RESEARCH PAPER

Harnessing benefit from targeting tumor associated carbohydrate antigens

Thomas Kieber-Emmons, Behjatolah Monzavi-Karbassi, Laura F. Hutchins, Angela Pennisi, and Issam Makhoul

Winthrop P. Rockefeller Cancer Institute, University of Arkansas for Medical Sciences, Little Rock, AR, USA

ABSTRACT

Integrating additive or synergistic antitumor effects that focus on distinct elements of tumor biology are the most rational strategies for cancer treatment. Treatments for breast cancer have increased overall survival, but remain limited by lack of efficacy in a subset of breast cancer patients. The real challenge is to define what elements of tumor biology make the most sense to be integrated. An emerging strategy is to consider a systems biology approach to impact multiple interactions in networks as compare with hitting a specific protein-protein interaction target. In this review, we consider how targeting tumor associated carbohydrate antigens (TACA) that are fundamental to signal pathways might be tailored to harness benefit from combination therapy of sustained immunity with chemotherapy. An approach we are developing makes use of a carbohydrate mimetic peptide (CMP) to induce polyspecific antibodies, which by their nature have numerous on and off target effects. Linking multi-target TACA recognition with mechanisms affecting tumor growth in the context of network heterogeneity and concepts of immune surveillance to tumor cells and the type of breast cancer patients that would benefit from such an approach provides a novel integrative treatment.

KEYWORDS

breast cancer; carbohydrate mimetic peptide; pathological complete response; tumor glycans; TACA; tumor vaccine

Introduction

Pharmacological treatment is a standard approach in the adjuvant and neoadjuvant therapy of breast cancer to reduce the risk of disease recurrence.¹ But the majority of women with relapsed or disseminated disease ultimately still expire from this disease.^{2,3} This should not be surprising. Common sense would dictate that any finding of residual cancer—in situ, invasive, or in axillary lymph nodes following neoadjuvant therapy—is likely associated with a worse prognosis compared with absolutely no evidence of residual disease. Notwithstanding that cancer is often molecularly and clinically a heterogeneous disease, cells may be resistant to drugs, forming the rational for both combinations of modalities of treatment as well as combination of molecular therapeutic strategies.⁴

The treatment of cancer by chemotherapy causes tumor cell death, mostly by apoptosis.⁵ Molecular mechanisms that regulate apoptosis and the mediators that either prevent or trigger cell death are an area of intense research. Consequently, apoptosis regulators have emerged as key targets for the design of therapeutic strategies aimed at modulating cellular life-and-death decisions.⁶ Proof-of-principle evidence obtained in several animal models confirms the validity of strategies targeting apoptosis, revealing the potential for therapeutic intervention. Nevertheless, there is a high failure and relapse rate for single-agent targeted therapies because most cancers have multiple inputs into cellular pathways that serve to bypass the intended outcome of single target therapies.

Cellular pathways have complex interactions and when perturbed exhibit redundant mechanisms for survival.⁷ Developing targeted agents for one pathway or target at a time often causes

rebound activation of backup pathways that bypass targeted molecules. Hence, targeting a specific, overexpressed, protein in a malignant cell may not result in an optimal outcome except for instances such as Her2 overexpression in breast cancer⁸ or BCR-Abl expression in chronic myelogenous leukemia.⁹ Combinations of the anti-human epidermal growth factor receptor 2 (HER2 or c-erbB2) antibody trastuzumab and chemotherapy lengthen survival in metastatic HER2-overexpressing breast cancer.¹⁰ However, progressive disease typically occurs because of genetic mutation of the single target.

There is a need to counter the heterogeneity and evolution of tumors. Mutation or a genetic event affecting the target of the drug renders the drug useless where a pathway is still able to drive the growth of the tumor, despite the continued presence of the drug. Interestingly, the epitome of targeted therapy is to identify a mutated oncogene and target it with a small molecule. This has led to the conceptual addiction to oncogenes.^{11,12} This concept was invoked to explain how some cancers that contain multiple genetic, epigenetic, and chromosomal abnormalities are dependent on or ‘addicted’ to one or a few genes for both maintenance of the malignant phenotype and cell survival. Genomics was developed with this in mind. So every disease was divided into a large number of diseases based on this premise and the search commenced for “The Agent” for this mutated oncogene. In vitro models supported this concept but clinical trials disappointed. Since every patient may have a different mutation or mutations the term “personalized medicine” was born from this practical and intellectual bottle-neck.¹³

It is clear a new approach is needed to harness the benefit of cell death. In contrast to targeting singular proteins we are

pursuing the strategy of pan immunotherapy, with roots in systems immunobiology and in understanding how Tumor Associated Carbohydrate Antigens (TACA) can be effective-targets to mediate cancer cell death. This innovative concept is coincident with mounting evidence that combination targeted therapies will be more effective than single agents. Systems biology, has revealed that human cells and tissues are composed of complex, networked systems with redundant, convergent and divergent signaling pathways.¹⁴⁻¹⁸ For example, the redundant function of proteins involved in cell-cycle regulation¹⁹ has inspired efforts to intervene simultaneously at multiple points in these signaling pathways.^{20,21} An approach consonant with a systems biology framework, and complementary to the target-based approach, entails identification of pathway points that can be targeted to perturb cellular signaling networks associated with apoptosis.

One set of molecular targets associated with a variety of biological functions is TACA. Interestingly, TACAs and glycans in general can be described as pan antigens as they are found on many proteins and lipids on many cancer cells. Aberrant glycosylation of proteins and lipids is one of the characteristic features of malignantly transformed cells, expanding the diversity of the proteome enormously. So how do we target TACA to interrupt cell survival signaling? A lesson can be learned from the polyspecific nature of natural antibodies and from lectins.²²

The polyspecific characteristics of natural antibodies see TACA as pan tumor antigens since natural antibody can recognize multiple glycans as part of the innate immune surveillance system. Naturally arising IgM antibodies to apoptotic cell determinants are present from birth and can be further induced by apoptotic cell challenge. Efficient clearance of cells undergoing apoptotic death is crucial for normal tissue homeostasis and for the prevention of autoimmunity.²³ Natural polyreactive IgM autoantibodies, encoded by unmutated germline Immunoglobulin (Ig) V genes, represent a major fraction of the normal circulating IgM repertoire. Studies have suggested a role for normal IgM in controlling cell death and proliferation, and imply a possible therapeutic role for IgM in autoimmune and lymphoproliferative disorders,²⁴ which can be extended to targeting TACA.²⁵

Harnessing the polyspecific nature of antibodies overcomes the singular nature of targeted therapeutics as well as the drawback from genetic mutations because TACA are a necessary component of all proteins and lipids. Targeting the multiple drivers of pathways simultaneously should therefore aid in removal of malignant cells and cancerous cells in the tumor microenvironment as well as being oncogene mutation-agnostic. A vaccine targeting TACA is likely to elicit an immune response that should be able to spread to other antigens that carry some similarity with the original epitope that was initially selected, something that “targeted therapy” could never do, leading to improved patient survival. The possibility to use this rationale for creating immunotherapies for cancer is examined.

Perspective on combination therapy

The idea that chemotherapy may increase tumor immunogenicity leading to better efficacy is old. Following cancer cell death, tumor antigens are released then processed by antigen

presenting cells and presented to immune cells. The adaptive immune response that follows is considered crucial for cancer control and it is perhaps one of the most important mechanisms through which chemotherapeutics exert their effect, as this effect may be abrogated in case of defective adaptive immunity.²⁶ However, not all types of chemotherapy-induced cell death are immunogenic. Immunogenic cell death (ICD),²⁷ a special case of regulated cell death, can trigger an adaptive immune response and is associated with the translocation of calreticulin to the plasma membrane and the release of type I interferon, High-mobility group box 1 protein and Adenosine triphosphate that can induce the production of proinflammatory cytokines leading to the recruitment and activation of antigen presenting cells and local T-cells against the tumor associated antigens. These signals are collectively called “damage associated molecular patterns - DAMPs” and were recently used to predict response to chemotherapeutics in the clinical setting.²⁸

Combining biologic and chemotherapies are now considered to aim at multiple therapeutic targets to improve treatment response, minimize development of resistance or minimize adverse events.²⁹ Considering the multiplicity of constitutively activated pathways and the possibility of redundancy in most cancer it becomes a daunting task to find combinations of biological agents that are likely to cure cancer. This represents a major advantage to immune and chemotherapy because both can act in a mutation agnostic way provided that the appropriate cell death mechanisms are operational.

Many different combinational cancer immunotherapies are being tested in various cancer models.³⁰⁻³² Most tumor immunologists focus on the establishment of a durable pool of T cells that have potent antitumor activity.³² The underlying causes for the failure of current immune therapies are 1) the failure of the immune system to recognize the cancer as abnormal, 2) the persistent of immune suppression in the tumor microenvironment that drives paralysis of pre-existing antitumor T cells and 3) the failure of cell death mechanisms. The interplay of immunotherapy and chemotherapy as a means to harness potential synergies therefore principally focus on T cells and checkpoint blockade³² but combination therapy with immune modulatory drugs might also prove effective.^{33,34} These drug types, like Lenalidomide, can stimulate Natural Killer (NK) cell activity that could be highly effective in combination with therapeutic monoclonal antibodies capable of inducing NK-cell-mediated antibody dependent cell cytotoxicity thereby enhancing the efficacy of the monoclonal antibody. Bortezomib, a novel proteasome inhibitor, causes G₂-M cell cycle arrest and apoptosis. But it also enhances anti-tumor T cell immunity via death receptor-mediated apoptosis.³⁵

Combination of chemotherapy and immunotherapy should strive to maximize the immunogenic effects of chemotherapeutics. Many questions should be raised when such combinations are being considered; 1) since the immunogenic effects of chemotherapeutics are pleotropic (ICD, antigen presentation, adaptive and innate immune effector cells, antibody response etc.) it is important to know all of them in order to create synergy between the chemotherapy and the immunotherapy, 2) what doses of the chemotherapeutics should be used, 3) what is the optimal sequence of chemotherapeutics

and immunotherapy and 4) what end points we should use to assess the efficacy of the intervention? Surrogate biological, immunological, radiological or pathological markers should be developed but ultimately, it is by proving that these interventions are capable of improving survival and decrease disease recurrence that they can be validated.

Glycans as targeted antigens for cell death

Glycans and lectins govern cell fate.³⁶ Among glycans are simple ones like *O*-GlcNAcylation with more than 1,000 *O*-GlcNAcylated proteins identified suggesting that targeting this epitope might impact on multiple signaling pathways.³⁷ Growing evidence reveals that *O*-GlcNAcylation has extensive crosstalk with phosphorylation either on the same or adjacent sites of various proteins.³⁸ *O*-GlcNAc modifies proteins in a similar time scale as phosphorylation; modifications that may regulate the cellular signaling pathways involved in cell death.³⁹ However, there can be differences in the expression of glycans dependent on types of tumors.⁴⁰ The GalNAc β 1 $\rightarrow$ 4GlcNAc (LacdiNAc or LDN) group at the nonreducing termini of both N- and O-glycans is not generally found in mammalian cells but whose expression is associated with the progression of human prostate, ovarian, and pancreatic cancers but not with breast cancer.⁴⁰

In tumor cells, alterations of the components comprising the 'glycosylation machinery' generate aberrant *O*-glycans and N-glycans on Fas and TRAIL receptors, which modulate apoptosis. Upregulation of GALNT3, FUT and GDP-FUC, as a result of aberrant DNA methylation, increases tumor cell sensitivity to extrinsic apoptosis through TRAIL. α 2-6-linked sialic acid decorating Fas receptor on tumor cells is known to block Fas ligand internalization, Fas-FADD complex formation and activation of caspase-8 and -3, thus attenuating cell death via the extrinsic pathway. Intracellular and extracellular galectins can modulate survival and apoptotic signaling pathways in tumor cells. Intracellular galectin-1 decreases Akt activity and induces apoptosis. By contrast, intracellular galectin-3 either free or associated with C2-ceramide cooperates with PI3K/Akt to increase tumor cell survival.

Understanding how lectins regulate cell viability and function can broaden our knowledge of the roles of such molecules in basic biological processes but can also facilitate development of therapeutic applications. Models for triggering apoptosis of tumor cells mediated by glycan recognition are those associated with lectins.^{22,41,42} For example, Mistletoe lectins are reported to induce apoptosis in different cancer cell lines in vitro and to show antitumor activity against a variety of tumors in animal models. *Viscum album* var *coloratum*, (VCA)-induces apoptosis by downregulation of Bcl-2 and telomerase activity and by upregulation of Bax through p53- and p21-independent pathway in hepatoma cells.⁴² In other studies it was observed that the induction of apoptotic cell death could be mediated through activation of caspase-3 and the inhibition of telomerase activity through transcriptional downregulation of hTERT in VCA-treated cells.⁴³ Choi et al also demonstrated that the dephosphorylation of Akt leads to inhibition of telomerase activity and concluded that VCA induces apoptotic cell death through Akt signaling pathway, correlated with the inhibition of telomerase activity, and the activation of caspase-3.⁴³

In contrast, other lectins mediate cytotoxicity through cell internalization.⁴¹ The mechanisms of cytotoxic activity of *Griffonia simplicifolia* 1-B4 (GS1B4) and wheat germ agglutinin (WGA) lectins against various murine tumor cell lines have been described.⁴¹ Interestingly, Tumor cells that lack lectin-binding carbohydrates can be resistant to lysis by these lectins. However, some cells that do express GS1B4 lectin-binding sites display low sensitivity to lysis, suggesting that the presence of lectin-binding epitopes, while essential, is not sufficient for tumor cell lysis. Such results suggest that some intracellular mechanisms are involved in the regulation of lectin-mediated cytotoxicity and lectin internalization is probably required for their lysis.

Natural lectin models are the Galectin family. Galectins can act either extracellular or intracellular to exert effects on cell growth and apoptosis. The Galectin family uses different mechanisms associated with their proapoptotic activity. On cancer cells *O*-glycans on cell-surface glycoproteins regulate cell sensitivity to Galectin-1 (Gal-1) induced cell death.⁴⁴ Gal-1 binds to LacNAc and polyLacNAc ligands that constitute attractive targets for molecular imaging and potential biomarkers for cancer.⁴⁵ In contrast, several lines of evidence indicate that galectin-7 (Gal-7) effect on apoptosis is not due to the lectin functioning extracellular through interactions with cell surface glycoconjugates.⁴⁶ In fact, this lectin is found to localize in nuclei and cytoplasm of the transfectants and the transformed keratinocyte line HaCaT. Therefore, galectin-7 is a pro-apoptotic protein that functions intracellular upstream of JNK activation and cytochrome c release.⁴⁶

Binding of galectin-8 modulates integrin interactions with the extracellular matrix and thus regulates cell adhesion and cell survival.⁴⁷ It was proposed that galectin-8 acts as an integrin binding-protein that exerts down-modulatory effects on integrin receptor functions.⁴⁷ Several signal transduction pathways were implicated in mediating anti-adhesive example, by a Ras/Raf- initiated MAP kinase pathway that suppresses integrin activation.⁴⁸ In Jurkat T cells Gal 8 induces a complex phospholipase D/phosphatidic acid signaling pathway.⁴⁹ It was demonstrated that Gal-8 increases phosphatidic signaling, which enhances the activity of both ERK1/2 and type 4 phosphodiesterases (PDE4), with a subsequent decrease in basal protein kinase A activity. The resulting strong ERK1/2 activation was purposed for the expression of the death factor Fas ligand and caspase-mediated apoptosis.⁴⁹

Galectin-9 (Gal 9) possess the anticancer properties by regulating various cellular functions, such as cell adhesion, cell proliferation, and induces apoptosis through the calcium-calpain- caspase-1 pathway.⁵⁰ It has been suggested Gal9-induced apoptosis is mediated by the activating transcription factor 3 (ATF)-Noxa pathway, but is independent of p53 or Bcr-Abl.⁵¹

Antibodies as mimics of lectins

Numerous studies have shown that antibodies can substitute for lectins and mediate tumor cell death.^{24,25,52} There is an emerging awareness that immune surveillance mechanisms that include antibodies and effector cells are intimately related to TACA reactivity that provides a template for developing

strategies for cancer immunotherapy because of the display of glycans in the context of pattern recognition. Both natural antibodies and vaccine-induced antibodies with reactivity to TACA have demonstrated oncolytic properties.^{24,25,52–54} The fact that multiple proteins and lipids on cancer cells are modified with the same carbohydrate structure creates a powerful advantage for TACAs as cancer targets in immunotherapy strategies. Most anti-glycan antibodies recognize epitopes of 2 or 3 sugars. Consequently, antibodies can cross-react with various terminal structures. This property of recognizing epitopes “shared” by different molecules is characteristic of anti-glycan antibodies, and can be considered an example of “antigen mimicry.”

Human hybridoma technology for isolating antibodies from cancer patients indicated that tumor-specific antibodies were germ-line coded and belonged nearly exclusively to the IgM class.²⁵ Furthermore, they all bound to new carbohydrates on post-translationally modified cell surface receptors on malignant cells. The IgM-induced cell death involves classical features of apoptosis such as nuclear fragmentation and activation of caspases.^{24,25,52} Monoclonal antibodies, such as anti-GD2 antibodies, can mediate apoptotic pathways extracellularly, whereby apoptosis signals were transduced via reduction in the phosphorylation levels of focal adhesion kinase (FAK) and the activation of a MAPK family member, p38, upon the antibody binding.⁵⁵ Knock down of FAK resulted in apoptosis and p38 activation.

The inhibition of p38 activity blocked antibody-induced apoptosis, indicating that p38 is involved in this process. Immunoprecipitation-immunoblotting analysis of immune precipitates with anti-FAK or anti-integrin antibodies using an anti-GD2 mAb revealed that GD2 could be precipitated with integrin and/or FAK. These results suggested that GD2, integrin, and FAK form a huge molecular complex across the plasma membrane. Taken together with the fact that GD2+ cells showed marked detachment from the plate during apoptosis, GD2+ small cell lung cancer cells seemed to undergo anoikis through the conformational changes of integrin molecules and subsequent FAK dephosphorylation.⁵⁵

Gangliosides are also sialic containing moieties. Gangliosides are ubiquitous membrane glycosphingolipids modulate cell proliferation, adhesion, migration, and differentiation probably through their effects on transmembrane signaling. Exogenous expression of sialic acid binding *Haliotis discus discus* lectin (HddSBL) induced apoptosis in several cancer cell lines, probably through down-regulating anti-apoptosis factor Bcl-2.⁵⁶ The same is observed for VCA that binds to galactose- and *N*-acetyl-D-galactosamine. Combining VCA with doxorubicin (DOX) suggests that downregulating Bcl-2 makes cells susceptible to chemotherapy.⁵⁷ Therefore, downregulating Bcl-2 by a panspecific response – reactivity to sialic acid moieties that includes gangliosides and/or galactose- and *N*-acetyl-D-galactosamine would be an approach to make these cells susceptible to chemotherapeutics.

We have taken an active therapy approach to targeting TACA using carbohydrate mimetic peptides (CMPs).^{53,58–61} In the development of our CMPs we reasoned that CMPs function as pan immunogens inducing antibodies that recognize multiple TACAs. Immunization with a CMP functions like a polyvalent TACA-based vaccine but the immunogen is a single entity.

Immunization with a common ligand would then induce subpopulations of antibodies that could see a variety of carbohydrate epitopes on various determinates. This approach would therefore induce a broad-spectrum of antibodies with varying specificities that would recognize a distinct yet relevant set of TACA.

We tested the hypothesis that mimicking the hydrogen bond pattern through amino acid substitutions in a CMP, to be coincident with that for the carbohydrate ligand, will enhance the ability of CMPs to elicit the desired anti-TACA antibodies with high titers and association constants.⁶⁰ In this exercise we developed the CMP, termed P10 with primary structure, GVVWRYTAPVHLGD from a random peptide library screen using the anti-GD2/GD3 antibody ME36.1.⁵⁹ Conformational and docking studies suggested that variants of P10 could form more hydrogen bonds with ME36.1 that are in common with the GD2 hydrogen bond interaction pattern with ME36.1. This increased level of mimicry would suggest that a more specific immune response to GD2 should be enhanced. Subsequently, a shortened version of the CMP P10 (WRYTAPVHLGDG) developed which showed anti-tumor activity in mice.⁶² Still, we observed that P10s reacted also with various lectins and with an anti-LeY antibody. This indicated that P10s, as an immunogen, could have induced a broad spectrum of antibodies with TACA reactivity's suitable for pan immunotherapy.

In our studies P10s was shown to bind to human antibody fractions with polyspecificity for a mistletoe determinate, in addition to lactose, fucosyl-lactose, sulfate fucosyl-lactose, sLNLex, Tn, blood group B, blood group A, GD3, GD2, GM2 and LeX, LeY along with several more glycans.⁶³ The antibody fraction isolated by the mimetic peptide also block migration of cancer cells.⁶³ In a phase I clinical trial P10s immunization led to antibodies that mediated cell death extracellularly.⁵⁴ Other CMPs were shown to mediate apoptosis of cancer cells intracellularly, meaning the antibodies are internalized.⁵³ Consequently, P10s and CMPs can induce antibodies that function like lectins mediating cell death.

Extracellular mediation of cell death may be through several pathways. Since the mimetic peptide reacts and induces antibodies reactive with GD2/GD3/GM2 reactivity it is hypothesized that these antibodies might be associated with mechanisms associated with anti-GD2 monoclonal antibodies operating through FAK or through down regulation of BCL-2. It is possible that peptide-induced antibodies play a role in sensitizing tumor cells for more efficient activity of some therapies.⁵⁴ Docetaxel not only inhibits microtubule formation but can also downregulate expression of Bcl-2, a known antiapoptotic oncogene. It is possible that peptide reactive antibodies contribute to downregulating BCL-2 and/or FAK silencing known to promote the *in vitro* efficacy of docetaxel in both taxane-sensitive and taxane-resistant cell lines and may serve as a novel therapeutic approach. The same is observed for Pertuzumab and for Trastuzumab. Combination of antibodies induced by P10s-PADRE and at least docetaxel might have a clear beneficial result.

Picking patient populations to benefit from TACA targeting

Breast cancer diagnosis and clinical trials have provided insight on the molecular characteristics of breast cancer. Treatment

combinations targeting receptor signaling that block the cross-talk between pathways and eliminate escape routes have been proven highly effective in preclinical models. Results of recent clinical studies, while partly supporting this approach, also highlight the need to better identify a priori the appropriate patients whose tumors are most likely to benefit from specific co-targeting strategies. Cancer cells on the brink of apoptosis are more likely to respond to certain chemotherapy agents than cancer cells that have yet to reach that stage.⁶⁴ It has been proposed that tumor growth may be more accurately determined by the outcome of the balance between tumor cell proliferation as indicated by the high expression of the proliferative marker Ki67 on the one side and apoptosis as indicated by elevated activation of caspase-3 on the other.⁶⁴

Gene expression profiling has led to the molecular classification of breast cancer characterized by 4 intrinsic subtypes: basal-like, HER2-positive, *luminal-A*, and *luminal-B*.^{65,66} Although *luminal-A* and *B* subtypes are frequently treated as similar entities, there are obvious differences in the tumor's biological and prognostic characteristics. An important major difference is that *luminal-B* has lower expression of estrogen receptor (ER)-related genes and higher expression of proliferative genes.^{67,68} Traditional classification systems regarding biological characteristics, such as tumor size, lymph node involvement, histological grade, patient's age, ER, progesterone receptors (PR) and HER2 or c-erbB2 status, may have limitations for patient-tailored treatment strategies. Among ER-positive types of breast cancer, the *luminal-A* subtype breast cancer has been shown to exhibit good clinical outcomes with endocrine therapy, whereas the *luminal-B* subtype represents the more complicated type, diagnostically as well as therapeutically. This subtype has a higher recurrence rate and lower survival rates after relapse compare with *luminal-A* subtype. For *luminal-A* type breast cancers, the most common subtype that represents 50–60% of all breast cancers, the addition of chemotherapy to endocrine therapy generally provides little benefit.⁶⁹

An effort to associate clinical benefit of treatment strategies relative to molecular subtype is perhaps best typified by studies in the neoadjuvant setting. Neoadjuvant systemic therapy is a standard of care for patients with inflammatory and locally advanced breast cancer, and is increasingly being used for early-stage disease⁷⁰; mostly with the aim of increasing the chance of achieving breast-conserving surgery. Moreover, the neoadjuvant setting is being increasingly used to study the activity of new drugs or new regimens because the primary endpoint of the trial is reached earlier in a neoadjuvant study compare with adjuvant trials or trials in patients with metastatic breast cancer.

Prominent for clinical benefit is the shrinkage of a tumor, i.e. pathological response, prior to its removal. Studies have demonstrated that pathological complete response (pCR) is the most significant prognostic factor in patients with breast cancer treated with neoadjuvant therapy.^{71,72} Overall, patients with breast cancer who experience a pCR with neoadjuvant chemotherapy have significant improvements in both disease-free survival (HR 0.48, 95% CI: 0.37–0.63) and overall survival (HR 0.48, 95% CI: 0.33–0.69) compared with patients with residual invasive disease.⁷³ Therefore, pCR is considered by some to

represent a valid surrogate end-point for long-term outcomes, including progression-free survival and overall survival that usually serve as primary end-points in trials in adjuvant or metastatic disease settings.⁷¹ Changing the pCR rate can affect standard of care. The FDA has accepted the proof of activity of an experimental agent in neoadjuvant trials with pCR as the primary end-point as the evidence for approval,⁷⁴ as exemplified for subcutaneous trastuzumab by the HannaH trial.⁷⁵ More recently, the FDA used the findings from a meta-analysis showing an increase percentage of pCR rates⁷¹ to support the approval of pertuzumab (Perjeta) in the neoadjuvant setting.⁷⁶

There is an important practical consideration for neoadjuvant therapy. FDA guidance renders pCR as an adequate predictor for studying combination therapy in high-risk populations comprised compare with those that hormone receptor positive but of low grade. Triple Negative and *luminal-B* populations typically have Ki67 expression levels of > 14% in keeping with the idea that chemotherapy is most effective at killing cells that are rapidly dividing. While displaying a high cell proliferation rate as determined by the Ki67 biomarker, the emphasis on this population is because of the unfavorable prognosis with existing therapy compare with low-grade, hormone receptor-positive tumors (so called *luminal-A*) that have a more favorable long-term prognosis and are more likely to do well with currently available therapy. The *luminal-A* tumors have low proliferation rate (Ki67 <14%), hence it takes longer to acquire all the needed changes and to reach the clinical detectability level while the triple negative is more proliferative.

Luminal-A cancers, however, represent a significant continuing challenge, because if patients with these tumors present with a high tumor burden with 4 or more positive nodes, their outcome with endocrine therapy remains largely unsatisfactory. The benefit from chemotherapy is either very minimal or non-existent suggesting that *luminal-A* tumor's and some *luminal-B* tumors are *de novo* resistant to chemotherapies. The effectiveness of endocrine therapy is limited by high rates of *de novo* or intrinsic resistance (existing before any treatment is given) and acquired resistance during treatment (resistance that develops during a given therapy after an initial period of response). One third of patients will have recurrent disease within 15 y after being treated with tamoxifen for 5 y. About 50% of patients with metastatic disease do not respond to initial endocrine treatment.⁷⁷ Inevitably the vast majority of patients with ER-positive advanced breast cancer will become refractory to endocrine therapy. Consequently, *luminal-A* tumors do not derive much benefit from chemotherapy, and pCRs in this subgroup are not very predictive of outcomes. If we were to find a way to sensitize tumor cells to standard chemotherapy will pCR for this subpopulation become predictive?

TACA are involved in tumor cell dissemination and in dormancy.⁷⁸ Circulating Tumor cells can evolve with the expression of different TACA moieties that affect distant tumor cell dissemination and organ colonization. These disseminated tumor cells (DTCs) carry the same indolent nature of the primary tumor but as they are evolving independently from the primary tumor and are undergoing different environmental pressures they end up acquiring different genetic/epigenetic changes; so, even if you have a treatment that kills all the

primary cancer you may not be able to eradicate the DTCs or micrometastases.

Altered sialylation has long been associated with metastatic cell behaviors including invasion and enhanced cell survival; however, there is limited information regarding the molecular details of how distinct sialylated structures or sialylated carrier proteins regulate cell signaling to control responses such as adhesion/migration or resistance to specific apoptotic pathways.⁷⁹ Sialic acid-binding lectin (SBL) is a multi-functional protein that is isolated from oocytes of *Rana catesbeiana* which displays caspase 3 dependent killing of cancer cells.⁸⁰ Selective treatment of cell lines with SBL revealed that SBL induces cell death on estrogen receptor (ER)-positive breast tumors but not on ER-negative breast tumors.⁸¹ The anti-tumor effect of SBL-treated ER-positive breast tumors is accompanied by the down-regulation of ER and Bcl-2.⁸¹ They also showed that Bcl-2 overexpression, but not Bcl-XL overexpression, significantly inhibits the effect.⁸¹

Gene expression profiling of *luminal-A* type cells suggest that Bcl-2 is upregulated in these cells, which indicates that they are perhaps resistant to cytotoxic chemotherapy. Reduced sensitivity to various chemotherapeutic drugs is well known to be mediated by high levels of the anti-apoptotic protein Bcl-2⁸² and a low Ki67 expression profile, typical for *luminal-A*. If we can overcome Bcl-2 resistance we can optimize for other breast cancer subtypes. Breast tumor cells overexpressing Bcl-2 is hypothesized to become more resistant to chemotherapy, but they have a less aggressive behavior (they are less able to produce metastasis), and for this reason primary tumors containing Bcl-2 overexpression can be related to a better survival. While Bcl-2 inhibits apoptosis, its overexpression is associated with hormone refractory cancer.⁸³ The antagonism of Bcl-2 in ER⁺ tumors might enhance the effectiveness of predominately proapoptotic treatments even more than it does from combination with endocrine therapies. Targeting sialic acid moieties using antibodies that function like SBL or using SBL directly might be synergistic with chemotherapies or with targeted therapies. Because diminished Bcl-2 expression in cancer confers increased sensitivity to cytotoxic chemotherapy, it is possible that breast cancer patients with endocrine-resistant disease could achieve significant therapeutic benefit from cytotoxic agents when used as a second-line treatment. Sensitizing tumor cells with TACA reactive antibodies that facilitates downregulation of Bcl-2 or interrupts survival-signaling pathways to enhance the cytotoxicity of standard Chemotherapy would provide a new molecular platform for the development of therapeutic strategies effective against solid tumors and might change treatment paradigms associated with breast cancer.

Summary

Chemotherapy may be ineffective when faced with cancer cells that are not already close to death. Chemotherapies activate multiple signaling pathways that can lead to different cell death outcomes. Understanding how these pathways cooperate and interfere is essential for the design of rationally based chemotherapeutic combinations. Targeting TACA highlights the goal of maximizing the induction of pCR in patient populations through combination therapy. The rationale for which lies in

studies of lectins used to explore particular signaling pathways that are important to cancer cell survival. Improving pCR in populations whose pCR rates are intrinsically low is a valid strategy in the development of new treatment regimens that may correlate with clinically meaningful improvement in cancer therapies.

Abbreviations

TACA	Tumor Associated Carbohydrate Antigens
NK cellpCR	Natural Killer
ICD	Immunogenic cell death
DAMPs	Damage associated molecular patterns
DOX	Doxorubicin
ER	Estrogen receptor
VCA	Viscum album var. coloratum
GS1B4	Griffonia Simplicifolia 1-B4
WGA	Wheat germ agglutinin
FAK	Focal adhesion kinase
HddSBL	Haliotis discus discus lectin
SBL	Sialic acid-binding lectin
CMPs	Carbohydrate mimetic peptides
PR	Progesterone receptors
HER2 or c-erbB2	Human epidermal growth factor receptor 2
Ig	Immunoglobulin

Disclosure of potential conflicts of interest

TKE, BMK, and UAMS have a financial interest in the technology discussed in this manuscript. This interest has been reviewed and approved in accordance with the UAMS conflict of interest policies. The other authors have no financial interest to declare. A patent has been filed on the vaccine.

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