was performed for three times and a two-week interval. Both animals' weight and size of the tumor were evaluated continuously when the mice were lethargic or the tumors size increased up to about 1000 mm3. The tumor size significantly decreased

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 30 September 2020 doi:10.20944/preprints202009.0734.v1

by DDC-P +CpG (P.value < 0.0001). Furthermore, in this group the survival time increased significantly. (P.value<0.05). (fig.6, table 3)

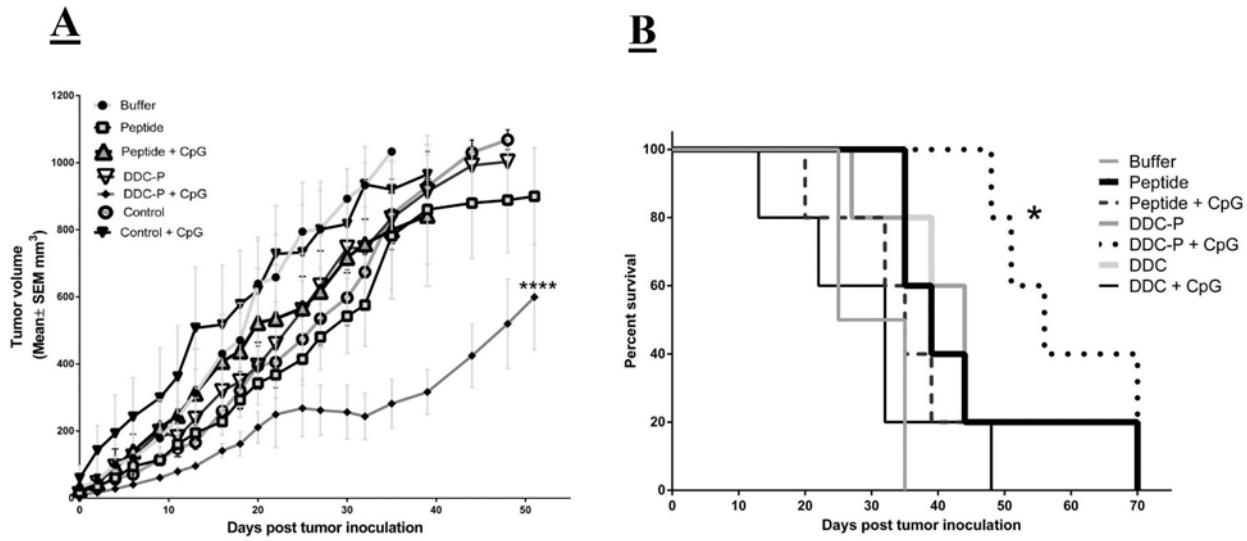

Fig. 6.A sheds light to Therapeutic effects of administered formulations in BALB/c mice against a TUBO tumor model. 5 ×105 TUBO cells per mice were subcutaneously injected. After observation of touchable tumor, the vaccination schedule started and was performed three times with two-week intervals and tumor sizes were calculated. The values are expressed as the means of tumor size ± SEM. (n = 5). DDC-P + CpG group significantly decreased the size of tumor. P.value < 0.0001.

Fig. 6.B represents Survival analysis of groups. Effects of the treatments on survival time were monitored for a period of 70 days among BALB/c mice. (n = 5). DDC-P + CpG group prolonged the survival time of animals significantly versus buffer. (P.value < 0.05).

Table 3: Therapeutic efficacy data of different liposomal vaccine formulations in TUBO tumor mice model

(n = 5).

Formulation	MST[a](Day)	**TTE[b](Day)± SD**	**TGD[c] (%)**
Peptide	39	49±26	67
Peptide + CpG	35	45±32	53
DDC-P	44	39±8	35.5
DDC-P + CpG	56	68±18	132
DDC	39	40±5	36
DDC+ CpG	32	40±33	34

 doi:10.20944/preprints202009.0734.v1

Buffer	30	29±6	0

[a] Median survival time.

[b] Time to reach end point.

[c] Tumor growth delay

3. Discussion

In the previous study of ours, the peptide was encapsulated in liposome, whereas here, the peptide was linked to liposome to get better effects [[20]]. AE36, as a sequence of HER2 protein, was employed for development of a liposomal base vaccine for prophylaxis as well as HER2+ breast cancer therapy. In fact, the hybrid peptide AE37 includes Ii-Key (LRMK) plus AE36 (GVGSPYVSRLLGICL). Ii-Key, as a section of (MHC) class II invariant chain (Ii), facilitates the epitopes to be charged to MHC class II molecules [[24]]. The vaccines have demonstrated immunologic responses to AE37 in vitro and in vivo, and also AE36 with or without GM-CSF (Granulocyte-macrophage colony-stimulating factor) in a dose-dependent manner [[19]]. Not only does GM-CSF impose costs, but it is also associated with many adverse effects when applied in clinical settings; for instance, fever, myalgia, malaise, rash and reactions at the site of injection [[25]].

In the present study, AE36 immunogenicity without Ii-Key peptide increased when linked to a positively charged liposome (DOTAP/DOPE/Cholesterol). As reported previously, positively charged liposomes can greatly induce cytotoxic T lymphocyte compared to the neutral or negatively charged liposomes [[26]]. CD4+ T helper activation has implied remarkable effects on cancer immunotherapy done by AE37. CD4+and CD8+ immune responses were observed for AE37 and AE36 peptide in most patients [[27]].

DOTAP phospholipid in the formulations produced positively charged liposomes leading to promotion of antigen transportation to macrophages and dendritic cells, which is due to quaternary ammonium components exhibits native adjuvanticity. Moreover, dendritic cells and chemokine induction could be stimulated with DOTAP, which stimulates migration of dendritic cells to lymph node and results in appropriate responses to antigen-specific CD8+T lymphocyte [[28]].

Cholesterol in liposomal formulations enhance the in vivo liposomes stability. The more inflexible the structure is, the more CD8+T-cell responses would be stimulated. On top of that, secretion of antigens from cytoplasm increases with cholesterol. It also prevents vesicle lysosomal degradation. In the liposome formulation, DOPE as a pH sensitive lipid is effective for presenting antigens to MHC I pathway as well as inducing CTL response [[29-32]].

The immune system of vertebrates seems to have protective immune responses to CpG motifs that are shown to be Th1-based. Toll-like receptor 9 is expressed via dendritic and B cells for generation of an innate immune response to CpG motifs detectable through Th1 and pro inflammatory cytokines generation. CpG-ODN is a vaccine adjuvant in tumor immunization. CpG DNA is of lower toxicity than other adjuvants and stimulates stronger immune responses [[33-35]].

Liposomes defend peptides from being degraded to the target area, like lymphocytes and cells which have antigen presentation. AE36 is able to attach to MHC II molecules of APC resulting in generation of T cell responses against peptides in cancer mouse models [[36, 37]].

Results of the present paper indicated that the stable particles and the liposomes sizes were between 186 - 200 nm before adding CpG and 290 - 303nm after adding it, CpG was found to be suitable to be appropriately delivered to the lymphatic system. Liposomes over 100 nm can generate a Th1 type of immune response versus those below 100 nm [[38, 39]]. Furthermore, particles net charge reduced

 doi:10.20944/preprints202009.0734.v1

after addition of CpG to liposomes in view of the negatively charged CpG, which can electrostatistically bind to the liposome surface followed by neutralization of the DOTAP charge.

4. Materials and Methods

4.1. Materials

Peptide AE36 with the purity of more than 95% was produced by China Peptides Co. (Shanghai, China). N- [1-(2, 3-Dioleoyloxy) propyl]-N,N,N-trimethylammonium methyl-sulfate (DOTAP) and dioleoylphosphatidylethanolamine (DOPE) were obtained from Us. Avanti Polar Lipids, Inc. The needed cholesterol was also provide from Sigma-Aldrich, Germany. Cytofix/CytopermTM Plus, PMA/Ionomycin mixture, anti-CD8a-PE-cy5, anti CD4-PE-cy5, anti-IFN-γ- FITC, and anti-IL-4-PE were obtained from BD Biosciences (San Diego, USA). The U-Cytech (the Netherlands) provided the mouse anti-IL-4 and anti IFN-γ ELISpot kits. The rest of reagents or solvents were applied as chemical grades.

4.2. Animals

Four to six-week-old female BALB/c mice were supplied by Pasteur Institute, Iran. The Ethics Committee and Research Advisory Committee of Mashhad University of Medical Sciences approved our research protocol regarding emplying animals (Approval code: 922610). TUBO cell line was delivered by Dr. Pier-Luigi Lollini (Department of Clinical and Biological Sciences, University of Turin, Ozrbassano, Italy), and was cultured in DMEM (Dulbecco's Modified Eagle's Medium) with fetal bovine serum. CT26, which is a murine colon carcinoma cell line, was bought from Pasteur Institute (Tehran, Iran) and cultured in RPMI-1640 medium supplemented with 10% FBS.

4.3. Conjugation of AE36 peptide to Maleimide -PEG2000-DSPE

AE36 was conjugated to Maleimide-PEG2000-DSPE via covalent binding between pyrrole group of maleimide and thiol group of cysteine in peptide with a molar ratio of 1.2:1 (AE36 peptide: Maleimide-PEG2000-DSPE) in DMSO: Chloroform (1:1) solution. The covalent binding reaction was completed in 72 hours at room temperature. Thin layer chromatography (TLC) was utilized to confirm the formation of the covalent binding. The solution was dried to a thin film by rotary evaporation (Heidolph, Germany) under vacuum. Subsequently, the thin film of lipids was freeze-dried (VD-800F, Taitech, Japan) overnight and the micelle was prepared by adding water.

4.4. Liposome preparation

Solution of lipids were mixed, dried to a thin film by rotary evaporation, and freeze-dried as mentioned before [[20]]. Afterwards, hydration in HEPES/ dextrose buffer (10 mM HEPES with 5% dextrose, pH=7) was done. Lipid dispersed in buffer properly by vortexing and sonicating. Large vesicles were extruded with a mini extruder (Avestin, Canada) and polycarbonate membranes (200 and 100 nm) in order to form nanoparticles. Ultimately, 10μg peptide in micelle with 100μl of liposome were co-incubated at 50 oC for 4 hours under stirring. Liposomes were then put at 4° C under argon [[21]].

4.5. Liposome characterization

Physical properties such as particle size, zeta potential and polydispersity index of liposomes were determined by dynamic light scattering (Malvern Instruments, Malvern, UK). Phosphate assay method was used to determine the amount of phospholipid in formulations [[22]].

4.6. Immunization

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 30 September 2020 doi:10.20944/preprints202009.0734.v1

BALB/c mice (Five mice per group) were immunized with different formulations (with or without CpG), three times with two-week intervals. Each dose of injection was 10 μg peptide for per mice. Some of the formulations were mixed with CpG ODN, 30 minutes before injection. The amount of CpG was 15μg for per animal. Two weeks after the last vaccination, three mice per group were sacrificed and their spleens were aseptically collected for evaluation of immune responses. Liposomes hydration buffer and liposome without any peptide (Empty liposomes) were used as control groups.

4.7. In vitro analysis of T-Cell immune responses

ELISA test was done by anti-mouse IFN-γ and anti-mouse IL-4 ELISA kits, according to the manufacturer's instruction. Preparing the ELISA kits, ELISA 96-well plates were coated with anti-mouse IL-4 and anti-mouse IFN-γ antibodies, one day ahead of the test. Afterwards, the serum (of 3 mice of each group) was collected to estimate the amounts of IFN-γ and IL-4.

4.8. Intracellular cytokine assay via flow cytometry analysis

Concerning the intracellular cytokine assay using flow cytometry, splenocytes stimulated with AE36 peptide or PMA/Ionomycin in medium containing 1 μl/ml GolgiPlugTM were incubated for 4 hours at 37 ºC. 105 splenocytes were transferred into tubes of flow cytometry. Splenocytes was washed with staining buffer (2% fetal bovine serum (FBS) in PBS). Staining of splenocytes was done by 2μl anti-CD8a-PE-cy5 antibody and 2 μl anti CD4-PE-cy5 antibody in separate tubes for 30min at 4 °C. After the washing, splenocytes were fixed with Cytofix/CytopermTM solution following two times washing with Perm/WashTM buffer. Subsequently, the splenocytes were stained with antibody (anti-IFN-γ- FITC) at 4 °C for 30 min. In addition, CD4 cells were stained with anti-IL-4-PE antibody. Washing with Perm/WashTM buffer was done and splenocytes were suspended in 300 μl staining buffer for flow cytometry analysis (BD FACSCalibur™, BD Biosciences, San Jose, USA).

4.9. In vitro CTL assay

TUBO and CT26 cells, as the target cells, were combined with Calcein AM (Calcein acetoxymethyl ester, USA) after being incubated (37°C/1 h) in a dark room. In order to remove the excess Calcein AM, DMEM–20% FBS was utilized for washing the cells. We cultured and incubated (37°C/4 h) TUBO cells (1.2 × 105 cell/well) using various splenocytes. A 96-well dark plate was applied to collect the supernatant. BioTek FLx800 Microplate Fluorescence Reader was employed to determine the fluorescence of supernatants utilizing excitation/emission wavelengths of 485/538 nm. Using below equation, the specific lysis was computed:

[(ITest− Iblank)/ (I100%− Iblank)] × 100

Where ITest, and I100% indicate Calcein AM intensity in splenocytes, and thoroughly lysed with 2% TX100, respectively. Iblank also represents the untreated cells intensity.

4.10. Prophylactic Model of TUBO Challenge

Five mice from each group were vaccinated three times with a two-week interval with different formulations. 14 days after the third vaccination, each mouse was subcutaneously injected with 5 × 105 TUBO cells in 50 μl PBS buffer. After five days, using a caliper, 3 orthogonal diameters a, b, c was calculated. Tumor volume was calculated with the following formula: [length × width × height] × 0.52) [[23]]. Calculating the tumor size and studying survivals of mice continued until one of these conditions for euthanizing was observed: the tumor volume found to be over 1000 mm3; the body weight showed less than 15% of primary mass or lethargic or sick mice which were impossible to be nourished.

4.11. Therapeutic Model of TUBO Challenge

Primarily, five mice per group were challenged with subcutaneous injection of 5 × 105 TUBO cells in 50 µl PBS buffer in the right flank. When the dimension of the tumor reached the approximate size of 3 mm, the immunization schedule was subcutaneously done three times with a two-week interval. The tumor volume was measured employing this formula: ([length × width × height] × 0.52). The mice without any tumor were considered as tumor-free at the end of the experiment. The mice were euthanized provided that the tumor volume was greater than 1000 mm3, or the body weight reached below 15% of initial mass or the mice became lethargic, sick or unable to feed.

4.12. Statistical analysis

Two-way analysis ANOVA followed by Tukey's post-test were done in order to evaluate the significance of the differences among various formulations. The survival data was analyzed by log-rank test between the groups. $P < 0.05$ was considered to be significant. Graph Pad Prism 6 Software was applied for data analysis. The significant differences are shown as P. values of less than 0.05, 0.01, 0.001 and 0.0001 flagged with one to four stars, respectively.

5. Conclusions

In conclusion, the present findings implied that the anti-tumor immune activity of AE36 significantly increased by liposomal formulations rather than the control groups. Moreover, the mentioned formulations were found beneficial in the treatment as well as prophylaxis of breast cancer. Accordingly, they could be regarded as the appropriate and promising candidates for vaccine delivery which could delay the tumor growth rate of HER2+ breast cancer. Further studies are needed to confirm the clinical aspects of AE36 peptide linked liposomal formulations.

Author Contributions: "Conceptualization, M.J.; methodology, M.J.; software, A.N.; validation, N.B.,formal analysis, N.B. and A.R; investigation, N.B.; resources, N.B.; data curation, N.B.; writing—original draft preparation, N.B.; writing—review and editing, M.M. and A.N; visualization, M.M.; supervision, M.J. and J.B and A.B and F.M; project administration, J.B. and M.J; funding acquisition, J.B. All authors have read and agreed to the published version of the manuscript.

Funding: We are grateful for the financial support from Mashhad University of Medical Sciences, Biotechnology Research Center, Nanotechnology Research Center and Student Research committee (Grant number: 922610)

Acknowledgments: We would also like to thank Azam Abbasi and Zahra Saberi for their excellent technical assistance. This study was a part of N.Barati's PhD dissertation.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Aguiar, O.; Gollücke, A. P. B.; de Moraes, B. B.; Pasquini, G.; Catharino, R. R.; Riccio, M. F.; Ihara, S. S. M.; Ribeiro, D. A., Grape juice concentrate prevents oxidative DNA damage in peripheral blood cells of rats subjected to a high-cholesterol diet. *British journal of nutrition* **2011,** 105, (05), 694-702.
2. Hanson, H. L.; Donermeyer, D. L.; Ikeda, H.; White, J. M.; Shankaran, V.; Old, L. J.; Shiku, H.; Schreiber, R. D.; Allen, P. M., Eradication of established tumors by CD8+ T cell adoptive immunotherapy. *Immunity* **2000,** 13, (2), 265-276.

3. Matsui, K.; O'Mara, L. A.; Allen, P. M., Successful elimination of large established tumors and avoidance of antigen-loss variants by aggressive adoptive T cell immunotherapy. *International immunology* **2003,** 15, (7), 797-805.
4. Polyak, K., Breast cancer: origins and evolution. *The Journal of clinical investigation* **2007,** 117, (11), 3155-3163.
5. Consortium, U., Reorganizing the protein space at the Universal Protein Resource (UniProt). *Nucleic acids research* **2011**, gkr981.
6. Zamani, P.; Teymouri, M.; Nikpoor, A. R.; Navashenaq, J. G.; Gholizadeh, Z.; Darban, S. A.; Jaafari, M. R., Nanoliposomal vaccine containing long multi-epitope peptide E75-AE36 pulsed PADRE-induced effective immune response in mice TUBO model of breast cancer. *European Journal of Cancer* **2020,** 129, 80-96.
7. Disis, M. L.; Calenoff, E.; McLaughlin, G.; Murphy, A. E.; Chen, W.; Groner, B.; Jeschke, M.; Lydon, N.; McGlynn, E.; Livingston, R. B., Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. *Cancer Research* **1994,** 54, (1), 16-20.
8. Perez, S. A.; Peoples, G. E.; Papamichail, M.; Baxevanis, C. N., Invariant chain-peptide fusion vaccine using HER-2/neu. In *Cancer Vaccines*, Springer: 2014; pp 321-336.
9. Alving, C. R., Liposomes as carriers of antigens and adjuvants. *Journal of immunological methods* **1991,** 140, (1), 1-13.
10. Arab, A.; Behravan, J.; Razazan, A.; Gholizadeh, Z.; Nikpoor, A. R.; Barati, N.; Mosaffa, F.; Badiee, A.; Jaafari, M. R., A nano-liposome vaccine carrying E75, a HER-2/neu-derived peptide, exhibits significant antitumour activity in mice. *Journal of drug targeting* **2018,** 26, (4), 365-372.
11. Gabizon, A.; Goren, D.; Cohen, R.; Barenholz, Y., Development of liposomal anthracyclines: from basics to clinical applications. *Journal of controlled release* **1998,** 53, (1-3), 275-279.
12. Dillman, R. O.; Cornforth, A. N.; Nistor, G., Cancer stem cell antigen-based vaccines: the preferred strategy for active specific immunotherapy of metastatic melanoma? *Expert opinion on biological therapy* **2013,** 13, (5), 643-656.
13. Knutson, K. L.; Wagner, W.; Disis, M. L., Adoptive T cell therapy of solid cancers. *Cancer Immunology, Immunotherapy* **2006,** 55, (1), 96-103.
14. Hale, D.; Perez, S.; Sears, A.; Clifton, G.; Vreeland, T.; Holmes, J.; Ardavanis, A.; Pistamaltzian, N.; Rellias, G.; Ponniah, S., P1-13-01: An Update of a Phase II Trial of the HER2 Peptide AE37 Vaccine in Breast Cancer Patients To Prevent Recurrence. AACR: 2011.
15. Perez, S. A.; Anastasopoulou, E. A.; Tzonis, P.; Gouttefangeas, C.; Kalbacher, H.; Papamichail, M.; Baxevanis, C. N., AE37 peptide vaccination in prostate cancer: a 4-year immunological assessment updates on a phase I trial. *Cancer Immunology, Immunotherapy* **2013,** 62, (10), 1599-1608.
16. Shariat, S.; Badiee, A.; Jaafari, M. R.; Mortazavi, S. A., Optimization of a method to prepare liposomes containing HER2/Neu-derived peptide as a vaccine delivery system for breast cancer. *Iranian journal of pharmaceutical research: IJPR* **2014,** 13, (Suppl), 15.
17. Bode, C.; Zhao, G.; Steinhagen, F.; Kinjo, T.; Klinman, D. M., CpG DNA as a vaccine adjuvant. *Expert review of vaccines* **2011,** 10, (4), 499-511.
18. Nikoofal-Sahlabadi, S.; Riahi, M. M.; Sadri, K.; Badiee, A.; Nikpoor, A. R.; Jaafari, M. R., Liposomal CpG-ODN: An in vitro and in vivo study on macrophage subtypes responses,

biodistribution and subsequent therapeutic efficacy in mice models of cancers. *European Journal of Pharmaceutical Sciences* **2018,** 119, 159-170.

19. Holmes, J. P.; Benavides, L. C.; Gates, J. D.; Carmichael, M. G.; Hueman, M. T.; Mittendorf, E. A.; Murray, J. L.; Amin, A.; Craig, D.; Von Hofe, E., Results of the first phase I clinical trial of the novel II-key hybrid preventive HER-2/neu peptide (AE37) vaccine. *Journal of Clinical Oncology* **2008,** 26, (20), 3426-3433.
20. Barati, N.; Nikpoor, A. R.; Razazan, A.; Mosaffa, F.; Badiee, A.; Arab, A.; Gholizadeh, Z.; Behravan, J.; Jaafari, M. R., Nanoliposomes carrying HER2/neu-derived peptide AE36 with CpG-ODN exhibit therapeutic and prophylactic activities in a mice TUBO model of breast cancer. *Immunology letters* **2017,** 190, 108-117.
21. Xu, M.; Kallinteris, N. L.; von Hofe, E., CD4+ T-cell activation for immunotherapy of malignancies using Ii-Key/MHC class II epitope hybrid vaccines. *Vaccine* **2012,** 30, (18), 2805-2810.
22. Stern, A. C.; Jones, T., The side-effect profile of GM-CSF. *Infection* **1992,** 20, (2), S124-S127.
23. Nakanishi, T.; Kunisawa, J.; Hayashi, A.; Tsutsumi, Y.; Kubo, K.; Nakagawa, S.; Nakanishi, M.; Tanaka, K.; Mayumi, T., Positively charged liposome functions as an efficient immunoadjuvant in inducing cell-mediated immune response to soluble proteins. *Journal of controlled release* **1999,** 61, (1-2), 233-240.
24. Perez, S. A.; Kallinteris, N. L.; Bisias, S.; Tzonis, P. K.; Georgakopoulou, K.; Varla-Leftherioti, M.; Papamichail, M.; Thanos, A.; von Hofe, E.; Baxevanis, C. N., Results from a phase I clinical study of the novel Ii-Key/HER-2/neu (776–790) hybrid peptide vaccine in patients with prostate cancer. *Clinical Cancer Research* **2010,** 16, (13), 3495-3506.
25. Firouzmand, H.; Badiee, A.; Khamesipour, A.; Shargh, V. H.; Alavizadeh, S. H.; Abbasi, A.; Jaafari, M. R., Induction of protection against leishmaniasis in susceptible BALB/c mice using simple DOTAP cationic nanoliposomes containing soluble Leishmania antigen (SLA). *Acta tropica* **2013,** 128, (3), 528-535.
26. Reddy, R.; Zhou, F.; Huang, L.; Carbone, F.; Bevan, M.; Rouse, B. T., pH sensitive liposomes provide an efficient means of sensitizing target cells to class I restricted CTL recognition of a soluble protein. *Journal of immunological methods* **1991,** 141, (2), 157-163.
27. Nair, S.; Zhou, F.; Reddy, R.; Huang, L.; Rouse, B. T., Soluble proteins delivered to dendritic cells via pH-sensitive liposomes induce primary cytotoxic T lymphocyte responses in vitro. *Journal of Experimental Medicine* **1992,** 175, (2), 609-612.
28. Chang, J.-S.; Choi, M.-J.; Cheong, H.-S.; Kim, K., Development of Th1-mediated CD8+ effector T cells by vaccination with epitope peptides encapsulated in pH-sensitive liposomes. *Vaccine* **2001,** 19, (27), 3608-3614.
29. Luo, L.; Li, Y.; Chang, J.-S.; Cho, S.-Y.; Kim, T.-Y.; Choi, M.-J.; Cheong, H.-S.; Kim, H.-J.; Ahn, H.-J.; Min, M.-K., Induction of V3-Specific Cytotoxic T Lymphocyte Responses by HIVgagParticles Carrying Multiple Immunodominant V3 Epitopes of gp120. *Virology* **1998,** 240, (2), 316-325.
30. Krieg, A. M., Immune effects and mechanisms of action of CpG motifs. *Vaccine* **2000,** 19, (6), 618-622.
31. Mansourian, M.; Badiee, A.; Jalali, S. A.; Shariat, S.; Yazdani, M.; Amin, M.; Jaafari, M. R., Effective induction of anti-tumor immunity using p5 HER-2/neu derived peptide

encapsulated in fusogenic DOTAP cationic liposomes co-administrated with CpG-ODN. *Immunology letters* **2014,** 162, (1), 87-93.

32. Weeratna, R. D.; McCluskie, M. J.; Xu, Y.; Davis, H. L., CpG DNA induces stronger immune responses with less toxicity than other adjuvants. *Vaccine* **2000,** 18, (17), 1755-1762.
33. Stark, B.; Andreae, F.; Mosgoeller, W.; Edetsberger, M.; Gaubitzer, E.; Koehler, G.; Prassl, R., Liposomal vasoactive intestinal peptide for lung application: protection from proteolytic degradation. *European Journal of Pharmaceutics and Biopharmaceutics* **2008,** 70, (1), 153-164.
34. Mittendorf, E.; Ardavanis, A.; Symanowski, J.; Murray, J.; Shumway, N.; Litton, J.; Hale, D.; Perez, S.; Anastasopoulou, E.; Pistamaltzian, N., Primary analysis of a prospective, randomized, single-blinded phase II trial evaluating the HER2 peptide AE37 vaccine in breast cancer patients to prevent recurrence. *Annals of Oncology* **2016,** 27, (7), 1241-1248.
35. Bachmann, M. F.; Jennings, G. T., Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. *Nature Reviews Immunology* **2010,** 10, (11), 787-796.
36. Badiee, A.; Khamesipour, A.; Samiei, A.; Soroush, D.; Shargh, V. H.; Kheiri, M. T.; Barkhordari, F.; Mc Master, W. R.; Mahboudi, F.; Jaafari, M. R., The role of liposome size on the type of immune response induced in BALB/c mice against leishmaniasis: rgp63 as a model antigen. *Experimental parasitology* **2012,** 132, (4), 403-409.
37. Nikpoor, A. R.; Tavakkol-Afshari, J.; Gholizadeh, Z.; Sadri, K.; Babaei, M. H.; Chamani, J.; Badiee, A.; Jalali, S. A.; Jaafari, M. R., Nanoliposome-mediated targeting of antibodies to tumors: IVIG antibodies as a model. *International journal of pharmaceutics* **2015,** 495, (1), 162-170.
38. Bartlett, G. R., Phosphorus assay in column chromatography. *Journal of Biological Chemistry* **1959,** 234, 466-468.
39. Huang, Z.; Jaafari, M. R.; Szoka, F. C., Disterolphospholipids: nonexchangeable lipids and their application to liposomal drug delivery. *Angewandte Chemie International Edition* **2009,** 48, (23), 4146-4149.