The Effect of Erlotinib on Human Angiogenesis

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Introduction

Angiogenesis is the process of new blood vessels developing from pre-existing ones. Physiologic angiogenesis only occurs under very specific circumstances in healthy individuals, such as in wound healing and formation of the placenta during pregnancy. However, angiogenesis can be pathologic as in cancer and some other diseases. The size of a developing tumor is limited by its blood supply; it is accepted that a tumor cannot expand beyond about 2 mm^3 in volume without recruiting vasculature.[1] Tumors utilize the process of angiogenesis to circumvent this size limitation. They do so by secreting growth factors that stimulate local blood vessels as well as overexpressing oncogenic tyrosine kinase receptors.[2]

Erlotinib HCl is designed to inhibit activity of one of the tyrosine kinase (TK) receptors of the ErbB family, the Epidermal Growth Factor Receptor (EGFR). Elevated EGFR expression has been associated with progression of many solid human tumors and poor prognosis. Erlotinib HCl has been approved by FDA for use in non-small cell lung cancer and pancreatic carcinoma. Our lab has identified several TK inhibitors, other than Erlotinib HCl that effectively inhibit angiogenesis in both human placental vein (HPV) and human neuroendocrine tumors (NETs). Preliminary testing of Erlotinib HCL in HPV showed a dose-dependent inhibition of angiogenesis in our model system. This is why we believe testing Erlotinib in inferior vena cava (IVC) and human NET liver metastases would provide data relevant for understanding the role of EGFR pathway in physiologic (placenta / IVC) as well as pathologic (tumor) angiogenesis.

This project seeks to explore an effect of Erlotinib HCl on human angiogenesis using human-tissue based fibrin-thrombin clot *in vitro* angiogenesis assay. We focus on two aspects of angiogenesis in human tissue, physiologic (in HPV and IVC) and pathologic (in liver NETs). An exciting prospect about this research is its applicability to diseases outside of cancer that also operate through angiogenesis such as rheumatoid arthritis, psoriasis, and ocular neovascularization. By preventing or at least limiting new blood vessel formation, we can possibly improve patient outcomes in both cancer and other angiogenesis-dependent diseases.

Hypothesis

Erlotinib HCl will inhibit both physiologic and pathologic angiogenesis in an in vitro angiogenesis model system.

Methods

The research protocols for this study were approved by Institutional Review Board of Louisiana State University Health New Orleans, LA, US.

Erlotinib HCl was evaluated in an *in vitro* angiogenesis assay cited in the lab's previous works.[3] Human tissue samples used in the assay were harvested from venous tissue (HPV and IVC) and liver NETs. For the purposes of the experiments, venous tissue modeled normal angiogenesis, while liver tumor samples modeled pathologic angiogenesis. The initial concentration of Erlotinib HCl treatment (10 μM) was selected based on previously reported effective concentrations for non-small cell lung cancer cells.[4] In total, three doses of Erlotinib HCl (1 μM, 10 μM, 100 μM) were selected for this project in order to determine the tissues' responses to Erlotinib HCl. Minced tumor fragments and punched vein discs were placed into wells of a 96-well plate pre-loaded with thrombin. The tissue was overlaid with a fibrinogen solution (0.3% fibrinogen, 0.5% caproic acid) to produce a clot. Erlotinib HCl was then added to the clot in a liquid overlay with growth media (M199, 20% FBS and an anti-microbial solution). Plates were incubated in a humidified environment at 37˚C with 6% CO_2. Media was replenished on day 7 and the experiment was terminated on day 14.

The effect of Erlotinib HCl on three parameters of angiogenesis (the angiogenic growth, the percent of initiation and the overall angiogenic effect) was assessed by use of an inverted phase-contrast microscope. Tissue in each well was visually divided into four quadrants and each quadrant was graded from zero to four based on the number, density and length of the new vessels. The four quadrant scores were summed to create an overall score for each well. The effect on angiogenesis was then evaluated by comparing the scores of Erlotinib HCl treated wells to those of untreated wells. Statistics were done using an unpaired t-test (Primer of Biostatistics).

After the angiogenesis was evaluated visually on day 14, supernatant and tissue were harvested separately from IVC and liver NET assays for both experimental groups. Supernatant from both tissue types was evaluated for angiogenesis-relevant ligands and soluble receptors using the human angiogenesis ligand and receptor arrays (Milliplex, EMD Millipore). High quality total RNA was extracted using RNeasy Mini kit (Qiagen US) from control and Erlotinib HCl treated samples. Corresponding cDNA was prepared via High-Capacity cDNA Reverse Transcription Kit (Life technologies, US) and used to analyze the gene expression levels in IVC and liver NET samples using TaqMan Array Human EGF Pathway (Life Technologies, Thermo Scientific).

Results

Human Placental Vein Angiogenesis Model Data

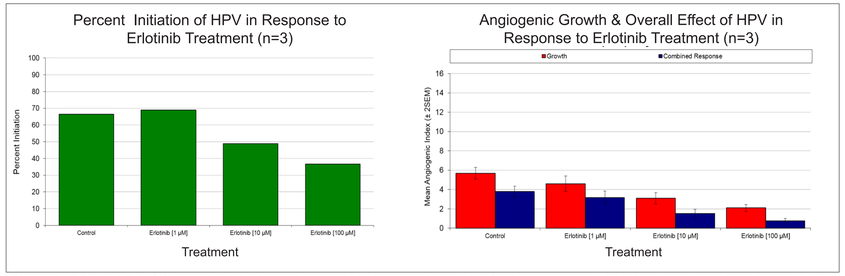

Figure 1. ***Effect of Erlotinib on physiologic angiogenesis in human placental vein.*** Average percent initiation (green), angiogenic growth (red) and overall angiogenic effect (blue) are represented for three placentas at a given Erlotinib HCl dose. For every placenta, each treatment group consisted of 30 tissue fragments. Using an unpaired t-test at $p<0.05$ (Primer of Biostatistics), we found that the Erlotinib HCl treated specimen group had statistically significant inhibition of Angiogenic Growth and Combined Response values for the 10μM and 100μM groups compared to the control.

Human Inferior Vena Cava Angiogenesis Model Data

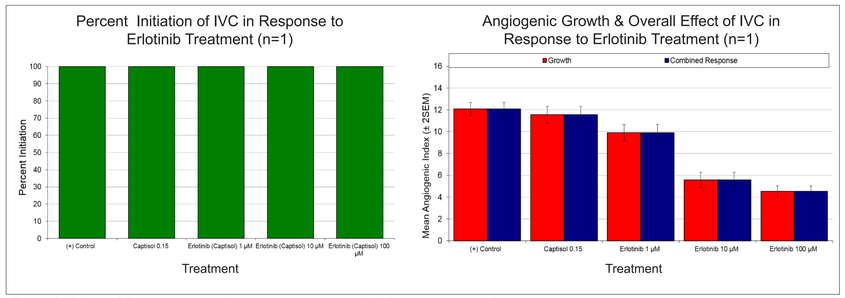

Figure 2. ***Effect of Erlotinib on physiologic angiogenesis in inferior vena cava.*** Percent initiation (green), angiogenic growth (red) and overall angiogenic effect (blue) are represented for each individual venous sample at a given Erlotinib HCl dose. Each treatment group consisted of 30 tissue fragments from the same IVC. Using an unpaired t-test at $p<0.05$ (Primer of Biostatistics), we found that the Erlotinib HCl treated specimen group had statistically significant inhibition of Angiogenic Growth and Combined Response values for all three Erlotinib HCl concentrations compared to control.

Human Tumor Angiogenesis Model Data

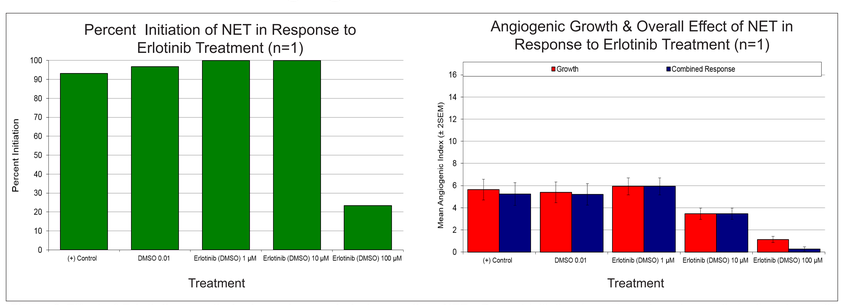

Figure 3 ***Effect of Erlotinib on pathologic angiogenesis in human liver NETs.*** Percent initiation (green), angiogenic growth (red) and overall angiogenic effect (blue) are represented for each individual tumor sample at a given Erlotinib HCl dose. Each treatment group consisted of 30 tissue fragments from the same liver NET. Using an unpaired t-test at $p<0.05$ (Primer of Biostatistics), we found that the Erlotinib HCl treated specimen group had statistically significant inhibition of Angiogenic Growth and Combined Response values for all three Erlotinib HCl concentrations compared to control. The effect of the highest dose of Erlotinib HCl on percent initiation in the liver NET was significant when compared to the control.

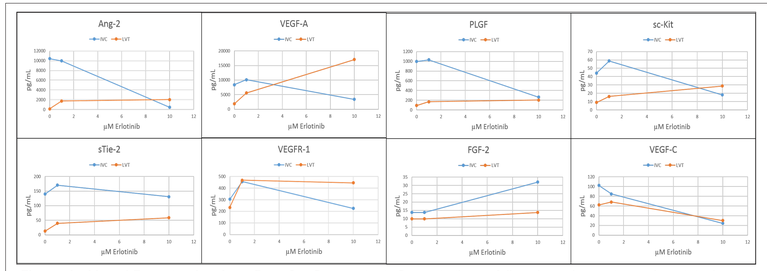

Figure 4. ***Ligand-Receptor Luminex Data for Supernatant.*** Concentrations of ligands and receptors relevant to human angiogenesis in Erlotinib HCl treated and untreated fragments from inferior vena cava (IVC) and neuroendocrine liver metastasis (LVT). Erlotinib HCl concentrations were 1μM and 10μM for the treated groups.

Sample Type:	Ang-2	sTie-2	VEGF-A	PLGF	VEGFR-1	VEGF-C	FGF-2	sc-Kit
IVC	↓	↓	↓	↓	↓	↓	↑	↓
LVT	↑	↑	↑	↑	↑	↓	↑	↑

Table 1. ***Luminex Data Trends for inferior vena cava and neuroendocrine liver tumor.*** Overall trend of supernatant protein concentrations in treated and untreated IVC and liver NET samples with increasing concentration of Erlotinib HCl. Highlighted elements represent ligand-receptor pairs.

Down-Regulated		Up-Regulated							
Gene	Fold Change	Gene	Fold Change	Gene	Fold Change	Gene	Fold Change	Gene	Fold Change
RHOD	10.64	RAC1	2.02	NCK1	2.12	MUC1	2.48	PIK3C2B	9.13
GAB1	5.15	PRKCZ	2.05	VAV3	2.34	CDH1	2.98	DIRAS3	9.13
CAV2	3.17	SRC	2.09	PDPK1	2.37	REL	9.13	PIK3CA	9.13
CAV1	3.10	NFKB1	2.10	VAV1	2.44	MAP3K1	9.13	PRKCQ	9.13

Table 2. ***Taqman EGFR Pathway.*** Changes in the EGFR pathway gene expression in liver NET in response to Erlotinib HCl treatment (10μM).

Gene	Fold Change	
	IVC	Liver NET
CAV2	32.75	3.17
GAB1	6.15	5.15
NCK1	2.67	2.12
PDPK1	4.10	2.37
MUC1	2.79	2.48

Table 3. ***EGF Pathway genes in common between IVC and liver NET in response to Erlotinib HCl.*** Up-regulated genes are shown in red, down-regulated genes are in green.

Conclusions

- Preliminary data from human placental vein indicated a dose dependent inhibition of angiogenesis with Erlotinib HCl; this trend was confirmed in human inferior vena cava tissue. Therefore, Erlotinib HCl is a dose dependent inhibitor of physiologic angiogenesis in our *in vitro* model system.
- In liver metastases of neuroendocrine origin, Erlotinib HCl effectively decreased angiogenic growth and percent initiation. Therefore, Erlotinib HCl also inhibited pathologic angiogenesis in a dose response manner in our *in vitro* model system.
- In inferior vena cava samples, an increase Erlotinib HCl concentration corresponded with a decrease in pro-angiogenic ligand and receptor pairs (Ang-2, sTie-2, VEGF-A, PLGF, VEGFR-1). In contrast, in liver NET tissue, these same pro-angiogenic pairs increased in concentration with the dose of Erlotinib HCl . This indicates that these proteins may have a different role in two types of angiogenesis, physiologic and pathologic.

Future Research

- Perform cell survival and proliferation assays in human umbilical vein endotheial cells (HUVEC) and human neuroblastoma cells (IMR-32) using Erlotinib HCl .
- Expand current study to include more patients and different metastatic sites to better understand changes in phenotype and gene expression that occur in response to Erlotinib HCl treatment.

References

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