The Affect of Pyrvinium, a Potent Small Molecule Wnt Inhibitor, on MSC Biology

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KEYWORDS. Mesenchymal stem cells, Wnt signaling pathway, pyrvinium

BRIEF. Inhibition of the Wnt signaling pathway promotes favorable tissue regeneration and remodeling.

ABSTRACT. Stem cells play a vast role in the maintenance and homeostasis of the human body. Stem cell therapy is a treatment that replaces dysfunctional tissues with stem cells. In order to improve this therapy, we hypothesized that controlling the Wnt signaling pathway would delay stem cell commitment to allow for cell growth at the outset of treatment. To test our hypothesis, the differentiation of MSCs in the presence of pyrvinium, Wnt inhibiting molecule, was monitored using specific gene markers. Overall, the study showed that pyrvinium successfully inhibited Wnt signaling and decreased differentiation of MSCs. While previous studies have targeted Wnt inhibition, pyrvinium is the first therapeutic tool today able to demonstrate similar results.

INTRODUCTION.

Adult stem cells play a great role in the maintenance and homeostasis of the human body. To list the ways in which stem cells are important is a broad task as stem cell use is virtually limitless. They have vast potential to treat a number of diseases and conditions that people have been struggling with for centuries. Adult stem cell research has been groundbreaking are in treating cancer, Type 1 diabetes mellitus, Parkinson's disease, Huntington's disease, Celiac Disease, cardiac failure, muscle damage and neurological disorders, and many others [1]. What makes these cells so truly promising rests in three main characteristics. First, stem cells can renew and proliferate themselves almost indefinitely [2]. Second, stem cells have the special ability to differentiate into cells with specialized characteristics and functions [2]. Lastly, stem cells themselves are largely unspecialized cells, which then give rise to specialized cells [2]. With these defining features a degeneration-regeneration cycle is created that is able to revitalize the tissue of target and maintain the tissue's functions.

In order to completely understand the potential of stem cells in many of the diseases listed, the advantages and disadvantages of stem cells therapy has to be considered. Stem cell treatments require intervention of new cells to settings where tissue has been damaged. These new cells should able to replace the previous ones as they differentiate into the tissue of interest [3]. However, a major problem arises with this therapy like in any other invasive treatment. The tissue has a difficult time accepting the cells that are newly introduced cells [3]. Stem cells can simply be added, though no guarantee that the cells well adapt to fit their surroundings can be promised [3]. The best way improve this process is not adding more and more cells, wasting valuable money and resources. However the key is to allow the stem cells additional time to proliferate and grow [3].

In turn, to study how development in stem cell therapy can be made, we specifically looked into bone marrow derived mesenchymal cells (MSCs), which have been widely accepted by both the scientific community and public alike. They not only have major potential, but they also do not bring up the ethical debate that embryonic derived stem cells bring about. Mesenchymal stem cells are plastic adherent stem cells that play a significant role in the regeneration of musculoskeletal tissues, such as bone and cartilage [4]. The first to observe the differentiation with MSCs was Pittenger et al., demonstrating that MSCs from bone marrow have the potential to differentiate into cartilage, bone, and fat cells [4].

One morphogenesis factor that is necessary for the commitment and differentiation of MSCs to osteocytes and chondrocytes is the Wnt signaling pathway [5]. Proteins from the Wnt family are essential in a wide array of developmental and physiological processes [5]. Wnt signaling can particularly control various cellular and biological processes, ranging from cell adhesion, stem cell self renewal and cancer development, to cell polarity, cell migration, and cell proliferation [5]. The increase of β-catenin levels is the key element associated with the Wnt signaling pathway [5]. When the Wnt ligand cannot bind to the receptor on the cell's surface, the cell maintains low cytoplasmic β-catenin levels due to its foremost degradation. β-catenin degradation is dependent on a complex containing glycogen synthase kinase 3 (GSK3), Casein Kinase 1α (CK1α), Adenomatous Polyposis Coli (APC), and Axin [5]. This "destruction complex" phosphorylates β-catenin by GSK3 and then targets for degradation by the uniquitin-proteasome pathway (UPP) [5]. However, when Wnt signaling pathway is active and the Wnt ligand is able to bind to the Frizzled and low-density lipoprotein-related receptor 5/6 (LRP5/6), the β-catenin "destruction complex" is inhibited and the scaffold protein Axin is degraded [5]. As a result, with the presence of the Wnt ligand there is an increase in cytoplasmic β-catenin [5]. The β-catenin is able to enter the nucleus and interact with other elements to activate TCF/LEF1 transcription factors to promote specific gene expression [5].

Many studies have been done on the Wnt signaling pathway to show that its inhibition promotes favorable remodeling and regeneration of MSCs. In a previous paper, when the expression of a natural Wnt modulator, Secreted frizzled-related protein 2 (sFRP2), was increased, MSC proliferation also enhanced consistently and notably when compared to similar molecules not specific to Wnt inhibition, such as Dkk1, sFRP3, and sFRP4 [6]. In addition, MSC engraftment vascularitiy of MSC deposited granulation tissue had both increased with in vivo models, identifying sFRP2 as the key MSC-derived factor mediating myocardial survival and repair [6]. Similarly in another study, Sfrp2 was also found to modulate Wnt signaling and demonstrate that cardiomyocytes treated with sFRP2 increased cellular β-catenin and up-regulated expression of antiapoptotic genes [7]. With these studies and many others, sFRP2 was targeted because of its nature as a negative regulator of the Wnt signaling pathway and the positive repair and remodeling outcome it promoted [9,1].

Despite the overwhelming studies done on the Wnt signaling pathway, there are no pharmacological tools yet to date that are able to specially inhibit the Wnt pathway [5]. If there was compound that could carry out the same tasks that sFRP2 does genetically, then the MSCs could simply be treated with the compound and injected to the wound site. While sFRP2 may sound like the answer to inhibit Wnt, the cost and possibility of gene therapy must be considered. Gene therapy is the changing or removing of genes within an individual's cells and biological tissues to treat disease. However, gene therapy today has exorbitantly high costs and therefore not an option for most families [8]. Unfortunately, gene therapy has also not been proven very successful in numerous randomized clinical trials [9]. This change at the very base of our genetics also raises a number of ethical questions. While the Food and Drug Administration (FDA) is still not quite sure how to regulate genetic testing and therapy, there is an obvious lack of government oversight on the process and its dependability [9]. The more likely solution would be to move around using gene therapy. While the demand to improve stem cells is increasing day by day, the gene therapy availability is seemingly out of sight. A Wnt inhibiting compound would create groundbreaking advances in the medical field.

In the present study, we utilized a screened FDA-approved small molecule inhibitor pyrvinium, a drug that has been identified to inhibit Wnt signaling [10]. Pyrvinium, compound 30, is able to work by activating and targeting Casein kinase 1 [10]. Casein kinase is the main enzyme that phosphorylates β-catenin and causes its proteolytic degradation [10]. In a recent study by Curtis et al, a

chemical screen showed that pyrvinium inhibited Wnt signaling. A TOP-flash reporter assay was done showing the absorbance of the luciferase gene, which is up-regulated when β-catenin progresses to the nucleus and gets transcribed. In the presence of pyrvinium, Wnt signaling was illustrated through the down regulation of luciferase as concentration of pyrvinium is increased. However when compared to its molecular analog compound 211, that is not specific to Wnt inhibition, the luciferase signals remained the same.

In Saraswati, *et al.*,MSC proliferation was quantified in vitro and in vivo increased with the presence of pyrvinium. In the in vitro study the same concentration of pyrvinium and compound 211 were added to MSCs, and checked for proliferation using BrdU assay. The results demonstrated pyrvinium increased proliferation. The in vivo experiment investigated cellularity via sponges loaded with MSCs injected with pyrvinium or 211 and was quantified using rt-PCR of GFP labeled MSCs. The results showed the MSC engraftment increased with pyrvinium and GFP levels in the pyrvinium treated cells had increased.

From these previous studies, we can conclude that pyrvinium inhibits Wnt signaling and increases the proliferation of MSCs, similarly to sFRP2. However, the differentiation has yet to be fully examined. The differentiation of the MSCs treated with pyrvinium is a key element in order to examine the complete effect of the compound on the development of stem cells, as differentiation is of the main characteristics responsible for their distinguished qualities. This study investigates and addresses one of the most important factors, differentiation, in order to improve MSCs for treatments without gene therapy. To fulfill their potential in the medical field we hypothesis that an immediate decrease of MSC differentiation is beneficial to buy time for cells to maximize proliferation.

MATERIALS AND METHODS.

MSC Culture.

The source of the MSCs was bone marrow derived mouse MSCs. These stem cells were initially grown as monolayer cultures in Dulbecco's modified Eagle's medium containing 10% defined fetal bovine serum (Hyclone, Mediatech) and antibiotics in 10 cm2 plates. In order to propagate the cells, MSCs were trypsinized with 0.25% trypsin (CellGro, Mediatech) after washing the monolayer with phosphate buffer saline (PBS). The cells were counted using a hemocytometer and seeded at a density of 8 x 105 in a 10-cm2 plates for propagation. *Insect Model.*

MSC Differentiation.

In order to observe differentiation, cells were plated at a density of about 6.0 x 103 cells/well in 6 well tissue culture plates and tissue culture slides (BD Falcon) in freshly made differentiation media. The osteogenic differentiation media contained 10% hyclone FBS, 0.1 mM ascorbic acid, 10 mM β-Glycerophosphate, 10-7 M dexamethasone, Antibiotics, and fungizone in low glucose Dulbecco's modified Eagle medium. The chondrogenic media consisted of 10% hyclone FBS, 0.01 ug/ml transforming growth factor beta (TGF-β), 10-7 M dexamethasone, antibiotics, and fungizone in low glucose Dulbecco's modified Eagle Medium. The cells were differentiated for 8 days at 37 °C in the presence or absence of treatments. The osteogenic and chondrogenic media with or without treatments were changed every 3 to 4 days.

Treatment.

During differentiation the MSCs were treated with 100nM concentration of pyrvinium in the presence or absence of Wnt3a (R & D). Wnt3a (50ng/ml) or compound 211 (100 nM) alone or in combination was used to treat the cells for positive and negative controls of Wnt inhibition, respectively. Pyrvinium and compound 211 were added every 3 to 4 days and Wnt3a was added every day for 8 days.

RNA Isolation and Quantitative Real-time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR).

The osteogenic and chondrogenic differentiation was stopped on the 8th day. The cells were washed with PBS and 500 ul of the TRIZOL reagent (Invitrogen) using the Gibco Life Technologies protocol. For both the osteogenic and chondrogenic studies, the cells were harvested on day 8. The total RNA was isolated from the cells using TRIZOL reagent (Invitrogen) and first strand cDNA was synthesized with reverse transcriptase and oligo(dT) priming (iScript, BioRad), from total RNA. One ug of cDNAs was used for PCR amplification using individual primers (Integrated DNA Technologies) specific to collagen XI and ostecalcin including the constitutive gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Real-time RT-PCR was performed in triplicate for each sample with a commercial system (iCyler; BioRad) and fluorescence detection (FastStart SYBR Green;Rocher). Each reaction was normalized against 18S.

Alizarin Red and Toluidine Blue Staining.

MSCs cultured on the tissue culture slides in the presence of osteogenic media were stained with alizarin red to detect calcific deposition. Cells were fixed on tissue culture slides in 100 % ethanol following a wash in PBS for 15-20 minutes at room temperature. Fixation solution was then removed and cells were washed with PBS. Filtered 2% Alizarin Red stock solution (pH 4.2) adjusted to have a pH of 6.4 was added to the fixed cells and left at room temperature for 1 hour. Alizarin Red Solution was removed and cells were rinsed once again with PBS. Chondrogenic cultures were stained with toluidine blue, which detects bone and cartilage deposition. Cells were then hydrated and washed with distilled water and fixed in 10% fomalin. Filtered 1% toluidine blue stock solution was adjusted to have a pH of around 2.4 with 1% sodium chloride. Working solution was added to cells for 2-3 minutes and then washed with distilled water. The images were acquired with a digital camera (Pixera, Los Gatos, CA).

Protein Isolation/Western Blot.

Following osteogenic and chondrogenic differentiation, MSCs were harvested in Radio-Immunoprecipitation Assay (RIPA, Sigma-Aldrich) buffer on the 8th day . The protein was then taken from the 4oC and fractionated by ultracentrifugation at 100,000 X g into the cytoplasmic and nuclear fractions. Cellular proteins were extracted with RIPA buffer. After BCA Protein Assay (Pierce), proteins were resolved by SDS/PAGE (12%) and transferred to nitrocellulose (BioRad). Membranes were blocked in 5% milk-TBST at 4°C overnight and then incubated with primary antibody diluted in 5% milk-TBST at 4°C overnight. Species-specific secondary antibodies were used and chemiluminesence (PerkinElmer, NEL104) was detected by film. β-actin (Santa Cruz) was used as a loading control.

Statistical Analysis.

The statistical significance between experimental groups and control was determined by unpaired Student's t-test, Mann Whitney Test, or ANOVA followed by Newman-Keuls post-test as designated using GraphPad Prism. $P<0.05$ was considered statistically significant.

RESULTS.

Pyrvinium reduces the cytoplasmic β-catenin levels of MSC during differentiation: Western blot analysis of the cytoplasmic extracts of MSCs differentiated into chondrogenic and osteogenic lineages revealed that MSC treated with pyrvinium had decreased cytoplasmic β-catenin levels indicating suppression of Wnt activity, when compared to control (Figure 1). However, in osteogenic and chondrogenic differentiation of MSCs treated with compound 211 did not result in a change of β-catenin levels, demonstrating that Wnt activity was not changed. These results indicate that pyrvinium inhibits Wnt signaling during osteogenic and chondrogenic differentiation.

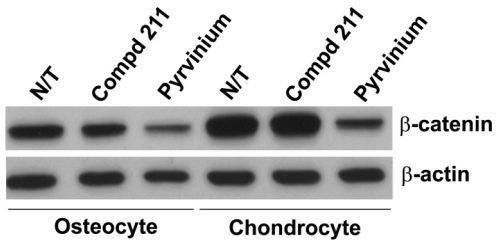

Figure 1. Pyrvinium reduces the cytoplasmic β-catenin levels of MSC during differentiation. MSCs were cultured in the osteogenic and chondrogenic media in the presence or absence of compd 211 or pyrvinium for 8 days. Cytoplasmic fraction was separated from the membrane fraction by ultracentrifugation at 100,000 X g for 2 hrs. Fifteen ug of the cytoplasmic proteins were separated on a SDS-PAGE and analyzed by western blot analysis using anti-β-catenin monoclonal Ab . β-actin was used to analyze equal loading.

Pyrvinium reduces the differentiation of MSCs into Osteocytes: (Figure 2b) The transcript levels of the osteogenic differentiation marker, osteocalcin, progression into the nucleus decreased ~3 fold in the presence of pyrvinium ($p<0.05$) and did not change in the presence of the control compound 211. Wnt, used as a positive control, enhanced the osteocalcin levels by ~2.7 fold. In addition, cells treated with pyrvinium in the presence of Wnt had ~3.45 fold decreased levels of osteocalcin when compared to the osteocalcin levels of the cells treated with Wnt alone; the similar effect was not observed in the cells treated with compound 211 in the presence of Wnt (Figure 2a). In addition, Alizarin red staining shows that osteogenic differentiation of MSCs decreased when in the presence of pyrvinium in both with and without treatments of Wnt. However, Osteogenic differentiation of MSCs stayed consistent with the control compound 211 in both cases Wnt treatment.

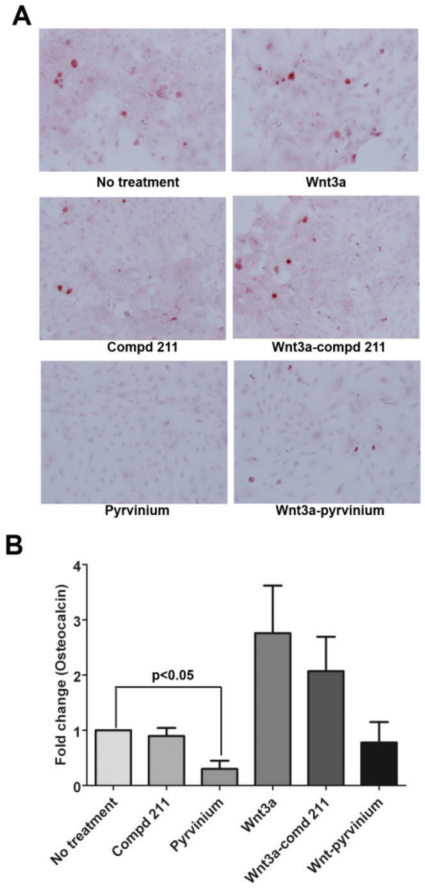

Figure 2. Pyrvinium reduces the differentiation of Mesenchymal stem cells (MSCs) into Osteocytes. MSCs were cultured for 8 days in the osteogenic differentiation media in the presence or absence of compound 211, pyrvinium, or Wnt3a. Treatment was also performed with Wnt3a in combination with compd 211 or pyrvinium. **A**) Representative images [20x] of Alizarin red stained MSCs. **B**) Fold change was calculated following quantitative real-time PCR for osteocalcin (osteocyte differentiation marker) with the cDNA made from the RNA isolated from the treated and untreated MSCs.

Pyrvinium reduces the differentiation of MSCs into Chondrocytes: (Figure 3b) The transcript levels of the chondrogenic differentiation marker, collagen XI, decreased by ~2.6 fold with the pyrvinium treatment ($p<0.05$). However, collagen XI levels in MSCs treated with compound 211 were only marginally affected. The collagen XI levels did not change much in the presence of Wnt, which was used as positive control. Nonetheless, ~1.5 decrease in the osteocalcin levels was identified in the cells treated with Wnt in presence of pyrvinium ($p<0.05$) when compared with the collagen XI levels of the cells treated with Wnt alone. The effect was not observed in the presence of compound 211, which maintained constant differentiation. (Figure 3a) In addition, toluidine blue staining shows that chondrogenic differentiation of MSCs decreased in the presence of pyrvinium regardless of Wnt treatments. However, chondrogenic differentiation of MSCs stayed consistent with the control compound 211.

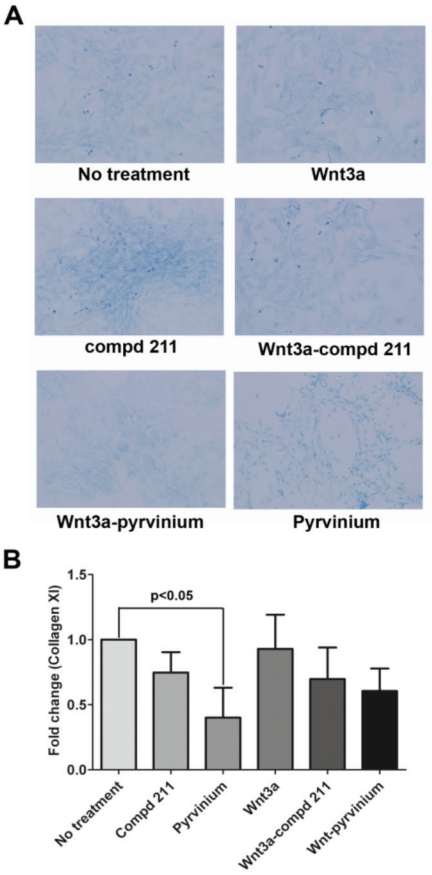

Figure 3. Pyrvinium reduces the differentiation of MSCs into Chondrocytes. MSCs were cultured for 8 days in the chondrogenic differentiation media in the presence or absence of compound 211 (Compd 211), pyrvinium, or Wnt3a. Treatment was also performed with Wnt3a in combination with compd 211 or pyrvinium. **A**) Representative images [20x] of Toluidine blue stained MSCs. **B**) Fold change was calculated following quantitative real-time PCR for collagen XI (chondrocyte differentiation marker) with the cDNA made from the RNA isolated from the treated and untreated MSCs.

GAPDH Control: In order to rule out the possibility that the inhibition of osteogenic and chondrogenic lineage markers in the presence of pyrvinium is due to cell death, a qRT-PCR was done on the GAPDH housekeeping gene. The levels of GAPDH stayed within ~1 fold when both the treatments of pyrvinium and compound 211 were added to the MSCs with osteogenic and chondrogenic media. These results further emphasize that pyrvinium inhibits MSCs to undergo osteogenic and chondrogenic lineage commitment and does not affect the housekeeping gene GAPDH.

DISCUSSION.

Studies before had shown that pyrvinium was able to inhibit Wnt signaling [10] and that inhibiting Wnt signaling would affect proliferation of MSCs [11]. However, the direct affect of pyrvinium on differentiation has not been studied. Our results demonstrate that when pyrvinium is added there is a beneficial decrease of both osteogenic and chondrogenic differentiation. We hypothesized that the ability for MSCs to initially proliferate and then differentiate is crucial to create productive stem cells. Typically when stem cells are injected they differentiate immediately and are rarely accepted by the tissue [3]. However, if differentiation could be delayed to allow time for stem cells proliferation and

adjustment of target site, both the number of MSCs for differentiation will increase and the new MSCs will be more familiar to the damaged tissue [3].

In addition, to insure Wnt signaling was the target of pyrvinium in differentiation, cytoplasmic ß-catenin levels were monitored. The beta-catenin levels decreased with the treatment of pyrvinium showing the degradation of β-catenin as a result of Wnt signaling inhibition. Lastly GAPDH, an important housekeeping gene, was used to confirm that the lack of differentiation was not due to cell death, but truly a result of pyrvinium.

Improving MSCs is an important goal for scientist and doctors alike today. The positive results that pyrvinium has illustrated with both with proliferation in previous studies and now with differentiation could be ground breaking in confronting many of the world's biggest health concerns.

While the control of various proteins, such as Sfrp2, are able to inhibit Wnt signaling as well, high prices and risky conditions make this process impracticable [9]. Not only is gene therapy impossible for most families, however a safe and reliable procedure has yet to be identified [9]. This study has shown that there is more than just one way to target Wnt signaling pathway. Pyrvinium is just one example of what possibly be many more compounds able to decrease immediate differentiation by aiming at the inhibition of Wnt signaling. This steppingstone not only provides for better understanding of MSC development but it paves the way for medical and economic improvement in MSC therapies.

However, to fully understand the therapeutic value of Wnt inhibition on MSCs using pyrvinium is limited by the compounds toxicity when used in high levels. The basis for pyrvinium's toxicity, as well as that of other small molecular Wnt inhibitors is not clearly established. The CK1α family of serine/threonine kinases is evolutionarily conserved in eukaryotes and is associated with a wide range of cellular processes that includes cell cycle, apoptosis, and Wnt signaling [12]. It is not clear whether the toxicity that is associated with pyrvinium is due to its effects on CK1α or to its potential alkylating activity. Nevertheless, pyrvinium presents an example of a small molecule Wnt inhibitor that was able to decrease differentiation and increase proliferation without cell death. Pyrvinium is only toxic when used with high concentration levels. In the future, investigating the affects of pyrvinium on MSC differentiation with in vivo studies will provide for further observation on the overall effects of pyrvinium on a living subject in light of toxicity. In addition, characterizing newly identified small molecule Wnt inhibitors and antibody-based inhibitors will improve our understanding of the mechanistic basis for adverse effects of systemic Wnt inhibition. Identification of a non-toxic Wnt inhibitor will enable us to more rigorously test the utility of Wnt inhibitors as therapeutic agents to enhance repair and regeneration.

The universal goal of improved stem cells is one that all researchers and medical advocates have their hope set upon. The closest and most ethical door to improvement lies in MSCs, as new studies such as the one Jiang et al. published in Nature showing that the once prized pluripotency characteristic of embryonic stem cells can now be found in MSCs derived from bone marrow. With more groundbreaking research knocking at the door for MSCs day-by-day, MSC therapy must be prepared and compound like pyrvinium, with results showing positive regeneration and remodeling can equip MSCs to do just that.

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SUPPORTING INFORMATION.

Figure S1. Effect of Pyrvinium on Wnt Signaling Transduction Pathway.

Figure S2. GAPDH Control.

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