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Article

Zinc-related treatments combined with chloroquine and gemcitabine for treating pancreatic cancer

Larissa Ma, Leya Joykutty
American Heritage School, Plantation, Florida

SUMMARY

Pancreatic cancer is one of the deadliest cancers, with a 10% 5-year survival rate. A prominent issue with the treatment mainly used today, gemcitabine, is that it is unable to penetrate the stroma around the pancreatic cancer tumor. The stroma is a dense network of cells that promotes cancer development and metastasis and creates difficulty for chemotherapy treatments to be delivered effectively to the tumor. Targeting the pathways responsible for both the stroma (namely the Hedgehog signaling pathway) and the tumor (the Ras/MAPK pathway) could potentially lead to more effective treatments. The dysregulation of zinc has been implicated in disease progression through these pathways, however its exact effect is unclear. TPEN is a metal ion chelator that is able to induce zinc deficiency. We hypothesized that if various dosages of TPEN and zinc are used in combination with Chloroquine and Gemcitabine, then combinations of medium to high doses of zinc, Chloroquine, and Gemcitabine will be the most effective at reducing cell proliferation. Several treatment combinations were created in a drug matrix and tested using a cell proliferation assay. To mimic the stroma-tumor interaction that would occur *in vivo*, we took the conditioned medium from pancreatic cancer associated fibroblasts (mCAF) and cultured pancreatic cancer cells (PANC-1). The results showed that while TPEN was able to significantly lower cell proliferation, it was not more effective than the current treatment. However, when combined with Chloroquine and Gemcitabine, zinc and TPEN both significantly lowered cell proliferation compared to Gemcitabine, suggesting a synergistic effect that resulted in a more cytotoxic treatment. Further research and clinical trials on this topic are needed to determine whether this could be a viable treatment for pancreatic cancer. This repurposed treatment could potentially increase survival rates and become a more affordable alternative to novel drug development.

INTRODUCTION

Pancreatic ductal adenocarcinoma, also known as pancreatic cancer, is a cancer that originates in the pancreas, an organ that releases enzymes into the bloodstream. It is one of the deadliest cancers with a 10% 5-year survival rate (1). There is very poor patient prognosis because of the often-late diagnosis due to the lack of symptoms and inconvenient screening techniques. Pancreatic cancer is typically caught in stage 4, when the tumor has already metastasized to other organs throughout the body. Therefore, surgical removal is no longer an option up to 80% of the time (2). A network of cells, called the stroma, surrounds the tumor, and is composed of stellate cells, immune cells, blood vessels, growth factors, and extracellular proteins. It takes part in immune suppression and has poor vascularization (3). These cells act as a protective barrier, making it harder for chemotherapy to reach the tumor and prove effective. Therefore, more effective chemotherapy drugs are needed. The issue with the current standard chemotherapy treatment, Gemcitabine, is that it is unable to penetrate the stroma around the pancreatic tumor (3).

Several pathways are associated with promoting the growth of these cells. Activating the Hedgehog pathway leads to the growth of fibrous tissue, or desmoplasia, creating the stroma. The Hedgehog pathway is regulated by a ligand called Sonic Hedgehog. When Sonic Hedgehog binds, it activates Smoothened which activates Gli, allowing for the Hedgehog signal to pass to the cytoplasm. This signaling leads to fibroblasts that promote cancer development and increases gemcitabine resistance. They provide a tumor environment suitable for pancreatic cancer development (4). In addition, the stroma and poor vascular density make it hard for chemotherapy to be delivered effectively to the tumor. Sonic Hedgehog expression levels are upregulated in cancer stem cells, which are resistant to many chemotherapies. Inhibition of this pathway has been shown to induce apoptosis and block proliferation (5). One of the most prevalent links to pancreatic cancer growth is the mutation of KRAS, an oncogene which is involved with 90% pancreatic cancer cases. The K-Ras protein functions as an on/off switch for pathways that regulate cell proliferation, growth, and death (6). The dysregulation of this protein leads to the growth and continuation of pancreatic cancer. Directly targeting K-Ras in treatment is difficult, so there is an effort to target downstream signaling pathways, such as the RAS/MAPK/ERK and the PI3K/AKT/mTOR pathways (7). The RAS/MAPK pathway regulates cell growth, differentiation, proliferation, apoptosis, and migration functions. When these functions are dysregulated, it causes the cells to grow out of control (8). In the RAS/MAPK/ERK pathway, MEK and ERK phosphorylate

other proteins to activate the pathway. The MEK/ERK pathway can also directly activate the Hedgehog pathway through non-canonical signaling.

Zinc is the second most abundant transition metal in the human body, and it plays an important role in cancer cells. Zinc is a signaling molecule, a structural component for many enzymes and proteins, and is found in the zinc finger domain of DNA binding proteins. Zinc is critical for cell proliferation, cell cycle regulation, differentiation, and apoptosis. The homeostasis of zinc is very important to cancer cells. Zinc overload and zinc deficiency are responsible for shutting down different pathways that are crucial to the cancer cells (9). In addition, zinc levels in serum are often decreased in cancer patients; however, measuring the zinc levels in tumors themselves have shown inconsistent results. In pancreatic cancer, the upregulation of the zinc transporter ZIP4 is linked to cell proliferation and migration (10). Other zinc transporters, such as ZIP3, are downregulated, implying the dysregulation of zinc levels (11). However, the effects of zinc levels on the Hedgehog and MAPK pathways in pancreatic cancer have been minimally described in literature. Previous research suggests that zinc regulates the MAPK pathway in T-cells. Although the mechanisms are not solidified, elevated intracellular free zinc led to inhibition of the phosphorylation of ERK and a decrease in MAPK-dependent cytokine production in T-cells (12). On the other hand, in breast cancer cells, zinc has been shown to stimulate the MAPKs ERK1 and ERK2 through the zinc-dependent activation of the G protein-coupled estrogen receptor. This leads to EGFR stimulation in which the downstream signaling leads to ERK activation (11). Furthermore, zinc can inhibit the auto processing and generation of the Hedgehog ligand in astrocytes (13). Because of the contrasting roles of zinc on the MAPK/ERK pathway and the Hedgehog pathway in other cells, the effect of zinc in pancreatic cancer cells needs to be characterized.

TPEN is an ion chelator that can remove metals from the body. It has a high affinity for zinc and is membrane permeable, allowing for intracellular zinc depletion. TPEN, when used in pancreatic cancer, depleted zinc, and promoted cell death by increasing reactive oxygen species and inhibiting pro-survival autophagy (14). In this study, we used this drug to induce zinc deficiency.

Gemcitabine is the current standard treatment for late-stage pancreatic cancer. Gemcitabine's mechanism of action starts when gemcitabine triphosphate is incorporated during DNA synthesis by DNA polymerase, causing the polymerase to be unable to continue and therefore synthesis is stopped, leading to apoptosis. In addition, gemcitabine activates the MAPK pathway to trigger apoptosis in tumor cells, but not in normal cells. However, gemcitabine can have difficulties passing the barrier around the tumor. The dense stroma and poor vascularization from the Hedgehog pathway led to poor drug delivery and lowered drug efficacy. In addition, tumors can develop chemoresistance to gemcitabine (15).

Chloroquine is a drug originally used to treat malaria. It has many anticancer effects, such as: inhibiting autophagy, the Hedgehog pathway, and the phosphorylation of MEK/ERK and enhancing zinc uptake. In cells, chloroquine enters the lysosome and then increases the pH, inhibiting the digestive enzymes of the lysosome, which prevents autophagy from occurring. Chloroquine inhibits Hedgehog signaling by reducing the production of Smoothened, a protein that is important to Hedgehog signaling. Chloroquine is also a zinc ionophore, meaning that it allows zinc to transport across the membrane and concentrate inside the lysosome (16), which is similar to the mechanism of the drug PDTC, which has been shown to synergize with gemcitabine and zinc (17).

These treatments could work together in a variety of ways. Using a combination of the drugs Gemcitabine, TPEN, zinc, and Chloroquine would help inhibit the Hedgehog pathway and the MAPK pathway. Chloroquine and different levels of zinc inhibit the Hedgehog pathway through canonical and non-canonical signaling. The MAPK/ERK pathway, which also leads to Hedgehog activation, is inhibited by Chloroquine and different levels of zinc. Zinc and Chloroquine also are known to have synergistic effects on each other. Chloroquine enhances zinc uptake, while zinc enhances the cytotoxicity of Chloroquine. Gemcitabine has been shown to synergize with drugs similar to Chloroquine and Zinc (namely, Disulfiram and zinc.) When these drugs are applied together, there is the potential that the stroma will be inhibited, allowing for the efficient administration of gemcitabine and repressed cell proliferation.

In this study, we examined how varying amounts of zinc deficiency by TPEN and zinc overload by zinc impact cell proliferation of PANC-1 cancer cells. We hypothesized that medium to high doses of zinc would be most effective in reducing proliferation of PANC-1 cells. Furthermore, we hypothesized that if TPEN and zinc are used in combination with Chloroquine and Gemcitabine, then combinations of medium to high doses of zinc, Chloroquine, and Gemcitabine would be most effective at inhibiting cell proliferation of PANC-1 cells.

RESULTS

Cell proliferation of the PANC-1 cells after 24 hours of treatment was measured using the WST-8 cell proliferation assay. This assay is a colorimetric assay in which tetrazolium salt, WST-8, is reduced to a soluble purple formazan by trans-plasma membrane electron transport from NADH. Greater absorbance of this color indicates higher cell proliferation. The PANC-1 cells were treated with conditioned medium from pancreatic cancer associated fibroblasts (mCAFs) to mimic tumor-stroma interactions in which soluble factors from the fibroblasts may affect pancreatic cancer cell proliferation.

The results from the varying concentrations of zinc were analyzed by t-tests comparing treated samples to the untreated control. Following up with a Bonferroni Correction, the significance level was adjusted to 0.0166. We did not find any statistically significant differences after treatment

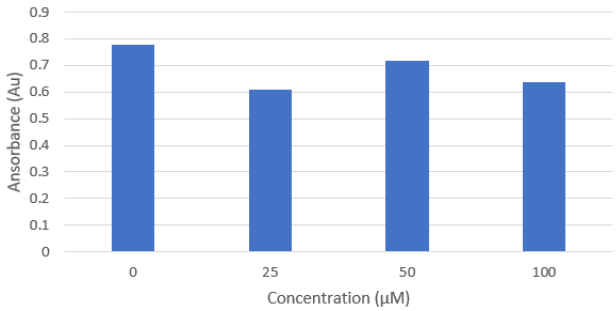

Figure 1. Cell proliferation 24 hours after zinc application. The average absorbance from the WST-8 cell proliferation assay was measured after 24-hour treatment of PANC-1 cells with one of four doses of zinc (25 μM, 50 μM, 100 μM) from four trials. Multiple t-tests were performed comparing the doses of Zinc to the negative control. Adjusted α = 0.0166. No statistically significant differences were observed (Student's t-test and Bonferroni Correction).

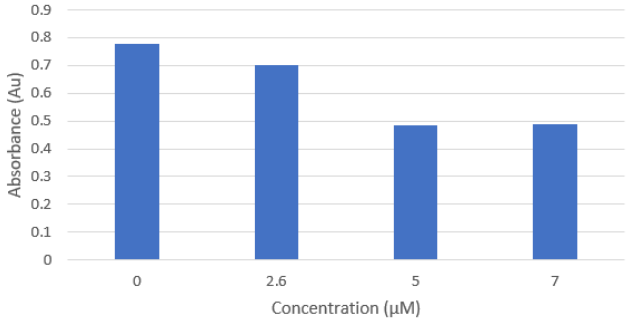

Figure 2. Cell proliferation 24 hours after TPEN application. The average absorbance from the WST-8 cell proliferation assay after 24 hours of varying doses of TPEN (0 μM, 2.6 μM, 5 μM, 7 μM) from four trials. Multiple t-tests were performed comparing the doses of TPEN to the negative control. Adjusted α = 0.0166. No statistically significant differences were observed (Student's t-test and Bonferroni Correction).

with three concentrations of zinc compared to untreated controls (p=0.14, p=0.35, p=0.20 for low, medium, and high doses of zinc respectively) **(Figure 1)**. We did not find any statistically significant differences after treatment with 3 concentrations of TPEN compared to untreated controls (p=0.33, p=0.019, p=0.024 for low, medium, and high doses of TPEN respectively) **(Figure 2)**. A single-factor ANOVA was performed to compare the combination treatments and Gemcitabine. There was a statistically significant difference between groups (p<0.0001). Tukey's post hoc test was used to compare each combination to Gemcitabine, the positive control. We found that the treatment of TPEN and Chloroquine (p=0.0367) and the treatment of zinc, chloroquine, and gemcitabine (p=0.0444) both significantly lowered absorbance compared to Gemcitabine **(Figure 3)**.

DISCUSSION

In the present study, we looked at the effect of zinc-related treatments with Chloroquine and Gemcitabine on PANC-1 cell proliferation. These treatments were chosen because of their unclear effect on disease progression and associated pathways. Namely, the tumor-stroma interactions are a current difficulty with the standard treatment, Gemcitabine. To mimic these interactions, a conditioned medium from mCAFs was used to test the impact of various treatments on cell proliferation. We found that zinc alone did not have any statistically significant effect on cell proliferation as compared to untreated cells. However, medium to high concentrations of TPEN, a zinc chelator, were effective in lowering cell proliferation as compared to untreated cells. When comparing combined treatments to Gemcitabine, treatment with TPEN and Chloroquine and treatment with zinc, Chloroquine, and Gemcitabine significantly decreased cell proliferation compared to Gemcitabine alone. Although it is still unclear the exact role that zinc addition and depletion plays in cell proliferation, we have discovered two effective treatments that lowered the cell proliferation as compared to Gemcitabine.

A limitation of our study was the relatively small sample size and that only one assay was conducted. Several other assays should be conducted in future studies to measure the effects of these treatments on the MAPK and Hedgehog pathways, such as a migration assay and measurement of intracellular reactive oxygen species (ROS) levels. These processes are associated with the growth of the stroma. Although condition medium was used, it does not completely model the tumor-stroma interactions that would occur in vivo. Lastly, the treatments could potentially interact with healthy cells in the body in unknown ways. While more research is required, this study provides a better understanding of potential pancreatic cancer treatments.

Although we found treatments that inhibit cell proliferation in this model, future research should be done in different models to confirm our findings and test for long-term effects and consequences. Different cell lines could be used, including other pancreatic cancer cell lines and chemotherapy resistant cell lines. An in vitro 3D tumor model of fibroblasts and

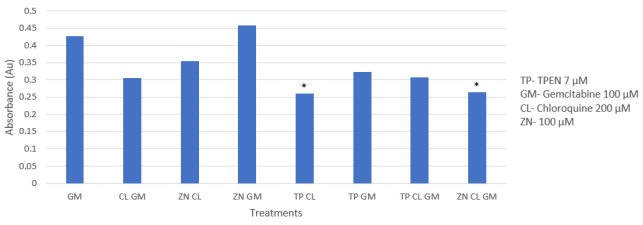

Figure 3. Effect of combination treatments on cell proliferation. The average absorbance from the WST-8 cell proliferation assay after 24 hours of combination treatments and the positive control from four trials. An ANOVA and further Tukey post hoc tests were performed comparing the combinations to the positive control. Treatment with TPEN and chloroquine and treatment with zinc, chloroquine, and gemcitabine significantly decreased cell proliferation compared to gemcitabine alone. *p<0.05.

pancreatic cancer cells could mimic more closely the tumor-stroma microenvironment in an actual tumor. The treatments could also be tested in animal models to investigate possible consequences, and eventually clinical trials. Future research should also use additional assays to specifically measure the effects of these treatments on the expression of the RAS/MAPK and Hedgehog Signaling pathways. Additionally, other drugs could be added to the combination and tested for possible synergistic effects.

Our results provide potential treatments that can effectively decrease pancreatic cancer cell proliferation compared to the current treatment. In addition, these treatments could potentially be applied to other cancers with strong stroma-tumor interactions, such as lung, breast, ovarian, and colorectal cancers. Finally, creating a drug concoction with drugs that are already on the market, also known as drug repositioning, is a more affordable alternative to new drug development and marketing for pancreatic cancer. Together, these results have potential to advance the current treatment options for patients.

METHODS

Cell Line and Maintenance

The pancreatic cancer cell line used was PANC-1 and received as a donation from the Morehouse School of Medicine. Human pancreatic cancer associated fibroblasts (mCAF) cell lines were received as a donation from the Fox Chase Cancer Center. These cells were used to recreate the tumor-stroma interactions occurring in a tumor, such as the effect of the mCAF-derived soluble factors on PANC-1 cell growth and response to treatment. Both cell lines were maintained under culture with Dulbecco's Modified Eagle's Medium (DMEM) and supplemented with fetal bovine serum (FBS) at a concentration of 10% and Penicillin-Streptomycin at a concentration of 1% at 37°C and in 5% CO_2.

Drug Treatments

The conditioned medium from the mCAF cells was collected after 24 hours from nearly confluent T-25 flasks. The media was spun down, and the supernatant was used in treatments. The treatments were dissolved using dimethyl sulfoxide (DMSO) and then further dissolved using the conditioned medium. The PANC-1 cells were then exposed to zinc, TPEN, gemcitabine, and/or Chloroquine for 24 hours. We tested the following conditions: TPEN low (2.6 μM); TPEN medium (5 μM); TPEN high (7 μM); Zinc low (25 μM); Zinc medium (50 μM); Zinc high (100 μM); Gemcitabine; Chloroquine; Chloroquine and Gemcitabine; Zinc and Chloroquine; Zinc and Gemcitabine; TPEN and Chloroquine; TPEN and Gemcitabine; TPEN, Chloroquine, and Gemcitabine; and Zinc, Chloroquine, and Gemcitabine. The concentrations used for the combined treatments were 100 μM Gemcitabine, 200 μM Chloroquine, 100 μM Zinc, and 7 μM TPEN. The experiment was conducted four times for all treatment groups.

Cell Proliferation Assay

Cell proliferation was examined using the WST-8 Cell Proliferation Assay Kit from Cayman Chemical. PANC-1 cells were seeded at 104 cells per well in 100 μL of culture medium with the treatments being tested. The cells were cultured in a CO_2 incubator at 37°C for 24-48 hours. 10 μL of the WST-8 Mixture was added to each well, and the cells were incubated for two hours at 37°C in a CO_2 incubator. Results were measured using a microplate reader at a wavelength of 450 nm.

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