Creating a Dead End for Cancer: The Role of LPA Receptors 1/3 and 2 in Ovarian Cancer Metastases

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Abstract—Lysophosphatidic Acid (LPA), a natural phospholipid, has been implicated as a signaling molecule of migration in invasive malignancies like ovarian cancer. The migration of ovarian cancer is a process that is poorly understood. This study investigated the role of LPA Receptors 1/3 and 2 in the migratory response and protein signaling pathways of ovarian cancer cells (OVCAR) by using a LPA Receptor 1/3 antagonist (Ki16425) and a LPA Receptor 2 agonist (dodecylphosphate). Using a modified Boyden migration assay, it was determined that OVCAR cells migrate in a dose response fashion towards LPA compared to untreated control ($p<0.05$). It was also determined that the LPA Receptor 1/3 antagonist inhibited migration toward LPA in a dose response fashion compared to untreated control ($P<0.05$). LPA Receptor 2 agonist inhibited migration towards LPA; combined treatment of LPA Receptor 1/3 antagonist and LPA Receptor 2 agonist inhibited 95 percent of OVCAR migration ($p<0.05$).

Vascular Endothelial Growth Factor (VEGF), an angiogenesis regulator, Epidermal Growth Factor Receptor (EGFR), a known migration regulator, and RhoA, a suggested cell cytoskeleton activator, were investigated to determine the effect of LPA Receptors 1/3 and LPA Receptors 2 on protein quantities. Exogenous LPA up-regulated VEGF secretion and had no effect on RhoA activation or production of EGFR. LPA Receptors 1/3 were shown to inhibit VEGF secretion, stimulate RhoA activation, and have no effect on production of EGFR ($p<0.05$). LPA Receptor 2 was shown to stimulate VEGF secretion and RhoA activation. In summary, it was determined that LPA Receptors 1/3 are pro-migration, and LPA Receptor 2 is anti-migration- perturbing both receptors simultaneously resulted in a 95 percent reduction in OVCAR cell migration suggesting its potential as a treatment for this highly invasive cancer.

Index Terms- ovarian cancer, LPA, LPA receptor 1/3, LPA Receptor 2, cell migration, VEGF, EGFR, RhoA

1 INTRODUCTION

THE trademark of cancer is its stealthy ability to spread through out the body, invading normal, healthy tissue with abnormal, malignant cells. The cancers that metastasize rapidly tend to be the most devastating, affecting multiple organs in addition to the primary site of the tumor. Death from cancer is more often the result of metastases, than invasion at the origin of the tumor. (World Health Organization, 2009) At the time of diagnosis, the disease has often already metastasized in the individual, which is especially true of ovarian cancer, known for its high metastases rate. If the invasiveness of these cells can be curtailed, it may be possible to develop a localized target for cancer treatment. Because symptoms of ovarian cancer are usually vague and also present in many benign digestive conditions, diagnosis of the cancer is usually made after it has progressed into its advanced stages (Bast et al., 2009). Understanding the spread of ovarian cancer will provide an opportunity to better elucidate the aggressive nature of the cancer and thereby treat it.

Recent literature has shown that elevated levels of Lysophosphatidic Acid (LPA) were frequently found in the ascites fluid of women with ovarian cancer, a finding that will better enable us to understand this disease. Because of this, researchers suggested LPA to be a biomarker to possibly aid in early detection of the ovarian malignancy (Shen et al., 2002). Additionally, LPA itself is a naturally occurring phospholipid that has been implicated as a mitogenic agent and growth factor as well as a chemotactic signal for migration in processes such as embryological development, osteogenesis, the immune response, and in invasive cancers such as ovarian cancer and pancreatic cancer (Ohta et al, 2003). It is also known that there is an LPA family of cellular receptors (LPA1/Edg-2, LPA2/Edg-4, and LPA3/Edg-7, being similar in structure), but the specific function of each of the individual LPA receptors has yet to be fully elucidated (Komachi, M, et. al, 2010). Investigations into the relationship between the LPA family of receptors and their ligand, LPA, have the potential to shed light on the behavior of the cancer's aggressive nature and propensity to metastasize. The pathways in which LPA affects migration are poorly understood and must be clarified and the molecular interactions of other migratory signaling molecules are vague and must be investigated (Dorsam et al., 2007). Research of this type may then lead to a potential immobilization, preventing the spread, and allowing for a more defined target for aggressive treatment modalities, such as radiation, chemotherapy, and immunotherapy.

In the research presented here, the migratory response (a mechanism of metastasis) of an ovarian cancer cell line (OVCAR) was investigated in relation to the G-protein coupled transmembrane receptors, Lysophosphatidic receptors 1, 2, and 3 (LPA1/Edg-2, LPA2/Edg-4, and LPA3/Edg-7). To do this, an LPA Receptor 1/3 antagonist (Ki16425) (Ohta et al.,2003) and an LPA Receptor 2 agonist (dodecylphosphate) known to function at 700nM (Chen et al., 2006) were utilized to determine receptor roles in OVCAR cell migration. In past studies, the downregulation of the LPA 2 receptor has been attempted with interference RNA. However, OVCAR cells seem to lend themselves to only a transient transfection. (Komachi et al., 2010) shRNA downregulation was attempted in this study as well, but only a transient transfection was achieved as reported by other researchers, and determined not appropriate for the study reported here.

Additionally, several factors that have been shown to mediate the migratory response in cancer cells, Vascular Endothelial Growth Factor (VEGF), Epidermal Growth Factor Receptor (EGFR), and RhoA activation were also investigated.

VEGF is a protein that promotes angiogenesis, the creation of new blood vessels. Continuous blood flow to the tumor is essential to its survival, proliferation and subsequent migration. (Research VEGF, 2010) Therefore, this protein was measured to determine if LPA Receptors 1/3 and/or LPA Receptor 2 ligand binding has an influence over its production hoping to further elucidate the function of these individual receptors in OVCAR cell migration.

Phosphorylation of EGFR is believed to activate pathways that lead to angiogenesis and migration. (Jiang et al., 2006) To determine whether LPA Receptor 1/3 or LPA Receptor 2 cross-talks with EGFR, once it is bound, to stimulate migration, total EGFR production and phosphorlyated EGFR was measured.

The formation and presence of microtubules and microfilaments were also investigated in this study. How the cancer cell utilizes different components of its cytoskeleton in order to migrate remains obscure. Therefore, a third protein, RhoA (Ras homolog gene family, member A), was also measured. RhoA has been shown to stimulate and activate the actin cytoskeleton in cancer cells. It may also be responsible for the rearrangement of F-Actin in the cell. Rearrangement of the cytoskeleton is essential to cellular migration and metastasis. The exact function of this protein is under

investigation and remains controversial (Heasman et al., 2010). This protein was studied to determine if a correlation between cell migration and RhoA activation is present in the OVCAR cell line. A recent study found that cells in culture produce extensions that carry actin or actin mesh. These forms of actin were found to be necessary for the invadopodia, or protrusions from the cell membrane that extend into the extracellular matrix, which allow the cell to move and is thought to be the initial step of metastases (Schoumacher et al., 2010). Thus, activated RhoA was quantified in order to further elucidate how each individual receptor (LPA Receptor 1/3 and 2) utilizes components of the cytoskeleton to achieve cell motility.

EGFR, VEGF, and RhoA in relation to LPA receptors were studied to best understand the pathway in which ovarian cancer cell migration takes place. The cascade of events that takes place before migration and metastasis has yet to be elucidated in many cancers. The role of each individual LPA receptor (1, 2, and 3) and the connection they have with these proteins remains unclear and often controversial as well.

The study reported here aimed to create a more concrete understanding of the aforementioned crosstalk between the LPA receptors 1, 2, and 3 and the protein signaling pathways in ovarian cancer. Understanding the exact function of these receptors may lead to treatments that prevent this highly metastatic cancer from spreading through out the body.

2 MATERIALS AND METHODS

Cell Culture: NIH:OVCAR-3 cells from (ATCC NO. HTB-161™), an ovarian adenocarcinoma cell line, were obtained from American Type Culture Collection (ATCC, Manassas, Virginia, USA). LPA 1 receptors, LPA 2 receptors, and enzymes regulating the production and degradation of LPA are aberrantly expressed by ovarian cancer cells. (Yu et al, 2009) Cells were maintained in RPMI-1640 medium supplemented with L-glutamine (Invitrogen, Calsbad California, USA) and 10% fetal bovine serum (Invitrogen). Cells were incubated at 37°C with 5% CO_2. The cell cultures were expanded when they reached near confluency utilizing 0.05% trypsin-EDTA (Invitrogen) 75cm^2 rectangular cell culture flasks, 96-well plates, 24-well plates, centrifuge tubes, and 50mL tubes were obtained from Corning (Corning, New York, USA). CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay was obtained from Promega (Madison, Wisconsin, USA). Cells were plated at a concentration of 500,000 cells/ mL (as determined by trypan blue exclusion) or 50,000 cells/well in a 96 well plate.

Ki16425- (LPA Receptor 1 antagonist) (Cayman Chemical,10012659, Ann Arbor, MI, USA) This chemical will be referred to as LPA Receptor 1 antagonist in paper and is an antagonist to the receptor. Cells were treated for 24 h with the antagonist for viability, migration, and ELISA assays. LPA receptor 1 antagonist was added to cells for twenty minutes before the addition of LPA receptor 2 agonist and/or exogenous LPA.

MTS Cell Viability Assay-(Promega, Madison, WI, USA) This 24 h colorimetric assay was used to determine reagent toxicity. The conversion of tetrazolium salts to formazan by metabolically active cells is responsible for the color change. The absorbance at 490 nm is measured with a BioTek ELx800 microplate reader. This was done to determine the effect of LPA 1 antagonist, LPA 2 agonist, and exogenous LPA on the viability of OVCAR in order to eliminate apoptosis or necrosis as a factor in protein production or migration behavior.

Migration Assay (Neuro-Probe,Inc., Gaithersburg, MD,USA): A modified Boyden migration assay (8µM pore size, 24 h) was used to quantify migration of cells with and without LPA receptor 1 antagonized. Plates were manually counted using a standard microscope with sufficient magnification to view the migrated cells. The cells with LPA receptor 1 antagonized were compared to untreated cells (control). The Neuro-Probe migration chamber used is shown below:

EGFR ELISA (R&D Systems, DYC1095-2, Minneapolis, MN, USA): The presence of total and phosphorylated epidermal growth factor receptor was quantified using ELISA method in treated and untreated cells (24 h). Cell lysates were collected after 24 hours. The assay was carried out using manufacturer's protocol. Absorbance was determined at excitation 540 nm / emission 600 nm and excitation 360 nm / emission 450 nm.

VEGF ELISA/ASSAY (Assay Designs, 900-080): The secretion of Vascular Endothelial Growth Factor was measured using ELISA method in treated and untreated cells (24 h). The assay was carried out using manufacturer's protocol. Absorbance was determined at 450 nm.

Dodecylphosphate- (LPA Receptor 2 agonist)- (Enzo Lif45e Sciences, LP-105) This reagent was used to agonize LPA receptor 2 to determine the receptor's role in migration and protein production in ovarian cancer cells. It was dissolved in DMSO and was used at concentration of 1.86 x 10^{-4} mg/mL (700 nM). Dodecylphosphate has been reported by literature to be a functional LPA Receptor 2 agonist (by acting as a ligand to this receptor) at the 700 nM concentration. This concentration had no significant effect on the viability of OVCAR.

Rho-A Activation Assay (Cytoskeleton, BK124) The amount of activated Rho-A in OVCAR cells was quantified using this ELISA in treated and untreated cells (24 hours). Cell lysates were collected after 24 hours. The assay was carried out using manufacturer's protocol. Absorbance was determined at 490 nm. Rho-A is a protein that activates and regulates the cytoskeleton of cancer cells. It is believed to aid cell migration.

Data analysis. Data were analyzed using Microsoft Excel 2010 (Microsoft, Redmond, Washington, USA). All assays were repeated at least twice. The unpaired Student's t-test was used to determine significance with 5 replicates. Alpha was set at 0.05. Standard deviation of data sets was determined and the assays were performed more than twice with similar findings.

3 Resul ts

Each following experiment was performed with NIH:OVCAR-3 cells.

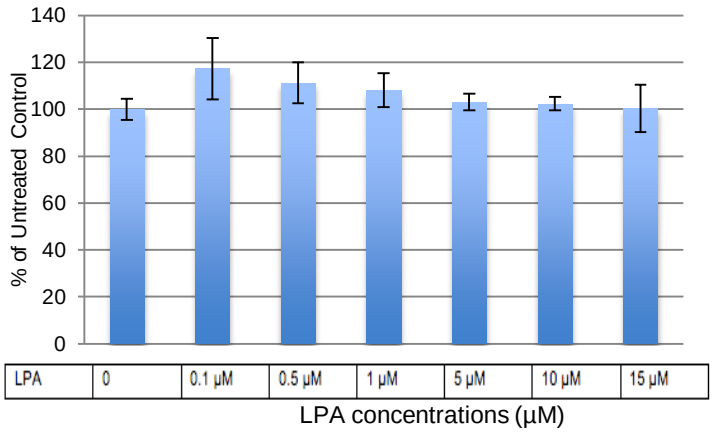

Fig 1. The effects of exogenous LPA on viability of OVCAR

The MTS Assay was used to measure viability of cells after they had been incubated with LPA for 24 h in order to eliminate toxicity of the molecule as a variable. LPA concentrations ranged from 0.1 µM to 15 µM. Incubation with LPA resulted in proliferation at concentration of 0.1 µM, which was not significant, but is likely due to a low dose stimulation. There was no significant effect on viability at all other concentrations of LPA when compared to untreated control. Data are represented as means ±S.D. (n = 5).

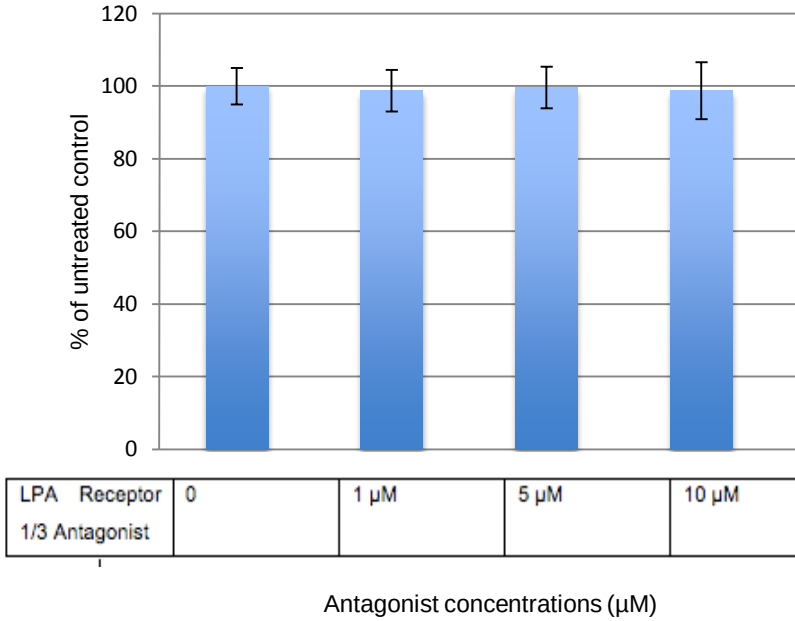

Fig 2. The effect of LPA Receptors 1/3 Antagonist on viability of OVCAR cells

The MTS assay was used to measure viability of cells after they were incubated with LPA Receptor 1/3 antagonist (Ki16425) for 24 h in order to eliminate toxicity of the molecule as a variable. Antagonist concentrations ranged from 1 µM to 15 µM. There was no significant change in viability across the concentrations when compared to untreated control. Data are represented as means ±S.D. (n = 5).

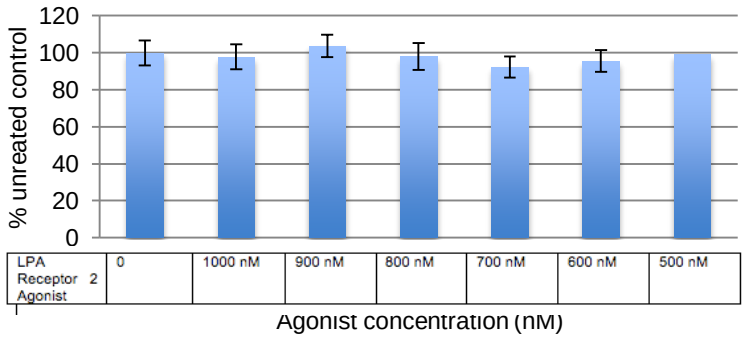

Fig 3. The effect of LPA Receptor 2 agonist on viability of OVCAR

The MTS assay was used to measure viability of cells after they were incubated with LPA receptor 2 agonist (dodecylphosphate) for 24 h in order to eliminate toxicity of the molecule as a variable. Agonist concentrations ranged from 500 nM to 1000 nM. There was no significant change in viability across the concentrations when compared to untreated control. Dodecylphosphate is reported to act as an agonist at 700 nM. (Enzo Life Sciences) Data are represented as means ±S.D. (n=5).

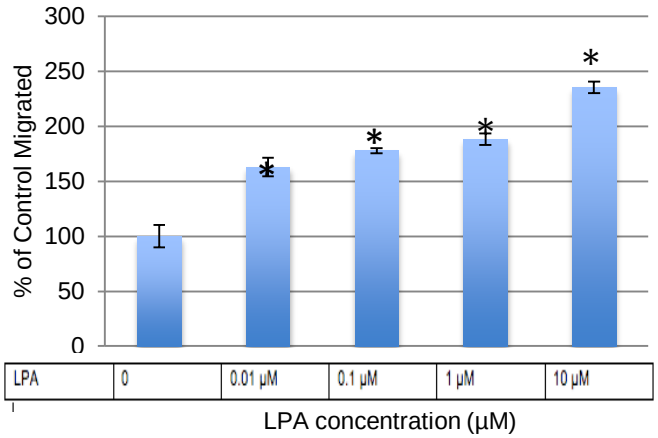

Fig 4. The Chemotactic Response of OVCAR to LPA
Migration of ovarian cancer cells was observed and quantified using a modified Boyden migration assay over 24 h. Bottom chamber contained increasing concentrations of LPA (0.01 µM to 10 µM). Cells were added to top of the chamber and migrated cells passed through the 8µM pore size filter that was placed in the center of the chamber. Migration of cells increased as concentrations of LPA increased. This dose dependent response is similar to that found in previous literature (Bast, R, 2009). Data are represented as means ±S.D. (n = 5). * = p<0.05 for statistical significance when compared to untreated control.

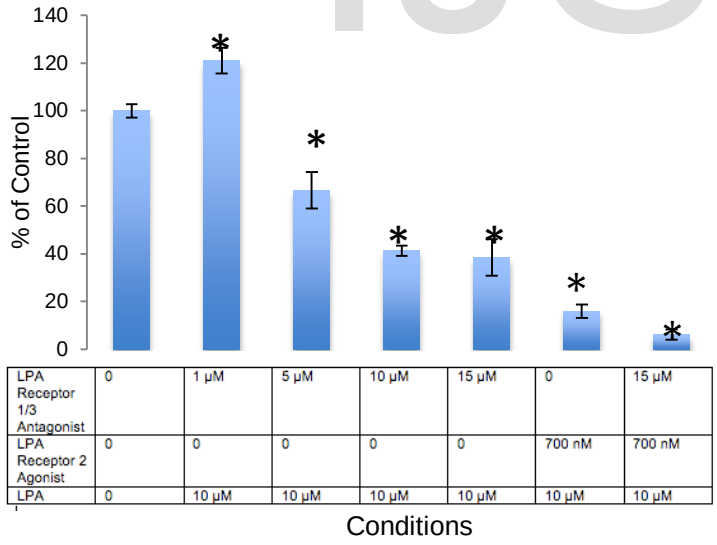

Fig 5. The chemotactic response of OVCAR to LPA with LPA Receptors 1/3 antagonized and LPA Receptor 2 agonized
Migration of ovarian cancer cells was observed using a modified Boyden migration assay over 24 h. Bottom chamber contained LPA (10 µM). Cells with varying concentrations of LPA Receptors 1/3 antagonist (1 µM-15 µM) and LPA Receptor 2 agonist (700 nM) were placed in the top of the chamber and migrated cells passed through the 8µM pore size filter that was placed in the center of the chamber. Migration decreased as concentrations of LPA Receptors 1/3 antagonist increased. At 1 µM of antagonist there was a significant increase in migration and is most likely the result of the phenomenon of low dose stimulation. Cells with LPA Receptor 2 agonist demonstrated a significant decrease in migration, and cells with both LPA Receptor 2 agonist (700 nM) and LPA Receptors 1/3 antagonist (15µM) showed an even greater decrease in cell migration (95 percent of cells did not migrate) Overall, this implies that LPA Receptor 1/3 may play a role in stimulating OVCAR migration and LPA Receptor 2 appears to play a role in the inhibition of OVCAR migration. Data are represented as means ±S.D. (n = 3). * = p<0.05 for statistical significance when compared to untreated control.

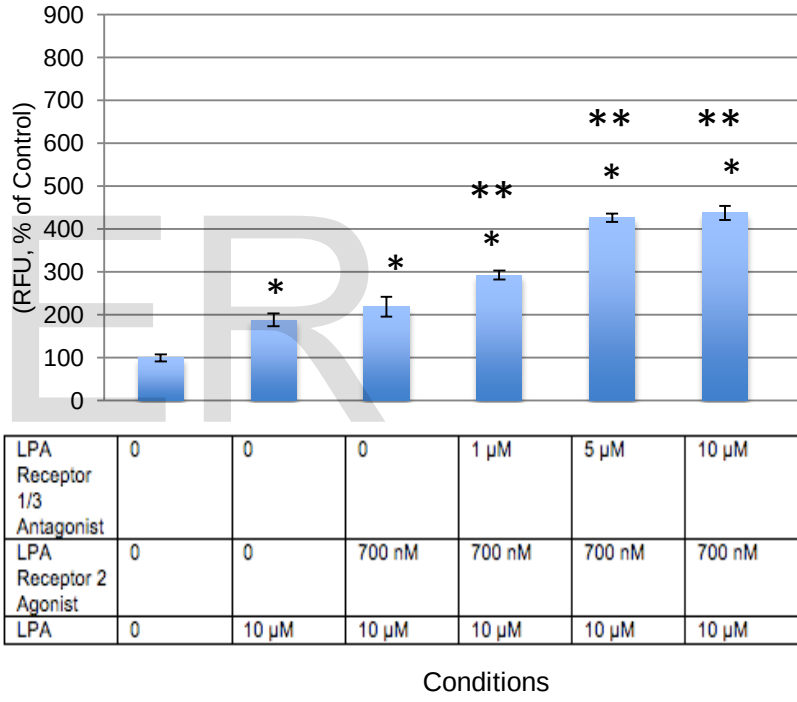

Figure 6: The effect of LPA Receptor 1 antagonist and LPA Receptor 2 agonist on VEGF secretion of OVCAR
VEGF secretion by OVCAR was determined by ELISA. Cells were induced with varying concentrations of LPA Receptor 1/3 antagonist or LPA Receptor 2 agonist (700nM). After 20 min, each was treated with exogenous LPA (10 µM). In combinatorial treatment, antagonist preceeded agonist by 20min. Supernatants were collected after 24 hours for ELISA. Cells that were induced with 10 µM LPA alone showed a significant increase in VEGF secretion. Treatment with LPA Receptor 2 agonist (700 nM) alone or LPA Receptor 2 agonist in combination with LPA Receptor 1/3 antagonist demonstrated an increase of VEGF secretion in a dose response fashion. These findings suggest that LPA Receptors 1/3 mediates VEGF secretion in an inhibitory fashion while LPA Receptor 2 stimulates VEGF secretion when treated with

LPA. LPA receptors 1/3 appear to act in an inhibitory fashion on VEGF secretion. Data are represented as means ±S.D. (n = 3). * = P<0.05 for statistical significance when compared to untreated control. Also, ** = p < 0.05 when comparing bar 3 to bars 4-7.

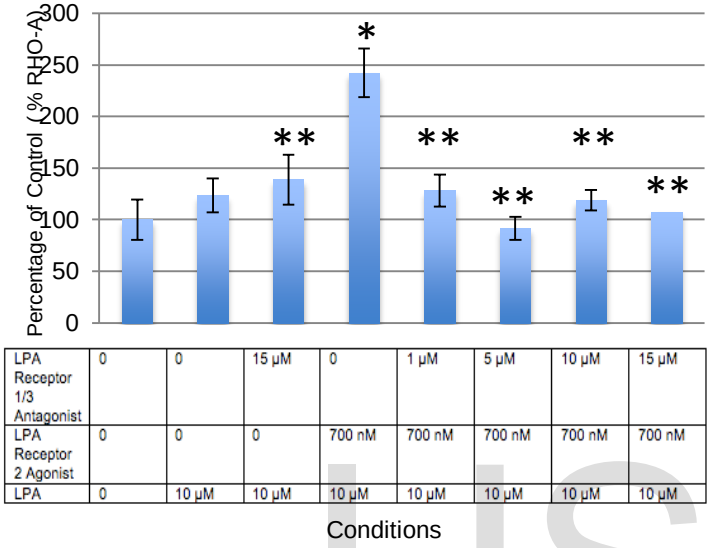

Fig 7. The effect of LPA Receptor 1 antagonist and LPA Receptor 2 agonist on activated RhoA

RhoA activation was determined using ELISA. Cells were treated with varying concentrations of LPA Receptor 1/3 antagonist (1 µM- 15 µM) or LPA Receptor 2 agonist (700 nM) and after 20 min were induced with exogenous LPA (10 µM). In combinatorial treatment, antagonist proceeded agonist by 20 min. Cell lysates were collected after 24 hours for ELISA. The administration of LPA Receptor 2 agonist resulted in a significantly greater level of RhoA activation. The combinatorial treatment of LPA Receptor 2 agonist and LPA Receptor 1/3 antagonist demonstrated a reduction in levels of RHO-A activation near constitutive amounts. While there seems to be a trend towards LPA stimulated activation of RhoA, the exogenous treatment of LPA (10 µM) may be below the threshold necessary for significant activation of RhoA. This data suggests that LPA Receptor 2 and LPA Receptors 1/3 may promote RhoA activation (due to significant increase with LPA Receptor 2 agonist and significant decrease with LPA Receptor 1/3 antagonist) in OVCAR cells. Data are represented as means ±S.D. (n = 3). *= P<0.05 for statistical significance when compared to untreated control. Also, ** = p < 0.05 when comparing bar 4 to bars 3,5-8.

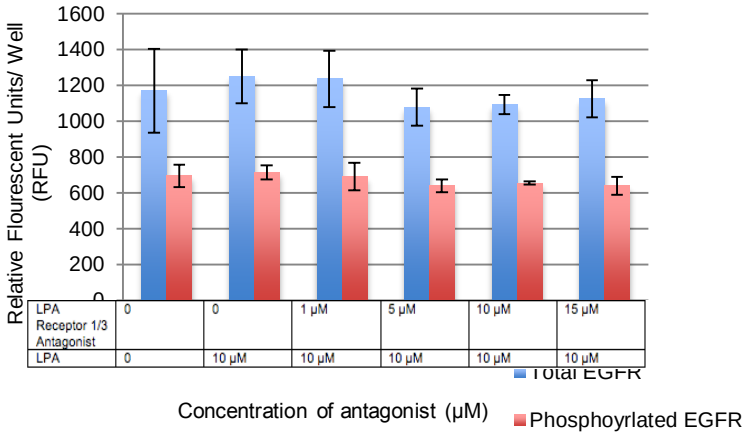

Fig 8. The effect of LPA Receptor 1/3 Antagonist on total and phosphorylated EGFR

ELISA method was used to quantify number of total and phosphorylated Epidermal Growth Factor Receptor of OVCAR cells that had been induced with 10 µM LPA and increasing concentrations of LPA Receptor 1/3 antagonist (1 µM- 15 µM). Antagonist was added to cells for 20 minutes before administration of 10 µM LPA. There was no significant change between total EGFR and phosphorylated EGFR in untreated control and cells that had been administered with 10 µM LPA. This suggests that LPA ligand binding does not influence production and activation of EGFR. LPA stimulated migration is achieved without the involvement of Epidermal Growth Factor Receptor. Addition of LPA Receptor 1/3 antagonist also did not affect the production of EGFR. This suggests that LPA Receptors 1/3 operate independently of the EGFR pathway to stimulate migration. Data are represented as means ±S.D. (n = 3).

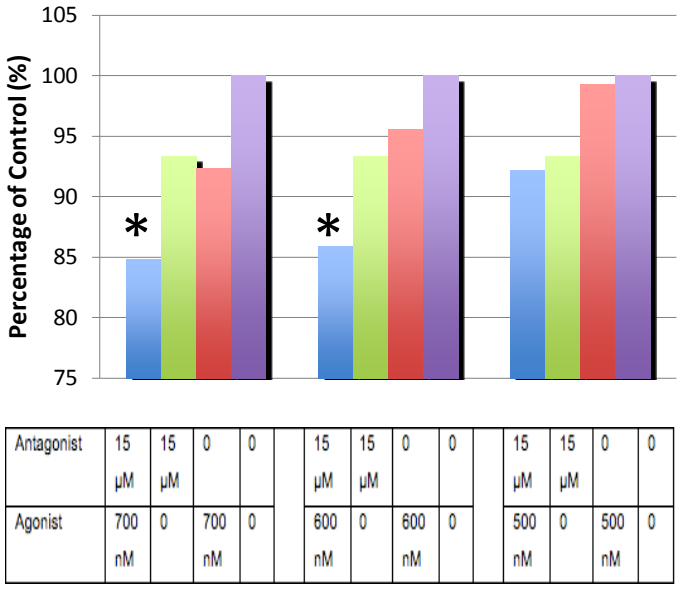

Fig. 9: The Effect of LPA Receptor 2 Agonist and LPA Receptor 1 Antagonist on Viability of OVCAR

When LPA Receptor 2 is agonized, there is a trend toward a decrease in viability suggesting that when LPA Receptor 2 is bound, there is a signal for an inhibitory effect on viability or proliferation. The binding of the individual experimental ligands does not produce a statistically significant effect on viability until they are administered in concert, These molecules in combination suggest a potential therapeutic option for the treatment of ovarian cancer of this nature. This mechanism of action deserves further investigation, but it seems there is a trend suggesting LPA Receptors 1/3 may promote proliferation while LPA Receptor 2 may inhibit it. Data are represented as means ±S.D. (n = 3). P<0.05 for statistical significance compared to untreated control.

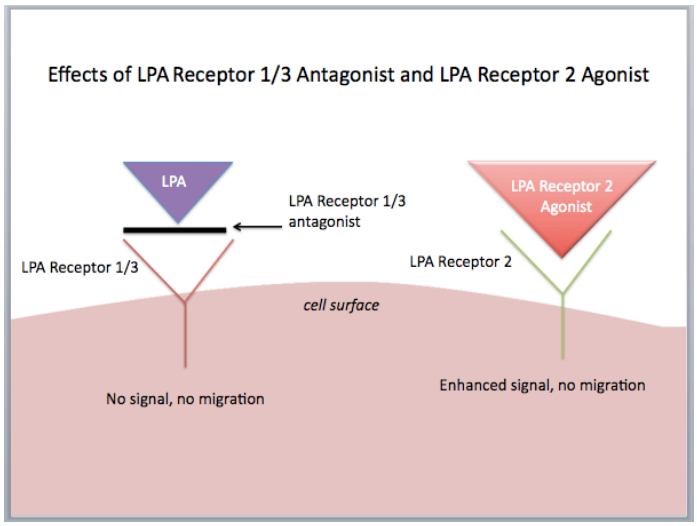

Fig 11. The effects of LPA Receptor 1/3 Antagonist and LPA Receptor 2 Agonist

Table 1: Summary of LPA Receptor 1/3 and LPA Receptor 2 Function

	Migration	VEGF	EGFR	RhoA	Proliferation
LPA Receptor 1/3	+	_	No effect	+	No effect
LPA Receptor 2	_	+	N/A	+	_
LPA (10 µM)	+	+	No effect	No effect	No effect

"+" stimulatory effect **"–"** inhibitory effect **"No effect"** for concentrations tested

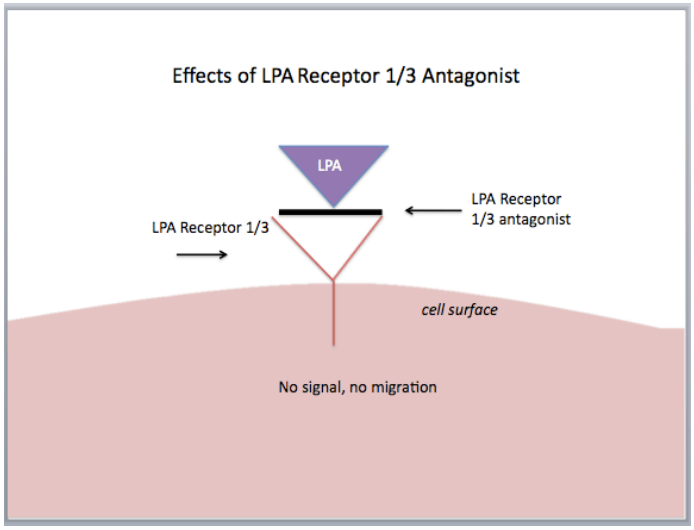

Fig 10. The effect of LPA Receptor 1/3 Antagonist

4 Conclusion

Much of the literature pertaining to the LPA family of receptors remains contradictory. The individual function of each receptor as well as their role in different cancer cell lines remains obscure and poorly understood.

In the study reported here, LPA Receptors (1 and 3) 1/3 appear to promote OVCAR cell migration and have no effect on viability in the concentrations tested (1-15 µM). This receptor was shown to inhibit VEGF secretion, stimulate activation of RhoA (evidenced by the significant downregulation when antagonized), and have no effect on total EGFR and phosphorylated EGFR. LPA Receptor 2 appears to inhibit cell migration and proliferation (shown in Figure 9). This receptor also promotes VEGF secretion and RhoA activation. The LPA ligand appears to promote cell migration and VEGF secretion.

LPA Receptors 1/3 seem to stimulate cell migration by operating through the RhoA pathway. RhoA is a protein that has been shown to aid in cell migration by causing F-actin rearrangement. (Heasman et al., 2010) The upregulation of RhoA via LPA Receptor 2 agonist (interesting given its inhibitory effect on migration) may be explained by the fact that RhoA controls several cystoskeletal processes that are necessary for both cell survival and cell migration, i.e., movement of protein encapsulated vesicles within the cell for maintenance of homeostasis. (Heasman et al., 2010) However, other literature has also reported the possibility of RhoA activation leading to an inhibition of cell migration. (Sugimoto et al., 2005) This upregulation of RhoA by LPA Receptor 2 could also be explained by this hypothesis, as an LPA Receptor 2 related inhibition of cell migration was observed in the study reported here. Because data shows that LPA Receptors 1/3 lead to cell migration, it is possible that the cell

is focusing on the physical mechanism of this process. The cell may be investing its energy in movement, which is why an upregulation in VEGF is not seen (as one might suspect with those molecules traditionally associated with the migratory response of cancer cells). Because LPA Receptor 2 inhibits cell migration, the OVCAR cell may work to secure gases and nutrients for the cell via the VEGF pathway, which promotes angiogenesis. In summary, it is possible that a moving cell focuses on the cytoskeleton activation, while one that is not migrating, focuses on other proteins that increase survival and the maintenance of homeostasis. Each individual receptor appears to have a distinct function that affects the cancer cell's response in the migration process. It is interesting to note that LPA Receptors 1/3 and LPA Receptor 2 appear to have opposing functions.

Other studies have reported an inhibition of migration related to LPA Receptor 1 ligand binding. This finding was reported in the CHO (Chinese hamster ovary cell line). One study found a stimulation of migration via LPA Receptor 1 and an inhibition via LPA Receptor 2 in a pancreatic cancer cell line (Komachi et al., 2010), similarly found in this study performed on the OVCAR cell line. A study done with the epidermal growth factor receptor found that LPA upregulated EGFR in an ovarian cancer cell line- a finding that was not found in this study (Oyesanya et al., 2010). It is possible that LPA Receptors 1/3 and LPA Receptor 2 have distinct functions that are exclusive to different cancer cell lines.

The results of this study show for the first time that combination treatment of LPA Receptor 1/3 antagonist (15 μM) and LPA Receptor 2 agonist (700 nM) prevented a remarkable 95 percent of ovarian cancer cells from migrating. This novel finding demonstrates that perturbing both these LPA receptors may inhibit ovarian cancer cells from migrating from the primary site of the tumor, and thus have potential to be a treatment modality for this highly invasive cancer.

5 Future work

To better understand the relationship between the LPA ligand/receptor family and the metastatic ability of ovarian cancer, the effect of LPA receptors 1/3 and 2 on other proteins found in the traditional migration pathways will be investigated. The presence of other receptors that affect migration will also be clarified to determine whether one specific type has a larger impact than another on OVCAR cells.

Additionally, OVCAR cells can be stained for F-actin to understand how LPA receptors 1/3 and 2 affects the physical mechanisms of the cell. This will allow us to understand how the cancer cell utilizes its cytoskeleton to migrate and if this pictorial data confirms RhoA results found in the study. Other cell lines will be investigated to determine if the role of LPA receptors in migration are exclusive to each individual cell line or can be applied to a range of cell lines.

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