

Characterization of Novel Drugs that Target the Hedgehog Signaling Pathway in Tumor-Induced Bone Disease

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KEYWORDS. Bone metastases, Hedgehog Signaling Pathway, Gli2

BRIEF DESCRIPTION. This research presents information about optimized transfection conditions for MDA-MB-231 cells, as well as cell viability data on known antagonists.

ABSTRACT. Breast cancer is the second leading cause of cancer deaths and has a propensity to metastasize to bone. Patients with skeletal metastases experience increased pain and fracture with a dramatic reduction to survival. While palliative therapies such as bisphosphonates improve bone quality, there are no cures for skeletal metastases. Previous studies have established that once tumor cells establish in bone they begin to express Parathyroid Hormone related Protein (PTHrP), a factor that can stimulate bone destruction cells. As the bone is destroyed, growth factors are released from the bone matrix that promote tumor proliferation, thus feeding into the vicious cycle of bone destruction. Our previous studies have demonstrated that PTHrP expression and bone destruction are regulated by the transcription factor, Gli2, while other groups have demonstrated that Gli2 contributes to tumorigenesis [1]. Therefore, we hypothesize that inhibition of Gli2 will reduce bone destruction and tumor growth. To test this hypothesis, we will test a panel of six small molecule antagonists that have been shown to inhibit pathways that regulate Gli2 expression and function. Identifying effective Gli inhibitors could potentially lead to new therapeutic approaches for treating skeletal metastases that reduce bone destruction and tumor growth, thus improving patient outcomes.

INTRODUCTION.

Breast cancer is the second leading cause of cancer deaths [2]. Breast cancer patients have a propensity to develop metastasis to the bone thereby decreasing their chance of surviving significantly [3]. Patients have access to palliative care that increases bone mass, increasing time to fracture and improving quality of life, but there are currently no therapeutic cures that target the tumor established in the bone.

The bone environment is tightly regulated by osteoclasts and osteoblasts. Osteoblasts are cells that synthesize the bone while osteoclasts degrade bone cells therefore maintaining homeostasis. In healthy bone, the osteoblast and osteoclast maintain homeostasis, degrading and reforming bone at a harmonious rate. However, in bone metastasis from breast cancer, there is a dysregulation in the activity of the osteoclasts [4]. Factors like the Parathyroid Hormone related Protein (PTHrP), increase the activity of osteoclasts causing bone destruction [5]. Key factors important in regulating bone metastases are TGF- β , PTHrP, and RANKL [5]. This research focuses on the pathway that regulates these growth factors: Hedgehog signaling pathway.

The Hedgehog (Hh) pathway is an important process in cell regulation, growth, and maintenance in healthy cells. The Hh signaling pathway has been reported to play a vital role in the destruction of the bone during bone metastases [6]. Gli2, a Hh signaling molecule is upregulated due to TGF- β stimulation. Previously published studies also show that Gli2 is an upstream transcription factor of PTHrP and is implicated in osteoclast mediated bone destruction. If Gli2 is inhibited, inhibition of Gli2 could prevent future bone degradation caused due to tumor establishment.

In this report, a library of six small molecule inhibitors that inhibit the Hh pathway were characterized using a bone tropic metastatic breast cancer cell line, MDA-MB-231. This work will lead to further studies to elucidate the molecular mechanisms of the drugs used to inhibit specific molecules like Gli2. Successful

inhibition of Gli2 inhibitors would a novel approach to developing therapeutic treatments for tumor-induced bone disease.

METHODS.

Culturing of MDA-MB-231 Cells

Cell culturing was performed to grow and maintain cells in a controlled environment outside of their normal conditions. The human breast cancer cell line used was a MDA-MB-231 cell, and they were obtained from ATCC [1]. In addition, MDA-MB-231 cells were stably transfected with green fluorescent protein (GFP) expressing plasmid to create a MDA-231-GFP clone. MDA-MB-231 and MDA-231-GFP cells were incubated 37° C at 5% CO₂ in 1X DMEM (Cellgro) supplemented with 10% heat inactivated fetal bovine serum (FBS, Atlas) and 1% Pencillin/ streptomycin (Complete DMEM media). Cells were split and expanded by rinsing with a phosphate buffered saline (PBS) and then incubating in trypsin, an adhesion cleaving protein.

Novel Drugs used for Characterization

Novel Drugs were used to determine IC₅₀ values using an MTS assay (Promega). A total of six antagonists were characterized. Hedgehog Pathway Inhibitors (HPI) 1, HPI 3, HPI 4, and GANT58 (or G58) are small molecule drugs that inhibit the Hedgehog Signaling Pathway as previously published [7]. In addition to HPI 1, HPI 3, HPI 4, and GANT58, two compounds were extracted from *Exoecaria agallocha*. *Exoecaria agallocha* is a plant that the compounds were obtained from, and the compounds are known to be Gli-mediated transcriptional inhibitors in other metastatic cell lines: Compound 1 and Compound 2 [8].

Transfections with Effectene and Lipofectamine 2000 at Optimized Amounts

A transfection optimization was performed to find the best reagent and conditions for MDA-MB-231 cells. The optimal transfection concentration will allow for better analysis of Gli2 when they are treated with drugs. Two reagents were used to transfect MDA-MB-231 cells: Effectene and Lipofectamine 2000. MDA-MB-231 cells were plated at a density of 6.5x10⁴ per well in a 48 well plate. The cells were then prepped to be transfected with a GFP plasmid and reagent per each manufacturer's protocol. Transfections were done with two reagents to determine which of the reagents transfected the highest amount of MDA-MB-231 cells. The transfections were performed at the manufacturer's suggestion as well as optimized concentrations. The DNA (μ g) to Effectene (μ L) ratios were 1 to 12.66, 1 to 21.33, 1 to 25, 1 to 37.33, 1 to 50, and 1 to 53.33. The DNA (μ g) to Lipofectamine (μ L) ratios were 1 to 0.5, 1 to 1.4, 1 to 2.6, 1 to 3.8, 1 to 4.7, and 1 to 5. Abbreviations for the concentration are listed in Table S1.

Determining GFP Expression in Transfected Cells

GFP expression was determined in order to view the results of the transfected cells. After 17 and 36 hours, the 48-well plate with the transfected cells was read using the Synergy2 plate reader (Biotek) to quantify the amount of cells transfected by the GFP luminescence.

Determining IC₅₀ of the Novel Drugs by performing a MTS Assay

Performing a MTS assay helped us characterize the drugs affect on the viability of MDA-MB-231 cells, and determine the IC₅₀ value. Cell viability was done

in vitro by performing an MTS assay using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit (Promega). Four drug concentrations were tested for each inhibitor: 0 μ M, 25 μ M, 50 μ M, and 100 μ M. Four thousand cells were plated on a 96-well plate in triplicates and growth was measured hourly for 24 hours at 450 nm on a Synergy2 plate reader per manufacturer's instructions.

Statistical Analysis

All statistical analyses for cell culture experiments were performed using Prism 6. Values are presented as mean \pm standard error of the mean (SEM), and p-values determined using unpaired t-test, one-way ANOVA, and two-way ANOVA tests, where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ unless otherwise stated.

RESULTS.

Optimal Transfection Efficiency Obtained using Effectene and Lipofectamine 2000

In order to determine the optimal transfection approach, we used two different reagents with varying ratios of DNA and reagent at two time points 17 and 36 hours. Effectene ratio 1 μ g to 25 μ L and Lipofectamine 2000 ratio 1 μ g to 2.6 μ L were statistically compared as shown in Figure 1. This comparison helped determine which reagent is better for the MDA-MB-231 cells based on the standard ratio. The transfection showed that the fluorescence values for Effectene were significantly higher than Lipofectamine 2000 at 17 hours; however, fluorescence values weren't found to be significant at 36 hours.

Optimized concentrations of the Effectene and Lipofectamine 2000 reagent were performed to determine the best concentration for MDA-MB-231 cell transfection. The fluorescence readings in Figure 2 are shown for the Effectene and Lipofectamine 2000 reagents at 17 and 36 hours. Compared to the standard ratio, the optimized Lipofectamine 2000 ratios were not as significant as Effectene. Also, the overall GFP fluorescence value was higher for Effectene than Lipofectamine 2000.

Not only did we optimize the reagent ratio for the transfections, but we also optimized with increased DNA as shown in Figure 3. Increased amounts of plasmid were used to determine whether a change in the amount of plasmid affected the amount of cells transfected with the concentration ratios remaining unchanged. The GFP fluorescence with Effectene and Lipofectamine 2000 is higher with the increased amounts of DNA.

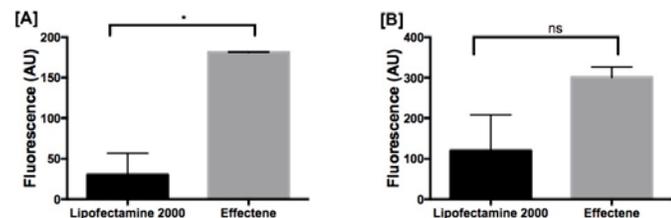


Figure 1. MDA-MB-231 cells were used for transfections per suggested manufacturer's concentration, which is 1 μ g to 25 μ L for Effectene and 1 μ g to 2.6 μ L for Lipofectamine 2000. Transfection optimization was left for 17 hours [A] and 36 hours [B]. An unpaired t-test was performed between with the suggested manufacturer's ratio for Effectene and Lipofectamine 2000 with * $p < 0.05$ and ns (nonsignificant).

IC50 values of Novel Drugs using a MTS Assay

HPI 1, HPI 3, HPI 4, Compound 1, and Compound 2 are recently discovered drugs that have never been tested in a bone metastatic line like MDA-MB-231. Characterization of these drugs on cell viability is presented in the values presented in Figure 4. Four different concentrations for each drug were used to determine the best IC₅₀ value. The cells treated with the drugs at the 12 hour time point and 24 hour time point presented no IC₅₀ values. The cells' viability increased for the majority of the drugs as the cells were treated with a higher drug concentrations; however, the standard error bars show the different ranges in the values.

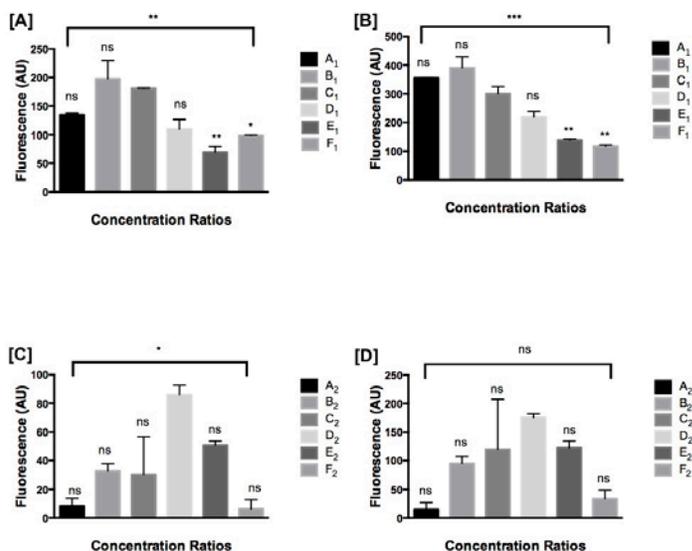


Figure 2. Effectene fluorescence values at 17 hours [A] and fluorescence values at 36 hours [B] are shown for the optimized concentration ratios. Lipofectamine 2000 fluorescence values at 17 hours [C] and fluorescence values 36 hours [D] are shown for the optimized concentrations ratios. A one-way ANOVA test was performed compared to the suggested manufacture amount with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0003$, and ns (nonsignificant).

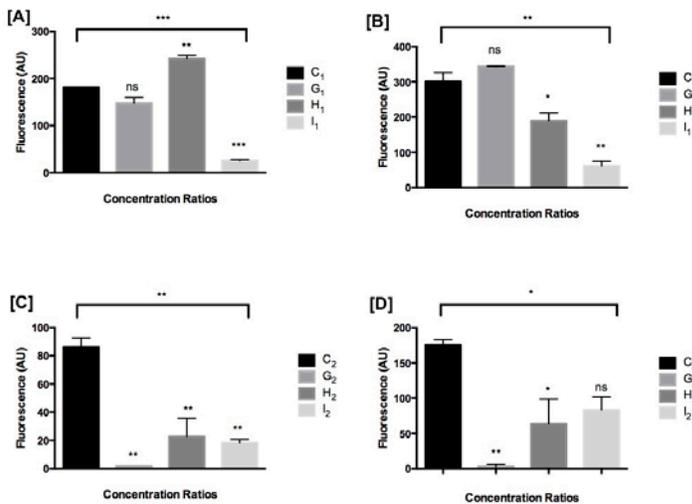


Figure 3. Effectene and Lipofectamine 2000 best ratio was compared to the ratios with the increased DNA. The data presented for Effectene at 17 hours [A] and Effectene at 36 hours [B] showed a significant change in 1 to 25 and 1 to 53.33. The data presented for Lipofectamine 2000 at 17 hours [C] showed a significant change in all concentrations with increased plasmid, and Lipofectamine 2000 at 36 hours [D] showed a significant change in only 1 to 0.5 and 1 to 2.6. A one-way ANOVA test was performed to compare the best ratio to the concentrations with the increased DNA with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0003$, and ns (nonsignificant).

DISCUSSION.

Bone metastases greatly decrease survival chances among breast cancer patients. Once the tumor metastasizes from the primary site to the bone, there are no therapies targeting the tumor in the bone microenvironment. Studies have discovered drugs that target tumor metastasis in the Hedgehog Signaling Pathway of different cancer cell lines [7]. This research focuses on determining the best conditions for transfections and the best concentration of the drugs for MDA-MB-231 cell line. By finding the optimized conditions with MDA-MB-231, the appropriate concentrations for the drugs and transfections would

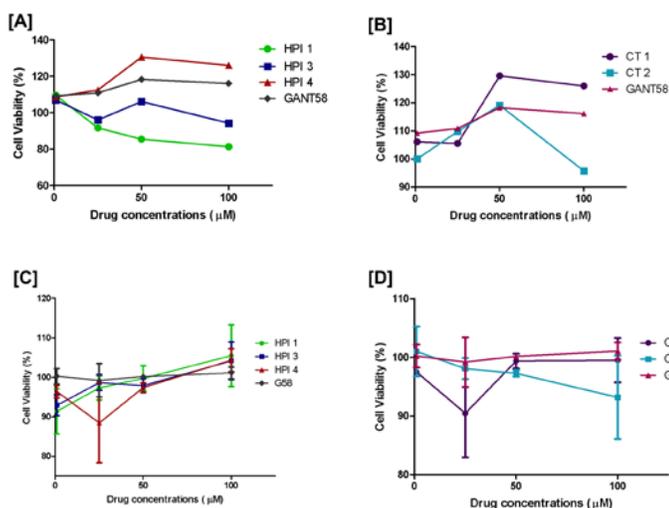


Figure 4. This figure shows the cell viability for HPI 1, HPI 3, HPI 4, GANT 58, Compound 1, and Compound 2. Each inhibitor was traced using four different concentrations 1 μM , 25 μM , 50 μM , and 100 μM . [A] and [B] showed the cell viability for the MDA-MB-231 cells after at a 12 hour treatment period. [C] and [D] showed the cell viability after a 24 hour treatment period.

provide the best treatment option. Characterizing HPI drugs with different cell lines provides a better understanding of the effects on different mechanisms.

The performance of a transfection optimization on MDA-MB-231 cells concluded that Effectene had a higher fluorescence value than Lipofectamine, which leads us to believe that using Effectene provides the best transfection for the cells. Effectene concentration of 1 μg to 25 μL and Lipofectamine 2000 concentration of 1 μg to 2.6 μL provided an overall better DNA to reagent ratio for Effectene and Lipofectamine 2000. Transfection with Effectene increased DNA seemed to provide a significant increase in fluorescence values; however, Lipofectamine 2000 with increased DNA had significant decrease in fluorescence values. With the transfection optimization for the MDA-MB-231, Effectene is concluded to be the better reagent for transfecting MDA-MB-231 at a 1 to 25 concentration ratio with increased DNA. With the optimized transfection concentration, experiments such as drug treatment and analysis of Gli2 expression can be conducted.

Analyses of the drugs were taken to determine the effect on cell viability. The MTS assay was performed to determine the IC_{50} values for HPI 1, HPI 3, HPI 4, GANT58, Compound 1, and Compound 2. The IC_{50} values for all of the drugs were not determined for treating MDA-MB-231 cells. Figure 4 didn't show that the cell viability decreased as the drug concentration increased as we expected. From this data, we determined that the drugs did not have a direct effect on the cells' mechanism to proliferate. To conclude the results found, another MTS assay should be conducted to further analyze the drugs because of the large error bars to receive consistent values in the experimental and biological replicates at the different concentrations.

With the conclusions from the optimized transfection concentration and MTS assay, studies can be conducted to look at the Gli2 expression in MDA-MB-231 cells. The optimized transfection reagent and concentration was concluded in this research along with a better characterization of the novel drugs. Further studies can be done to determine gene expression of osteolytic factors such as TGF- β and PTHrP. Since Gli2 is upstream of PTHrP, then with PTHrP stimulation there should be a noticeable increase in the Gli 2 expression. TGF- β is stimulated in the presence of metastatic breast cancer tumor, which therefore also induces increased Gli 2 expression.

Finding an optimal transfection concentration as well as finding accurate drug concentrations can provide a better understanding of the mechanisms in MDA-MB-231. The optimization provided the concentration that should be used

during the next transfection, and then those cells can be treated with the different drug concentration from the MTS assay. This research can then be used to determine the future targeted areas in the Hedgehog Signaling Pathway. The characterization of the novel drugs used in this research serve as preliminary data that can be further analyzed to provide future treatments to patients with tumor-induced bone disease that not only provide palliative treatment, but also inhibit the tumor from continuing to metastasize. The overall goal is to try and find a treatment that targets the metastasized cancer in the bone of MDA-MB-231 cells, and this research provides the preliminary studies needed to get one step closer.

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SUPPLEMENTAL INFORMATION.

Figure S1. This image shows the relationship between the main components in bone metastasis. PTHrP in the metastatic tumor is increased which then releases RANKL. RANKL upregulates the production of osteoclasts that then deteriorates the bone. Gli2 is necessary for the activation of TGF- β fueling the expression of PTHrP in an endless cycle.

Table S1. The concentrations used for Effectene and Lipofectamine 2000 are matched with a letter abbreviations in the table with G, H, and I being the reagents with more DNA. Future figures refer to the abbreviations for the concentrations ratios listed.



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