TITLE: Neutralization of Prolactin Receptor Function by Monoclonal Antibody LFA102, A Novel Potential Therapeutic for the Treatment of Breast Cancer


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Abstract

Numerous lines of evidence suggest that the polypeptide hormone prolactin (PRL) may contribute to breast and prostate tumorigenesis through its interactions with the prolactin receptor (PRLR). Here we describe the biological properties of LFA102, a humanized neutralizing monoclonal antibody directed against the extracellular domain of PRLR. This antibody was found to effectively antagonize PRL-induced signaling in breast cancer cells in vitro and in vivo and to block PRL-induced proliferation in numerous cell line models, including examples of autocrine/paracrine PRL activity. A single administration of LFA102 resulted in regression of PRL-dependent Nb2-11 tumor xenografts and significantly prolonged time to progression. Finally, LFA102 treatment significantly inhibited PRLR signaling as well as tumor growth in a carcinogen-induced, estrogen receptor (ER)-positive rat mammary cancer model as a monotherapy and enhanced the efficacy of the aromatase inhibitor letrozole when administered in combination. The biological properties of LFA102, elucidated by the preclinical studies presented here, suggest that this antibody has the potential to be a first in class, effective therapeutic for the treatment of PRL-dependent cancers.

Introduction

PRL was initially described in the 1930s as a pituitary-derived polypeptide hormone that had the capacity to stimulate mammary gland development and lactation, but it was not until the 1970s that PRL was first recognized as having the ability to contribute to the initiation and promotion of mammary tumors in rodents. More recently, high circulating
levels of PRL in humans have been correlated with an increased risk for developing breast cancer, especially ER-positive (ER+) disease (1). Pharmacological agents which are known to increase systemic PRL are also associated with an increased risk for breast cancer (2). Further pointing to the tumorigenic potential of PRL, transgenic expression of this hormone in mouse mammary glands induced intraepithelial neoplasms and invasive tumors (3). Biological and molecular characterization studies suggested that the majority of these tumors (irrespective of ER status) most closely resembled estrogen-insensitive human tumors of the luminal subtype, suggesting that PRL may contribute to tumor growth in instances where estradiol does not function (or no longer functions) as a mitogen (4).

PRL mediates its physiological functions through engagement of the extracellular domain of PRLR, a type I cytokine receptor expressed with high prevalence in human breast cancer (5). PRLR lacks intrinsic kinase activity, but upon interaction with its ligand, the dimerized receptor undergoes a conformational change which activates other intracellular signaling partners to produce its ultimate cellular effects. In addition to human PRL (hPRL), human growth hormone (hGH) and placental lactogen are also known ligands of human PRLR (hPRLR) (6,7). The signaling pathway most often associated with PRLR function is the Jak2/Stat5 pathway, although other pro-survival and anti-apoptotic signaling cascades are also known to be triggered in cancer cell lines, including Jak1/Stat3, PI3-kinase/Akt and Raf/MEK/ERK (8-10). PRLR has also been observed to cross-talk with other growth factor and steroid receptor signaling pathways, including IGF1R, EGFR, HER2 and ERα (11-15), suggesting opportunities
for synergy between an anti-PRLR therapeutic and other targeted agents in human breast cancer.

Early clinical investigations of agents which act by inhibiting PRL release from the pituitary gland (e.g., the dopamine receptor agonist bromocriptine) in the treatment of human breast cancer were mostly inconclusive (16;17), potentially due to the fact that non-pituitary (dopamine receptor independent) sources of PRL synthesis exist, including adipose, mammary, prostate and endometrial tissues, as well as breast and prostate tumors themselves (18-22). The presence of tumor autocrine PRL has recently been reported to be significantly correlated with either high tumor grade and/or poor prognosis in breast, endometrial and prostate cancers (22;23). Thus, true systemic abrogation of PRL function would require a therapeutic capable of effectively blocking endocrine, autocrine and paracrine PRL action within a tumor. To this end, mutant PRL peptide antagonists acting directly on PRLR have been developed and utilized as key preclinical tools to investigate PRLR biology (24-27), however, several inherent liabilities of these antagonists have made their use as therapeutics in humans difficult (25;28). Here we report the development and characterization of a neutralizing monoclonal antibody against the human PRLR, designated LFA102, which is capable of inhibiting the biological functions of both endocrine and autocrine PRL. LFA102 potently antagonizes PRLR signaling and function in vitro and inhibits the growth of PRL-sensitive cancer models in vivo, suggesting this antibody could have utility for the treatment of PRL-dependent neoplasms in the clinic.
Materials and Methods

Materials

T47D and MCF7 cells were obtained from American Type Culture Collection (ATCC) and were authenticated using short tandem repeat profiling. T47D-T2 cells were derived from T47D cells (obtained from EG&G Mason Research Institute) though serial passage in immunocompromised mice to optimize in vivo growth characteristics and were authenticated using SNP analysis. Nb2-11 cells were obtained from the European Collection of Cell Cultures and were authenticated using DNA barcoding and DNA profiling. BaF3 cells were obtained from the German Collection of Microorganisms and Cell Cultures and was authenticated using standard and multiplex-PCR. All cell lines were used fewer than 6 months after resuscitation and were not re-authenticated. Primary human breast tumor specimens were obtained from MT Group in accordance with appropriate protocols and patient consent. Cell culture conditions and procedures for generating engineered cell lines are described in the Supplementary Methods. The parental mouse hybridoma to LFA102 was generated from mice immunized with recombinant extracellular domain of PRLR; LFA102 was generated by humanization of the mouse antibody using Human Engineering (HE™) technology at XOMA, LLC (Berkeley, CA). Sources of recombinant proteins, commercially available antibodies, compounds and vehicle formulations used for in vitro and in vivo studies can be found in the Supplementary Methods.
**Fluorescence-activated cell sorting (FACS) analysis of LFA102 binding to live cells**

To evaluate the levels of PRLR expressed on the plasma membrane and the extent of anti-PRLR antibody binding, adherent cells were detached from tissue culture flasks using Enzyme-Free Cell Dissociation Buffer (Gibco) or were disaggregated from a primary tumor prior to staining as described in the Supplementary Methods.

**PRL competition assay**

Amine-reactive Alexa Fluor 647 dye (A647; Invitrogen) was used to label recombinant hPRL per manufacturer’s directions. T47D cells (in FACS buffer) were incubated with antibodies or 5 μg/mL unlabeled hPRL for 45 minutes on ice. A647-PRL was then added to a concentration of 0.5 μg/mL, and the cells were incubated for 20 minutes prior to washing and analysis by FACS.

**Signaling assays (Western blotting)**

T47D and MCF7 cells were seeded into 6-well tissue culture plates overnight, prior to use. BaF3/hPRLR, BaF3/hPRLR/hPRL or dissociated primary human breast cancer cells were seeded and utilized in the assays without overnight incubation. Cells were incubated with LFA102 or human IgG1 isotype control antibody in serum-free RPMI for 30 minutes at 37°C prior to stimulation with hPRL (50 ng/mL) for 30 minutes. Details of cell lysate preparation and antibodies used for Western blotting are described in the Supplementary Methods. Experiments were performed in triplicate.
**Cell Proliferation and antibody dependent cell-mediated cytotoxicity (ADCC) assays**

For cell proliferation analyses, cells were seeded as indicated in the Supplemental Methods and were then treated for 4-7 days with hPRL or hGH in the presence or absence of anti-PRLR antibodies LFA102 or MAB1167. For ADCC analysis, T47D cells were co-cultured with fresh human NK cells, as described in the Supplementary Methods. CellTiter-Glo® and a luminescence counter (Wallac Trilux) were utilized to assess cell numbers upon completion of the assays. Luminescence counts from wells containing untreated cells (100% viability) were used to normalize treated samples. EC$_{50}$ values (half maximal inhibitory concentration of antibody) were calculated using GraphPad Prism 4 software. Experiments were carried out in triplicate.

**Evaluation of pharmacodynamic (PD) effects of LFA102 in T47D-T2 tumors**

T47D-T2 cells (7.5x10$^6$) were injected in the right mammary fat pad of female SCID mice. Mice with approximately 125 mm$^3$ T47D-T2 tumors were injected intravenously (i.v.) with 10 mg/kg of LFA102 or human IgG1 control antibody. Forty-eight hours later, mice were injected intraperitoneally (i.p.) with 100 μg ovine PRL (oPRL) or saline (5 per group). Tumors were collected 60 minutes post-PRL injection to evaluate phosphorylated Stat5 (p-Stat5) levels by immunohistochemistry (IHC) and whole blood was collected for serum isolation to determine LFA102 concentrations (see Supplementary Methods).
In vivo PD and efficacy evaluation of LFA102 in Nb2-11-luc tumor-bearing mice

Female SCID mice were injected subcutaneously (s.c.) in the right flank with 5x10^6 Nb2-11-luc cells. To examine the impact of LFA102 on PRL-induced signaling Nb2-11 tumors, mice were treated in a manner similar to the T47D PD study, except that 20 μg oPRL was utilized instead of 100 μg (described in the Supplementary Methods). For the in vivo efficacy evaluation, tumors were randomized into groups based on bioluminescence four days post-implant (tumor burden of approximately 4x10^7 photons/second; 8 per group). A single administration of LFA102 (0.01 to 10 mg/kg) or human IgG1 control antibody (3 mg/kg) was given i.p. Bioluminescence imaging is described in the Supplementary Methods. Statistical significance was determined using the Kruskal-Wallis One-Way ANOVA on Ranks, and pair-wise analysis was performed with Dunn’s post-hoc test. Time to progression was defined as the day animals required euthanasia for clinical signs of disease burden and statistical significance was determined using the Log Rank test.

Evaluation of LFA102 pharmacokinetics (PK), PD and efficacy as a single agent, and activity in combination with letrozole in a DMBA-induced mammary tumor model

Mammary tumors were established and treated with LFA102 as a single agent (300 mg/kg twice weekly) prior to analysis of tumor size, p-Stat5 and LFA102 serum levels (described in Supplementary methods). The activity of LFA102 and letrozole as single agents and in combination was assessed by enrolling animals onto the study when tumor volumes reached an average of approximately 200 mm^3 (n =15 /group). LFA102 was dosed i.p. at 300 mg/kg three times weekly. Letrozole was administered orally two
times daily at a total dose of 10 μg/kg. A partially efficacious dose of letrozole was utilized to maximize the potential to detect a combination effect of the two agents. Statistical analysis of tumor volumes was performed by one way ANOVA followed by the Tukey or Holm-Sidak post hoc test.

Results

Characterization of LFA102 binding to PRLR

LFA102 is a humanized IgG1-kappa antibody derived using mouse hybridoma and Human Engineering (HE™) technology. To demonstrate binding of the antibody to PRLR expressed in its native conformation on the surface of cells, a FACS assay was employed. In addition to binding to the human ER+ luminal breast cancer cell lines T47D and MCF7, LFA102 was also found to bind to freshly isolated primary human breast cancer cells and to the rat pre-T cell lymphoma line Nb2-c11, indicating rat PRLR (rPRLR) cross-reactivity of this antibody (Figure 1a). As a demonstration of specificity, LFA102 did not bind to PRLR negative BaF3 cells but did bind to BaF3 cells expressing exogenous hPRLR (BaF3/hPRLR). The degree of LFA102 binding to hPRLR on each cell line correlated with PRLR expression levels assessed by Western blotting (Supplementary Figure 1). LFA102 did not bind to cells expressing mouse PRLR (data not shown).
Epitope mapping studies determined that LFA102 binds to a conformational epitope within the D2 domain of hPRLR (data not shown), which contains the putative dimerization interface of the receptor and the WSXSX motif hypothesized to be a “molecular switch” for PRLR activation (29). Binding within the D2 domain of PRLR also suggests that LFA102 will bind to the majority of the isoforms of PRLR described previously (30). LFA102 is not thought to directly interact with the D1 domain of the receptor, which contains the majority of the ligand binding pocket (31).

To characterize the LFA102 binding mode in relation to PRL-PRLR interactions, a cell-based ligand competition assay was developed to examine the impact of antibody binding on the ability of Alexa647-labeled PRL (A647-PRL) to associate with PRLR. Figure 1b depicts the mean fluorescence signal measured for cells pre-treated with anti-PRLR antibodies (LFA102 or MAB1167) or unlabeled hPRL (specificity control). MAB1167 significantly displaced fluorescent signal from cells, indicating that the binding of this antibody influences the association of hPRL with hPRLR. In contrast, LFA102 had no significant effect on A647-PRL binding to PRLR, even when saturating concentrations of the antibody were added. Thus, LFA102 does not bind to PRLR in a ligand-competitive manner.

Neutralization of PRLR signaling in breast cancer cell lines in vitro, primary breast tumor cells ex vivo and T47D xenograft tumors in vivo.

To assess the impact of LFA102 on PRLR signaling in vitro, T47D and MCF7 cells were utilized. As determined by Western blotting with phospho-protein specific antibodies,
LFA102 was found to efficiently neutralize hPRL-induced phosphorylation of Stat5 (p-Stat5), Akt (p-Akt) and p42/p44 (p-ERK1/2) signaling in a concentration-dependent manner in T47D cells (Figure 2a). In contrast to T47D cells, MCF7 cells only exhibited clear p-Stat5 activation in response to hPRL, which was efficiently antagonized by LFA102 treatment. Importantly, no induction of PRLR signaling was seen in either cell line when treated with LFA102 alone, indicating that the antibody is devoid of measurable agonistic activity.

To confirm that primary human breast tumor cells also have the capacity for PRL-induced signaling which can be abrogated with LFA102 treatment, a fresh primary human breast tumor specimen (characterized as ER+/progesterone receptor+/HER2- and PRLR+) was utilized. The dissociated tumor cells demonstrated hPRL-induced p-Stat5 induction (Figure 1d) which was completely neutralized by LFA102 at a concentration of 10 μg/mL.

To demonstrate that LFA102 can neutralize PRLR activity in a breast carcinoma in vivo, mice with T47D-T2 tumors were administered a dose of LFA102 or a non-specific human IgG1 (control antibody), followed 48 hours later by a bolus of oPRL to stimulate PRLR (mouse PRL does not stimulate hPRLR), or a control injection (Figure 2c) 1 hour prior to assessment. Tumors from mice not treated with oPRL displayed little to no p-Stat5 while mice administered control antibody prior to oPRL stimulation showed significantly elevated p-Stat5 levels in tumors. In contrast, LFA102 treatment prior to oPRL stimulation resulted in no detectable intra-tumoral p-Stat5, demonstrating that LFA102 efficiently blocked PRLR signaling in this human breast cancer model. Serum levels of LFA102 in the treated mice ranged from 30-56 μg/mL at the time of sample
collection (data not shown), a concentration anticipated to be achievable in humans (32).

Abrogation of endocrine and autocrine PRL-induced cell proliferation by LFA102

To evaluate the ability of LFA102 to antagonize PRL-induced proliferation, T47D cells were exposed to increasing concentrations of hPRL in the presence of either LFA102 or control antibody, and changes in viable number were examined. Exposure of PRL-treated T47D cells to LFA102, but not control antibody, completely neutralized the effects of PRL on cell proliferation (Figure 3a). LFA102 was able to antagonize the pro-growth effects of hPRL at physiological levels (10-200 ng/mL) and super-physiological levels (up to 2000 ng/mL). An ability to antagonize PRLR in the presence of high levels of ligand may be important for a potential therapeutic, in that pathway inhibition may lead to hyper-prolactinemia (33). LFA102 was also found to completely inhibit the survival of the PRL-dependent rat pre-T cell lymphoma cell line Nb2-11 (Figure 3b).

To explore the activity of LFA102 in other contexts, an engineered hPRLR-expressing and hPRL-dependent cell line (BaF3/hPRLR) was utilized. Both LFA102 (non-PRL competitive) and MAB1167 (PRL competitive) antibodies inhibited the hPRL-dependent growth of BaF3/hPRLR cells, (EC$_{50}$ of 0.5 vs 3.9 μg/mL, respectively) (Figure 3c). Interestingly, only LFA102 efficiently suppressed PRL-induced cell proliferation in the presence of higher concentrations of this ligand (Figure 3d), despite both antibodies having a similar binding affinity ($K_D$) for recombinant human PRLR (LFA102 = 2 nM, MAB1167 = 3 nM; data not shown). In addition to hPRL, hGH can also directly bind
and activate hPRLR and has been hypothesized to play a role in breast cancer (34). Therefore, we examined the ability of LFA102 to antagonize hGH-mediated activation of PRLR in BaF3/hPRLR cells (which do not express GH receptor). In these studies, LFA102 potently neutralized the effects of hGH on hPRLR, similar to its impact on the actions of hPRL (Figure 3e). Thus, LFA102 has the capacity to block cell proliferation induced by multiple PRLR ligands.

Given that extra-pituitary sources of PRL include mammary tissue and tumors, it has become apparent that tumor cells may be exposed to autocrine or paracrine sources of PRL within their microenvironment. To assess the ability of LFA102 to inhibit PRLR function in these contexts, BaF3/hPRLR cells were engineered to be dependent on autocrine/paracrine hPRL through the expression of a hPRL transgene. This cell line, termed BaF3/hPRLR/hPRL, displays an auto-activated PRLR signaling apparatus, as measured by baseline p-Stat5 levels in the absence of exogenously added cytokines. Incubation of the cells with LFA102 (but not MAB1167 or control antibody) strongly suppressed this baseline signal (Figure 3f) and only LFA102 was found to inhibit the growth and survival of BaF3/hPRLR/hPRL cells in proliferation assays (Figure 3g). These results indicate that ligand-competitive PRLR antagonists may be inefficient at neutralizing PRLR function in instances where local PRL concentrations may be high, consistent with previous observations (25).

ADCC induced by LFA102
Through their interactions with Fcγ receptors on immune effector cells, antibodies of the human IgG1 isotype have the potential to elicit tumor cell lysis via a process known as ADCC. Primary human natural killer (NK) cells and T47D cells (which express PRLR and Her2) were used to assess the ADCC potential of LFA102. LFA102 was found to mediate T47D cell killing with a similar range of activity as the anti-Her2 antibody trastuzumab (maximum cell killing = 50%; EC$_{50}$ of 0.13 μg/mL and 0.02 μg/mL for LFA102 and trastuzumab, respectively) (Figure 3h). Therefore, LFA102 has the capacity to wield ADCC as an additional mechanism of action in vivo.

LFA102 pharmacokinetics (PK) and pharmacodynamics (PD) in rats

Given that LFA102 binds and neutralizes rat PRLR (rPRLR), rats were utilized for in vivo PK and PD analyses of LFA102. In the dose ranges examined, LFA102 clearance ranged from 1.45 - 0.92 ml/hr/kg in male rats and 13.5 - 3.93 mL/hr/kg in female rats (Supplementary Table 1). The mean estimated half-life ranged from 1.43 – 8.99 days in males and from 0.12 – 4.23 days in females. These results suggest that LFA102 has a rapid clearance mechanism in female rats that is not observed in male rats. In contrast, the T$_{1/2}$ of LFA102 in female mice was between 7.1 and 8.5 days, typical for a human IgG1 antibody (35). A likely explanation for the gender difference in LFA102 PK in rats is target mediated distribution of LFA102 to female rat liver, which expresses significantly more PRLR than that found in male rat liver or livers from either gender of mouse, cynomolgus monkey or human ((36) and data not shown). Consequently, we
expect that in humans the PK of LFA102 will be similar to that found in cynomolgus monkeys and dissimilar to that seen in female rats.

To examine the PD effects of LFA102 in the rat mammary gland, the antibody was administered at 50 mg/kg to compensate for the abnormally high rate of LFA102 clearance in this species, prior to rat PRL (rPRL) stimulation and tissue collection. Similar to the T47D-T2 PD study described above, LFA102 strongly antagonized rPRLR signaling in mammary tissue (Supplementary Figure 2).

Because PRLR knockout mice are hyperprolactinemic, suggesting the existence of a compensatory physiological reaction to PRLR signal ablation (33), we hypothesized that serum PRL levels may serve as a marker of PRLR pathway inhibition by LFA102. To remove the highly variable influence of the estrous cycle on baseline PRL levels, ovariectomized rats were used to test this hypothesis. Rats were given daily administrations (days 0-4) of LFA102 (50 mg/kg) or vehicle. Mean serum rPRL levels in LFA102 treated rats were significantly elevated on days 1-7, relative to controls (Supplementary Figure 3; p<0.05). Analysis of LFA102 levels in the serum showed a positive correlation with elevated PRL (Supplementary Figure 4). This data indicates that elevated PRL levels could represent a viable biomarker for LFA102 activity in humans. Based on the results shown in Figure 3d, it is anticipated that the peripheral effects of elevated PRL would be adequately antagonized by LFA102 in this situation.
Inhibition of PRLR signaling and tumor growth by LFA102 in the Nb2-11-luc rat pre-T cell lymphoma tumor model in mice

It has been recognized that human breast cancer cell lines are not very dependent on PRL, likely due to the fact that bovine PRL (bPRL) found in fetal bovine serum (FBS) does not activate hPRLR (37;38). Thus, human cell lines have been maintained under conditions where initially PRL-dependent cells will ultimately not survive. As bPRL does activate rPRLR, Nb2-11 cells have maintained their PRL dependence during culture in FBS, and for this reason they were used as a mechanistic model of PRL dependent human cancer. To examine the ability of LFA102 to inhibit PRL signaling and tumor growth in this model, mice with established subcutaneous Nb2-11-luc xenografts were utilized. Baseline p-Stat5 levels in these tumors were low and variable, potentially due to the inefficiency of mouse PRL (mPRL) in activating rPRLR, or to the contribution of the estrous cycle to systemic mPRL levels. Therefore, an injection of oPRL was administered to stimulate intra-tumoral p-Stat5 levels. LFA102 treatment efficiently blocked oPRL-induced Stat5 phosphorylation in Nb2-11-luc tumors (Figure 4a). To assess anti-tumor activity of LFA102, mice with established tumors received a single dose of LFA102 (ranging from 0.01 mg/kg to 10 mg/kg) or control antibody. Disease burden was evaluated by bioluminescence imaging through day 14 post-dosing, and time to progression assessed for 4.5 months. A single treatment of LFA102 at dose levels ≥0.3 mg/kg resulted in disease regression by day 3 post-dosing, with significant efficacy relative to the control group sustained until the final day of imaging (p<0.05; Figure 4b). The mean photon count on day 14 post-dosing in the IgG1 isotype control group was 1.7x10^9 photons/second, while that of the 10 mg/kg LFA102 treated group
was $5.6 \times 10^5$ photons/second, approximately the lowest level of detection (Figure 4c). Time to progression in the groups receiving $\geq 0.3$ mg/kg LFA102 was significantly longer relative to the control group ($p<0.05$; Figure 4d). These results indicate that LFA102 is highly efficacious in a PRL-dependent cancer model in mice.

**LFA102 PK/PD relationship and efficacy in DMBA-induced rat mammary tumor model**

A paucity of biologically relevant preclinical models, perhaps driven by the lack of hPRLR–activating PRL in standard cell culture media, has contributed to the difficulty in assessing the relevance of PRL as an oncogenic driver in human breast cancer (37). Additionally, as mPRL does not appreciably activate hPRLR (Figure 2), human breast cancer cells or primary tumors cannot be utilized as viable *in vivo* efficacy models in standard immunocompromised mice (39). Similarly, with the exception of MCF7 cells, few non-selected or non-engineered ER-positive human breast cancer cell lines have maintained estrogen sensitivity or are suitable for use as *in vivo* efficacy models.

DMBA carcinogen-induced rat mammary tumors represent widely utilized models of hormone sensitive breast cancer which have been used previously to justify the clinical use of aromatase inhibitors (40;41). A transplantable mammary tumor model expressing both PRLR and ERα (Figure 5a) was derived from a primary DMBA-induced tumor and used to perform a PK, PD and efficacy evaluation of LFA102 as a monotherapy. To compensate for the abbreviated serum half-life of LFA102 in female rats (Supplementary Table 1), multiple doses of the antibody were used to achieve an effective systemic exposure. Phospho-Stat5 levels in the tumors were analyzed by IHC
48 hours after the final LFA102 dose in this study. A correlation between LFA102 concentration in serum and inhibition of intra-tumoral p-Stat5 levels was observed (Figure 5b). A significant anti-tumor effect of LFA102 treatment was also observed from study days 10 through 27 post-dosing (Supplemental Figure 6; p<0.05), with 33% of tumors regressing with LFA102 treatment compared to 11% with vehicle treatment (1 case of spontaneous regression). In a second study, the efficacy of LFA102 and the aromatase inhibitor letrozole were evaluated singly and in combination. Treatment with LFA102 alone or in combination with letrozole resulted in significant efficacy relative to vehicle administration on days 9-20 post-dosing (Figure 5c; p<0.05). Notably, the activity of the combination treatment was higher compared with letrozole as a single agent on days 6-16 post-dosing (p<0.05). Tumor regressions were observed in both the LFA102 monotherapy and combination groups on day 20 (20% and 47% of tumors, respectively), however regressions were not observed in the animals treated with letrozole alone or with vehicle (Table 1). Additional studies are being performed to understand why some LFA102-treated tumors (as a single agent or in combination) completely regressed while others demonstrated only a growth inhibition. It is unclear whether or not even higher levels of LFA102 efficacy could have been achieved in these studies had antibody clearance and variability in exposure not been an issue in this species. No body weight loss or other clinical observations were observed in either study. Thus, LFA102 modulates PRLR signaling activity, inhibits tumor growth and is well tolerated in a mammary cancer model expressing both PRLR and ER.

**Discussion**
To date, antagonists targeting the PRL/PRLR signaling axis have had associated liabilities that precluded their use in understanding the role of prolactin in human cancer. The clinical utility of mutant PRL peptide antagonists, including G129R-hPRL, Δ1-9-G129R-hPRL and S179D-hPRL, has been limited by factors such as agonism, low potency and short systemic half-lives in animals (24;25;28;42;43). For example, although Δ1-9-G129R-hPRL does not exhibit agonism, it requires a circulating concentration of 1-2 μM to effectively inhibit autocrine PRL function in a mouse model of prostate cancer (25). This level of drug is likely to be a challenge to achieve chronically in humans given that the reported serum T1/2 of PRL and its peptide antagonists is approximately 20 minutes (44;45). Anti-prolactinemic agents that act directly on the pituitary gland, such as bromocriptine, fail to impact the effects of PRL derived from non-pituitary tissue sources (including tumor autocrine/paracrine PRL) or to inhibit hGH-mediated activation of PRLR, and thus fall short of fully neutralizing PRLR activity systemically (19). Here we have described the preclinical development of a monoclonal antibody which specifically binds and neutralizes PRLR with high potency. In vitro assays utilizing PRL-sensitive human breast cancer cell lines and PRL-dependent rodent cell lines have indicated that LFA102 is an effective antagonist of PRLR function. Furthermore, through the use of mechanistic cell line models, we have also shown that LFA102 is capable of neutralizing the effects of autocrine/paracrine PRL as well as high levels of exogenously added PRL in vitro, differentiating LFA102 from most strictly ligand-competitive PRLR antagonists. Based on the non-ligand competitive nature of LFA102 binding and the location of its epitope in the D2 domain of PRLR, we hypothesize that LFA102 antagonizes receptor function by either blocking receptor
dimerization or by locking the dimer into an inactive conformation. Further experimentation is needed to better define the structure-function relationship of LFA102-PRLR interactions.

LFA102 has demonstrated an ability to effectively antagonize PRL-induced signaling in 3 tumor xenograft models and to induce the regression of PRL-dependent Nb2-11-luc tumor xenografts, leading to prolonged time to progression after a single administration. We have also shown that at therapeutically relevant concentrations, LFA102 has the capacity to mediate ADCC, a mechanism of action shared by many clinically successful monoclonal antibodies (46;47). The observed LFA102-induced rise in serum PRL levels in rats is suggestive that systemic PRLR neutralization had been achieved in these animals and this endpoint is being explored further as a potential biomarker of LFA102 activity. High levels of LFA102 exposure were found to be extremely well tolerated in preclinical safety studies (data not shown), consistent with the lack of overt toxicity observed in PRLR knockout mice (48). This suggests there could be few significant toxicological consequences of abrogating PRLR signaling with this antagonist in humans.

The demonstration that LFA102 was able to achieve significant anti-tumor efficacy as a monotherapy, including tumor regressions, in the DMBA-induced rat mammary cancer model was particularly encouraging. Given that the \textit{in vivo} efficacy of letrozole may be enhanced through the addition of LFA102 indicates that a combination therapeutic regimen consisting of LFA102 with a standard of care (e.g. tamoxifen or an aromatase inhibitor) could lead to greater anti-tumor efficacy in patients, compared to either agent alone. The high dose levels of LFA102 used in studies with female rats (to compensate
for abnormal antibody PK in this species) are not expected to be needed to achieve effective PRLR neutralization in humans, based on LFA102 PK in male rats and primates (data not shown) and efficacious dose levels of LFA102 in mice. We are also investigating whether activation of the PRL/PRLR signaling axis could constitute a mode of tumor escape from the effects of anti-estrogens or other breast cancer therapeutics, in which case LFA102 combination approaches could have utility in stemming the emergence of drug resistance and prolonging the durability of clinical responses. The therapeutic properties of LFA102 described here suggest that this antibody has the potential to become a first-in-class anti-cancer medicine and to address unmet medical need through a unique targeted approach. A clinical trial has been initiated to examine the safety, pharmacokinetics and preliminary evidence of efficacy of this antagonist in patients with metastatic breast cancer.

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**Tables**

**Table 1: Tumor Response to LFA102 and Letrozole in the DMBA Tumor Model**

<table>
<thead>
<tr>
<th>Drug treatments</th>
<th>Mean tumor volume (+ SEM)</th>
<th>% Control tumor volume</th>
<th>% Tumor regressions*</th>
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<tr>
<td>Vehicle</td>
<td>1964 ± 243</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>LFA102 (300 mg/kg)**</td>
<td>809 ± 279</td>
<td>34</td>
<td>20</td>
</tr>
<tr>
<td>Letrozole (10 ug/kg)</td>
<td>1004 ± 196</td>
<td>45</td>
<td>0</td>
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<tr>
<td>LFA102 + Letrozole</td>
<td>436 ± 144</td>
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* 3 consecutive measurements < 50% of tumor volume at randomization  
** High dose level utilized to compensate for abnormally high LFA102 clearance in female rats

**Figure legends**

**Figure 1.** Characterization of LFA102 binding to PRLR-expressing cells. A, LFA102 (10 μg/mL) was incubated with T47D, MCF7, Nb2-11, PRLR-negative mouse pro B cells (BaF3), BaF3 cells expressing hPRLR (BaF3/hPRLR) or disaggregated fresh primary human breast tumor cells prior to FACS analysis. Open (dotted line) histograms = non-specific human IgG1 control antibody fluorescence signal; solid histograms = LFA102 fluorescence signal. B, Antibody-ligand competition was determined by pre-incubating T47D cells with anti-PRLR antibody (LFA102 or MAB1167), and then exposing cells to Alexa647-labeled hPRL (A647-PRL) for 20 minutes prior to fluorescence detection by FACS. Unlabeled hPRL was added to one sample (dark gray bar) to demonstrate specificity of A647-PRL binding to cells.

**Figure 2.** Inhibition of PRLR signaling by LFA102. A, Inhibition of PRL-induced signaling in T47D and MCF7 cells. Cells were pre-incubated with IgG1 control antibody
or LFA102 for 30 minutes prior to stimulation with hPRL (50 ng/mL) for 30 minutes and analysis of lysates by Western blotting. B, Fresh primary human breast cancer cells were disaggregated and treated as above. C, LFA102 inhibits PRL-induced p-Stat5 in T47D-T2 xenograft tumors. SCID mice with T47D-T2 mammary tumors were injected with an IgG control or LFA102 antibody (10 mg/kg). Forty-eight hours later, saline or oPRL was administered 60 minutes prior to tumor collection. Representative IHC images of p-Stat5 immunostaining are shown (200X).

**Figure 3.** LFA102 inhibits cell growth and viability *in vitro*. A, Inhibition of PRL-induced proliferation in T47D cells. Viable cell number was assessed using CellTiter-Glo® after 96 hours of exposure to hPRL in conjunction with LFA102 or IgG1 control antibody (both at 10 μg/mL). B, Inhibition of Nb2-11 cell survival. Relative number of viable Nb2-11 cells was assessed as described above. C, Inhibition of BaF3/hPRLR cell survival. BaF3/hPRLR cells were incubated with hPRL (50 ng/mL) in conjunction with LFA102, MAB1167 or species-matched IgG1 control antibodies for 96 hours prior to analysis. D, LFA102 efficiently neutralizes PRLR in the presence of high PRL concentrations. BaF3/hPRLR cells were incubated with antibodies (10 μg/mL) and increasing concentrations of hPRL for 96 hours prior to analysis. E, LFA102 inhibits hGH-induced hPRLR activation. BaF3/hPRLR cells were treated as above except in some cases hGH was added instead of hPRL (both at 50 ng/mL). F, Blockade of autocrine PRL signaling by LFA102. BaF3/hPRLR/hPRL cells were incubated with antibodies in the absence of exogenously added hPRL for 60 minutes prior to lysis and analysis of p-Stat5 and total Stat5 levels by Western blotting. G, LFA102 abrogates survival of
BaF3/hPRLR/hPRL cells. Cells were incubated with antibodies in the absence of exogenously added hPRL for 168 hours prior to analysis. H, Induction of ADCC by LFA102. Freshly isolated human NK cells were incubated with T47D cells at a ratio of 5:1 (effectors:targets) in the presence of LFA102, trastuzumab or IgG1 control antibody. Twenty-four hours later, plates were washed and the relative number of adhered viable cells was assessed. Values in A, B, C, D, E, G and H are means (+/- SD) of triplicate samples; Y-axis = % viable cell number relative to untreated cells or untreated cell mix (H, NK+T47D only).

Figure 4. LFA102 treatment inhibits PRLR signaling and tumor growth in the Nb2-11-luc xenograft model. A, LFA102 treatment inhibits PRL-induced p-Stat5 in Nb2-11-luc tumors. SCID mice with subcutaneous Nb2-11–luc tumors were injected with an IgG1 control antibody or LFA102 at a dose of 10 mg/kg. Forty-eight hours later, an injection of saline or oPRL was administered (60 minutes prior to tumor collection). Representative IHC images of p-Stat5 immunostaining are shown (200 X). B, LFA102 displays anti-tumor activity against Nb2-11-luc xenograft tumors. Treatment of tumor-bearing mice with a single dose of LFA102 resulted in dose-proportional efficacy (dosing time point indicated by arrow). Tumor bioluminescence is presented as mean +/- standard error of the mean (SEM; *p<0.05). C, Representative bioluminescence images of mice on day 14 post-randomization, administered a single dose of 3 mg/kg IgG1 isotype control antibody (top panel) or 10 mg/kg LFA102 (bottom panel). Bioluminescence is measured as photons/sec/cm^2/steradian (p/sec/cm^2/sr). D, Treatment of Nb2-11-luc tumor-bearing mice with a single dose of LFA102 significantly
increases time to progression (*p<0.05). The percentage of mice lacking clinical signs of disease progression in each group is plotted.

**Figure 5.** The DMBA-induced mammary tumor model expresses PRLR and ERα and is responsive to LFA102. A, Representative images of PRLR and ERα immunostaining in tumors (400X). B, The degree of inhibition of p-Stat5 in tumors correlates with LFA102 serum levels (data from LFA102 monotherapy study). Representative IHC images of p-Stat5 immunostaining of tumors from treated rats are shown (200X) with semi-quantitative p-Stat5 scores in the upper right hand corner (3=high, 2=med, 1=low, 0=negative); corresponding LFA102 serum levels are indicated below images. C, LFA102 inhibits the growth of DMBA-induced mammary tumors as a single agent and in combination with letrozole. Dosing initiation indicated by arrow. Tumor volume data are presented as mean +/- SEM. Statistically significant efficacy of LFA102 and the combination treatment was demonstrated on days 9-20 post-dosing (*p<0.05). The activity of LFA102 plus letrozole was significantly higher than letrozole alone on days 6-16 post-dosing (†p<0.5).
Figure 1.

A. 

B. 

- A647-PRL only
- A647-PRL + unlabeled PRL (10x)
- A647-PRL + LFA102
- A647-PRL + MAB1167
Figure 2.

A. | T47D | MCF7
---|---|---
IgG1: | 0 5 0 0 5 0 0 0 0 0 | 0 5 0 0 5 0 0 0 0 0
LFA102: | 0 0 5 0 0 0 0.05 0.1 0.5 1 5 | 0 0 5 0 0 0 0.05 0.1 0.5 1 5
PRL: | - - - + + + + + + + | - - + + + + + + +
p-Stat5 | | |
Stat5 | | |
p-Akt | | |
Akt | | |
p-ERK1/2 | | |
ERK1/2 | | |

B. | Primary human breast tumor (ER+/PR+/HER2-)
---|---
LFA102: | - - - + |
IgG1: | - - + - |
PRL: | - + + + |
p-Stat5 | | |
Stat5 | | |

C. | IgG1 control | IgG1 control + PRL
---|---|---
LFA102: | | |
LFA102 + PRL: | | |
Figure 3.

A. T47D

- Viable cell number (% of control) vs. PRL (ng/mL)
- Lines: NuIgG1, LFA102

B. NB2-11

- Viable cell number (% of control) vs. Antibody (µg/mL)
- Lines: NuIgG1, LFA102

C. BaF3/hPRLR

- Viable cell number (% of control) vs. Antibody (µg/mL)
- Lines: NuIgG1, LFA102, muIgG1, MAB1167

D. BaF3/hPRLR

- Viable cell number (% of control) vs. PRL (ng/mL)
- Lines: NuIgG1, LFA102, muIgG1, MAB1167

E. LFA102 (µg/mL) vs. p-Stat5

- Bar graph: LFA102 concentrations
- Lines: MAB1167

F. Beta-actin

- Western blot: p-Stat5, Stat5, Beta-actin

G. BaF3/hPRLR/hPRL

- Viable cell number (% of control) vs. Antibody (µg/mL)
- Lines: NuIgG1, LFA102, muIgG1, MAB1167

H. ADCC: T47D

- Viable cell number (% of control) vs. Antibody (µg/mL)
- Lines: IgG1, LFA102, Trastuzumab
Figure 4.

A. IgG1 control  IgG1 control + PRL

LFA102  LFA102 + PRL

B. Photon Emission

Days Post-Randomization

C. Luminescence

LFA102 10 mg/kg

D. % Animals Remaining

Time to Progression (Days)
Figure 5.

A. PRLR | ER-α

B. Vehicle

| Serum LFA102 | 202 μg/mL | 70 μg/mL |
| Serum LFA102 | 18 μg/mL | 12 μg/mL |

C. Mean Tumor Volume (mm³) ± SEM

- Vehicle
- LFA102 300 mg/kg
- Letrozole 10 μg/kg
- LFA102 + Letrozole

Days Post-Randomization
Molecular Cancer Therapeutics

Neutralization of Prolactin Receptor Function by Monoclonal Antibody LFA102, A Novel Potential Therapeutic for the Treatment of Breast Cancer

Jason S Damiano, Katherine G Rendahl, Christopher Karim, et al.

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