Design of HER2-specific Virus-Like Particles: the next step in targeted therapy

Joana Pinto dos Santos[1], MSc in Biomedical Engineering, joana.d.santos@tecnico.ulisboa.pt

[1] Instituto Superior Técnico, (IST), Universidade de Lisboa, Lisbon, Portugal

Abstract: In recent years, exciting new diagnosis and treatment strategies have emerged in the context of cancer research. Human Epidermal Growth Factor Receptor 2 (HER2) positive cancers are extremely aggressive, associated with a poor prognosis and are most commonly treated with trastuzumab. However, not all patients respond to this therapy. Virus-like particles (VLPs) arise as promising nanoplatforms to be used as drug delivery systems. They can acquire specificity through the expression, on their surface, of a cell target-specific molecule of interest, increasing the therapeutic efficacy of a drug. The main goal of this work was the development and characterization of an HIV-1 based VLP containing on the surface a single chain variable fragment (scFv) of an anti-HER2 antibody. The HEK- 293T cells were chosen to produce the HIV-1 based VLPs by transient transfection. *In vitro* studies were done to assess the presence of the fusion protein, binding to the HER2 target and the cytotoxic effects of VLPs. Our results showed the efficient production of HIV-1 based VLPs containing on the surface a scFv of an anti-HER2 antibody. More importantly, the preliminary results obtained, indicated binding between the receptor HER2 and engineered HIV-1 based VLP highlighting that there was no loss of specificity of trastuzumab scFv when expressed on the VLP surface. Further, no cytotoxic effect was detected when cells were incubated with the HIV-1 based VLPs. This work was the beginning for new targeted therapies for HER2 overexpressed cancers and anti-HER2 delivery systems.

Key words: HIV-1 based Virus-Like Particles; Human Epidermal Growth Factor Receptor 2 (HER2); Target Therapy; Therapeutic Delivery System; HER2-specific Virus-Like Particles

1. Introduction

Overexpression of cell receptors has been shown to play a key role as predictive biomarkers in cancer research and therapy being key targets for drug development (Twomey et al., 2017). Human Epidermal Growth Factor Receptor 2 (HER2) is involved in cell growth, differentiation, and survival and mediates several signalling pathways important to promote cell proliferation and prevent apoptosis (Tai et al., 2010; Iqbal & Iqbal, 2014;).

When *HER2* gene is amplified, the protein will be upregulated, which can cause a malignant cellular formation. There is a direct association between this and a poor clinical outcome in breast, gastric, ovarian, and prostate cancer, among other cancers. HER2 positive cancers are associated with tumor aggressiveness and a higher probability of relapse as well as metastasis (Advani et al., 2015; Dai et al., 2017). The most widely used drug for targeting HER2 is trastumuzab (Iqbal & Iqbal, 2014).

However, not all patients respond to this therapy and others develop intrinsic resistance to this drug. One way to enhance the therapeutic efficacy of a drug is to increase the specificity and affinity towards its target, the targeted therapies. Nanoparticles opened up new perspectives to the next-generation of targeted therapies due to their recognized ability to improve drug-packaging, delivery and targeting efficiency (Gulati et al., 2018). Virus- like particles (VLPs) appear as promising nanoplatform due to their biocompatibility and biodegradability (Steinmetz, 2010). VLPs are formed by the self-assembly of viral structural proteins, mimicking the organization and conformation of authentic native viruses (Zeltins, 2013). VLPs are non-infectious since they do not contain any of the virus genetic material and are unable to replicate bringing no safety concerns when administrated. Despite the absence of the virus genetic material, they still maintain the tropism and antigenicity characteristic of the native virus (Lua et al., 2014; Zdanowicz & Chroboczek, 2016; Forouhar-Kalkhoran, 2017). To get specificity VLP needs to express a cell target-specific molecule of interest on the surface. In this work the selected VLP is based on HIV-1 due its *in vivo* immunogenicity (Chen et al., 2020) and the selected target is HER2, that can be used as a marker for receptor mediated targeted drug delivery system, because this receptor is overexpressed in cancer cells and has an accessible extracellular domain (Lewis Phillips et al., 2008). HIV-1 based VLPs are complex nanostructures that can be constituted by a structural internal protein, a lipidic membrane and envelope proteins. The core is formed by Gag polyproteins (Lavado-García, Jorge, et al., 2021). The easy expression of gag, the stability and structure of these VLPs makes them a robust platform to be used as delivery systems (Cervera et al., 2019; Lavado-García et al., 2021).

Preliminary work was done where a biomimetic vector containing the single-chain variable domain fragment (scFv) of trastuzumab, an anti-HER2 antibody, as a targeting motif fused with HIV viral protein gp41 was designed and produced. Docking studies were performed to select the most favourable residue in the viral protein to be considered to fuse with a scFv from trastuzumab, after which DNA plasmid was produced. This DNA plasmid was transfected in HEK-293T cells and it was demonstrated that the plasmid construction we are using in this work is robust (Santos et al., 2021).

The main objective of this work was the development and characterization of an HIV-1 based VLP containing at the surface a scFv of an anti-HER2 antibody. The design of this nanoplatform will allow to obtain a delivery system able to bind to the receptor HER2, and thus deliver therapeutic and/or imaging molecules directly to the cancer cells overexpressing *HER2*. Currently, there are a few anti-HER2 systems described (Dhritlahre & Saneja, 2021). However, to my knowledge, this is the first time that an anti-HER2 delivery system has used an HIV-1 based VLP.

The model candidate presented in this work is a VLP formed by the HIV-1 Gag polyprotein and an envelope protein that translates a scFv from trastuzumab fused with gp41 from HIV (Santos et al., 2021). The expression of this VLP in a mammalian system, HEK-293T cell line, produces the VLP model present in Figure 1.

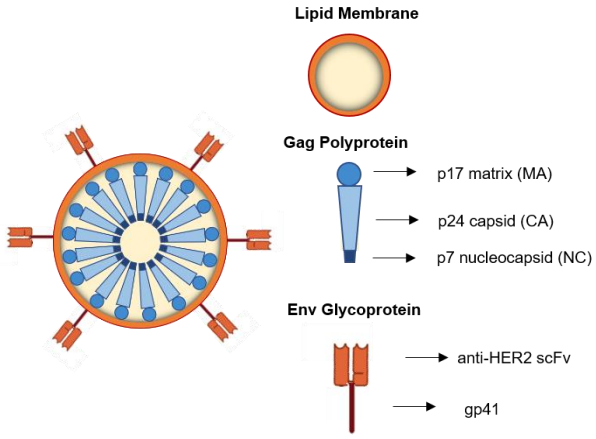

Figure 1: Schematic representation of the HIV-1 based VLP model used in this work.

2. Materials and Methods

2.1. Plasmids

The plasmid DNA (X1665+pcDNA3.1+) containing the anti-HER2 scFv and the viral protein gp41 sequence was synthetized in Synbio Tech (NJ, USA). From now on this plasmid DNA will be called pX1665. pMDLg/pRRE, pCMV-VSV and pRSV-REV (Addgene, USA), encodes the packaging proteins Gag-Pol, VSV-G and Rev respectively, were used to produce lentiviral particles for cell internalization. pHR_EGFPligand (Addgene, USA) encodes the EGFP surface ligand. This vector is adapted from the HIV and will integrate its genetic information in the VLP genome.

2.2. Cell culture

The cell line used to produce the HIV-1 based VLPs was the HEK-293T cells (human embryonic kidney-273 cells expressing the large T-antigen of simian virus 40). The cell lines chosen to study the characteristics of the HIV-1 based VLPs produced were SK-BR-3 (human breast adenocarcinoma cell line HER2-positive) and MDA-MB-231 (human breast adenocarcinoma cell line HER2-negative). Cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 6 mM L- glutamine, 1 nM sodium pyruvate, 0.1 nM MEM Non-Essential Amino Acids, 10% (v/v) fetal bovine serum and 1% (v/v) penicillin-streptomycin, all obtained from Cytiva (USA). HEK- 293T cells were maintained in exponential growth phase in disposable polycarbonate 75 cm^3 flasks at 37°C with 5% of CO_2, while SK-BR-3 and MDA-MB-231 cells were maintained in a disposable polycarbonate 25 cm^3 flasks in the same conditions as previously mentioned. Cell concentration and viability were assessed by haemocytometer cell counts using trypan blue exclusion dye (Merck, Germany). All cell lines were tested for mycoplasma using the LookOut® mycoplasma PCR Detection Kit.

2.3. HIV-1 based VLP production

HEK-293T cells were seeded on 6-well plate at a density of $1.0x10^6$ cells/well and incubated overnight at 37ºC and 5%CO_2. Lipofectamine™ 3000 (ThermoFisher Scientific®, MA, USA) was used to transfect the cells with 3 µg of DNA. For batch 1 a DNA ratio of 1:1:1:1 was chosen while batch 2 a ratio of 5:1:1:1 (pX1655: pMDLg/pRRE: pRSV-REV: pHR_EGFPligand ratio). Subsequently, the cells were incubated for 6 h at 37ºC and then the medium was replaced with fresh medium. At 48 h post-transfection, the supernatant was collected and centrifuged at 2000 rpm (Beckman, J2-21M, Beckman Coulter, Inc., USA) at room temperature for 10 min. The supernatant containing the VLPs produced was collected and stored at -80ºC for later use.

2.4. HIV-1 based VLP quantification – ELISA

Quantification of the HIV-1 based VLPs were determined by p24 ELISA using the

commercially available kit INNOTEST HIV Antigen mAb (Fujirebio). The assay was performed according to the manufacturer's instruction.

2.5. Western blot analysis

Expressed proteins in the supernatants were visualized by Western blotting. Samples were mixed with 1/6 of 6X SDS sample buffer and boiled for 10 min at 95°C. Then, were loaded per well and migrated on a 12.5% SDS-PAGE gel. After electrophoresis, the samples were transferred to a nitrocellulose membrane (BioRad). The membrane was blocked at room temperature for 45 min with 5% (w/v) non-fat dried milk in PBS buffer containing 0.2% (v/v) Tween 20. Then, was incubated for 60 min at room temperature with gentle agitation with anti-HA primary monoclonal antibody (Biolegend) diluted 1:2000 in 1% (w/v) non-fat dried milk in PBS buffer containing 0.2% (v/v) Tween 20. After washing with PBS Tween 0.2% (v/v), the membrane was incubated with the secondary antibody anti-mouse (BioRad) diluted 1:3000 in 1% (w/v) non-fat dried milk in PBS buffer containing 0.2% (v/v) Tween 20 for 60 min at room temperature. After, it was washed with PBS Tween 0.2% (v/v), the proteins were visualized using the ECL® reagent (GE Lifesciences) according to the manufacturer's instructions.

2.6. Flow cytometry

HER2 positive cells, the SK-BR-3, and HER-2 negative cells, the MDA-MB-231, were incubated with the HIV-1 based VLPs. 7.5 x10^4 SK-BR-3 cells and 5.0x10^4 MDA-MB-231 cells were plated in 24-well plates and incubated at 37ºC for 48 h. Then, different dilutions (1/2, 1/5 and 1/10) of the VLPs were added to the cells. After 24 h of incubation at 37ºC, the cells were collected with EDTA (10 mM) in PBS and centrifuged at 1623 rpm (Beckman, J2-21M, Beckman Coulter, Inc., USA) for 5 min. After, cells were re-suspended in 200 µL of PBS 1% BSA + EDTA (1 mM) and collected. Green fluorescence intensity present in the incubated cells was measured by flow cytometry using a Cytek™ Aurora system (Cytek Biosciences, Inc). Analysis from 10000 events per sample was done. First, SSC-A versus FSC-A and FSC-H versus FSC-A density plots were used to gate the individual cell population. Then, the GFP-positive population was assessed in a GFP-A histogram. Data were analysed with the FlowJo software (FlowJo, LLC).

2.7. Cytotoxicity assay - WST-1 proliferation test

Cell viability was evaluated using the WST-1 proliferation test. SK-BR-3 cells were seeded in 96-well plate at a density of 1.0x10^5 cells/well and incubated at 37ºC for 52 h. Each plate contained blanks, controls, and three dilution series (1/2, 1/5 and 1/10) with two replicates each. The different dilutions of VLPs were added to the cells. After 24 h of incubation at 37ºC, the inoculum from the wells was removed and replaced with 100 µL of fresh medium (DMEM 10% FBS) and 10 µL of a cell proliferation reagent WST-1 (Roche). After gentle mixing, the cells were incubated at 37ºC for 6 h. The absorbance was measured on a EZ Read 800 Microplate Reader (Biochrom) at 450/620 nm. Cytotoxicity of the VLPs was evaluated through the percentage of cell viability (measured as WST-1 reduction) compared to controls (Equation 1).

$$\% \, Cell \, Viability = \frac{Abs_{Incubated \, cells} - Abs_{VLPs}}{Abs_{Not \, incubated \, cells} - Abs_{Culture \, Medium}} \times 100\% \quad \textbf{(1)}$$

3. Results and Discussion

HIV-1 based VLP is an enveloped VLP, which needs a eukaryotic expression system to be produced (Chen et al., 2020). The mammalian HEK-293T cells were chosen to produce the HIV-1 based VLPs because they provide the proper protein folding and post-translational modifications required for its function. Further they also allow the collection of VLPs from the cell culture supernatant (Fuenmayor et al., 2017; Cervera et al., 2019; González-Domínguez et al., 2020; Nooraei et al., 2021).

3.1. HIV-1 based VLP production and quantification

HIV-1 based VLPs produced by transfecting the HEK-293T cells at 1.0x10^6 cells/well and harvest 48 hours post-transfection were quantified by p24 ELISA.

The correlation equation to quantify the different HIV-1 based VLPs batches is as follows:

$$y = -0.012x^2 + 9.356x + 89.077 \quad \textbf{(2)}$$

$$R^2 = 0.9986,$$

where (y) corresponds to the sample absorbance and (x) to the p24 concentration.

The concentrations of the different batches and main differences are presented in Table 1.

Table 1: p24 concentration of the HIV-1 based VLPs produced and the main difference between batches.

	Batch 1	**Batch 2**
pX1655: pMDLg/pRRE: pRSV-REV: pHR_EGFPligand ratio	1:1:1:1	5:1:1:1
[p24] (ng/mL)	0.53	0.61

Different ratios of pX1665 were studied to find out if increasing the availability of scFv-HER2_gp41 fusion protein in the cell, would facilitate the budding process and allow the production of more HIV-1 based VLPs with the anti-HER2 at the surface.

The transfection protocol presented in this work allowed the production of HIV-1 based VLPs around ng/mL range, which is agreeable to the ones found in the literature. Gonelli was able to produce 3.62 ng/mL Gag-Env VLPs (Gonelli et al., 2019). There is no significant difference in p24 concentration between the different batches. This is not expected since the quantity of gag in batch 2 is lower which theoretically would imply a lower p24 concentration. Since the *gag* gene is the one that expresses the Gag polyprotein that self-assembles and forms the VLPs. However, it can be seen that even when the amount of pMDLg/pRRE introduced into the cells is two times lower, it did not decrease the concentration of p24. These results may be caused by the adherent conditions of the

HEK- 293T cells which is a vital factor in the transfection, a highly demanding process (Lavado-García et al., 2020).

3.2. Presence of the trastuzumab scFv on the HIV-1 based VLP

In order to assess whether assemble of the Gag VLP with the scFv-HER2_gp41 fusion protein was successful, it was performed a Western blot protocol with the supernatant (Figure 2).

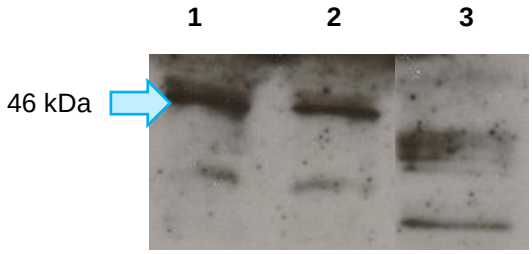

Figure 2: Western blot analysis. 1: batch 1, 2: batch 2, 3: positive control (protein extract containing HA). Western blot detection was performed using a primary monoclonal HA-antibody (1:2000) and secondary antibody anti-mouse (1:3000). Visualization of bands was carried out using the ECL® reagent.

The expected molecular weight of the recombinant protein is 47.1 kDa (Santos et al., 2021). The molecular weight of the protein present in the supernatant is about 46 kDa (Figure 2), slightly lower than the theoretical value. These results confirm the presence of the scFv-HER2_gp41 protein in the HIV-1 based VLPs.

The estimated quantities of p24 added to each well in the SDS-Page are 18.65 pg for batch 1 and 21.37 pg for batch 2. Looking at the bands from batch 1 and 2, which were produced on the same day with different ratios of pX1665, 1 and 5, respectively, batch 1 had a lower amount of p24 but the band intensity is slightly higher than batch 2, even though batch 1 was produced with a pX1665 ratio of one. This result may reveal that increasing the ratio of pX1665 plasmid will not increase the production of VLPs containing the scFv-HER2_gp41 fusion protein. Further studies need to be done to evaluate the role of the pX1665 ratio in the formation of VLPs containing the fusion protein.

3.3. HIV-1 based VLP binding to HER2 receptor

To assess if the HIV-1 based VLPs had the fusion protein on the surface and if the scFv bound to HER2 receptor in the cells, flow cytometry was performed. If the HIV-1 based VLP binds to HER2 receptor and is internalized into the cell, then the GFP genetic information will be integrated into the cell genome, and the cell will start expressing the GFP.

Both in Figure 3 and Figure 4, it can be observed a shift of fluorescence from left to right and an increase in the GFP positive population in the samples that were incubated with the VLPs. The samples that were incubated with the HIV-1 based VLPs are more GFP positive than the control group, which may suggest that the VLPs bound to the SK-BR-3 and were able to internalize in these cells.

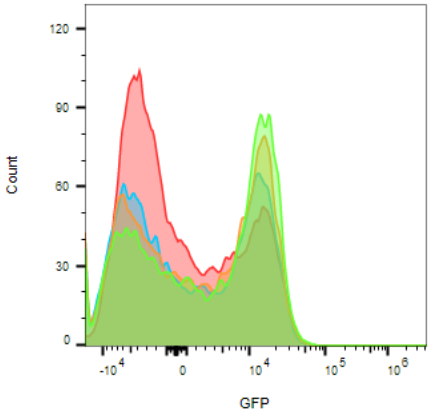

Figure 3: Assessment of GFP presence in the SK-BR-3 cells incubated with HIV-1 based VLPs (batch 1 - pX1665 ratio of one). Red: control group; blue: dilution 1:2; orange: dilution 1:5; green: dilution 1:10.

In Figure 3, it is also observed that, when a higher dilution was used to incubate the cells, an increase in the population (number of cells) that are GFP positive occurred. These results may be due to the fact that a higher quantity of VLPs entering the cells, and integrating the GFP genetic information in the cell genome, can translate in a higher cell death, which decreases the amount of fluorescence obtained in the flow cytometer. Further, the control group has a peak which occurs due to the auto fluorescence present in this sample. This auto fluorescence may be caused by the presence of dead cells in the population selected. To confirm and remove the dead cells from the population of interest, a viability dye must be added to the samples.

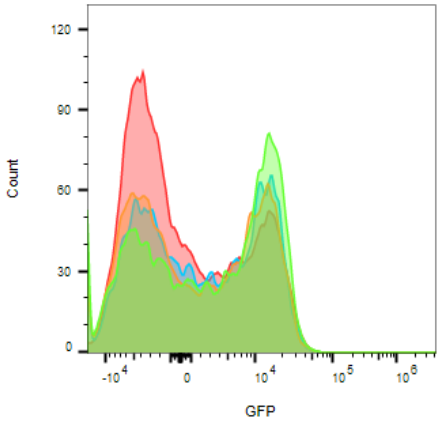

Figure 4: Assessment of GFP presence in the SK-BR-3 cells incubated with HIV-1 based VLPs (batch 2 - pX1665 ratio of five). Red: control group; blue: dilution 1:2; orange: dilution 1:5; green: dilution 1:10.

In Figure 4, it can be observed that there is a smaller population of GFP positive cells, when the samples were incubated with HIV-1 based VLPs produced with a pX1665 ratio of five which may suggest that using this ratio in the transfection, may lead to a reduction in the efficient production of competent VLPs. These results indicate that producing HIV-1 based VLPs with a pX1665 ratio of five may not bring any advantage compared to a ratio of one.

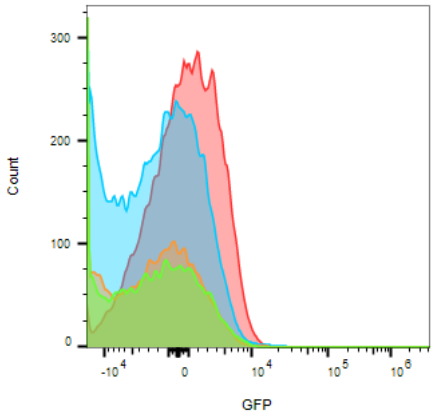

Figure 5: Assessment of GFP presence in the MDA-MB-231 cells incubated with HIV-1 based VLPs (batch 1 - pX1665 ratio of one). Red: control group; blue: dilution 1:2; orange: dilution 1:5; green: dilution 1:10.

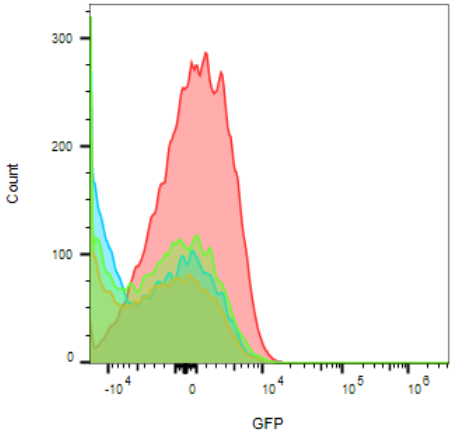

Figure 6: Assessment of GFP presence in the MDA-MB-231 cells incubated with HIV-1 based VLPs (batch 2 - pX1665 ratio of five). Red: control group; blue: dilution 1:2; orange: dilution 1:5; green: dilution 1:10.

In Figure 5 and Figure 6, the control group has the population with the highest GFP intensity which may imply that the VLPs did not bind to the MDA-MB-231 cells.

These preliminary results suggest that the HIV-1 based VLPs produced bound to the HER2 receptor of the SK-BR-3 cells and it did not bind to any other receptor as seen with MDA- MB- 231 cells. It indicates that there is specificity between the VLP and the HER2 receptor. Nevertheless, future optimizations to the process must be done and these results should be verified.

3.4. Evaluation of cytotoxic effect

The WST-1 proliferation test was performed to assess the cytotoxic effect of the HIV-1 based VLPs. The proliferation of cells incubated with the produced HIV-1 based VLPs was compared with the proliferation of cells that were not incubated. The cells chosen for this assay were the SK-BR-3 cells because they overexpress the HER2 receptor on their membranes and as was seen in the cytometry the VLPs bind to this receptor in these cells.

The results for the cell viability, calculated by Equation (1), are presented in Figure 7.

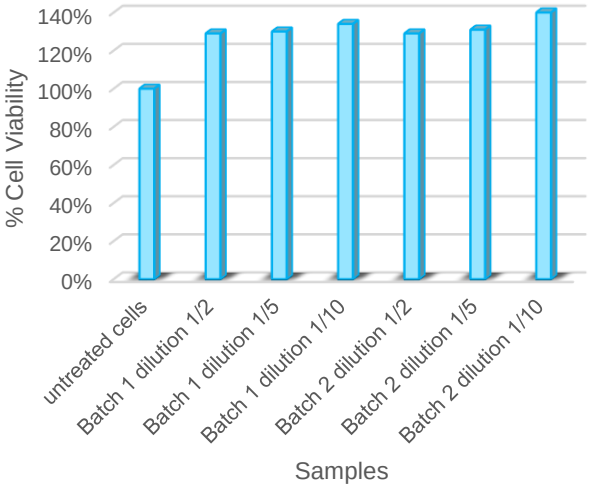

Figure 7: Cell viability of SK-BR-3 cells after incubation with HIV-1 based VLPs.

Figure 7 shows that the cell viability has increased when compared to the untreated cells (cells not incubated with the VLPs), which indicates that the VLPs produced are not cytotoxic to the cells. There is not a significant difference in the percentage of cell viability when incubating the cells with different batches, so no differences between the HIV-1 based VLPs in terms of the cytotoxic effects to the cells were observed. However, the difference between the cell viability of incubated and not incubated cells is around 30%, higher than the 10% value that is acceptable due to random experimental fluctuations. This result may indicate that the addition of the HIV-1 based VLPs to the cells stimulated the cell growth due to the fact that the supernatant containing the VLPs was spent medium of the HEK-293T cells. Despite the step of centrifugation where cell debris is removed from the supernatant that contains the VLPs, this supernatant can have exosomes, growth factors and other molecules of the HEK-293T cells that may influence the cellular growth of the SK-BR-3 cells. Beas-Catena studied the effect of spent medium on cell proliferation and concluded that when less than 50% of spent medium from a culture in mid-exponential growth phase is used, it improves the cellular growth when compared to the control culture grown in fresh medium (Beas-Catena et al., 2013). To validate the results obtained, it is necessary to repeat the experiment changing the supernatant where the VLPs are to PBS, for example, so the effect of the spent medium in the viability of the SK- BR- 3 cells can be eliminated.

4. Conclusions and Future Perspectives

Anti-HER2 delivery systems can update the HER2 positive cancers therapeutics, not only in the treatment but also in the diagnostic. Their characteristics allow a more specific treatment of the tumour and less toxic to the patient. Nanoparticles, as VLPs, are a novelty nanoplatform that have been studied for drug delivery systems due their biocompatibility and biodegradability. These VLPs can acquire specificity through the expression, on their surface, of a cell target-specific molecule of

interest. This work showed that it is possible to produce an HIV-1 based VLP containing on the surface a scFv of an anti-HER2 antibody with specificity to the target receptor HER2 without cytotoxic effect to the cells. These results are a starting point for a new targeted therapy for HER2 overexpressed cancers and anti-HER2 delivery systems. In the future, it would be interesting to characterize the morphology of the HIV-1 based VLP by performing a TEM analysis. Further, it would be advantageous to improve VLP production by studying the effect of different ratios of the pMDLg/pRRE or changing the packaging vector.

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