J. Serb. Chem. Soc. 84 (9) 951–960 (2019) UDC 546.19–31+547.922+577.161.2:616.7:615
JSCS–5238 *Original scientific paper*

Antiosteogenic effect of arsenic trioxide, cholecalciferol, lovastatin or their combination *in vitro*

JELENA GVOZDENOVIĆ-JEREMIĆ[1]*, EKATERINA VERT-WONG[1] and LJILJANA MOJOVIĆ[2#]

[1]*Nostopharma, LLC, 10319 Westlake Dr #241 Bethesda, 20852 Maryland, USA and* [2]*Faculty of Technology and Metallurgy, University of Belgrade, Karnegijeva 4, 11000 Belgrade, Serbia*

(Received 4 March, revised 16 June, accepted 17 June 2019)

Abstract: Pathological formation of bone in non-skeletal soft tissues or heterotopic ossification (HO), for which there is currently no effective treatment, is considered to be mediated by activation of Hedgehog (Hh) signaling pathway. Moreover, the biochemical mechanism of this pathological process is not fully understood. Here, we tested the efficacy of three chemical inhibitors of the Hh signaling pathway, arsenic trioxide (ATO), lovastatin (Lov) and cholecalciferol (Vitamin D) to hamper differentiation of mesenchymal stem cells (MSC) into osteoblasts or osteogenesis. Each of the three Hh inhibitors potently decreased alkaline phosphatase activity, suggesting effective suppression of osteogenic activity in Hh-impaired MSC. Gene expression analysis revealed a significant reduction in mRNA levels of chief Hh signaling marker, Gli1, following administration of Hh small molecule inhibitors. A functional link between Hedgehog and osteogenesis in native MSC cells is further established in studies involving the mix of three Hh inhibitors acting at different checkpoints of the Hh signaling pathway. Thus, a combination of small molecule inhibitors of the Hh pathway at their lower concentrations could be a novel approach for HO prophylaxis with increased efficacy and potentially reduced side effects.

Keywords: heterotopic ossification; Hedgehog inhibitors; combination therapy; mesenchymal stem cells.

INTRODUCTION

Heterotopic ossification (HO), either acquired or hereditary, is featured by the ectopic bone formation outside of the normal skeleton.[1,2] Acquired form of HO is a debilitating condition associated with a wide range of problems such as pain, loss of range of motion, or joint ankylosis. It is a costly complication of various types of traumatic events: musculoskeletal trauma, central nervous system injury, combat trauma, hip and elbow fractures, and total joint replacement

* Corresponding author. E-mail: jelenaj@nostopharma.com
# Serbian Chemical Society member.
https://doi.org/10.2298/JSC190304060G

surgeries. The incidence of HO varies and, for example, up to 7 % patients experience clinically significant HO after total hip arthroplasty (THA), while in case of total knee arthroplasty (TKA) only 1 % of patients are symptomatic.[3] Hereditary HO conditions, such as fibrodysplasia ossificans progressiva (FOP)[4,5] and progressive osseous heteroplasia (POH),[6,7] are ultra-rare but progressive and potentially life-threatening genetic disorders which result in severe disability secondary to extensive formation of heterotopic ossification in the soft tissue. There are around eight hundred fifty registered cases of FOP and approximately one hundred instances of POH documented worldwide.

Heterotopic ossification results from ectopic osteoblast differentiation of mesenchymal stem cells (MSC).[8] The inappropriate formation of cartilage or bone, in tissues such as skeletal muscle and adipose tissue[9] involves MSC that differentiate along the osteochondrogenic lineage to form HO.[10,11]

The characteristic features of MSC are their abilities to self-renew and to differentiate into all mesoderm-type lineages, including osteoblast, chondrocytes, adipocytes, smooth muscle cells, and myocytes. Osteogenesis is a process of differentiation of progenitor cells under the influence of growth factors into osteoblasts, bone precursor cells. Mesenchymal progenitor cells have been used already to produce bony implants and in the repair of skeletal defects.[12] Given that MSCs are a prime candidate population involved in HO formation[11], in this study, we used primary cultures of bone marrow stromal cells as a surrogate model system known for their favorable osteogenic differentiation potential.[13]

In addition, Hedgehog (Hh) signaling has been shown to be sufficient and necessary for HO formation in case of POH.[8] In this pathological condition, an inactivating mutation of heterotrimeric G protein $G\alpha_s$ led to a constitutive upregulation of Hh pathway and subsequent differentiation of MSC to osteoblasts.[8] Recently, it was shown that local dysregulation of Hh signaling participates in the pathophysiology of murine muscle injury model of HO.[14,15] Furthermore, these lineage tracing studies suggested an involvement of Hh target transcription factor, Gli1, in endochondral bone formation.[14,15]

Currently, there is no effective and safe HO prophylaxis treatment. Radiation therapy and indomethacin are the most widely used therapeutic approaches in the setting of post-surgical heterotopic ossification prophylaxis, with evidently many hazards and shortcomings.[3,16] The rationale behind our reasonable therapeutic strategy was based on all these studies showing that chondrogenic and osteogenic cell differentiation *in vitro* and *in vivo* actually requires a Hh pathway involvement. Many Hh pathway inhibitors are already approved for clinical use in several Hedgehog-mediated pathologies, like cancer.[17] For instance, exogenous addition of vitamin D has been shown to inhibit the Hh pathway in basal cell carcinoma (BCC) cell line.[18–22] Interestingly, many studies found cholesterol synthesis required for Sonic Hh (SHH) signaling transduction.[23–25] Thus, blocking

cholesterol synthesis with statins might be beneficial for HO patients. However, no effective treatment currently exists for HO.

In view of this, we explored the effect of very well known (arsenic trioxide, ATO), and other less known (lovastatin and vitamin D) Hh inhibitors on the regulation of osteogenesis in bone marrow derived MSC. The current study demonstrates that inhibition of Hh signaling pathway using ATO, Vitamin D, lovastatin, or their combination potently inhibited *Gli1* gene expression and osteogenesis in native mesenchymal progenitor cells.

EXPERIMENTAL

Arsenic trioxide preparation. Arsenic trioxide solution was prepared by placing 50 mg of ATO (Sigma) at the bottom of a 50 ml conical tube and dissolving it in 1ml of 1M NaOH. 48 ml of PBS was added and 1M HCl was used to adjust pH to 7.2. Working concentrations in the cell culture experiments were selected according to our previous experiments[8] and amounted to 5 and 10 μM.

Cholecalciferol (Sigma PHR1237) was dissolved in absolute ethanol in 10 mM stock concentration. Working concentrations were selected according to the previous studies[18] and amounted to 5 and 10μM.

Lovastatin (Calbiochem) was dissolved in absolute ethanol in 1 mM stock concentration. Working concentrations, used in the cell culture experiment, were selected according to our previous results and were 0.5 and 1 μM.

Mix preparation. Combination of agents was prepared by mixing single agents 5 μM ATO, 5 μM cholecalciferol and 0.5 μM lovastatin into a single mixture, immediately, with osteogenic media (OM).

Mouse bone marrow stromal cells isolation. Bone marrow stromal cells (BMSC) were isolated from six weeks old wild type mice. Femurs and tibias were dissected from surrounding tissues.[8] The epiphyseal growth plates were removed, and the marrow was collected by flushing with modified essential medium (DMEM, Gibco, Waltham, MA, USA) containing 100 U/ml penicillin, 100 μg/ml streptomycin (Pen Strep, Gibco, Waltham, MA, USA) and 10 % fetal bovine serum (FBS) with a 25 G needle. Single cell suspensions were prepared by passing the cell clumps through an 18 G needle followed by filtration through a 70-μm cell strainer. Cells were propagated in Alpha-MEM (Gibco, Waltham, Massachusetts, USA) media supplemented with 20 % fetal bovine serum, 2 mM L-glutamine (Gibco, Waltham, MA, USA), recombinant mouse prolactin protein[26] 1 ng/ml (Sigma-Aldrich), MEM non-essential amino acids (1x, Gibco, Waltham, MA, USA) and 100 U/ml penicillin/(100 μg/ml) streptomycin (Pen Strep, Gibco, Waltham, MA, USA) in a humidified atmosphere with 5 % CO_2 at 37 °C.

Human bone marrow cells. Human bone marrow derived cells (hBMSC) were obtained from ATCC (ATCC PCS 500-012, Manassas, VA, USA) and propagated in basal medium (ATCC PCS 500-030) supplemented with growth kit in a humidified atmosphere with 5 % CO_2 at 37 °C. For hBMSCs passage 1-3 was used for all experiments.

Osteogenesis. After the cells reached confluence, the medium was replaced with osteogenic differentiation medium (OM). Osteogenic differentiation medium (OM) consisted of: DMEM (Gibco, Waltham, MA, USA), 10 % FBS, 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco, Waltham, MA, USA), 2 mM glutamine (Gibco, Waltham, MA, USA), 10 μM L-ascorbic acid 2-phosphate (Wako Chemicals, USA) and 10 mM *β*-glycerol phosphate

(Sigma–Aldrich). Duplicate wells were exposed to differentiation media without any agent (appropriate vehicle only-control), single agent (cholecalciferol, ATO or lovastatin) or combination of agents. Media replacement occurred every 2–3 days with fresh differentiation medium (made each time during feeding) with or without single agents or their combination.

Alkaline phosphatase assay. Mouse BMSC cells were stained after four days of exposure in OM differentiation medium for alkaline phosphatase (AP) assay. Human BMSC were stained after six days of exposure in OM differentiation medium for AP assay. This is a colorimetric assay where 4-(benzoylamino)-2,5-dimethoxybenzenediazonium chloride hemi salt is hydrolyzed by alkaline phosphatase to yield a bluish-purple precipitate. After 4 (for mBMSC) to 6 (for hBMSC) days of culturing cells in OM media, the medium was removed, and cells were washed once with PBS. Cells were fixed with 4% paraformaldehyde for 5 min. After washing cells with PBS twice, one ml of One-step NBT/BCIP staining solution (Thermo Fisher 34042) per well was added and incubated in the dark for 30 min to 1 h at room temperature. The cells were washed with deionized water to stop the reaction. A digital camera (Motical 10.0 MP) attached to an inverted phase-contrast microscope was used for microscopic observations. For image quantification Image J was used or DAPI staining to evaluate the percentage of AP positive over total live cells.

RT–PCR. Total RNA was first isolated with trizol (invitrogen) and then with RNeasy kit (Qiagen). First strand cDNA was generated using the Abcam kit. Quantitative real-time (RT)-PCR was performed using Biorad cycler and 40 cycles of 95 °C for 15 s and 60 °C for 60 s. PCR product was detected using SybrGreen (BioRad). Primers for mouse BMSC cells used for amplification are: actin, forward 5'- CAC AGC TTC TTT GCA GCT CCT T-3′, reverse 5′-CGT CAT CCA TGG CGA ACT G-3′; Alk Phos, forward 5′- CAC GCG ATG CAA CAC CAC TCA GG -3′, reverse 5′- GCA TGT CCC CGG GCT CAA AGA -3′; Gli1, forward 5'-GAA AGT CCT ATT CAC GCC TTG A-3'; Gli1, reverse 5'-CAA CCT TCT TGC TCA CAC ATG TAA G-3. Primers for human BMSC cells used for amplifications are: GAPDH, forward 5'-GGC ATG GAC TGT GGT CAT GAG-3'; reverse 5'-TGC ACC ACC AAC TGC TTA GC-3'; Alk Phos, forward 5'-CTC CCA GTC TCA TCT CCT-3', reverse 5;- AAG ACC TCA ACT CCC CTG AA; Gli1, forward 5'-GTG CAA GTC AAG CCA GAA CA-3', reverse 5'-ATA GGG GCC TGA CTG GAG AT-3'.

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 hoc* Dunnett's test was used for pairwise comparisons among groups. Differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Given the critical role of Hh signaling pathway in bone formation, in this work the extent to which Hh inhibitors, arsenic trioxide (ATO), cholecalciferol (vitamin D) and lovastatin (Lov) modulate osteogenesis in bone precursors, mesenchymal stem cells was tested. For functional osteogenic studies, alkaline phosphatase (AP) assay was used, as AP is one of the very first enzymes to be activated and regulates early stages of osteogenesis.[27] A key to understanding the role of AP in mineralization is provided by studies of the phased expression of genes during osteoblastic differentiation and growth plate cartilage calcification.

In both tissues, bone and calcifying cartilage, AP must function in the early process of osteoblast formation.[28] Osteoblasts are integral to the creation of heterotopic bone through the production of alkaline phosphatase (AP).

Bone marrow stromal cells were isolated from wild type mice and plated at first passage (P1) to test osteogenic properties in the presence of ATO, vitamin D, lovastatin or their combination. The initial doses for ATO, vitamin D and lovastatin are selected based on previously validated and published *in vitro* data showing effective inhibition of the Hh pathway at these inhibitor concentrations.[8,18] Alkaline phosphatase levels, reflecting osteogenic activity, were detected in control mouse BMSC cells after four days of exposure to osteogenic media (Fig. 1). By contrast, additions of ATO, vitamin D, lovastatin or their combination significantly reduced (more than 2-fold) the alkaline phosphatase activity as compared to control BMSC (Fig. 1).

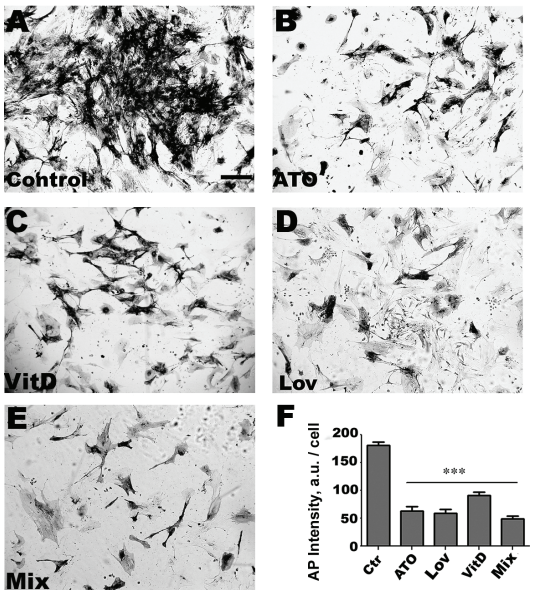

Fig. 1. Alkaline phosphatase microscopy assay of primary mouse BMSC after four days exposure in osteogenic media: A) control, no agent present; B) in the presence of 10 μM ATO during osteogenesis; C) in the presence of 10 μM cholecalciferol (vitamin D) during osteogenesis; D) 1 μM lovastatin (Lov) in media during osteogenesis; E) three agents combined (Mix), ATO (5 μM), cholecalciferol (5μM) and lovastatin (0.5 μM), present during osteogenesis. F) The intensity of the AP signal in MSC is quantified with Image J software and reported as arbitrary units (a.u.). Data, collected from three independent experiments (at least 60 cells per experimental group), are presented as mean ± SEM. Bar, 10 μm.

Strong modulatory action of Hh inhibitors on turnover of key osteogenic regulators was also observed in gene expression studies (Fig. 2). Real-time PCR analysis revealed that, compared to the control (osteogenic media present), all three Hh inhibitors or their combination significantly reduced (up to 9-fold) mRNA levels of alkaline phosphatase (Fig. 2). To further test the Hh role in osteogenesis of BMSC, changes in gene expression of Hh marker, Gli1, in control and Hh inhibitor-treated cells were measured. Arsenic trioxide and lovastatin significantly diminish (up to 2-fold) Gli1 mRNA levels relative to osteogenic controls (Fig. 2). Interestingly, a combination of the three Hh inhibitors (Mix) at

their reduced concentrations evoked equipotent inhibitory effect on Gli1 gene expression that was comparable to single, higher-dosage treatments (Fig. 2). However, vitamin D alone did not suppress the Hh pathway, at least not at Gli1 readout level. This result suggests that alternative, Hh-independent, pathway such as vitamin D receptor (VDR) may possibly downregulate osteogenic activity in MSC cells treated with vitamin D.[29]

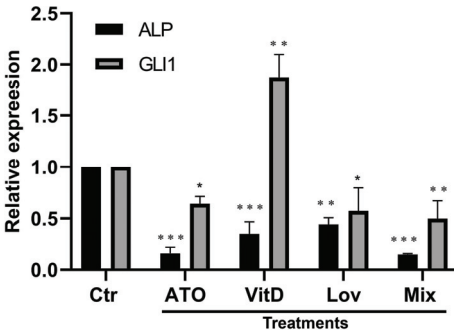

Fig. 2. The RT-PCR analysis of expression profile of an early osteogenic marker, alkaline phosphatase (AP) and Hedgehog signaling marker, Gli1, in wild type mouse BMSC. Following isolation, BMSC were cultured for four days in OM media without (control) or in the presence of single agents or their combination. mRNA was isolated and quantified using $2^{-\Delta\Delta Ct}$ method. Data (mRNA levels) are normalized to the housekeeping gene actin and expressed as mean ± SEM relative to the control (set to 1).

Next, human BMSC to test if Hh-mediated osteogenesis in murine progenitor cells is unique or somewhat conserved differentiation process were used. Analogous to the murine cells (Fig. 1), all inhibitors visibly reduced AP staining as compared to control (OM media only) in human BMSC (Fig. 3). To ensure that this decrease in AP staining is not due to the toxicity of Hh inhibitors in concentrations used, the cells were stained with a nuclear dye, DAPI, and a total number of AP-positive cells were counted (Fig. 3). Our combined fluorescen-

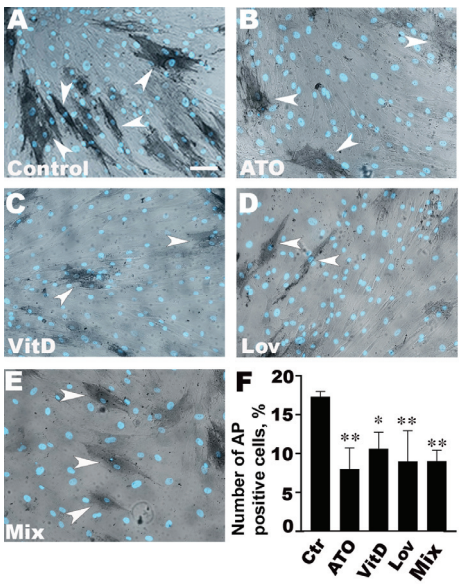

Fig. 3. Alkaline phosphatase assay and DAPI staining of primary human BMSC, six days after exposure in osteogenic media: A) control, untreated cells; B) in the presence of 10 μM ATO during osteogenesis; C) in the presence of 10 μM cholecalciferol (vitamin D) during osteogenesis; D) 1 μM lovastatin (Lov) in media during osteogenesis; E) Mix–ATO, vitamin D and lovastatin present during osteogenesis; F) quantification of AP-positive cells over total live cells, expressed as a percentage. Data, collected from three independent experiments, are presented as mean ± SEM. Bar, 10 μm. (Note a decrease in AP activity in cells treated with Hh inhibitors compared to the control).

ce/light microscopy approach revealed no visible signs of apoptosis such as shrinkage of cells, nuclear condensation or fragmentation in the treated or control cells. However, microscopic studies confirmed that there was a significant decrease (~2-fold) in the number of AP-positive cells and an overall reduction in AP signal in Hh-inhibitor treated cells as compared to the controls (Fig. 3).

Similar findings were obtained with gene expression analysis of Hh-markers in human BMSC (Fig. 4). Additions of all three Hh inhibitors, alone or in combination, to human BMSC during osteogenesis abrogated expression of AP gene, reflected by a significant decrease in AP mRNA levels in ATO, Lov or vitamin D-treated cells as compared to control cells (Fig. 4). Similar to mouse counterparts, ATO and lovastatin significantly diminished (up to 2-fold) Gli1 expression in human BSMC relative to controls, while supplementation of vitamin D had no modulatory effect on Gli1, a molecular readout of Hh signaling activity (Fig. 4). Published data demonstrate that Hh dependent pathway is predominantly involved in stem cells differentiation, while in the case of cancer, both Hh dependent and Hh independent pathway exist.[30] Lack of the Hh pathway inhibition by vitamin D and the fact that the combination of all three tested compounds does not provide complete cumulative effect suggest that these inhibitors inhibit osteogenesis of MSC *via* both Hh-dependent and Hh-independent pathways. However, Gli1 gene expression data suggest that ATO and lovastatin blocked osteogenesis in both mouse and human BMSC *via* common Hh-mediated mechanism.

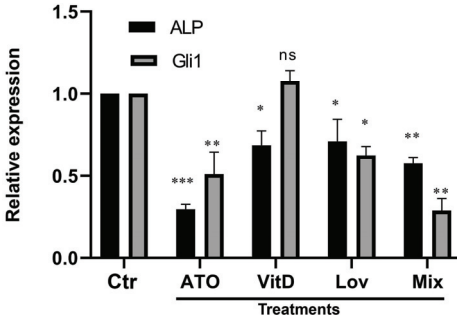

Fig. 4. The expression profile of an early osteogenic marker, alkaline phosphatase (ALP) and Hedgehog signaling marker, Gli1, in human BMSC cultured for six days in supplementation of OM media without (control) agents present, with single agents or their combination, calculated using $2^{-\Delta\Delta Ct}$ method. The histogram represents data ± SEM relative to the control that was set to 1.

Collectively, our study provides early evidence that ATO, lovastatin and vitamin D act as potent inhibitors of the otherwise rapid and irreversible MSC osteoblast differentiation triggered by Hh and related osteogenic signaling pathways. ATO, lovastatin and vitamin D each reduced early osteoblasts formation *via* inhibiting alkaline phosphatase activity and expression when used at their higher concentrations. Interestingly, the combined application of these three Hh inhibitors at their half-reduced concentrations elicited a comparable decrease in AP activity and Gli1 mRNA levels. This cooperative effect of ATO, Lov and vitamin D suggests that inhibitors target different checkpoints of the Hedgehog

signaling pathway, which may explain their enhanced efficacy against osteogenesis. A reduced dosage of these drugs may potentially improve selectivity and reduce their off-target effects *in vivo*.

CONCLUSION

In summary, our study showed that three examined chemical inhibitors of the Hh signaling pathway, arsenic trioxide (ATO), lovastatin (Lov) and cholecalciferol (vitamin D) impede differentiation of mesenchymal stem cells (MSC) into osteoblasts or osteogenesis. Each of them potently decreased alkaline phosphatase (AP) activity, suggesting effective suppression of osteogenic activity in Hh-impaired MSC. The combined application of these three Hh inhibitors at their half-reduced concentrations elicited a comparable decrease in AP activity and Gli1 mRNA levels. This effect suggests that the inhibitors target different checkpoints of the Hedgehog signaling pathway and indicates a promising potential utilization of this combined mixture for heterotopic ossification prophylaxis *in vivo*.

NOMENCLATURE

AP or ALP – Alkaline phosphatase;
ATO – Arsenic trioxide;
BCC – Basal cell carcinoma,
BMSC – Bone marrow stromal cells;
DAPI – 4',6-Diamidino-2-phenylindole dihydrochloride;
FOP – Fibrodysplasia ossificans progressive;
HO – Heterotopic ossification;
MSC – mesenchymal stem cells;
LOV – Lovastatin;
SHH – Sonic Hedgehog;
POH – Progressive osseous heteroplasia;
VDR – Vitamin D receptor

ИЗВОД

СМАЊЕЊЕ ОСТЕОГЕНЕ АКТИВНОСТИ *in vitro* У ПРИСУСТВУ АРСЕН-ТРИОКСИДА, ВИТАМИНА Д, ЛОВАСТАТИНА И ЊИХОВЕ КОМБИНАЦИЈЕ

ЈЕЛЕНА ГВОЗДЕНОВИЋ-ЈЕРЕМИЋ[1], ЕКАТЕРИНА ВЕРТ-ВОНГ[1] и ЉИЉАНА МОЈОВИЋ[2]

[1]*Nostopharma, LLC, 10319 Westlake Dr #241 Bethesda, 20852 Maryland, USA и* [2]*Технолошко–металуршки факултет, Универзитет у Београду, Карнегијева 4, 11000 Београд*

Патолошка формација кости ван скелетног система, у меканим ткивима, или хетеротопна осификација, за коју до сада није пронађен ефикасан третман, вероватно је посредована активацијом хеџхог сигналног пута. Биохемијски механизми овог патолошког процеса такође нису потпуно разјашњени. Испитивали смо ефикасност три хемијска инхибитора хеџхог (Hh) сигналног пута, арсен-триоксида (ATO), ловастатина (Lov) и холекалциферола (витамин Д) у спречавању процеса диференцијације прогениторских мезенхимских матичних ћелија у остеобласте, познатог као остеогенеза. Примена сваког од наведена три Hh инхибитора је резултирала у значајном смањењу активности алкалне фосфатазе као и смањењу остеогене активности у хеџхог дефектним

мезенхимским ћелијама. Анализа генске експресије показала је значајно смањење mRNA нивоа главног Hh сигнала, Gli1, након додавања нискомолекулских инхибитора хеџхог сигналог пута. Функционална веза између Hh сигналног пута и остеогенезе у прогениторским мезенхимским ћелијама показана је у студији са комбинацијом три инхибитора који делују на различитим местима Hh сигналног пута. Дакле, комбинација нискомолекулских инхибитора у ниским концентрацијама представља нов приступ у профилакси хетеротопне осификације са повећаном ефикасношћу и потенцијално смањеним нежељеним ефектима.

(Примљено 4. марта, ревидирано 16. јуна, прихваћено 17. јуна 2019)

REFERENCES

1. M. Pacifici, *Curr. Opin. Pharmacol.* **40** (2018) 51 (https://doi.org/10.1016/j.coph.2018.03.007)
2. D. S. Edwards, J. C. Clasper, *J. R. Army Med. Corps* **161** (2015) 315 (https://doi.org/10.1136/jramc-2014-000277)
3. E. Baird, Q. Kang, *J. Orthop. Surg. Res*. **4** (2009) 1 (https://doi.org/10.1186/1749-799X-4-12)
4. E. M. Shore , M. Xu, G. J Feldman , D. A. Fenstermacher, T. Cho, I. Choi, M. Connor, P. Delai, D. Glaser, M. LeMerrer, R. Morhart, J. G. Rogers, R. Smith, J. T. Triffitt, J. A. Urtizberea, M. Zasloff, M. A. Brown, F. S. Kaplan, *Nat. Genet.* **38** (2006) 525 (https://doi.org/10.1038/ng1783)
5. J. M. Wozney, V. Rosen, A. J. Celeste, L. M. Mitsock, M. J. Whitters, R. W. Kriz, R. M. Hewick, E. A. Wang, *Science* **242** (1988**)** 1528 (PMID: 3201241)
6. F. S. Kaplan, G. V. Hahn, M. A. Zasloff, *J. Am. Acad. Orthop. Surg*. **2** (1994) 288 (PMID: 10709021)
7. M. C. Eddy, S. M. Jan de beur , S. M. Yandow , W. H. McAlister , E. M. Shore , F. S. Kaplan, M. P. Whyte, M. A. Levine, *J. Bone Miner. Res*. **15** (2000) 2074 (https://doi.org/10.1359/jbmr.2000.15.11.2074)
8. J. Regard, D. Malhotra, J. Gvozdenovic-Jeremic, M. Josey, M. Chen, L. Weinstein, J. Lu, E. M. Shore, F. S. Kaplan, Y. Yang, *Nat. Med.* **19** (2013) 1505 (https://doi.org/10.1038/nm.3314)
9. E. M. Shore, F. S. Kaplan, *Nat. Rev. Rheumatol.* **6** (2010) 518 (https://doi.org/10.1038/nrrheum.2010.122)
10. L. Kan , Y. Liu , T. L. McGuire , D. M. Berger, R. B. Awatramani , S. M. Dymecki , J. A. Kessler, *Stem Cells.* **27** (2009) 150 (https://doi.org/10.1634/stemcells.2008-0576)
11. L. Kan, J. Kessler, *Orthopedics* **37** (2014) 329 (doi: 10.3928/01477447-20140430-07)
12. F. Barry, M. Murphy, *Nat. Rev. Rheumatol.* **9** (2013) 584 (https://doi.org/10.1038/nrrheum.2013.109)
13. L. Xu, L. Liu, Y. Sun, B. Wang, Y. Xiong, W. Lin, Q. Wei, H. Wang, W. He, B. Wang, G. Li, *Stem Cell Res. Ther.* **8** (2017) 1 (https://doi.org/10.1186/s13287-017-0716-x)
14. C. Kan., N. Ding, J. Yang, Z. Tan, T. L. McGuire, H. Lu, K. Zhang, D. M. Palila Berger, J. Kessler, L. Kan, *Stem Cell Res. Ther.* **10** (2019) 1 (https://doi.org/10.1186/s13287-018-1107-7)
15. C. Kan , L. Chen, Y. Hu, N. Ding, Y. Li, T. L. McGuire, H. Lu, J. A. Kessler, L. Kan, *Bone* **109** (2018) 71 (https://doi.org/10.1016/j.bone.2017.06.014)
16. N. Eisenstein, S. Stapley, L. Grover, *J. Orthop. Res.* **36** (2018) 1061 (https://doi.org/10.1002/jor.23808)
17. K. Udupa, J. Thomas, C. B. Udupa, V. S. Binu, P. Sharan, *Indian J. Hematol. Blood Transfus.* **33** (2017) 45 (https://doi.org/10.1007/s12288-016-0668-9)

18. J. Y. Tang, T. Y. Xiao, Y. Oda, K. S. Chang, E. Shpall, A. Wu, P. L. So, J. Hebert, D. Bikle, E. H. Epstein Jr., *Cancer Prev. Res.* **5** (2011) 744 (https://doi.org/10.1158/1940-6207.CAPR-10-0285)
19. A. Uhmann, H. Neimann, B. Lammering, C. Henkel., I. Hess, F. Nitzki, A. Fritsch, N. Prüfer, A. Rosenberger, C. Dullin, A. Schraepler, J. Reifenberger, S. Schweyer, T. Pietsch, F. Strutz, W. Schulz-Schaeffer, H. Hahn, *Mol. Cancer Ther*. **10** (2011) 2179 (https://doi.org/10.1158/1535-7163.MCT-11-0422)
20. M. Cortes, S. Y. Liu, W. Kwan, K. Alexa, W. Goessling. T. North, *Stem Cell Rep.* **5** (2015) 471 (https://doi.org/10.1016/j.stemcr.2015.08.010)
21. B. Linder, S. Weber, K. Dittmann, J. Adamski, H. Hahn, A. Uhmann, *J. Biol. Chem.* **290** (2015) 19614 (https://doi.org/10.1074/jbc.M115.646141)
22. B. Albert, H. Hahn, *Adv. Exp. Med. Biol*. **810** (2014) 329 (PMID:25207374)
23. Y. Zhang, D. P. Bulkley, Y. Xin, K. J. Roberts, D. E. Asarnow, A. Sharma, B. A Myers, W. Cho, Y. Cheng, P. A. Beachy, *Cell* **175** (2018) 1352 (https://doi.org/10.1016/j.cell.2018.10.026)
24. R. B. Corcoran, M. P. Scott, *Proc. Natl. Acad. Sci. U.S.A*. **103** (2006) 8408 (https://doi.org/10.1073/pnas.0602852103)
25. M. K. Cooper, C. A. Wassif, P. A. Krakowiak, J. Taipale, R. Gong, R. I. Kelley, F. D. Porter, P. A. Beachy, *Nat. Genet.* **33** (2003) 508 (https://doi.org/10.1038/ng1134)
26. T. L. Tsai, W. J. Li, *Stem Cell Rep.* **8** (2017) 387 (https://doi.org/10.1016/j.stemcr.2017.01.004)
27. R.S. Siffert, *J. Exp. Med*. **93** (1951) 415 (PMID: 14832392).
28. H. C. Tenenbaum, J. N. M. Heersche, *Calcif. Tissue Int.* **34** (1982) 76 (https://doi.org/10.1007/BF02411212)
29. Y. Wang, J. Zhu, H. F. DeLuca *J. Bone Miner. Res.* **29** (2014) 685 (https://doi.org/10.1002/jbmr.2081)
30. B. Stecca, A. R. Altaba, *J. Mol. Cell Biol.* **2** (2010) 84 (https://doi.org/10.1093/jmcb/mjp052).