An Investigation of Therapeutic Potential of Plant-Made Cholera Toxin B Subunit, an Orally Active Anti-inflammatory Protein, in a Mouse Model of Acute Colitis

Baily A. Nelson1, Joshua M. Royal2, Keegan Baldauf1, Calvin Kouokam3, and Nobuyuki Matoba1,2

1Department of Pharmacology and Toxicology, and 2James Graham Brown Cancer Center, University of Louisville School of Medicine, Louisville, KY 40202, and 3The Owensboro Cancer Research Program, Owensboro, KY 42303

Introduction

Cholera Toxin B Subunit (CTB)
- Non-toxic subunit of cholera holotoxin; 56kDa in size
- A component in an internationally licensed oral vaccine (Dukoral®)
- Induces strong anti-inflammatory response
- An identical CTB suppressed pathogenic immune responses associated with allergy and Crohn’s disease.1,2

Robust Plant Production System of CTB
- Our group has generated a variant of recombinant CTB (CTBp) in Nicotiana benthamiana plants3
- Remarkably high and uniform expression of CTBp in 5 days
- Demonstrated GM1 ganglioside binding affinity and oral immunogenicity equivalent to the original Dukoral vaccine antigen.

Mouse Model of Inflammatory Bowel Disease (IBD)
- CTB has been shown to protect against inflammation
- The present study utilized a model of ulcerative colitis (UC) in mice
- Dextran Sodium Sulfate (DSS) was given to induce immunological tolerance, regulatory T cells and the adjuvant effects of CTBp.
- CTBp protein levels in mice serum for each mouse were measured using CTB160 ELISA.
- Robust Plant Production System of CTB

Results

Conclusions and Future Studies

Future Studies
- Additional studies are ongoing with different dose levels and timing to find an effective dosing regimen of CTBp.
- Comprehensive analyses of TNFα and other cytokine levels in serum and tissue will be performed.

Acknowledgements

References


Conclusions
- Treatment with 3µg CTBp significantly decreased the DAI when compared to Infliximab and PBS-DSS treatment.
- However, treatment with 3µg CTBp did not significantly blunt colon inflammation or decrease percent body weight loss when compared to PBS-DSS treatment.
- Treatment with 3µg CTBp showed a trend of decrease in many inflammatory genes and chemokines compared to PBS-DSS, while a single dose of 5mg/kg Infliximab did not.

Future Studies
- Additional studies are ongoing with different dose levels and timing to find an effective dosing regimen of CTBp.
- Comprehensive analyses of TNFα and other cytokine levels in serum and tissue will be performed.

Materials and Methods

Animals and treatments: Eight week old female C57BL/6J mice were provided from Jackson Laboratory (Bar Harbor, ME). Animals were given oral (gavage) doses of CTBp (3µg) or PBS twice times during DSS exposure, or an intraperitoneal injection of Infliximab (5 mg/kg) on the third day of DSS exposure (see Figure 2).

Percent Body Weight Change
- Initial body weights were collected immediately prior to the initiation of DSS exposure.
- Body weights were collected in the same time frame daily and the percent change from the baseline (initial body weight prior to exposure) was calculated. 0% DSS was administered for 7 days and followed by a 2 day recovery period. Mice were sacrificed 9 days after the initiation of the experiment.

Disease Activity Index (DAI) Scoring
- The DAI score is based on a combination of stool consistency, percent body weight change, and the area of the rectum with blood test from Helena Laboratories (Beaumont, TX).
- Scoring guidelines are as follows:
  - <15% weight loss
  - + occult blood test (max color change)
  - Mice were sacrificed 2 weeks after the initiation of the experiment.

Acknowledgements

Research supported by a grant from University of Louisville Cancer Education Program (NUVHC-92S-CA134283), the Helmsley Charitable Trust Program and DoD/DUSA/USAMRICD/W81XWH-10-2-0082-CULQ. CTBp was produced and validated by Krystal Haney and Lauren Bennett at CorPharm.
In human cancers, rates of glycolysis have been shown to increase up to 200 times in order to achieve high rates of proliferation and survival. One key regulator, 6-phosphofructo-2-kinase (PFKFB3) phosphorylates fructose-6-phosphate (F6P) to produce fructose 2,6-biphosphate (F2,6BP), a potent activator of 6-phosphofructo-1-kinase (PFK1), that regulates an irreversible step of glycolysis. Oncogenes and various tumor suppressor genes regulate PFKFB3 (i.e. PTEN, RAF/BRAF, Hif1α). A promising drug, PFK-158 that inhibits PFKFB3 is currently in Phase I clinical trials. About half of metastatic melanomas express a mutant form of B-RAF (BRAFV600E) and those patients are treated with Vemurafenib (VEM) or Dabrafenib; both are specific inhibitors of mutant B-RAF kinase activity. However, up to 50% of patients treated with VEM respond, but then relapse while the other 50% are intrinsically resistant to VEM. Since BRAFV600E promotes glucose metabolism, survival and growth, and regulates Hif1α, we hypothesized that BRAFV600E regulates glycolysis through PFKFB3. Knockdown of BRAFV600E with specific siRNA’s mimicked the glycolytic effect of VEM including down-regulating PFKFB3. To our surprise, overexpressing BRAFV600E had little effect on PFKFB3 in cells expressing WT BRAF. Lastly, combination treatment with VEM and PFK-158 in VEM resistant cells that express BRAFV600E, resulted in synergistic cell death. Our data suggest VEM + PFK-158 may be a promising treatment option for metastatic melanomas resistant to Vemurafenib.

**Conclusions**

- BRAFV600E knockdown in A375 cells reduced PFKFB3 and F2,6BP levels suggesting BRAFV600E regulates PFKFB3
- Overexpression of BRAFV600E did not lead to an increase in PFKFB3 expression, F2,6BP levels, or an increase in glycolysis.
  - We hypothesize that endogenous wild-type BRAFV600E is having a dominant negative effect
  - Experiments to knockdown endogenous and overexpress BRAFV600E are underway
- Synergistic cell death was observed when the VEM resistant cell line (A2058) was treated with VEM and PFK158.
  - We hypothesize this is due to complete inhibition of glycolysis and will test this in the future.

**Acknowledgements**

Research supported by a grant from R25-CA-134283 from the National Cancer Institute, & the University of Louisville Cancer Education Program.
Circadian Disruption: distress and sleep quality in breast cancer patients

Thomas Packer, BA1, Lauren A. Zimmmer, BA1, Elizabeth Cash, PhD1,2, and Sandra E. Sephton, PhD1,2

1 Department of Psychological and Brain Sciences, University of Louisville, Louisville, KY. 2 J.G. Brown Cancer Center, University of Louisville, Louisville, KY

ABSTRACT

Breast cancer is the most common form of cancer and second leading cause of cancer deaths among women of all races. The distress accompanying a cancer diagnosis and its affect on circadian rhythmicity has not been fully explored. We examined the predictability of circadian activity measures based on the psychosocial factor of distress. Our assumptions were based on a model of tumor progression (Eismann, et al., 2010). Forty-eight breast cancer patients completed an Impact of Event Scale self-report to measure their subjective response to their cancer diagnosis. A multiple regression analysis controlling for age was used to predict each measure. Actigraphy is the recording of body movement that provides a noninvasive measure of rest-activity rhythms and sleep patterns. Participants wore a wristwatch-like device (Motionlogger) continuously for four days. Whenever the device moves, a piezoelectric beam generates voltage signals that are recorded in 60-second segments, creating a curve which are scored as “wake” or “sleep”. The circadian rhythm in activity was estimated using the autocorrelation coefficient calculated based on 24-hour time lags. We hypothesized that total distress would be associated with circadian rhythmicity, sleep efficiency, amount of time spent awake after sleep onset, amount of time spent sleeping, and the number of nightly awakenings. After statistical analysis, total distress scores alone were incapable of predicting circadian disruption in any of the measures tested. Total distress as reported from the IES did not show significant proportion of variance in circadian activity measures.

BACKGROUND

Breast cancer is the most common form of cancer and second leading cause of cancer deaths among women of all races. It is a slowly progressive disease throughout her lifetime. While about 5-10% of breast cancers can be explained by mutations in the BRCA1 and BRCA2 genes, mutations in circadian regulatory genes as are increasingly being studied as alternative causes for tumor development and metastasis. Circadian regulatory genes such as PER3 and NPAS2 are responsible for regulating the endogenous 24-hour sleep wake cycle (circadian rhythm) that maintains sleep, wakefulness, hormone biosynthesis, metabolism, and immune response. Abnormal circadian rhythms have been associated with higher risk of cancer development with faster tumor growth and shorter survival. Survival at 2 years was greater for those with a normalized activity rhythm. Disruption of circadian function has been correlated with poor sleep, fatigue, and depression. Patients with marked rest/activity rhythms also have better quality of life and report significantly less fatigue. Studies have shown that circadian activity correlates with several health related quality of life scales. A cancer diagnosis can be stressful for an individual. This is why cancer diagnoses are commonly accompanied by psychological distress caused by anxiety and depression some time after the initial diagnosis. Psychological distress is a known deregulator of endocrine and immune function, while possibly even affecting cortisol and circadian rhythms. Activity/Disruption will be associated with distress as measured by the self report Impact Event Scale (IES). The agency, influence, and control impaired in distress would likely be a result of a stressful event or change in the environment.

RESULTS

Total distress did not significantly predict actoocorrelation coefficient values, β = 0.063(0.422)−0.675 and did not show significant variance R² = 0.17(2.45)−0.388 p < 0.05

Total distress did not significantly predict overall sleep efficiency β = 0.11t(0.74)−0.463 and did not show significant variance R² = 0.012(2.45)−0.274 p < 0.05

Total distress did not significantly predict amount of time spent awake after sleep onset β = 0.063(0.422)−0.675 and did not show significant variance R² = 0.043(2.45)−1.007 p < 0.05

Total distress did not significantly predict the number of nightly awakenings β = 0.19t(0.33)−0.211 and did not show significant variance R² = 0.089(2.45)−0.552 p < 0.05

Grouped IES data was grouped and analyzed via MANOVA analysis but showed no significance within or between groups.

DISCUSSION

There are some possible explanations for this non-significant data.

*Extreme outliers made data analysis difficult because of the many of the measures did not account for normality.
*Since these patients have only recently been diagnosed with cancer, it is possible their health had not deteriorated to a point which compromised their circadian rhythmicity
*It’s possible there was error in collecting the self report data because of the mental health of the subject. This is an attractive explanation because all of the subjects were newly diagnosed cancer patients. The sudden mental distress may raise eyebrows on the accuracy of self report data of participants weren’t truly involved in data collection. In such situation it would be beneficial to have many different methods of self report data.

Future Projects:

*This experiment is being repeated with an expanded IES and more psychosocial variables such as anxiety, depression, coping, and social support.
*Measures such as the Perceived Stress Scale (PSS) and the Symptom Distress Scale (SDS) can offer a more reliable and comprehensive self report into patient distress.
*Measures such as the Beck Anxiety Inventory (BAI), Beck Depression Inventory (BDI), Pittsburgh Sleep Quality Inventory (PSQI) offer a chance to find more comprehensive relationships between variables such as anxiety, depression, and circadian disruption.
*There is interest in testing patients for mutations in circadian regulation genes PER3 and NPAS2

REFERENCES


Acknowledgments

Authors would like to thank National Cancer Institute grant R25-CA134283.
Plasma-Based MicroRNA Panel Specific for Colorectal Neoplasia

**Introduction**

- Colorectal cancer (CRC) is the third most common cancer worldwide.
- Detection of colorectal adenomas (CRAd) is key to reduce the incidence and mortality of CRC.
- Current methods of CRC screening (colonoscopy and fecal occult blood test) have many shortcomings thus there exists a need for a non-invasive biomarker with high sensitivity and specificity for the diagnosis of CRAd and CRC.
- microRNAs (miRNAs) are short, non-coding RNA molecules approximately 22 nucleotides in length, that bind to messenger RNA and block translation of proteins.
- miRNAs are thought to be involved in the development and progression of CRC, making them potential biomarkers of early disease.
- Numerous miRNAs have been reported in the literature to be significantly dysregulated in the plasma of many different cancer types (e.g. miR-21).

**Hypothesis**

We hypothesize that there exists a panel of miRNAs in plasma that is specific to colorectal neoplasia and would allow for a inexpensive, non-invasive and clinical useful detection method.

**Overall Study Design**

- **Discovery Phase**
  - Run 381 miRNA screening arrays to identify significantly dysregulated miRNAs.
  - Using individual assays on the same patients from the screening phase as well as additional patients to verify our findings.
  - Blindly validate dysregulated miRNAs in a different set of patients.

- **Test Phase**

- **Validation Phase**

**Discovery Phase Study Design**

- Peripheral Blood
- Isolate Plasma
- Extract Total RNA (Trizol LS)
- Reverse Transcription
- Preamplification
- Statistical Analysis (IBM SPSS 21)

**Discovery Phase Statistical Analysis**

- Each miRNA was normalized to the mean of U6 and miR-520d-5p (ΔCT).
- Three comparisons were performed:
  - CRC+CRAd+BC+LC+PC (n=60) vs. Controls (n=10)
  - CRC+CRAd (n=30) vs. BC+LC+PC (n=30)
  - CRC (n=20) vs. CRAd (n=10)
- For each comparison:
  - Only microRNAs with >90% expression were included in statistical analysis.
  - Two-tailed students t-test with equal variance was preformed to calculate p-value for ΔCT values.
  - ROC curves were made using logistic regression model.

**Discovery Phase Results**

- **Top 3 Significantly Dysregulated**
  - miR-29c
  - miR-192
  - miR-139-3p

- **Top 4 Significantly Dysregulated**
  - miR-374
  - miR-374-5p
  - miR-150
  - miR-486

**Conclusions and Future Directions**

The discovery phase for our plasma miRNA panel specific to colorectal neoplasia shows tremendous potential. These data will be verified and validated using individual miRNA assays in a larger test cohort (n=120) and double blinded validation cohort (n=150), respectively.

**Acknowledgments**

National Cancer Institute grant R25-CA134283, John W. and Caroline Price Family Trust as well as Donald and Irene Dizney.
INTRODUCTION

Prostate Cancer as a Public Health Problem

- Despite improvements in the early detection of prostate cancer (PCA) and treatment strategies, men with PCA stage 3 or higher have a 28% chance of survival 5 yrs after diagnosis.
- Among PCA patients with stage 4 disease, there is a 90% chance that the tumor will progress and become hormone refractory. Specific metastasis is an aggressive disease that is non-responsive to conventional treatment.
- New strategies are needed to improve the prevention/treatment of pre- and metastatic PCA.

HYPOTHESIS

- Quercetin is a flavonoid found in fruits (cranberry, black plums, strawberries, grapes, apples), vegetables (kale, leafy greens), herbs (dill, cilantro), grains (e.g., buckwheat) and red wine.
- This flavonoid has antioxidant, anti-inflammatory, anti-cancer properties.
- Quercetin inhibits cell invasion, migration, apoptosis, and/or cell proliferation in a metastatic PCA cell line (e.g., PC-3).
- Quercetin modulates the expression of genes involved in DNA repair, matrix degradation and tumor invasion, angiogenesis, apoptosis, cell cycle, matrix degradation, metabolism and glycolysis.
- Quercetin also acts as a bioavailability enhancer for many other substances by slowing their metabolic conversion to other substances.

Recent studies and clinical trials have confirmed claims that quercetin has activity against cancer tumors.

A few animal studies indicate that quercetin may alter the expression of oncogenic and tumor suppressing related miRNA in vitro studies.

However, it is not clear whether quercetin may be able to modulate the expression of miRNAs in a metastatic (i.e., PC-3) and primary PCA cell line derived from an African-American male (i.e., E006AA).

OBJECTIVES

- To evaluate whether quercetin may modulate the expression of miR-186 and the miR-25-106b cluster.
- To assess the impact of quercetin treatment (12.5-75µM) on cell proliferation and cell migration of E006AA (primary cancer cell line) as derived from an AA and a metastatic PCA prostate cancer cell lines.

HYPOTHESIS

- Quercetin will decrease prostate cancer cell proliferation and cell migration in the non- and metastatic prostate cancer cell lines.

- Quercetin will decrease the expression of oncogenic related miRNA (miR-106b, miR-186, miR-92a, miR-25).

- Alternatively, since miR-25 behaves as a tumor suppressor related miRNA, Quercetin treatment may down-regulate the expression of miR-25.

RESULTS

- Figure 1. E006AA (A) and PC-3 (B) cells were treated with 12.5-75µM quercetin for 24 hrs. Following treatment, cell proliferation and migration were increased by 20-30% relative to the DMSO treated cells using E006AA (50-97%) and PC-3 (20-64%) cell lines. However, at the highest quercetin dose (75µM), resulted in a non-significant 25% increase in cell viability compared to the DMSO control. There was a significant (72%) decrease in cell viability for E006AA cell lines treated with 75µM of quercetin compared to the 50µM concentration. An unpaired T-test was performed.

- Figure 2. Quercetin inhibits the migration of E006AA prostate cancer cells. Cells were treated with quercetin (12.5-75µM), cell migration assay was performed and migration of prostate cancer cells E006AA (P = 0.0001). Statistical significance was determined using the Mann-Whitney U test, adjusted for multiple pairwise comparisons using the Dunn’s test.

- Figure 3. E006AA cells were treated with 12.5-75µM quercetin. Cell movement were captured at times 0, 4, 8, 12, 16 hours. After 12 hour incubation with 50µM and 75µM of quercetin respectively, compared to Vehicle control (DMSO 0.075%) (P<0.0001). Statistical significance level was based on a Kruskal Wallis Test, adjusted for multiple pairwise comparisons using the Dunn’s test.

- Figure 4. E006AA (A) and PC-3 (B) cells were treated with 75µM quercetin for 24 hrs. Following treatment, cell viability was increased by 45% and metastatic PCA cell lines decreased by 20% in PC-3 cell lines. The expression of miR-106b was increased by 70% compared to controls. This result is a duplicate of two independent studies (P < 0.0001). Statistical significance level was based on a Kruskal Wallis Test, adjusted for multiple pairwise comparisons using the Dunn’s test.

DISCUSSION & FUTURE DIRECTIONS

- Quercetin treatment significantly inhibited cell migration and upregulated cell survival. Effect of miR-25 in E006AA cell lines.

- Relative to quercetin treatment at 50µM, we observed a significant (7%) decrease in cell viability at the 75µM concentration.

- Modify cell proliferation and cell migration assays using a wider quercetin dosage range (1.5µM-175µM) and lower %DMSO (i.e., 0.01%)

- Determine whether quercetin treatments will:
  - Down-regulate the expression of oncomiRs and up-regulate the expression of tumor suppressing miRs using next generation sequencing.
  - Reduce aggressive PCA phenotypes (i.e., cell proliferation, colon cancer formation, cell invasion) using metastatic PCA (i.e., LNCaP, DU145, MDA-PCA-2a, MDA-PCA-2b) and normal kidney epithelial (i.e., RWPE1, RWPE2) cell lines.
  - Reduce tumor size, tumor number, or metastasis using animal models.

- Modify miRNA targets and corresponding proteins using PCA or normal epithelial cell lines transfected with miRNA mimics or inhibitors.

- Evaluate whether a quercetin metabolite or quercetin analogs will have a more pronounced effect on modulating the expression of human miRs and/or PCA phenotype.

- Assess whether quercetin treatment combined with conventional/nutraceuticals may help increase survival rates among pre- or metastatic PCA patients.
**Analysis of Mutant Epidermal Growth Factor Receptor Trafficking and Signaling in Lung Cancer Cells**

Tejas N. Sangoi\(^1\), Adriana S. Bankston\(^2\), and Brian P. Ceresa\(^2\)

\(^1\)R25 Cancer Education Program, University of Louisville

\(^2\)Department of Pharmacology and Toxicology, University of Louisville

---

### Introduction

- Lung cancer is a major health concern in the Commonwealth of Kentucky, and there are more deaths in the U.S. from lung cancer than any other cancer (www.cancer.org).
- Specifically, over 95% of lung cancers are non-small cell lung cancer (NSCLC), and cells from NSCLC patients are used in this project. (www.cancer.org).
- Many NSCLC cells are characterized by activating EGFR mutations. EGFR is a receptor tyrosine kinase that is normally activated by ligand binding, and inactivated by either endocytic recycling or degradation. Mutant EGFR have constitutive kinase activity, which modulate downstream signaling pathways.
- Del19 EGFR mutations are in-frame deletions at exon 19 (E746-A750) that occur in 48% of all lung cancer cells from NSCLC patients are used in this project. (www.cancer.gov).
- Many NSCLC cells are characterized by activating EGFR mutations. EGFR is a receptor tyrosine kinase that is normally activated by ligand binding, and inactivated by either endocytic recycling or degradation. Mutant EGFR have constitutive kinase activity, which modulate downstream signaling pathways.

### Methods

- Methods: H1650 cells express mutant EGFRs, A549 cells express wild type EGFRs. Immunoblotting was used to test for time-dependent EGFR phosphorylation and degradation upon EGF stimulation. Receptor trafficking and endosomal accumulation at steady state were assessed by immunofluorescence staining of the total EGFR. The pattern of EGFR constitutive recycling of the receptor was examined by incubating with Ab1 antibody. Kinetics of H1650 EGFR endocytosis and ligand-mediated degradation were measured by radioligand binding assay.

### Results

- Results: Immunoflourescent for phosphorylated EGFR shows an increase in steady state EGFR phosphorylation, as well as elevated phosphorylation of effector molecules MAPK and MEK in H1650 cells. Steady state distribution of unliganded, mutant EGFR through immunostaining indicates localization in the early endosome. After 24 hours of Ab1 treatment, mutant EGFR show increased endosomal accumulation. Furthermore, mutant EGFRs exhibit a slower rate of recycling and degradation.

### Purpose

To better understand how activating mutations of epidermal growth factor receptor (EGFR) regulate trafficking and signaling of the receptor in lung carcinoma cells.

### Conclusions

- Activating mutants of EGFRs found in lung cancer cells have higher level of phosphorylation in EGFR Y1068, MAPK, and MEK. Localization of mutant EGFR is seen in the early endosome at steady state, as well as a defect in constitutive membrane trafficking after 24 hours. Less radioligand recycling and degradation in the mutant lung cancer cell lines indicate a defect in endocytic trafficking.

---

**Figure 1** Elevated EGFR Y1068, MapK, and Mek phosphorylation in mutant lung cancer cells

- A549 & H1650 cells were serum starved and treated for 0-180 minutes with 10 ng/mL of EGF. Cell lysates were harvested and equivalent amounts of protein were resolved through SDS-PAGE. Gels were transferred to nitrocellulose and probed for various proteins. Phosphorylation of EGFR at Y1068 is unchanged, whereas Y1086 is elevated in H1650 cells. The effector proteins MAPK, MEK, and ERK remain relatively unchanged. A full report is used as a loading control. The results shown are representative blots from an experiment performed n=3 times.

**Figure 2** Mutant EGFRs localize in the early endosome at steady state

- A549 & H1650 cells were serum starved and treated for 0-180 minutes with 10 ng/mL of EGF. Cell lysates were harvested and equivalent amounts of protein were resolved through SDS-PAGE. Gels were transferred to nitrocellulose and probed for various proteins. Phosphorylation of EGFR at Y1068 is unchanged, whereas Y1086 is elevated in H1650 cells. The effector proteins MAPK, MEK, and ERK remain relatively unchanged. A full report is used as a loading control. The results shown are representative blots from an experiment performed n=3 times.

**Figure 3** Mutant EGFRs show a defect in constitutive membrane

- In its unstimulated state, the wild type EGFR is a monomer on the cell surface. Upon ligand binding, the receptor undergoes a conformational change which leads to receptor dimerization and activation of the intracellular kinase domain (purple box). Receptor pairs trans-phosphorylate, yielding C-terminal phosphotyrosine; these phosphotyrosines serve as docking sites for downstream signaling proteins. In contrast, mutant EGFRs have constitutive kinase activity, and thus proceed with the dimerization, auto-phosphorylation, and effector association steps without a ligand present.

**Figure 4** Mutant cells show less secretion and degradation in 125I-EGF binding assay

- A549 & H1650 cells were incubated on ice with 0.05μg/mL 125I-EGF until steady-state cell surface binding was achieved. Free radioligand was removed and cells were placed at 37°C. At each time point, samples were collected to determine the total secreted, cell surface, intact, and intracellular 125I-EGF. Intracellular 125I-EGF was TCA precipitated to determine the amount of degraded and intact 125I-EGF. The data shown are average ± S.E.M. H1650, n=5; A549, n=3.

---

**Acknowledgements**

R25 Cancer Education Program, Ceresa Lab Members

Funding: NCI R25-CA134283, NIH R01-GM092374.
Introduction

One of the challenges to efficacious drug and gene delivery to solid tumors is inadequate penetration and distribution throughout the tumor vasculature. To overcome these challenges, nanotechnologies such as nanoparticles (NPs), can be utilized to protect agents during delivery, prolong delivery, and safely localize drugs and biologics to the tumor microenvironment. In addition to these attributes, NPs can be modified to enhance penetration and distribution throughout the tumor vasculature. Different factors including: NP surface charge, surface composition, size, and shape are integral to enabling drug delivery vehicles to withstand systemic interactions and to transport to the tumor site. The long-term goal of this study is to develop adaptable poly(lactic-co-glycolic acid) (PLGA) NPs with a variety of surface modifications and sizes to evaluate how each modification contributes to 3D distribution in a tumor spheroid model. In combination with mathematical modeling to predict formulations that will enhance distribution, experimental validation will enable us to rationally design NP formulations that successfully penetrate the tumor microenvironment. We hypothesize that ultra-small (<70nm) and surface-modified NPs will improve targeting of, and penetration to the tumor spheroid, and will significantly enhance NP uptake to individual cells. The experiments conducted here provide us with a preliminary assessment of design factors governing NP-tumor interactions in a 3D environment. We expect these and future experiments will provide insight to select efficacious modifications for increased tumor targeting and distribution.

Methods

In this study, we synthesized and characterized PLGA NPs encapsulating a fluorescent dye, Coumarin 6 (C6). To evaluate tumor penetration and distribution via fluorescent microscopy. To initiate our studies, three different NP formulations were synthesized: 1) PLGA unmodified, 2) PLGA ultra-small unmodified, and 3) PLGA surface-modified NPs.

Nanoparticle Fabrication

C6 NPs were synthesized using an oil-in-water single emulsion technique. For surface-modified NPs, avidin-palmitate was conjugated to the NP surfaces for subsequent reaction with biotinylated ligands (PEG, CPPs). For ultra-small NPs, supernatant after the first centrifugation was saved and further centrifuged to obtain sub-70nm NPs.

Tumor Spheroid Formation

HeLa cervical cancer cells were used to form tumor spheroids, which closely mimic the human tumor physiological environment in vitro. Tumor spheroid cultures were formed using the liquid overlay method. This method inhibits the attachment of cells to tissue culture plates and promotes cell-cell aggregation. Prior to spheroid formation, 24-well tissue culture plates were coated with a 1% (w/v) agarose gel to prevent cells adhering to the plate. Cells were subsequently added to each well and tightly shaken for 15 minutes. NP distribution experiments followed after incubation at 37°C for 14 days.

To visualize NP distribution within the tumor spheroid, a 1mg/ml solution of ultra-small and regular NPs were incubated with the spheroids at three different time points: 1hr, 6hr, and 24hr. After incubation, spheroids were washed with PBS and then transferred to a MatTek 14mm glass bottom imaging dish. For these experiments, we utilized both confocal microscopy and inverted epifluorescent microscopy.

Results: Nanoparticle Characterization

Figure 1: Scanning electron microscope (SEM) images of: (A) ultra-small C6 NPs and (B) regular C6 NPs.

Figure 2: Nanoparticle Sizing via SEM: (A) regular C6 NPs, (B) ultra-small C6 NPs and (C) PEG-modified NPs.

Figure 3: Nanoparticle surface charge.

Results: Spheroid Imaging

Figure 4: Tumor spheroid imaging using inverted epifluorescent microscopy for regular (A, C, E) and ultra-small (B, D, F) NPs. Images after 1hr. (A, B, D), 6hr. (C, D), and 24hr. (E, F) incubation times.

Figure 5: Tumor spheroid imaging using confocal microscopy for (A) ultra-small C6 NPs and (B) regular C6 NPs at 24hr.

Figure 6: Expected NP distribution from computational math model of: (A) Vimentin-targeted (CPP-homing) NPs, (B) PEG-modified NPs and (C) ultra-small NPs.

Conclusions

- At time 1hr, NPs become more dispersed and less punctate than at 6hr.
- Spheroids are saturated at a NP concentration of 1mg/ml. Lower concentrations (doses) may achieve successful penetration.
- Ultra-small NPs are able to penetrate the spheroid more efficiently than regular NPs.
- PEG-modified NPs are expected to navigate the tumor microenvironment and penetrate the tumor vasculature more efficiently than both ultra-small and regular NPs.
- NPs modified with a cell penetrating peptide are hypothesized to provide better penetration and uptake to tumor vasculature and individual tumor cells.
- Vimentin-targeting NPs (homing + CPP) are postulated to target, penetrate, and distribute throughout the tumor model.

Acknowledgements

Research is supported by the University of Louisville Cancer Education Program NIH/NCI R25-CA134283.