Tissue-Engineered "Metastases": Treatment of Hepatic Colon Tumors with a Dual Action Autotaxin Inhibitor-Lysophosphatidic Acid Receptor Antagonist

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Abstract: Lysophosphatidic acid (LPA) acts *via* G protein coupled receptors (GPCRs) to regulate critical cellular functions and pathophysiological levels of LPA or its receptors are linked to cancer initiation, progression and metastasis. LPA is biosynthesized by the lysophospholipase D activity of autotaxin (ATX/lysoPLD), a known factor for tumorigenesis. By attenuating both LPA signaling and LPA production, we expected to observe synergistic anti-cancer therapeutic effects. *In vitro*, treatment of human colon cancer cells (HCT 116) with BrP-LPA, a potent dual action ATX inhibitor and pan-LPA GPCR antagonist, significantly reduced cell proliferation, migration and invasion. Next, a tissue-engineered xenograft model to mimic hepatic metastasis of colon cancer was used to evaluate BrP-LPA efficacy *in vivo*. HCT 116 cells were suspended in Extracel™, a synthetic extracellular matrix (sECM), and injected directly into the livers of nude mice (n = 8). After 1 week, BrP-LPA in saline buffer was administered for two weeks by intraperitoneal injection (10 mg/kg) twice per week. Controls were injected with saline buffer only. The BrP-LPA treated group showed reduced liver tumor weight ($p < 0.05$) and reduced tumor volume ($p < 0.05$) relative to controls. This study is the first demonstration of the effects of a dual action ATX inhibitor/LPA antagonist on colon cancer cells, and the first example of a tissue-engineered hepatic colon cancer "metastases" as a platform for anti-cancer drug evaluation. The results suggest that attenuation of signaling through the LPA pathway offers a promising therapeutic target for reducing colon cancer growth and metastasis.

Keywords: LPA, signal transduction modifier, lysolipid, cell encapsulation, Extracel™, tumor engineering, injectable hydrogel, hyaluronic acid, HA.

INTRODUCTION

Colon cancer is the third most common form of cancer and the second leading cause of cancer-related death in the western world [1]. Treatment of primary colon cancer with surgical resection, combined in certain cases with chemotherapy or radiation therapy, is curative in many patients. However, nearly half of patients will develop liver metastases during the course of their disease. As with many types of cancer, death from colon cancer is often a result of metastatic disease [2, 3].

The liver is the most common site of distant metastasis from colorectal cancer. Approximately 60% of patients with colorectal cancer go on to develop hepatic metastases, and 15-25% of these present synchronous metastases [4, 5]. Only 10-25% of patients with colorectal cancer liver metastases alone are candidates for surgical resection. For the remaining 75-90% of patients with non-resectable, isolated colorectal cancer liver metastases, systemic chemotherapy is the only remaining, albeit inefficient, therapeutic option [6].

Treatment of metastatic disease has become an important target for drug development. Unfortunately, models for metastatic disease are poorly predictive of clinical outcome in patients. To create a versatile and simple model for metastatic disease, we hypothesized that tumors from the parent tumor cell type could be created as "engineered metastases" using a 3-D tissue-engineered xenograft approach. In this model, colon cancer cells would be encapsulated in a semi-synthetic extracellular matrix (sECM) product, and injected into the liver. The resulting tumor mass would mimic an established colon cancer metastasis to the liver. We then hypothesized that we would be able to reduce the size of this engineered metastasis using signal transduction modifiers.

Lysophosphatidic acid (LPA) [7] is a lipid mediator with diverse effects on various cells, including cell survival, proliferation, migration, and induction of cytokines and growth factors [8]. The physiological functions of LPA suggest that LPA could contribute to a number of pathophysiological states of cancer [9-11]. Signaling by LPA is mediated *via* six receptors - LPA_{1-6}, which are members of a family of G protein-coupled receptors, GPCR [12, 13]. Both LPA_2, and LPA_3, promote the proliferation in HCT 116 cells *via* inactivation of GSK-3β and nuclear translocation of β-catenin [14]. LPA_2 is the major LPA receptor that

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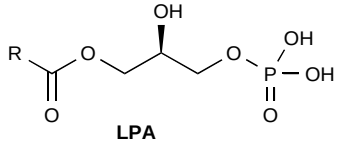
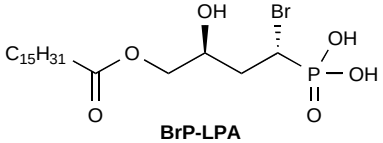
mediates mitogenic signals and cytokine induction in colonic epithelial cells. In addition, LPA has been found to be overproduced in colon cancer cell lines-SW 480 and HCT 116 [15]. However, expectations for anti-cancer agents based on the modification of signaling through the LPA signaling pathway [9, 16, 17] remain unfulfilled, despite the increasing understanding of molecular mechanisms in this pathway [18, 19].

In addition to targeting the action of LPA *per se*, targeting its production is a recognized target for therapeutic development. The lysophospholipase D (lysoPLD) activity of the tumor-associated protein autotaxin (ATX) converts lysophosphatidylcholine (LPC) to LPA [20, 21]. ATX is one of the 40 most upregulated genes in invasive cancers, and has been implicated in cell motility and tumor invasion, metastasis, and neovascularization [22]. In addition, ATX is feedback inhibited by its product LPA [23, 24]. Thus, the ideal anti-cancer drug targeting LPA signaling would simultaneously abrogate signaling through all GPCRs and attenuate LPA production by ATX [25]. Presently, computational and experimental studies have identified novel pharmacophores with potent ATX inhibition profiles [26, 27], including an unusual boronic acid based inhibitor [28]. Two crystal structures of ATX have further accelerated the search for clinically-useful ATX inhibitors as cancer therapeutics [29, 30].

Indeed, we previously observed that the palmitoyl α-bromomethylenephosphonate BrP-LPA (Figure **1**) was a pan–antagonist for LPA_1, LPA_2, LPA_3, and LPA_4 GPCRs [31]. This mixture of diastereomers also inhibited > 98% of ATX activity at 10 μM, suggesting it could have therapeutic utility as a pan-antagonist with dual function as a potential ATX inhibitor [32]. We recently synthesized and separately evaluated the two diastereoisomers, and found that the *anti*-relationship between the hydroxyl and the bromine resulted in greater potency towards the human breast cancer cells, MB-231 for *in vitro* proliferation, migration and invasion. Significant reduction of tumor size and vascularity was observed in an orthotopic breast cancer xenograft model [25]. In addition, the dual activity of BrP-LPA actively reduced tumor growth and angiogenesis in a 3-D xenograft model of A549 non-small cell lung carcinoma cells injected subcutaneously in nude mice [33]. We now report the results of using the signal transduction modified BrP-LPA, a pan-LPA antagonist and inhibitor of autotaxin (ATX) to reduce proliferation, migration and invasion of HCT-116 human colon cancer cells *in vitro*. In addition, we illustrate the ability of BrP-LPA to dramatically reduce the volumes of hepatic colon cancer tumors using a novel tissue-engineered xenograft model. In this "engineered metastasis" model, colon cancer cells are encapsulated in the sECM Extracel™, a hyaluronic acid and gelatin-based hydrogel used for 3D cell culture and cell therapy, and injected into the liver. The resulting tumor mass mimics an established colon cancer metastasis to the liver.



LPA



BrP-LPA

Figure 1: Chemical structures of LPA and BrP-LPA.

MATERIALS AND METHODS

Cells and Reagents

HCT 116 cells were purchased from the American Type Culture Collection (Manassas, MA). Medium and reagents were obtained from the following sources: McCoy's 5a medium (ATCC, Manassas, VA); penicillin-streptomycin (MP Biomedical, Solon, Ohio); fetal bovine serum (FBS) (ATCC, Manassas, MA); Accutase (MP Biomedical, Solon, OH); MTS (Promega, Madison, WI); Matrigel™ (Becton Dickinson Labware, Cambridge, MA); Extracel™ (Glycosan, Salt Lake City, UT), now HyStem®-C (BioTime, Alameda, CA). The LPA antagonist/ATX inhibitor BrP-LPA was synthesized as previously described [25].

Cell Proliferation

HCT 116 cells were maintained in McCoy's 5a medium supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 units/ml penicillin at 37 °C in 95% air, 5% CO_2. HCT-116 cells (4,000) were seeded in 100 μl media in each well of 96-well flat-bottomed microplates (BD Labware, NJ). BrP-LPA was added at final concentrations of 2 μM, 10 μM, and 20 μM to each well. At 72 h, 20μl MTS (Promega, Madison, WI) was pipetted into each well, and cells were further incubated for 2 h. The absorbance of the samples at

490 nm was measured using a 96-well plate reader (OPTI max, Sunnyvale, CA).

Transwell Matrigel™ Invasion Assay

The effect of BrP-LPA on HCT-116 cells was determined by 24-well Transwell™ permeable support (Corning, Lowell, MA) and basement membrane Matrigel™ (Becton Dickinson Labware, Cambridge, MA) invasion assay as described previously [34]. The 8 μm pore polycarbonate filters were coated with basement membrane Matrigel™ (50 μg/filter). HCT 116 cell suspensions were prepared in culture medium containing 50,000 cells/ml. Then, 0.75 ml of medium containing BrP-LPA at 2 μM, 10 μM, or 20 μM was added each well of the BD plate. Sterile forceps were used to transfer the support inserts and control inserts to the wells containing BrP-LPA, and 0.5 ml of HCT 116 cell suspension was added to the inserts. The invasion inserts were incubated for 22 h at 37°C, 5% CO_2. Non-invading cells and Matrigel™ were removed from the upper membrane surface using a cotton-tipped swab, and cells on the lower surface of the membrane were stained with Diff-Quik (IMEB Inc., San Marcos, CA). Using a light microscope at ×100 magnification, five randomly selected fields in each chamber were examined; each group had 6 inserts, and the mean number or cells invaded was calculated. Invasiveness was expressed as the percent invasion for each BrP-LPA concentration through Matrigel™ relative to migration through the control.

Scratch Wound Assay

HCT 116 (20,000 cells per well) were plated in a six-well plate. When the cells reached 100% confluence, the cells were treated with 10 μg/ml mitomycin C (Sigma, MO, USA) for 2 h, and then the monolayer was scratched using a 200 μl pipette tip. Medium and non-adherent cells were aspirated, the adherent cells were washed once, and new medium containing 2 μM, 10 μM, or 20 μM of BrP-LPA was added. Marked fields (n = 4) were photographed at 0, 16, 24 and 48 h. Wound closure was expressed as a percentage of the initial wound area and quantified using NIH ImageJ software.

Animals and Animal Protocol

The experimental protocol and animal care complied with the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, Washington, DC; National Academy Press, 1996), and were approved by the Institutional Animal Care and Use Committee of the University of Utah.

Four-week-old female athymic nude mice (Charles River Laboratories, Wilmington, MA) were used for colon cell implantation. All animals were maintained in a sterile environment. Cages, bedding, food, and water were all autoclaved. All animals were maintained on a daily 12 h light/12 h dark cycle. Each nude mouse was anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg) according to the protocol approved by the University of Utah Institutional Animal Care and Use Committee (IACUC).

After anesthesia induction, the aseptic surgical field was sterilized with iodine and alcohol swabs. In total, 1×10^6 HCT 116 cells in 50 μl of the sECM hydrogel Extracel™ [35] was directly injected into the livers of nude mice after they had been randomly assigned to one of the groups at the beginning of the experiment (8 mice/group). The control group was injected intraperitoneally (i.p.) with physiological saline twice per week starting at one week after the cell transplantation and ended at two weeks after the treatment. The treatment group was injected i.p. with 10 mg/kg BrP-LPA twice per week at the same times as the control group.

Mice were observed daily, and euthanized at 3 weeks post-injection using a carbon dioxide chamber. Body weights were measured and livers were excised. Liver weights and tumor diameters were subsequently determined as described previously [35, 36]. Colon tumors were measured using digital calipers, and the volume of the cancer was calculated according to the formula: Cancer Volume (CV) = $(d^2 \times D)/2$, where d and D are the shortest and the longest diameters, respectively [37]. After measurement, the tumor tissue was then harvested and placed in 10% formalin for paraffin embedding in preparation for the subsequent histological analyses.

Statistical Analysis

Data are presented as mean ± SD. Student's *t* test (unpaired) was used as indicated in the text to compare continuous variables. The statistical significance level was set at $p < 0.05$.

RESULTS AND DISCUSSION

Effect of BrP-LPA on HCT 116 Cells *In Vitro*

First, we examined the anti-proliferative effect of the LPA antagonist and ATX inhibitor BrP-LPA on HCT 116

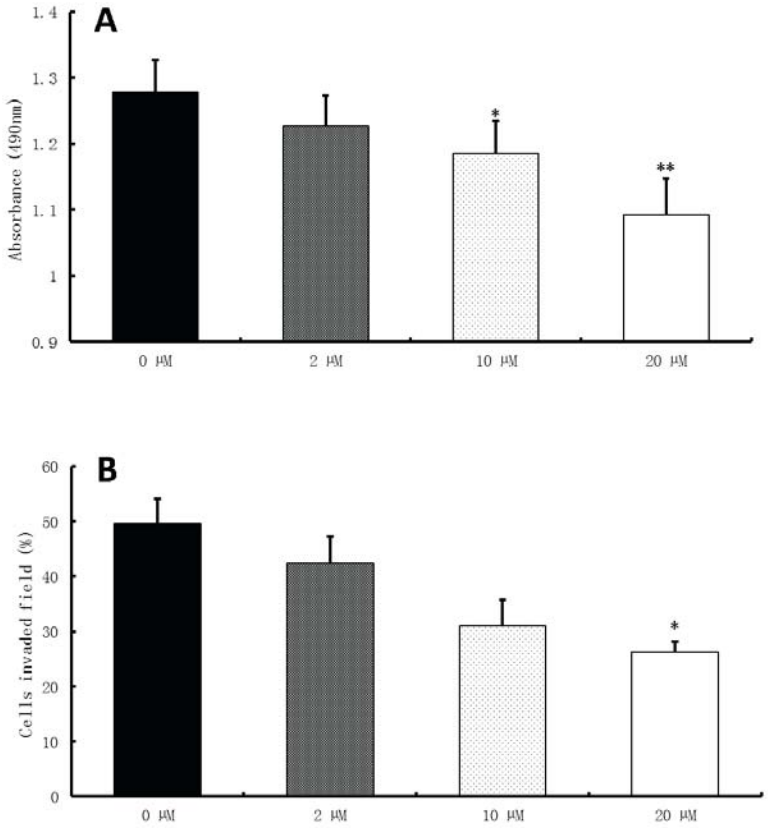


Figure 2: (**A**) BrP-LPA reduces HCT 116 cell proliferation (* $p < 0.05$, ** $p < 0.01$). (**B**) BrP-LPA reduces HCT 116 cell invasion (* $p < 0.05$, ** $p < 0.01$).

cells. Treatment of HCT 116 cells with BrP-LPA caused a dose-dependent inhibition in tumor cell proliferation, with the significant reduction of proliferation at 10 μM and 20 μM (Figure **2A**). Second, to assess the effect of BrP-LPA on the metastatic phenotype, cell invasion assays were performed using Matrigel™ as a chemoattractant. BrP-LPA inhibited the invasive capacity of HCT 116 cells in a dose-dependent manner, showing significant reduction of invasion at 20 μM (Figure **2B**).

Third, a scratch wound assay was performed to determine the ability of BrP-LPA to inhibit the migration HCT116 cells into a denuded area at 0, 16, 24 and 48 h after scratching the confluent cell layer. Figure **3A** shows the micrographs of the time- and concentration dependent inhibition of migration by BrP-LPA. Figure **3B** summarizes the results, showing statistically significant reduction of migration at 24 h by 20 μM BrP-LPA, and significant reduction by 10 μM and 20 μM BrP-LPA at 48 h.

Effect of BrP-LPA on Hepatic Tumor Growth

To evaluate the effects BrP-LPA on tumor growth of human colorectal cancer at the most common metastatic site (the liver), HCT 116 colon cancer cells were encapsulated in Extracel™, an sECM composed of *in situ*-crosslinkable thiolated hyaluronic acid and thiolated gelatin [38, 39], and the cell suspension was directly injected into the livers of eight nude mice. This tissue-engineered xenograft protocol has been successfully employed for creating engineered orthotopic tumors from human cell lines injected into the mammary fat pads, the colon serosum, the ovarian capsule, and the pancreas of nude mice [35, 40]. Based on the dosage required to achieve reduction of tumor size and vascularity by BrP-LPA in an orthotopic breast cancer model [25], we selected a two-week treatment course of 10 mg/kg, injected i.p. twice per week starting after one week of tumor growth.

All nude mice in both groups developed liver tumors. Autopsy showed that the implanted HCT 116 cells in Extracel™ grew rapidly and formed extensive tumor masses attached to the liver in the untreated group. In contrast, the tumor masses in the BrP-LPA treatment group were significantly smaller than those of the controls. Representative photographs of excised livers of control untreated animals are shown in Figure **4A**. The animals treated with the LPA antagonist

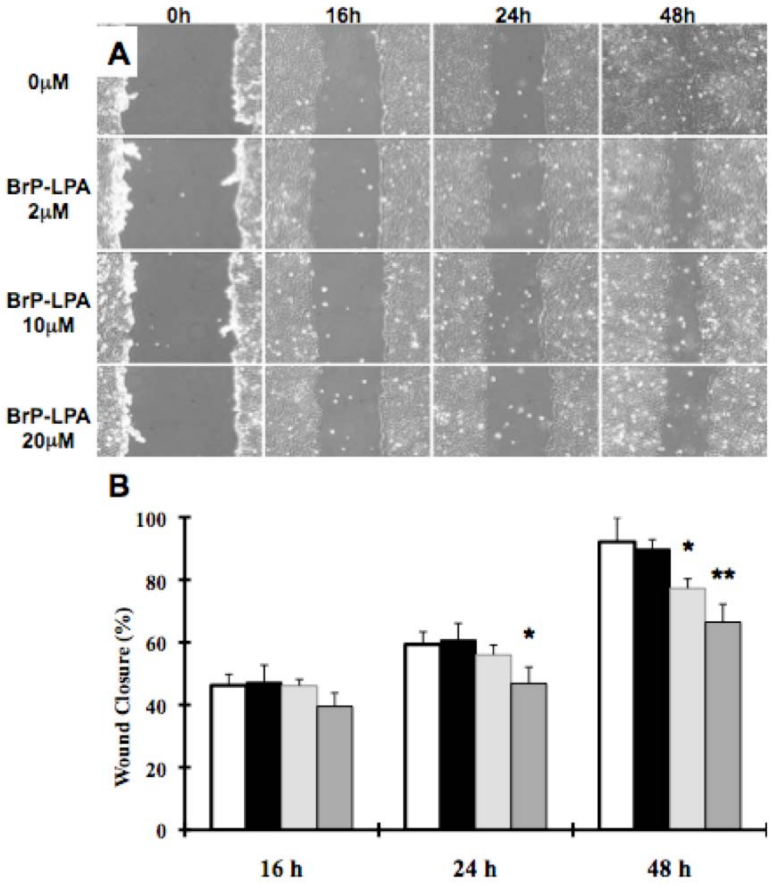


Figure 3: The LPA antagonist and ATX inhibitor BrP-LPA reduces HCT 116 cell migration. (**A**) Images at 0 (white), 2 (black), 10 (light grey), and 20 μM (dark grey) BrP-LPA; (**B**) Summary of data (* $p < 0.05$, ** $p < 0.01$).

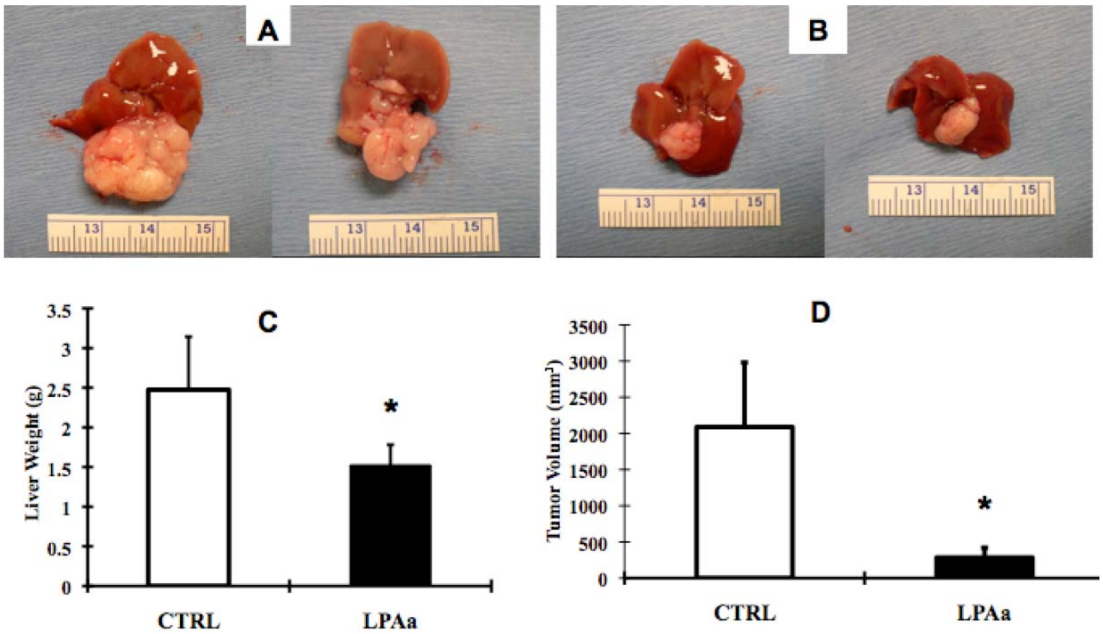


Figure 4: Effect of the LPA antagonist and ATX inhibitor BrP-LPA on hepatic colon cancer growth. (**A**) Representative images of excised livers from untreated control animals; all animals in both groups developed liver tumors. (**B**) Representative images of excised livers from animals treated with the LPA antagonist (LPAa) BrP-LPA (10 mg/kg i.p. x 4); (**C**) Reduction of tumor burden in BrP-LPA treated group (* $p < 0.05$). (**D**) Reduction (eight-fold) of tumor volume in BrP-LPA treated group (* $p < 0.05$). Key: LPAa, LPA antagonist BrP-LPA.

(LPAa) and ATX inhibitor BrP-LPA showed a marked reduction of hepatic tumor burden (liver weight; $p < 0.05$; Figure **4B** and **4C**). Treatment with BrP-LPA also led to a significant decrease in tumor volume ($p < 0.05$; Figure **4D**). Tumor volumes averaged 2,088 mm^3 in the control group and 283 mm^3 in treated group; importantly, no local or distal organ metastases were observed during this relatively short time course. Importantly, no significant difference was observed between the mean body weights after euthanasia of the control animals and those treated with BrP-LPA (LPAa).

A hepatic colon tumor (Figure **5A**) was sectioned, and H&E staining revealed a distinct interface between hepatic colon cancer and mouse native liver (Figure **5B**). The arrangement of cell in cancer section was irregular, and newly generated blood vessels were observed (Figures **5C**, **5D**). This preliminary study shows that the irregular cancerous regions are reduced in size and number in BrP-LPA-treated tumors (Figure **5D**) relative to the control "engineered metastasis" (Figure **5C**). Moreover, the BrP-LPA treated tumor showed qualitatively fewer blood vessels than in the control group. This qualitative histological data supports the quantitative reduction of tumor burden (Figure **4**) and is consistent with the reduced angiogenesis observed in BrP-LPA treatments for the orthotopic 3-D breast cancer model [25] and subcutaneous 3-D lung carcinoma model [33].

Signal Transduction Modifiers in the LPA Pathway

Lysophosphatidic acid (LPA) signalling contributes to multiple physiological and pathophysiological processes. The most biologically relevant pathway for LPA biosynthesis occurs by the action of a lysophospholipase D, also called autotaxin (ATX/lysoPLD), on lysophosphatidylcholine. By generating LPA, ATX/lysoPLD contributes to tumor progression by providing an invasive and vasculogenic microenvironment for tumor cells [9]. Aberrant production and degradation of LPA occur in cancer cells as well as in cancer patients [9,11,18, 19]. LPA-signaling has been linked to cancer in numerous ways. Dysregulation of autotaxin or the LPA receptors can lead to hyperproliferation, which contributes to oncogenesis and metastasis [8, 9, 11, 17]. Therefore, inhibitors of LPA signaling and LPA production act as signal transduction modifiers that have potential therapeutic value for the treatment of primary tumors as well as metastases [19].

Tissue-Engineered Orthotopic Xenograft Models

An appropriate animal model plays an important role in understanding of human cancer. Clinically

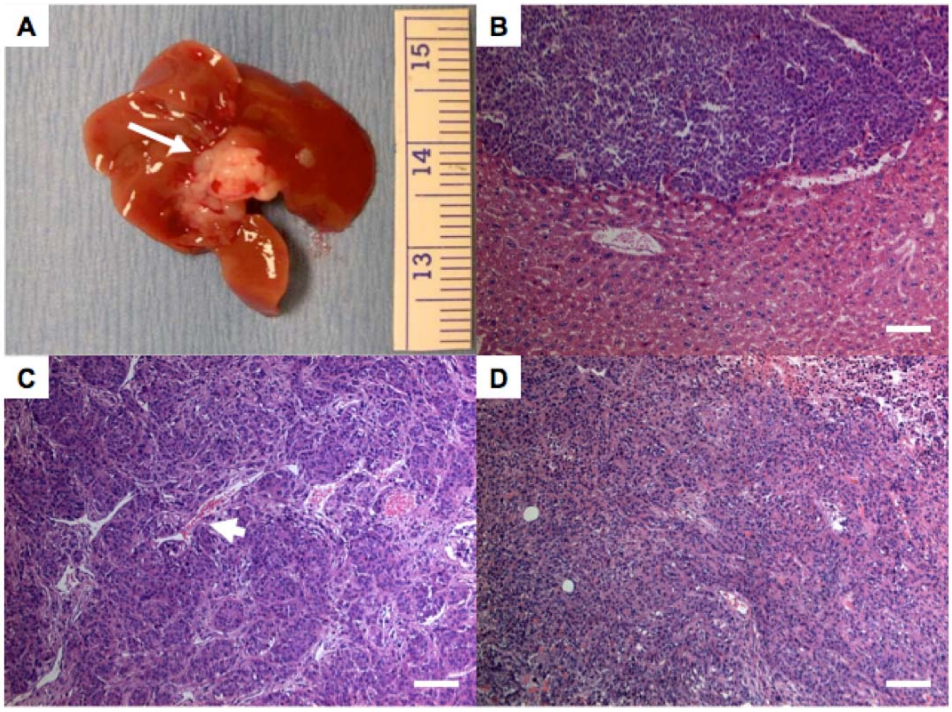


Figure 5: (**A**) Liver with involved colon tumor (arrow). (**B**) Pathohistology of tumor-liver interface. The upper region comprises the hepatic colon cancer growth, while the lower region shows the native nude mouse liver (100×). Representative H&E staining of a hepatic colon cancer in (**C**) an untreated control and (**D**) a BrP-LPA treated animal (100×). Scale bar = 50 μm.

relevant animal models of human cancer are necessary for understanding cancer biology, and invasion and metastasis in the context of the diseases presented by patient. Subcutaneous tumor implantation had been a standard methodology for establishing animal models for human cancer research for many years [41, 42]. However, subcutaneous tumor models are clearly do not reproduce the primary site of the common human cancers, nor do they represent the common sites of metastasis. It is increasingly recognized that organ microenvironment plays a major role in tumorigenesis. The specific organ microenvironment determines the extent of cancer cell proliferation, angiogenesis, invasion, and survival [43].

To address these inadequacies, we developed a method that we called "tumor engineering" to better mimic the tumor microenvironment [35]. We encapsulated six human tumor cell lines in Extracel™, a modular, hyaluronan (HA)-based, *in situ*-crosslinkable mimic of the ECM [38, 39] and delivered these cell suspensions orthotopically in nude mice: two breast cancer cell lines were injected into the mammary fat pad; two colon cancer cell lines were injected subserosally in the colon; and two ovarian cancer cell lines were injected into the ovarian capsule. The results were dramatic: tumor "take" increased dramatically, cells grew and proliferated in the synthetic ECM matrix, and single vascularized tumors formed. The engineered orthotopic tumors better mimicked the presentation of human cancer patients. The first uses of this tumor engineering technology to test drug efficacy compared the dual activity BrP-LPA to taxol [25].

Analogously, we created an "engineered metastasis" by implantation of human colon cancer cells in Extracel™ into the lobes of the livers of immune compromised mice. This model of orthotopic colon cancer liver metastases better mimics the morphology and microenvironment of colon cancer following establishment of a metastatic lesion. The value of this approach is that it bypasses the poor reproducibility and long lead time in typical metastasis models, while still allowing the study of the interaction of an otherwise healthy tissue with cells from a cancer of different tissue origin.

In summary, we report that a dual activity LPA antagonist – ATX inhibitor reduces the growth of HCT 116 hepatically implanted human colon cancer and reduces tumor angiogenesis. Results from these studies indicated LPA is an important mediator in the development and angiogenesis of colon cancer tumor. However, its precise role in this process remains to be elucidated.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

G.D.P held founder's equity in Glycosan BioSystems, holds stock in BioTime, Inc., advises Echelon Biosciences, and has a pending patent application on BrP-LPA.

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