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Article

Prostate Cancer Metastases Are Strongly Inhibited by Agonistic EphA2 Ligands in an Orthotopic Mouse Model

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Simple Summary: We demonstrate that pro-oncogenic EphA2 (ephrin type-A receptor 2) expression is activated in aggressive prostate cancers, and in mouse models of prostate cancers that are treated with enzalutamide. We also demonstrate in mouse models, that agonistic EphA2 targeting agents are very effective in suppressing cell migration and tumor metastases, hence anticipating the possible use of such agents in innovative anti-metastatic therapeutic modalities.

Abstract: The EphA2 tyrosine kinase receptor is highly expressed in several types of solid tumors. In our recent studies, we targeted EphA2 in pancreatic cancer with agonistic agents and demonstrated that suppression of EphA2 significantly reduced cancer-cell migration in cell-based assays. In the present study, we focused on targeting EphA2 in prostate cancer. While not all prostate cancers express EphA2, we showed that enzalutamide induced EphA2 expression in prostate cancer cells and in a patient-derived xenograft (PDX) animal model, which provides further impetus to target EphA2 in prostate cancer. Western blot studies showed that agonistic dimeric synthetic (135H12) and natural (ephrinA1-Fc) ligands effectively degraded EphA2 receptor in the prostate cancer cell line PC-3. The agents also delayed cell migration of prostate cancer (PC-3) cells, while an in vivo PC-3 orthotopic metastatic nude-mouse model also revealed that administration of ephrinA1-Fc or 135H12 strongly reduced metastases. The present study further validates EphA2 as an important target in metastatic prostate cancer treatment. Our results should incentivize further efforts aimed at developing potent and effective EphA2 synthetic agonistic agents for the treatment of EphA2-driven aggressive metastatic tumors including prostate, pancreatic, and breast cancer.

Keywords: EphA2; ephrinA1-Fc; prostate cancer; breast cancer; cell migration; metastasis

1. Introduction

Ephrin receptor signaling pathways control cell shape, migration, invasion, and formation of tissue boundaries by altering the organization of the actin cytoskeleton, integrins, and intercellular adhesion molecules [1–3]. The Eph receptors have an extracellular domain that is critical to their function by binding to their ligands, the ephrins. The intracellular domains contain a tyrosine kinase domain and other structural or regulatory domains [4]. While EphA2 is expressed at low levels in adult

normal tissues, it is overexpressed in several cancers, and this aberrant expression is often associated with poor prognosis [5,6] given that unbound EphA2 is pro-oncogenic and promotes cell migration and metastasis [7].

Recent advances in the management of advanced prostate cancer (PCa) with androgen signaling axis inhibitors and chemotherapy have improved patient outcomes. However, therapeutic resistance of prostate cancer makes it the second leading cause of cancer-related death in American men with about 191,930 new cases and about 33,330 deaths from prostate cancer anticipated in 2020 (www.cancer.org). Initially, most of these cancers are androgen dependent, and respond to androgen deprivation therapy (ADT). Androgen deprivation leads to substantial cancer-cell apoptosis. However, the malignancy invariably progresses to a castrate-resistant state (CRPC) characterized by recurrent disease that is more often a reflection of dysregulation of normal cell turnover mechanisms than of hyper-proliferation. Taxanes are generally effective in high-grade PCa [8], but also provoke a marked reduction of white blood cells, contributing to dose limiting concentrations that are overcome by the tumor. Despite prolonged survival resulting from taxane chemotherapy, CRPC is still poorly managed, as most patients with CRPC die within 3 years of diagnosis [9]. Hence, strategies to improve current taxane-based chemotherapeutics are urgently needed. The CHAARTED and STAMPEDE Phase III clinical trials of 790 and 1776 hormone-sensitive prostate cancer patients, respectively, demonstrated that combining the taxane docetaxel with ADT as first-line therapy resulted in significantly longer overall survival compared to ADT alone [10–12]. However, there were increased adverse events for the combination therapy in these patients compared to the patients that received ADT alone.

During PCa progression, the EphA2 receptor can gain ligand-independent pro-oncogenic functions due to Akt activation and reduced ephrin-A ligand engagement. The effects can be reversed by ligand stimulation, which suppresses oncogenic signaling of Akt and ERK pathways [13]. Moreover, activation of EphA2 by ephrin ligands causes receptor clustering, internalization, and degradation. Because the dimerization of the receptor is a first critical event in the activation process, dimeric versions of the ephrinA1 ligand (ephrinA1-Fc), and of synthetic agonistic compounds (agent 135H12 [14,15] and Supplementary Table S1) were used as agents to probe the effect of EphA2 suppression in cellular and in vivo studies. Indeed, the ephrinA1 ligand is less effective as an agonist compared to the Fc dimerized molecule [16], and similarly, we recently reported that the synthetic agonistic agent 135H11 [14,15] is significantly less effective in activating the receptor in cellular assays than its dimeric version 135H12 [14,15]. The description of these agents, their affinity and selectivity for the EphA2-ligand binding domain, including biophysical characterization and X-ray structure in complex with EphA2, can be found in our recent publications [14,15]. Over the years a few peptide-like molecules have been reported targeting the EphA2 ligand binding domain, and among these, 135H11 is a 12-mer peptide mimetic that binds potently to the EphA2 receptor with K_d values in the 100 nM range, with no appreciable binding to the most closely-related Eph receptors, namely EphA3 and EphA4 [14,15]. Recently, we reported that the dimeric version of 135H11 can efficiently suppress cell migration of pancreatic cancer cells [14,15]. In addition, we also reported previously that earlier generations of agonistic EphA2-targeting-peptide mimetics can be used as carriers for targeted delivery of chemotherapy to breast [17], pancreatic [15,18], and prostate cancer [19–21]. In the present study, we aimed at further evaluating the therapeutic potential of targeting EphA2 by agonistic agents 135H12 and ephrinA1-Fc to suppress tumor metastasis in an orthotopic nude-mouse model of prostate cancer.

2. Results

2.1. EphA2 Expression in Prostatic Epithelia is Responsive to ADT

To support our central hypothesis that EphA2 is a potential target for combination therapy for PCa, we monitored EphA2 expression in ADT (enzalutamide) treated mice in patient-derived xenograft studies and prostatic epithelia (Figure 1a,b). The PDX was derived from prostatectomy tissues that were histologically evaluated from frozen section and grafted into the subrenal capsule of

immunocompromised mice. Of note, prostatectomy subjects had not been treated prior to surgery and as the tissues were never propagated in mice prior to this study, each graft had both cancerous and benign tissues. Mice hosting the PDX were treated with vehicle control or enzalutamide ($n = 6$). Immuno-histochemical studies showed EphA2 elevation in cancer epithelia of enzalutamide-treated mice (Figure 1a). Moreover, while not all cell prostate cancers highly express EphA2 (indeed EphA2 does not seem to represent a reliable prognostic for prostate cancer from the protein-atlas database [22], we found that ADT treatment elevates EphA2 in prostate cancer cell lines CWR22Rv1 and ARCaP_M (Figure 1b). Finally, mining of multiple datasets through the CBioPortal for Cancer Genomics demonstrated significant amplification of EphA2 in neuroendocrine prostate cancer (NEPC) patients (Figure 1c) [23]. These observations further support the hypothesis that agonistic EphA2 agents could provide a therapeutic benefit for the treatment of prostate cancer.

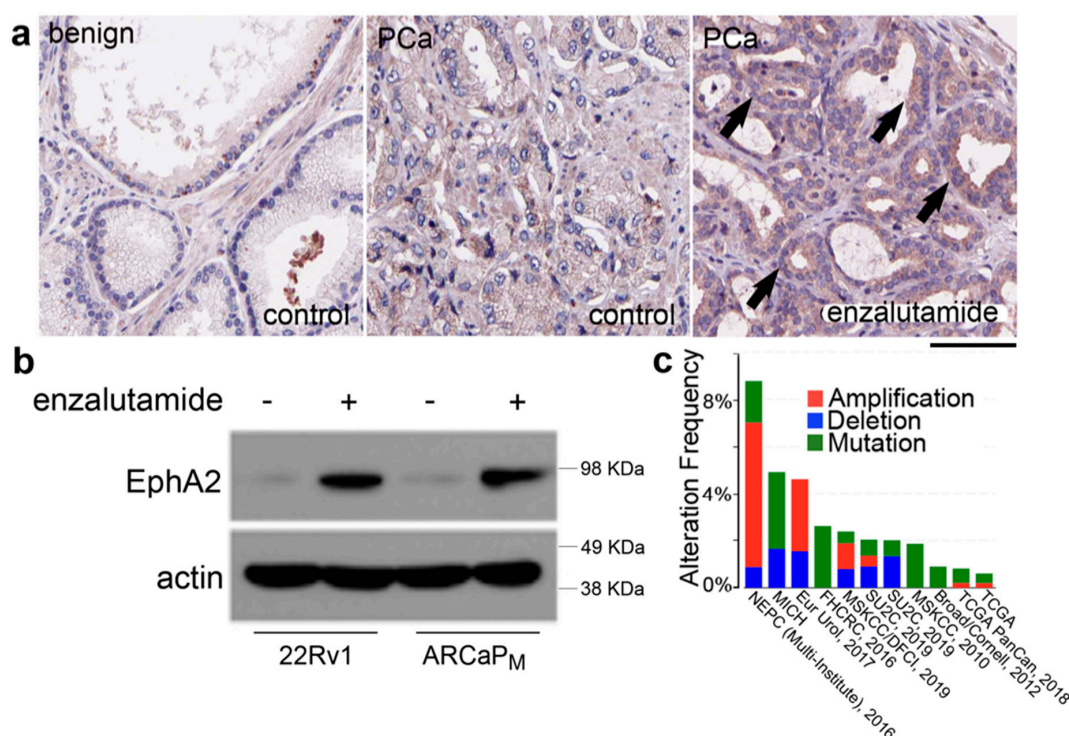


Figure 1. EphA2 (ephrin type-A receptor 2) expression in prostatic epithelia is responsive to ADT. (a) Mice hosting patient-derived xenografts (PDX) were treated with vehicle control or enzalutamide for 4 consecutive days ($n = 6$). Immuno-histochemical detection of EphA2 (brown) was greater in the cancer epithelia of enzalutamide-treated mice (arrows). Scale bar represents 50 μ m. (b) Western blotting shows EphA2 elevation in CWR22Rv1 and ARCaP_M cells with enzalutamide treatment for 72 h at 5 μ M (c) Search of multiple publicly-available data sets demonstrated an amplification of EphA2 in neuroendocrine prostate cancer (NEPC) patients.

2.2. Cellular Effects of Targeting EphA2 Signaling by Agonistic Agents

In the present study, in order to test the ability of EphA2 agonistic agents to reduce EphA2 levels over time, we initially treated prostate cancer PC-3 cells with 10 μ M of the agonistic synthetic agent 135H12, or with the dimeric EphA2 ligand ephrinA1-Fc (at 1 μ g/mL corresponding to \sim 22 nM of the monomer). We chose PC-3 cells given that this cell line expresses EphA2 and a PC-3-GFP (green fluorescent protein) cell line is available in our laboratories to test the antimetastatic properties of the agents in an orthotopic model, as discussed below. Cells lysates were probed for total EphA2 using anti-EphA2 antibody (1C11A12; Thermo Fisher Scientific) (Figure 2). Both 135H12 and ephrinA1-Fc were very effective in causing EphA2 degradation, compared to control (Figure 2a). Analysis of possible

downstream-signaling pathways also indicated that both 135H12 and ephrinA1-Fc caused similar initial rapid dephosphorylation of Akt (Figure 2a). This is in agreement with several previous studies with ephrinA1-Fc and with 135H12 with other cancer cells lines. To ascertain whether the agents could induce a sustained degradation of EphA2, we also monitored EphA2 levels for up to 3 days after treatment with either 135H12 (at 10 μ M) or ephrinA1-Fc (in this experiment we used 2 μ g/mL, or 44 nM, again to assess the ability of ephrinA1-Fc to induce receptor degradation over longer period of times; Figure 2b). While both agents seemed very active, ephrinA1-Fc caused a more sustained and extensive decrease in EphA2 levels even at day 3 after treatment, compared to prostate cancer cells treated with 135H12, where EphA2 levels returned after 2 days of treatment (Figure 2b). These observations have been made several times with similar agents and a variety of cell lines [14,15,17,18,21], including PC-3 [19,20,24], for example reported in Supplementary Figure S1. While generally we observed that 135H12 has agonistic activity in the low-to sub-micromolar range [14,15], we tested it at 10 μ M against PC-3 to assess the ability of the agent to cause a sustained internalization of the receptor over longer periods of time (Figure 2), in view of its anticipated use in vivo.

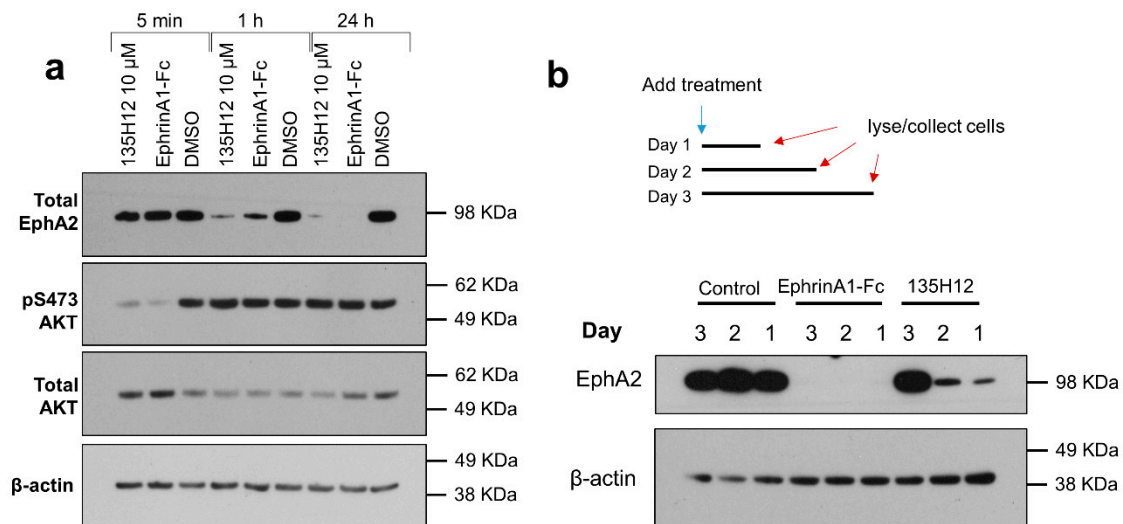


Figure 2. EphA2 ligands 135H12 and ephrinA1-Fc degrade EphA2 receptor in prostate-cancer PC-3 cells. (a) EphA2 ligands down-regulated EphA2 receptor in PC-3 prostate-cancer cells. The agents also dephosphorylated signaling protein AKT after 5 min, but the protein levels returned to normal at the 1 h time point. (b) PC-3 cells were treated with EphA2 ligands for one, two, or three days. 2 μ g/mL ephrinA1-Fc eliminated EphA2 levels even after three days from treatment, while 10 μ M 135H12 completely degraded EphA2 for two days. 3, 2, and 1 day correspond to 72 h, 48 h, and 24 h, respectively. Original uncropped files are provided as Supplementary Figures S2–S5.

These results support the conclusion that both dimeric agents induce EphA2 receptor degradation, with ephrinA1-Fc being more effective than 135H12 in the prostate-cancer cell line PC-3.

To further examine the effect of EphA2 on prostate-cancer cell migration, we used live-cell analysis of a scratch wound assay (IncuCyte S3, Sartorius, Göttingen, Germany) (Figure 3). For this assay, homogeneous scratch wounds were created on plated PC-3 prostate cancer cells. This was facilitated by a 96-pin mechanical device (WoundMaker, Sartorius, Göttingen, Germany). Plated cells were subsequently imaged over time to determine the rate of wound closure, under various treatments conditions. Cells were treated with either ephrinA1-Fc, Fc or DMSO as controls, or with various concentrations of 135H12 (Figure 3a). Cell migration was significantly attenuated in cells treated with ephrinA1-Fc and at the highest concentration of 135H12 (10 μ M; Figure 3a,b). However, lower concentrations of 135H12 were not effective in producing a significant inhibition of the migratory properties of PC-3 prostate cancer cells in this assay (Figure 3c). EphrinA1-Fc induced the largest decrease in the rate of migration compared to the Fc control (Figure 3). This may be due to the fact that

ephrinA1-Fc is a non-selective EphA2-targeting agent; it is rather a promiscuous agonist for other EphA or EphB receptor subtypes [25]. On the contrary, we reported previously that 135H12 is more exquisitely selective for the EphA2 subtype [14]. Nonetheless, our recent EphA2-KO data with pancreatic cancer cells suggested that EphA2 subtype alone contributes to the pro-migratory properties [15]. Moreover, previous studies reported that low-EphA2-expressing LNCaP prostate-cancer cells acquired increased invasion properties when transfected with EphA2 [26]. It is also notable that normal tissues generally do not present high expression of unliganded EphA2. In summary, both ephrinA1-Fc and 135H12 (at the highest concentration) can revert the pro-migration signaling associated with EphA2 expression in PC-3 prostate cancer cells, while cell proliferation was apparently not significantly affected by either treatment with 135H12 or by ephrinA1-Fc.

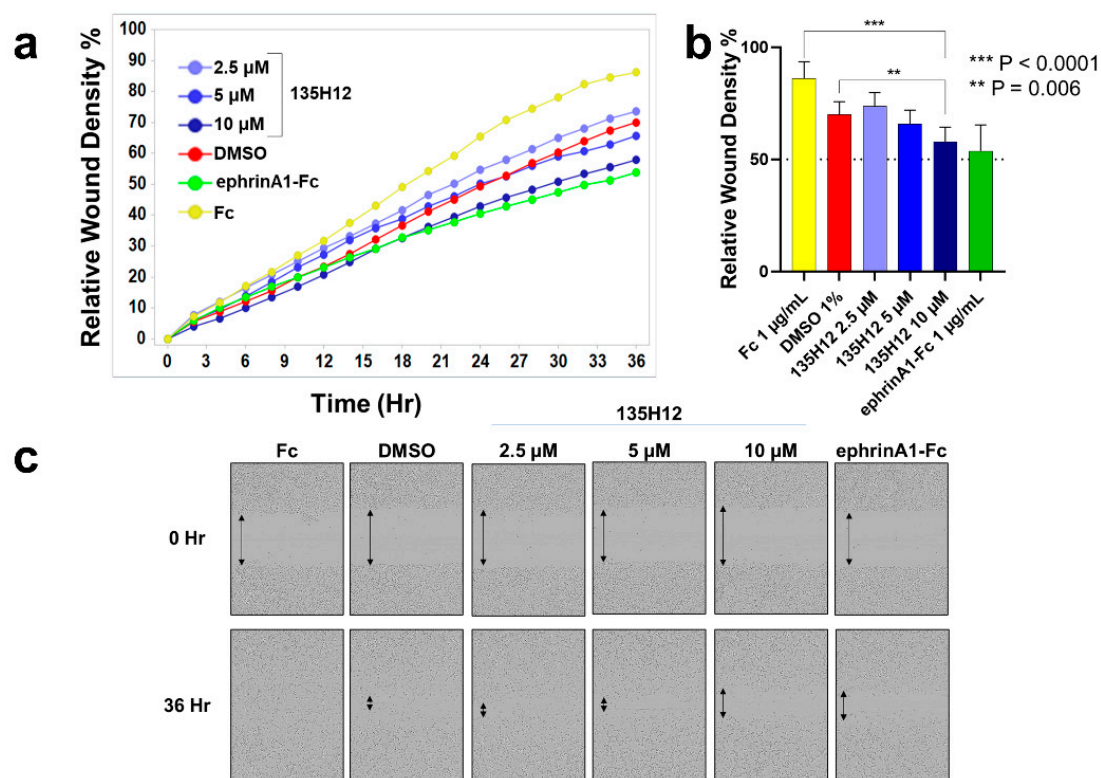


Figure 3. EphA2 ligands inhibit PC-3 prostate cancer cell migration. (a) Mean relative wound density curves illustrate migration of PC-3 cells with different treatments (in quadruplicates). Cells were plated, scratched and treated with several concentrations of 135H12, 1 μ g/mL of ephrinA1-Fc, Fc, or 1% DMSO (dimethyl sulfoxide). (b) Plot of relative mean (quadruplicate repeats) wound density % at 36 h shows the highest concentration of 135H12 can significantly inhibit PC-3 migration similar to ephrinA1-Fc. Error bars indicate standard deviation for the replicates. (c) Field pictures (10 \times magnification) of representative wells of PC-3 prostate cancer cells taken at time 0 and 36 h after treatment.

2.3. Efficacy of Agonistic Agents on Tumor Metastasis in an Orthotopic Model of Prostate Cancer

To further assess if suppression of EphA2 signaling by agonistic agents can suppress tumor metastasis *in vivo*, we used the PC-3-GFP human prostate cancer cell line in an orthotopic xenograft model. The orthotopic models (5 mice per cohort) were treated with either ephrinA1-Fc, 135H12, or vehicle control, for 7 days. Body weight of mice in each group was monitored at day 0, 5 and 7, and no significant differences in weight between mice in the treated groups versus the untreated control group were observed (Figure 4a). Similarly, no significant differences were observed in the primary tumor volumes measured at day 7 (Figure 4b) between the treated and untreated groups. Both results are not unexpected given that the agents are not generally cytotoxic and do not affect proliferation of PC-3 prostate cancer cells. However, the groups differed significantly in the metastases observed as

monitored at day 7 via the FluorVivo fluorescence imager (Indec Biosystems, Santa Clara, CA, USA) (Figure 4c,d). After necropsy, imaging was performed in the thorax and abdomen for inspection of metastases in each organ. These images revealed significantly reduced metastases (Figure 4c) in the cohorts treated with either ephrinA1-Fc or 135H12. This can be appreciated by representative images from representative mice from each group (Figure 4d).

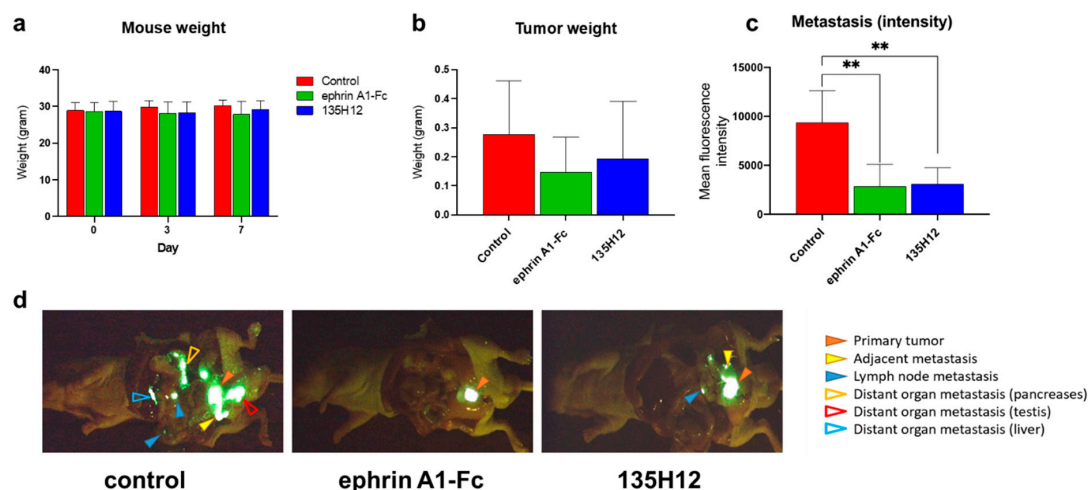


Figure 4. Treatment with ephrinA1-Fc or 135H12 on an orthotopic mouse model of prostate cancer with PC-3-GFP cells ($n = 5$ mice per treatment group). (a–c) show body weight, tumor volume, and mean fluorescence intensity related to metastases, respectively. Panel (d) representative images taken at day 7 from mice in each group, control (the solvent formulation used for 135H12), ephrinA1-Fc treated, 135H12 treated. Error bars represent standard deviation. $** p < 0.01$.

Prostate-cancer metastases were identified by measurements of intra-vital fluorescence intensities at the end of the experiment. In the control group significant fluorescence was detected in lymph nodes (4/5 mice), in areas adjacent to the primary tumor (4 out of 5 mice), and in distant organs including the pancreas (3 out of 5 mice), the liver (2 out of 5 mice), and the testis (4 out of 5 mice). The number of detectable metastasis was reduced in number and, most importantly, in intensity (Figure 4b) in mice treated with either ephrinA1-Fc or 135H12, as follows: lymph nodes in 1 and 3 out of 5 mice, respectively; in areas adjacent to the primary tumor in 2 and 3 out of 5 mice, respectively; in the pancreas in 1 and 2 out of 5 mice, respectively; in the liver in 0 and 1 out of 5 mice, respectively; and in the testis in 0 out of 5 mice, with both treatments. The total mean fluorescence intensities in these sites is plotted in Figure 4c, while representative images taken at day 7 from mice in each of the groups, control, ephrinA1-Fc treated, and 135H12 treated, are reported in Figure 4d.

Hence, at the doses and regimens used in this experiment (~60 mg/Kg daily for 7 days, i.p. for 135H12; ~100 μ g/Kg daily i.p. for 7 days for ephrinA1-Fc) showed significant efficacy in suppressing of the spread of PC-3 prostate-cancer metastases. We conclude that further development studies with 135H12, possibly optimization steps to further increase its affinity for EphA2 ligand binding domain, could translate these findings into novel and effective anti-metastatic therapeutics agents for prostate cancer and possibly other indications, including pancreatic and breast cancer.

3. Materials and Methods

3.1. Cell Culture

The PC-3 cell line was purchased from the American Type Culture Collection (ATCC). PC-3-GFP was available from AntiCancer, Inc. All culture media and supplements were purchased from ThermoFisher and media were supplemented with 10% FBS (Fetal Bovine Serum) and 1% Pen Strep. PC-3 cells were cultured in complete DMEM (Dulbecco's Modified Eagle Medium). Anti-EphA2

antibody (1C11A12) was purchased from ThermoFisher (Waltham, MA, USA) and AKT antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). β -Actin antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

3.2. Cell Migration Assays

Cells were plated at 40×10^3 cells/well in 96-well ImageLock plates (Sartorius, Göttingen, Germany). The following day, cells were scratched using the WoundMaker (Sartorius, Göttingen, Germany) and washed three times with PBS. Subsequently, cells were treated with the indicated agents in DMEM. Treatments included ephrinA1-Fc (1 $\mu\text{g}/\text{mL}$) and test agents 135H12 (2.5 μM , 5 μM , and 10 μM , in quadruplicates). Plates were imaged (10 \times magnification) every two hours using IncuCyte S3 (Sartorius, Göttingen, Germany), and relative wound areas were analyzed using the algorithm of the imager cell migration software module.

3.3. Immunoblotting Assays

Cells were lysed with cell lysis buffer (20 mM Tris, pH 7.4, 120 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1% IGEPAL, 5 mM EDTA, supplemented with EDTA-free Protease Inhibitor Cocktail and PhosStop from Sigma-Aldrich, St. Louis, MO, USA) for 10 min on ice. Cell lysates were then centrifuged to clear off cell debris for 10 min at 13,000 rpm at 4 °C. Samples were prepared and loaded into 4–12% NuPAGE Bis-Tris Precast Gels and transferred to PVDF (polyvinylidene difluoride) membranes as indicated previously [14]. The membrane was blocked with 5% non-fat milk in TBS (tris buffered saline) buffer and 0.1% Tween for 1 h, then incubated with primary and secondary antibodies and visualized using a Clarity Western ECL kit (BIO-RAD, Hercules, CA, USA). The membranes were stripped using Restore Western to blot with a loading control antibody.

3.4. Animal PDX Studies

Male NOD-SCID-gamma (NSG) mice (Jackson Labs), 6–12 weeks old were used. Human derived PDX (patient derived xenografts) tissues from prostatectomies, were resected, and necrotic tissues removed and mechanically sectioned into smaller fragments ($\sim 1\text{--}2 \text{ mm}^3$ per graft size) and subsequently these were xenografted into the sub-renal capsule. Mice were treated 1 week after with enzalutamide daily for 4 consecutive days at 1 mg/mouse by oral gavage. These studies were conducted according to the Cedars-Sinai Institutional Animal Care & Use Committee (CSMC IACUC) #IACUC007440.

3.5. Immuno-Histochemical Analyses

EphA2 staining was performed by immunohistochemistry. Briefly, paraffin-embedded sections from PDX xenografts were deparaffinized in xylene, rehydrated through graded ethanol, and then submerged into citric acid buffer for heat-induced antigenic retrieval. Samples were subsequently blocked with 10% bovine serum albumin, incubated with the EphA2 primary antibodies at 4 °C overnight, and developed using the DAKO ChemMate Envision Kit/HRP (Dako-Cytomation). They were then counterstained with hematoxylin, dehydrated, cleared, and mounted.

3.6. Database Meta Search

The genomic status of EphA2 in prostate cancer patients were mined through cBioPortal for Cancer Genomics [27]. Genomic data types integrated by cBioPortal included somatic mutations, DNA copy-number alterations. This portal contains several data sets for prostate cancer and, among them, a multi-institutional dataset for neuroendocrine prostate cancer that was used to mine for the amplification of EphA2 [23].

3.7. Orthotopic Metastasis Nude-Mouse Model of PC-3 Prostate Cancer

A total of 20 nu/nu male mice, 4–6 weeks old, were used in the study. Test animals were from AntiCancer Inc. (San Diego, CA, USA). All animals were weighed using an electronic balance (APX-203, Spectrum, Gardena, CA, USA) and given a clinical examination to ensure that they were in good condition. The mice were housed 5 per cage. An inspection was performed to ensure their suitability for the study before tumor orthotopic implantation. The animals were maintained in a HEPA-filtered environment in a Micro-VENT full ventilation rodent housing system (Allentown Caging Equipment Co., Allentown, PA, USA) at AntiCancer, Inc. according to IACUC approved protocols (AntiCancer Inc. Animal Care and Use Committee approval #AC2020007). Vivarium room controls were set to maintain temperature and relative humidity at $22\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ and $55\% \pm 15\%$, respectively. The vivarium rooms were lit by artificial light for 12 h each day. Cages and bedding were autoclaved. Water was purified by Milli-Q Biocel System (Millipore, Billerica, MA, USA), autoclaved and supplied ad libitum to each cage via water bottles. Autoclavable rodent diet 5010 was obtained from PMI Nutrition International Inc. (Brentwood, MO, USA).

PC-3-GFP cells, at a concentration of 2.0×10^6 cells in 150 μL , were injected into the flank of nude mice to make subcutaneous tumor stock. After growth, the subcutaneous tumors were excised, inspected and any grossly necrotic or suspected non-GFP tumor tissue was removed. Tumor tissues were subsequently cut into small fragments. 20 nude mice were implanted by surgical orthotopic implantation (SOI) using PC-3-GFP tumor fragments of 1 mm^3 harvested from the tumor stock animals. The animals were anesthetized with a mixture of ketamine, acepromazine, and xylazine. The surgical area was sterilized using iodine and alcohol. After proper exposure of the prostate gland following a lower-midline abdominal incision, 2 pieces of 1 mm^3 tumor fragments per mouse were implanted on the prostate gland. An 8-0 surgical suture was used to penetrate these small tumor pieces and suture them on the prostate gland. The prostate gland was then returned to the abdominal cavity. The incision in the abdominal was closed with a 6-0 surgical suture in one layer. Animals were kept in a barrier facility under HEPA filtration. Body weight was measured by an electronic scale. Primary tumor sizes were measured by calipers on the sacrifice day and were estimated by measuring the perpendicular minor dimension (W) and major dimension (L). Approximate tumor volume (mm^3) was calculated by the formula $(W \times L) \times \frac{1}{2}$.

The in vivo fluorescence imaging system (Index Biosystems) was used for whole body imaging and to observe in vivo tumor growth. After necropsy, final open imaging was performed in the thorax and abdomen for inspection of metastases in each organ was performed. 135H12 was dissolved in a formulation containing PBS (phosphate buffer saline):EtOH (ethanol):PEG400 (polyethylene glycol 400): DMSO (dimethyl sulfoxide) at a ratio 50:20:20:10, 10 mg/mL concentration and administered i.p. (~150 μL to obtain 60 mg/kg for each of the treated mice, for 7 days). EphrinA1-Fc was dissolved in PBS at 15 $\mu\text{g}/\text{mL}$ and injected i.p. (150 μL) in the treated mice for 7 days.

4. Discussion and Conclusions

Recent studies with EphA2 transfected cells demonstrated increased cells migration of prostate cancer cell lines [26]. Moreover, the pro-migratory properties of cancer cells were further enhanced by transfecting the with EphA2 mutants that were designed to prevent EphA2 dimerization and activation [7]. Hence, we probed whether EphA2 dimerizing agents such as ephrinA1-Fc or 135H12 could effectively revert EphA2 pro-migratory properties, in cell culture, and metastasis in vivo. We observed that both 135H12 and ephrinA1-Fc caused a sustained reduction of the receptor over time. However, unlike ephrinA1-Fc treatment, cells treated with 135H12 displayed some EphA2 expression 1 day after treatment. Nonetheless, both agents significantly suppressed cell migration of PC-3 prostate cancer cells, with ephrinA1-Fc being slightly more effective. 135H12 required a relatively high concentration to elicit a significant suppression of migration (10 μM) compared to ephrinA1-Fc (1 $\mu\text{g}/\text{mL}$ corresponding to ~22 nM concentration of the dimeric macromolecule). Expression of EphA2 in prostate cancer correlates with aggressiveness of the tumor [26]. However, we found that

treatment with ADT induced EphA2 expression in cell lines or in PDX models. These results suggest that targeting EphA2 in prostate cancer with agonistic agents may have a significant therapeutic potential. To preliminarily assess the ability of the two selected agonistic agents in suppressing tumor metastases, we used a PC-3-GFP orthotopic model of metastatic prostate cancer and mice were treated for 7 days post implantation of the xenografts. A significant reduction of metastases was observed in the group treated with both ephrinA1-Fc or 135H12 treatments. Therapeutic targeting of the EphA2-LBD has been pursued in recent years by several different approaches [6,21,28–39] and most studies envisioned the use of agonistic agents as vehicles for targeted delivery of chemotherapy [17–21,24]. Indeed, we had recently demonstrated that an earlier generation EphA2-targeting-peptide, when conjugated with paclitaxel, was remarkably effective in inhibiting lung metastases in a syngeneic mouse model of breast cancer [17]. Such remarkable anti-metastatic activity was attributed to the ability of the drug conjugate to capture and kill circulating tumor cells [17]. The present study, however, suggests that suitable dimeric agonistic agents capable of inducing a sustained reduction, presumably via its activation, internalization and degradation, of EphA2, like ephrinA1-Fc, could be translated to potential therapeutics to reduce tumor metastases *in vivo*, without drug conjugation. This is presumably attributable to the suppression of cell migration induced by agonistic EphA2 agents. In addition to attenuation of cell migration, EphA2 suppression by agonistic agents can also alter how cancer cells interact with the tumor microenvironment and their ability to adhere to other tissues. This is a property of this receptor that has been well documented and can involve also other Eph receptor subtypes [40–44]. We noted such effect previously with earlier agonistic agents with a melanoma mouse model of cancer-cell lung adhesion [21]. Hence, interference with cancer cell adhesion may be more pronounced in the mice treated with ephrinA1-Fc compared to the mice treated with 135H12, given that the former agent is potently promiscuous against a variety of other Eph receptors. This may partly explain why the effective *in vivo* concentration of 135H12, which is much more selective for the EphA2 subtype, is much greater than that of the ephrinA1-Fc, which is rather promiscuous. These considerations would open the question on whether a therapeutic agent should possess broad-spectrum pan-Eph agonistic activity, and, if so, what additional receptors subtypes may be needed to be suppressed concomitantly with the subtype EphA2 to attain the same effects elicited by ephrinA1-Fc. At the same time, normal-cell expression of the EphA2 subtype is very low, making the case for an EphA2-specific agent, like 135H12, perhaps to balance efficacy with potential adverse effects. In addition, recent studies with the LNCaP prostate cancer cell line, that expresses low levels of EphA2, demonstrated that the sole acquisition of EphA2 expression via transfection, resulted in a cell line with markedly increased pro-invasion characteristics, strongly corroborating that EphA2 alone is likely the most relevant target among the members of this receptor tyrosine kinase family in prostate cancer [26]. While additional experimental studies are necessary on the effect of these agents on the tumor microenvironment, and on normal tissues *in vivo*, these considerations and the present study suggest that further drug development and optimization of synthetic and specific EphA2 agonistic agents may be warranted. For the ligand 135H12, these optimizations, perhaps aided by our recently determined crystallographic structure [14] and structural analyses of 135H12 and related agents in complex with the EphA2 ligand binding domain [15], could lead to novel agents that could be rapidly translated in innovative and effective anti-metastatic therapeutic strategies for prostate cancer and potentially other cancers including pancreatic [15,18] and breast [17] cancers.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2072-6694/12/10/2854/s1>, Figures S1–S5: uncropped western blots and densitometry analysis. Table S1: chemical structure and affinities for the cited agents 135H11 and 135H12.

Author Contributions: M.P., N.A.B., R.M.H., and A.F.S., designed the research strategy. L.G. synthesized and purified 135H12. A.F.S. conducted cellular experiments reported in Figures 2 and 3, helped assemble Figure 4, and wrote the methods sections and figure captions related to these experiments. S.B., under the supervision of N.A.B., prepared the data relative to Figure 1, and associated methods sections and figure caption. Y.S., M.Z., H.O., and R.M.H. performed the experiment reported in Figure 4 and provided related methods sections and figure caption.

M.P. analyzed data with all authors, and wrote the manuscript, while R.M.H., N.A.B., S.B., and A.F.S. helped with final editing. All authors have read and agreed to the published version of the manuscript.

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