INTRODUCTION

Hepatocellular carcinoma (HCC) is the 5th most common malignancy in the world, and the third leading cause of cancer death due to a rapid progression, poor prognosis and high rate of mortality. Even in countries of historically low incidence rates particularly the US have experienced a steady increase in the occurrence of HCC. Yet in the US it has been reported that 15-50% of HCC patients had no exposure to established risk factors like heavy alcohol consumption, viral infection or aflatoxin B1 exposure. This highlights the role of predisposing factors such as diet and potential exposure to occupational or environmental carcinogens as being critical factors in HCC development and progression. Obesity, which has been on the rise for the last 30 years in the West, and high fat diets have been experimentally and epidemiologically linked to an increased risk of HCC incidence and mortality. Therefore the goal of our study was to investigate the carcinogenic potential of an environmental carcinogen 4,4’-methylene-dianiline (MDA) both singly, and in combination with obeseogenic dietary components.

Sixty-day old male F344 rats were exposed to either a normal diet, a high fructose diet (30% in drinking water), or a high fat high cholesterol diet (Western diet, Open Source Diets, 17% of calories from protein, 43% of calories from carbohydrates and 41 % from fat) with and without exposure to MDA. Those animals receiving MDA were administered a single oral gavage of 10 mg/kg MDA each week for eight weeks after four weeks of exposure to each respective diet. Livers obtained from these rats were subjected to immunohistochemical and Western blot analysis. Specifically, the effects on the hepatic cell cycle regulator cyclin D1 and the detoxification enzyme glutathione S-transferase (GSTP1) which are established pre-neoplastic and neoplastic markers of HCC were examined. Both the HCC neoplastic markers were observed to be increased in response to MDA in animals receiving normal diet according to immunohistochemical staining. Importantly, a further increase in the expression of these markers was observed as a consequence of the combinatorial effect of MDA and diet. Overall, our findings suggest that carcinogenic potential of environmental occupational/carcinogenic could be significantly heightened by dietary components.

RESULTS

• As expected rats fed the Western diet and the high fructose diet had significantly higher body weights in both the treatment group as well as the control group. Both the treated and control groups gained approximately the same increase in weight with the Western diet resulting in the largest gains.
• Both of these diets also caused the percent liver weight to body ratio to increase significantly when compared to the normal diet control. Interestingly both the Western and high fructose diets had approximately the same increase in liver to body weight ratios, suggesting that both diets cause an approximately equal increase in hepatic steatosis.
• Immunohistochemical staining showed both a greater overall positive staining of hepatic tissues in rats receiving MDA in combination with either the high fructose or Western diets. However rats receiving MDA and a normal diet showed a few localized regions of positive staining.
• Metamorph analysis of immunohistochemical staining revealed that both pre-neoplastic markers, GSTP1 and Cyclin D1 were increased by the high fructose and Western diets alone, yet the increase was greater in those animals treated with MDA in combination with the Western diet. Cyclin D1 expression was marginally increased by a combination of high fructose diet and MDA exposure.

CONCLUSIONS

• Our hypothesis was validated as MDA causes pre-neoplastic events in the livers of rats, and these events are greatly amplified in rats fed a Western diet.
• Pre-neoplastic events were detected even using a dosage of MDA that is markedly lower than similar studies, highlighting the importance of the influence of predisposing factors, like diet, in addition to carcinogenic exposure.
• Rats receiving high fructose showed only marginal differences in the amount of cyclin D1 expression and showed no increase in GSTP1.
• The experimental data strongly suggests that the type of diet along with exposure to environmental or occupational carcinogens, like MDA, could greatly enhance the risk of developing HCC in obese and overweight individuals.

ACKNOWLEDGEMENTS

• Funding provided by NCI Grant R25-CA134283
Abstract

Combination of Withaferin A and Cisplatin Eliminates Ovarian Cancer Stem Cells
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University of Louisville, Louisville, Kentucky

Currently first round of chemotherapy for patients with ovarian cancer includes a platinum based drug called cisplatin. This treatment is often effective in 70% of women, however after a few months of treatment tumor relapse occurs and patients become platinum resistant, which happens in 70 to 90% of patients. ALDH1 activity and tumor renewal has been reported due to cancer stem cells that are present in small numbers in cancer. The purpose of the present study is to determine the effects of cisplatin in combination with withaferin A (WFA), a bioactive plant product. We looked for cancer stem cells that express high levels of aldehyde dehydrogenase 1 (ALDH1), a marker for cancer stem cells. The expression levels of ALDH1 were tested in vivo by using immunohistochemistry and western blot of mouse ovarian cancer tissues after drug treatment. In vitro analysis includes western blot, ALDEFLUOR flow cytometry, and sphere forming assay of ALDH+ and ALDH- sorted cells. In vivo results suggest that cisplatin resulted in a significant increase in ALDH1 expression levels, whereas WFA when used alone or in combination with cisplatin significantly suppressed the expression of ALDH1, suggesting that cisplatin increases the number of cancer stem cells. These results help better understand the high probability of secondary platinum resistant cancer in patients. In vitro results show that both cisplatin and WFA reduce ALDH1 expression and act synergestically in combination. The difference between in vivo and in vitro studies could be that cisplatin treatment may not have enough time to act on the cancer stem cells in vitro or the microenvironment could affect expression. The sphere forming assay shows that only ALDH1+ cells are able to form spheres on ultra low attachment plates in stem cell media. In conclusion, we have shown that ALDH1 expression is up regulated with cisplatin treatment and down regulated with withaferin A combination treatment. Cancer stem cells also appear to normally high ALDH1 production in order to survive. Research supported by the NHVCC R25-CA-134263 grant.

Introduction

- Ovarian cancer is the leading cause of mortalities in gynecological malignancies.
- It is very difficult to detect early due to symptoms that are mild at first and often mimic other diseases. Most patients have advanced ovarian cancer when it is detected.
- 75% of women respond to the first round of platinum based chemotherapy; however 75% of those patients who responded will relapse. If relapse occurs within six months the cancer is determined to be platinum resistant.
- Only about 30% of women survive beyond five years.
- The combination drug therapy of withaferin A (WFA) and cisplatin (CIS) offers an alternative to the current treatments in clinical practice.
- Aldehyde dehydrogenase 1 (ALDH1) is an enzyme which converts intracellular aldehydes into carboxylic acids.
- Increased ALDH1 activity has been linked to cancer stem cell properties in many cancer types.
- The goal of this present study was to analyze ALDH1 levels in response to WFA and CIS treatments and to understand the role of ALDH1 in ovarian cancer.

Methods

- Cell Culture: Cell line A2780 maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotic solution. Cells were cultured at 37°C in humified air with 5%CO2.
- Drug Treatment: A2780 cells were seeded into 6 well plates and treated with WFA, CIS or a combination of WFA and CIS in varying concentrations for 48 hours. Cells were lysed and collected after 48 hours.
- Protein Assay: BSA used a standard for protein concentration determination of cells via spectrophotometry.
- Western Blot: Protein from the cell samples were resolved on SDS-PAGE gel and transferred to nitrocellulose paper. Diluted primary antibodies were applied to detect concentrations of ALDH1. Peroxidase labeled secondary antibodies applied and detected using HRP chemiluminescence. Nitrocellulose paper was then washed and re-probed with β-actin to normalize protein concentrations.
- FACS Analysis and Cell Sorting: ALDEFLUOR kit was used to detect ALDH+ and ALDH- populations. The two subpopulations were sorted and collected. The using flow cytometry.
- Sphere Forming Assay: Cells collected from flow cytometry were plated on ultra low attachment plates with stem cell media. Growth factors added to the media every three days. Aldehyde dehydrogenase 1 (ALDH1) is an enzyme which converts intracellular aldehydes into carboxylic acids.
- Chemiluminescent immunoassay: Preciously xenografted tumors were sectioned and fixed in paraffin. Slides were prepared using ALDH1 antibodies and photographs were taken.

Results

Figure 1: Combination Drug Therapy Immunohistochemistry of ALDH1

- Control tumor
- CIS 6mg/kg
- WFA 2mg/kg
- ALDEFLUOR + DEAB (Negative Control)
- ALDEFLUOR

Figure 2: In Vivo Western Blot Analysis

- Lane 1: Control
- Lane 2: WFA 2mg/kg body weight
- Lane 3: CIS 6mg/kg body weight
- Lane 4: (WFA 2mg + CIS 6mg)/kg body weight

Figure 3: FACS Analysis of ALDH1 Positive Cells

- FACs analysis of ALDEFLUOR kit treated cells. Diethyalminobenzaldehyde (DEAB) is an inhibitor of ALDH1 activity and was used as the control to show baseline fluorescence. The cells that appear above the indicated line for ALDEFLUOR were collected as ALDH+ and the cells below the line collected as ALDH-.

Figure 4: In Vitro Western Blot Analysis of A2780 Cells

- Lane 1: Control
- Lane 2: WFA 0.5µM
- Lane 3: CIS 1.5µM
- Lane 4: CIS 20µM
- Lane 5: CIS 50µM
- Lane 6: WFA 0.5µM + CIS 20µM
- Lane 7: WFA 1.5µM + CIS 20µM
- Lane 8: WFA 1.5µM + CIS 50µM

Figure 5: Sphere Forming Assay

- ALDH+ and ALDH+ cells that were sorted from FACs analysis were plated on ultra low attachment plates with stem cell media. Pictures shown taken after 1 week and 2 weeks of growth. ALDH+ cells are able to form spheres while ALDH+ cells are unable to form spheres.

Conclusions

- ALDH1 is an important biomarker for ovarian cancer stem cells
- Only cancer stem cells with high ALDH1 activity are able to form spheres on ultra low attachment plates in stem cell media.
- Cisplatin increases ALDH1 levels
- Withaferin A decreases ALDH1 levels
- Combination therapy of CIS + WFA decreases ALDH1 synergistically
- Further studies need to be performed on the roles of signaling pathway molecules with ALDH1 (Notch 1, HES1, HEY1, NF-κB) as well as generation of tumor in mice by injecting ALDH1+ cells into SCID mice and subsequent treatment with cisplatin, withafarin A or in combination.

References

Acknowledgements

Research supported by the NHVCC R25-CA-134263 grant and the University of Louisville Cancer Education Program
A Proposed Treatment Algorithm for Stage III Pancreatic Adenocarcinoma
Thomas Brenzel, Robert CG Martin II, MD, PhD
Department of Surgery, Division of Surgical Oncology, University of Louisville, Louisville, KY

Introduction
Pancreatic adenocarcinoma has seen new advancements in therapy in recent years, but the 5-year overall survival remains at 5%. About 50,000 cases of pancreatic adenocarcinoma are diagnosed each year in the United States. Of these cases, 30% present as locally advanced, stage III. The management of stage 3 pancreatic adenocarcinoma requires a challenge in oncology. Balancing quality of life management with active and effective therapies remains the key goals in optimal care.

Hypothesis and Aims
Hypothesis:
- There is currently no standardized treatment or staging algorithm for unresectable locally advanced pancreatic adenocarcinoma.

Aims:
- Evaluate current reported strategies in the care and treatment of locally advanced unresectable pancreatic adenocarcinoma.
- Propose and establish an acceptable treatment strategy to optimize diagnosis and treatment outcomes.

Methods
- A literature review was conducted using the PubMed, Embase, and Library of Congress Database for articles with the title containing, “Pancreatic Cancer” Locally Advanced”, “Stage 3 Pancreatic Cancer”, “Locally Advanced Pancreatic Adenocarcinoma”.
- For the purpose of this analysis articles were excluded if:
  - they were not published in the last 7 years or not published in English.
  - Abstracts of the remaining articles were assessed and case studies, previous meta-analysis articles, and articles not treatment or outcome focused were removed.
- The selection process is demonstrated in Figure 1. Fourteen Articles met the criteria and were then summarized in Table 1.

Results
- Currently, there is no well-defined and established treatment algorithm for stage III pancreatic cancer. In the last 7 years patients have been treated by chemotherapy alone, chemoradiotherapy (CRT), induction chemo followed by CRT, or CRT followed by chemo. Other patients received irreversible electroporation (IRE) or ablation.
- Only a limited number (n = 3) of randomized control trials were found for specifically stage 3 PDAC.
- Patients who become eligible for surgical resection have the greatest survival benefit.
- Gemcitabine was the most common chemo agent used. Only 9 of the 14 articles define extensively the chemotherapy agents used, the dosages, and durations.
- New combinations of chemotherapy drugs are being studied for patients with stage 3 PDAC. Notably FOLFIRINOX, which is 5-fluorouracil [5-FU], oxaliplatin, irinotecan, and leucovorin.
- The most frequent dose of radiation given in patients with chemo-radiation therapy was 54.0 Gy. These articles show a consensus of recommending between 50-60 Gy.
- Treatment with CRT has higher toxicity than chemo alone.
- CRT has no clear survival advantage over chemo alone.
- Chemo followed by CRT reported the highest OS.
- The single article reporting on FOLFIRINOX showed an improved progression free survival but did not calculate OS.

Table 1: Analysis Articles

<table>
<thead>
<tr>
<th>Article</th>
<th>Study Type</th>
<th>Study Design</th>
<th>Stage</th>
<th>Treatment</th>
<th>OS/FS</th>
<th>DFS</th>
<th>Toxicity</th>
<th>Toxicity</th>
<th>Conclusion</th>
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<td>Phase II</td>
<td>III</td>
<td>Chemo</td>
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<tr>
<td>B</td>
<td>Prospective</td>
<td>Randomized</td>
<td>III</td>
<td>CRT</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>C</td>
<td>Retrospective</td>
<td>Phase II</td>
<td>III</td>
<td>CRT</td>
<td>Yes</td>
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</tbody>
</table>

Table 1 Legend:
- OS = overall survival; DFS = disease free survival; CRT = chemoradiation therapy; Chemo = chemotherapy; IRE = irreversible electroporation; FU = fluorouracil.

Discussion
- No established or well-defined treatment algorithm was found for Stage III (locally advanced unresectable) pancreatic cancer. Stage III should be treated uniquely from stage II or stage IV by the proposed algorithm.
- Pancreatic adenocarcinoma should be staged with a triphasic CT scan and diagnostic laparoscopy.
- CRT should be the second-line treatment in patients that have stable disease or response following 4-6 cycles of induction chemotherapy.
- Patients with progressive disease should be treated with chemotherapy.
- FOLFIRINOX is the recommended first-line chemosensitization. Reports have demonstrated improved survival in metastatic patients.

Acknowledgements
National Cancer Institute 5R25CA134283-03 – Cancer Education Program Grant
Mechanistic Insight Into Vinyl Chloride-Induced Liver Injury.

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1 Department of Pharmacology and Toxicology, 2 Department of Medicine, University of Louisville Health Sciences Center, Louisville, KY 40292, USA, 3 Louisville VAMC

ABSTRACT

Background. Vinyl chloride (VC) is a ubiquitous environmental contaminant and the main byproduct of the ATRIA (chloroalkane) and chloroethane breakdown products. These compounds have been shown to cause liver injury and affect cell membrane permeability, increasing the risk of liver disease. Since VC is a potent inducer of oxidative stress, this study evaluated the effects of VC on the cellular and molecular mechanisms underlying liver injury.

Materials and Methods. Eight-week-old male C57BL/6J mice were treated with or without VC (300 ppm) for 2 weeks. All mice were sacrificed by isoflurane anesthesia and liver tissue was collected for further analysis. VC exposure did not affect liver mass, tissue morphology, or histology compared to controls. Plasma glucose and lactate levels were increased in VC-treated mice compared to controls. VC exposure increased levels of aspartate and alanine aminotransferases (ALT and AST) in plasma samples. VC treatment resulted in decreased liver glycogen levels and increased liver weight compared to controls. VC treatment resulted in decreased liver glycogen levels and increased liver weight compared to controls. VC exposure did not alter levels of plasma transaminase activity.

Results. VC exposure resulted in decreased liver glycogen levels and increased liver weight compared to controls. VC treatment resulted in decreased liver glycogen levels and increased liver weight compared to controls. VC exposure did not alter levels of plasma transaminase activity.

Conclusion. These findings suggest that VC exposure can cause liver injury through mechanisms that include oxidative stress, decreased liver glycogen levels, and altered membrane permeability.

REFERENCES


FUNDING SUPPORT

This research was supported by NIEHS (NCATS) K24 ES011558 (NIAAA), NIEHS (NIAAA), and NIAAA (NIAAA).
Development of a shRNA library for high-throughput screening of the role of sphingolipids in tumorigenesis.


J. B. Speed School of Engineering, Departments of Pharmacology and Toxicology and Medicine, James Graham Brown Cancer Center, University of Louisville, Louisville, KY.

We seek to develop a comprehensive and unbiased resource that will allow us to study the role of sphingolipid metabolism in lung cancer biology. We are creating a library of viral vectors that will facilitate knockdown of every protein involved in sphingolipid metabolism. This library will be utilized in cellular models to identify sphingolipid genes involved in response of lung cancer to standard of care chemotherapeutics and will allow the scientific community to interrogate the entire sphingolipid metabolic pathway in an unbiased and comprehensive manner. This will increase our understanding of the biological processes regulated by sphingolipids and may lead to the identification of novel therapeutic targets.

**Basic Sphingolipid Metabolism**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceramide synthase</td>
<td>Synthesis of ceramide from sphingosine and palmitoyl-CoA</td>
</tr>
<tr>
<td>Ceramide desaturase</td>
<td>Conversion of ceramide to sphingosine</td>
</tr>
<tr>
<td>Phosphatidylcholine synthase</td>
<td>Synthesis of phosphatidylcholine from phosphatidic acid and choline</td>
</tr>
<tr>
<td>Sphingomyelin synthase</td>
<td>Synthesis of sphingomyelin from ceramide and choline</td>
</tr>
</tbody>
</table>

Sphingosine 1-phosphate (S1P) is a key regulator of cell survival.

**Balance of Sphingolipids Dictates Cell Survival**

- Unbalanced sphingolipid metabolism in cancer cells results in an altered ratio of glycosphingolipids to ceramides, increasing resistance to apoptotic stimuli.

**Glycosphingolipids**

- S1P is a key regulator of cell survival.

**Summary and future directions**

- Complete optimization and perform primary screens.
- Initial targets will be validated using several different established and primary lung cancer cell lines.
- Using the shRNA libraries as novel tools, we will identify which genes involved in sphingolipid metabolism alter the response of lung cancer cells to standard of cancer treatments.
- This high throughput technique will be invaluable resources that we, and others, can utilize for a myriad of applications.

**Acknowledgements**

This work was supported by NCI grant R25 CA134283 to the University of Louisville. This work is also supported by the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health (5R01DK093462) to L.J.S., Kosair Pediatric Cancer Research Program award (to L.J.B.) and the James Graham Brown Cancer Center.
The Hepatic "Matrisome" Responds Dynamically to Stress: Novel Characterization of the ECM Proteome

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ABSTRACT

Background: Chronic liver injury leads to a dramatic increase in hepatic matrix and remodeling of extracellular matrix (ECM) proteins. There are no data on how the hepatic matrisome (i.e., all matrix proteome) responds dynamically to stress. Moreover, outside the context of liver diseases, the nature and impact of these matrix remodeling processes are poorly understood.

Methods: We used high performance liquid chromatography (HPLC) on four weeks of diet, alcohol and/or lipopolysaccharide (LPS) to determine the effects of diet, alcohol and/or LPS on the matrisome. Liver sections from these exposure groups were analyzed by western blotting and immunohistochemistry to compare to separate proteins by age and cross-linking. Probes in the resulting panels were identified using high-throughput proteomics techniques. Changes were assessed relative to control groups. Liver sections from these exposure groups were stained with Sirius red to determine ECM deposition in a manner similar to previously described techniques. Drug discovery was performed using primary anti-body arrays, high-resolution sequences, and protein-identification databases used to investigate the matrisome. These results suggest that this approach can be used to document qualitative changes in the ECM proteome (i.e., presence and absence). These data further support the use of the matrisome in understanding the hepatic response to injury.

Background: The hepatic matrisome (i.e., all matrix proteome) responds dynamically to stress. Moreover, outside the context of liver diseases, the nature and impact of these matrix remodeling processes are poorly understood.

Methods: We used high performance liquid chromatography (HPLC) on four weeks of diet, alcohol and/or lipopolysaccharide (LPS) to determine the effects of diet, alcohol and/or LPS on the matrisome. Liver sections from these exposure groups were analyzed by western blotting and immunohistochemistry to compare to separate proteins by age and cross-linking. Probes in the resulting panels were identified using high-throughput proteomics techniques. Changes were assessed relative to control groups. Liver sections from these exposure groups were stained with Sirius red to determine ECM deposition in a manner similar to previously described techniques. Drug discovery was performed using primary anti-body arrays, high-resolution sequences, and protein-identification databases used to investigate the matrisome. These results suggest that this approach can be used to document qualitative changes in the ECM proteome (i.e., presence and absence). These data further support the use of the matrisome in understanding the hepatic response to injury.

Materials and Methods: Western blot and immunohistochemistry were used to determine the effects of diet, alcohol and/or LPS on the matrisome. Liver sections from these exposure groups were analyzed by western blotting and immunohistochemistry to compare to separate proteins by age and cross-linking. Probes in the resulting panels were identified using high-throughput proteomics techniques. Changes were assessed relative to control groups. Liver sections from these exposure groups were stained with Sirius red to determine ECM deposition in a manner similar to previously described techniques. Drug discovery was performed using primary antibody arrays, high-resolution sequences, and protein-identification databases used to investigate the matrisome. These results suggest that this approach can be used to document qualitative changes in the ECM proteome (i.e., presence and absence). These data further support the use of the matrisome in understanding the hepatic response to injury.

REFERENCE


Viability of Losartan as a Combination Therapy with Oncolytic Adenovirus

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Department of Surgery, Brown Cancer Center, Departments of Pharmacology and Toxicology, Microbiology and Immunology, University of Louisville School of Medicine

Abstract
Cancer-selective oncolytic adenoviruses have great conceptual potential as an emerging lung cancer therapy. However, one of the major obstacles in oncolytic virotherapy is maximizing the spread of the virus therapy throughout the tumor. Tumors have an extracellular matrix that is dense enough to retard or outright halt the distribution of therapeutic nanoparticles like viruses. Combination treatments aimed at degrading, or limiting extracellular matrix production hold great promise.

Losartan is a clinically-approved hypotensive drug with anti-fibrotic properties; it has been shown in mouse models to increase the distribution of some therapeutic nanoparticles by limiting extracellular matrix production. In vitro experiments were performed to verify the suitability of Losartan as a combination treatment with oncolytic adenoviruses. Toxicity tests showed that Losartan had no anti-cancer properties of its own. Various cell lines in culture were then treated with a combination of Losartan and adenovirus. The effects were verified through visual examination of AdGFP fluorescence expression, crystal staining of adherent cells, Western blot analysis for viral protein expression, and virus titre produced in infected cells.

In several cell lines, the combination treatment of Losartan and adenovirus was markedly more effective than the adenovirus alone. H327 cells responded particularly well to the combination treatment, with positive results in each of the four analytical methods used. More testing is needed to understand the full range of Losartan’s effects and how to maximize its positive effect on viral efficacy. Losartan appears to be a viable option to increase adenovirus distribution in tumors.

Introduction
Oncolytic viruses ought to be an effective and self-sustaining weapon against cancer. However, the dense extracellular matrix in tumors prevents therapeutic nanoparticles from spreading through the tumor and greatly limits the efficacy of adenoviral therapy. Moreover, tumors grown in immunocompetent mice have also been demonstrated to form capsule structures. These structures appear to be an active tumoral response to viral infection that serves to contain the spread of virus particles. Given all this, increasing the distribution of viruses in tumors is vital to increase virotherapy efficacy. Preliminary tests were run to verify the drug Losartan’s suitability for this purpose.

Conclusions
• AdGFP experiments show that Losartan increases the initial penetration and distribution of the virus.
• Viral replication appears to be increased in the presence of Losartan, as shown by the virus titers.
• Losartan exhibits a positive effect on viral cytotoxicity and increases the efficacy of adenovirus therapy, particularly at low MOI’s.

Acknowledgements
Research was partially supported by NCI grant R22 CA134283 to the University of Louisville (Dr. D. Heim).