

Drexel University

Annual Progress Report: 2011 Formula Grant

Reporting Period

July 1, 2014 – December 31, 2014

Formula Grant Overview

Drexel University received \$1,320,271 in formula funds for the grant award period January 1, 2012 through December 31, 2014. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

An Interdisciplinary Approach to Directly Identify Changes in the miRNA-targeted mRNA Population Induced by HBV Infection – This project will identify specific changes in the population of RISC-associated micro(mi)RNAs and their targeted mRNAs in HBV-infected hepatocytes. Further, bioinformatic analyses and data-mining will be used to define signaling networks that are affected by these alterations in miRNA targeting. We will use these results to propose mechanistic studies to determine how HBV controls miRNA signaling networks in ways that promote HBV replication and sensitize hepatocytes to the continued inflammatory insults associated with chronic HBV infection, ultimately leading to liver cancer. An understanding of these regulatory networks will help to identify new points of intervention in the treatment of HBV-induced progressive liver disease.

Duration of Project

1/1/2012 – 12/31/2014

Project Overview

This project will define how alterations in the expression of specific microRNAs (miRNAs) that are induced by infection of primary human hepatocytes (PHHs) with hepatitis B virus (HBV) can lead to changes in cell physiology that promote viral replication and deregulation of pathways that control cell proliferation. miRNAs are known to be key players with a role in pathways that govern fundamental cell processes such as proliferation, differentiation, apoptosis, and response to stress. Changes in miRNA expression have been reported in hepatocytes upon infection with HBV or overexpression of HBV X protein (HBx). Despite the identification of some miRNA-targeted mRNAs, there is no comprehensive understanding of the mRNAs that are regulated by this process in infected cells.

We hypothesize that HBV infection changes the population of miRNA-targeted mRNAs to alter normal hepatocyte physiology and create an environment that favors HBV replication and contributes to hepatocellular carcinoma (HCC) development. To test our hypothesis we will address the following aims:

Aim 1: Establish photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) technology and data analysis parameters for direct identification of RISC-associated miRNAs and targeted mRNAs in cultured PHHs.

Aim 2: Characterize changes in the miRNA population and miRNA-targeted mRNAs induced by HBV infection of PHHs.

Aim 3: Determine the impact of miRNA targeting of specific mRNAs identified in Aim 2 on HBV replication and hepatocyte physiology. We will use a new technology, PAR-CLIP to directly identify and quantify the mRNAs that are targeted by RISC-associated miRNAs in HBV-infected PHHs.

Bioinformatic analyses of our results and data mining of open access gene expression data will be combined to develop a signaling-system interaction network and a set of interlinked signaling modules to predict pathways that are affected by HBV infection and drive HCC development. The role of miRNAs that are predicted to occupy key nodal points in the interaction map will be experimentally validated by altering their expression level, using miRNA mimics or inhibitors in PHHs, and characterizing the effects on HBV replication and hepatocyte physiology.

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Expected Research Outcomes and Benefits

This project will help us to understand the mechanisms by which HBV infection alters miRNA expression patterns, the cell signaling pathways that they mediate, and the extent to which these alterations benefit the virus or promote disease progression. Although chronic HBV infection is recognized as a major risk factor for liver disease, including fibrosis, cirrhosis, and hepatocellular carcinoma (HCC), the cellular mechanisms that underlie disease progression are incompletely understood. Studies of changes in miRNA expression have been informative, particularly in providing biomarkers for the later stages of liver disease and HCC. However, liver disease progresses over decades and is often only detected at late stages when treatment options

are limited or ineffective. We expect that our studies will provide new information on how cellular signaling networks are disrupted at early stages of viral infection in ways that alter the cellular response to continuing inflammatory insults, thereby setting the stage for the development of liver disease. By identifying the mRNA targets of specific miRNAs whose expression or function is altered by viral infection, we will uncover potential therapeutic targets and opportunities for earlier and more effective therapeutic intervention in the devastating liver diseases caused by chronic HBV infection. By increasing our understanding of the cellular pathways that are deregulated in infected hepatocytes, our studies may also point to common therapeutic targets for other causes of HCC.

Summary of Research Completed

Aim 1: Establish PAR-CLIP technology and data analysis parameters for direct identification of RISC-associated miRNAs and targeted mRNAs in cultured primary human hepatocytes (PHHs).

Progress: During this funding period, we continued to attempt to optimize conditions for conducting PAR-CLIP in hepatocytes. However, it became apparent that studies in cultured primary human hepatocytes would require optimization of the procedure that required excessive number of primary human hepatocytes. We therefore altered our primary model to cultured primary rat hepatocytes (Drexel IACUC approved; protocol #20057), which we routinely isolate and are more readily available. Unfortunately, however, incorporation of 4SU was quite low in cultured primary hepatocytes, and might not suffice for incorporation into the pool of miRNAs. We therefore changed our strategy to the use of high-throughput sequencing of RNA isolating by crosslinking immunoprecipitation (HITS-CLIP). The overall strategy in this procedure is quite similar to the PAR-CLIP procedure except that 4SU is not added to the culture medium and all miRNAs in the RISC complex are crosslinked to their targeted mRNAs regardless of whether or not they are newly synthesized (as would be required for PAR-CLIP with 4SU). It also became apparent that PAR-CLIP and HITS-CLIP procedures ask and answer different questions: PAR-CLIP would only identify newly made miRNAs and their newly made targets, whereas HITS-CLIP would identify all miRNAs interacting with mRNAs regardless of whether or not they were newly made. When considering the relatively low level of 4SU that was incorporated into cultured primary hepatocytes, the HITS-CLIP procedure became an attractive alternative. Unfortunately, as was the case for the PAR-CLIP procedure, most previous HITS-CLIP studies had been conducted in cells with transfected, over-expressed components of the RISC complex that were often tagged with epitopes to facilitate immunoprecipitation. Because we were attempting to isolate endogenous miRNA-mRNA complexes in normal hepatocytes, the transfection of constructs for overexpression of the components of RISC was not acceptable. We consulted with researchers familiar with the HITS-CLIP procedure (Klaus Kaestner, U. Penn) but after repeated attempts, could achieve no level of consistency with the HITS-CLIP procedure in cultured primary hepatocytes. Our studies suggest that there is a relatively low level of expression of components of RISC in cultured primary hepatocytes such that the number of hepatocytes required for a single experimental parameter was unreasonable, increasing the cost of such an experiment beyond the level of funding provided. Hence, to at least attain some characterization of HBV effects on miRNA and mRNAs in primary hepatocytes, we have, unfortunately, been forced to use more traditional methods (see below)

Aim 2: Characterize changes in the miRNA population and miRNA-targeted mRNAs induced by HBV infection of PHHs.

Progress: Although we had previously reported some changes in miRNAs in established cell lines expressing HBV, the ultimate purpose of the studies funded in this proposal was to determine if the same would be true in primary hepatocytes. Due to the failure of the PAR-CLIP and HITS-CLIP procedure in cultured primary hepatocytes, we isolated miRNAs from HBV-infected primary rat hepatocytes. To transduce the HBV genome into primary rat hepatocytes, which cannot be directly infected with HBV, we infected these hepatocytes with a recombinant adenovirus expressing green fluorescent protein (GFP) and the entire HBV genome (AdHBV) such that HBV RNAs are expressed and HBV replication occurs in these cells; the control for these experiments is a recombinant adenovirus that expresses GFP (AdGFP). To analyze miRNA expression level changes, total RNA was isolated from primary rat hepatocytes at 24 and 48 hours post-AdGFP or AdHBV infection. RNA was isolated using the mirVana RNA isolation kit (Life Technologies) following the total RNA procedure. miRNA expression was measured using the Liver miFinder miScript PCR array (Qiagen) for 81 liver-enriched miRNAs. Fold change was determined by $(\Delta\Delta Ct)$ between AdHBV and AdGFP at each time point with data normalized to endogenous small RNA controls included on each plate. While significant expression differences were seen in four miRNAs (miR-148a-3p, miR-221-3p, miR-222-3p, miR-378a-3p) at 24 hours post-infection, this differential expression was lost at 48 hours post-infection, when none of the analyzed miRNAs showed significant expression differences.

Aim 3: Determine the impact of miRNA targeting of specific mRNAs identified in Aim 2 on HBV replication and hepatocyte physiology.

Progress: Because the results of Aim 2 studies did not identify HBV-induced changes in miRNA profile except for at the initial 24 hour time point in primary hepatocytes (unlike our previous studies in established cell lines), we did not test the effects of miRNAs on HBV replication and hepatocyte physiology in normal, untransformed hepatocytes.

Research Project 2: Project Title and Purpose

Novel Anti-Pancreatic Cancer Peptides from the ras-p21 Protein – We will establish that a kinase super-complex with oncogenic k-ras forms in pancreatic cancer, but not in normally proliferating cells, that is disrupted by peptides synthesized from oncogenic ras-p21 containing oncogenic amino acid substitutions that induce stereotypical changes in the three-dimensional structures of six domains of the oncogenic proteins. Two such peptides corresponding to these domains, PNC-2 and PNC-7, will then be tested to selectively arrest pancreatic cancer cell growth. We will test PNC-2 and PNC-7 for their ability to eradicate human pancreatic cancers transplanted into nude mice. We will also test the ability of these peptides to eradicate a syngeneic pancreatic cancer utilizing an established murine model.

Duration of Project

1/1/2012 – 12/31/2014

Project Overview

Most pancreatic cancers are caused by a mutant, oncogenic protein called ras-p21. This protein, but not its normal form, causes normal human cells to transform into malignant ones and causes egg cells (oocytes) to divide. We have found in oocytes that the mutant protein causes cell division in an uncontrolled manner by forming large super-complexes with other proteins that stimulate cell division and maintains them in the complex so that they continuously tell the cell to divide. In this project, we will lyse human pancreatic cells and immunoprecipitate (IP) k-ras from the lysates using a k-ras-specific monoclonal antibody and blot the IPs for each of the component proteins (ras, raf, MEK, MAPK, JNK) with the appropriate antibody. Detection will be accomplished using anti-mouse secondary antibody with an ECL chemiluminescence detection kit. We will further blot the IPs for the phosphorylated forms of each of these kinases in the super-complex using phospho-protein-specific antibodies. Since we have developed an untransformed pancreatic acinar cell line (BMRPA1) in our laboratory, we will perform the same studies on lysates from these cells as controls to demonstrate that no such complex forms in these cells. TUC-3 cells are ras transformed rodent BMRPA1 cells.

Secondly, we have developed two new peptides, called PNC-2 and PNC-7 that are from parts of ras-p21 that block cell division caused by the mutant ras-p21 protein but have no effect on the normal form of this protein, as previously shown in our oocyte model. We will test the effect of these peptides on complex formation and alternate pathway activation in pancreatic cancer cell lines. Treated cells will be lysed and the lysates subjected to the above protocols to determine the level of each protein in the complex and alternate pathway activation. Moreover, we will also test whether these peptides break up the large complexes with mutant ras-p21. We will also test another peptide GST 35-50 that blocks the activation of one of the important targets of mutant ras-p21, called JNK, which likewise selectively blocks oncogenic, mutant ras-p21 in oocytes. Lastly, we will test these peptides on pancreatic cancers that grow in animal models, i.e., nude mice and syngeneic mouse models that have pancreatic cancer.

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Victor Adler, MD, PhD – consultant

Expected Research Outcomes and Benefits

We anticipate that selected pancreatic cancer cells will have super-complexes of the activated kinases with k-ras. Since we have developed and obtained untransformed pancreatic cell lines (BMRPA1) in our laboratory, we will perform the same studies on lysates from these cells during growth as controls to demonstrate that no such complex forms in these cells. In cells treated with PNC 2 and PNC-7, we expect that the supercomplex levels will be reduced significantly over 48 hours and that the alternate pathway will persist in TUC-3 cells possibly explaining their phenotypic reversion. We will further perform the same experiment with our GST 35-50 peptide. This peptide should block tumor cell growth but not affect levels of the super-complex; rather we anticipate that it will reduce phosphorylation of the kinases in the super-complex. In our animal models, human pancreatic cancer cells (i.e., MIA-PaCa-2 and PANC-1) will be transplanted in the midline of the back (upper thoracic) of nude mice. At an adjacent site, we will implant Alzet pumps that will administer two different concentrations of each peptide over a two week period. Concurrently, we will treat one set of the nude mice with transplanted tumors with a control peptide, PNC-29, that has no effect on pancreatic cancer cell growth. To test if our anti-pancreatic cancer peptides are tolerated immunologically, we will then perform identical experiments on immunocompetent C57 bl/6 mice (5-6 weeks old). For both sets of experiments, we anticipate that, within 2 weeks, tumors will either undergo eradication or reversion to the untransformed phenotype. We also anticipate minimal toxicity in either the nude or syngeneic mouse tumor models. These preclinical studies will provide important data for developing a novel, non-toxic, targeted approach against pancreatic cancer.

Summary of Research Completed

Specific Aim 1. Completed

Specific Aim 2. We will then test PNC-2 and PNC-7 for their ability to eradicate pancreatic cancers transplanted into nude mice. We will also test these peptides for their ability to eradicate a syngeneic pancreatic cancer utilizing an established murine model.

Progress:

Modification of proposal to specific Aim 2 was instituted to create an improved translational metastatic intraperitoneal pancreatic cancer tumor model which more closely mimics actual presentation of disease in humans. Our institution has acquired an *In Vivo* Imaging System (IVIS) Lumina XR Series III (Perkins Elmer, Waltham, MA) which allows for developing and testing treatment response in murine tumor models. We believe that this tumor model, rather than the originally proposed model, offers more accurate and translationally applicable model for pre-

clinical testing. As detailed below, developing this model using colon cancer cells offers distinct advantages over pancreatic cancer cells for the purpose of developing and refining this model. We now can test new derivatives, PNC-27 (treatment) and PNC-29 (control) peptides in both xenogeneic and syngeneic pancreatic peritoneal carcinomatosis models.

Transfection of human murine colon cancer cell-line, CT-26 with the luciferase gene.

CT-26 murine colon cancer cells were grown to confluence in RPMI-1640 Media (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS in a 24-well plate. After establishing confluence, media was replaced. In separate Eppendorf tubes, the transfection complex mixtures were assembled. This mixture consisted of 0.7µg pGL4.50 [*luc2*/CMV/hygro] (Promega, Madison, WI), 50 µl of serum-free RPMI-1640 Media, and varying amounts of TransPass D2 transfection reagent (New England Biolabs, Ipswich, MA), ranging from 0.7µl to 2µl. After 30 minutes of standing at room temperature, the transfection mixture was added to cells and allowed to incubate at 37°C overnight. The following day, after four hours of incubating in fresh growth media, hygromycin (Life Technologies, Grand Island, NY) was added to the wells at a final concentration of 100µg/ml. After an additional 24h of incubation, cells were trypsinized and transferred to separate 10cm² tissue culture dishes. Dishes were checked daily for cell growth and media was changed per lab routine. Growth media was supplemented with 100µg/ml hygromycin. When adequate growth was observed, cells were trypsinized and transferred to a 75ml tissue culture flask and grown to confluence. Once confluence was achieved, luciferase transfection was assessed using the Luciferase Assay (Promega, Madison, WI) per manufacture instructions. In brief, cells were harvested and lysed using a lysis reagent. Lysate was added to the assay reagent in a 96-well plate. Luminescence was read using a plate reader, confirming transfection.

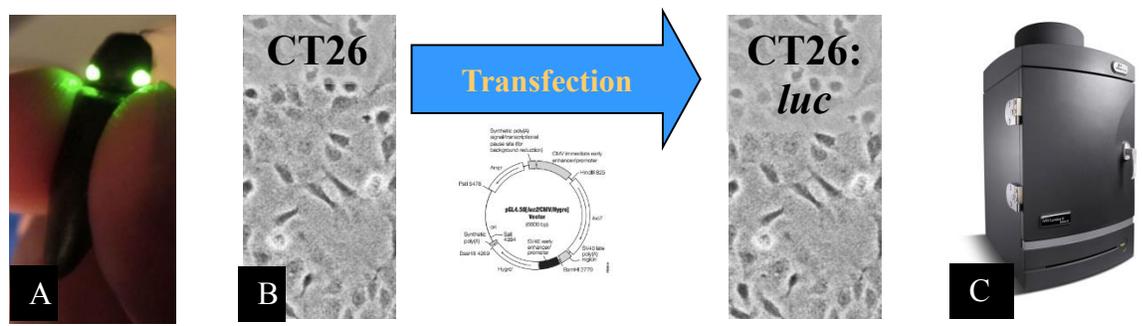


Figure 1A (left): The *luc2* gene for luciferase expression is naturally found in click beetles and fireflies. **Figure 1B (middle):** A schematic of CT26 cells in culture transfected with *luc2*-containing plasmid, thereby becoming capable of bioluminescence. **Figure 1C (right):** The *In Vivo* Imaging System (IVIS) helps capture and quantify bioluminescence signal.

Xenogeneic Model

1X10⁶ CT-26 cancer cells were injected into the peritoneal cavity of nu/nu mice in 0.5 to 1 mL of PBS using a tuberculin syringe and 28g needle. After three days of established peritoneal carcinomatosis, were divided into 2 experimental groups: I) PNC-27 peptide treatment and II) PNC-29 control peptide treatment. PNC-27 and PNC-29 peptides were injected into the

peritoneum for 14 days at dose 100ug peptide/gram mouse (estimated 2 mg/mouse/day). Mice were euthanized on day 15. Tumor nodules were harvested and histologically examined. A treatment-blinded rater reviewed H&E-stained slides to quantify necrosis in tumor nodules and to determine if necrosis took place in non-tumor-bearing tissues.

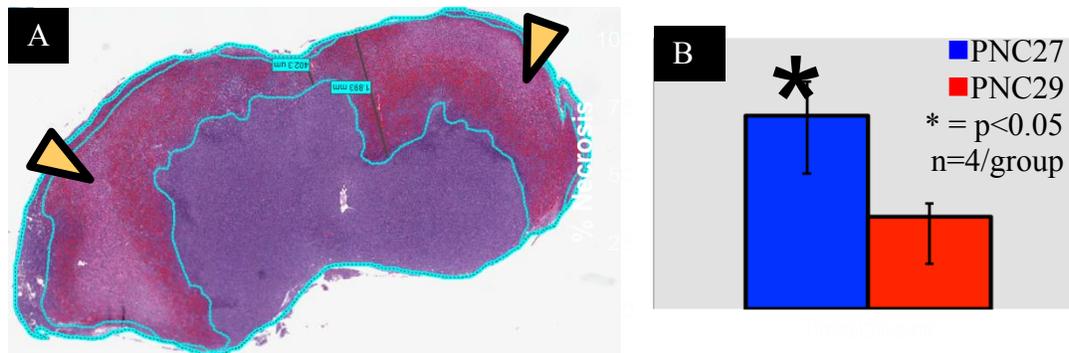


Figure 2A (Left): H&E-stained tumor nodule from PNC27-treated mouse demonstrating peripheral coagulation necrosis (gold arrows). **Figure 2B (Right):** PNC-27 induces significant necrosis compared to control peptide

Syngeneic Model

1X10⁶ CT-26 cancer cells were injected into the peritoneal cavity of nu/nu mice in 0.5 to 1 mL of PBS using a tuberculin syringe and 28g needle. Tumor response was monitored every two days with the *In Vivo* Imaging System (IVIS) Lumina XR Series III (Perkins Elmer, Waltham, MA). Prior to imaging, mice received 50 ul of D-luciferin at 75 mg/kg in PBS followed by general anesthesia (isoflurane- induction dose 3-4%; maintenance dose 1-2%). Bioluminescence Imaging (BLI) signals were quantitated and analyzed using Lumina XR software. Mice were euthanized on day 15. At necropsy, intraperitoneal tumor burden was quantified using by tumor weights, tumor volumes, and peritoneal carcinoma index (PCI). PCI was scored according to a point rating scale indicating large (> 3 mm: 3 points), moderate (1-3 mm: 2 points), small (<1 mm: 1 point) or no tumor (0 points) for seven abdominal regions (pelvis, omentum, transverse colon, mesentery, left and right subdiaphragmatic area, subhepatic area). These scores were added to give a PCI (maximum score 21 points). Terminal BLI signals were correlated with conventional measures of tumor burden and a correlation coefficient was calculated.

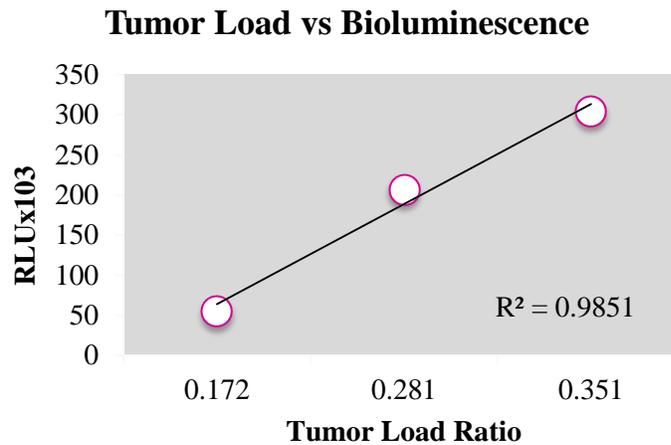
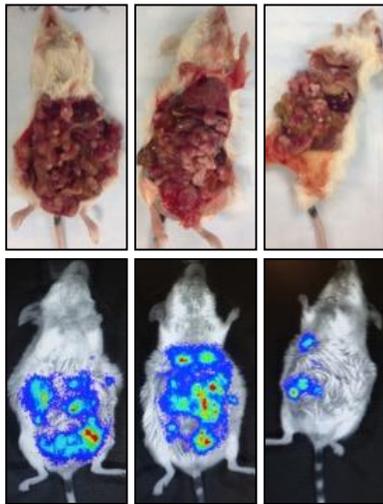


Figure 3(left). Necropsy specimens (top row) and IVIS images demonstrating bioluminescence (bottom row) **Figure 3(Right).** Significant correlation between composite measure of tumor burden and bioluminescence signal (RLU = relative luminescence units)

Research Project 3: Project Title and Purpose

Impaired T Cell Immunity and Survival in Neonatal Influenza Virus Infection – In order to simulate more closely neonatal infection and determine the neonatal responses to viral infection, we have developed a neonatal mouse model to examine acute influenza infection at day 2-3 of life. Preliminary data shows that the neonatal C57Bl/6 mice exhibit significant mortality compared to adult mice. The specific aims of this project are to: 1) determine the mechanism of increased mortality in viral pulmonary infection of neonatal mice and 2) investigate influenza virus-specific CD8+ T cell responses in neonates.

Duration of Project

1/1/2012 – 12/31/2013

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.pa.gov/MyRecords/Health-Research/CURE>.

Research Project 4: Project Title and Purpose

Epigenetic Modulation in an Animal Model of Depression – The purpose of this project is to understand the neuroadaptive mechanisms that occur in the central nervous system (CNS) in response to chronic inflammation and immune dysregulation and contribute to a depressive behavioral phenotype. We will study a model of chronic inflammation in the mouse where immunization with Bacille Calmette Guerin leads to activation of the innate immune response and the development of a depressive phenotype. We expect that the findings from these studies will suggest novel targets for treating patients with inflammation related Major Depressive Disorder (MDD).

Duration of Project

1/1/2012 – 6/30/2013

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.pa.gov/MyRecords/Health-Research/CURE>.

Research Project 5: Project Title and Purpose

Characterization of the Effects of Non-thermal Plasma on Liquids and Cells – In order to exploit the potential for clinical applications, from enhancing wound healing, to sterilizing tissues, to inducing localized apoptotic cell death in tumor tissue, a mechanistic understanding of the interaction of non-thermal plasma with living tissues is required. Our initial studies using mammalian cells in culture revealed that non-equilibrium plasma (NEP) has dose-dependent effects that range from increasing cell proliferation to inducing apoptosis; these effects are primarily due to formation of intracellular reactive oxygen species (ROS). We propose to study the effect of NEP on molecules in solution and on DNA damage pathways to better understand the biological effects.

Duration of Project

1/1/2012 – 6/30/2013

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.pa.gov/MyRecords/Health-Research/CURE>.

Research Project 6: Project Title and Purpose

Novel Antagonists of CX₃CR1 to Prevent Skeletal Metastasis – The overall goals of this proposal are to synthesize the first small-molecule, non-peptide antagonists of CX₃CR1 and produce pre-clinical evidence that these compounds, by interfering with CX₃CR1-FKN interactions, impair the dissemination of breast cancer cells to the skeleton. We propose a radical switch in the current standard of care for breast cancer patients, with the implementation of systemic therapeutic measures to be started immediately after breast surgery and maintained during the time preceding second surgery or local adjuvant treatments.

Duration of Project

1/1/2012 – 6/30/2013

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.pa.gov/MyRecords/Health-Research/CURE>.

Research Project 7: Project Title and Purpose

Astrocyte Senescence as a Component of HIV-related Neurodegeneration – Aging of the HIV-infected population places the disease in a distinctive set of biological and psychosocial influences. Our main purpose is to identify common factors and mechanisms in the interplay of aging and HIV. We find that astrocytes enter into senescence in response to oxidative stress and HIV-1 proteins and that the brains of aged individuals and individuals with Alzheimer's disease are highly populated by senescent astrocytes. Because HIV infection is associated with the release of neurotoxic factors which induce cell stress, we plan to evaluate the impact of viral factors on astrocyte senescence. Our studies will provide information for successful therapeutic efforts to alleviate HIV-associated neurocognitive disorders during aging.

Duration of Project

1/1/2012 – 12/31/2014

Project Overview

Aging of the human immunodeficiency virus type 1 (HIV-1)-infected population places HIV/AIDS in the context of aging. One of our research objectives is to identify common factors and mechanisms between aging and HIV. Astrocytes, the most abundant cell type in the brain, perform vital functions in the central nervous system. Astrocytes are host cells for HIV-1 infection and we postulate that changes in astrocyte function may contribute to the neuropathogenesis of HIV-associated neurocognitive disorder (HAND). Recently, we have reported that human

astrocytes are highly sensitive and trigger a senescence program in response to oxidative stress or cellular insult. We have evidence indicating that the number of senescent astrocytes increases in the brain with age, and further in patients suffering from Alzheimer's disease. HIV infection is associated with the release of neurotoxic factors, such as HIV-gp120, and we predict that this stressful environment will trigger the senescent program in astrocytes. Preliminarily, we found that HIV-gp120 protein induces astrocyte senescence. We hypothesize that senescent astrocytes negatively impact the microenvironment, contributing to HAND in older patients living with HIV/AIDS.

Aim 1. Determine the impact of HIV-1 on astrocyte senescence during aging of infected patients.

Rationale: We will perform a comprehensive analysis of brain tissue of patients infected by HIV-1 at different ages. We will quantify senescent astrocytes using a variety of markers of senescence in several brain areas from tissue samples from the National NeuroAIDS Tissue Consortium (NNTC). We will also study effects on neurodegeneration and microglia.

Aim 2. Evaluate the influence of HIV-1 viral products on the astrocyte senescence program and the secretion of pro-inflammatory mediators.

Rationale: Astrocytes are highly sensitive and we hypothesize that viral products trigger the senescent program. We will evaluate effects of HIV-1 gp120 /Tat proteins on astrocyte senescence, and the secretory pattern. In addition, we will evaluate effects of pharmacologic intervention to block senescence, by inhibiting mTOR and p38MAPK. These targets will provide preclinical data for further testing models or pharmaceuticals to block senescence associated cellular changes that affect neural physiology.

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Expected Research Outcomes and Benefits

Based on our data indicating that human astrocytes trigger the senescent program in response to a variety of stressors in vitro, the increased population of senescent astrocytes in brain tissue during aging and Alzheimer's disease, and our results showing the ability of viral products such as HIV-1 gp120 to induce astrocyte senescence; we expect to find a significant increase in the population of senescent astrocytes in human brain of patients infected with HIV-1. HIV infection is associated with a stressful environment including the release of neurotoxic factors, oxidative stress, and inflammation, favorable conditions that could trigger the senescent program in astrocytes and

create a loss in homeostasis in the CNS. We anticipate that the amount of senescent astrocytes will be higher in older HIV-1-infected patients compared with younger HIV-1 infected patients. We anticipate finding a higher abundance of senescent astrocytes, neurodegeneration and microglia activation in those areas preferentially affected by HIV-1 infection. We assume that the pattern of gene expression and biomarkers of senescence altered during *in vitro* astrocyte senescence will be similarly affected in brain tissue during HIV-1 infection.

Viral products such as HIV-1 gp120 and tat induce oxidative stress and inflammation in astrocytes. Given this, we hypothesize that they will induce the expression of markers of senescence and an altered secretion pattern including releasing of cytokines, proteases, and reactive oxygen species. We propose mTOR and p38MAPK as target pathways of the HIV-1-induced astrocyte senescence, and we anticipate that pharmacologic intervention to block these pathways will modulate senescence, reduce the secretion pattern of astrocytes, influence neural physiology and be potential therapeutic targets to improve HAND during the aging of these patients.

Summary of Research Completed

Aim 1. Determine the impact of HIV-1 on astrocyte senescence during aging of infected patients.

Rationale: We will perform a comprehensive analysis of brain tissue of patients infected by HIV-1 at different ages. We will quantify senescent astrocytes using a variety of markers of senescence in several brain areas from tissue samples from the National NeuroAIDS Tissue Consortium (NNTC). We will also study effects on neurodegeneration and microglia.

Progress:

As neurocognitive impairments and neuroinflammation have shifted to cortical regions of the brain in the CART era as opposed to sub-cortical regions pre-CART, we investigated whether these phenomena correlated with an increase in senescence. We initially stained tissue from 9 HIV patients with (HAD) and without (HIV) HIV associated dementia for p16, a senescence marker, using immunohistochemistry (IHC). We randomly imaged 20 fields from each slide and quantitated the p16 positive (brown) and negative (blue) nuclei. There was a trend towards higher senescence in the HAD tissues, however this did not reach statistical significance ([Figure 1](#)).

The pattern of p16 positivity in the mid frontal gyrus, however was not uniform. Therefore, the random fields chosen from each slide were not representative of the whole tissue. p16 positivity was contained in certain areas of each tissue cross section. We therefore imaged and quantitated areas of high and low p16 positivity separately for 4 patients from each condition. p16 positivity was drastically different between these regions as shown in [Figure 2](#). Percent p16 positivity was, however, when compared between high and low positive regions, similar for the HIV and HAD cases ([Figure 3](#)). Random fields from each tissue and comparison of the positivities between high and low p16 staining regions have not shown a difference between HIV infection with and without dementia (HAD). However, the extent of the p16 high positivity regions in each tissue slide has so far not been taken into consideration. It is possible that despite p16 positivity being similar within these regions, the regions are more numerous or cover a larger area in the HAD tissue. We will count the total number of these regions in the same slides next.

γ H2AX is a phosphorylated histone variant that is normally a marker of DNA double strand breaks. However, it was also observed to dramatically increase in p21-dependent senescence in transformed rodent fibroblasts³, and this increase was prevented by the use of rapamycin, which is a known suppressor of senescence. Moreover, the increase in γ H2AX happened without the induction of DNA double strand breaks. We therefore investigated whether there was an increase in γ H2AX foci in HAD as compared to HIV without dementia in frontal cortex tissue of patients using immunofluorescence. Flash frozen paraffin embedded frontal cortex sections from 4 HIV and 4 age matched HAD patients from 3 different age groups were probed with antibodies to γ H2AX and the neuronal marker MAP2 (Millipore). These antibodies were in turn reacted with secondary antibodies carrying red (γ H2AX) or green (MAP2) fluorophores and the slides were imaged ([Figure 4A](#)). The HAD tissue had dramatically higher γ H2AX staining in two age-matched pairings. No significant difference was observed in one pairing, while the HAD tissue had fewer γ H2AX+ neurons in the oldest age group (53 years) ([Figure 4B](#)). These results indicate a real difference in the extent of senescence in HAD vs. HIV, with an overall trend for increased senescence in HAD. This conclusion and a possible age-related effect on γ H2AX positivity need to be verified by analyzing tissue samples from more patients, which we have available. Also, 53BP1 was seen to be associated with DNA damage response (DDR) induced, but not senescence induced γ H2AX foci formation³. Co-staining of γ H2AX with 53BP1 is needed to determine the nature of γ H2AX positivity seen in HIV patients.

Aim 2. Evaluate the influence of HIV-1 viral products on the astrocyte senescence program and the secretion of pro-inflammatory mediators. Rationale: Astrocytes are highly sensitive and we hypothesize that viral products trigger the senescent program. We will evaluate effects of HIV-1 gp120 /Tat proteins on astrocyte senescence, and the secretory pattern. In addition, we will evaluate effects of pharmacologic intervention to block senescence, by inhibiting mTOR and p38MAPK. These targets will provide preclinical data for further testing models or pharmaceuticals to block senescence associated cellular changes that affect neural physiology.

Progress:

Induction of astrocyte senescence by these HIV drugs may play a critical role in the chronic inflammatory process observed in HIV-infected patients. Our results indicate that abacavir/lamivudine increases NF- κ B activity ([Figure 5](#)), which could trigger the increase in interleukin-8 mRNA that we observed in response to these drugs ([Figure 5](#)). This increase in inflammatory response seems not to be associated with changes in chromatin remodeling as indicated in [Figure 6](#).

We have started studies to evaluate the effects of clinically used drugs combinations on premature senescence induction in human astrocytes, adjusting concentrations to levels found in brain tissue. We used abacavir (ABC) 3 μ M, lamivudine (3TC) 1.9 μ M, atazanavir (ATV) 50 nM, ritonavir (RTV) 300 nM, tenofovir (TDF) 100 nM, emtricitibine (FTC) 1.2 μ M and efavirenz (EFV) 125 nM. In [Figure 7](#), we examined whether HAART treatment induces senescence-associated beta galactosidase (SA-Beta Gal) in human astrocytes. Over the course of 3 weeks, SA-Beta Gal positive cells were significantly increased for each HAART combination used. This suggests that HAART drugs can induce some senescence-like phenotypes in human astrocytes.

We explore this further in [Figure 8](#) by looking at reactive oxygen species (ROS) production since ROS can contribute to senescence induction. We used several combinations of HAART drugs treatment for up to 2 weeks and saw increases in total ROS to various extents. We next wanted to both look at the effects of a longer treatment as well as examine mitochondrial ROS. In [Figure 9](#), treatment for 4 weeks resulted in an increase in both cellular and mitochondrial ROS. This suggests that stress from HAART drug treatment causes ROS induction, which may lead to senescence.

Lastly in [Figure 10](#), we looked at IL-6 secretion, a component of the senescence-associated secretory phenotype (SASP) since it has the potential to disrupt the surrounding microenvironment and contribute to HIV-associated neurocognitive disorders (HAND) *in vivo*. Initially, for [Figures 10A-B](#), we used 100 nM RTV in our combos and saw secretion over a 1 week time course but not at 3 and 4 weeks. This experiment was repeated with 300 nM RTV in [Figure 10C](#) and we saw increased secretion over the course of 4 weeks. These results demonstrate that HAART drugs can induce IL-6 secretion and a senescence-like phenotype in human astrocytes.

FIGURES

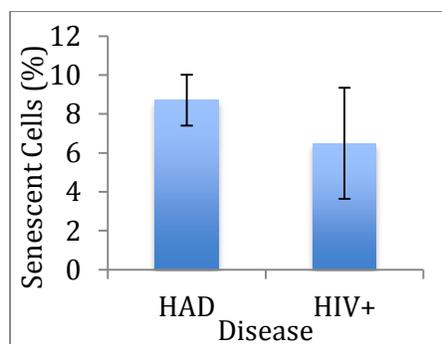


Figure 1. Percent p16+ cells in the mid-frontal gyrus of HIV patients with and without dementia.

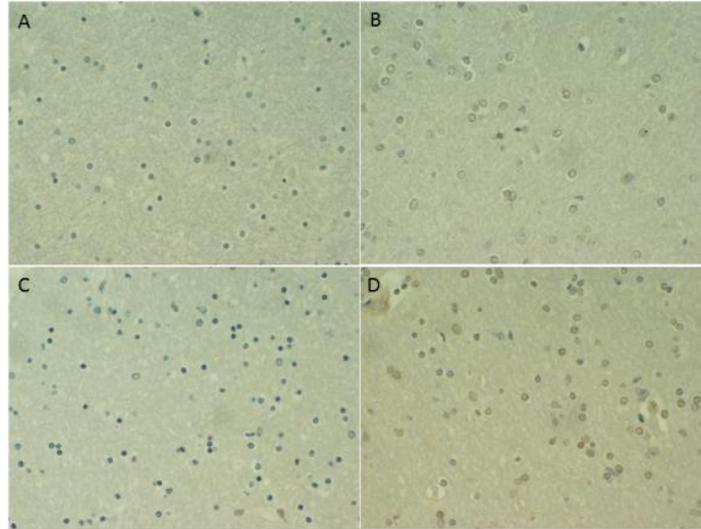


Figure 2. p16 staining in mid frontal gyrus of patients with and without HAD. Tissue sections were probed with p16 antibody at a 1:100 dilution and bound antibody was detected using the DAB substrate (brown). Cell nuclei were stained with hematoxylin (blue). A) 45 year old HIV patient region of low positivity, B) same patients region of high positivity, C) 35 year old HAD patient region of low positivity, D) same patients region of high positivity.

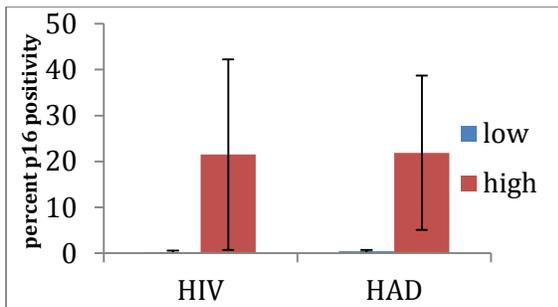


Figure 3. Quantitation of p16 positivity in the high and low p16 positive regions from HIV and HAD tissue. Results from all patients were averaged. Error bars represent standard deviation between patients.

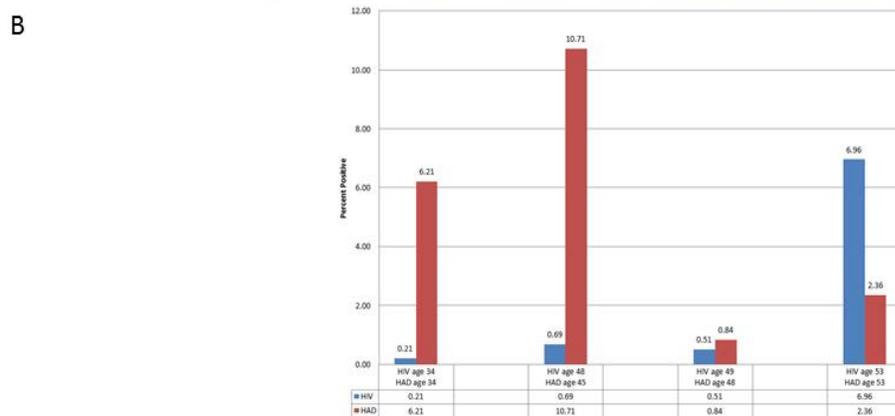
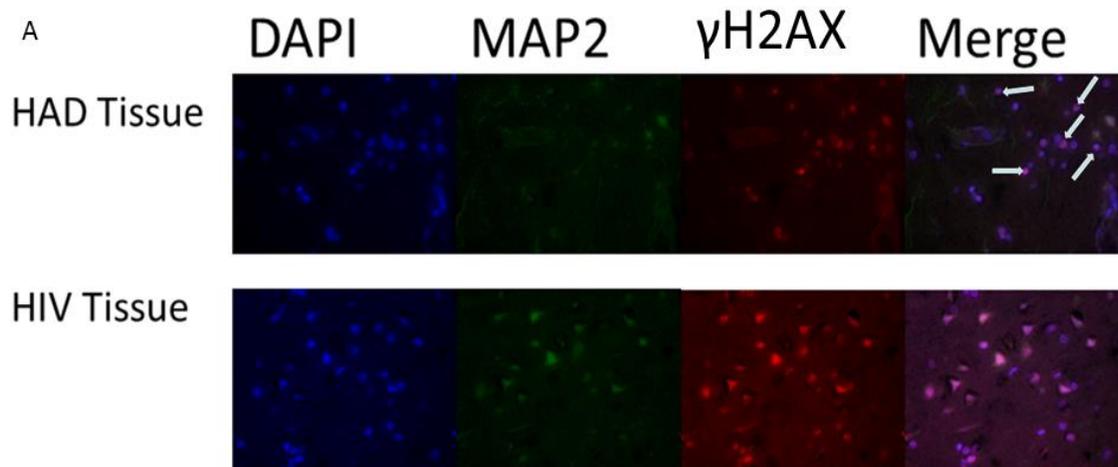


Figure 4. γ H2AX staining in neurons in HIV infection with and without dementia. In A) separate channels for nuclei (blue), MAP2 (green), γ H2AX (red) and a composite of all three (merge) from representative images are shown. Nuclei with γ H2AX foci are indicated with white arrows. In B) percentages of γ H2AX positive neurons over all neurons (MAP2+ cells) are shown for each age-matched pair are shown. HIV numbers are represented by blue columns, HAD numbers by red columns.

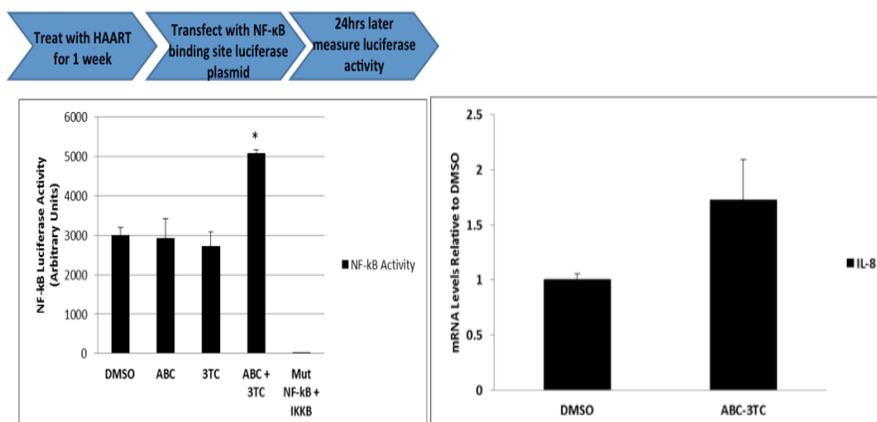


Figure 5. Astrocytes were treated 1 week with 3 μ M abacavir (ABC) and 1.9 μ M lamivudine (3TC). Left, NF- κ B activity, cells were transfected with luciferase plasmid under the control of an NF- κ B binding site

or scrambled (Mut). 24 h later luciferase activity was assayed. Right, IL-8 mRNA assayed by RT-PCR. * = p value < .05, # = p value < .001, error bars from 3 biological replicates.

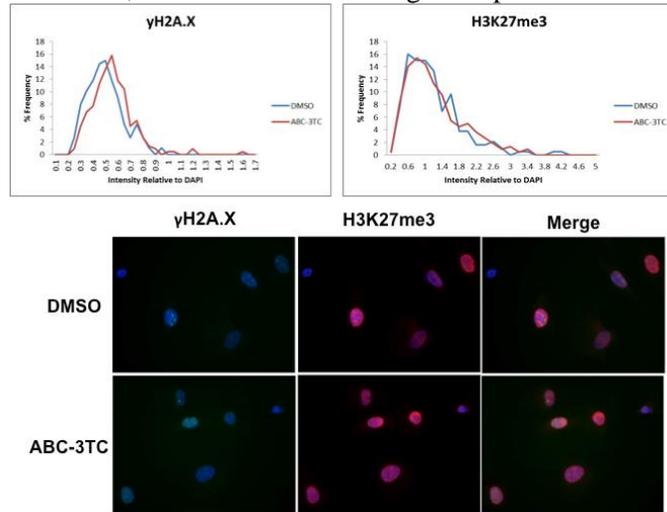


Figure 6. Human astrocytes were treated with a combination of abacavir (ABC) and lamivudine (3TC) for 1 week in complete astrocyte media. Cells were then stained overnight on coverslips with γ H2A.X and H3K27me3 antibodies and visualized with immunofluorescent microscopy. Intensity was quantified using Cell Profiler software and presented as % frequency on a histogram distribution curve.

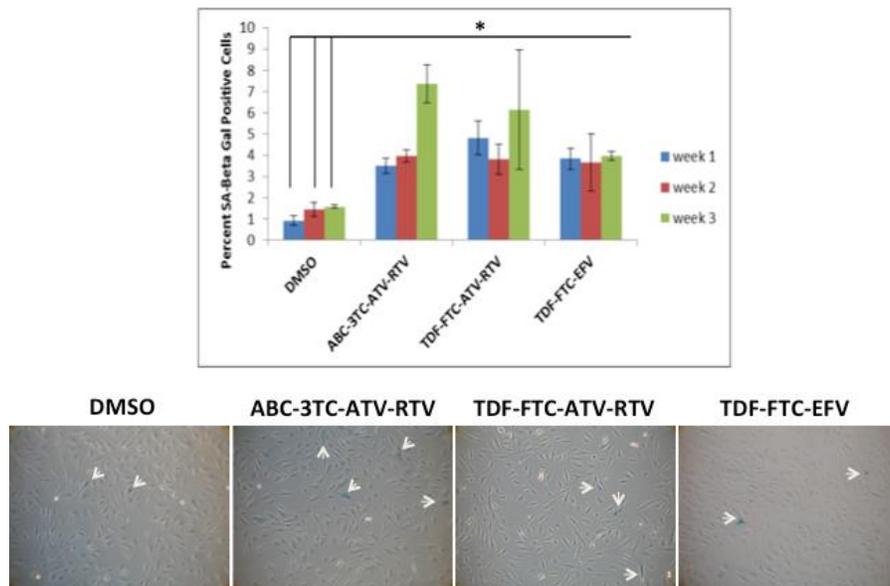


Figure 7. HAART Combos Induce Senescence-Associated Beta Gal. Human astrocytes were treated with HAART combinations involving abacavir (ABC), lamivudine (3TC), atazanavir (ATV), ritonavir (RTV), tenofovir (TDF), emtricitibine (FTC) and efavirenz (EFV) for up to 3 weeks in complete astrocyte media. Following treatment, cells were stained overnight for SA-Beta Gal and counted for positive staining. * = p value < .05, error bars represent the mean of 3 biological replicates.

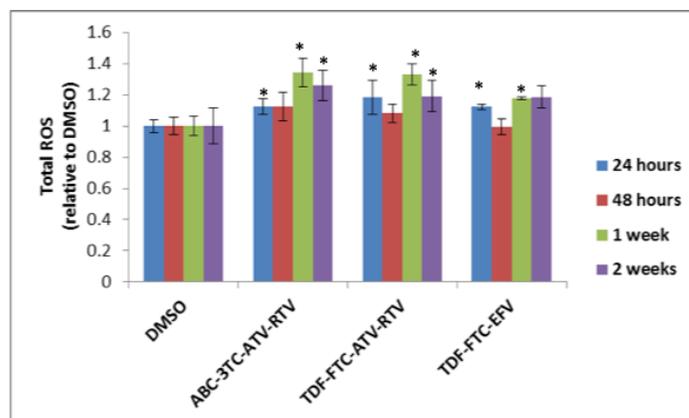


Figure 8. HAART Combos Increase ROS in Human Astrocytes. Human astrocytes were treated with HAART combinations involving abacavir (ABC), lamivudine (3TC), atazanavir (ATV), ritonavir (RTV), tenofovir (TDF), emtricitibine (FTC) and efavirenz (EFV) for up to 2 weeks. Fluorescent measurement of cellular ROS was done by flow cytometry after staining with DCFDA (total ROS) for 30 min. before measurement. * = p value < .05, error bars represent the mean of 3 biological replicates.

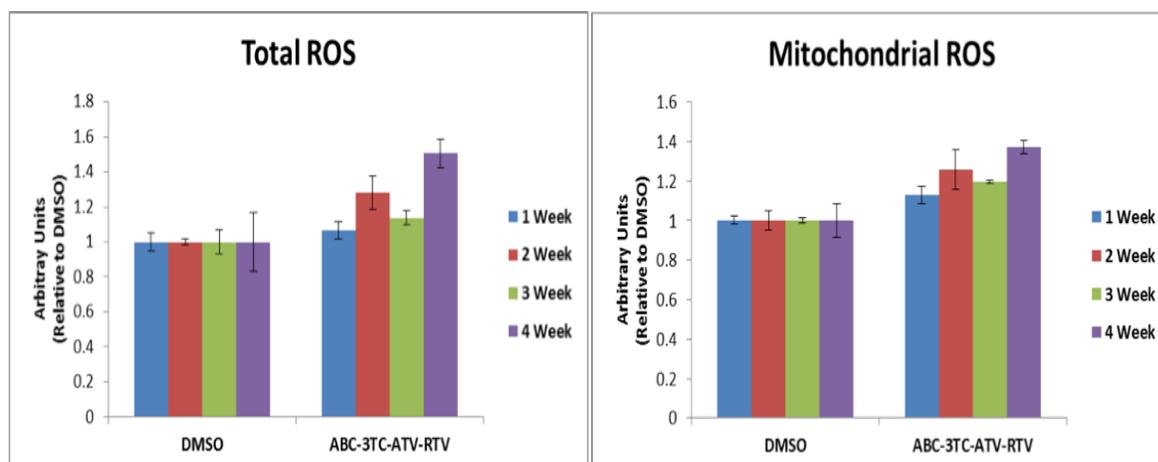


Figure 9. HAART Drugs Induce Total and Mitochondrial ROS in Human Astrocytes. Human astrocytes were treated with a combination of abacavir (ABC), lamivudine (3TC), atazanavir (ATV) and ritonavir (RTV) for up to 4 weeks. Fluorescent measurement of cellular and mitochondrial reactive oxygen species measurement was done by flow cytometry after staining with DCFDA (total ROS) or MitoSox (mitochondrial ROS) for 30 min. before measurement. * = p value < .05, error bars represent the mean of 3 biological replicates.

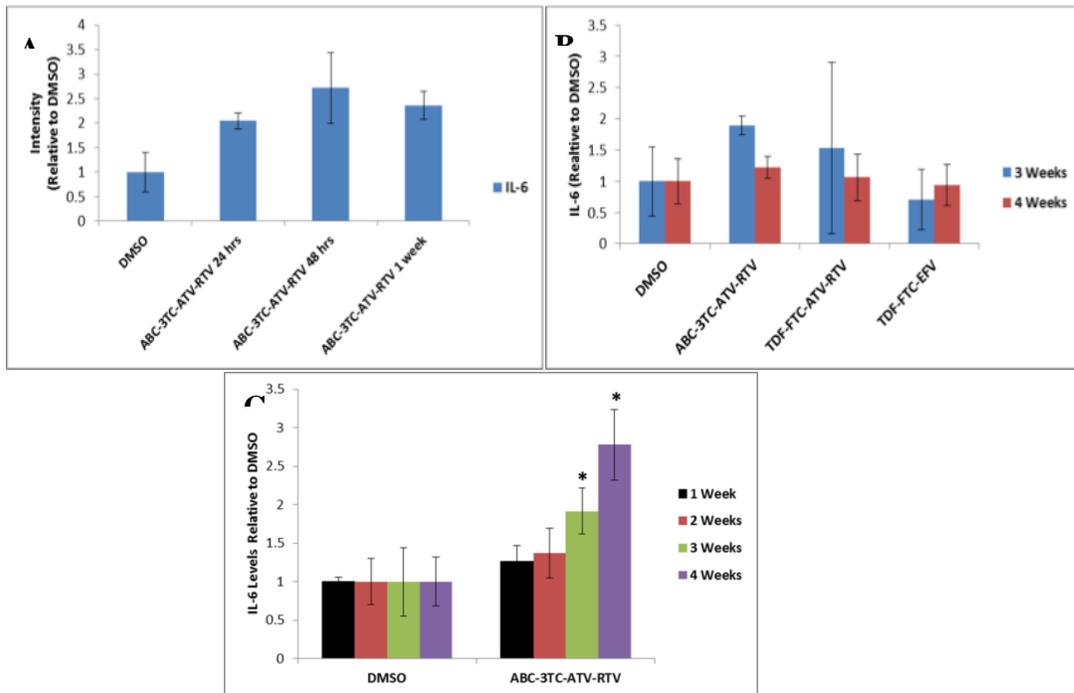


Fig. 10. HAART Drugs Induce IL-6 Secretion in Human Astrocytes. Human astrocytes were treated with a combination of abacavir (ABC), lamivudine (3TC), atazanavir (ATV) and either 100 nM ritonavir (RTV) (A-B) or 300 nM (C) for up to 4 weeks in complete astrocyte media. Cells were then incubated with serum-free astrocyte media for 24 hours and the conditioned media was subjected to IL-6 ELISA. * = p value < .05, error bars represent the mean of 3 biological replicates.

Research Project 8: Project Title and Purpose

Aβ Peptide and Vascular Dysfunction in Alzheimer's Disease – Vascular dysfunction occurs in Alzheimer's disease, however, the role of vascular dysfunction in Alzheimer's disease initiation and progression is not known. Furthermore, the effect of the mechanical environment on brain microvascular endothelial cells has not been extensively studied. This project will determine the effect of Aβ peptide on vascular endothelial cell response to fluid shear stress and angiogenesis. While current medications can slow the progression of dementia symptoms, there are currently no treatments to prevent, halt, or cure Alzheimer's disease. These fundamental studies will elucidate new roles for Aβ peptide and the microvasculature in the disease, and lead to new targets for pharmaceutical therapies.

Duration of Project

1/1/2012 – 6/30/2013

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.pa.gov/MyRecords/Health-Research/CURE>.

Research Project 9: Project Title and Purpose

Interactive Roles of Interstitial Flow and Hepatitis B Virus in Liver Cancer Progression – The purpose of the project is to determine how interstitial flow affects the invasive behavior of primary liver cancer cells, and whether this process is potentiated by hepatitis B infection. Liver cancer is one of the most common cancers worldwide, resulting primarily from chronic hepatitis B infection, and incidence in the United States is rising. The processes underlying the link between liver cancer and hepatitis B remain poorly understood. At the same time, the role of biomechanical forces in cancer progression is increasingly being appreciated. Our project will elucidate interactions between biomechanical forces and hepatitis B infection to understand their contributions to liver cancer invasion, and develop targeted therapies to halt these processes.

Duration of Project

1/1/2012 – 12/31/2013

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.pa.gov/MyRecords/Health-Research/CURE>.

Research Project 10: Project Title and Purpose

Encapsulation Systems with Tunable Permeability for Improved Stability and Release Profile of Encapsulated Materials of Biomedical Importance – Oxidation of encapsulated bioactive molecules such as drugs and vitamins results in loss of their activity. The oxidation process within these encapsulation systems is initiated by transport of oxidants such as free radicals generated in the aqueous phase and transported across the interfacial layer of the encapsulation system and into the encapsulation system. In the present research, we propose to design encapsulation systems with tunable permeability to minimize the transport of these free radicals across encapsulation system, thus reducing the oxidation of encapsulated materials of biomedical importance. Tunable permeability will also enable enhanced control over the release profile of these encapsulated bioactive molecules.

Duration of Project

1/1/2012 – 6/30/2013

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.pa.gov/MyRecords/Health-Research/CURE>.