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Review

Recent advances in the field of anti-cancer immunotherapy

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ABSTRACT

Background: The main goal of anti-cancer therapy is to specifically inhibit the malignant activity of cancer cells, while leaving healthy cells unaffected. As such, for every proposed therapy, it is important to keep in mind the therapeutic index — the ratio of the toxic dose over the therapeutic dose. The use of immunotherapy has allowed a means to both specifically block protein–protein interaction and deliver cytotoxic events to a tumor-specific antigen.

Review scope: It is the objective of this review to give an overview on current immunotherapy treatment for cancers using monoclonal antibodies. We demonstrate three exciting targets for immunotherapy, TNF-α Converting Enzyme (TACE), Cathepsin S and Urokinase Plasmogen Activator and go over the advances made with one of the most used monoclonal antibodies in cancer therapy, Rituximab; as well as Herceptin, which is used for breast cancer therapy. Furthermore, we touch on other venues of immunotherapy, such as adaptive cell transfer, the use of nucleic acids and the use of dendritic cells. Finally, we summarize some ongoing studies that spell tentative advancements for anti-cancer immunotherapy.

General significance: Immunotherapy is at the forefront of anti-cancer therapies, allying both a high degree of specificity to general high effectiveness and fewer side-effects.

Contents

1. Introduction

The 21st century has ushered in an era of great scientific progress and discoveries, resulting in a surge of interest by the general public in

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all manner of research. Modern science looks to improve lives, focusing not only in the eradication of disease, but also in extending the average lifespan of humans. Unfortunately, as people live longer, new problems arise. As age increases, an individual is more likely to develop complications, namely degenerative diseases, such as cancer.

In cancer biology, tumors are described as complex tissues comprised of heterogeneous neoplastic cells interwoven with tumor-associated stroma. The characterization of proteins associated with tumors presents opportunities for targeted therapeutic intervention. This approach is called "targeted therapy". However, the heterogeneity of tumors dictates that, in order to achieve successful clinical treatment, it is necessary to employ a combination of targeted therapies. The most specific targeted therapies currently in use are monoclonal antibodies.

In the last decade, the use of antibody therapy in the field of oncology has shown very promising results [1]. Due to their high specificity, antibodies represent a promising method for interfering with a single target molecule, with high selectivity. Back in 1980, the first patient with relapsed lymphoma was treated using a therapeutic antibody approach. While the antibody was shown to be clinically ineffective, the therapy was deemed innocuous and was well-tolerated [2,3]. These safety and tolerated rationales built up the groundwork that led to the use of therapeutic antibodies in the treatment of cancer.

During the past few years, attention has turned to using antibodies to target different tumor-associated antigens. These include surface glycoproteins associated with clusters of differentiation, CTLA-A, or pathways regulated by growth factors [4]. Furthermore, while the use of monoclonal antibodies monotherapy has had a tremendous impact on cancer treatment, namely in non-Hodgkin's lymphoma, their efficiency has been further improved through the combination of chemotherapy along with monoclonal antibodies [5].

However, many of the studies presented ambiguous or insufficient criteria for clinical objective response. Results from such studies may improperly imply effectiveness when compared to historical controls. This emphasizes the need for thoughtful changes in the application of cancer treatment approaches, such as a combination of multi-targeting antibody-based therapy [6–8].

2. Monoclonal antibody immunotherapy

One of the most promising and exciting fields in modern anti-cancer therapy involves the use of monoclonal antibodies which, once administered to the patient, will selectively and efficiently, target a particular protein involved, in some way, with the proliferation of tumor cells. A large number of monoclonal antibody therapies have already been approved and are currently in use, as described in Table 1.

In the cases described below – TACE/ADAM17, Cathepsin S and Urokinase Plasminogen Activator – the proteins show an abnormally high expression in cancer cells. This makes them the perfect targets for inhibition through the use of monoclonal antibodies.

Furthermore, we also take a look at Rituximab, one of the principal antibodies used in anti-cancer therapy, as well as Herceptin, the only antibody therapy approved by the FDA that targets the human epidermal growth receptor 2 protein.

2.1. TNF-α Converting Enzyme (TACE)

Many growth factors and cytokines require proteolytic release from the cell surface for their activation [11]. TNF-α converting enzyme (TACE), also known as A Disintegrin and Metalloprotease 17 (ADAM17); is a transmembrane metalloprotease responsible for solubilizing many pathologically significant membrane substrates and is an appealing therapeutic target for the treatment of several diseases [11]. In terms of structure, mature ADAM-family ectodomains contain a globular metalloprotease catalytic domain, a disulfide-dependent disintegrin-cysteine rich (Dis-Cys) domain and, in some cases, an epidermal growth factor (EGF)-like domain [11].

Initially, TACE was described as an enzyme, whose function was attributed to solubilizing membrane-associated pro-TNF-α [12] — a process named "ectodomain shedding". Since then, TACE has been described as capable of cleaving epidermal growth factor receptor (EGFR) ligands [13,14], extracellular Notch1 [15], adhesion molecules [16] and cell-surface receptors [17]. Ever since proteolytic cleavage has been proven to be indispensable for the activation of many of these substrates, TACE has been studied as a target in the treatment of cancer [18] and rheumatoid arthritis [19]. Furthermore, dysregulation of ectodomain shedding has been linked to autoimmune and cardiovascular diseases, neurodegeneration, infection and inflammation [20].

Several studies have demonstrated that TACE is over-expressed in various tumor cells, such as those from ovarian cancer, breast cancer, pancreatic ductal adenocarcinoma, colorectal carcinoma, gastric cancer stem cells, gastrointestinal stromal tumors (GIST), non-small cell lung carcinoma and head and neck cancer [21]. This protein has also been associated in governing endothelial cell migration and pathological angiogenesis, which are equally relevant to tumor growth [21]. Chemotherapy may activate TACE, leading to growth factor shedding, which contributes to resistance in colorectal cancer models, as well as contributing to resistance to trastuzumab in breast cancer [21].

There is a very high homology (96%) between the human and mouse TACE ectodomains, which makes the antibody selection and production process even more important. Adding to that, there is the need to adhere to the therapeutic requirements for human antibodies and the desire to avoid metzicin active site immunoreactivity. For these reasons, antibody phage-display presents an attractive technology for producing a specific TACE inhibitor [22].

Antibody phage-display is a powerful *in vitro* selection technology capable of producing fully human antibodies against human antigens. A flowchart of the main steps in phage-display technology is present in Fig. 1.

This technique can be used to direct antibodies towards desired epitopes, due to the biochemical control available during selection conditions. Solution-phase phage display typically produces antibodies with non-linear (conformational) epitopes. Thus, intricate macromolecular cross-domain binding might be hypothetically achieved through this technology, an ideal scenario for an ADAM inhibitor [11]. In fact, antibodies have been produced through phage display, due to recent technical advances, capable of recognizing multiple distinct antigens [23] and different conformations of the same antigen [24,25].

Table 1
Monoclonal antibodies currently in use in anti-cancer immunotherapy, targets and respective cancer types, as well as date of approval both by the Food and Drink Administration and by the European Medicines Agency.
Adapted from Chames, P., et al. Br. J. Pharmacol, 2009 [9] and Oldham, R. K. and Dillman, R. O. Journal of Clinical Oncology, 2008 [10].

Monoclonal antibodies used in cancer immunotherapy					
Generic name	Commercial name	Target	Cancer type	FDA approval	EMEA approval
Rituximab	Rituxan	CD20	Non-Hodgkin's lymphoma	26/11/1997	2/6/1998
Trastuzumab	Herceptin	Erb B2 (HER-2)	Breast	25/9/1998	28/8/2000
Alemtuzumab	Campath	CD52	Chronic lymphocytic leukemia	7/5/2001	6/7/2001
Cetuximab	Erbitux	EGFR	Colorectal	12/2/2004	29/6/2004
Panitumumab	Vectibis	EGFR	Colorectal	27/9/2006	19/12/2007
Bevacizumab	Avastin	VEGF	Colorectal	26/2/2004	12/1/2005

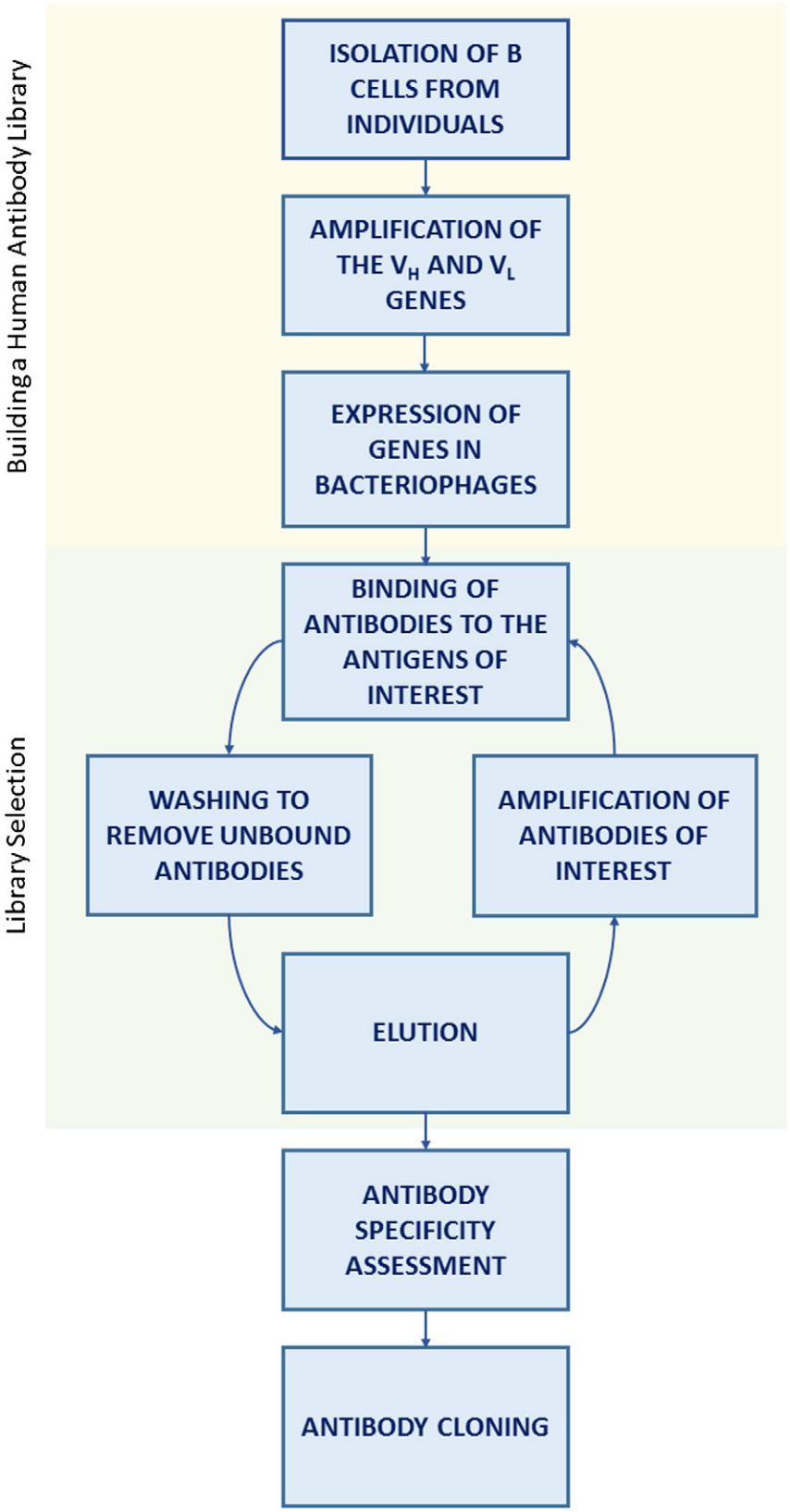

Fig. 1. Flowchart for the protocol for Phage Display Technology. V_L and V_H refer to variable light and variable heavy chains in antibodies. Various genes responsible for encoding the variable regions of antibodies are amplified from human B-cells and used to build an antibody library. The library is cloned for display on the surface of the phage. In a procedure similar to the two-hybrid system, the antibody fragment is expressed in fusion with the virus coat protein. The phage display library goes through a process of selection, whereupon those that do not bind to the selected epitopes are washed away. The ones that do are eluted and amplified by infection of *Escherichia coli*. After an adequate number of selection series, the specificity of the desired antibody can be assessed through Enzyme-Linked Immunosorbent Assay (ELISA) or Fluorescent-Activated Cell Sorting (FACS). Once the achieved specificity is satisfactory, the genes corresponding to the antibody's variable regions can be cloned into whole human IgG expression vectors and transfected into cells, such as HEK293, which will produce fully human mAbs (hmAbs). The antibodies will be expressed into the cell medium. At that point, the supernatant can be collected for antibody purification.

D1(A12) is a monoclonal antibody developed through antibody phage-display, which targets the TACE ectodomain. Studies have allowed a comprehensive understanding of the biochemical properties of D1(A12), through the use of assays on human cancer cells. Furthermore, it has been confirmed through xenograft analyses, in *in vitro* as well as *in vivo*, that D1(A12) serves as a potent inhibitor of human ADAM17's activity [11,21]. However, later studies have demonstrated that this antibody was unable to lower the concentration of human TNF-α circulating in the bloodstream. These results suggest that, following the inhibition of ADAM17 in an *in vivo* environment, other factors may replace the concentration of TNF-α [21]. One possible culprit is ADAM10, as this enzyme has shown sheddase activity towards TNF-α in murine fibroblasts that were deficient in ADAM17. In certain types of lymphoma, ADAM10 is also responsible for the solubilization of TNF-α [21]. Recently, it was determined that the D1(A12) antibody can successfully inhibit the proliferation and motility of cancer cells in head and neck squamous cell carcinoma (HNSCC), by reducing the overall amount of circulating EGFR ligands [26]. These results further prove, not only the promising future applications of this particular antibody in cancer therapy, but also the importance of cancer immunotherapy, moving forward.

Studies continued, in an effort to identify an antibody possessing cross-reactivity between human and mouse antigens. This is important, particularly in pre-clinical trial conditions, to ensure the safety of the proposed therapy. Thus, a method was proposed that alternates selection rounds between human and mouse antigens [22]. The discovery of such an antibody would allow research to proceed into a purely *in vivo* environment. With these conditions in mind, work continued, resulting in the identification of A9, an antibody clone that demonstrated mostly non-competitive inhibition [22].

Subsequent experiments revealed that A9 was an allosteric inhibitor, which could bind to a secondary site outside the catalytic cleft of TACE, thus disturbing its ability to bind to the active site [22]. In fact, experiments developed in the presence of CT1746 – a hydroxamate inhibitor of metalloproteinases that interacts with TACE's active site Zn [26] – demonstrated that the binding of ligands to the active site of TACE affected the A9 binding site on the protein. In other words, the affinity of A9 to TACE was reduced in the presence of CT1746 [22]. This data suggests that the inhibition of TACE by A9 is not purely non-competitive, but rather a mixed form of inhibition.

It is important to consider that there are approximately 70 known metzincin metalloproteases that possess Zn in their active site [27]. Therein lies the problem of small molecule inhibitors of TACE: the lack of selectivity in these inhibitors would lead to off-target toxicity [28]. Hence, the significance of A9: a non-Zn-binding inhibitor, specific for the TACE protein.

Due to the importance of this protein in a cancer environment and the promising results described above, this area and, in particular, TACE inhibition; has proven itself to be rife with possibilities on the path of cancer research and eventual eradication.

2.2. Cathepsin S

Another promising target being investigated is Cathepsin S, a proteolytic enzyme. This protein functions predominantly as an endopeptidase within the endolysosomal vesicles of healthy cells, and is involved in many physiological processes, such as differentiation, protein turnover, degradation and apoptosis. In many cancer cell lines, Cathepsin S has been demonstrated to be highly expressed or upregulated, contributing to the development and progression of the cancer phenotype [6].

In colorectal cancer patients, Cathepsin S associates with the cell membrane, providing an opportunity for antibody-dependent cellular cytotoxicity. In fact, the targeting of Cathepsin S, in this case, through the use of a humanized antibody with an immune effector function, has resulted in natural killer cell targeted tumor killing, with a 22% cytotoxicity. In this case, the antibody treatment inhibits the breakdown of the extracellular membrane around the extracellular periphery of tumor cells, resulting in the attenuation of tumor cell invasion through the extracellular membrane. This leads to an inhibition of tumor cell invasion, growth and neovascularization [29].

A recent antibody, Fsn0503h, has been developed which can inhibit Cathepsin S. The *in vivo* results are promising, and include the suppression of angiogenesis and metastasis, effectively halting cancer progression. While there is still much to be uncovered regarding this lysosomal cysteine protease, Cathepsin S is thought to contribute to resistance against more common types of cancer therapy, such as radio and chemotherapies, making it an important research subject in the field of immunotherapy [34].

2.3. Urokinase Plasminogen Activator

Mammary carcinoma and lung cancer are the most common type of malignant tumors in adult women and an undeniable concern for general public health. Unfortunately, some of the issues with finding a solution to human breast cancer include its high genetic heterogeneity, different molecular profiles and varied clinical behavior. One of the targets being focused on, in an attempt to eradicate this type of tumor, is the urokinase plasminogen activator protein [31].

The urokinase plasminogen activator (uPA) system is composed of uPA, a specific cell receptor for uPA (uPAR), and serpin inhibitors of uPA, such as plasminogen activator inhibitor-1. Among the roles of this system, are the release and processing of latent growth factors located on the extracellular membrane, such as FGF-2, VEGF, HGF and TGF-β. There is a great number of papers describing the crucial role of uPAR in the evolution of some solid cancers, including breast, colon, prostate, pancreatic, ovarian, lung and brain, as well as several hematologic malignancies such as acute leukemia and myeloma [31,32]. This has led to the recent identification and development of a monoclonal antibody that specifically targets uPAR. This therapy has proven effective in a number of different animal tumor models, without blocking the interaction between uPA and uPAR [32].

Recently, a novel therapeutic uPAR antibody was developed, ATN-658, which has been capable of exhibiting reliable anti-tumor effects across a variety of tumor models. ATN-658 has been proven to inhibit invasion, metastasis and tumor proliferation as well as induce apoptosis. In uPAR's DIII domain, there is a small 6-mer disulfide loop near the glycolipid anchor, which serves as the antibody's epitope. The antibody closely mimics the interaction of CD11b. CD11b-positive cells act as suppressors to diminish cytotoxic T-cell response, allowing tumors to progress, as well as secrete factors that drive that development. ATN-658 blocks the CD11b–uPAR interaction, which leads to the hypothesis that uPAR may actually function to promote metastasis [32].

Beyond the use of antibodies, a study has been described that uses aptamers, specifically RNA aptamers, which selectively bind to human uPA, by targeting the active site of the enzyme. This inhibition effectively halts the activation cascade of pro-uPA, while not interfering with any uPA that are already active, or even other serine proteases. The effects of using the above aptamer, named upanap-126, include a reduction in tumor dissemination and cell invasion [37].

As a result, the further study of the uPA system may be an important future endeavor in the fight against cancer.

2.4. Rituximab

One of the existing anti-cancer therapies that has found some success, is the monoclonal antibody Rituximab.

Rituximab, also known as IDEC-C2B8 [33], is used for treatment directed against the B-cell-specific antigen CD20 expressed on non-Hodgkin's lymphomas. It revolutionized the clinical treatment of B-cell non-Hodgkin's lymphoma, being the first monoclonal antibody drug

antibody that specifically binds to the CD20 antigen on the surface of normal and tumor B-cells [33,34]. As CD20 is absent from hematopoietic stem cells, normal B-cells are able to regenerate after the Rituximab treatment and return to pretreatment levels within several months or years [35].

However, treatment with Rituximab has been linked to moderate to severe first-dose side-effects, notably in patients with high numbers of circulating tumor cells [36]. These side-effects include fever, rigors, bronchospasm and hypoxemia, concomitant with rapid reduction and laboratory evidence of tumor destruction [37]. In some cases, tumor lysis syndrome has been detected in the 24 h period following the first infusion with the antibody. This condition is characterized by a rapid reduction of the tumor followed by acute renal failure, hyperkalemia, hyperuricemia, hyperphosphatemia, hypocalcemia and, on occasion, death. Other risks include a high number of circulating malignant cells or high tumor burden. Should tumor lysis occur, electrolyte imbalances should be corrected while monitoring renal functions and fluid balance [38].

When considering patients with autoimmune diseases, it is important to keep in mind the unknown, but possible development of malignancies with the administration of Rituximab. This is especially compounded in elderly patients, as the recovery to normal levels of B-cells can be delayed. Prolonged immunosuppression has been associated with increased incidence of cancer [39]. Even so, the use of Rituximab in autoimmune diseases is rapidly increasing [40]. While the efficacy and safety vary among different autoimmune diseases, the use of this treatment is ultimately beneficial, as is suggested by most studies [39].

In cases of idiopathic neuromyelitis optica, a demyelinating disease of the central nervous system characterized by the co-occurrence of transverse myelitis and optic neuritis, treatment with Rituximab was well tolerated and patients experienced less exacerbation than expected, based on their historical data for attack rates [41]. In the case of 35-year old woman, who had developed Burkitt's lymphoma during early pregnancy, treatment with Rituximab and CHOP therapy was safely administered, without causing any malformation, developmental retardation or immune dysfunction in the child, while producing complete remission in the mother [42].

It has been suggested that complement and complement inhibitors are likely to play a role in the heterogeneity of the response to Rituximab *in vivo* [43]. Administration of Rituximab results in a prompt activation of the complement system, resulting in cytokine release. This, in turn, activates macrophages and mast cells, which are capable of releasing cytokines themselves as well as complement activation products, which can function as anaphylatoxins, and might be thus responsible for some of the side-effects [36]. It is likely that the mechanism of action of Rituximab *in vivo* is to some extent affected by the complement. This means that studies to improve the safety as well as the efficacy of these treatments should keep in mind the role of the complement in the treatment [36,44].

2.5. Herceptin

However, Rituximab is not the only monoclonal antibody therapy in use today.

In 20% to 25% of invasive breast cancers, the human epidermal growth receptor 2 protein (HER-2) has been found to be overexpressed [45,46]. HER-2 is a tyrosine kinase that is related to the epidermal growth factor receptor EGFR [45]. Its structure is composed of an extracellular domain, a transmembrane domain and an intracellular domain with tyrosine kinase activity [46]. This protein has the ability to transform normal fibroblasts and, when overexpressed, produce breast cancer in transgenic mice. This enzyme has become an important therapeutic target in breast cancer, as higher levels are closely linked with higher pathogenesis and worse prognosis of breast cancer. Due of toxicity of HER-2 targeting drugs is decreased, as it is present in much higher proportion in said tumor cells, compared to healthy cells [45].

Herceptin, also known as trastuzumab, is a recombinant humanized monoclonal antibody, directed against the extracellular domain of the HER-2 protein. Currently, Herceptin is the only therapy approved by the United States Food and Drug Administration that targets HER-2. Before treatment with this mAb therapy, the American Society of Clinical Oncology recommends the evaluation of HER-2 status in all primary breast tumor, both at the time of diagnosis and upon recurrence, as this affords both prognosis information, as well as being determinant of the response to Herceptin [45].

Although the exact mechanisms by which Herceptin is capable of inhibiting HER-2 are not yet completely understood, some of its effects have been observed, both *in vitro* and *in vivo*, such as diminished receptor signaling, induction of apoptosis, inhibition of angiogenesis and inhibition of DNA repair [45].

Some of the mechanisms that may be used by therapeutic antibodies to combat cancer cells are exemplified in Fig. 2.

Initial phase I clinical trials with this antibody proved it to be safe and with reliable pharmacokinetics, giving response rates of up to 34%. A later study observed that combining Herceptin with doxorubicin plus cyclophosphamide produced longer time to progression, higher response rates and improved survival rather, as opposed to simple chemotherapy. However, this also caused severe cardiac dysfunction [48]. At the same time, research groups are attempting to identify new means of increasing Herceptin efficiency while decreasing cardiotoxicity. The solution might include a multidisciplinary care approach, with both cardiology and oncology specialists providing a risk–benefit assessment and proper patient education on how to manage the disease, the cure and on adopting a healthy lifestyle [49].

One of the main issues with this mAb therapy lies in the fact that objective response rates, when in a monotherapy regimen, are low, ranging from 12% to 34% for a duration of 9 months. Because of this, Herceptin is usually administered in combination with chemotherapies, such as paclitaxel or docetaxel, which increase response rates, time to disease progression and overall survival [50]. Unfortunately, patients who demonstrate an initial response to Herceptin-based regimens, generally acquire resistance within one year [45].

Some mechanisms have been proposed that explain how tumors avoid the cytotoxicity caused by this therapy. One such possibility are mutations in the *her2* gene (also called *erbB-2*), – which encodes for the HER-2 protein – resulting in an inability for the antibody to recognize its epitope and, therefore, to bind to HER-2 [45,51]. Another mechanism revolves around the fact that EGFR type I growth factor receptor tyrosine kinase family consists of EGFR, HER-2, HER-3 and HER-4. Although it is possible that Herceptin is, indeed, inhibiting cell signaling through HER-2 binding, it will not reduce signaling through the other HER receptors. Fortunately, new antibody therapies are being developed to counter this mechanism [45].

Moving forward, more research is being done with Herceptin in order to maximize its potential as an immunotherapy. This includes combining Herceptin with novel agents, such as the anti-EGFR tyrosine kinase inhibitor gentifibin, which has produced complete remission of BT474 breast tumor xenograft; as well as developing novel strategies for targeting HER-2, like the recombinant humanized HER-2 mAb pertuzumab, capable of blocking the dimerization of HER-2 with other HER receptors, thus avoiding one of the mechanisms described above through which patients develop resistance to Herceptin therapy [45].

Upon observing how much effort is being placed into ensuring the effectiveness of anti-HER based treatment, it is clear that this mAb therapy may have lost the battle, but is still far from losing the war. This is especially true if new active targeted agents are developed,

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