RESEARCH ARTICLE

Preparation of Immunotoxin Herceptin-Botulinum and Killing Effects on Two Breast Cancer Cell Lines

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Abstract

Background: Worldwide, breast cancer is the most common cancer diagnosed among women and a leading cause of cancer deaths. The age of onset in Iran has become reduced by a decade for unknown reasons. Herceptin, a humanized monoclonal antibody, is a target therapy for breast cancer cells with over expression of HER2-neu receptors, but it is an expensive drug with only 20% beneficial rate of survival. This study introduces a novel approach to enhance the efficacy of this drug through immunoconjugation of the antibody to botulinum toxin. Decreasing the cost and adverse effects of the antibody were secondary goals of this study. Materials and Methods: Botulinum toxin was conjugated with Herceptin using heterobifunctional cross linkers, succinimidyl acetylthiopropionate (SATP) and sulfo-succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) according to the supplier's guidelines and tested on two breast cancer cell lines: SK-BR-3 and BT-474. Toxin and Herceptin were also used separately as controls. The cytotoxicity assay was also performed using the new bioconjugate on cultured cells with Alamar blue and a fluorescence plate reader. Results: Herceptin-Toxin bioconjugation significantly improved Herceptin efficacy on both breast cancer cell lines when compared to the control group. Conclusions: Toxin-Herceptin bioconjugation can be a potential candidate with increased efficiency for treating breast cancer patients with over expression of the HER2 receptor.

Keywords: Breast cancer - herceptin - botulinum toxin - HER2 - SK-BR-3 - BT-474

Asian Pac J Cancer Prev, **16 (14)**, 5977-5981

Introduction

Worldwide, breast cancer is the most common cancer found among women and the leading cause of deaths. According to the American Cancer Society, approximately 232,340 new of invasive cases and 39,620 deaths are expected to occur among US women in 2013. One in 8 women in the United States will develop breast cancer in her lifetime. The rate of incidence rates increased slightly among African American women; decreased among Hispanic women; and remained stable among whites, Asian Americans/Pacific Islanders, and American Indians/ Alaska natives from 2006 to 2010 (Althuis et al., 2005). Global differences in incidence rates and fluctuations in rates within a country are both affected by changes in risk factor for prevalence and secular trends in diagnosis (DeSantis et al., 2014).

The peak incidence of breast cancer in Iran is between the ages 40-50, which is a decade younger than that of the western countries (Hosseini et al., 2013). In Iran, it ranks first among cancers diagnosed in women and the fifth most common cause of death (Taghavi et al., 2012). Although the exact cause is not known, genetic and environmental factors seem to play roles.

The human epidermal growth factor receptor-2 gene C-Erb2/neu (also known as HER2/neu) encodes 1255 amino acids to a membrane glycoprotein receptor of 185KD from the tyrosine kinase family (Kaufmann et al., 2011). HER2 receptor regulate cell growth and differentiation through specific signal transduction pathways. HER2 gene amplification on chromosome 17 accounts for about 25% of over expressions leading to tumor growth, lengthening the S phase in cell cycle, aneuploidy and down-regulation of estrogen and progesterone receptors (d'Agnano et al.,

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1996; Alshenawy, 2012).

Herceptin (Trastuzumab) is a humanized monoclonal IgG1 kappa antibody that selectively binds with high affinity to the extracellular domain of HER2 receptors with high affinity (Beuzeboc et al., 1999). Trastuzumab induces down modulation of HER2 receptor and, as a result, inhibits critical signalling pathways (i.e. ras-Raf-MAPK and PI3K/Akt) and blocks cell cycle progression by inducing the formation of p27/Cdk2 complexes. Trastuzumab also inhibits HER2 cleavage, preceding antibody-induced receptor down modulation that in turn contribute to its antitumor activity in some tumor cells. Moreover, trastuzumab have shown to inhibits angiogenesis and induces antibody-dependent cellular cytotoxicity (Albanell et al., 2003).Immunotheraputic administration of Herceptin is a promising new approach in breast cancer treatment, complementing surgery, and other therapy such as chemotherapy, radiation and anti-hormonal therapy (Untch et al., 2003). Inspite of these advances Herceptin is an expensive intervention with side effects such as: cardiovascular adverse events (Tsai et al., 2014) leukopenia, hypotension, dyspnea, anemia, fatigue, headache, fever, diarrhea (Dang et al., 2010), and skin rash (Adachi et al., 2011). Nevertheless its activity is largely restricted to treatment and management of breast cancers cases with the highest level of HER2 gene amplification (Albanell et al., 2003).

Botulinum toxin is a neurotoxin produced by the bacterium Clostridium Botulinum (Yoneyama et al., 2008). To date eight serotypes of this toxin (A through H) have been identified. The toxin endocytosed after binding to neurons and inhibits acetylcholine release via the photolytic activity of its catalytic domain (Ray, 1993). Some medical applications of Botulinum Toxin include treating children with cerebral palsy (Nezu and Iwasaki, 2013), overactive bladder(Ravindra et al., 2013), excessive sweating (Naumann et al., 2002), chronic migraine prevention of salivary stagnation after surgery of oral cavity cancer (Corradino et al., 2012) and controlling pain after mastectomy (Layeeque et al., 2004). Botulinum Toxin is also shown to induce apoptosis in T47D breast cancer cell line through caspase-3 and -7 pathways (Bandala et al., 2013).

In this study we conjugated Herceptin to Botulinum to enhance its efficiency and possibly reducing the therapeutic dose of the drug.

Materials and Methods

Herceptin preparation and activation:

100 mg Herceptin was dissolved in deionized water and transferred to a dialysis bag containing 50 mM phosphate buffer (pH: 7.5), contains 1 mM EDTA, and kept for 12 hours at 4˚C to remove small molecules from Herceptin. 300μl Herceptin (5mg/ml) was mixed with 20μl SATP (N-succinimidyl-S-acetylthiopropionate) solution and incubated for 60 minutes at room temperature and passed through superdex G-10 chromatography column to remove excess SAPT from antibody solution. 50 μl Hydroxyl amine solution was also added to 500 μl Herceptin-SATP to protect the –SH groups. After 2 hours of incubation the solution was dialyzed in 50 mM phosphate buffer (pH: 7.5), contains 1 mM EDTA, for 12 hours at 4˚C.

Botulinum Toxin preparation and activation:

250 μl toxin aqueous solution (1mg/ml) was dialyzed in 0.1 mM phosphate buffer (pH: 7.5), contains 1 mM EDTA, for 12 hours at 4˚C and ultra-centrifuged at 4˚C to remove the accompanying materials.

Immunotoxin preparation:

150μL concentrated Botulinum Toxin was added to the same volume of Herceptin-SATP (molar ratio1:1) in a microtube and the mixture was wrapped in aluminum foil and kept for 18 hours at room temperature (Li et al., 2008).

High-Performance Liquid Chromatography (HPLC) Testing:

Diol-200-8.0X500-DL20S05-5008WT column was used to confirm the bioconjugation reaction by HPLC analysis. For column preparation, 0.1 M phosphate buffer including 0.15 NaCl was flown through the column at least for 1 hour. Then 10 μl of each samples was run for 50 minutes, and sample concentration was calculated by OD measurement at 280nm.

Cell culture

Two breast cancer cell lines; SK-BR-3 and BT-474; were purchased from Pasteur Institute of Iran and cultured at standard conditions at 37˚C, 95% humidity and 5%. The culture medium was replaced at 48 and 72 hour intervals, to feed the cell lines and maintain the cell growth. Each cultured cell line was divided into four groups, based on the following treatments: Group 1: Cells + medium + Botulinum toxin (20 μg/ml); Group 2: Cells + medium + Herceptin (20 μg/ml); Group 3: Cells + medium + Botulinum-Herceptin(20 μg/ml); Group 4: Cells + medium

Alamar blue (25μl) was added to the wells after 5 days and the fluorescence intensity was measured using a fluorescence reader at wavelengths 530nm (excitation) and 605nm (emission) to monitor the cell viability.

Results

HPLC procedures: The HPLC analysis of the bioconjugate preperation was performed according to the figure legends to find out various HPLC peaks for Trastuzumab, the toxin and the bioconjugate (immunotoxin) to ensure proper conjugation of two components (antibody and toxin).

As it is shown in figure 1, the second peak (Retention Time=28.542min) corresponds to the toxin. Peak 1 represents oligomeric form of the toxin, peak 2 corresponds to the monomeric form of the toxin, Peaks 3 and 4 correspond to the small molecules in the toxin buffer matrix.

Figure 2 shows the chromatogram of Herceptin, in which only one peak can be seen to respect the antibody monomer without any other contaminations or component.

Figure 3 shows the HPLC Analysis of the conjugation

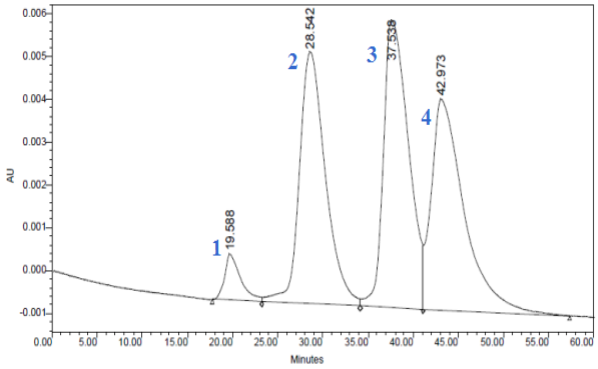

Figure 1. Chromatogram of the Toxin; Peak 1: Oligomeric Protein; peak 2: Monomeric Protein; Peak 3&4: Small Molecules. Column: YMC-Pack Diol-200-8.0X500-DL20S05-5008WT, Mobile phase: 0.1M Phosphate buffered saline (PBS) with 0.15M NaCl. Injection volume: 10μl. Runtime: 50 minutes. Detector: Ultraviolet Wavelength: 280nm. Flow rate: 0.5 ml/min

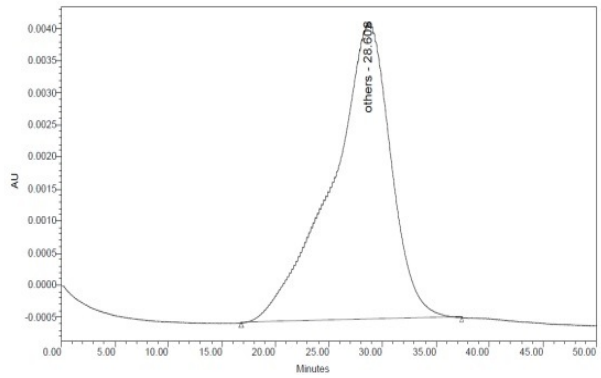

Figure 2. HPLC chromatograph of Herceptin: the peak Corresponds to Purified Herceptin (Retention Time: 28.608min). All the Chromatographic Conditions were the Same as Figure 1

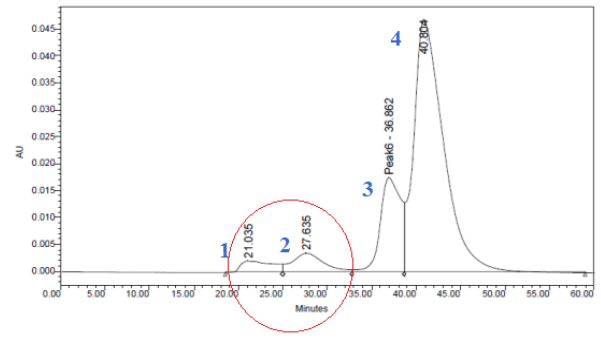

Figure 3. HPLC chromatograph of the Bioconjugate; Peaks 1&2: Toxin-antibody Bioconjugate; Peaks 3&4: Small Bounded Co-molecules

reaction mixture. Chromatographic conditions were the same as figure 1. The first and second peaks correspond to the bioconjugat molecules at two different molecular ratio. The conjugation precedures combine 1-3 molecules of toxin per antibody molecules. The two peaks here, coresponds to 1-3 bioconjugate molecules based on the molecular ratio of both molecules, and the 3rd and 4th peaks corespond to non-conjugated biomolecules.

Cell culture results:

We observed a substantial reduction in the number

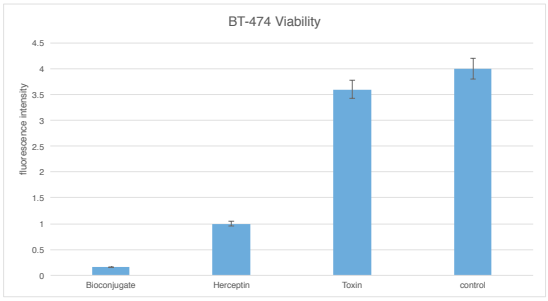

Figure 4. Fluorescence Intensity (average +/- standard deviation) of BT-474 Cell Line in Different Groups shows Significant Reduction (94.96%) in Cell Viability in the Bioconjugate Group (P<0.001)

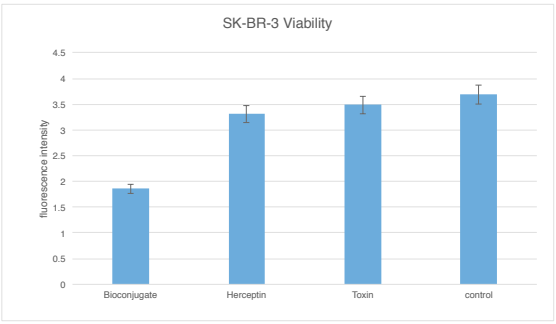

Figure 5. Fluorescence Intensity (Average +/-Standard deviation) of SK-BR-3 Cell Line in Different Groups Shows Significant Reduction (95.8%) Cell Viability in the Bioconjugate Group (P < 0.001)

of cells (94.96% and 95.8% in BT-474 and SK-BR-3 respectively) in both cell lines when treated with the bioconjugate when compared to the untreated control. Herceptin alone showed higher efficacy on BT-474 cell line (figure 4) compared to a condition where the antibody tested on SK-BR-3 cells (figure 5). Decrease in cell viability was also more dramatic when BT-474 cells treated with the bioconjugate.

Discussion

Resistance to Herceptin therapy has been documented in majority of patients with HER2 over expression (Ahmad et al., 2014). Only 20% of patients with amplified HER2/neu response to Herceptin. Moreover; A cardio toxicity profile has been described in more than 10 percent of the patients who used Herceptin, thus its dosage needs to be carefully adjusted and tailored to each patient, especially in elderly patients with cardiovascular problems (Sparano, 2001).

The new bioconjugate (immunotoxin) showed significant reduction in the viability of BT-474 and SK-BR-3 cell lines compared to both Herceptin and botulinum toxin individually. This result indicates significant synergistic reduction effect of bioconjugated drug on cancer cells viability 94.96% and 95.8% in BT-474 and SK-BR-3 respectively.

The efficacy of conventional cytotoxic medications is directly related to the ratio of dividing tumor cells to healthy cells, as they are distributed to all tissues through blood due to their low molecular weight and

lack of specificity. This will in turn lead to adverse side effects and resistance. In the last decade recombinant biopharmaceuticals have contributed in many novel therapeutics with higher specificity which renders them more effective and more tolerable (Olver, 2008). Researchers attach cytotoxins to macromolecules (e.g. antibodies, serum proteins, lectins, peptides, growth factors and synthetic polymers) to enhance their efficacy and reduce the unwanted effects. Antibodies play an important role in cancer therapies, their activity however, is short lived (Meyer et al., 2014).

Immunotoxin consist of a toxin attached to an antibody or a growth factor with a specific target to enter the cytoplasm and to mediate their effects on the normal cells (Avila et al., 2007; Singh et al., 2007). The bioconjugate improves the therapeutic profile of the antibody by increasing its tissue distribution, reducing its side effect on the healthy cells and increasing the half-life of the medication. In vivo experiments and clinical trials have established the effectiveness of new bioconjugates (Dao et al., 2012).

The first effective immunotoxins were plant derived toxins like ricin that could inhibit protein synthesis via inhibiting ribosomal RNA (Chan et al., 2004) and vascular injuries associated with this drug ameliorated by genetic modifications of Ricin A chain (Mohanraj and Ramakrishnan, 1995). Other herbal cytotoxins include: Saporin, Gelonin and Poke weed. Immunotoxins can inhibit cell growth through inhibiting protein synthesis and inducing apoptosis. (Houchins, 2000) Anti-c-Met/PE38KDEL for instance, targets stomach cells in this manner (Wei et al., 2011).

Breast cancer is the most prevalent cancer among women worldwide and the most frequent malignancy among Iranian women over the past few decades (Afsharfard et al., 2013), with an increasing trend. Herceptin (Trastuzumab) is a monoclonal antibody against the extracellular domain of HER2 with humanized complementary-determining regions (CDRs) of monoclonal antibody 4D5 (Albanell and Baselga, 1999). Herceptin therapy adjuvant to chemotherapy has shown to increase therapeutic response and survival in HER2/neu positive patients (Perez, 2001)

Barok et al investigated the effect of a new conjugate trastuzumab-DM1 on four gastric cancer cell lines (N-87, OE-19, SNU-216 and MKN-7). N-87 and OE-19 showed greater sensitivity compared to Herceptin alone. MKN-7 showed moderate response while a limited efficacy was observed in SNU-216. The study was performed in in-vitro setting where its analysis supported our data (Barok et al., 2011).

Lattová et al used Herceptin in combination with Lipoplex mixed with plasmid DNA against Herceptin resistant cell lines and showed a positive response when measured by trypan blue after 72 hours (Lattova et al., 2010). In this study we used Alamar blue which is more sensitive than trypan blue.

Similar in-vitro and in-vivo experiments have confirmed the efficacy of immunotoxins in treatment of different types of malignancies. Moreover we can isolate the light chain of the toxin responsible for its protease activity and utilize the Herceptin to function as the toxin heavy chain for cell targeting and endocytosis.

Finally, this study demonstrated an increase in the efficacy of Herceptin when bound to Botulinum toxin. This could in turn lead to a lower dose of administration and therefore lower rates of adverse side effects.

For further study, in animal model, the L-Chain of the Botulinum Toxin needs to be separated from cell-bind domain and conjugated into antibody in order to prevent destructive effect on normal cells.

Acknowledgements

We would like to thank all our study participants for their commitment to this study. Financial support was provided by Iran University of Medical Science and Iran Food and Drug Organization (IR.FDA).

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