Specific Aims

Breast cancer is a complex disease driven by the interaction of heterogeneous tumor and tumor-interacting cells. Metastatic spread drives the vast majority of breast cancer mortality and, to do so, requires tumor cells to both disseminate and avoid clearance by the immune system.[7] Inhibiting TAMRs has shown promising results in models of breast carcinoma by blocking tumor cell dissemination, preventing resistance to chemotherapy and HER2-targeted drugs, reprograming the tumor microenvironment, and relieving immune suppression (Fig. 1).[3,4,8–11] Based on these results, the first therapies targeting these receptors are now in early clinical studies.[9,12] We lack a molecular understanding of exactly how, when, and where these receptors are activated, however. Identifying the cues activating TAMRs in each of the various TAMR-expressing cell populations is essential to identify the patients who will benefit from existing therapies and develop effectively targeted inhibitors.

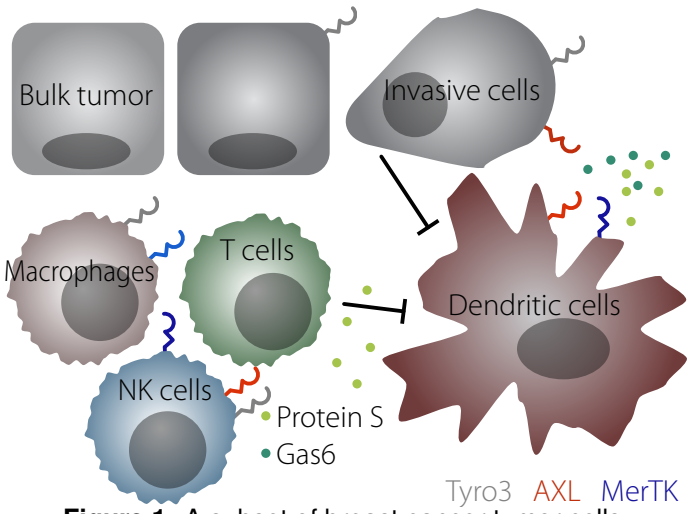

Figure 1: A subset of breast cancer tumor cells overexpress AXL, making them invasive and resistant to therapy. TAM (Tyro3, AXL, MerTK) receptor (TAMR) activation within dendritic cells (DCs) potently inhibits the innate immune response.[1] T cell release of Protein S (ProS) further dampens the immune response.[2] Activation of TAMRs inhibits natural killer (NK) cell-mediated lysis.[3] Each of these cell populations express distinct and dynamic combinations of TAMRs, modulating functional changes in microenvironmental response.[3–6]

Here, we propose to assemble mechanistic models for activation of these receptors and to examine the contexts in which TAMR activation occurs. With models of TAMR activation and tool compounds of specific, well-characterized effect, we will revisit an *in vivo* model of breast carcinoma known to depend on TAMR signaling through multiple processes. Investigating the efficacy and consequences of these inhibitors will allow us to deconvolve the pleiotropic role of these receptors *in vivo*.

Year 1: Utilize mechanistic kinetic modeling to identify the effects of differing TAMR targeting strategies Challenge: Poor understanding of the ligand-mediated TAMR activation mechanism limits our ability to identify tumors dependent upon TAMR signaling and effectively target the receptors.

- Measure the binding interaction kinetics of murine and human TAMR for ProS/Gas6
- Parameterize kinetic models of receptor activation using stimulation and affinity measurements
- Examine the influence of receptor identity and activation mechanism on targeting strategies by modeling the effects of decoy receptor fragments (DRFs)
- Validate model predictions regarding the ability of each DRF to inhibit specific TAMRs or activation contexts

Year 2: Validate model-predicted targeting *in vivo* by comparing the effects of DRFs to existing TAMR-targeted compounds Hypothesis: DRFs will demonstrate superior TAMR targeting due to their unique selectivity profiles.

- Compare the effect of selected DRFs to R428 and LDC1267 in an immunocompetent model of breast carcinoma with pleiotropic TAMR-dependent effects
- Quantify the consequences of each therapeutic strategy on immune cell infiltration, cytokine expression, and metastatic burden
- Deconvolve the *in vivo* mechanisms of TAMR-targeted therapeutic efficacy through multivariate modeling

The benefit of these efforts will be two-fold: (1) The tool compounds developed here and their derivatives will be inhibitors of exceptional specificity and potency, with potential therapeutic value. (2) Characterizing the processes by which TAMRs are activated by tumor cells and the mechanism of therapeutic benefits will suggest measurements that can be employed for determining which patients will benefit from these therapies.

Scientific & Technical Merit

Significance

Decoding the complex roles of TAMRs in breast cancer Tyro3, AXL, and MerTK comprise the TAMR receptor tyrosine kinase (RTK) family. While activating TAMR mutations have not been documented in breast cancer, targeting the receptors by genetic or pharmacological means potently blocks metastasis and enhances survival in murine models.[3,9,13] TAMR ligands, ProS and Gas6, bind to the receptors via two independent sites, and ligand bridges two receptors to induce activation (Fig. 2).[14] Both TAMR ligands contain Gla domains that are post-translationally modified by γ-carboxylation, a vitamin K-dependent process, enabling phosphatidylserine (PS) binding.[15–17] PS is important for activation of TAMRs and the compliment of TAMRs expressed can modulate dependence on PS for activation.[15,17,18] The complex and multifactoral activation mechanism of this receptor family has made identifying when the receptors are active challenging. This in turn complicates identifying which patients will benefit from therapies that inhibit TAMRs.

These receptors have diverse roles in cancer progression (Fig. 1). In breast cancer cells, AXL expression occurs in response to epithelial-to-mesenchymal transition, and is essential for metastasis even when the receptor's effects on the primary tumor growth are less substantial.[8,20] Basal B subtype, triple negative breast carcinoma cell lines (BCCLs) show striking overexpression of AXL, while tumors *in vivo* show much greater molecular heterogeneity.[21] Accordingly, while *in vivo* overexpression of AXL within the bulk tumor is not striking, AXL overexpression in disseminating cells corresponds to a poor prognosis, positive lymph node status, and metastatic capacity.[8,21] AXL activation also acts as a means of resistance to chemotherapy and targeted therapies such as those against HER2 and PI3k activation, and so these therapies may select for tumor cells primed to disseminate.[10,11,22,23]

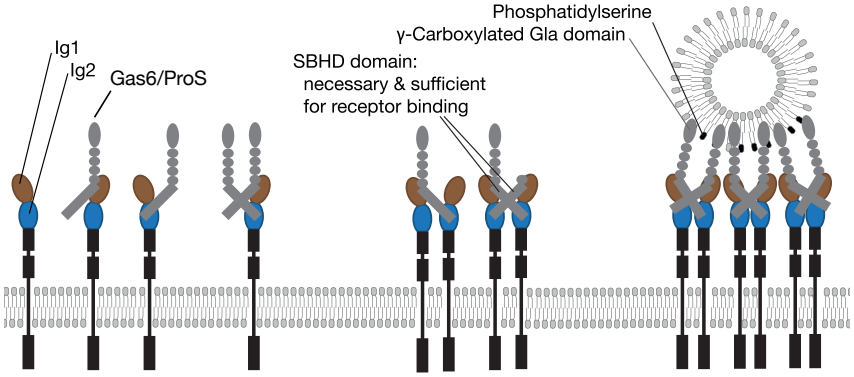

Figure 2: Diagram of TAMR-ligand interaction. Receptor and ligand interact through two (Ig1, Ig2) binding interfaces. These interactions, only mediated through the SBHD domain, lead to receptor dimerization with no receptor-receptor ectodomain interactions. PS interaction via the Gla domain is known to be important to activation. Adapted from [19].

TAMRs simultaneously drive breast carcinoma through tumor-extrinsic processes as well. These receptors negatively regulate the innate immune system when activated in macrophages (MØs) and DCs, and similarly block NK cell activation.[1,2,24] Knockout of MerTK within leukocytes, or of all three TAMRs within NK cells, potently blocks metastasis.[3,4,6] Specifically within the post-partum breast, mammary involution creates an abundance of apoptotic debris, leading to a prometastatic environment through activation of MerTK in tumor-infiltrating immune populations.[6] TAMR activation ultimately also regulates the adaptive immune response by regulating DC interactions with B and T cells.[2,25,26] Some specificity toward effects on metastases, rather than the primary tumor, may indicate that the subpopulation of actively disseminating cells benefit most from TAMR activation.[3,4,13]

Despite these many promising results largely from genetic manipulation of TAMRs in murine models, to design effective therapies we still need an integrated understanding of when TAMR activation drives breast cancer within the various TAMR-expressing cell populations. Within cancer cells, the exact processes that AXL overexpression contributes to in establishment of metastases—whether enhanced survival of disseminating cells, invasion, or immune escape—has not been elucidated. Immune cells display distinct complements of TAMRs (e.g. DC and NK cells can express all three), and modulate their expression in response to inflammatory cues, likely modulating the response to and consequence of PS interaction.[3,15,17,18] While genetic studies have to date only examined systemic inhibition of receptor activation, dependence upon PS exposure likely indicates TAMR activity is highly localized to sites of interaction with cellular debris.[3,6] It is unclear the extent to which systemic inhibitors exert their beneficial effects through direct breast cancer cell targeting versus promoting immune surveillance. Understanding

the role of these receptors in tumor progression is critical for designing optimal TAMR-targeted therapies, knowing which patients will benefit, and combining these therapies with other agents.

Rationally designed TAMR inhibitors of improved potency and specificity The inhibitors developed here will have the unique capacity to inhibit both Gas6 and ProS-mediated TAMR activity across the family, have quantitatively predictable effects on signaling and ligand engagement, and show exceptional selectivity (Fig. 3). These features will arise through the unique design for these protein fragments with well-characterized interaction, our molecular engineering efforts to characterize their effect (Aim 1), and our *in vivo* validation (Aim 2). Each feature will be critical to translating the benefits of inhibiting TAMRs in preclinical models to patients.

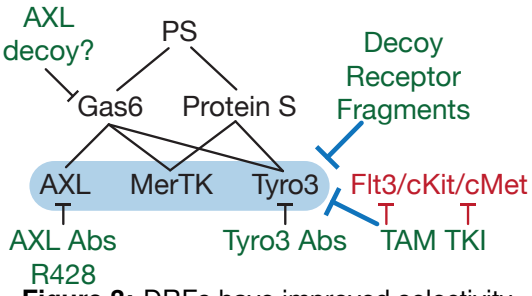

Figure 3: DRFs have improved selectivity and potency for inhibiting TAMRs. Green indicate inhibition strategies. Blue lines indicate inhibition of all three TAMRs. Red indicate undesirable off-target inhibition. Black indicate inhibition of individual entities. DRFs are unique in their ability to inhibit all three TAMRs without inhibiting other RTK families, their ability to inhibit ligand binding and receptor activation in different combinations, and well-characterized effect. These features will be critical for studying the role of TAMRs *in vivo*, designing effective and potent TAMR-targeted therapies, and identifying the patients who will benefit from these therapies.

Selectivity may be essential to targeting the TAMRs; knocking out all three receptors results in viable animals, suggesting a promising therapeutic window with minimal toxicity.[27] The compounds here should have minimal effects on other processes as their only known binding partners are the two TAMR ligands through their TAMR-interacting sites. In contrast, small molecule inhibitors of TAMRs (with the possible exception of R428) all possess off-target inhibition of the other RTKs cKit, Flt3, and/or cMet.[9,28] Inhibiting these receptors without therapeutic tumor effects is undesirable due to the receptors' roles in hematopoetic (cKit) and dendritic cell (Flt3) differentiation and development,[28–30] and consequent hepatic and nephrotic toxicity (via cMet).[28,31]

In contrast to the benefits of selectivity among all RTKs families, broad inhibition of all three TAMRs, activated by either ligand, will be necessary for potently inhibiting TAMR-dependent effects. Every entirely TAMR-specific compound, including R428, a decoy AXL receptor, antibodies, and receptor-binding aptamers, only inhibit a subset of TAMR activity (Fig. 3).[13,32–34] Mammary epithelial cells frequently express some amount of MerTK and/or Tyro3, in addition to AXL, which will consequently likely act as a resistance mechanism to just inhibiting AXL.[6,35–37] Immune cells similarly express combinations of all three TAMRs, and dynamically change their TAMR expression in response to cues.[18] Therefore, being able to inhibit all three family members in combination will likely improve treatment efficacy.

A final benefit of the compounds proposed here is that they are a somewhat diverse set of TAMR inhibitors with well-characterized effect. Through our efforts in Aim 1, we will be able to make quantitative predictions about the extent of TAMR inhibition in particular cell populations. Additionally, since each fragment will vary in its effect, we can compare different treatments in order to learn about the role of TAMR activation *in vitro* or *in vivo*. Through these benefits, developing this family of DRFs will be a valuable step toward improving our understanding of TAMRs in cancer and effectively inhibiting this receptor family.

Identify predictive measurements of TAMR activity Maximizing the effectiveness of targeted therapies has generally required pairing with diagnostic technologies to identify patients in which the targeted dysregulation is maximally active.[38] For non-TAMR receptors that respond to ligand concentration or have activating mutations, measuring these two factors has been effective for this diagnostic.[39] The more complex activation mechanism for TAMRs, however, indicates that other measurements are likely necessary to predict whether these receptors are active.[15,19] The modeling results assembled here will be immediately helpful for determining what set of measurements will be maximally informative.

Innovation

TAMRs are implicated in multiple processes promoting breast carcinoma progression, including metastasis, resistance to chemotherapy and targeted agents, and immunosuppression.[3,4,8–11] Yet, we are currently unable to determine where and when these receptors are activated. This limits our ability to identify measurable factors that predict which patients will benefit from TAMR-targeted therapies, and to rationally design therapies targeting TAMR activation in the relevant cell populations.

Engineering TAMR activity This proposal combines the strengths of computational and experimental approaches to enable prediction and manipulation of TAMR activity in a more precise fashion. By directly measuring the affinities of both interaction sites for each receptor-ligand pair, we will be able to limit the parametric uncertainty of our modeling efforts. Further parameterizing our mechanistic kinetic models of TAMR activation with quantitative measurements of receptor activation in controlled experiments will allow us to predict the effect of defined interventions on multiple TAMR-expressing populations simultaneously. The approach taken here of directly measuring the requisite affinities, and then using Bayesian methods to assess model uncertainty throughout, will be critical to properly interpreting the results of our modeling, and identifying additional experiments that might address uncertainties. While borrowing methods from the study of other RTKs and quantitative observational fields, the approach here is unique in combination and will ensure rigor while quickly assembling a complete TAMR activation model.

This combination of techniques will be critical to understanding where and when these receptors are activated. All three TAMRs are expressed in distinct combinations across immune and tumor cell populations. These three receptors bind two ligands with differing combinations of affinities, which then can bind to PS-presenting moieties. If these factors vary in abundance at just three levels with different behavior, combinations of these factors can give rise to as many as 3^6 or 729 different responses. Thus, experimentally manipulating these factors alone or even in combination is unlikely to fully capture the range of possible behaviors without a theoretical underpinning for these observations.

Combinations of precisely characterized tool compounds for querying *in vivo* TAMR function Part of our limitation in understanding where and when TAMR activation influences breast cancer progression arises from our limited ability to manipulate the receptor family. Currently, one is limited to genetic manipulations, small molecule inhibitors with critical off-target issues, and biologic inhibitors of sparsely characterized effect.

A core novelty of our approach, building upon efforts such as an affinity enriched AXL ectodomain,[13] is to split each receptor into fragments with a single ligand interaction. Having a single interaction site greatly improves our ability to predict the effect of these protein fragments. Our preliminary results already have shown the diversity of responses these fragments offer in inhibiting distinct TAMR members and activation contexts (Fig. 5). Thus, this strategy is both novel and a critical development toward studying the *in vivo* environment.

Research Strategy

Aim 1: Utilize mechanistic kinetic modeling to identify the effects of differing TAMR targeting strategies

Rationale Knowing the molecular details of how TAMRs become activated will allow us to determine in which cell populations these receptors are active within different patients, then predict the effect of particular inhibition schemes.

1.1. Measure the binding interaction kinetics of murine and human TAMR for ProS/Gas6 Measurements of TAMR-ligand binding have not taken into account the presence of two distinct binding interfaces with unique affinities.[14] Recent studies of TAMR function from our lab and others have shown that the asymmetry in affinity between each binding interface is critical for the PS sensing function of these receptors.[19] This has been most extensively investigated for the case of AXL-Gas6 interaction, where the high affinity binding interface is of picomolar affinity and ligand is found almost constitutively bound. Despite this, the lower affinity receptor-ligand interaction is rate limiting, making PS interaction essential for activation.[13,15,17] Further, while ligand and receptor

from mouse and human is frequently used interchangeably, species-dependent activity differences have frequently been observed.[15] For example, in the case of ProS binding to Tyro3, the overall murine receptor-ligand interaction is known to be 100-fold stronger than in humans.[40]

To understand how each ligand and receptor function within a unified system, we will express the His-tagged Ig domains of each receptor in baculovirus. We will then measure interaction of Gas6 and ProS with each domain by kinetic surface plasmon resonance analysis and in equilibrium binding assays. A concern with using fragments of each receptor is the fragment may not be representative of full length protein due to conformational changes induced between Ig domains. To validate our measurements, we will verify that the affinities measured correspond to those for the two domains expressed together. Receptor-ligand binding is known to be influenced by the endosomal pH, and so we will also measure the effect of pH on affinity.[41]

As preliminary validation of our approach, we have successfully expressed each DRF and measured the equilibrium binding of each TAMR Ig domain-Gas6 interaction (Fig. 4a). Importantly, our results are consistent with previous measurements of Gas6 binding to the full monomeric Tyro3 extracellular domain (Fig. 4b).[33] This result provides us with confidence that our affinity measurements are representative of the binding kinetics driving receptor activation in cells. These measurements of binding kinetics will be essential for determining the consequences of particular interventions and interpreting these effects across species. Further, the affinities of many of these fragments, in the 1–20 nM range, support their use as competitive inhibitors of receptor activation.

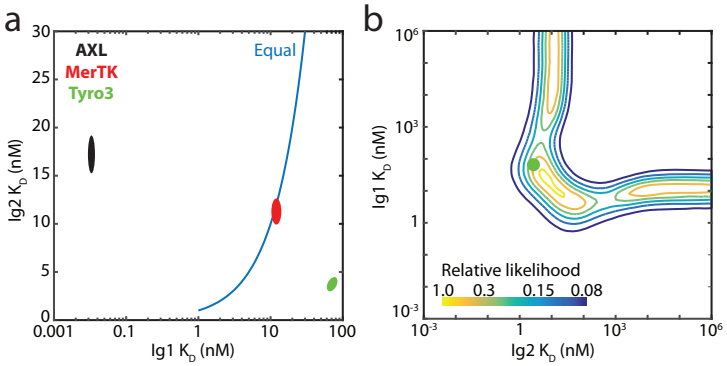

Figure 4: Preliminary measurement of human TAMR-ligand interaction. **a)** Resulting fits to equilibrium binding measurements for Gas6 and each TAMR Ig fragment. Ellipses show the std. err. of each K_D. **b)** Each contour line represents the relative likelihood of different Ig1/Ig2 affinity combinations by reexamining previous measurements of the overall affinity between human Tyro3 and Gas6.[33] The green ellipse indicates our measurement of each individual Gas6-Tyro3 Ig domain affinity.

1.2. Parameterize kinetic models of receptor activation using stimulation and affinity measurements TAMR interaction with their cognate ligands is known from crystal structures of the interacting complex.[14,42] In work published last year, we used this information to develop a mechanistic ordinary differential equation model that simulates activation of a TAMR by ligand when only one receptor is expressed and one ligand is present (Fig. 5a). We then used this model to capture the activation kinetics of cancer cells expressing AXL stimulated by Gas6.[19] In order to account for localized stimulation of receptor, we extended our reaction model to incorporate diffusion of receptor within a radially symmetric geometry. This allowed us to take into account effects of local stimulation driven by PS interaction and to examine differences in sensitivity to particular therapeutic approaches. This work showed that relocalization of AXL to regions of high receptor concentration can drive activation, and that this localization can explain the ability of PS to potently activate AXL.[19] A model for cells expressing multiple TAMR receptors simultaneously exhibits additional combinatorial complexity due to the higher order effects of heterodimerization. However, with measurements of each receptor Ig domain's affinity for each ligand, the model with multiple receptors and ligands has no additional parameters, or parametric uncertainty, due to detailed balance.[43]

In order to specify a model, we additionally need information about the kinetic parameters for each trafficking process (endocytosis, synthesis, degradation, recycling). These have been roughly estimated in prior work, but will be allowed to vary within an order of magnitude to account for variation between cells.[19] To make sure any uncertainty is taken into account, we will utilize Markov Chain Monte Carlo, a Bayesian approach to fully sample parameter space, fitting our published measurements of AXL activation dynamics in response to Gas6 in BCCL.[19,44] Any predictions derived in subsequent analyses will take into account parameter uncertainty by deriving the predictions over the entire parameter posterior distribution.

1.3. Examine the influence of receptor identity and activation mechanism on targeting strategies by modeling the effects of DRFs With models for activation of TAMRs within various cell types, we will then examine strategies specifically targeting each. We will simulate the effect of adding different competitively binding proteins, including decoy receptor (each Ig domain) (Fig. 5). We will quantify (a) the specificity of each intervention toward either cancer or immune cell signaling, (b) whether the intervention preferentially blocks signaling from localized (PS-dependent) or uniform ligand stimulation, and (c) whether the intervention leaves PS debris tethered to the cell surface. Specificity will arise from distinct receptor expression in each cell type, differences in the dependence on PS for activation, and differences in whether a phenotypic outcome relies on just debris binding or also TAMR activation. We expect that some interventions will show specificity toward PS-mediated activation of TAMR receptors within NK and/or other immune cells, while others will show specificity toward PS-mediated activation of AXL within tumor cells. Therefore, by comparing the effect of these different ligand and receptor fragments, we can determine which processes are therapeutically important. While we will test the *in vivo* effect of DRFs in Year 2, this modeling effort will provide confidence in the molecular mechanism of effects we observe, provide us the basis for selecting particular DRFs, and direct us to further optimize the receptor fragments as inhibitors.

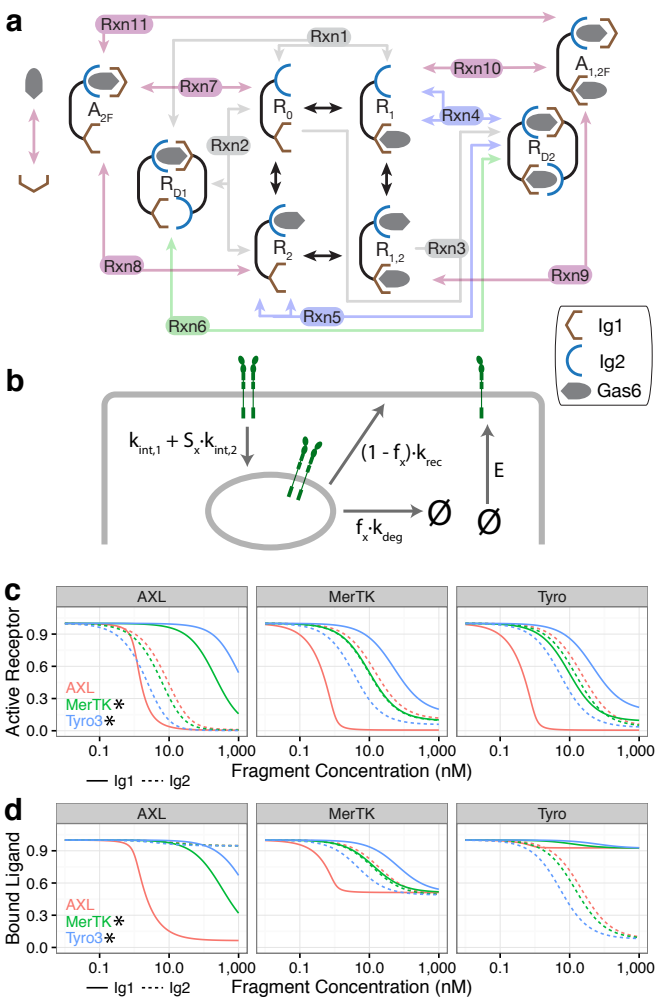

Figure 5: Preliminary predicted effects of DRF without PS effects. **a)** Schematic of TAMR signaling model incorporating one receptor and ligand along with the effect of a DRF. Ligand binds at two sites of distinct affinity (brown/blue). Receptor dimerization is driven by ligand interaction. Heterodimerization of TAMRs can be accounted for but is not shown for simplicity. **b)** Receptor and ligand trafficking was assembled from knowledge about other receptors. **c)** Predicted effects of each DRF on phosphorylated receptor for simulated cells expressing just the indicated TAMR. **d)** Predicted effects of each DRF on surface TAMR-bound ligand. Stars indicate fragments that should also have effects on ProS-mediated activation.

1.4. Validate model predictions regarding the ability of each DRF to inhibit specific TAMRs or activation contexts To experimentally evaluate our computational predictions, we will use the Ig domain fragments produced for TAMR ligand affinity measurement as competitive inhibitors. To test inhibition of PS-independent TAMR activity, we will treat a panel of AXL-expressing BCCLs with increasing amounts of each protein (0–250 nM), in the presence of 1 nM ΔGla Gas6 (AXL does not bind ProS), along with warfarin. (Warfarin inhibits synthesis of vitamin K, inhibiting γ-carboxylation of Gas6 and thus interaction of the Gas6 Gla domain with PS.) As a positive control, we will inhibit AXL kinase activity with 1 μM R428.[9] We will lyse cells after 4 hrs of treatment, immunoprecipitate each receptor, and then quantify associated phosphotyrosine. We will then also test the DRF inhibitory effects with PS present by stimulating with PS-containing vesicles and full-length ligand (without warfarin). Parallel to quantifying the abundance of phosphorylated receptor, we will capture the molecular consequences of AXL inhibition through a panel of downstream pathway activation measurements (incl. pAkt, pErk, pcJun, & pP38) by multiplexed ELISA.

The predicted effects of each DRF will additionally be validated in TAMR-expressing primary naïve immune populations. Whole blood will be obtained from healthy human donors, and peripheral blood mononuclear cells will be isolated by density gradient centrifugation. We will isolate naïve monocyte and NK cell populations by negative selection, and differentiate a portion of the monocyte population into macrophages through M-CSF

stimulation for 7 days. We have successfully performed this isolation to characterize the abundance of each TAMR in these populations. In the background of no exogenous ligand, 1 nM Gas6, or 10 nM ProS, with exogenous addition of PS-containing vesicles, the consequence of a pretreated dose response for each DRF will be evaluated both through quantification of phosphorylated TAMR and through quantification of cell-associated ligand. Ligand concentration will be measured by ELISA in each case to account for autocrine ligand production as well. In addition to our receptor-proximal measurements we will quantify STAT1 phosphorylation and SOCS1/SOCS3 abundance to assess TAMR-mediated immunosuppression.[1]

Potential pitfalls & alternative approaches Parameterization of ordinary differential equation models, even for simple reaction systems, can be challenging due to nonlinear effects and redundancy in the set of parameters that produce what is experimentally observed. Parameter uncertainty may obscure specific predictions. To reduce uncertainty in Aim 1.2, we may need to further constrain the models with additional data such as the amount of surface receptor, cell-associated ligand, or phosphorylated receptor with different ligand stimulation conditions.

In the absence of parametric uncertainty, our modeling efforts may fail to accurately predict TAMR activity due to missing factors in our model. However, this would suggest interesting and important biology to be uncovered. In this case, we will use the model as a hypothesis-generating tool for identifying these new factors. Lacking a predictive model for TAMR activation, we would still be able to experimentally characterize the effect of each DRF on TAMR activation within each cell population, and so do not believe such a situation would prevent the subsequent tasks described here.

Year 2: Validate model-predicted targeting *in vivo* by comparing the effects of DRFs to existing TAMR-targeted compounds

Rationale The work proposed in this Aim will apply the modeling and tool compounds developed up to this point to manipulate *in vivo* TAMR signaling in a controlled manner. We expect that the DRFs used here will serve as valuable reagents to precisely manipulate TAMR activity and will have unique therapeutic value.

2.1. Compare the effect of selected DRFs to R428 and LDC1267 in an immunocompetent model of breast carcinoma with pleiotropic TAMR-dependent effects To evaluate different TAMR targeting strategies, we will use the immunocompetent, syngeneic breast carcinoma xenograft BALB/c-4T1-luciferase. Importantly, this and a similar model show pleiotropic benefits from targeting TAMR signaling through the tumor cells themselves,[8,9,13,20] the tumor microenvironment,[4,6] and NK cells.[3]

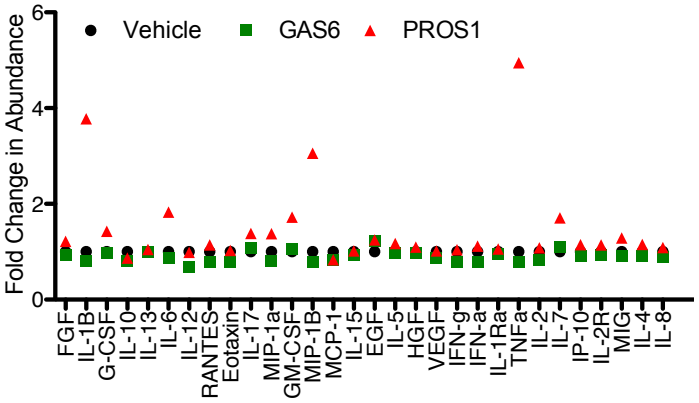

Figure 6: Cytokine changes measured from M2-polarized (using PMA/IL-4/IL-13) MØs after treatment for 24 hrs with Gas6 or ProS.

We will evaluate the efficacy of differing TAMR inhibition strategies in parallel: (1-2) two DRFs, or (3-4) two TAMR-targeted kinase inhibitors in preclinical and clinical development. We will select two of the six DRFs based upon the relative affinities of each for Gas6/ProS and relative potency towards blocking DC, tumor cell, and NK cell TAMR signaling. The effects of each DRF fragment are derived from its affinity for Gas6/ProS and binding location (Ig1 vs. Ig2). Some weight will be assigned toward selecting DRFs of differing properties (e.g. differing combinations of TAMRs) over simply the affinity of each, in order to use the observed effects as a means for probing the relevant cellular processes leading to therapeutic efficacy *in vivo*.

To determine tolerability of each DRF, 6-wk-old BALB/c mice will be injected intravenously via the tail vein with a 10 mg/kg body weight dose twice a week for 4 wks.[13] Animal weight will be measured over the course of the study. After 28 days, mice will be killed, and complete blood counts and chemistry panels performed. Lung, liver and kidney tissue samples will be harvested and stained with hematoxylin and eosin (H&E) for histological analysis of toxicity, evaluated by a veterinary pathologist. To measure pharmacokinetics, DRFs will be injected

intravenously via the tail vein, and blood samples will be taken periodically for 48 hrs. The abundance of each over time will be measured by ELISA.

Animals will be injected with 10^6 4T1-luciferase cells, allowed to establish for 1 wk, and then randomized into treatment groups. We will include 17 mice in each group, sufficient for power at 0.8 at a significance level of $p < 0.05$ given a moderate effect size (true difference in mean equal to std. dev., two-sided t-test). Dosing schedules and amounts for each small molecule compound will follow previously established protocols.[3,9] If well tolerated, each DRF will be administered at 10 mg/kg twice a week via intravenous tail vein injections for 30 days. The dosing schedule may be adjusted to higher frequency if required by the observed pharmacokinetics to sustain 10% of the maximal serum concentration and well tolerated. Tumor volume will be measured twice weekly and calculated according to $\frac{1}{2}(w \cdot h \cdot l)$. Every other week, each mouse will additionally be characterized by IP injection with luciferin substrate, anesthesized, and then imaged in an IVIS Imaging System. Animals will be euthanized on day 31, or at a tumor burden end point dictated by IACUC protocol. In addition to standard reporting of statistical significance, we will include confidence intervals in our analysis and conduct an additional post-hoc power analysis to ensure our conclusions are sufficiently powered.

2.2. Quantify the consequences of each therapeutic strategy on immune cell infiltration, cytokine expression, and metastatic burden We expect that the therapeutic benefits of DRFs arise through: (1) selectivity for TAMRs without the potential to inhibit other RTKs such as FLT3 and cKIT and (2) the ability to inhibit all three TAMRs activated by Gas6 or ProS. In order to investigate the cellular response consequences and any benefits of the DRF compounds over existing therapeutic strategies, we will evaluate the functional consequences of each *in vivo*.

We will examine the consequences of each therapy through a combination of measurements. Primary tumors will be split into two fractions, with one disassociated and fixed as single cells and another lysed. Various tissues including the lungs, bone, rib cage, lymph nodes, ovaries, kidneys, liver, brain, intestine, and spleen will be examined for metastases by H&E staining. Using CD3/CD19/Ter119/NK1.1/EMR1/PDCA1/CD11c staining by multicolor flow, we will examine the composition and abundance of tumor infiltrating immune populations.[29] We expect to see suppression of DC abundance with LDC1267 due to its off-target inhibition of Flt3 and cKit, while all four treatments may lead to beneficial tumor infiltration through the multiple effects of TAMR inhibition. We will additionally examine the abundance of a wide panel of cytokines within the tumor and metastasis lysates by multiplexed ELISA (Fig. 6). Both DRFs, then LDC1267, are expected to have the largest cytokine expression effect due to the coordinate inhibition of all three TAMRs.

2.3. Deconvolve the *in vivo* mechanisms of TAMR-targeted therapeutic efficacy through multivariate modeling As TAMR-targeted therapies will likely benefit only a subset of cancer patients, molecular diagnostics are needed to identify these patients before treatment. Doing so will require a better understanding of the cell populations and molecular changes that lead to the benefits these therapies provide.

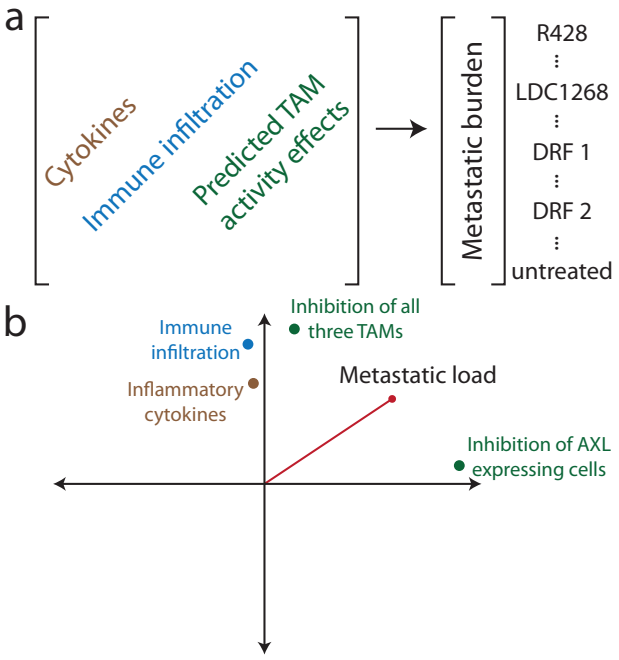

Figure 7: Schematic of partial least squares regression (PLSR) analysis. **a)** Layout of regression. Measurements from each individual mouse will be used as each observation. The luciferase signal outside the primary tumor will be used as the output measurement of metastatic burden. Input measurements will be assembled from measurements of cytokine abundance, immune infiltration to the primary tumor, and the predicted TAMR inhibition of each treatment. **b)** Schematic of expected results loading plot. Projection of the phenotype, metastatic burden, shown in red. We expect that the distinct effects of each therapy will show differing efficacy in blocking metastasis, and that this will be reflected in our molecular measurements. By modeling these coordinate changes, we will be able to deconvolve the multiple molecular and cellular changes leading to a reduction in metastases.

With our coordinate molecular and cellular measurements, we will be able to compare the consequences of these distinct therapies. Using principle components analysis, we will examine the multivariate similarities and differences between each TAMR-targeting strategy. By regression against our measurements of metastatic burden using PLSR, we will identify immune infiltration and cytokine features that correlate with improved response between these distinct targeting strategies (Fig. 7). If, for example, the therapeutic benefits of each therapy are strongly correlated to the magnitude of changes in immune infiltration and cytokine expression, this would serve as suggestive evidence that even AXL-targeted inhibitors such as R428 largely enact their therapeutic benefits via immunological consequences. Further, this would suggest that cytokine response measurements may serve as an effective signature for response to these therapies.

Potential pitfalls & alternative approaches An inherent assumption of our PLSR modeling as proposed is that a conserved molecular program drives the therapeutic benefits of each TAMR-targeted therapy. If, for example, R428 effectively blocks metastasis by a fundamentally different mechanism than the other treatments, this will be reflected by poor predictive capacity of the model. In this case, we can remove treatments that are poorly predicted and build models of the remaining treatments. Knowing the extent to which the different therapies work via the same or different mechanisms would still be extremely helpful. For example, if R428 and DRFs work via different mechanisms, combination treatments may show greater efficacy.

We may determine that, in contrast to our expectations, the small molecule inhibitors are more effective than DRF treatment. In this case, the paired molecular and outcome information, along with the modeling, will still be extremely helpful for understanding how these therapies are beneficial *in vivo*.

Another concern for the efforts in Aim 2.3 is that each treatment may be effective as compared to the controls, but that each treatment may show only minor differences when compared to one another. An important factor to keep in mind, however, is that the regression will be performed across all treatments, rather than comparing between treatments. Therefore, even very minor effects will be sufficiently powered (corr. $r > 0.3$ at $d = 0.8$). This means that even if the different treatments show similar outcomes on blocking metastasis, our molecular measurements will still be helpful in determining the mechanism of those effects.

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BCCL breast carcinoma cell line. 2, 5, 6

DC dendritic cell. 1, 2, 7, 8

DRF decoy receptor fragment. 1, 3, 5–9

H&E hematoxylin and eosin. 7, 8

MØ macrophage. 2, 7

NK natural killer. 1, 2, 6, 7

PLSR partial least squares regression. 8, 9

ProS Protein S. 1–8

PS phosphatidylserine. 2, 4–6

RTK receptor tyrosine kinase. 2–4, 8

TAMR TAM (Tyro3, AXL, MerTK) receptor. 1–9