UDC 575.
https://doi.org/10.2298/GENSR1901347G
Original scientific paper

DRUG REPOSITIONING FOR A RARE GENETIC DISORDER *PROGRESSIVE OSSEOUS HETEROPLASIA (POH)*

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Gvozdenović-Jeremić J. and Lj. Mojović (2019): *Drug repositioning for a rare genetic disorder progressive Osseous heteroplasia (POH)*.- Genetika, Vol 51, No.1, 347-355.

Progressive osseous heteroplasia (POH) is an ultrarare genetic disease of progressive ectopic ossification caused by heterozygous inactivating mutations of GNAS, the gene encoding the alpha subunit of the G-stimulatory protein of adenylyl cyclase (Gsα). Extensive ossification of the deep connective tissues can result in ankylosis of affected joints and growth retardation of involved limbs. Inhibition of main molecular signaling, Hedgehog (Hh) pathway, by pharmacological methods may reduce the severity of ectopic bone formation in POH patients. Hh inhibitors currently used or known for other conditions may be potential candidate drugs for treating this debilitating disease. In this study, three potential Hedgehog pathway inhibitors such as arsenic trioxide, statin, and vitamin D and their combinations were tested on subcutaneous mesenchymal progenitor (SMP) cells of $G\alpha_s$ *f/f* mice model for possible therapeutic application for POH. The combination of these three drugs at their significantly reduced concentrations retained anti-osteogenic activity in SMP cells with aberrant Hedgehog activity. In that light, we propose here a potential new approach of the drug combination in order to reduce potential toxicity, the side effect and increase success rate for Hh inhibitors drug repositioning.

Keywords: drug repurposing, ectopic ossification, Hedgehog pathway inhibitors, mesenchymal progenitor cells, *Progressive Osseous Heteroplasia*

INTRODUCTION

Progressive Osseous Heteroplasia (POH) is a rare genetic condition, where heterotopic ossification tends toward the formation of mainly intramembranous bone tissue in response to inactivating mutations in the GNAS locus (EDDY *et al.* 2000; KAPLAN *et al.* 1994, SHORE. *et al.* 2002). This mutation is at the core of pathophysiology of heterotopic ossification, which affects

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mesenchymal progenitor cells fate determination, leading to a painful formation of bone in skeletal muscle and adipose tissues. Inappropriate bone formation in soft tissues predominantly occurs through an intramembranous process, and ectopic osteoblasts differentiate from mesenchymal progenitors independently of chondrocytes in POH disease (PIGNOLO *et al*, 2015). POH patients show variable malformation of natural skeletal elements, and recognizing the causative genes and their associated signaling pathways as key mediators of skeletal development is of ultimate importance, in addition to regulating cell-fate decisions by adult stem cells.

Because POH often goes unrecognized or misdiagnosed, the prevalence of this disorder in the general population is hard to access. Clinically, symptoms are presented during infancy with dermal and subcutaneous ossifications that progress during childhood into skeletal muscle and deep connective tissues. In the most severe cases, signs are usually apparent at birth or within the first few weeks of life. Over time, ectopic ossification leads to ankylosis of affected joints and growth retardation of the affected limb. There are an estimated one hundred patients globally diagnosed with severe POH (Online Mendelian Inheritance in Man #166350). Lack of pharmacological treatment option is a significant unmet medical need in these children. Understanding the cellular mechanisms responsible for these rare disorders might lead to the development of therapeutic approaches relevant to common conditions of excessive and insufficient bone formation.

Recently, it has been demonstrated that loss of GNAS causes heterotopic ossification by activating Hedgehog signaling molecular mechanism regulated by $G_s\alpha$ and inducing POH in our mice model (REGARD *et al.* 2013). Regard *et al*. have demonstrated that Hedgehog signaling activation is both, necessary and sufficient, to induce heterotopic ossification and that Hedgehog signaling must be actively suppressed by $G_s\alpha$ to ensure spatial restriction of bone formation to the normal skeleton.

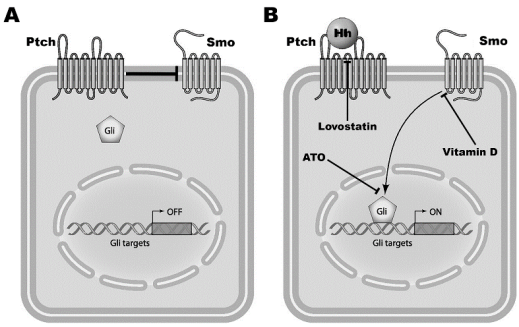

Fig 1. A proposed mechanism of Hedgehog pathway regulation by Hh inhibitors: **A**. In absence of Hh ligand, Hh pathway is suppressed in normal SMP cells **B**. In SMP cells with aberrant Gsα function and active Hh signaling pathway, the SMP differentiation into osteoblasts is initiated via Gli mediated transcriptional mechanism. Illustration depicts a different checkpoint of a proposed regulatory actions of Hh inhibitors.

In order to design an effective therapeutic treatment for the disease, the underlying molecular mechanism is necessary to be elucidated and explained. $G_s\alpha$ has emerged as a seminal regulator of mesenchymal progenitors in the skeletal system. $G_s\alpha$ is a physiological activator of PKA, an inhibitor of Hedgehog signaling that governs a wide variety of processes during development (JIANG *et al*. 1995; JIANG *et al* 2008. TUSON *et al*. 2011)). Emerging evidence points to aberrant Hedgehog (Hh) signaling pathway as an important etiological factor in *Progressive osseous heteroplasia* (REGARD *et al*. 2013). Mammalian Hh signaling pathway includes specific ligands (sonic Hh, Indian Hh, and desert Hh), patched receptor (PTCH1), signal transducer smoothened (SMO), and transcription factors (Gli1, Gli2, Gli3). In the absence of ligands, SMO signaling in cells is inhibited by PTCH1 (Fig1A). Upon binding of an active Hh ligand, this inhibition is released, allowing SMO to activate downstream Gli class of transcription factors (Fig1B). Gli molecules are known regulators of many oncogenes. Gli1 and Gli2 are also upregulated in bone forming progenitor cells, just prior osteoblastic differentiation (KAN *et al*. 2018; REGARD *et al.* 2013). Anticancer agent, arsenic trioxide (ATO) (FDA approved as "Trisenox"), directly binds to Gli1 protein and inhibits its transcriptional activity thereby silencing expression of Gli target genes, mainly essential for osteoblast differentiation in POH (Fig1). Recently, the evidence has been provided that previously identified Hedgehog signaling inhibitors, particularly Gli inhibitors that have been developed for cancer therapy, may be repurposed to treat heterotopic ossification and possibly other diseases caused by reduction of α- subunit of the G protein ($G_s\alpha$) that stimulates adenylyl cyclase activity (REGARD, *et al.* 2013). Thus, arsenic trioxide, as one of them, has the potential to suppress ectopic ossification. However, this inhibitory action was achieved at the high ATO concentrations that may be toxic to patients.

Furthermore, all known Hh proteins are secreted molecules, some of which act locally and some act as hormones and target distal tissues during development. Hh protein precursors undergo post-translational modifications or auto-cleavage to release the N-terminal fragment [HhN], which covalently associates with intracellular cholesterol at its C-terminal. This modification is essential to facilitate Hh binding to PTCH receptor. Thus, it is crucial to limit free (membrane) cholesterol availability and therefore to achieve low cholesterol binding to the Hh molecule immediately after secretion. This, in turn, will restrict Hh molecule binding to PTCH and further activation of Hh signaling in the cell at the site of pathologic bone formation. The inclusion of a statin in POH drug formulation is projected to improve inhibition of the Hedgehog signaling, which in turn will improve therapeutic outcome. Another, potentially effective, component to be included in POH drug formulation is an active form of Vitamin D3 (Calcitriol), which was shown to potently reduces aberrant regulation of Hh pathway in basal cell carcinoma (BCC) cell lines (TANG, *et al*. 2011). It was also reported that Vitamin D3 acts as a potent inhibitor of major Hh target gene *Gli1* and consequently downregulates its expression. Vitamin D is a fat-soluble prohormone whose primary biologic function is to maintain serum calcium and phosphorous homeostasis. It promotes calcium absorption in the gut and reabsorption from the kidneys and inhibits the secretion of parathyroid hormone. Vitamin D, therefore, enables the normal mineralization of bone by regulating bone growth and remodeling the activity of osteoblasts and osteoclasts in endochondral bone.

The purpose of this work was to determine a novel therapeutic potential of Hedgehog pathway inhibitors such as arsenic trioxide, statin and vitamin D and their combination for POH treatment. Therapeutic switching of already identified compounds and drugs combination is an alternative approach for increased success of new therapeutic applications such as POH.

MATERIAL AND METHODS

Cell culture and osteogenic differentiation

Subcutaneous mesenchymal progenitor cells (SMP) were isolated from GS alpha LoxP (*Gsα f/f*) mice subcutaneous skin tissue containing adipose deposits, following the procedure previously published (REGARD, *et al.* 2013). *Gsα f/f* mice were generated so that the metabolic effects of $G_s\alpha$ deficiency could be examined in specific tissues by addition of Adeno Cre virus or crossing with Cre specific mice lines (Exon1, which is specific for $G_s\alpha$, was surrounded with loxP recombination sites). SMP cells were further cultured in Alpha MEM (Invitrogen) media containing 20% Fetal Bovine Serum (Gibco), 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco) and 2mM L-Glutamine (Gibco) at 37ºC in a humidified incubator with 5% CO_2 in air until 80 % confluency. At this point cells were infected with Cre recombinase of adenovirus ($\sim 10^{10}$ pfu/ml) at 1:1000 dilution for four hours for the removal of LoxP cassette in cultured SMP cells. After that time, cells were rinsed with Alpha MEM media overnight. Next day, SMP cells were switched to osteogenic induction media containing: DMEM (Invitrogen), 10% lot-selected Fetal Bovine Serum (Gibco), 100 U/mL penicillin, 100 µg/mL streptomycin (Gibco), 2 mM glutamine (Gibco), 10^{-4} M L-ascorbic acid 2-phosphate (Wako) and 10 mM β-glycerol phosphate (Sigma Aldrich). In treated cells, osteogenic media was supplemented with Hh inhibitors and replaced every other day for total duration of six days.

Preparation of Hh inhibitors

Arsenic Trioxide (ATO) preparation: 50 mg of ATO was placed on the bottom of a 50 ml conical tube and dissolved it with 1 ml of 1M NaOH. 48 ml of PBS was then added to the tube and 0.82 ml of 1.2 N HCl was added to adjust pH to 7.2. The freshly made stock solution was further diluted in osteogenic media to 10 µM for an application in experiments.

Cholecalciferol (Vitamin D3) preparation**:** Cholecalciferol (Sigma Aldrich) was dissolved in ethanol at 10 mM stock solutions. Final concentration in experiments was 10 µM, freshly prepared in osteogenic media.

Statin (Lovastatin) preparation: Lovastatin, sodium salt (Calbiochem) was dissolved in ethanol to 100mM stock solution. Further dilution to 100 µM in ethanol was performed right before the experiment. Final concentration of 1 µM, diluted in osteogenic media, was used in the experiments.

Mix; Mixing all three components at a final concentration: Arsenic trioxide 5 µM, Lovastatin 0.5 µM and Calcitriol 5 µM directly in osteogenic media.

Gene expression analysis: The total RNA was isolated first with Trizol (Invitrogen) and then with the RNeasy Kit (Qiagen) with on-column DNAse digestion. The RNA concentrations were estimated on the basis of A_{260}. The RNA samples were reverse transcribed with High capacity RNA kit for quantitative reverse transcription-polymerase chain reaction (RT-PCR;

Applied Biosystems) using dNTP and random hexamers. Real-time,RT-PCR analysis was performed using iTaq Universal SYBR Green Supermix (BioRad) and BioRad CFX96 Realtime PCR cycler for 40 cycles of 95°C (15 seconds) and 60°C (for 60 seconds.). Primers used for amplification are provided in Table 1. The housekeeping gene, β-actin was used as a control for RNA loading samples. Relative gene expression was calculated with the $2^{(-\Delta\Delta CT)}$ formula.

Table 1. Primer sequences for RT-qPCR

Gene	Forward primer (5'-3')	Reversed primer (5'-3')
β-Actin	CAC AGC TTC TTT GCA GCT CCT T	CGT CAT CCA TGG CGA ACT G
ALP	CAC GCG ATG CAA CAC CAC TCA GG	GCA TGT CCC CGG GCT CAA AGA

Alkaline phosphatase staining: After six days of exposure in osteogenic media that contained or lack Hh inhibitors, SMP cells were rinsed with PBS and fixed in 4 % paraformaldehyde for ten minutes. Cells were then rinsed two times with PBS before exposure to 1-Step NBT/BCIP (Thermo Fisher) substrate solution for a one hour in the dark. This is colorimetric assay where 4- Benzoylamino-2,5-dimethoxybenzenediazonium chloride hemi salt is hydrolyzed by alkaline phosphatase to yield a bluish-purple precipitate. A digital camera attached to an inverted phase-contrast microscope (Motical 10.0 MP) was used for microscopic observation of biomineralization in control and Hh treated cells.

Statistics

All experiments and assays were performed in triplicate. Data are expressed as mean ± the standard error of mean (SEM). One-way analysis of variance (ANOVA) followed by *post hock* Dunnett's test was used for pairwise comparisons among groups. Differences were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

New drug therapies development remains time-consuming and costly. New approaches, strategies, and technologies are needed to accelerate new drug discovery and to improve the favorable outcome of drug development. An alternative approach for developing new therapeutics quickly, for many diseases that currently have no effective treatments, is repositioning of existing drugs or drug candidates. Additionally, the favorable outcome of new drug discovery and development does not adequately address the unmet clinical need for disease treatments. Here we explore the novel application of three Hedgehog pathway inhibitors: arsenic trioxide, statin, and vitamin D3 and their combinations to suppress differentiation of subcutaneous mesenchymal progenitor (SMP) cells into osteoblast and thus osteogenesis. For this purpose, we used SMP cells isolated from *Gsα f/f* mice, a recognized murine model for POH. All three Hh inhibitors visibly reduced mineralization (an absence or reduced blue staining) in SMP cells induced by osteogenic media (Fig. 2). The mix of all three single agents (1:2 dilution) also effectively inhibited mineralization of SMP cells (Fig 2). This is first time that

action and synergistic-like effect of Hh inhibitors was tested and shown to be equally effective against osteogenesis at their reduced concentrations.

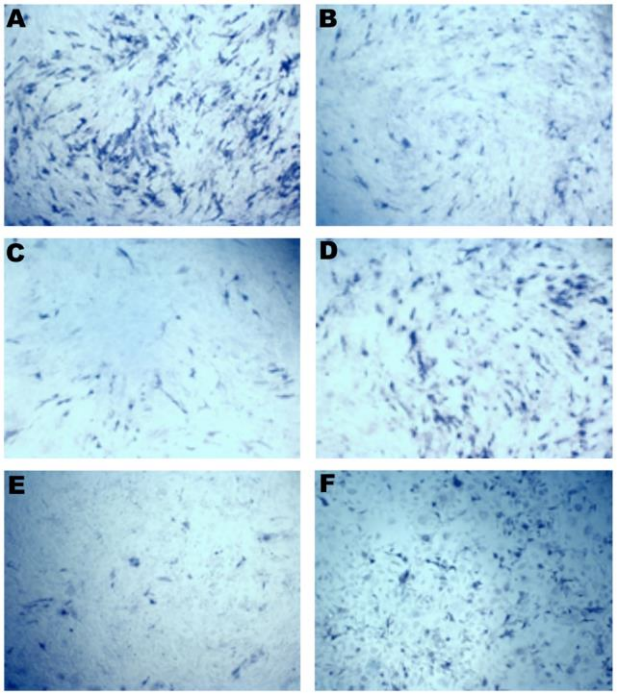

Fig 2. In vitro biomineralization - Alkaline Phosphatase assay. This assay was performed on subcutaneous mesenchymal progenitor (SMP) cells of $G\alpha_s$ f/f mice model infected with Adeno Cre virus (except F). Data shows inhibited mineralization (an absence or reduced blue staining) in the presence of single agent treatment (B) Arsenic trioxide 10μM; (C) Calcitriol 10μM; (D) Statin 1μM and (E) mix of all three single agents in 1:2 dilution or (F) SMP cells infected with Adeno GFP virus or (A) control (osteogenic media only with no drug present).

Figure 3 quantitatively characterizes the effect of the applied drugs alone or in a mixture on an early osteogenic marker- alkaline phosphatase (ALP) gene expression analysis, relative to the control.

This approach of approved drugs repositioning has recently increased in number, especially for the development of new therapeutics for diseases that lack any effective drug treatment. Drug combinations of two or more compounds with different mechanisms of action are an alternative approach to increase the success rate of drug repositioning (SUN *et al.* 2016). In agreement with drug repositioning strategy, the combination of Hh inhibitors had a comparable effect on the gene expression of the early osteoblast differentiation marker, alkaline phosphatase

(AP), as compared to individual treatments (Fig3.). Thus, inhibition of cell differentiation and aversion of ectopic ossification was retained even at lower concentration of inhibitors.

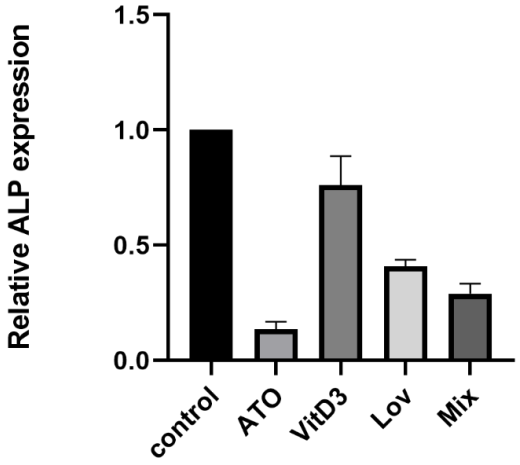

Fig 3. Alkaline phosphatase (ALP) gene expression analysis relative to the control (osteogenic media only) in presence of single agents: Arsenic trioxide (ATO) 10 μM; Vitamin D3(VitD3) 10μM; Lovastatin (Lov) 1 μM or their mix two-fold diluted, four days after exposure to osteogenic media and the addition of Ade Cre virus. All experiments are performed in triplicates. Data (mRNA levels) are normalized to the housekeeping gene actin and expressed as mean ± SEM relative to the control (set to 1).

In summary, combination approach comprising Cholecalciferol, Arsenic trioxide and statin may prove to be beneficial in the treatment of POH patients in which ectopic ossification is associated with aberrant Hh signaling activity. The advantage of using this multi-component approach lies in its unique ability to target different checkpoints of the Hedgehog signaling pathway (Fig. 1) thereby efficiently inhibiting trans-differentiation of subcutaneous mesenchymal stem cells into osteoblasts. The synergistic-like effect of such drug combination is enabling a significant reduction of required individual drug concentrations in a final combination (Fig. 2 and Fig. 3), which will likely minimize new combination drug side effects.

CONCLUSION

The novel, combined approach, consisting of three clinically tested Hh inhibitors with complementary mechanisms of action may increase their success rates against POH with reduced off target effects. This drug repositioning approach might offer a new hope for POH patients with no present therapy.

ACKNOWLEDGMENT

Dr. L.Weinstein from NIH NIDDK kindly provided mouse tissue from *Gsα f/f* mice.

Received, October 10th, 2018
Accepted February 18h, 2019

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PREUSMERAVANJE MEDIKAMENATA ZA PRIMENU U LEČENJU RETKE GENETSKE BOLESTI- PROGRESIVNE HETEROPLASTIČNE OSIFIKACIJE

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Izvod

Progresivna heterotopna osifikacija je ultra retka bolest kod koje je progresivna ektopična osifikacija posledica heterozigotne inaktivirajuće mutacije GNAS gena koji kodira alfa subjedinicu G- stimulacinog proteina adenilat ciklaze. Intenzivna osifikacija unutrašnjeg vezivnog tkiva može dovesti do anklioze zahvaćenih zglobova i retardacije rasta zahvaćenih ekstremiteta. Inhibicijom glavnog signalnog Hedgehog puta, farmakološkim metodama može se smanjiti obim stvaranja ektopične kosti kod pacijenata sa ovom bolešću. U ovoj studiji su ispitana tri potencijalna inhibitora Hedgehog signalnog puta kao što su arsen trioksid, statin i vitamin D, kao i njihove kombinacije u cilju pronalaženja adekvatnog tretmana. Ispitivanja su vršena na potkožnim progenitorskim mezenhimalnim ćelijama $G_s\alpha_{f/f}$ miša. Ispitivanja su pokazala da je jako dobre efekte i potencijal pokazala kombinacija gore navedena tri leka. Ova kombinacija ima značajne prednosti jer je njenom primenom moguće smanjiti toksične i neželjene efekte pojedinačnih visokih doza.

Primljeno 12.X.2018.
Odobreno 18. II. 2019.