A preliminary study to establish the transfected CHO cell lines which highly express Trastuzumab - A biosimilar product of Herceptin

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ABSTRACT

Human epidermal growth factor receptor 2 (HER2) has been identified as a molecular target for breast cancer therapy, such as Trastuzumab (Herceptin®). This has been shown to improve patient survival substantially. The current study is aiming to locally produce an anti-HER2 monoclonal antibody (named Trastuzumab) which has an equivalent biological properties in comparison with the original version, Herceptin®). *In silico* design and construction of recombinant vectors, as well as the establishment of transfected cell lines with high expression of Trastuzumab were performed. Based on the protein sequences obtained from the Drugbank, the DNA sequences encoding for the light chain (Tras-Lc) and heavy chain (Tras-Hc) of Trastuzumab were optimized and integrated into *pNanogen-Hygro* and *pNanogen-Puro* vectors, respectively. The Neon Transfection System was used to co-transfect the *pNanogen-Tras-Lc-Hygro* and *pNanogen-Tras-Hc-Puro* constructs into CHO cells. Different co-transfected single-cell-colonies selected on media supplemented with hygromycin and puromycin were used for ELISA and SDS-PAGE assays to identify the CHO cell lines which highly express Trastuzumab. Based on the present results, 30µg of both constructs were suitable for DNA co-transfection. After 07 days of culture, the highest amount of Trastuzumab (561 µg/ml) was obtained from the H06LD68 cell line.

1. Introduction

Breast cancer is the most commonly diagnosed cancer among females. Breast cancer refers to cancer originating from breast tissue, characteristically resulting in a mass or lump (American Cancer Society, 2019; Sun et al., 2017). Notably, it commonly transfers to distant organs such as bone, liver, lung, kidney, etc. According to the etiological factors, several genes, including oncogenes and tumor suppressor genes, have been identified to be significantly associated with breast cancer (Feng et al., 2018; Sun et al., 2017). Among them, the overexpression of HER2 (Human epidermal growth factor receptor 2), also known as *c-erbB-2*, has been investigated in

approximate 20% of primary breast tumors, leading to the increase of breast tumor initiation and progression (Elizalde, Russo, & Chervo, 2016; Sun et al., 2017). Therefore, HER2 receptors, located in the surface of cells, have been used mainly for therapeutic purposes for the control or reduction of tumor malignancy growth in breast cancer (Tai, Mahato, & Cheng, 2010; Akbarzadeh-Sharbaf, Yakhchali, Minuchehr, Shokrgozar, & Zeinali, 2012; Mitri, Constantine, & O'Regan, 2012). Therefore, the development of HER2-target therapies has been substantially developed. One successful therapy that has been developed is to disable HER2 receptors by using Trastuzumab (Herceptin®; F. Hoffmann-La Roche Ltd.). Trastuzumab (Herceptin®) was the first antibody-targeted therapy for breast cancer which was approved by the US Food and Drug Administration (FDA) in 1998 (Siegel, 1998). Up to now, many clinical trials have established that a combination Trastuzumab (Herceptin®) with chemotherapy, compared to other treatments, increases the time of disease progression and Overall Survival (OS) in both the metastatic and adjuvant settings. Therefore, the addition of Trastuzumab (Herceptin®) to chemotherapy are inform in the treatment of patients with HER2+ early breast cancer. So, due to the large demand for using Trastuzumab in breast cancer treatment as well as decreasing the cost of Trastuzumab in developing countries, including Vietnam, producing biosimilar biological drugs was necessary. Therefore, aiming to produce the anti-HER2 monoclonal antibody (Trastuzumab) with biological properties equivalent to the original drug Herceptin, which could be further used in the treatment of breast cancer, we performed the *in-silico* design and construction of recombinant vectors for producing Trastuzumab in CHO cell line, then, established the transfected cell line with high expression of Trastuzumab.

2. Materials and methods

2.1. Construction of recombinant vector containing the light chain and heavy chain of Trastuzumab in E. coli TOP10F'

The Tras-Lc and Tras-Hc were individually integrated into a pNanogen-Hygro and pNanogen-Puro vector, which contain sequences of the promoter, enhancer, DHFR, and antibiotic resistance gene (Hygromycin resistance, and Puromycin resistance), to create the recombinant vector of pNanogen-Tras-Lc-Hygro and pNanogen-Tras-Hc-Puro. They were transfomed into TOP10F' chemically competent *E.coli*.

2.2. Preparation recombinant vectors for transfection

The recombinant vectors were amplified, extracted, and purified by Purelink Expi Endotoxin Free Maxi Plasmid Purification Kit (Thermofisher, A31232).

2.3. Co-transfecting recombinant vectors into CHO cell line, and determining the higher Trastuzumab-producing CHO cell line by ELISA assay

The Neon Transfection System was used to co-transfect pNanogen-Tras-Lc-Hygro and pNanogen-Tras-Hc-Puro into CHO cell line according to the manufacturer's guideline. CHO cells were co-transfected with the following amount of the plasmid: 10, 20, and 30µg in a ratio of 1:1 of pNanogen-Tras-Lc-Hygro and pNanogen-Tras-Hc-Puro.

After transfection, Trastuzumab concentration in supernantant was determined by ELISA assay, the protocol was as follows: Dilute 2-fold of Herceptin (start with 0.5 µg/ml) and 10-fold of the sample (start with 1X) in PBS solution. Incubate for overnight at 02 - 08^{o}C. Wash 01 time with 200µl of PBS solution per well. Block plate with 100µl per well with BSA 0.5% for 01 hour at room temperature. Wash 01 time with 200µl of PBS per well. Add 100µl of anti-Trastuzumab

(1/500) into each well. Incubate for 01 hour at room temperature. Wash 03 times with 200µl of PBST per well. Add 100µl of mouse IgG HRP - conjugated antibody (1/5000) into each well. Incubate for 01 hour at room temperature. Wash 03 times with 200µl of PBST per well. Following the wash, invert and tap on absorbent paper to remove excess liquid. Add 100µl of TMB substrate solution to each well. Incubate the plate for 05 - 15 minutes at room temperature. Add 50µl of Stop solution to each well. Measure the absorbance at 450nm within 15 minutes of adding the Stop solution. Base on a standard curve of Herceptin to quantify the concentration of samples.

2.4. Screening recombinant CHO cells expressed the highest Trastuzumab concentration

CHO cells were cultured in SFM4CHO without serum containing two antibiotics of Hygromycin and Puromycin for 14 days in the presence of 37°C, 5% CO_2, and 75% humidity. Antibiotics concentration using screening based on the kill curve of each antibiotic.

2.5. Selection and expansion of clones post-transfection

After transfection, the CHO cell line expressed the highest Trastuzumab was selected single clone. The end-point dilute assay was applied to set up the recombinant CHO cell culture assay within 1 recombinant cell per 100µl culture medium containing Hygromycin and Puromycin. Plating was performed on 96-well with a density of 01 cells/100 µl/well. The single-cell colonies were scaled up using 24-well and 6-well plates with 14-day culture in each step of scaling up. At the final scaling up to 6-well plates, Trastuzumab concentration was evaluated by ELISA assay.

2.6. Detection Trastuzumab by SDS-PAGE electrophoresis

CHO cell lines with higher Trastuzumab expression will be transferred to suspension culture supplemented with antibiotics in a 50ml culture medium within 14 days. Antibody production was monitored during each round of amplification using the SDS-PAGE method: 300µl each test sample was centrifuged at 13,000 rpm/min for 1min. The supernatant was collected into new tube. For reducing condition: Add 35µl reducing sample buffer 4X into 100µl test sample. Incubate tubes in boiling water for 5min. Incubate tubes in ice for 10min. For non-reducing condition: Add 35µl non-reducing sample buffer 4X into 100µl test sample. Before loading into a gel, test samples were centrifuged at 13,000 rpm/min for 1min. 10µl prepared samples were pipetted into the well of the gel 12%. The gel was run at 120V until the dye front reaches the bottom of the gel.

3. Results and discussion

3.1. Construction of recombinant vector of pNanogen-Tras-Lc-Hygro and pNanogen-Tras-Hc-Puro

Based on protein sequence (Drugbank accession number: DB00072), the nucleotide sequences, encoded the Tras-Lc, and Tras-Hc were optimized by GenScript, were integrated into pNanogen-Hygro and pNanogen-Puro, respectively. The recombinant vector pNanogen-Tras-Lc-Hygro and pNanogen-Tras-Hc-Puro were cloned, then sequencing. As the results, the sequences of the light chain and heavy chain of Trastuzumab in recombinant vectors were similar by 100% to the optimized sequences from Genscript. (Figure 1). Thus, it could be concluded that Tras-Lc and Tras-Hc were successfully integrated to pNanogen-Tras-Lc-Hygro and pNanogen-Tras-Hc-Puro.

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(A)

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(B)

Figure 1. The comparison of the sequence alignment between (A) Tras-Lc; (B) Tras-Hc and the origin sequences downloaded from NCBI

3.2. Co-transfecting recombinant vectors into CHO cell line, and screening recombinant CHO cells

The CHO cells were co-transfected and transfected with different amounts of recombinant vector in a ratio of 1:1 of pNanogen-Tras-Lc-Hygro and pNanogen-Tras-Hc-Puro. As the results, the concentration of Trastuzumab in three experiments indicated that the average concentration of

Trastuzumab was proportional to the total amount of heavy and light chain DNA. The higher concentration of Trastuzumab was 0.535 ± 0.035 µg/ml, which was observed in the experiment of 30µg recombinant vectors **(Table 1).**

Table 1

The concentration of Trastuzumab after 03 days of transfection

	pNanogen-Tras-Lc-Hygro : pNanogen-Tras-Hc-Puro (1:1)		
Total amount of recombinant vector (µg)	10	20	30
Concentration of Trastuzumab (µg/ml)	0.236 ± 0.021	0.347 ± 0.029	0.535 ± 0.035

Source: Data analysis result of the research

The two recombinant vectors pNanogen-Tras-Lc-Hygro and pNanogen-Tras-Hc-Puro carried Hygromycin and Puromycin resistance genes, which encoded proteins functioned as degrading Hygromycin and Puromycin, respectively. In the case of successfully co-transfected with pNanogen-Tras-Lc-Hygro and pNanogen-Tras-Hc-Puro, CHO cells can survive in the medium containing Hygromycin and Puromycin. However, the concentration of Hygromycin and Puromycin were needed to be modified to find out the appropriate concentration. In the current study, the concentrations of Hygromycin and Puromycin were investigated in a range of 50 to 350 µg/ml, and 0.5 to 3.5 µg/ml, respectively, then followed up to 14 days. As the result, the selection of Hygromycin-based, and Puromycin-based survival CHO cells was indicated in **Figure 2, 3**. The results indicated that the concentration of antibiotics and the percentage of viable cells was inversely proportional to each other. The concentration of Hygromycin and Puromycin were 350 µg/ml and 3.5 µg/ml, respectively, causing the complete death of CHO cells after the 05-day culture. Thus, it could be concluded that the appropriate concentration of Hygromycin and Puromycin for selection were 350 µg/ml and 3.5 µg/ml, respectively.

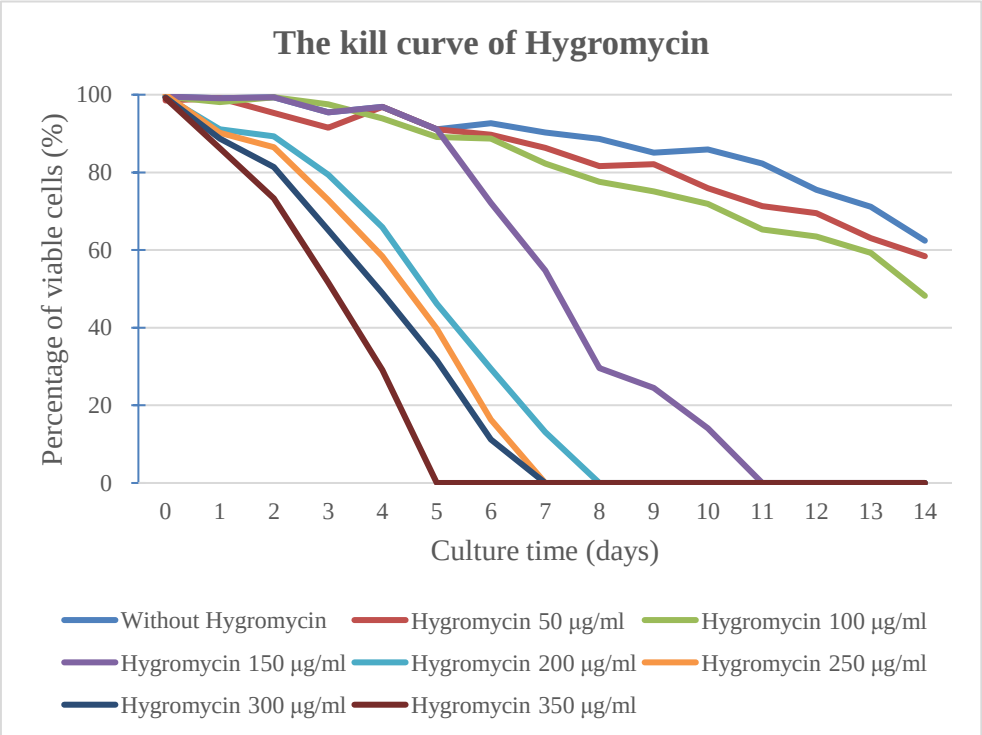

Figure 2. Dose response curve for Hygromycin selection of co-transfected CHO cells

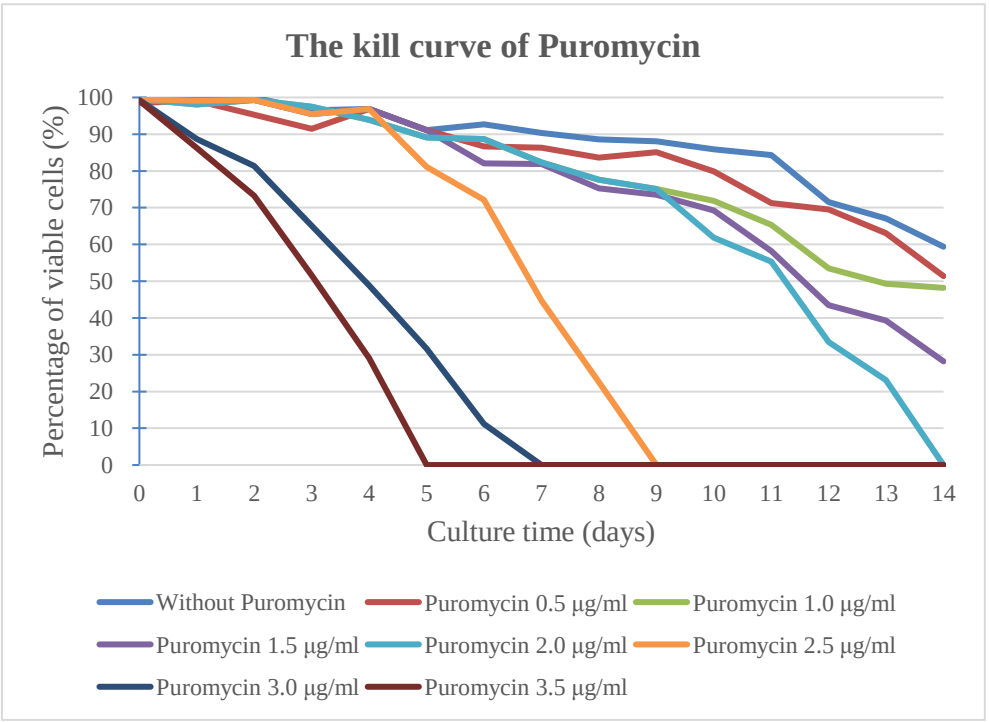

Figure 3. Dose response curve for Puromycin selection of co-transfected CHO cells

3.3. The higher Trastuzumab-producing CHO cell line

The final single-cell colonies were scaled up using 6-well plates within 14-day culture, and the concentration of Trastuzumab was determined to establish the eight CHO cell lines with higher Trastuzumab expression. As the results, the eight CHO cell line with higher Trastuzumab expression were established after following the end-point dilute assay, shown in **Table 2**.

Table 2

The eight CHO cell lines with higher Trastuzumab expression

No.	Cell line	Concentration of Trastuzumab (µg/ml)
1	H06LD02	0.720
2	H06LD08	0.821
3	H06LD19	0.772
4	H06LD27	0.641
5	H06LD68	0.766
6	H06LD75	0.648
7	H06LD81	0.725
8	H06LD90	0.645

Source: Data analysis result of the research

During the 50-ml suspension culture, each cell line was monitored for evaluation of proliferation every day. Notably, the medium was collected on the 3rd and 7th day to determine the cell density, Trastuzumab concentration, and perform an SDS-PAGE electrophoresis assay. The concentration of Trastuzumab, which was expressed in single-cell colonies cultured in the volume of 50-ml, were indicated in **Table 3**.

Table 3

The concentration of expressed Trastuzumab in single-cell colonies was cultured in the volume of 50-ml

Cell line	Group	Day 3			Day 7		
		(1)	(2)	(3)	(1)	(2)	(3)
H06LD02	B	1.82 ± 0.24	219 ± 33	120 ± 14	2.78 ± 0.21	504 ± 24	181 ± 8
H06LD08	B	3.90 ± 0.24	278 ± 29	71 ± 8	1.92 ± 0.20	355 ± 16	185 ± 11
H06LD19	A	4.46 ± 0.15	346 ± 39	78 ± 8	5.70 ± 0.31	533 ± 26	94 ± 6
H06LD27	A	5.14 ± 0.21	313 ± 24	61 ± 7	4.50 ± 0.17	446 ± 29	99 ± 8
H06LD68	B	2.80 ± 0.16	349 ± 29	125 ± 7	2.22 ± 0.17	561 ± 12	253 ± 15
H06LD75	A	4.62 ± 0.33	429 ± 24	93 ± 12	2.84 ± 0.30	686 ± 36	242 ± 16
H06LD81	A	4.56 ± 0.30	261 ± 26	57 ± 8	5.72 ± 0.24	410 ± 29	72 ± 7
H06LD90	B	3.58 ± 0.16	284 ± 19	79 ± 8	4.64 ± 0.22	427 ± 18	92 ± 8

Note: (1) Cell density (106 cell/mL); (2) Concentration of Trastuzumab (µg/ml); (3) Protein synthesis efficiency (pg/cell)
Source: Data analysis result of the research

As indicating in Table 3, after 3-day culture, eight higher CHO cell lines with higher Trastuzumab expression could be divided into two groups: group A had a cell density of over 04 million cells/ml, group B had a cell density of fewer than 04 million cells/ml. The cell line of H06LD75 and H06LD68 were the highest in each group with a concentration of Trastuzumab of 429 µg/ml and 349 µg/ml, and protein synthesis efficiency of 93 pg/cell and 125 pg/cell, respectively. By day 07, the cell density of the H06LD75 line (group A) decreased by approximately 40% while that of the H06LD68 cell line (group B) only decreased by 20%. However, the protein synthesis efficiency of the H06LD68 cell line was increased to 242 pg/cell, compared to day 03.

The SDS-PAGE assay was applied to determine the presence of Trastuzumab, which is expressed in the cell line of H06LD68 on day 03 and day 07. As the results, the SDS-PAGE assay conducted in both non-reducing and reducing conditions was shown in Figure 3. Indicating in Figure 3, the results of SDS-PAGE electrophoresis of H06LD68 cell line cultures on day 03 and day 07 in both non-reducing and reducing conditions showed lines equivalent to Herceptin. In the non-reducing condition, the disulfide bridge between the heavy chain and the light chain was not broken, thus only one band of >150kDa was observed. In the reduced condition, because the link between the two chains is broken, the Trastuzumab protein will exist in the form of a single heavy chain and a light chain with sizes of about 50 and 23kDa, respectively. Based on those results, it was concluded that we successfully established the H06LD68 cell line with the characteristic of higher expression of Trastuzumab.

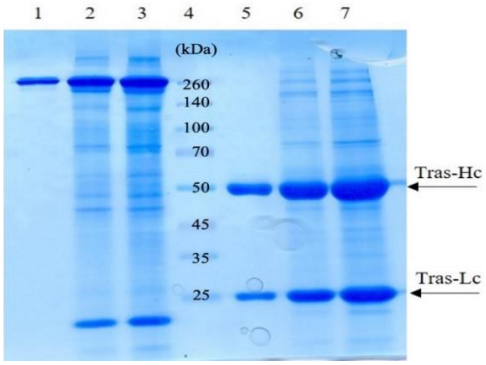

Figure 4. The result of SDS-PAGE of the cell line of H06LD68. (1) Herceptin in a non-reducing condition; (2) The cell culture fluid on day 03 in a non-reducing condition; (3) The cell culture fluid on day 07 in a non-reducing condition; (5) Herceptin in a reducing condition; (6) The cell culture fluid in day 03 in reducing condition; (7) The cell culture fluid in day 07 in reducing condition; (4) Protein ladder (Fermentas)

4. Conclusion

We successfully constructed two recombinant vectors pNanogen-Tras-Lc-Hygro and pNanogen-Tras-Hc-Puro to produce the Transtuzumab in the CHO cell line. The total amount of vectors of 30µg were suitable for gene co-transfection. Additionally, we successfully established the H06LD68 cell line with the characteristic of higher expression of Trastuzumab.

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