In vivo and in vitro determinates that regulate BCLxl’s apoptotic potency

Amy Song and Levi J. Beverly

Department of Medicine, James Graham Brown Cancer Center
University of Louisville, Louisville, KY 40202, USA.

Abstract

BCLxl, a member of the B-cell lymphoma-2 (BCL-2) family, is a protein that plays a key role in cell survival by preventing mitochondrial outer membrane permeabilization (MOMP). The ability of BCLxl to block apoptotic signals in the cell has been positively linked to tumorigenesis. Earlier in vivo experiments in the lab using mouse models showed the significance of individual domains within BCLxl’s protein structure for dictating oncogenic potency. The Bcl-2 homology (BH)-4 domain of BCLxl has been shown to be essential in the anti-apoptotic functionality of BCLxl and certain residues within the BH4 domain of BCLxl are significant to the pro-survival potency of the protein. Site-directed mutagenesis was used to identify point mutations of conserved residues within the BH4 domain of BCLxl. Successful mutations would be used in ongoing in vitro experiments to further analyze the biochemical functions of BCLxl.

Introduction

The B-cell lymphoma 2, or BCL2, family of proteins plays a large role in the determination of cell death and cell survival. The family proteins are divided into pro-apoptotic proteins, such as BAX and BAK, and anti-apoptotic proteins, such as BCL2 and BCLxl. Pro-apoptotic proteins promote cell death via mitochondrial outer membrane permeabilization (MOMP) whereas anti-apoptotic proteins inhibit MOMP and can drive tumorigenesis. Thus the ability to regulate cell death is a critical area of research in developing novel therapeutics for cancer. Anti-apoptotic members of the BCL2 family have four conserved BCL2 homology domains: BH1, BH2, BH3, and BH4. The structural variations between the members of the BCL2 family is likely responsible for the difference in functional activity of these proteins. The first three homology domains are highly conserved, whereas the BH4 is much less well conserved, suggesting that the BH4 domain is significant in determining the potency of the individual BCL2-like gene. Chimeric genes were developed to further determine the role of BH4 in dictating in vivo oncogenic potency of BCLxl.

Methods

Site Directed Mutagenesis

Figure 3. Point mutations of conserved residues in the BH4 domain of BCLxl are proposed to significantly impair the anti-apoptotic functionality of BCLxl. These mutations include replacements at the 6th, 9th, and 17th residues with glutamine, alanine, and tryptophan, respectively.

Figure 4. Structures of BCLxl (construct 2) and BCLb (construct 1) and chimeric constructs. Construct 5 includes the BH4 and loop domains of BCLb: construct 7 only includes the loop domain of BCLb.

Previous studies have also shown that substitutions of residues in the BH4 domain of BCL2 significantly impaired function, reducing the cell survival activity. Homologous residues were identified in the BH4 domain of BCLxl in order to perform a similar experiment to test cell survival with a mutant BCLxl gene. Site directed mutagenesis was performed using oligonucleotides containing the specific altered residues.

Conclusions and Future Directions

Point mutations via site directed mutagenesis will be utilized in future experiments to further examine the potency of mutated BCLxl. Once mutated BCLxl DNA is successfully generated, cell viability assays may be used to test cell survival.

Further studies will also be done to resolve the low expression of MIT BCLxl/BCLb constructs.

Acknowledgements

Research is supported by grant R25-CA-134283 from the National Cancer Institute. Many thanks to Dr. Levi Beverly and members of the Beverly lab for their mentorship and guidance.
Recombinant Expression of Codon-Optimized ANAPC2 and ANAPC11
James A. Stewart, Mark Doll, and J. Christopher States

Pharmacology and Toxicology, University of Louisville School of Medicine

Abstract
The current mitosis disrupting chemotherapeutics on the market today are spindle poisons that attack mitotic spindle function and  
activate the spindle assembly checkpoint (SAC). Activation of the SAC causes inhibition of the Anaphase Promoting Complex/Cyclosome (APC/C), 
anaphase-promoting complex/cyclosome (APC/C). APC/C catalyzes the ubiquitylation of anaphase-promoting complex/cyclosome (APC/C), the master  
complex. Evidence of displacement will demonstrate the APC/C as an effective target for the treatment of cancer. A key step in developing  
the APC/C as an effective target for the treatment of cancer is the identification of compounds that can disrupt the ANAPC2/ANAPC11 binding.  
Thermoflour assays can be used to test compound binding affinities.  Compound binding to ANAPC2 with purified recombinant ANAPC2 will allow binding assays to be run to test compound binding affinities.  Compounds binding to ANAPC2  
with high affinity may displace ANAPC11 from ANAPC2/ANAPC11 complexes. Evidence of displacement will demonstrate the APC/C as  
a new target for future chemotherapeutic drugs. Partially supported by NCI grant R25 CA149268 to the University of Louisville.

Introduction
Cancer remains the second leading cause of death in the United States with 585,720 new deaths estimated in 2014. Cancer is the unregulated growth of cells. While early detection and treatment continue to increase likelihood of survival, there remains a great need for better drugs.

Current mitosis disrupting chemotherapeutics such as paclitaxel target mitotic spindle microtubules and arrest cells in metaphase.  
Another class of mitosis disrupting chemotherapeutics, the spindle poisons that attack mitotic spindle formation and activate the spindle  
assembly checkpoint (SAC). At this stage inhibition of the ANAPC11 binding will be measured to determine the effectiveness of the compound.  
Thermoflour assays can be used to test compound binding affinities.  Compound binding to ANAPC2 with purified recombinant ANAPC2 will allow binding assays to be run to test compound binding affinities.  Compounds binding to ANAPC2  
with high affinity may displace ANAPC11 from ANAPC2/ANAPC11 complexes. Evidence of displacement will demonstrate the APC/C as  
a new target for future chemotherapeutic drugs. Partially supported by NCI grant R25 CA149268 to the University of Louisville.

Methods

Figure 1. APC/C Role in Mitosis

Figure 2. Cloning Genes Into The Vector

Figure 3. Protein Expression and Purification

Figure 4. Restriction digest to isolate optimized ANAPC2 cassette and ANAPC11 cassette

Figure 5. Restriction digest to isolate ANAPC11 cassette. A triple digest (Ndel, BamHI, EcoRI) of the ANAPC11 cassette was digested.  
The ANAPC11 plasmid was used to isolate the ANAPC11 cassette. The band at the  
lanes at 0.7 kbp for Lane 2. Sample analysis from protein purification. Cell cultures were grown and  
extracted. Isolated fragments were  
ligated to pTYB21 vector isolated in

Figure 6. Time course for optimal protein expression. Samples of cell cultures were used directly in SDS sample buffer, run as SDS-PAGE and probed with an anti- 
fragment antibody, and visualized using secondary antibody conjugated to horseradish peroxidase, incubate with ECL detection reagent, and image captured on a film.  
Lane 1: 0 h induction (ANAPC2); Lane 2: 1 h induction (ANAPC2); Lane 3: 3 h induction (ANAPC2); Lane 4: 1 h induction (ANAPC11); Lane 5: 2 h induction (ANAPC11); Lane 6: Blank; Lane 7: pTYB21 positive control for CBD. Sample in Lane 2 added into Lanes 1 and 3.

Figure 7. Sample analysis from protein purification. Cell cultures were grown and expression induced with IPTG. Cells were collected by centrifugation and resuspended for electrophoresis and blotting. Gel bands were removed by centrifugation and the supernatant was run on a  
chitin column to isolate the fusion proteins. Columns were incubated with TDT to induce cleavage by the intein to liberate the ANAPC proteins.  
Lane 1: Beads from column after cleavage (ANAPC11 immediate induction); Lane 2: Beads from column after cleavage (ANAPC11, 2°C, 1 h); Lane 3: Lysozyme added to column for Lane 1; Lane 4: Lysozyme added to column for Lane 2; Lane 5: Beads from column after cleavage (ANAPC2, 37°C, 10); Lane 6: Lysozyme added to column for Lane 5. Lanes 3, 4, and 5 are from the same gel but at a longer exposure time.

Results

Conclusions
Expression of ANAPC2 is most effective at 37°C with shorter expression times in order to make the protein soluble.

Expression of ANAPC11 is most effective at 37°C with 1 hour of growth after addition of IPTG at any concentration.  
\n
Future Work
Large scale purifications with the T7 express cells expressing the optimized ANAPC2 and ANAPC11 proteins.

Acknowledgement
Partially supported by NCI grant R25 CA134283 to the University of Louisville.
Evaluation of coated gold nanoparticles targeted with Syndecan-1 for detection of pancreatic adenocarcinoma

Christopher Ullum, Anil Khanal, Shaniee Hudson, Lacey R. McNally

Department of Medicine, University of Louisville, Louisville, KY

ABSTRACT

Purpose: Mortality of pancreatic cancer remains unchanged for the past four decades due in part to the inability to detect early-stage tumors. Presently, there is no gold standard for early identification of pancreatic adenocarcinoma. Therefore, we sought to create a contrast agent which would identify pancreatic tumor cells using a newly emerging imaging system, Multi-Spectral Optoacoustic Tomography (MSOT). Because this technology detects the thermoacoustic expansion of highly absorbing particles, we developed two fully functional and stable gold nanorods to serve as this contrast agent.

Methods: Gold nanorods (GNRs) were synthesized via the seed-mediated method to overcome the common detriment of gold nanoparticle aggregation. The GNRs were coated with mesoporous-silica (MS) or poly-acrylic acid (PAA). Coated GNRs were conjugated to Syndecan-1 peptide to facilitate detection of pancreatic cancer cells. Synthesized nanoparticles were characterized by Transmission Electron Microscopy (TEM), UV-Visible Spectroscopy (UV-vis), and Zeta-potential. The cellular uptake of these nanorods was evaluated using CytoSoft Hyperspectral Imaging (HSI) assessment of coated and targeted GNRs as potential contrast agents to detect pancreatic cancer was determined using tissue phantoms within the MSOT. Subsequently, mice bearing orthotopic pancreatic tumors were injected with Syndecan-1 MS-GNR followed by MSOT imaging.

Results: The GNRs have an aspect ratio of 30:7 with MS-GNR and PAA-GNR containing a 10 nm mesoporous-silica shell and 3 nm PAA shell, respectively. While the encapsulation of mesoporous-silica decreased the zeta-potential from -23 mV (CTAB-GNR) to -32 mV (MS-GNR), zeta-potential of PAA-GNR was -62.2 mV. Upon coating of mesoporous-silica and PAA, the UV-visible spectra showed a red-shift of 32 nm and 15 nm, respectively. Neither the MS-GNR nor PAA-GNR demonstrated aggregation for 2 days in comparison to the CTAB-GNR particles which aggregated within 1 h at pH 7.4. The Syndecan-1 MS-GNR particles were observed on the cellular membrane and targeted detection of pancreatic cancer cells within the tissue phantom using MSOT. Syndecan-1 MS-GNR particles facilitate detection of aggregated gold nanoparticles.

Conclusion: Syndecan-1 targeted MS-GNR could serve as a potential contrast agent to facilitate detection of pancreatic tumors using Multi-Spectral Optoacoustic imaging. Further studies will be conducted to conclude the location of GNR during cellular uptake.

RESULTS

Figure 2: Transmission Electron Micrograph shows GNRs with poly-acrylic acid shell (PAA) and mesoporous-silica (MS) shell. (A) AuNRs were made with a gold core ratio 30:7 and coated with a 3 nm poly-acrylic acid shell (coated by -2 shell). (B) AuNRs were encapsulated with a 10 nm mesoporous-silica shell.

Figure 3: Absorption spectra of CTAB-GNR, PAA-GNR, and MS-GNR. (A) Poly-acrylic acid and mesoporous-silica coating was confirmed by a small red-shift of 15 nm and 15 nm, respectively. (B) Instability and aggregation of CTAB-GNR is apparent after 1 hr. (C) Stability of PAA-GNR is shown up to 2 days. (D) Stability of MS-GNR is shown up to 2 days.

Figure 6: Visualization of ligand-targeted coated GNR in cells using phantoms with MSOT. Cells incubated with MS-GNR Phantoms are imaged with the MesoGone Vision tool (software) on the left and the positive control on the right. Cylindrical phantoms with a diameter of 2 cm were prepared using a gel made from distilled water. After drying, a 0.5% solution of ICG was added to the gel and the gel was placed within the phantom.

FUTURE DIRECTIONS

To determine the exact cellular location of the GNR cell wall (mesoporous silica), treated cells will undergo standard fixation and sectioned for Transmission Electron Microscopy. More in vivo studies will be conducted to determine the appropriate concentration of conjugated GNR and optimize imaging timing.

ACKNOWLEDGEMENTS

This work was supported by NIH/NCI R25-CA134283 and CA15000.
Modulating Epidermal Growth Factor Receptors via Small Targeting Peptides
Adrienne L. Voelker¹, Luis F. Neves², and Brian P. Ceresa²
¹R25 Cancer Education Program, University of Louisville
²Department of Pharmacology and Toxicology, University of Louisville

Abstract

Purpose: To better understand the epidermal growth factor receptor (EGFR) in the endocytic pathway and the effect of a small Sprouty2-derived peptide on the rate of degradation of EGFR, which impacts cell proliferation and wound healing.

Methods: Liposomes were synthesized and peptide (FITC-Ahx-IRNTNE{pTYR}TEGPTV) was encapsulated in the liposomes via freeze and thaw cycles. Liposomes were incubated with the S2VP10 human pancreatic cancer cell line. S2VP10 cells were also treated with digitonin to permeabilize the membrane and incubated with peptide at different concentrations. Efficiency of peptide uptake into the cell was determined by fluorescence microscopy. EGFR degradation levels upon EGF ligand stimulation at time points 0, 15, 60, and 120 minutes were measured through immunoblotting.

Results: Dynamic Light Scattering (DLS) and spectral evaluation of the liposomes confirm that synthesis of the liposomes and encapsulation of the peptide was successful. Fluorescent microscopy shows that liposomes offer an efficient delivery of the peptide into the cell. EGFR activity levels, quantified through immunoblotting, do not suggest an effect induced by the liposomes.

Conclusions: The methodology used for the synthesis and encapsulation of the peptide allows for successful encapsulation of the peptide. Liposomes are an effective method of delivering the peptide into the cell in a controlled, targeted manner. Western Blot results do not reveal a significant effect of the peptide on EGFR activity levels in the cells. Future experimentation is required to determine the efficacy of the peptide.

Introduction

Cancer is the second leading cause of death in the US, accounting for one in every four. In 2014, 1,665,550 people are expected to be diagnosed with cancer, and 585,720 of these cases are expected to lead to death (www.cancer.org). Overexpression of the epidermal growth factor receptor (EGFR) is found in many carcinomas. Receptors may be endocytosed and follow the endocytic pathway from the early endosome to the lysosome for degradation, or they may be recycled back to the membrane by transport vesicles via transcytosis. C-Cbl binds to the phosphorylated receptor, which targets proteins for ubiquitination, leading to degradation of the receptor-ligand complex in the lysosome. Sprouty2 is a large protein that has been found to act as a c-Cbl inhibitor.

Hypothesis: Binding a small Sprouty2-derived peptide to c-Cbl will sequester it, preventing receptor ubiquitination, causing EGFR to be recycled to the plasma membrane and promote proliferation.

By learning how to manipulate basic cell mechanisms, we can better understand how to slow proliferation and make advances in cancer treatment. Liposomes will be used to deliver the peptide into the cells by encapsulating the peptide in the core of the liposome. Internalization of liposomes into the cell may occur through endocytosis, lipid exchange, receptor-mediated endocytosis, or fusion. Liposomes: A Practical Approach. Liposomes will promote internalization of large molecules that would not otherwise be efficiently taken into the cell. Smaller liposomes, ideally 200 nm or smaller, have a longer circulation half-life and enhanced ability to internalize into the cells when compared with larger vesicles. The objectives of this project are to: (1) synthesize liposomes with encapsulated Sprouty2-derived peptide; (2) internalize the liposomes in S2VP10 cells to release the peptide, which will in turn sequester c-Cbl; and (3) prevent EGFR from degrading in the lysosomes and promote recycling of the receptor back to the membrane.

Flow Chart

Liposome Synthesis & Peptide Encapsulation
1. Synthesis
2. Peptide Encapsulation
3. Extrusion
4. Dialysis

Figure 1

Liposome Size Distribution

Figure 2

Spectral Evaluation of Liposomes

Dialysis uses a concentration gradient to remove the free peptide from the liposomes. Spectral evaluation of the liposomes was performed to verify the presence of peptide (4). The peak at 410 nm indicates peptide in solution, due to the FITC tag attached to the peptide. The presence of peptide following dialysis confirms that the peptide was successfully encapsulated in the liposomes.

Figure 3

Peptide Delivery via Digitonin Treatment or Liposomes in S2VP10 Cells

A. Digonitin Treatment
B. Liposome Delivery

Figure 4

Evaluation of Receptor Activity Upon Ligand Stimulation

Control
Liposomes
IB: EGFR
IB: α-tubulin

Conclusions

- Freeze and thaw cycles facilitate encapsulation of the peptide in the liposomes, while also leading to the formation of smaller vesicles
- Extrusion is an effective method to control the size of the liposomes
- Liposome delivery is an effective method of delivering the peptide into the cells and is comparable to high peptide concentration delivery
- Liposome treatment did not result in sustained EGFR activity as seen through immunoblotting
- Sustained receptor dephosphorylation was observed in the liposome treated cells

Future Studies: Optimization of liposome encapsulation protocol; use of scrambled peptide to determine specificity of Sprouty2-derived peptide; binding cell penetrating peptides to the surface of the liposomes to improve efficacy of liposome uptake into the cell

Acknowledgements

R25 Cancer Education Program, Dr. Ceressa’s Lab Members
Funding: NIH/NCI R25-CA134283, NIH-GM092874
Is there a Relationship between Patient Worry and Follow-up Care Preferences after Curative Treatment for Lung Cancer?

Ariel M. Washington B.S., Karen Kayser, MSW, PhD, Lisa C. Smith, MSSW, Smita Ranjan, MS, MSN Goetz Kloecker, M.D, MBA, MSPH, FACP
University of Louisville - Kent School of Social Work and School of Medicine

Background

While new methods and techniques are being developed to increase the survivorship rate of lung cancer patients, there is a vast need to aid Kentucky’s cancer survivors.

- Kentucky has the highest incidence rate of lung cancer in the country.
- 97 out of 100,000 residents are diagnosed with lung cancer[1][2] compared with the national average of 61 per 100,000.
- Lung cancer survivorship is only 18%.[1]

- There lacks a general guideline for oncologists to follow in aiding patients during the transition from curative treatment to surveillance.
- Guidelines of five leading medical organizations (ASCO, ACCP, ESMO, Group) differ about the frequency and the level of imaging and how often follow-up appointments should occur.
- Little consideration is given to the survivor’s preferences for follow-up—an important component of patient-centered care.

This study was conducted in an effort to understand the worry that patients experience about recurrence after curative treatment and whether it affects their preferences for treatment.

Research Aims

The aims of this study are to:

1. Describe patients’ preferences for medical and supportive care during follow-up treatment.
2. Determine if a relationship exists between patients’ preferences for medical follow-up care and their level of fear of recurrence; and
3. Determine if a relationship exists between patients’ supportive care preferences and their level of fear of recurrence.

Methods

This is a prospective observational study conducted with the James Graham Brown Cancer Center and UofL’s Kent School of Social Work. The study is part of a larger longitudinal study investigating the preferences for follow-up care of patients and their primary support persons. After patients had completed their curative treatment and were assessed to be cancer free they were given a survey at their next clinic visit. The questionnaire consisted of several measures focused on their preferences for follow-up regime, quality of life, cancer recurrence worry, physical, emotional and social well being. The Stress and Coping Model was used as a conceptual framework (see Figure 1).

Results

Figure 1: Conceptual Model of Stress and Coping

Adapted From: Folkman, S., and Lazarus, R.S. 1980

Table 1: Patient Demographics (N=28)

<table>
<thead>
<tr>
<th>Variable</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>15</td>
</tr>
<tr>
<td>Female</td>
<td>13</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>50-59</td>
<td>10</td>
</tr>
<tr>
<td>60-69</td>
<td>13</td>
</tr>
<tr>
<td>Marital Status</td>
<td></td>
</tr>
<tr>
<td>Married</td>
<td>19</td>
</tr>
<tr>
<td>Single</td>
<td>9</td>
</tr>
<tr>
<td>Employment</td>
<td></td>
</tr>
<tr>
<td>Employed</td>
<td>19</td>
</tr>
<tr>
<td>Unemployed</td>
<td>9</td>
</tr>
<tr>
<td>Education</td>
<td></td>
</tr>
<tr>
<td>Some college</td>
<td>14</td>
</tr>
<tr>
<td>Bachelor</td>
<td>10</td>
</tr>
<tr>
<td>Graduate</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 2: Correlation of Patient Preferences for Medical-related Follow-up Care with Level of Worry

Preferecnes for Medical-related Follow-up Care | Fear of Recurrence – Worry for Care

<table>
<thead>
<tr>
<th>Preference</th>
<th>Fear of Recurrence</th>
<th>T-Value</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Discuss options for follow-up</td>
<td>0.428*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fear of recurrence</td>
<td>0.363*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Discuss treatments and treatment</td>
<td>0.048</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fear of recurrence</td>
<td>0.410*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Discuss plan for future</td>
<td>0.450**</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Frequencies of Patient Preference for medical appointments

<table>
<thead>
<tr>
<th>Preference</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Every Three Months</td>
<td>16</td>
</tr>
<tr>
<td>Every Six Months</td>
<td>8</td>
</tr>
<tr>
<td>Every Month</td>
<td>17</td>
</tr>
<tr>
<td>Every Two Months</td>
<td>11</td>
</tr>
<tr>
<td>Two Months</td>
<td>4</td>
</tr>
<tr>
<td>Taxi Months</td>
<td>14</td>
</tr>
<tr>
<td>Taxi Miles</td>
<td>11</td>
</tr>
<tr>
<td>Taxi Mileage</td>
<td>11</td>
</tr>
<tr>
<td>How often would you like to have your follow-up appointments?</td>
<td>N (%)</td>
</tr>
<tr>
<td>Every Three Months</td>
<td>16</td>
</tr>
<tr>
<td>Every Six Months</td>
<td>8</td>
</tr>
<tr>
<td>Every Month</td>
<td>17</td>
</tr>
<tr>
<td>Every Two Months</td>
<td>11</td>
</tr>
<tr>
<td>Taxi Months</td>
<td>4</td>
</tr>
<tr>
<td>Taxi Miles</td>
<td>14</td>
</tr>
<tr>
<td>Taxi Mileage</td>
<td>11</td>
</tr>
</tbody>
</table>

Table 4: Correlation of Patient Preferences for Psychosocial Supportive Care with Level of Worry

Preferences for Psychosocial Supportive Care | Fear of Recurrence – Worry for Care

<table>
<thead>
<tr>
<th>Preference</th>
<th>Fear of Recurrence</th>
<th>T-Value</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Discuss how my family is coping.</td>
<td>0.346†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Help with managing feelings of anxiety or sadness.</td>
<td>0.363†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Receive information about what supports are available for my family.</td>
<td>0.428*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Discuss how I can get help at home.</td>
<td>0.450**</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: *p≤.05, **p≤.01

There were significant correlations between preferences of psychosocial support and worry.

Conclusions

Despite the small sample size, there were some significant relationships between patients’ worries about recurrence and their preferences for medical-related follow-up and psychosocial care needs. In particular, those patients who had high levels of worry were more likely to prefer that their follow-up appointments help them and their loved ones to cope better and manage their distress. However, it is noteworthy that very few patients expressed an interest in seeing the social worker for help, although their needs were primarily of a psychosocial nature. Our future research will examine physician preferences for patient follow-up care, screening and referral for psychosocial issues during follow-up, and the development of guidelines for surveillance decision-making.

Acknowledgments

This study was supported by the University of Louisville Cancer Education Program, along with a grant from NIH/NCI

References

Examination of the Cell Cycle Effects of Small Molecule Inhibition of PFKFB4

Lindsey J. Wattley, Jennifer Clark, BA, Erin Ballard, BA, and Sucheta Telang  MBBS
Telang Lab, Molecular Targets
Brown Cancer Center, University of Louisville

Abstract
Glycolysis despite the presence of oxygen (i.e., the Warburg effect) is a metabolic hallmark of cancer cells. 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase 4 (PFKFB4), a glycolytic regulating protein, is responsible for converting fructose-6-phosphate (F6P) to fructose-2,6-bisphosphate (F2,6BP) which in turn activates the enzyme 6-phosphofructo-1-kinase (PFK-1).

The requirement of PFKFB4 for cancer cell growth makes this protein a rational drug target. Through in silico screening, 5MPN was identified as an inhibitor of the substrate binding site of PFKFB4. We observed that 5MPN decreases cell proliferation by arresting cancer cells in the G0/G1 stage of the cell cycle and sought to further examine the effect.

We used two lung cancer cell lines chosen from an initial screen. We used siRNA to knock down PFKFB4 in each line. Then, we treated each cancer cell line with 5MPN, and the cell cycle effect of the drug was found to mimic siRNA treatment specific to PFKFB4. Finally, we used overexpression of PFKFB4 to rescue the effect of the drug on the cell cycle. Through these analyses we determined that 5MPN decreases cell proliferation in cancer cells by arresting the cells in the G0/G1 phase.

Introduction

Studies have shown that cancer cells take up glucose at a much higher rate than normal cells. According to the Warburg effect, cancer cells preferentially use glycolysis, rather than mitochondrial cellular respiration, even in an abundance of oxygen (1). Therefore, glycolytic regulating proteins have been the targets for new research.

The enzymes PFKFB1-4 are metabolic regulation proteins responsible for interconverting fructose-6-phosphate (F6P) and fructose-2,6-bisphosphate (F2,6BP). F2,6BP in turn activates the enzyme 6-phosphofructo-1-kinase (PFK-1), which increases the rate of change of F6P to fructose-1,6-bisphosphate (F1,6BP), a key step of glycolysis (2).

PFKFB4 has been found to be overexpressed in several cancers particularly under conditions of hypoxia, indicating that this enzyme may play an important role in regulating glycolysis in cancer (3).

In an attempt to inhibit the activity of PFKFB4 and in turn decrease cancer cell growth, an inhibitor (termed 5MPN) was found. Through examination of enzyme kinetic activities, 5MPN has been shown to inhibit the kinase activity of PFKFB4 (unpublished data, Telang et al). 5MPN may serve as a future anti-cancer therapeutic.

NHBE, A549, H460, H1299 + 5MPN
NHBE, A549, H460, and H1299 cells were treated with 5MPN for 72 hours. The cells were then lifted and counted. The H460 and H1299 had the lowest IC50 of the cell lines examined and therefore were chosen for further investigation.

H460 and H1299 + 5MPN
H460 (a) and H1299 (b) cells were treated with 5MPN for 48 hours. The cells were then lifted, fixed in ethanol, plasma and nuclear membranes disrupted by shearing and stained with PI for analysis of cell cycle by flow cytometry.

H460, H1299 siRNA
H460 and H1299 cells were transfected with PFKFB4 silencing RNA and nonsense RNA for 6, 12, and 18 hours. The cells were then lifted and cell cycle analyzed by flow cytometry.

H460, H1299 Overexpression
H460 and H1299 cells were treated with 2.5 μM 5MPN for 48 and 72 hours. After 24 hours of treatment, the cells were transfected with a vector containing PFKFB4 or an empty vector. After 48 and 72 hours, cells were lifted and cell cycle analyzed by flow cytometry. Representative Western blot confirming overexpression shown (a), % of cells in G0/G1 in H460 (b, 72h) and H1299 cells (c, 48h) shown.

PFKFB4 Role in Glycolysis

In conclusion, treatment with 5MPN causes a decrease in cell proliferation by means of a G0/G1 phase arrest. The cell cycle effect of 5MPN mimics the affect of PFKFB4 siRNA, by increasing the amount of G1 and decreasing both S and G2 phases. This cell cycle effect was able to be rescued at low concentrations of 5MPN through an overexpression of PFKFB4.

Conclusion

Acknowledgements

References

Support of the Summer Research Intern Lindsey Wattley was partially supported by NCI grant R25 CA134283 to the University of Louisville.

It's Happenig Here.