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Combined treatment of human MCF-7 breast carcinoma with antibody, cationic lipid and hyaluronic acid using ex vivo assays

Bartusik, Dorota; Tomanek, Boguslaw; Lattova, Erika; Perreault, Hlne; Fallone, Gino

Abstract

The effective targeting of malignant cell surface antigens is essential in cancer therapy. Resistance to treatment and rapid invasion of cancer cells are the main causes of cancer mortality. Despite intense research efforts, treatments often have demonstrated insufficient outcomes in clinical applications.

The aim of the present study was to determine whether combined administration of monoclonal antibody (Herceptin^{}, trastuzumab) and anti-HER-2 (clone CB11) with hyaluronic acid (HA) and lipoplex (containing lipofectamine (LipA) and plasmid DNA) can produce a synergistic reaction to increase the therapeutic effect of monoclonal antibodies. To assess the treatment response, we cultured a 3-D MCF-7 cell line overexpressing HER-2 and CD44 receptors. The high density 3-D cell aggregation in the hollow fiber bioreactor (HFB) used for the cell culture was monitored with the use of proton magnetic resonance imaging (¹H MRI). In addition, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) was used in combination with HPLC (high performance liquid chromatography) to evaluate structural changes in the proteins contained in treated cells. The study showed that incorporation of antibodies into targeted lipoplex results in more efficient delivery of the complex to tumor cells. The viability of cells decreased mostly due to cellular uptake of lipoplex and binding of the antibodies to the cellular surface receptor. The data also demonstrate that HA could be used to enhance treatment efficacy of trastuzumab and anti-HER-2 (clone CB11) in breast cancer cell cultures.

Keywords: MCF-7 breast cancer cells; Immunotargeting; Three-dimensional cell culture; MRI; MALDI-MS

Abbreviations: MRI, magnetic resonance imaging; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry

1. Introduction

HER-2 positive breast tumor cells have more extensive invasion with frequent metastasis than HER-2 negative cells [\[1\]](#). Moreover, HER-2 receptors are colocalized and coimmunoprecipitated with hyaluronan receptor CD44 in all mammary carcinoma cell lines [\[2\]](#). In previous studies it was demonstrated that both receptors are physically linked and interact with each other via interchain disulfide bonds [\[3\]](#). However simultaneous targeting of HER-2 and CD44 receptors has not been studied in breast MCF-7 carcinoma.

HER-2 is a transmembrane oncoprotein, belonging to the epidermal growth factor receptor family, with tyrosine kinase activity [4]. Overexpression of this protein has an impact on intracellular signaling and activation of genes involved in cell growth. This impact is associated with shorter survival, enhanced aggressiveness and other poor prognostic factors [4]. CD44 is a transmembrane glycoprotein classified as a member of the hyaladherin-receptor family. Multiple functions and cellular responses have been attributed to HER-2 and CD44, including growth promotion, invasion and cell adhesion [5].

Immunotargeting of the HER-2 receptor is essential for studying the viability of cancer cells in majority of human carcinomas. Immune-based treatments to target HER-2 can consist of administering monoclonal or modified polyclonal antibodies. However, in clinical practice most patients who achieve an initial response to antibody treatment generally acquire resistance within 1 year [6]. HER-2 transmits information from breast cancer cell surface into cytoplasm and is crucial in growth inhibition and cellular adhesion [7]. Breast cancer cells overexpressing HER-2 and CD44 receptors tend to be much more elastic than normal cells, as they exhibit a much stiffer extracellular matrix (ECM) [8]. These properties of cancer cells are responsible for cellular invasion in healthy tissue, which induces and controls tumor growth. Although breast cancer surface antigens are studied extensively, there is almost no information available regarding the binding of hyaluronic acid (HA) to CD44, both known to be involved in growth inhibition and in a variety of tumor growth processes [9].

It has already been shown that Lipofectamine (LipA) used in vitro transfers the transfected material into the cell through lipid membrane [10]. This process results in the direct delivery of the protein to the target without interfering with cell membrane and influences several cancer cells' activities, including growth and adhesion. LipA is composed of the polycationic lipid 2,3-dioleoyl-*N*-[2(sperminecarboxymido)ethyl]-*N,N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE) in membrane filtered water. It is known that LipA is lipophilic and interacts electrostatically with negatively charged plasmid DNA or other proteins and enables passing through the hydrophobic cell membrane [11]. Therefore, we used cationic LipA–plasmid DNA complex (lipoplex) combined with antibodies, directed against cell surface molecules, to assess their efficiency.

Cellular adhesion is closely associated with tumor invasiveness. HA by interactions with cell surface receptors such as CD44 and receptor for hyaluronic acid (HA) is involved in cell adhesion processes [12]. The effect of HA on healthy cells has been studied extensively [13], however, little is known about HA's effect on malignant cells *ex vivo*. Therefore, *ex vivo* matching of cancer cells to the appropriate drugs would increase the effectiveness of cancer therapy.

In the present work we targeted CD44 and HER-2 to determine if HA or antibody binding had any correlation with growth inhibition and its efficacy. We used proton magnetic resonance imaging (MRI) for non-invasive monitoring of the three-dimensional (3-D) high density cell culture. Breast cancer cells within the human body interact with neighboring cancer cells and with ECM components to establish a unique 3-D organization [14]. The 3-D cell cultures are essential to induce *ex vivo* formation of multicellular tissue-like structures [15]. Therefore the

present paper compares the properties of breast cells together with treatment efficacy and provides some insight in the therapeutic use of antibodies in human MCF-7 cell cultures.

2. Experimental

2.1. Reagents

For targeting HER-2 we used Herceptin[®] (trastuzumab) (Genentech Inc., San Francisco, CA) and anti-HER-2 (clone CB11) (Abcam, Cambridge, MA) alone and with lipoplex containing Lipofectamine (LipA) and plasmid (Innovita Inc., Geithesburg, MD). All compounds for the cell culture were supplied by Fisher Scientific (Oakland, ON). Phenylhydrazine and 2,5-dihydroxybenzoic acid were obtained from Sigma (St. Louis, MO). Trypsin and the enzymatic assay kit were from Promega (Madison, WI). Trypan blue was supplied by Sigma–Aldrich (Oakville, ON).

2.2. Three-dimensional cell culture

Human malignant breast epithelial (MCF-7) cells were obtained from the American Type Cells Collection (Manassas, VA). HER-2 and CD44 overexpression of these cell lines was confirmed by cytometric analysis [\[16\]](#) and [\[17\]](#). The MCF-7 cultures were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with a 5% fetal bovine serum (FBS), 2 mM l-glutamine, 15 g/l sodium bicarbonate, 10 mM sodium pyruvate and 100 IU penicillin/100 µg streptomycin and 50 µg/ml gentamycin. For the study we used the Hollow Fiber Bioreactor (HFB, FiberSystem Cell Inc., Frederic, MD) which contains a specific fiber to allow cells to grow on their surfaces. In our study we used one fiber with 0.1 µm diameter pores. Nourishing elements and waste are delivered in a controlled manner through the fibers' pores [\[14\]](#). We used collagen solution to create an ECM between cells and fiber. The polysulfone fiber was flushed with 10 ml of solution containing 1 mg collagen per 1 ml PBS. In this manner MCF-7 cells growing originally in suspension build up a 3-D solid tumor. During 4 weeks of culturing, the media were replaced each week. The media flow rate was maintained at 14 ml/min and controlled by a peristaltic pump. Twelve HFBs were used for the study: 2 for controls (untreated cells), 2 for each tested drug (5 drugs). Two untreated (controls) HFBs were continuously exposed to fresh media without any drug.

The number of cells was determined using Trypan blue exclusion method [\[18\]](#). Briefly, MCF-7 cells were harvested from HFB, seeded in 6-well microplates and exposed to 0.4% (w/v) Trypan blue dye solution. Cell numbers were determined manually with a hemacytometer chamber (Hausser Scientific, Horsham, PA). Cells were cultured in the HFB to reach 5×10^9 cells/ml densities.

2.3. Treatment of CD44 with hyaluronic acid

MCF-7 cells (10^9 cells/ml) in two HFBs were incubated with HA (0.05, 0.5, 5, 10, 50, 100, 200, 500 and 1000 µg/ml) for 72 h at 37 °C in 5% CO₂ and 95% air.

2.4. Treatment of HER-2 receptor and transfection

Trastuzumab (0.05, 0.5, 5, 10, 50, 100, 200, 500 and 1000 µg/ml) and anti-HER-2 (clone CB11) (0.05, 0.5, 5, 10, 50, 100, 200, 500 and 1000 µg/ml) were used for the 72 h treatment of MCF-7 cells cultured in HFB. After 72 h of treatment, the culture media in HFB was refreshed.

The transfection of MCF-7 cells using cationic LipA–DNA complex (lipoplex) was also investigated. One day before transfection the human MCF-7 cells were maintained in serum-free growth media without antibiotics. Each sample was treated with trastuzumab and anti-HER-2 (clone CB11). The ratio of LipA/trastuzumab and LipA/anti-HER-2 (clone CB11) was 3/1 (w/w) [19].

Plasmid DNA containing β-gal was diluted in serum-free media and added to the LipA/trastuzumab and LipA/anti-HER-2 (clone CB11) at a DNA/LipA ratio of 1/9 (µg/nmol). Then the solution was mixed with the medium for 30 min to produce lipoplexes:trastuzumab–plasmid DNA complex or anti-HER-2 (clone CB11)–plasmid DNA complex. The transfection was performed with a 3 ml volume of complexes of 10^9 cells/ml. After 48 h, serum-free media were replaced with serum containing media. The cells were washed with PBS and the β-gal activity was measured by enzymatic assay (Promega, E2000) following the manufacturer's recommendation. Moreover, viability of MCF-7 cells was measured after 72 h treatment with LipA alone and plasmid DNA containing β-gal without antibody. The concentrations of LipA were at ratio of 3/1 (w/w) to previously used antibody concentrations (0.05, 0.5, 5, 10, 50, 100, 200, 500 and 1000 µg/ml), as described above. Plasmid DNA containing β-gal was used at ratio of 1/3 to antibody concentration.

2.5. Treatment of HER-2 and CD44 receptors

Trastuzumab (0.05, 0.5, 5, 10, 50, 100, 200, 500 and 1000 µg/ml) and anti-HER-2 (clone CB11) (0.05, 5, 10, 50, 100, 200, 500 and 1000 µg/ml) were mixed with HA in 1/1 ratio. A 5 ml volume was used to target MCF-7 culture with density 5×10^9 cells/ml.

2.6. Cell adhesion

After 72 h of exposure to HA, two HFBs were used for the 5-h study of cell adhesion. Briefly, 50 ml of media with single MFC-7 cells (2×10^5 cells/ml) were perfused through the HFB with 5×10^8 cells/ml using a flow from 5 to 14 ml/min for 45 min. Single cells were obtained using 10 ml of 0.1% trypsin solution. Briefly, 1 mg of MCF-7 cells was harvested from HFB and resuspended in trypsin water solution [18]. The temperature in HFB and media reservoir was 37 °C. The number of adhesive cells (NACs) was calculated according the formula (Eq. (1)).

(1) $NAC = NAC_0 - NAC_{45}$ where NAC is the number of single MCF-7 cells attached to cancer tissue in HFB cartridge; NAC_0 is the number of single cells at time 0 (min) in reservoir media; NAC_{45} is the number of single cells in reservoir media at 45 (min) after addition of adhesive media with single cells.

2.7. Assessment of serum

The influence of different concentrations of FBS to the cell growth/inhibition with trastuzumab was assessed by plating 4×10^4 cells per 35 mm Petri dish containing growth media. Trastuzumab (0.05, 0.5, 5, 10, 50, 100, 200, 500 and 1000 $\mu\text{g/ml}$) was added separately to the medium supplemented with 1%, 5% and 10% of FBS.

2.8. MRI and aggregation

All MR images were collected with 9.4 T/21 cm magnets (Magnex, UK) and TMX console (NRC-IBD). The HFBs with cell cultures were placed in a 10 mm diameter transmit/receive radio frequency (rf) coil within the magnet bore. For imaging, a spin echo pulse sequence was used with echo time (TE)/repetition time (TR) = 12.4 ms/5000 ms. One slice of 1 mm was acquired with matrix size of 256×256 and field of view $3 \text{ cm} \times 3 \text{ cm}$. All imaging parameters were the same for each HFB. Throughout the MRI experiments, the HFBs were maintained under incubator-like conditions (37 °C, 5% CO₂ and 95% air). Region of interest (ROI) was drawn around the region of the tumor cells before treatment. All remaining voxels within this region were counted and multiplied by the voxel dimensions to produce an enhancement volume.

2.9. HPLC and MALDI-MS analysis

Prior to analysis, cell samples were digested with trypsin in 25 mM ammonium bicarbonate buffer, pH 7.8, for 20 h at 37 °C, at a substrate-to-enzyme ratio of 50:1. Aliquots of the digests were fractionated on a System Gold HPLC chromatograph equipped with a System Gold 166 Ultra violet (UV) detector and 32-Karat software (Beckman–Coulter). For reversed phase HPLC, the analytical column Vydac 218 TP54 Protein & Peptide C18 (Separation Group, Hesperia, CA) was used. The samples (5 μL injected) were eluted with 5% acetonitrile (ACN) in water as solvent A and 90% ACN in 0.1% trifluoroacetic acid as solvent B at a flow rate 0.5 ml/min. An elution gradient was applied from 5% to 70% ACN over 60 min. UV detection was performed at 245 nm. All fractions were collected manually and concentrated in vacuo prior to MS analysis. MALDI-MS and MS/MS spectra were acquired on the Manitoba/Sciex prototype quadrupole/TOF (QqTOF) mass spectrometer (commercial model sold as QSTAR by Applied Biosystems/MDS Sciex, Foster City, CA). Typically, the samples were spotted onto 2,5-dihydroxybenzoic acid (matrix) predeposited on the surface of a MALDI target. Peptide mass fingerprinting was performed in positive MS mode and peptide identifications were achieved with Mascot database search (www.matrixscience.com). Search inputs in MS/MS included the measured precursor and fragment ion masses. The error tolerances used for the monoisotopic ions were 0.2 Da and the query was made for human and mammals.

2.10. Statistical analysis

Results were expressed as a mean $\pm$ SD. Differences between groups at each time point were identified by one-way ANOVA. Statistical comparison between two independent variables was determined by two-way ANOVA with Dunnet's correction performed post hoc to correct multiple comparisons. The *p*-values <0.05 were considered statistically significant. All data reported here are from sets of six separate experiments. Error bars in all graphs represent the standard error of the mean. Data were analyzed using the Sigma Stat Soft (Chicago, IL) software.

3. Results

3.1. Cell growth and treatment of HER-2 and CD44

[Fig. 1](#)A and B shows a typical proton MR image of MCF-7 cells cultured in the HFB after 4 weeks of the continuous culture. The cell images were used for the visualization of the regions of cells' harvesting. The cells were able to adhere to the fiber during 5 weeks. For the continuous cell growth with the appropriate density the media flow was maintained at 14 ml/h. [Table 1](#) shows the characterization of the MCF-7 cells in the HFB device. The 3-D cultures started with cell densities of 5×10^5 cells/ml and after 4 weeks cells reached a density of 5×10^9 /ml. During the time period between 1 and 4 weeks, the volume of the cells increased from 15 ± 0.5 to 55 ± 0.9 mm³.

Fig. 1.

An MR image of the MCF-7 cells in the hollow fiber bioreactor at 9.4 T. SE pulse sequence (TR/TE = 5000 ms/12.8 ms, FOV = 3 cm × 3 cm, slice thickness 1 mm and matrix 256 × 256) was used. The red solid lines indicate the cells area before treatment (A) and after 72 h (B) of treatment with trastuzumab/lipoplex. Yellow solid line is the fiber.

Table 1. 3-D cell cultures characterization.

Time [week]	1	2	3	4
Cell number [cell/ml]	5×10^5	5.2×10^6	6.5×10^8	5×10^9
Viability [%]	93 ± 4	91 ± 6	90 ± 3	88 ± 2
Cells volume [mm ³]	15 ± 0.5	21 ± 0.5	42 ± 0.6	55 ± 0.9

We observed that more HER-2 positive cells responded differently to antibodies than to lipoplex or HA. Moreover, effects on cell growth inhibition appeared to correlate with an improved delivery of trastuzumab to targets. Within 72 h the viability decreased from $94 \pm 1\%$ to $53 \pm 5\%$ at the trastuzumab concentration of 0.05–1000 µg/ml ([Fig. 2A](#)) and from $96 \pm 2\%$ to $60 \pm 2\%$ for 0.05–1000 µg/ml of anti-HER-2 (clone CB11) ([Fig. 2B](#)) and from $93 \pm 1\%$ to $47 \pm 5\%$ for 0.05–1000 µg/ml of HA ([Fig. 2C](#)).

Fig. 2.

Growth inhibition of the MCF-7 cells using (A) trastuzumab (0.05, 0.5, 5, 10, 50, 100, 200, 500 and 1000 µg/ml), (B) anti-HER-2 clone CB11 (0.05, 0.5, 5, 10, 50, 100, 200, 500 and 1000 µg/ml) and (C) HA (0.05, 0.5, 5, 10, 50, 100, 200, 500 and 1000 µg/ml). Statistically significant differences to control are indicated with an asterisk (*) and to initial time points are indicated with (#).

Treatment of MCF-7 cells with 50, 100, 200, 500 and 1000 µg/ml of trastuzumab, significantly decreased the growth with respect to control at 24, 48 and 72 h ([Fig. 2A](#)). For these trastuzumab concentrations the viability of 3-D MCF-7 cells at 6 and 72 h decreased from $80 \pm 3\%$ to $62 \pm 2\%$, from $86 \pm 7\%$ to $59 \pm 6\%$, from $84 \pm 5\%$ to $55 \pm 8\%$, from $82 \pm 9\%$ to $56 \pm 9\%$ and from $82 \pm 3\%$ to $53 \pm 5\%$, respectively. There were no significant changes in cell growth for trastuzumab concentrations lower than 50 µg/ml ([Fig. 3A](#)).

Fig. 3.

Viability of the MCF-7 cells after (A) 6 h and (B) 72 h exposure to trastuzumab, trastuzumab/lipoplex and trastuzumab/HA. Statistically significant differences to control are indicated with an asterisk (*) and trastuzumab alone vs. trastuzumab/lipoplex are indicated with (+).

The exposure of cells to anti-HER-2 (clone CB11) showed significant growth-inhibitory effects for concentrations of 200 µg/ml and higher. After 72 h, the MCF-7 cells treated with anti-HER-2 (clone CB11) showed viability equal to $89 \pm 2\%$, $84 \pm 4\%$, $80 \pm 2\%$, $77 \pm 5\%$, $73 \pm 5\%$, $73 \pm 7\%$, $64 \pm 2\%$, $62 \pm 9\%$ and $60 \pm 2\%$ for concentrations of 0.05, 0.5, 5, 10, 50, 100, 200, 500 and 1000 µg/ml, respectively (Fig. 3B). We observed that trastuzumab effectiveness was higher than anti-HER-2 (clone CB11) at each time point.

After 72 h exposure to trastuzumab/lipoplex (3/1) cell viability decreased and corresponded to the observation made with an increased concentration of trastuzumab. MCF-7 cells treated with trastuzumab/lipoplex (3/1) showed increased transfection efficiency with fourfold increase of β -gal expression as compared with non-treated one. We did not observe decreases in the growth of MCF-7 control cells treated with LipA only. However, viability of cells treated with plasmid DNA containing β -gal reached values higher than cell treated with trastuzumab/lipoplex and lower than cells treated with trastuzumab, after 72 h. The viability of cells treated with plasmid DNA containing β -gal was $83 \pm 2\%$, $80 \pm 4\%$, $78 \pm 3\%$, $75 \pm 6\%$, $73 \pm 5\%$, $70 \pm 2\%$ and $69 \pm 2\%$, after 72 h. Cell treatment with anti-HER-2 (clone CB11)/lipoplex (3/1) caused significant changes in viability for concentrations 200 µg/ml or higher, as compared to control (Fig. 4A and B).

Fig. 4.

Viability of the MCF-7 cells (A) after 6 h and (B) 72 h exposure to anti-HER-2 clone CB11 and anti-HER-2 clone CB11/lipoplex and anti-HER-2 clone CB11/HA. Statistically significant differences to control are indicated with an asterisk (*) and anti-HER-2 clone CB11 alone vs. anti-HER-2 clone CB11/lipoplex (+).

Exposure of MCF-7 cells to trastuzumab/HA (1/1) showed a continuous decrease in growth which was significant compared with that of control experiments for concentrations of 50 µg/ml and higher (Fig. 4A and B). After 72 h, trastuzumab/HA (1/1) showed more than 20% growth-inhibitory effect as compared with trastuzumab alone and more than 10% as compared with

efficacy of HA. On the other hand, anti-HER-2 (clone CB11)/HA decreased the growth by 20% and 5% more when compared to anti-HER-2 (clone CB11) and HA treatments, respectively. These results suggest that HA binding to CD44 is more effective when HER-2 was treated with trastuzumab than with anti-HER-2 (clone CB11). HA combined with trastuzumab or anti-HER-2 (clone CB11) significantly decreased cells viability when compared to trastuzumab and anti-HER-2 (clone CB11) alone or HA alone. After 72 h, trastuzumab/lipoplex (3/1) showed more than 20% and 10% higher decreases in cell growth, as compared with trastuzumab and anti-HER-2 (clone CB11)/lipoplex, respectively. In general cells were much more sensitive to trastuzumab than to anti-HER-2 (clone CB11) treatments. The effect of trastuzumab/lipoplex was higher than anti-HER-2 (clones CB11)/lipoplex but it did not differ significantly (p -value >0.05) even at 50 $\mu\text{g/ml}$ and higher drug concentrations. The tests of different serum concentrations (1–10%) showed that trastuzumab induced cell growth inhibition was independent of serum concentrations. Therefore cells were treated with Herceptin and anti-HER-2 (clone CB11) in media with 5% serum. To avoid interference of LipA with serum for trastuzumab/lipoplex and anti-HER-2 (clone CB11)/lipoplex treatments, cells were treated in serum-free media.

Using variable laminar flow from 5 to 14 ml/h in the HFB chamber we also examined the adhesion ability of single MCF-7 cells to 3-D tumor tissue. The single cancer cells showed 30% adhesion to cancer tissue in the HFB, after 45 min. The adhesion increased about 2% at each 1 ml/h flow increment.

3.2. HPLC and MALDI-MS analysis

The changes in targeted cells after treatment at the protein level were next studied by HPLC and MALDI-MS analysis. The HPLC–UV chromatograms of trypsin digested samples differed not only between those original untreated cells and trastuzumab or anti-HER-2 (clone CB11) treated cells, but the influence of lipoplex on transfection of cells was also clearly observed ([Fig. 5](#)). To verify the composition of compounds in individual HPLC separated peaks, all fractions were examined by MALDI-MS. Numerous peaks from all samples corresponded mainly to peptides originated from immunoglobulin, T-cell receptors and other common proteins. However, HPLC-profiles and intensities of peaks detected in MS spectra of single fractions indicated variations in products after digestions. For example, the most abundant peak in the MS spectra ([Fig. 6](#)) of HPLC fractions with elution time 28 min ([Fig. 5A](#) and [B](#)) in both samples treated with anti-HER-2 (clone CB11) antibody was detected at m/z 1117.5164 and was not observed in the spectra of untreated cells. This peak could be assigned to a peptide originated from antigen T-cell receptor. According to data obtained, the peak at m/z 1499.8047 ([Fig. 6A](#)) matched to the amino acid sequence HDFEVRSGDVVNGR ($M_{r_{\text{calc.}}}$ 1498.7277) described in the breast and ovarian cancer susceptibility protein [\[19\]](#). In the spectrum of the corresponding fraction from anti-HER-2 (clone CB11)/lipoplex treated sample ([Fig. 6B](#)), additional peaks at m/z 974.4629 and 1529.6974 most probably matched to the tryptic peptides DTPVPGDGTGR ($M_{r_{\text{calc.}}}$ 973.4465) and SCTVIDSRVLGSHR ($M_{r_{\text{calc.}}}$ 1528.7780) found in the heavy chain immunoglobulin. Peaks detected exclusively in fractions obtained from samples treated with trastuzumab in the presence LipA observed at m/z 1086.5, 1235.5, 1495.6 and 1527.6 corresponded to tryptic peptides originating from Fibrillin ([Fig. 7](#)). In MCF-7 cell samples treated with trastuzumab alone or in combination with lipoplex additional peaks appeared at m/z 2634.1, 2796.2 and 2958.2 in

comparison with peaks detected in samples of trypsin digested MCF-7 cells only. These additional peaks corresponded to glycopeptides, which were more abundant in samples treated with antibodies in combination with lipoplex. Other glycopeptides were observed at m/z 2431.1, 2593.2 and 2650.2 and were not detected in other MCF-7 samples (Fig. 8). MS/MS-fragmentation patterns of these ions corresponded to glycoforms of a peptide with EEQYNSTYR amino acid sequence and bearing neutral biantennary fucosylated glycans on the N residue. These glycoforms originated from human IgG [20] and [21]. It is important to point out that complete sequencing of proteins and identification of all proteins were not the primary intentions of this work and are well beyond this study. However, MS data of selected compounds in the samples analyzed provided good evidence that there is an influence by incorporating antibodies into MCF-7 cells under the conditions described in this paper.

Fig. 5.

HPLC–UV chromatograms obtained from trypsin digested total MCF-7 cells treated with: (A) anti-HER-2 (clone CB11), (B) anti-HER-2 (clone CB11)/lipoplex, (C) trastuzumab and (D) trastuzumab/lipoplex.

Fig. 6.

MALDI-MS spectra recorded from the HPLC fractions with elution time 28 min (Fig. 5), obtained from trypsin digested MCF-7 cells treated with: (A) anti-HER-2 (clone CB11) only (Fig. 5A) and (B) anti-HER-2 (clone CB11)/lipoplex (Fig. 5B). All ions are $[M+H]^+$.

Fig. 7.

MALDI-MS spectrum of the HPLC fraction (elution time 20 min), obtained from trypsin digested MCF-7 cells treated with trastuzumab/lipoplex. All labeled ions are $[M+H]^+$.

Fig. 8.

MALDI-MS spectrum of glycopeptides recorded from a HPLC fraction obtained from trypsin digested MCF-7 cells treated with trastuzumab/lipoplex. (A) At m/z 2796.1, all ions are $[M+H]^+$ and (B) tandem mass spectrum of glycopeptide observed in MS spectrum. Symbols: $\circ$, mannose (Man); $\bullet$, galactose (Gal); $\blacksquare$, *N*-acetylglucosamine (GlcNAc); $\blacktriangle$, fucose (Fuc).

4. Discussion

Breast cancer cells overexpress many receptors and biomarkers, which can be used as drug targets. There is a clinical evidence that trastuzumab, a monoclonal antibody targeting the HER-2 receptor, is an important component of the first-line treatment for patients with metastatic breast cancer. However, the clinical issue of resistance to HER-2 directed therapy still remains unsolved [22]. As a result of the resistance, overexpression of HER-2 has been associated with more aggressive tumor behavior and poorer prognosis. Therefore, the combined administration of trastuzumab with other non-toxic and cost-effective agents is required.

Previous studies of breast carcinoma reported that trastuzumab alone did not inhibit the growth by more than 30–50% in relation to doses of 10–1000 $\mu\text{g/ml}$ over 72 h [23]. Moreover, when trastuzumab was combined with other cytotoxic agents (e.g. cisplatin) it was difficult to evaluate the combined effect by using conventional in vitro techniques because cytotoxic agents not only affect the viability of cancer cells but also affect trastuzumab [24]. To avoid unwanted toxicity

effects, we used biocompatible and non-toxic agents, HA and lipoplex combined with antibody. The combined treatments of HER-2 based on trastuzumab or anti-HER-2 (clone CB11) and lipoplex produced a significant growth-inhibitory effect higher than the monoclonal antibody alone.

The other findings of the present study suggest that HA appears to be a growth-inhibitory agent in breast carcinoma cells due to binding to CD44 receptors. HA is used in clinical treatment due to its unique viscoelastic and hydrophilic properties along with its biocompatibility and non-immunogenicity [25]. Previous studies [25] showed that the in vitro administration of more than 320 mg/ml of HA inhibited proliferation of murine melanoma cells by 50–90%. In particular, naturally occurring high levels of HA in colon cancer cells significantly inhibit the tumor growth [26]. However, the inhibitory effect of HA on cell growth can be masked by the fact that the HA is degraded to unknown forms in the cancer cells by naturally occurring hyaluronidases (HYAL1). It is still controversial whether HYAL1 is a tumor promoter or a suppressor. It was reported lastly that HYAL1 may provide a new class of anti-cancer therapeutics without side effects [27]. Binding of HA to a CD44 can activate the HER-2 tyrosine kinase activity and thus speed up the rate of cancer progression [28].

The results also indicate possible interactions between HER-2 and CD44 during cancer cell growth. Various responses observed in MCF-7 cells may reflect changes in hydrophobicity of the substrate or variations in the molecular interactions between drugs and cells. In general HA and lipoplex showed a good affinity for MCF-7 cells that express high levels of the CD44 and HER-2 receptor.

The results presented here showed variability of carcinoma cells viability when treated with different antibodies. The findings indicate that the combined use of antibodies and resistance to treatment can be studied easily and cost-effectively in HFB that mimics in vivo tumor condition. The CD44 receptor, such as the HER-2 receptor is expressed in a variety of tumors (breast, colon, intestinal and brain), as well as melanoma, basal cell carcinoma, and stem cells [29]. Therefore, simultaneous and combined treatments may be extended to several other targets through the proper choice of biocompatible agents. Moreover the co-expression of HER-2 and CD44 in JIMT-1 breast cancer cells [30] and ovarian tumor cells [28] was already studied.

MRI based measurement provides an alternative to assess volume of tumor. In this work, we investigated the ability to obtain tumor volume by ROI-based measurements. For the ROI-based measurement, the tumor area was calculated by the summation of all tumor pixels in each axial slice and multiplication by the slice profile. However, another method to calculate tumor volume is the diameter-based measurements. For the diameter-based measurements [31], the three tumor diameters (longitudinal, anteroposterior and lateral) in each orthogonal measurement plane were measured. The tumor volume was computed using the formula for ellipse. The longitudinal diameter along the long axis was measured on the sagittal images; the anteroposterior diameter (orthogonal to the longitudinal diameter) was measured on the sagittal images; and the largest lateral diameter was measured on the axial images.

In spite of the positive outcome of these experiments, the data indicate that successful combined delivery of antibodies to HER-2 receptor and plasmid DNA to cell nuclei decreases cells

viability. More specifically LipA plays a direct role in transfection by promoting DNA fusion and membrane destabilization that allows more efficient surface targeting using antibodies.

5. Conclusion

Firstly, our findings showed that it is possible to grow human MCF-7 breast carcinoma 3-D culture ex vivo suitable for MRI and MALDI-MS techniques. Secondly, monoclonal antibody may be targeted with MCF-7 cells using a cationic lipid. Thirdly, the observed decreases in MCF-7 cell growth were more evident when the treatment with HER-2 and CD44 was combined.

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