Putative PSAT1 Small Molecule Inhibitors Decrease Breast Cancer Cell Proliferation and Synergize with Anti-Estrogen Therapies

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Abstract

Despite aggressive treatments, a significant proportion of individuals with estrogen-receptor-positive breast cancer relapse after becoming resistant to current therapies. Therefore, the investigation of combinatorial therapies that could re-sensitize these cancers is necessary to identify further treatment options for these patients. It has been previously demonstrated that phosphoserine aminotransferase 1 (PSAT1) is up-regulated in ER+ endocrine-resistant and triple-negative breast cancer. Thus, the serine pathway may be necessary to protect resistant breast cancer cells from common therapeutics, drugs, including anti-estrogens and cytotoxic agents. We hypothesize that specific targeting of PSAT1 in combination with common chemotherapeutic agents may prove beneficial in treating endocrine-resistant and triple-negative breast cancers. In silico modeling of PSAT1 identified several chemical compounds that may suppress PSAT1 activity. Further analysis revealed two molecules that exhibited inhibitory activity on recombinant PSAT1. We now examined the ability of these antagonists to decrease proliferation of MDA-MB-468 breast cancer cell lines. Additionally, combinatorial therapy with the anti-estrogens, Tamoxifen and Fulvestrant, and common cytotoxic breast cancer cell lines, revealed two molecules that exhibited inhibitory activity on recombinant PSAT1. We now examined the ability of these antagonists to decrease proliferation of MDA-MB-468 breast cancer cell lines. Additionally, combinatorial therapy with the anti-estrogens, Tamoxifen and Fulvestrant, and common cytotoxic breast cancer cell lines, revealed two molecules that exhibited inhibitory activity on recombinant PSAT1.

Introduction

- Phosphoserine aminotransferase 1 (PSAT1) catalyzes the second step within the serine biosynthetic pathway via conversion of 3-phosphoglycerate to phosphoserine.
- Prior computational modeling analysis identified several putative PSAT1 inhibitors (Figure 1).
- Tamoxifen is a selective estrogen receptor modulator and fulvestrant is a pure anti-estrogen.
- Doxorubicin is a DNA intercalator and generator of free radicals.
- Cyclophosphamide is an alkylating agent that induces cell death.
- Patițaxel exhibits cytotoxic activity in tumor cells.
- Analyzing the synergy of each chemotherapeutic drug with designated PSAT1 inhibitors could lead to proposition of new combinatorial drug strategies.

Figure 1. In silico identification of putative PSAT1 small molecule inhibitors.

Results

LY2 cells and MDAMB-468 cells were plated in 250 µL of media in 48-well plates at experimentally determined densities, 5% and 10% per well, respectively, and placed in an 37°C incubator to grow. After 24 hours, the anti-estrogens, cytotoxic agents, and putative PSAT1 inhibitors were added in each well to bring the volume to 500 µL at final concentrations determined by previously published papers and/or experimental results.

Figure 2. Effect of PSAT1 Inhibitors on Endocrine Resistant LY2 cells in combination with Tamoxifen or Fulvestrant. LY2 cells were treated with PSAT1 Inhibitor 1 (A & C) or PSAT2 Inhibitor 2 (B & D) with or without different concentrations of Tamoxifen (A & B) or Fulvestrant (C & D).

Figure 3. LY2 Growth Inhibition. LY2 cell growth at specific concentrations of PSAT1 Inhibitor 2 and Tamoxifen (A) or Fulvestrant (B).

MDA-MB-468: Triple Negative Cells

Figure 4. Effect of PSAT1 Inhibitors on Triple Negative 468 cells in combination with Doxorubicin, Patițaxel, or Cyclophosphamide. 468 cells were treated with PSAT1 Inhibitor 1 (A, C, & E) or PSAT2 Inhibitor 2 (B, D, & F) with or without different concentrations of Doxorubicin (A & B), Patițaxel (C & D), or Cyclophosphamide (E & F).

Acknowledgments

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Conclusions

- Putative PSAT1 inhibitors 1 & 2 are effective in decreasing both ER+ and triple negative breast cancer cell proliferation in a dose-dependent manner.
- Both compounds appear to cooperate with the anti-estrogens tamoxifen and fulvestrant in reducing ER+ breast cancer cell growth.
- Combination of non-effective concentrations of PSAT1 inhibitor 2 and either anti-estrogen synergizes in suppressing proliferation of LY2 breast cancer cells.
- Addition of the clinically used cytotoxic agents, doxorubicin, patițaxel, or cyclophosphamide, did not exhibit any additive effects in combination with the putative PSAT1 inhibitors against MDA-MB-231 triple negative cell growth.

Future Directions

- Correlate effects observed with these PSAT1 small molecule inhibitors to the effects of specific depletion of PSAT1 by siRNA mechanisms in order to determine potential off-target effects of the compounds on cell proliferation.
- Expand investigation into other cytotoxic agents, such as cisplatin or oxaliplatin.
- Examine additional putative PSAT1 inhibitors identified through computer screening on growth potential of breast cancer cells, including potential derivatives of inhibitors 1 & 2.
Porphyromonas gingivalis induction of EMT transcriptional factors in gingival epithelial cells

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Abstract

Objective

To determine the effect Porphyromonas gingivalis (Pg) on transcriptional regulation of epithelial mesenchymal transition (EMT) in host epithelial cells.

Background

Oral Squamous Cell Carcinoma (OSCC) has 50,000 new cases and 13,500 deaths per year in the United States. EMT is a critical process in the growth of tumors. Oral bacteria are epidemiologically associated with OSCC tumors. Pg, an oral anaerobe, is consistently shown to be harbored at higher rates on tumor surfaces than normal gullets. Pg has also been shown to impact expression of host genes related to apoptosis and cell cycle progression.

Hypothesis

Pg infection of epithelial cells will cause differential expression of EMT transcriptional regulators Zeb1, Zeb2 and Snail2.

Methods

The TIGK gingival epithelial line was infected with Pg at a multiplicity of infection (MOI) 10, 50, or 100 for various time points. RNA was extracted, and EMT transcriptional factors were quantitated with qRT-PCR. To show protein expression, cells were stained with antibodies for ZEB1 and examined by confocal microscopy. To assess the role of Pg fimbriae, TIGK cells were infected with a knockout strain defective in the gene encoding the major fimbrial protein of Pg (ΔfimA).

Results

Pg infection was shown to increase transcription of Zeb1, Zeb2 and Snail2 over a range of MOI and infection times. Pg infection also increased the amount of Zeb1 protein localized in the nucleus. When infected with the ΔfimA mutants TIGK cell expression of Zeb1, Zeb2 and Snail2 mRNA was significantly reduced compared to wild type.

Conclusions

Pg increases expression of transcription factors that control EMT indicating that the organism could contribute to neoplasia in the oral cavity. FimA may have value as a potential therapeutic target and/or biomarker.

Introduction

Oral and oropharyngeal cancers have over 50,000 new cases every year and 13,500 deaths per year. Despite advanced therapeutic techniques, the 5-year survival rate for oral squamous cell carcinoma is estimated at 50%.

Previous studies have shown that oral bacteria can disrupt molecular pathways with relevance to cancer development and can enhance cancerous tumor growth. Oral bacteria are also epidemiologically associated with cancers such as pancreatic, colorectal, and oral squamous cell carcinomas (OSCC), and high levels of certain salivary bacteria have been shown to be possible indicators of OSCC. Moreover, OSCC surfaces harbor significantly higher levels of Porphyromonas gingivalis (Pg) compared with healthy mucosa, and immunohistochemistry with Pg antibodies revealed higher levels of staining in OSCC compared with healthy gingiva.

The goal of this project is to determine the effect of Pg on the epithelial–mesenchymal transition (EMT)-Zeb1, Zeb2, and Snail2 transcription factors which play an important role in controlling EMT and markers of the EMT. One of the major proteins that allows Pg to interact with and invade host cells is the major fimbrial protein A (FimA). The role of FimA in the regulation of EMT transcription factors was investigated.

Methods

Grow Porphyromonas gingivalis

Cell infection

TIGKs and Pg were grown, the TIGKs were infected with the Pg and infections were done separately for confocal imaging.

Results

Fluorescent confocal microscopy of TIGK cells (stained with TTRC-phalloidin, red) infected with Pg confirmed increased expression of ZEB1 protein (stained with antibodies, green) in the nucleus (blue) where it is functionally active.

Conclusions

- Infection of epithelial cells by Pg causes EMT transcriptional regulators to express at significantly higher levels.
- FimA significantly enhances Pg induced expression of ZEB1, ZEB2 and SNAIL2.
- FimA, which is unique to Pg may have value as an early biomarker of OSCC or as a potential therapeutic target.

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Role of sphingosine kinase 1 and 2 in MYC-induced leukaemogenesis

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ABSTRACT

Sphingosine kinases (SKs) catalyze the conversion of sphingosine to sphingosine-1-phosphate (SIP), a lipid mediator of inflammation, cell proliferation, angiogenesis, and other pro-survival cellular processes. Altered levels of key sphingoids have been observed in a wide variety of cancers; our lab is interested in the role of altered sphingoid metabolism with regards to acute myeloid leukemia. Previously, our lab developed an in vivo screen in mice for identification of potential phase 1 drugs for leukemia. Using this screen, we identified the two SK isoforms, SK1 and SK2, as cooperators with the oncogenic MYC in the induction of leukemia. The long-term purpose of this project is to determine the specific domains within the SK proteins that are required for cooperation with MYC in the induction of leukemia. To this end, we created mutations in specific regions of the SKs, including the catalytic domains, and phosphorylation sites. The impact that these mutations had on expression and activity was analyzed in vitro in HEK293 cells. Expression of WT and mutant forms of SK1 and SK2 were examined via real-time PCR and western analysis and compared to the enzymatic activities that were quantified using a radiolabeled assay. Now that our mutant forms of SK1 and SK2 have been characterized, future experiments will involve co-expressing them with MYC in our Tet-O-MYC model system of leukemia. Further characterizing the SK-MYC cooperation will provide an understanding of leukemogenic mechanisms for identification of potential therapeutic targets. This research was supported by National Cancer Institute grant R25-CA134283.

METHODS

Plate cells Transfect with constructs Harvest cells, make plates End point assays (WB, RT-PCR, activity assay)

SK activity assay (from Pitman, Pham, and Pitson Methods Mol Biol 2012)

1. Incubate at 37°C for 60 minutes

2. Add KCl and chloroform to create phase separation

3. Spot lower phase onto TLC plate, then develop plate in butanol/H2O/acetate acid

4. Expose to phosphor screen and read with phosphorimager

RESULTS

Fig. 3. Our in vivo model shows that expression of SK1 and SK2 accelerates MYC-induced leukemogenesis. BCLxL is shown as a positive control for a potent MYC cooperator and GFP is shown as a negative control.

CONCLUSIONS & FUTURE DIRECTIONS

We were able to characterize the relative expression activity of our mutant SK1 and SK2 isoforms in vitro. With this quantification as a guide, future directions include:

• Co-expressing the wild type and mutant SK isoforms with MYC in our TET-O-MYC mouse model of leukemia
• Determining the specific domains that interact with MYC to potentiate leukemogenesis

ACKNOWLEDGEMENTS

This research was supported by the R25 University of Louisville Cancer Education Program (NCI grant R25-CA134283).
Localized delivery of drug and contrast agent has the potential to address the current inadequacies of pancreatic adenocarcinoma diagnosis and treatment. With a five-year survivability of 6%, this phenotype is the deadlast form of cancer. The poorly ordered vasculature of pancreatic tumors combined with high expression of certain receptors makes this tumor difficult to diagnose and treat effectively. We created 25 nm, pH responsive, peptide targeted, mesoporous silica nanoparticles (MSNs) to act as a diagnostic nanodelivery system that preferentially delivers contrast to pancreatic tumors. The MSNs were conjugated with chitosan and V7 pH low insertion peptide to impart pH responsiveness and pancreatic tumor targeting, respectively. It was determined that loading the MSNs with IR 780 iodide dye did not appreciably affect the absorbance at 780 nm (loaded dye 784 nm), though slight attenuation was observed. Pancreatic cancer cell lines Panc1 and S2VP10 were assessed for MSN loading at physiological pH 7.4 and at cancerous pH 6.6. It was found that the MSNs showed 45.6x the fluorescence at pH 6.6 that was seen at pH 7.4, confirming pH responsive cell binding. These results suggest that the MSN system is a good candidate for future use with in vivo models of pancreatic adenocarcinoma.

Methods

Synthesis: Mesoporous silica nanoparticles (MSNs) were synthesized using a dual surfactant method, with cetyltrimethylammonium chloride (CTAC, 3.6 g, 8.14 mmol) as the pore-scaffolding and triblock copolymer Fluronic F127 (2.0 g, 0.159 mmol) as the growth limiting agent. Surfactants were dissolved in 50 mL of distilled water (pH 7.4) and stirred for 1 hour. TX-100 (10 mL) was added, followed by addition of sodium metasilicate (4.0 g, 0.159 mmol), sodium hydroxide (3 M, 30 mL), and ethanol (95%, 50 mL). The solution was heated to 90°C for 1 hour in a water bath. Complete precipitation was observed by the formation of a white solid. The solid was collected, washed with ethanol and distilled water, and dried in a vacuum at 60°C for 24 hours.

Chitosan Addition: Pure chitosan (0.1 g) was resuspended in ethanol (10 mL). 2% v/v functionalized MSNs were added and stirred at room temperature for 12 hours. The reaction was pushed to completion by addition of acetic acid (2 mL) and washed with ethanol and distilled water. The resulting C-MSNs were collected by centrifugation and dried in a vacuum.

Dye Loading: Suspended C-MSNs were mixed with a solution of IR 780 iodide dye (3.3 mM) in dimethylformamide (DMF). This solution was acidified to pH 3 with HCl (5 M) and stirred for 24 hours to allow dye to equilibrate within the MSNs. The solution was then diluted to pH 7.4 with NaOH (1 M) to close the dye into the particles. Dye loaded MSNs were purified by dialysis for 6 hours in a 1.8 mL dialysis tube. The final solution was dialyzed for 4 days against sodium phosphate buffer.

pH-Responsive Release: In Vitro Assay: Two pancreatic cancer cell lines, Panc1 and S2VP10, were seeded and allowed to reach confluence, then treated with pH specific media (pH 7.4 or 6.6) for 3 hours. Cells were then treated with C-MSN and loaded IR 780 dye nanoparticles for 10 minutes. Media was removed after incubation and cells were washed with PBS (pH 7.4 or 6.6). After the wash, cells were imaged on the Leica Odyssey.

Conclusions

In vitro trials with IR 780 iodide show significant differences in fluorescence from nanoparticles that have an absorbance curve very similar to that of the unloaded dye. A dual surfactant method of synthesis limits particle growth and allows for the creation of small mesoporous silica nanoparticles.

Future Studies

In the future, we would like to explore variable chitosan coating thicknesses for optimized contrast retention and release kinetics in mesoporous silica. We hope to plan to begin in vivo trials with mouse models, using Multidetector Computed Tomography as the primary imaging modality. This imaging modality will enable us to track in vivo dye release kinetics in real-time without sacrificing the animal. This will allow for comparison with in vitro experiments and the potential to begin Gemcitabine trials in vivo. Future in vivo testing of these nanoparticles will move targeted therapeutics one step closer to clinical translation.

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Genetic polymorphisms in 5-FU related enzymes predict complete pathologic response in rectal cancer

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Patients with locally advanced rectal cancer undergo preoperative neoadjuvant chemoradiation with approximately 70% exhibiting pathologic downstaging in response to treatment. Of these, 15-20% of patients exhibit “complete pathologic response” with no remaining viable cancer. There is currently no accurate test to identify patients who are complete responders to neoadjuvant therapy who may be able to avoid undergoing radical surgery.

5-Fluourouracil (5-FU) is an antimetabolite drug that is widely used in the neoadjuvant treatment of rectal cancer. Genetic polymorphisms affect the activity of enzymes involved in 5-FU metabolism, such as thymidylate synthase (TYMS), and may also account for differing responses to neoadjuvant treatment seen in rectal cancer patients. Detection of such polymorphisms might permit identification of patients likely to have a complete response to neoadjuvant therapy.

Methods

1. DNA was isolated from whole blood taken from patients with newly diagnosed rectal cancer who received neoadjuvant therapy (n=54) and whole genome amplification was performed.
2. Medical records were reviewed and a tumor regression grade (TRG) was given based on pre- and post-operative TNM staging (Ryan et al., 2005).

<table>
<thead>
<tr>
<th>Description</th>
<th>Tumor Regression Grade</th>
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<tbody>
<tr>
<td>No viable cancer cells or single cells/small groups of cancer cells</td>
<td>1</td>
</tr>
<tr>
<td>Residual cancer outgrown by fibrosis</td>
<td>2</td>
</tr>
<tr>
<td>Significant fibrosis outgrown by cancer or no fibrosis with extensive residual cancer</td>
<td>3</td>
</tr>
</tbody>
</table>

3. Polymerase Chain Reaction (PCR) was performed using previously published primers targeting the promoter region of TYMS (Pullarkat et al., 2001).

Forward: 5'-GTGGCTCCTGCGTTTCCCCC-3'
Reverse: 5'-GCTCCGAGCCGGCCACAGGCATGGCGCGG-3'

4. PCR products were separated by electrophoresis on a 3% agarose gel to visualize if a patient was homozygous for a double-tandem repeat (2R), a triple-tandem repeat (3R), or heterozygous (2R/3R). A single nucleotide polymorphism (SNP) may also be present in the second repeat unit of the 3R allele.

5. Restriction fragment length polymorphism (RFLP) assays were performed on patients with at least one 3R allele using HaellII, a restriction enzyme that recognizes the GGCC sequence and cleaves between the second and third nucleotides. The fragments were separated by electrophoresis.

Results

Figure 3 (left): TS PCR Gel Electrophoresis. The 2R allele band appears at 220-bp, while the 3R allele band appears at 250-bp.

Figure 4 (right): RFLP Gel Electrophoresis. SNPs were determined using a combination of PCR and RFLP gel electrophoresis results.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>2R/2R</th>
<th>2R/3R</th>
<th>3R/3R</th>
</tr>
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<tbody>
<tr>
<td># of patients</td>
<td>14</td>
<td>28</td>
<td>10</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>SNP</th>
<th>3C</th>
<th>3G</th>
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<tr>
<td>% of 3R population</td>
<td>26</td>
<td>20</td>
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Figure 5 (left): Frequency of TS Alleles and SNPs.

Figure 6 (right): TS Allele Frequency vs. TRG. Patients with at least one TYMS 3G allele were more likely to have complete or partial pathological response to 5-FU neoadjuvant therapy (OR 9.6 95% CI 1.2 – 75.7) (p=0.02).

Conclusions

Identification of patients with specific genetic polymorphisms in enzymes involved in 5-FU metabolism appear to predict the likelihood of complete response of rectal cancer to preoperative 5-FU based neoadjuvant therapy and may alter surgical decision making.

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Combined Therapy of Oncolytic Adenovirus and Temozolomide Enhances Lung Cancer Virotherapy In Vitro and In Vivo

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Introduction

- Lung cancer remains the leading cause of death from cancer worldwide. Most chemotherapy and molecular therapies are based on the induction of apoptosis. However, tumors frequently develop resistance to apoptosis prior to or during cancer treatment.

- Oncolytic adenoviruses (OAds) are very promising for the treatment of lung cancer. However, OAd-based monotherapeutics have not been effective during clinical trials. Therefore, the effectiveness of virotherapy must be enhanced by combining OAds with other therapies.

- In this study, the therapeutic potential of OAd in combination with temozolomide (TMZ) was evaluated in lung cancer cells in vitro and in vivo. The combination of OAd and TMZ therapy synergistically enhanced cancer cell death; this enhanced cancer cell death may be explained via three related mechanisms: apoptosis, virus replication, and autophagy. Autophagy inhibition partially protected cancer cells from this combined therapy. This combination significantly suppressed the growth of subcutaneous H441 lung cancer xenograft tumors in athymic nude mice.

- In this study, we have provided an experimental rationale to test OAds in combination with TMZ in a lung cancer clinical trial.

Results

1. Evaluation of oncolytic adenovirus-mediated cytopathic effect in permissive and non-permissive lung cancer cells and determination of TMZ IC50.

2. Evaluation of apoptosis induction.

3. Effect of TMZ on oncolytic adenovirus replication in permissive, non-permissive, and lung non-cancerous cells.


5. Evaluation of the therapeutic potential of combined therapy of Adhz60 with TMZ in subcutaneous lung cancer mouse model.

Conclusions

In summary, TMZ-induced autophagy provides a better cellular environment for adenovirus replication. The combination of both TMZ and Adhz60 enhances their potency reciprocally. This study represents a potential alternative to lung cancer therapy, because the increased oncolytic adenovirus replication induced by TMZ may facilitate the virus spread within the lung tumors.

Acknowledgements

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