Volume 6, Issue 5, 2016, 1445-1449 ISSN 2069-5837
Biointerface Research in Applied Chemistry
www.BiointerfaceResearch.com
Review Article Open Access Journal
Received: 04.05.2016 / Revised: 15.08.2016 / Accepted: 15.09.2016 / Published on-line: 13.10.2016

3-D cell cultures as a tool for studying cellular aspects of trastuzumab treatment

Dorota Bartusik [1,*], David Aebisher [2], Jacek Tabarkiewicz [3]
[1]Southern Polytechnic State University, Department of Biology and Chemistry, 1100 South Marietta Parkway, Marietta, Georgia 30060, USA
[2]Shorter University, Natural Sciences Department, 315 Shorter Ave, Rome, Georgia 30165, USA
[3]University of Rzeszów, Centre for Innovative Research in Medical and Natural Sciences, Warzywna 1A, 35-959 Rzeszów, Poland
*corresponding author e-mail address: ***dorota.bartusik@gmail.com***

ABSTRACT
Human epidermal growth factor receptor-2 (Her-2) is a promising target in immunotherapy. Monoclonal antibody drugs such as Trastuzumab target Her-2 and are currently used in immunotherapy. Targeting overexpressing Her-2 receptors on the surface of human tumors provides the imputus to develop improved Her-2 targeted drug delivery systems. We summarize recent examinations of Trastuzumab at the cellular level and the basic nature of Trastuzumab-cell interactions.
Keywords: *three dimensional cell cultures, cancer, trastuzumab, treatment, Her-2 receptor.*

1. INTRODUCTION

The reasons for the lack of effectiveness of many cancer drugs are often unknown therefore new approaches are needed to examine drug-cell interactions. Direct drug delivery to cancer cells is a critical approach in the application of new therapies to treat tumors. Common immunotherapies employ monoclonal antibodies such as Trastuzumab which effectively treats Her-2 positive breast carcinoma (Figure 1).

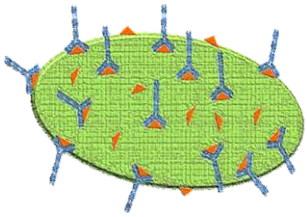

Figure 1. Trastuzumab targeting Her-2 receptor on the surface of cancer cell.

The tumor oncogenic tissue is characterized by an increased expression of human epidermal growth factor 2 (Her-2). Binding of Trastuzumab to Her-2 receptors prevents the binding of growth factors to receptors resulting in tumor growth inhibition, lower Her-2 expression and finally Her-2 inhibition [1,2]. Trastuzumab in combination with chemotherapy has been demonstrated to be more effective than chemotherapy alone [3]. Recently, research has shown that Trastuzumab, by blocking the Her-2 pathway, has significant clinical benefits in metastatic breast cancer treatment. Although Trastuzumab is an efficient drug, resistance to Trastuzumab is a common and challenging problem. Some breast cancer cells make (overexpress) too many copies of a particular gene known as Her-2. The Her-2 gene makes a protein known as a Her-2 receptor. These Her-2 receptors receive signals that stimulate the cells to grow and multiply breast cancer cells. Cells with Her-2 receptors receive excess growth signals and start growing and multiplying abnormally. Breast cancer cells that overexpress the Her-2 gene are said to be Her-2-positive. Several studies have shown that the Her-2 receptor is overexpressed in tumors of up to 30% of breast cancer patients [1-4]. The known Her-2 overexpressing breast cancer cell lines like SK-BR-3, BT-474, MDA-MB-361, and MDA-MB-453 are used in multiple preclinical studies. Research shows that Trastuzumab reduces breast cancer recurrence by ~50% [5]. Also, Trastuzumab therapy is a treatment option for patients with ovarian cancers when the tumor is Her-2 overexpressing [6]. The merging of immunotherapy and tumor detection can result in the development of new drug delivery systems. Trastuzumab can target Her-2 receptors wherever they may be in the body. The drug works on the surface of cells but can still enter the cell when it is modified in a drug delivery system with carrier particles and other chemotherapeutics. Trastuzumab containing particles are efficiently internalized into cells and contained within internal structures called endosomes [7]. Thus, the next critical issue in research is to construct modified Trastuzumab that results in an increased killing effect inside the cell with enhanced therapeutic effect. Currently, over 50 new antibody drug conjugates are in clinical development [8]. These compounds display cytotoxicity lower than standard chemotherapeutic alone. To improve Trastuzumab efficiency, current methodology links Trastuzumab with others reagents which improve delivery and efficiency. Her-2 is one of the most promising targets in immunotherapy. Her-2, which is a member of the ErbB family of receptors, is overexpressed on the surface of tumors. The family of ErbB consists of EGFR (ErbB-1), Her-2 (ErbB-2), Her-3 (ErbB-3), and Her-4 (ErbB-4) [9-11]. Members of the ErbB family have a similar structure, with an extracellular binding domain, a transmembrane segment, an intracellular tyrosine-kinase domain (except for Her-3), and an intracellular C-terminal tail with multiple tyrosine residues. Ligand binding to the extracellular domain induces dimerization of two receptors (homodimerization if two identical receptors, heterodimerization if not), which activates the tyrosine-kinase domains, phosphorylating the tyrosine residues of its binding partner [12, 13]. Her-2 overexpression in breast cancer confers increased tumor aggressiveness [4]. Moreover, Her-2 overexpression/amplification

is a necessary condition for trastuzumab activity. Two diagnostic techniques are currently approved for assigning Her-2 status in clinical practice as follows: immunohistochemistry (IHC) and in situ hybridization (ISH). Whereas IHC uses an antibody to evaluate Her-2 protein expression, ISH determines the number of Her-2 copies per nucleus only or as a dual-probe technique, where hybridization of a chromosome 17 allows determination of the Her-2/chromosome 17 centromere probe. The low density of Her-2 receptor on cancer cells limits targeting detection [13]. Therefore, monitoring of the Her-2 receptor treatment requires a highly sensitive technique. In view of these limitations, many molecular probes have been designed to improve Her-2 treatment and determination of treatment efficacy. Trastuzumab works by attaching itself to the Her-2 receptors on the surface of breast cancer cells and blocking them from receiving growth signals. By blocking the signals, Trastuzumab can slow or stop the growth of the breast cancer. Trastuzumab is an example of an immune targeted therapy. Various drugs have been studied for the treatment of the Her-2 receptor, however, Trastuzumab was the first immunotherapeutic treatment targeting the Her-2 receptor [3, 14]. Although Trastuzumab is an efficient drug, resistance to Trastuzumab is a common and challenging problem. As suggested above, *in vitro* effects of these drugs may be cell line context dependent. Trastuzumab can also help fight breast cancer by alerting the immune system to destroy cancer cells onto which it is attached.

2. CELLULAR STUDIES

Cellular model systems suitable for preclinical testing must not only reproduce the pathology and behavior of human tumors, but must also be highly reproducible to continue drug testing in similar environments. Differences in cell line, experimental conditions, time of treatment and cellular density are known to impact Her-2 levels [15, 16]. So far, Trastuzumab has been reported to promote accumulation of cells in the G0-G1 phase of the cell cycle and prevents production in phase S [16]. The mechanism of cellular action of Trastuzumab consists of several approaches. Trastuzumab is known to (1) prevent Her-2 dimerization, (2) down regulates the Her-2 receptor, (3) induces cellular cytotoxicity, (4) and inhibits constitutive Her-2 cleavage mediated by metalloproteases [17]. Some studies have demonstrated that Trastuzumab has the properties of being Her-2 agonists, inducing phosphorylation of Her-2 and at times, transiently activating downstream signaling events [16, 17]. Trastuzumab, is transported with blood to the surface of the cancer cells and inhibits cancer cells growth. Trastuzumab, known as a monoclonal antibody targeting the Her-2 receptor, is used together with chemotherapy in the treatment of metastatic breast cancer in patients whose tumors overexpress the Her-2 receptor. Although this combination therapy has extended disease-free survival in a significant number of patients, not all Her-2 overexpressing tumors respond to this treatment, and some develop resistance after an initial response. For *in vitro* testing of drugs such as Trastuzumab a large numbers of cells are required, which can sometimes be a limiting factor because this often cannot be achieved until after several passages of standard two dimensional (2-D) cultures. These standard 2-D culture methods produce rather low cell concentrations. Moreover, tumor growth in animal model is *hormone independent* with much lower levels of receptors than human tumors.The morphology of most mouse tumors cannot be classified in an equivalent manner to the standard human tumor [18]. Although it is tempting to use cells molecularly engineered to express these receptors. Therefore new technologies should be used to enhance research ability to improve existing models or create new models for mammary carcinogenesis. Therefore three dimensional (3-D) cell cultures grown in a bioreactor was developed to provide a controlled environment and a high cell concentration to study cancer cells and cancer drugs. 3-D cancer cell cultures offer a controlled and systematic way to investigate cellular and molecular properties associated with disease. They represent an unlimited self-replicating source that can be grown in large quantities and are easily replaced from frozen stocks if lost through contamination. Mammalian cells viability and functional performance can be significantly enhanced when grown in 3-D. When compared to conventional monolayer cultures, 3-D culture models tissues are better in terms of structural and functional properties. Many cells can aggregate into 3-D when cultured in suspension. Moreover, model systems suitable for preclinical testing must reproduce the pathology and behavior of human tumors, but must also be highly reproducible. Therefore, an experimental system that provides controlled conditions and allows for reproducible experimental set-up as well as quantification of processes is needed for viability studies. The Hollow Fiber Bioreactor (HBR) can produce cultures of 10^9-10^{10} cells/ml. Also, spheroids models are widely used as avascular tumor models for metastasis and for therapeutic screening. The majority of cell culture process development techniques focus on novel analytical devices or novel line development [19-21]. Most cells within the human body interact with neighboring cells and extracellular matrix (ECM) components to establish a unique 3-D organization.These cell-cell and cell-ECM interactions form a complex communication network of biochemical and mechanical signals which are critical for normal cell physiology. As a result, loss of tissue-specific properties is common for cells grown in 2-D monolayer cultures. Numerous attempts have been made in the last two decades to develop 3-D cell culture models to bridge the gap between cell-based assays and animal studies in the hope of reducing experimental uncertainties arising from monolayer cultures and hence the cost of subsequent drug screening processes [13]. Biologists at present have several types of 3-D cell models at their disposal. The model most resembling native tissues are organotypic explants; a small piece of tissue freshly dissected

from experimental animals and maintained *in vitro*. Tissue explants have been exploited in nearly all biomedical research fields and probably will not be replaced by other 3-D culture models in the near future. However, such a model faces its own major obstacles, such as difficulty in obtaining specimens. In addition, tissue explants are typically several millimeters in size, which hampers mass transport to central regions and results in poor tissue viability. In view of these problems, a 3-D culture model that is simple to prepare and on the scale of 100–500 µm would be a useful alternative to tissue explants. One such model is the multicellular spheroid (MCS) using a self-assembly process from suspended cells. Most MCS exhibit higher similarity to real tissues in many aspects than that of monolayer cells, and therefore have recently gained increasing recognition in biomedical research. Cellular heterogeneity within 3-D tumor models caused by mass transport limitations, resembling the multiple phenotypes found in solid epithelial tumors is far more realistic than the cellular homogeneity found in monolayer culture. The primary advantage of 3-D cultures is their well-defined geometry which enables the possibility of structure analyses. Only 10^5 cells/ml (MCF-7/Her-2, MCF-7/Neo4, CEM and HMEC) are seeded in the HBR to start the culture [20]. The HBR is a closed loop system that consists of a porous hydrophilic hollow fiber and polysulfone tubing. The pH in the space of HFB device can be measured throughout the duration of experiments. A 10-fold increase in the number of cells is observed in the HBR device when compared to 2-D cultures.The use of HBR allows continuous and controlled delivery of therapeutics as well as removal of substances from the cell compartment. Additionally, in the HBR device, human cells are exposed to drug concentration profiles simulating *in vivo* pharmacokinetics *in vitro*. Therefore, 3-D cultures can improve our understanding of the role of the microenvironment in the modulation of drug resistance in solid tumors.There are several methods of culturing cancer cells, such as the spillage technique, differential centrifugation technique or different types of bioreactors (e.g. HBR or rotating wall vessel (RWV)). The HBR simulates an *in vivo* environment favorable for tissue growth and recapitulates particularly well the spatial organization of solid tumors. Nutrients are delivered and waste disposed in a controlled manner. Depending on their growth characteristics, these cultures can be maintained for extended periods of time. The first description of hollow fiber systems for cell culture was provided by Knazek et al., who sought a method to grow cells at densities similar to those found *in vivo* i.e., 10^8 cells/ml or higher as opposed to the 10^6 cells/ml or so achieved using standard cell culture techniques [21]. Hollow fiber modules can provide large surface area in a small volume. This allows a large number of cells to attach in a very small volume. Extremely efficient exchange of nutrients and waste products occurs across the fiber wall and filtrating characteristics of the fiber can be adjusted to retain particular proteins. Another fundamental difference between HBR and other cell culture techniques is that hollow fibers form a porous support for cell attachment. Culture parameters such as temperature, pH, biochemical gradients and mechanical stress are constantly controlled. The RWV device allows for the creation of spheroid cell cultures that represent the behavior of the early stage of a tumor *in vivo*. This model captures particularly well the spatial organization of solid tumors, in particular the necrotic center and the preferential location of invasive cells close to the tumoroid surface. However, the RWV allows growth of a tumor 1 mm in diameter. The most straightforward technique of growing cancerous tissue is the spillage technique which involves cutting the tumor and collecting the cells that spill out from the cut surface. The majority of other protocols have relied on enzymatic dispersal of tumor fragments following mechanical disaggregating. The sandwich culture in which dissociated breast tumor cells are sandwiched between two microscope slides is also known. The slides are immersed in culture medium which fills the gap between two slides creating a natural diffusion gradient for oxygen, nutrients and metabolism waste products. Targeted therapy based on the well-studied biology of malignancies is resulting in advances in cell cultures techniques. Individual therapeutics with a defined target help to make these strides, but major impacts will likely continue to come from the combinations of therapeutic agents. In clinical oncology, combinations of drugs are often chosen empirically; often simply on the basis of feasibility of safe delivery when non-overlapping toxicities are associated. The advantages of the use of a 3-D cultures devices are as follows: (1) continuous supply of nutrients and oxygen to cells; (2) continuous detoxification of the culture; (3) cultivation of cells in high cell density; (4) support for 3D growth of cells; (5) creation of an *in vivo* like microenvironment; (6) controlled delivery of substances to cells to realize time dependent profiles; (7) microscopy; (8) monitoring of the viability of the cultured cells without disturbing the culture at any time point; (9) concentration of soluble factors, pH, oxygen content and gas composition; (10) small culture volume to improve toxicity testing *in vitro* [20-22]. The function of biological cells is intimately associated with their internal organization, in which multiple subcellular structures have specialized functions. Such subcellular structures hold the secrets to normal cellular function and progression of disease, and the interactions among biomolecules. Research on Trastuzumab is increasing (Figure 2). The PubMed database shows that as of April 2016, 220 research papers have been published.

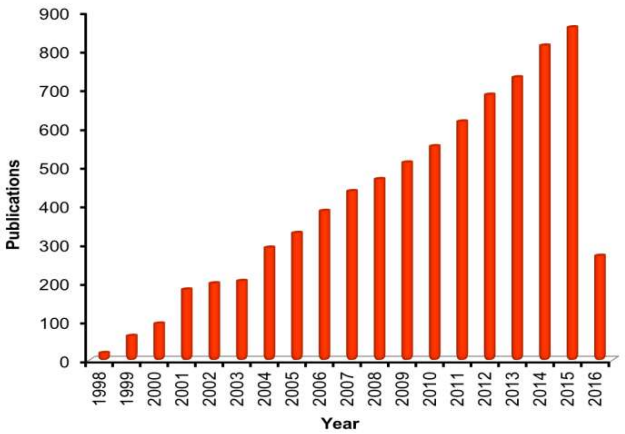

Figure 2. Trastuzumab research publications.

3. RESULTS OF TREATMNTS

The number of papers reporting Trastuzumab applications in clinic and research published during 1998-2015, has increased 13 times.The studies showed that Trastuzumab with vinorelbine were more efficient than Trastuzumab and taxanes [23]. A 75% response rate was reported for Trastuzumab combined with vinorelbine in Her-2 overexpressing breast cancer patients. The combination therapy of Trastuzumab with paclitaxel [24] and Trastuzumab with docetaxel [25] showed 61% and 63% response rates, respectively. Trastuzumab/cyclophosphamide combination therapy was mild [1-2]. In another study, Trastuzumab administered with anthracycline gave a new adjuvant regimen for breast cancer; however, further research is needed to determine the efficacy of this new treatment therapy [26]. Over the last decade, there has been a rapid increase in the number of clinical trials involving Trastuzumab in Her-2 therapies. It is worthwhile to notice that the main trend in research is to use Trastuzumab in combined therapy [27]. The main challenge is designing new Trastuzumab conjugates to improve drug efficiency [28, 29]. In fact, the highest efficiency recently found in gemcitabine conjugates is 54.6 ± 3.50 % for breast cancer [30].The cellular and molecular parameters confer sensitivity or resistance to Trastuzumab. Another study showed that the combination of Trastuzumab with ICI 182, 780, a pure antiestrogen that also down regulates ER, resulted in enhanced growth inhibition over either drug alone using ML-20 cells reported to express a high level of ER and moderate level of Her-2 [31]. Recent work by Hoffmann and coworkers showed that cancer tissue grown in the 3-D spheroid model mimics both tumor characteristics and the stromal microenvironment. The authors suggest that the 3-D spheroid is a valuable screening model for pharmaceutical drug development such as Trastuzumab [32]. Moreover, human mammary adenocarcinoma cell lines as spheroids treated by Trastuzumab showed that 3-D cell organization is promising to study cellular resistance to Trastuzumab [33]. The response of the Her-2 overexpressed cells to Trastuzumab was highly dependent on whether the cells were cultured in 2-D or 3-D cell cultures. The authors concluded that 3-D cultures of Her-2 overexpressed breast cancer cell lines had significantly increased sensitivity to Trastuzumab [34]. This study showed that 3-D cell cultures reflect *in vivo* aspects of Her signaling and can be used to further improve the understanding of molecular mechanisms of Trastuzumab [35].

4. CONCLUSIONS

Trastuzumab is a monoclonal antibody targeting the Her-2 receptor used together with chemotherapy in the treatment of metastatic breast cancer in patients whose tumors overexpress the Her-2 receptor. The introduction of Trastuzumab to therapy markedly improved the poor prognosis associated with Her-2-amplified cancers. Although this combination therapy has extended disease-free survival in a significant number of patients, not all Her-2-overexpessing tumors respond to this treatment, and some will develop resistance after an initial response. Here, we review current approaches in cancer cell culture formation, their biomedical applications, and recent advances in spheroid culture, manipulation, and analysis techniques.There are many benefits to be gained from using 3-D cell cultures. In some cases, cells are only maintained in culture for a finite period of time and have little opportunity to undergo the transformations that are seen in the long term culture of immortalized cell lines. For the review of cellular studies we conducted an electronic literature search in PubMed.

5. REFERENCES

[1] Pegram M., Hsu S., Lewis G., Pietras R., Beryt M., Sliwkowski M., Coombs D., Baly D., Kabbinavar F., Slamon D., Inhibitory effects of combinations of HER-2/*neu* antibody and chemotherapeutic agents used for treatment of human breast cancers, *Oncogene*, 18, 2241–2251, **1999.**

[2] Pegram M.D., Konecny G.E., O'Callaghan C., Beryt M., Pietras R., Slamon D.J., Rational combinations of trastuzumab with chemotherapeutic drugs used in the treatment of breast cancer, *Journal of National Cancer Institute*, 96, 739–749, **2004.**

[3] Slamon D.J., Leyland-Jones B., Shak S., Use of chemotherapy plus a monoclonal antibody against Her-2 for metastatic breast cancer that overexpresses Her-2, *New England Journal of Medicine,* 344, 783–792, **2001.**

[4] Nowsheen S., Cooper T., Bonner J.A., LoBuglio A.F., Yang E.S., Her-2 overexpression renders human breast cancers sensitive to PARP inhibition independently of any defect in homologous recombination DNA repair, *Cancer Research*, 72, 18, 4796-4806, **2012.**

[5] Piccart-Gebhart M.J., Procter M., Leyland-Jones B., Goldhirsch A., Untch M., Smith I., Gianni L., Baselga J., Bell R., Jackisch C., Cameron D., Dowsett M., Barrios C.H., Steger G., Huang C.S., Andersson M., Inbar M., Lichinitser M., Lang I., Nitz U., Iwata H., Thomssen C., Lohrisch C., Suter T.M., Ruschoff J., Suto T., Greatorex V., Ward C., Straehle C., McFadden E., Dolci M.S., Gelber R.D., Herceptin Adjuvant (HERA) Trial Study Team, Trastuzumab after adjuvant chemotherapy in Her-2-positive breast cancer, *New England Journal of Medicine*, 353, 16, 1659-1672, **2005.**

[6] McAlpine J.N., Wiegand K.C., Vang, Ronnett B.M., Adamiak A., Köbel M., Kalloger S.E., Swenerton K.D., Huntsman D.G., Gilks B., Miller D.M., Her-2 overexpression and amplification is present in a subset of ovarian mucinous carcinomas and can be targeted with trastuzumab therapy, *BMC Cancer*, 9, 433, **2009.**

[7] Ferris R.L., Jaffee E.M., Ferrone S., Tumor antigen-targeted, monoclonal antibody-based immunotherapy: clinical response, cellular immunity, and immunoescape, *Journal of Clinical Oncology*, 28, 4390–4399, **2010.**

[8] de Goeij B.E.C.G., Lambert J.M., New developments for antibody-drug conjugate-based therapeutic approaches, *Current Opinion in Immunology*, 40, 14–23, **2016.**

[9] Harris A.L., Nicholson S., Sainsbury R., Wright C., Farndon J., Epidermal growth factor receptor and other oncogenes as prognostic markers, *Journal of National Cancer Institute,* 11, 181-187, **1992.**

[10] Davies D.E., Chamberlin S.G., Targeting the epidermal growth factor receptor for therapy of carcinomas, *Biochemistry Biophysic search Communicate,* 51,1101-1110, **1956.**

[11] Goldenberg M.M., Trastuzumab, a recombinant DNA-derived humanized monoclonal antibody, a novel agent for the treatment of metastatic breast cancer, *Clinical Therapy*, 21, 309-318, **1999.**

[12] Brennan P.J., Kumogai T., Berezov A., Murali R., Greene M.I., Her-2/Neu: mechanisms of dimerization/oligomerization, *Oncogene*, 19, 6093-6101, **2000.**

[13] Marcucci F., Bellone M., Rumio C., Corti A., Approaches to improve tumor accumulation and interactions between monoclonal antibodies and immune cells, *MAbs,* 5, 34-46, **2013.**

[14] Sliwkowski M.X., Lofgren J.A., Lewis G.D., Hotaling T.E., Fendly B.M., Fox J,A., Nonclinical studies addressing the mechanism of action of trastuzumab (Herceptin), *Seminin Oncology,* 26, 60–70, **1999.**
[15] Kornilova E.S., Taverna D., Hoeck W., Hynes N.E., Surface expression of erbB-2 protein is post-transcriptionally regulated in mammary epithelial cells by epidermal growth factor and by the culture density, *Oncogene*, 7, 511–519, **1992.**
[16] Taverna D., Antoniotti S., Maggiora P., Dati C., De Bortoli M., Hynes N., E. erbB-2 expression in estrogen-receptor-positivebreast-tumor cells is regulated by growth-modulatory reagents, *International Journal of Cancer*, 56, 522–528, **1994.**
[17] Baselga J., Albanell J., Molina M.A., Arribas J., Mechanism of action of trastuzumab and scientific update, *Semin in Oncology*, 28, 4-11, **2001.**
[18] Tuveson D., Hanahan D., Translational medicine: cancer lessons from mice to humans, *Nature,* 471, 316–317, **2011.**
[19] Bartusik D., Tomanek B., Lattova E., Perreault H., Fallone G., Ex vivo assays of CEM cells cultured and treated in the three dimensional cultures, *Biomedicine and Pharmacotherapy,* 64, 390–395, **2010.**
[20] Kirstein M.N., Brundage R.C., Elmquist W.F., Remmel R.P., Marker P.H., Guire D.E., Yee D., Characterization of an in vitro cell culture bioreactor system to evaluate anti-neoplastic drug regimens, *Breast Cancer Research and Treatments*, 96, 203–301, **2006.**
[21] Knazek R.A., Gullino P.M., Kohler P.O., Dedrick R.L., Cell culture on artificial capillaries: an approach to tissue growth in vitro, *Science*, 178, 65-66, **1972.**
[22] Abbott A., Cell culture, biology new dimension, *Nature,* 424 870–872, **2003.**
[23] Burstein H.J., Kuter I., Campos S.M., Gelman R.S., Tribou L., Parker L.M., Manola J., Younger J., Matulonis U., Bunnell C.A., Partridge A.H., Richardson P.G., Clarke K., Shulman L.N., Winer E.P., Clinical activity of trastuzumab and vinorelbine in women with Her-2-overexpressing metastatic breast cancer, *Journal of Clinical Oncology*, 19, 2722-2730, **2001.**
[24] Seidman A.D., Fornier M.N., Esteva F.J., Tan L., Kaptain S., Bach A., Panageas K.S., Arroyo C., Valero V., Currie V., Gilewski T., Theodoulou M., Moynahan M.E., Moasser M., Sklarin N., Dickler M., D'Andrea G., Cristofanilli M., Rivera E., Hortobagyi G.N., Norton L., Hudis C.A., Weekly trastuzumab and paclitaxel therapy for metastatic breast cancer with analysis of efficacy by Her-2 immunophenotype and gene amplification, *Journal of Clinical Oncology*, 19, 2587-2595, **2001.**
[25] Esteva F.J., Sahin A.A., Cristofanilli M., Arun B., Hortobagyi G.N., Molecular prognostic factors for breast cancer metastasis and survival, *Seminars in Radiation Oncology*, 12, 4, 319-328, **2012.**
[26] Shen B-Q., Bumbaca D., Saad O., Yue Q., Pastuskoas C.V., Khojasteh S.C., Tibbits J., Kaur S., Wang B., Chu Y-W., LoRusso P.M., Girish S., Catabolic fate and pharmacokinetic characterization of trastuzumab emtansine: an emphasis on preclinical and clinical catabolism, *Current Drug Metabolism,* 13, 901-910, **2012.**
[27] Zanardi E., Bregni G., de Braud F., DiCosimo S., Better Together: Targeted Combination Therapies in Breast Cancer, *Seminarium in Oncology*, 42, 887-895, **2015.**
[28] Ecker D.M., Jones S.D., Levine H.L., The therapeutic monoclonal antibody market, *MAbs*, 7, 1, 9-14, **2015.**
[29] Lambert J.M., Antibody−Drug Conjugates (ADCs): Magic Bullets at Last, *Molecular Pharmaceutics,* 12, 1701-1702, **2015.**
[30] Shin D.H., Koo M.J., Kim J.S., Kim J.S., Herceptin-conjugated temperature-sensitive immunoliposomes encapsulating gemcitabine for breast cancer, *Archives of Pharmacalogical Research,* **2016.**
[31] Kunisue H., Kurebayashi J., Otsuki T., Tang C.K., Kurosumi M., Yamamoto S., Tanaka K., Doihara H., Shimizu N., Sonoo H., Anti-Her-2 antibody enhances the growth inhibitory effect of antiestrogen on breast cancer cells expressing both oestrogen receptors and Her-2, *British Journal of Cancer*, 82, 46–52, **2000.**
[32] Hoffmann O.I., Ilmberger C., Magosch S., Joka M., Jauch K.W., Mayer B., Impact of the spheroid model complexity on drug response, *Journal of Biotechnology*, 205,14-23, **2015.**
[33] Rodríguez C.E., Reidel S.I., Bal de Kier Joffé E.D., Jasnis M.A., Fiszman G.L., Autophagy Protects from Trastuzumab-Induced Cytotoxicity in Her-2 Overexpressing Breast Tumor Spheroids, *PLoS One*, 10, **2015.**
[34] Weigelt B., Lo A.T., Park C.C., Gray J.W., Bissell M.J., Her-2 signaling pathway activation and response of breast cancer cells to Her-2-targeting agents is dependent strongly on the 3-D microenvironment, *Breast Cancer Research and Treatments,* 122, 35-43, **2010.**
[35] Pickl M., Ries C.H., Comparison of 3-D and 2-D tumor models reveals enhanced Her-2 activation in 3-D associated with an increased response to trastuzumab, *Oncogene,* 28, 461-468, **2009.**