Assessment of Hedgehog/GLI2 Signaling Inhibitors Within Metastatic Breast Cancers

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BRIEF. Hedgehog pathway inhibitors were established as potential remediation treatments for tumor-induced bone disease. Additionally, hypotheses concerning inhibition methods were established for future investigation.

ABSTRACT. The inappropriate activation of the Hedgehog (Hh) signaling pathway within metastatic breast cancers promotes cancer growth as well as severe bone degradation. The overexpression of an upstream component, Gli2, and has been correlated to the progression of both effects. Five compounds including synthetic Hh pathway inhibitors(HPI 1, HPI 3, and HPI 4) and Excoecaria agallocha tea extracts (Tea 1 and Tea 8) were isolated as Hh/GLI2-mediated inhibitors and surveyed for potency and ideal dosages within metastatic breast cancer cells overexpressing the Gli2 gene. Reduced cell mRNA levels of Gli2-mediated expression of parathyroid hormone-related protein (PTHrP) demonstrated satisfactory inhibition by four of the five compounds. Additionally, HPI 3 was found to possibly inhibit by targeting Gli2 activation while HPI 1, HPI 4, and Tea 1 likely inhibit Gli2 transport into the nucleus. The discovery, qualification, and standardization of this drug inhibition aids in the possible reduction and remediation of tumor-induced bone disease within metastatic breast cancer

INTRODUCTION

The bone is a common target region of metastasized breast cancer leading to tumor-induced bone disease which causes bone destruction leading to severe pain and fracture, dramatically impacting patient quality of life. Breast cancer patients face increased mortality and morbidity at a 5 year survival rate of only 29% [1]. Canonical Hedgehog (Hh) signaling pathway transmits information to embryonic cells for proper development and plays a vital role in cell differentiation and survival [2]. The inappropriate activation of this signaling pathway can, in various cancers, promote oncogenesis and result in the formation of bone lesions. Hedgehog pathway inhibitors (HPI) have been isolated as both synthetic and natural compounds and found to be viable within an array of different cancers, including human pancreatic and prostate cancer cells [3]. However, HPIs are relatively novel and their role in the complex relationship between Hh inhibition and tumor promotion is not well understood, particularly for breast cancer. This study expands the understanding of HPIs and their role as therapeutic approaches to the reduction of bone disease and the pervasiveness of metastatic breast cancers. Drug viability studied in this work indicates distinct inhibition methods which provide insight into the future direction for remedial treatment.

The Hh signaling pathway serves a complex role in the metastasis of breast cancer. Within the bone environment, the Hh ligands (Sonic Hedgehog (SHH), Indian Hedgehog (IHH), or Desert Hedgehog (DHH)) bind to the membrane receptor Patched1 (Ptch1), which activates Smoothen (Smo), an inhibitor of SuFu. Inhibited SuFu releases its repression of Gli2 protein within the cytoplasm. With Gli2's translocation into the nucleus, this protein is converted to a transcriptional activator and promotes upregulation of expression of the osteolytic factor, parathyroid hormone receptor protein PTHrP [1][3]. The resulting bone degradation by osteoclasts prompts the release of growth factors such as TGF-β, increasing tumor growth in a self-promoting cycle [1]. Hedgehog pathway blockage occurring at the Gli2 point can inhibit PTHrP expression, therefore lessening bone destruction. Though Gli2 does not affect the tumor directly, its downstream effects encourage growth factor support for metastatic tumors and bone lesions. Inhibition of Gli2 will result in lower growth factors and less space for tumor expansion due to the decreased number of bone lesions [1].

Gli2's role in the activation of the Hedgehog pathway has made Gli2 a recent drug target for reducing tumor-induced bone disease. Future work is necessary to characterize Gli2 inhibitors' efficacy, the ability to produce a desired biological effect, and potency, the minimum concentration required to produce a biological effect. Within this study, three synthetic and two naturally occurring Hedgehog pathway inhibitors were targeted as possible additions to the investigation into Gli2 blockage. Hh/GLI- mediated transcriptional inhibitors (naturally occurring) have been isolated from Exocoecaria agallocha leaves and Zizyphus cambodiana [4], [5]. Further research has revealed that compounds, such as epigallocatechin-3-gallate (EGCG), in Japanese green teas can have inhibitory effects on the Gli family, especially Gli2 [6]. Thus, two tea compounds extracted from Exocoecaria agallocha were incorporated into the purpose of this study. Overall, four of these proposed inhibitors were successful in furthering research into this issue with the realization of possible optimal concentrations and inhibition mechanisms.

MATERIALS AND METHODS.

Cells.

The human breast cancer line, MDA-MB-231, was acquired from the American Type Culture Collection (ATCC). A bone metastatic variant was generated in the lab to be used for in vivo procedures [1]. The MDA-MB-231 cells were maintained in Dulbecco's Modified Eagle Medium plus 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin. The cells were incubated at 37 °C with 5% CO2.

Hedgehog Pathway Inhibitors (HPI).

A total of five HPIs were evaluated for potency and efficacy. HPI 1, 3, and 4 were purchased from Specs. The Excoecaria agallocha extracts, Quercitrin (Tea 1) and Kaempferol 3-O-L-arabinofuronoside (Tea 8), were purchased from Carbosynth.

Drug Treatments.

MDA-MB-231 cells were plated in triplicate in a 6 well tissue culture plate at 3.6 x 105 cells per well and incubated at 37°C for 24 hours before treatment. Cells were treated with an array of concentrations based upon known IC50 values, the concentration at which the inhibition level is at 50% (Table 1) [4]. This value is commonly discovered with the use of a dose response assay measuring Hedgehog pathway inhibition as a result of varying drug concentration over a large range. Dimethyl sulfoxide (DMSO) was used as a vehicle control and administered at the highest concentration for each drug treatment. Treated cells were incubated at 37°C for 24 hours prior to downstream molecular assays.

Quantitative Real-time PCR.

Briefly, cells were harvested with trypsin 24 hours after drug treatment and total RNA was extracted using the RNeasy Mini Kit (Qiagen). The qScript cDNA supermix (Quanta, VWR) was used to synthesize cDNA using 1 µg total RNA, per manufacturer's instructions. Quantitative Real-time PCR (qPCR) was performed using PTHLH (Hs00174969_m1) and Gli2 (Hs01119974_m1) Taqman gene expression assays run on the 7500 Real-Time PCR System (Applied Biosciences). Specifically, cDNA was serially diluted to create a standard curve. The cDNA samples and standard curve were combined with TaqMAn Universal PCR Master Mix (Applied Biosystems), and primer: PTHLH (Hs00174969_m1), Gli2 (Hs01119974_m1), or TaqMan Euk 18S rRNA. Samples were loaded onto a clear 96 well plate and the

Q-PCR reaction was performed under the following cycling conditions: 95ºC for 10 min, followed by 40 cycles of 95ºC for 15sec, 60ºC for 1 min. Gene expression changes were calculated using the absolute quantification method using 18S as an internal control.

Table 1. Drug concentration ranges. An * denotes the IC50, the concentration at which there is 50% inhibition

Drug	Concentrations (μM)				
HPI 1	0	2	4*	6	8
HPI 3	0	30*	60	75	100
HPI 4	0	30*	60	75	100
Tea 1	0	0.25	0.5*	0.75	1
Tea 8	0	2*	4	5	6

Western Blot.

The cells were lysed with radioimmunoprecipitation (RIPA) lysis buffer containing a cocktail of protease and phosphotase inhibitors (ThermoScientific). Equal protein concentrations of 16.25 μl were prepared for loading with NuPAGE sample buffer (Life Technologies) and separated on a 10% SDS-PAGE gel (BioRad). Each protein sample was loaded in duplicate. Proteins were transferred onto a Polyvinylidene fluoride (PVDF) membrane in transfer buffer [(25 mmol/L Tris, 192 mmol/L glycine, 20% (v/v) methanol (pH 8.3)] at 30 V at 4ºC overnight. The membranes were blocked with Odyssey Blocking Buffer containing TBS (Tris Buffered Saline) consisting of 1% Tween 20 for 1 hour (Li-Cor). The primary antibody Gli2 (1:1000, Santa Cruz) was then added to the membrane and allowed to incubate overnight at 4˚C. After washing with TBST, the blots were incubated with anti-goat IgG horseradish peroxidase secondary antibodies (AbCam) at a dilution of 1:1000 for 1 hour. The membrane was washed and the signal was detected by enhanced chemiluminescence using an In-Vivo MS FX Pro (Bruker). α GAPDH antibody (Santa Cruz) was used as a loading control.

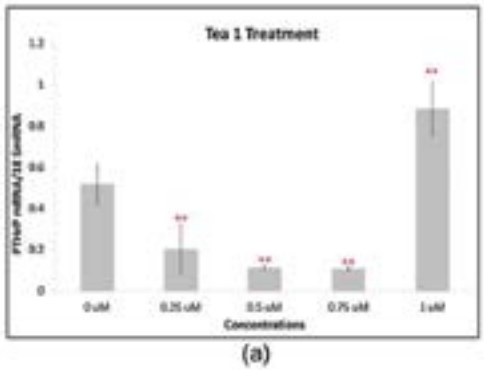
Statistical Analysis.

Instat GraphPad software was used to analyze the PTHrP mRNA results for each drug treatment. A one way ANOVA was used to compare the means of each drug concentration to that of the respective DMSO control.

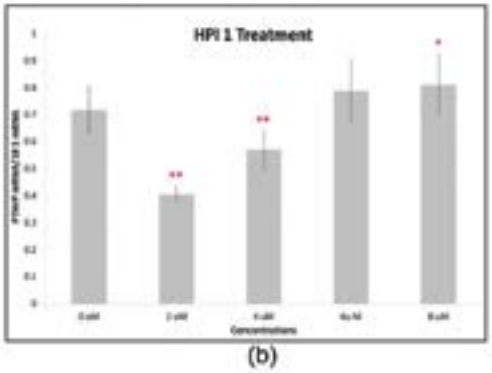
RESULTS.

Suppression of PTHrP mRNA.

Gli2 induces expression of parathyroid hormone receptor protein. Therefore, the inhibition of PTHrP mRNA in the MDA-MB-231 cells was determined using qPCR. Figure 1 presents the mean values of PTHrP mRNA/18 S mRNA levels for HPI 1, HPI 3, HPI 4, and Tea 1 at each assessed concentration over three trials. Tea 8 exhibited little to no inhibition of PTHrP mRNA. The mRNA suppression by Tea 1 is displayed in Figure 1a, it was also found to carry out satisfactory suppression of PTHrP mRNA. The effective dose for Tea 1 was found to be 0.5 μM. The concentration of 0.5 μM had the least variable inhibition levels and was also found to have a p-value of P<0.01. The results of the HPI 1 drug treatment are presented by Figure 1b. The concentration of 2 μM was found to be the most effective as it had a levels of reduction with a p-value of P<0.01. HPI 1 additionally exhibited variations of PTHrP that corresponded to the characteristics of a standard dose response curve. HPI 3 and HPI 4 also displayed apparent inhibition though their drug treatments had higher levels of variation. The effective dosage of HPI 3 was determined to be 75 μM. As exhibited by Figure 1c, the concentration of 75 μM had significantly lower PTHrP mRNA levels, with a p-value of P<0.01, and had a lower standard deviation.



(a)



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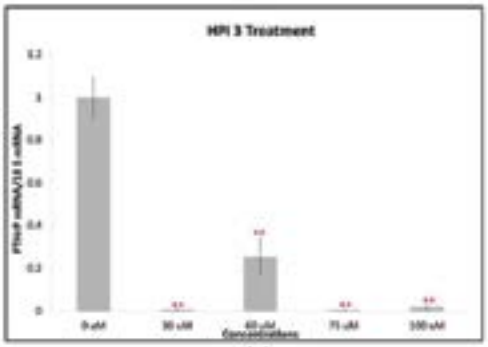

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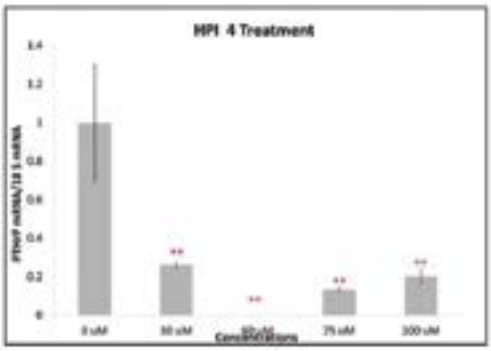

(d)

Figure 1(a-d). HPI 1, HPI 3, HPI 4, and Tea 1 suppression of PTHrP mRNA with 18S used as a loading control. The bars represent the average of triplicate samples. The asterisks represent the degree of statistical significance in comparison to the 0 μM concentration.

Likewise, for HPI 4, 75 μM was found to have a p-value of $P<0.01$ and possessed a small standard deviation (Figure 1d). Though HPI 4 does appear to be carrying out complete inhibition at 60 μM, this result is likely to experimental error during the qPCR amplification of DNA as complete inhibition in this case is unlikely.

Gli2 Protein.

The inhibitors' ability to target the Gli2 protein at different steps of the Hedgehog pathway was assessed using a Western blot for total Gli2 protein within a drug treated MDA-MB-231 cell (Figure 2). HPI 1, HPI 4, and Tea 1 did not significantly affect the amount of total Gli2 protein within the cell. Their Gli2 protein levels were not found to be much higher or lower than the protein levels of the control. However, HPI 3 showed signs of Gli2 protein reduction that is most likely stemming from Gli2 protein reduction within the cytoplasm.

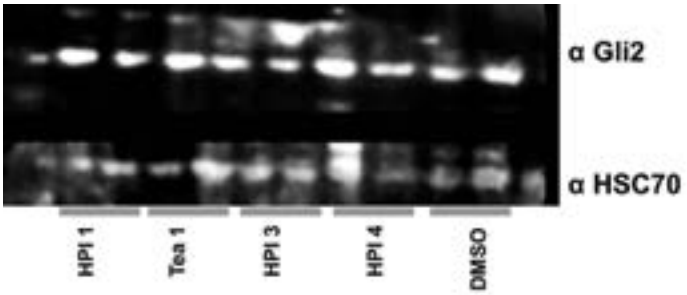

Figure 2. Western blot showing changes in total Gli2 protein for duplicate samples of each drug. HPI 3 reduced Gli2 protein which suggests inhibition of Gli2 activation.

DISCUSSION.

Overall, for each inhibitor, PTHrP mRNA was expected to be the lowest at concentrations recognized previously as established IC50 values, or concentrations of 50% inhibition. At higher concentrations, PTHrP levels were expected to rise as the cells became saturated with the drug, thus leaving it ineffective. For Tea 8, no conjectures concerning optimal concentration could be made from the data received from its testing. However, Y. Rifai et al found it to be relevant in the suppression of Gli2 activity though this study focused in inhibition of preceding proteins along the Hh pathway [4]. The effective dose of the HPI 1 drug also differed from previously established IC50 values [3]. HPI 1 was found to inhibit best at 2 μM, a concentration lower than previous studies conducted by J.M Hyman et al. HPI 3 and HPI 4 were found to inhibit best 75 μM, which fell into the range determined by this study [3]. The variation in the HPI 1 findings could be due to the different methods used in determining inhibition. This study focused on PTHrP mRNA levels while J.M Hyman et al. focused on inhibition at the promoter level. It is essential that a standard should be confirmed between these differing readings. There is a possible risk of underdosing or causing the cells to become saturated. Additionally, it is more opportune for drugs to be more effective at lower concentrations, which reduces cost and inefficiency.

The results of the Gli2 Western blot procedure provided insight into the inhibition mechanisms of the successful drugs. The Western blot analyzed the total amount of Gli2 protein in the cell. There were two main points of Gli2 targeting identified within this study; these are described in Figure 3. Gli2 protein can be targeted within the cytoplasm of the cell, where it is concentrated before nuclear transport. At this point, inhibitors can stunt Gli2 activation, cancelling any downstream effects. Alternately, drugs can be active along the route of Gli2's nuclear transport into the cell, restricting the transcription of PTHrP. In relation to a control protein, cells treated with HPI 3 were found to have reduced levels of Gli2 protein. This leads to the hypothesis that HPI 3 is effective in inhibiting Gli2 activation within the cytoplasm. Reduced Gli2 shows degradation, which is most likely occurring in the cytoplasm. On the other hand, the total protein levels of HPI 1, HPI 4, and Tea 1 were higher and suggested later inhibition of nuclear transport. The Gli2 protein does not seem to be degrading so its function may be the target of the inhibitors. These findings coincide with previous conclusions about Tea 1 which is thought to be a transcriptional inhibitor [2].

This study has established the viability to HPI 1, HPI 3, HPI 4, and at least one Excoecaria agallocha extract. This research has also had the needed purpose of testing the efficacy of small molecule inhibitors that target downstream effects of the Hedgehog pathway [1]. Pathways of targeting Gli2, and therefore PTHrP, were also established and in need of further investigation.

CONCLUSIONS AND FUTURE DIRECTIONS.

This work clarified potential pathways of Gli2 inhibition, previously unknown. These mechanisms markedly differed for at least one of the drugs, HPI 3. Additional data about these findings can be attained by conducting Western blots that separately gauge the amounts of nuclear and cytoplasmic Gli2 protein. The concentration of Gli2 in each area after drug treatment can confirm inhibition in the cytoplasm or along the pathway of nuclear transport. It is necessary for these inhibition mechanisms to be better understood for coordination of drug treatments; drugs targeting different points of Gli2 activity can doubly restrict its effects and further reduce bone degradation.

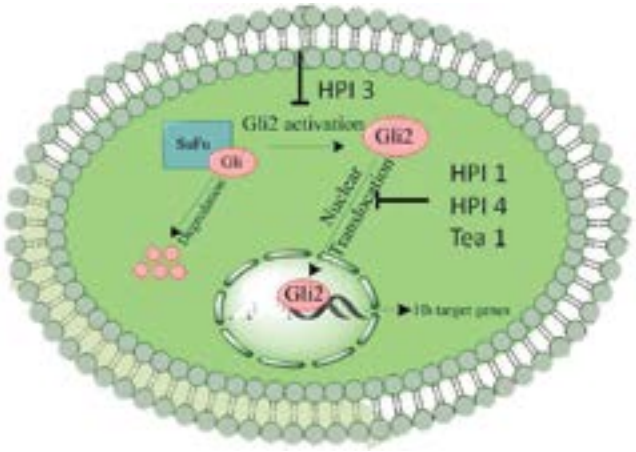

Figure 3. The possible points of inhibition by each drug are exhibited. From the results of this study, HPI 3 is thought to affect Gli2 activation while HPI 1, HPI 4, and Tea 1 are thought to antagonize Gli2 activity along the route of nuclear translocation.

Many questions about the mechanisms of Gli2 inhibition still remain unclear. Future directions include further investigation of HPI 3, HPI 4, and Tea 8 to provide more certain conclusions on effectiveness. A luciferase reporter assay with a PTHrP construct should be conducted to understand the inhibition of Gli2 at the promoter level and ensure the validity of the effective concentrations. Additionally, a cytotoxicity assay should be carried out to ensure that the drugs are targeting Gli2 and not simply killing the cells. In view of future progression in application, the viability of these drugs should be assessed within the bone environment as it is believed that skeletal structure has an effect on tumor growth [7][8]. Knowledge of these drugs' inhibition of bone destruction will significantly affect patient quality of life and potentially survival.

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