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Review Article

Activin Receptor-Ligand Trap for the Treatment of β-thalassemia: A Serendipitous Discovery

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Abstract. β-thalassemia is a hereditary disorder caused by defective production of β-globin chains of hemoglobin (Hb) that leads to an increased α/β globins ratio with subsequent free α-globins. Alpha globin excess causes oxidative stress, red blood cells membrane damage, premature death of late-stage erythroid precursors, resulting in ineffective erythropoiesis.
The transforming growth factor β (TGF-β) superfamily signaling acts on biological processes, such as cell quiescence, apoptosis, proliferation, differentiation, and migration, and plays an essential role in regulating the hematopoiesis. This pathway can lose its physiologic regulation in pathologic conditions, leading to anemia and ineffective erythropoiesis. Activin receptor-ligand trap molecules such as Sotatercept and Luspatercept downregulate the TGF-β pathway, thus inhibiting the Smad2/3 cascade and alleviating anemia in patients with β-thalassemia and myelodysplastic syndromes.
In this review, we describe *in extenso* the TGF-β pathway, as well as the molecular and biological basis of activin receptors ligand traps, focusing on their role in various β-thalassemia experimental models. The most recent results from clinical trials on sotatercept and luspatercept will also be reviewed.

Keywords: β-thalassemia; TGF-β.

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Introduction. β-thalassemia is a hereditary disorder caused by defective production of β-globin chains of hemoglobin (Hb)[1] that leads to an increased α/β globins ratio with subsequent free α-globins that precipitate in the red blood cells (RBCs). Excess of α-globin aggregates in erythroblasts lead to maturation arrest, oxidative stress, membrane damage, and premature death of late-stage erythroid precursors and, in turn, to a reduced RBCs half-life.[2]

Transforming growth factor β (TGF-β) superfamily signaling acts on cell quiescence, apoptosis, proliferation, differentiation, and migration and plays a crucial role in the regulation of hematopoiesis.[3] In selected pathological conditions, including β-thalassemia, this pathway can lose its physiologic regulation leading to anemia and ineffective erythropoiesis. Activin receptors ligand traps such as Sotatercept and Luspatercept downregulate the TGF-β pathway, thus inhibiting the Smad2/3 cascade and alleviating anemia in patients with β-thalassemia and

also myelodysplastic syndromes.[3]

In this review, we describe *in extenso* the TGF-β pathway, starting from the role of activins and their cognate receptors to get to the description of signal effectors. We also summarize the molecular and biological basis of activin receptors ligand traps, focusing on their role in various β-thalassemia experimental models. The most recent results from clinical trials on sotatercept and luspatercept will also be reviewed.

Activins and Activin Receptors. Activins are typical proteins members of transforming growth factor β (TGF-β) superfamily and control many cellular processes involved in cell proliferation, death, metabolism, homeostasis, differentiation, immune response, and endocrine function.[4,5]

Activin A, initially described as a regulator of reproductive processes, is an erythroid differentiation factor of hematopoietic progenitor cells *in vitro* and *in vivo*.[6]

Activins are biologically active in all tissues as βA and βB homodimers, or βA and βB heterodimers, whereas βC and βE are predominantly expressed in the liver. Activins are synthesized as larger precursor proteins containing a prodomain and a mature region. Pro-domains, non-covalently bound to their mature regions, are essential for the dimer stabilization, its secretion, and association with heparan sulfate residues of proteoglycans of target cells, allowing activins to concentrate near their receptors and protecting themselves from proteolysis. Activin B lacks residues fundamental for binding heparan sulfate and shows a lower affinity for their receptors. The mature regions contain a cysteine-rich domain which forms intra- and inter- disulfide bonds, well conserved in other members of TGF-β family, including TGF-β1, TGF-β2 e TGF-β3, BMP2 e BMP7 and probably responsible for the characteristic open hand configuration of Activin.[7]

Activins initiate their biological signaling by binding specific type II A (ActRIIA) or B (ActRIIB) receptors (for activin A or B respectively) serine/threonine kinases on the surface of target cells. Both type II receptors present an extracellular domain, the transmembrane domain, and the cytoplasmic domain carrying kinase activity. The interaction of Activin with a dimer of type II receptors is necessary for recruitment and phosphorylation of the activin type I receptor-like kinase dimers (ALK4 or ALK7) at their glycine-serine-rich domain. Once activated, ALK 4/ALK7 binds and phosphorylates the cytoplasmic Smad (Smad2 and Smad3) proteins, thus allowing signal transduction to the nucleus.[8]

Smad proteins are a family of transcription factors that regulate more than 500 target genes in a cell-specific and dose-dependent manner. They are divided into three groups: (i) receptor-regulated Smad (R-Smad) consisting of Smad2 and Smad3 activated by TGF-β and activins and Smad1, Smad5 and Smad8 activated by BMP, (ii) inhibitory Smad, i.e., Smad6 and Smad7, (iii) and the common mediator Smad4. Once phosphorylated, R-Smad binds another R-Smad or Smad4, and then the complex translocates into the nucleus where it binds the target DNA consensus sequence triggering activin signaling dependent transcription (**Figure 1A**).[9]

Modulation of Activin Signaling. The activin pathway is regulated and modulated at various levels, including extracellular binding proteins (follistatin and protein encoded by FLRG), molecules which antagonize their bind with the receptor (inhibins), co-receptors of activin antagonists (beta-glycan), inhibitory Smad proteins, and other proteins ligands of activin receptors (myostatin -or GDF8- and GDF11).

The glycoprotein follistatin exists in two isoforms of 288 (FS288) and 315 (FS315) aminoacid residues due to alternative RNA splicing. FS288 is a potent extracellular negative regulator of Activin; in particular, its FS-1 domain, consisting of ten cysteine residues involved in binding the cell surface, is essential to abolish activin biological activity. Follistatin acts by masking the activin regions crucial for the interaction with ActRII and ALK4.[10]

Another follistatin family member is the follistatin-related gene FLRG, differing from follistatin because it lacks the FS-3 domain, and it is considered a homolog, not totally functional, of the alternative spliced circulating isoform of follistatin (FS315). FLRG is highly expressed in the placenta, testis, and cardiovascular tissue, while follistatin expression is higher in the pituitary and ovary.[11] Nevertheless, the protein encoded by FLRG shows functional similarity to follistatin in inhibiting erythropoiesis, being follistatin a repressor of Activin A-induced erythroid differentiation.[12] Follistatin and the protein encoded by FLRG inhibit not only activins but also GDF11 and GDF8.[13]

Activins beta A and beta B subunits can also heterodimerize with inhibin α-subunit to form inhibin A or B. Inhibins share the same binding site of ActRII; however, their affinity is tenfold lower than activins. Beta glycan is the co-receptor for inhibins, which increases their affinity by 30 times for ActRII, forming a complex, which prevents ActRII from binding to ALK4.[14] Beta glycan is expressed in rat brain, pituitary gland, and gonads confirming its modulatory role on inhibins.[15,16] Betaglycan is also an accessory receptor for TGF-β ligands 1, 2, and 3.

Inhibitory Smads bind stably with type I and type II receptors. Smad7 strongly inhibits Activin, BMP, and TGF-β signaling,[17] but Activin upregulates its expression, BMP, and TGF-β representing a feedback

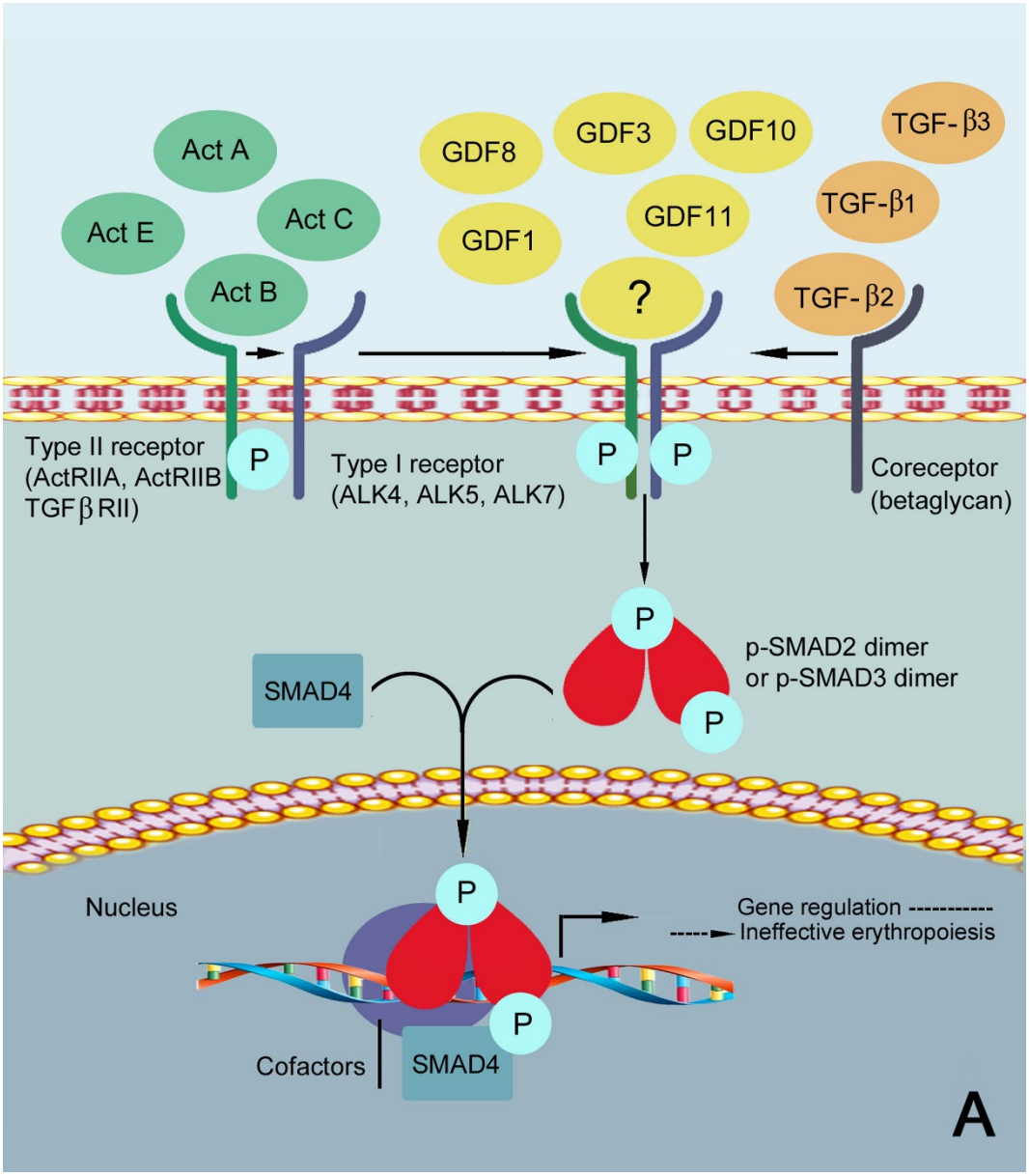

Figure 1. TGF-β pathway and activin receptors ligand traps. A) Canonical signaling trough Smad2/3 activation. Ligand binding induces dimerization of type II receptors and to oligomerization with type I receptors; the activated multimers activate Smad2/3s by phosphorylating them and triggering the formation of the complex with Smad4. pSmad2/3-Smad4 complex translocates to the nucleus regulating specific gene expression. Dimeric ligands and receptors appear as monomers only to simplify the picture.

control mechanism of extracellular signaling.

Activin signaling can also be modulated via post-translational degradation of Smads when Smad-ubiquitination-regulatory factors Smurf interacts with Smad, targeting their ubiquitin proteasome-mediated degradation. While Smurf1 modulates the BMP pathway targeting Smad1 and Smad5, Smurf2 interferes with all BMP, TGF-β and activin signaling because of its broad interaction with Smad. It is noteworthy that Smad4, regulated by BMP and activin/TGF-β competing with each other, is free from ubiquitination regulated by Smurf.[18]

GDF8, a negative regulator cytokine of muscle mass, expressed in cardiac and smooth cells, adipose tissue, mammary gland, and placenta, shares ActRII and ALK4 for intracellular signaling with Activin. GDF8 is expressed in blood cells and promotes differentiation in hematopoietic cell lines, supporting the idea that it may be an autocrine/paracrine factor involved in hematopoiesis control. Its activity could be partially redundant with the activin pathway.[19]

GDF11, whose amino acid sequence is 90% identical to GDF8, is another essential ligand for ALK4 and ActRII involved in neurogenesis, kidney, endocrine pancreas development, and heart. It has been recently identified as a negative regulator of late-stage maturation of erythroid precursors. GDF11 is overexpressed in β-thalassemia, and it has been hypothesized to lead to ineffective erythropoiesis through a deleterious autocrine amplification loop involving oxidative stress and alpha-globin precipitation.[20]

TGF-β and TGF-β Receptors. TGF-β, the founding member of the complex TGF-β superfamily signaling, is a critical crucial and multifunctional cytokine existing in three mammalian isoforms (TGF-β1, TGF-β2 and TGF-β3). Each isoform is produced in an inactive form made by a latency-associated peptide (LAP) and the active TGF-β fraction which is covalently associated with latent TGF-β binding protein (LTBP) with significant consequences on TGF-β localization and activation. Although various stimuli have been proposed (heat, acid pH, wound, reactive oxygen species), the key activators of TGF-β are integrins. Integrins are cell adhesion and signaling receptors formed by one of 18 α and one of eight β subunits of a transmembrane receptor, thus creating 24 types of different integrins. Only four have been shown to liberate active TGF-β. It is noteworthy that TGF-β2 lacks the integrin-binding motif; consequently, another mechanism might be involved in TGF-β2 activation.[21]

The pivotal role of TGF-β in regulating proliferation of hematopoietic stem cells (HSCs) has been well demonstrated. Indeed, TGF-β1 is essential for controlling the quiescence of HSCs, and the production of TGF-β by both HSCs and niche stromal cells can contribute to the maintenance of the stem cell compartment. Opposite to TGF-β1, TGF-β2 is a positive regulator of hematopoietic stem cells, while TGF-β3 only functions as an inhibitor on primitive hematopoietic cells. Generally, TGF-β inhibits growth *in vitro* by transcriptional downregulating the growth-stimulating protein c-Myc and receptors for hematopoietic cytokines (GM-CSF, IL-3, IL-1), and inducing the cyclin-dependent kinase inhibitors (CDKIs) p15, p21, p27 and p57, and stem cell factor. Indeed, TGF-β has been considered dispensable for regulating hematopoietic stem cells *in vivo*, probably because of the existence of redundant signals in the whole system.[22]

TGF-β isoforms signaling acts via different expression functions by sharing the same receptors, TGF-β type II receptor (TGF-βRII) and type I receptors (ALK1 and ALK5), and it is sustained by Smad2 and Smad3 at the cytoplasmic level. As for activins, also in the case of TGF-β the binding of R-Smad with Smad4 stimulate the transcription of target genes. Otherwise, it has been shown that the ubiquitous nuclear protein Transcriptional Intermediary Factor 1 gamma (TIF1-γ) binds Smad2/3 selectively competing with Smad4. Whereas the association of Smad2/3 with Smad4 inhibits the proliferation of hematopoietic stem cells, the interaction of TIF1-γ with Smad2/3 stimulates erythroid differentiation, suggesting that hematopoiesis could be controlled by these two distinct branches of TGF-β pathway.[23]

Finally, TGF-β can modulate other signaling pathways without engaging the canonical Smad system: TGF-β activated kinase 1 (TAK1), a component of mitogen-activated protein kinase (MAPK), activates c-Jun-N terminal kinase (JNK) and p38. Other downstream transducers directly activated by TGF-β are the extracellular signal-regulated kinase (ERK), phosphatidylinositol three kinases (PI3K), and RHO-like small guanosine triphosphatases. The role of these systems is not yet well defined in hematopoietic stem cells.[24]

Activin Receptor II Ligand Traps. Misregulation of the activin pathway has been implicated in anemia, myeloma-associated osteolysis, metastatic bone disease, and carcinogenesis.[25] Activin and activin receptors, because of their diverse biological processes, have been extensively studied as potential therapeutic targets in several pathological conditions.[26] In particular, the inhibition of the TGF-β pathway is the mechanism by which a therapeutic effect can be achieved, acting on the Smad2/3 cascade. Two such agents are Sotatercept (ACE-011) and Luspatercept (ACE-536), ligand-trapping fusion proteins containing the modified extracellular domain of activin receptor type IIA (ActRIIA) or IIB (ActRIIB), respectively, fused to the Fc domain of IgG1.[27,28] Both of them sequestrate the ligand before it can interact with the receptor, thus inhibiting the signal transduction cascade.

It has been demonstrated that in *ex vivo* conditions, ACE-011 binds Activin A and B, GDF8, GDF11, and other BMPs (such as BMP6, BMP7, and BMP10) with different affinities.[27] Luspatercept, on the other hand, has shown high binding capacity with activin B, GDF8, and GDF11, but not with Activin A.[28,32] Both of them do not interact with TGF-β1, TGF-β2 nor TGF-β1. These activin ligand traps show similar binding ligand-binding profiles, but ACE-536 shows a higher ligand selectivity making this specific molecule more suitable for treating anemia and ineffective erythropoiesis.

Preclinical Studies on the Effect on Hematological Parameters. The murine counterpart of Sotatercept (namely RAP-011) was firstly studied to evaluate the role of Activin and its related ligands in bone metabolism,[29] and ACE-011 showed an unexpected effect in increasing hematocrit and Hb levels[30,31] during clinical trials for post-menopausal osteoporosis. Different studies are ongoing in the attempt to elucidate the molecular mechanisms underlying Hb elevation. Wildtype mice treated with RAP-011 showed a rapid increase of hematocrit, Hb, and RBC count.[33] Similarly, in $Hbb^{th1/th1}$ mouse model of β-thalassemia intermedia, RAP-011, or RAP-536 treatment resulted in higher RBC count, hematocrit, total Hb concentration, as well as reduced reticulocytosis.[34] Also, thalassemia RBC morphology ameliorates under RAP-011 treatment, confirming this molecule's effect on erythropoiesis and

the effect observed in clinical studies.[35] In treated Hbb$^{th1/th1}$ mice, physiologic erythroid differentiation was partly restored, showing a decrease in the number of basophilic erythroblasts and an increase in orthochromatic erythroblasts and reticulocytes, both in bone marrow and spleen. Also, the degree of splenomegaly improved, thus acting on ineffective erythropoiesis.[34,35] These results indicated that these molecules promote erythropoiesis, augmenting the terminal erythroid differentiation by inhibiting the ActRII pathway.

In the attempt to identify the specific precursor targeted by activin ligand traps, the numbers of BFU-E and CFU-E were also assessed in wild type mice treated with RAP-011 and ACE-536.[32,33] The results were controversial since RAP-011 was able to increase the percentage of bone marrow BFU-E in bone marrow, inducing the formation of larger colonies.[33] On the other side, by 48h, ACE-536 reduced both bone marrow and spleen BFU-E and CFU-E, followed seven days after by an increased number of these progenitors. EPO acts synergically with these molecules since studies have shown that the EPO antibodies can decrease hematological parameters such as hematocrit, Hb level, and total red blood cells while cotreatment with ACE-536 rescued this blockage. EPO and ACE-536 acted additively since their combined effect was even more significant than the sum of the single-agent' effects, and that suggests that activin ligand traps act on later stage differentiation while EPO sustains RBC production by increasing the availability of early-stage progenitors.[32]

Despite the proven effects in mouse models and clinical trials, the target of these molecules remains unknown. Some works have shown, with the use of ex-vivo models, such as CD34+ cells treated with the molecule in liquid cultures, that there is not a measurable effect of these agents on the stimulation of growth or differentiation of erythroid progenitors or precursors, thereby suggesting that the surrounding environment could mediate the effect. Thus, the use of co-cultures or conditioned media obtained by administration of the RAP-011 or ACE-011 to stromal or long term bone marrow cultures[33,36,37] could be a potential model. In these settings, factors secreted by bone marrow and/or stromal cells probably mediate the effects of RAP or ACE-011 on erythroid differentiation, thus creating a microenvironment that is more permissive for erythropoiesis.

Modulation of Iron Homeostasis and ROS. It was also observed that RAP-011 and RAP-536 modulate iron homeostasis in the Hbb$^{th1/th1}$ mouse model since treated mice did show a reduction in serum iron, transferrin saturation, and ferritin,[34,35] as well as a restored splenic architecture. Since iron parameters are not affected in wildtype animals, it is conceivable that the ligand traps act indirectly on iron homeostasis. Suragani et al. did investigate the expression of two essential genes in the regulation of iron homeostasis: Hepcidin (Hamp) and Bmp6. RAP-536 did increase Hamp liver expression in treated versus untreated Hbb$^{th1/th1}$ mice, while Bmp6 expression was elevated versus wild type mice and unchanged in both of them. The authors hypothesized that Bmp6 expression could remain elevated since it enhances Hamp expression until liver iron reaches average values.

Further information on the mechanism of action was obtained by evaluating α-globin aggregates, ROS, and hemolysis since erythrocytic damage and hemolysis caused by unpaired α-chain aggregates and oxidative stress are key features of β-thalassemia. It was seen that both RAP-011 and RAP-536 reduced ROS and α membrane-associated aggregates levels.[34,35] α-globin gene expression was also decreased[35] (personal data). Also, treated mice display improved hemolysis parameters such as a reduction in the mean concentration of total bilirubin and increased erythrocyte life span.[34]

The Identification of a Specific Ligand. Further studies have investigated the molecular mechanisms aiming to identify the potential candidates that mediate activin receptor ligand traps' action. Most of these studies focused their attention on known ligands, such as Activins A and B, GDF8, and GDF11, involved in the TGF-β pathway and mediating the pathway activation through Smad2/3 phosphorylation. RAP-011 is able to block phosphorylation of Smad 2/3 induced by activin A and GDF11.[33] ACE-536 displayed quite the same activity also towards GDF8 but did not inhibit signaling induced by activins. Among these ligands, the role of GDF11 as a potential target of activin receptors ligand traps and as a new regulator of erythropoiesis has been studied in different disease models. In a β-thalassemia mouse model, GDF11 expression is prominent in the red pulp area, the spleen's erythropoietic niche, and it was abundant in the sera from β-thalassemia as well as myelodysplastic syndrome (MDS) patients. GDF11 expression seems to be higher in immature erythroid precursors and then progressively decreases as maturation proceeds. Wild type mice treated with GDF11 developed mild anemia with reduced RBC parameters and increased the spleen weight, which is indicative of ineffective erythropoiesis. *Ex vivo* treatment with GDF11 caused a reduction of mature erythroblasts.[32] At the same time, treatment with GDF11 antibodies (Ab) promoted terminal erythroblast maturation in the Hbb$^{th1/th1}$ β-thal mice model, suggesting that GDF11 negatively regulates erythroid maturation.[35] Thus, GDF11 seems to inhibit differentiation and maintain the survival of immature progenitors, and as it decreases, erythroid maturation proceeds. The concomitant use of ACE-536 also

reduced the effect mediated by GDF11,[32] and RAP-011 in Hbb$^{th1/th1}$ β-thal mice model also reduced GDF11 expression and reduced ROS levels indicating that in Hbb$^{th1/th1}$ β-thal mouse GDF11 is overexpressed, and it represents a characteristic of ineffective erythropoiesis.[35] The treatment with RAP-011, RAP-536, or ACE-536 in wild type and Hbb$^{th1/th1}$ mouse models and, also in ex-vivo cellular models, inhibited the Smad2/3 phosphorylation promoting terminal erythroid differentiation and the effect on RBC indices.[32-34] Thus, these studies identified GDF11 as a putative key member of TGF-β family directly implicated in late-stage differentiation inhibition through increased oxidative stress.

Nevertheless, this proposed model is controversial, since ACE- and RAP-536 have shown their effectiveness in healthy humans and mice; although GDF11 is not overexpressed,[34,38] GDF11 could be a player, but not the principal one, in this game. A recent study by Guerra et al. excluded GDF11 as the master target of RAP-536, using different genetic approaches. They evaluated hematological parameters in tamoxifen-inducible Cre/Lox recombinase conditional GDF11-knock-out Hbb$^{th3/+}$ mice compared to those of a GDF11-KO-Hbb$^{+/+}$ control mouse. The absence of GDF11 did not improve anemia: GDF11-knock-out Hbb$^{th3/+}$ mice did not show an improvement of hematological parameters, since no change in RBC numbers, Hb, hematocrit, and the percentage of reticulocytes was noted versus Hbb$^{+/+}$ control mice. Testing the efficacy of RAP-536, Guerra et al. also reported that GDF11- knock -out Hbb$^{th3/+}$ and the wild type mice responded in the same way, increasing RBC, Hb, and Hct parameters. Also, GDF11 expression was evaluated in erythroid progenitors derived from healthy and β-thal CD34+ cells, and although GDF11 was very low in both conditions, it was higher in β-thal cells than control cells, even though this difference was not significant.

Similarly, low expression was observed in Ter119^{+} spleen late erythroblasts and also after RAP-536 treatment.[39] Likewise, in our unpublished data, GDF11 mRNA levels were low in CD34^{+}-derived erythroid cultures from β-NTDT patients, but at the same time, the relative levels of GDF11 were higher compared with healthy cultures. RAP-011 treatment did not induce any difference, consistent with Guerra et al. results. Altogether, the results from Guerra et al. excluded that RAP-536 exerts its action exclusively through GDF11 blockage and that this latter is the master effector of TGF-B inhibition of late erythropoiesis.

Modulation of GATA1 and TIF1-γ by ACE-536. Another work addressing the molecular mechanism beyond ACE-536 action focused on pSmad2/3-mediated inhibition of erythroid differentiation. Martinez et al., using differentiating MEL cells and GDF11, induced overactivation of the smad2/3 pathway saw a higher nuclear localization of pSmad2/3 and Smad4 and a concomitant reduced nuclear localization and expression of GATA1 and TIF1-γ. GDF11-induced overactivation of Smad2/3 led to an increase in cells with a wider range of cell size, suggesting an accumulation of more immature cells, increased ROS levels, and reduced cell viability and hemoglobin levels. ACE-536 was able to revert these effects and increased nuclear localization of TIF1-γ and expression of GATA1. Moreover, the transcriptome analysis of splenic erythroblasts from Hbb$^{th3/+}$ mice treated with RAP-536 revealed that different genes were differentially expressed; among them, GATA1 was found. A gene set enrichment analysis of GATA1 activator against RAP-536 data identified other genes that were significantly upregulated. Specifically, specific GATA 1 target genes, regulated by RAP-536, were involved in erythroid differentiation, heme biosynthesis protein quality control, and proliferation, and cell death. At present, it is not still clear if increased GATA1 expression and availability could be a direct effect of RAP-536 or an induced effect due to reduced oxidative stress and cell death.[40] Our unpublished data are consistent with this result, since in RAP-011-treated cultures, at day seven, we observed a higher expression of GATA1 and α-globin compared to untreated cells ($p<0.01$ and $p<0.05$, respectively), which was associated with a transitory reduction in β/a globin ratio. Interestingly, on day 14, GATA1 and α-globin expression were reduced in RAP-011-treated cells, associated with a re-balance of β/a globin ratio compared with untreated cultures ($p<0.005$) (unpublished data).

Martinez and coauthors conclude that higher pSmad2/3 facilitates the complex with Smad4. The treatment with RAP-536 lowers of pSmad2/3 and favors the formation of pSmad2/3-TIF1-γ complexes, influencing GATA1 nuclear availability and expression and other key genes (**Figure 1B**).[40] Thus, they indicate GATA1 as a possible key effector of ACE-536 action in alleviating the ineffective erythropoiesis in MDS cellular and β-thal mouse models.

Activin Receptor-Ligand Traps in Clinical Trials. Both sotatercept and luspatercept have been tested in clinical trials.

Sotatercept was first tested in single and multiple doses in post-menopausal healthy women volunteers, inducing an increase in bone mineral density and bone formation biomarkers. Moreover, clinically significant, dose-dependent increases in Hb, hematocrit, red blood cells (RBCs), and reticulocytes were observed.[30,41] Similarly, luspatercept was evaluated in post-menopausal female healthy volunteers, and they showed an increase in Hb, RBCs, and reticulocytes in a dose-related manner.[38] A phase IIa study evaluated the safety and tolerability of sotatercept and its effects on bone

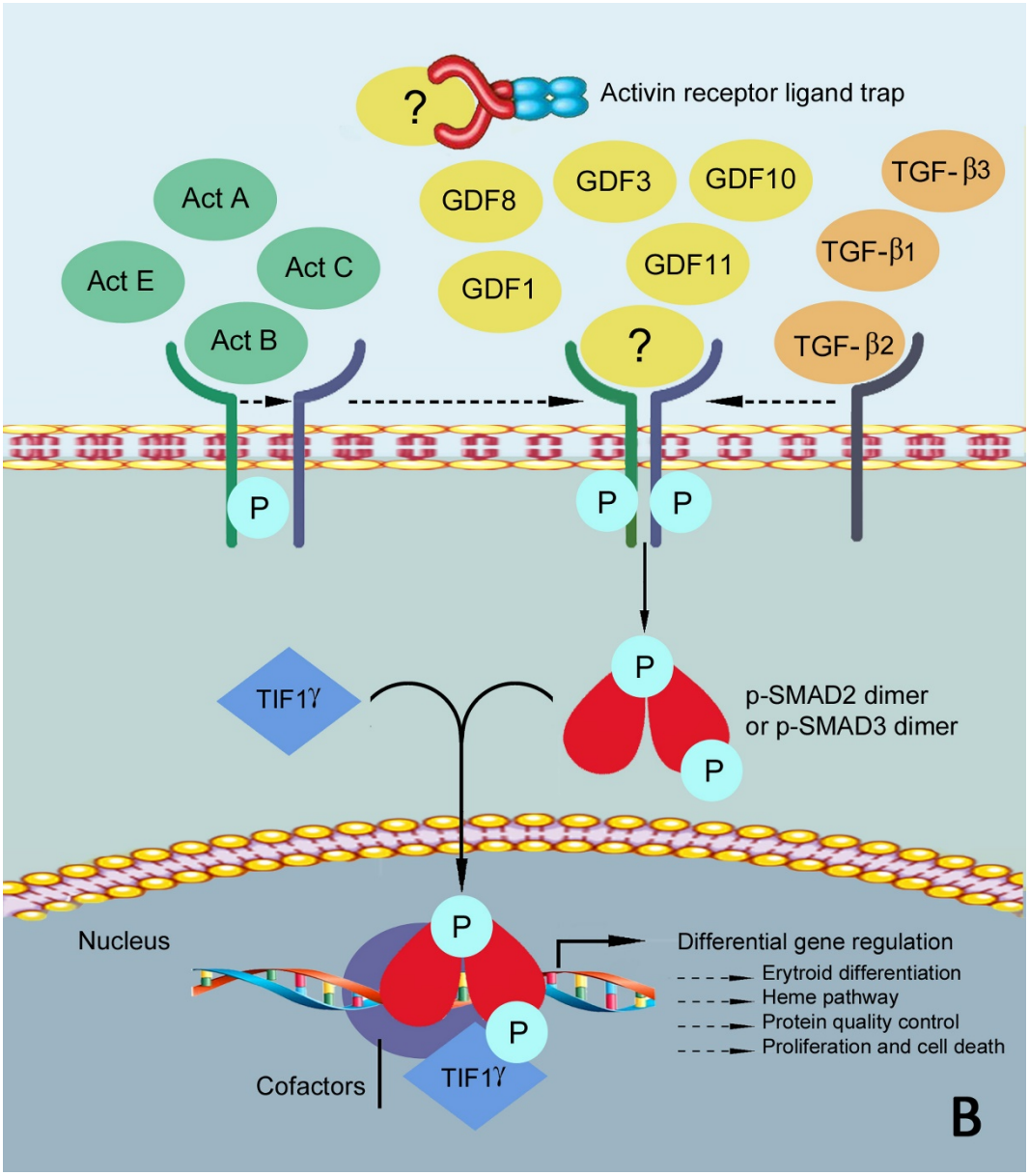

Figure 1. TGF-β pathway and activin receptors ligand traps. B) Signaling inhibited by Activin receptors ligand traps. Activin receptors ligand trap sequestrate the ligand, inhibiting the pSmad2/3-Smad4 complex formation and favoring the interaction with TIF1-γ. In the nucleus, this complex should exert its action by inducing a different transcriptional response. The specific ligand targeted has not been identified yet. Dimeric ligands and receptors appear as monomers only to simplify the picture.

metabolism and hematopoiesis in newly diagnosed and relapsed multiple myeloma patients. Anabolic improvements in bone mineral density and bone formation were observed, whereas bone resorption was minimally affected. Among sotatercept-treated patients, 71% had at least one dose interruption, mainly due to increases in Hb levels, which was dose-dependent.[42]

Activin receptor-ligand trap for hematological disorders. Given the increase in Hb levels, these compounds have been tested in the hematological setting as a potential treatment for patients with ineffective erythropoiesis. Sotatercept was tested for chemotherapy-induced anemia in breast and lung cancer; the studies were terminated early because of slow accrual, but approximately half of the patients receiving sotatercept achieved Hb increase of more than 1 g/dL.[43] Moreover, both were tested in either thalassemia or myelodysplastic syndrome (MDS), resulting in the approval of luspatercept by the Food and Drug Administration (FDA) in November 2019 for thalassemia, April 2020 for MDS, and in July 2020 by European Medicines Agency (EMA).

Sotatercept and luspatercept for β-thalassemia. A multicenter international phase 2 study using sotatercept was conducted on 16 TDT and 30 NTDT patients (NCT01571635).[2] They were treated with sotatercept at doses ranging from 0.1 to 1.0 mg/kg subcutaneously every three weeks. In the TDT group, 63% of the patients achieved a transfusion burden reduction of ≥ 20% sustained for ≥ 24 weeks, 44% a reduction of ≥33%,

and 13% a reduction of ≥50%. In these patients, the mean change in Hb level from baseline to the end of treatment was 0.7 g/dL, and the effective dose of sotatercept was ≥ 0.5 mg/kg. In the NTDT group, 60% achieved a mean Hb increase of ≥1.0 g/dL, and 37% of ≥1.5 g/dL sustained for ≥12 weeks.[2] Sotatercept exhibited an overall good safety profile and was tolerated by most patients.

Luspatercept has been first approved by the FDA to treat TDT patients at the recommended starting dose of 1 mg/kg every three weeks by subcutaneous injection.[44] In a multicenter international phase 2 dose-finding study in β-thalassemia patients (NCT01749540), luspatercept was administered subcutaneously every three weeks at doses ranging from 0.2 to 1.25 mg/kg. In the NTDT group (n=33), 58% of the patients achieved a mean Hb increase from baseline of ≥1.5 g/dL over 14 consecutive days. In the TDT group (n=31), 81% achieved a transfusion burden reduction of ≥20% over any 12 weeks on study compared with the 12 weeks before baseline.[45] These findings paved the way to a randomized Phase 3 clinical trial.

The BELIEVE study is a phase 3 multicenter international randomized, double-blind, placebo-controlled trial that enrolled 336 adult TDT patients randomized in a 2:1 ratio to receive luspatercept plus best supportive care (BSC) versus placebo plus BSC every three weeks for at least 48 weeks (NCT02604433). The primary endpoint was the percentage of patients who had an erythroid response, defined as a reduction in the transfusion burden of at least 33% from baseline (the 12 weeks before the first dose of luspatercept or placebo) during weeks 13 through 24 plus a reduction of at least two red cell units over this 12-week interval. Forty-eight out of 224 (21.4%) in the luspatercept group achieved the primary endpoints compared to the placebo group (4.5%) (P<0,001). Also, 75% had at least a 33% reduction in transfusion burden during any rolling 12-week interval with luspatercept. Transfusion independence was achieved by 11% of the patients in the luspatercept group during any 8-week interval. Adverse events of transient bone pain, arthralgia, dizziness, hypertension, and hyperuricemia were more frequent with luspatercept than placebo.[46] A 5-year open-label extension phase is ongoing to provide long-term efficacy and safety data.

A phase 2 trial (BEYOND) in adults with NTDT is ongoing (NCT03342404). The primary endpoint is the proportion of patients with an increase in mean Hb concentration of ≥ 1 g/dL in the absence of RBC transfusion from weeks 13-24 vs. baseline.

Luspatercept for myelodysplastic syndrome. A phase II multicenter dose-finding study was conducted with sotatercept to treat anemia in low- or intermediate-1-risk MDS and transfusion-dependent anemia failing anemia erythropoiesis-stimulating agent (ESA) (NCT01736683). Sotatercept was administered once every three weeks at a dose ranging from 0.1 to 2.0 mg/kg. Approximately half of the patients achieved hematological improvement-erythroid (HI-E), according to the International Working Group 2006 criteria. Treatment was well tolerated.[47]

Luspatercept has been recently approved by the FDA for the treatment of anemia failing an ESA and requiring two or more RBC units over eight weeks in adult patients with very low- to intermediate-risk MDS with ring sideroblasts (MDS-RS) or with myelodysplastic/myeloproliferative neoplasm with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T).[44] In phase 2, multicenter, dose-finding study (PACE-MDS) (NCT01749514, extension study NCT02268383), patients with low or intermediate 1 risk MDS or non-proliferative chronic myelomonocytic leukemia received luspatercept subcutaneously once every three weeks at dose concentrations ranging from 0.125 mg/kg to 1.75 mg/kg. An erythroid response (defined as a reduction in red-cell transfusions of ≥4 units per eight weeks in patients with a baseline transfusion burden of ≥4 units per 8 weeks or as an increase in the hemoglobin level of ≥1.5 g/dL per deciliter over eight weeks in patients with a baseline transfusion burden of <4 units per eight weeks) was observed in 63% of luspatercept-treated patients and 38% had transfusion independence for eight weeks or longer.[48] Since the overall erythroid response rate was higher among patients with ringed sideroblasts (≥15% ring sideroblasts) than other subtypes of MDS, the phase 3 enrolled patients with lower-risk MDS with ring sideroblasts who had been receiving regular RBCs transfusions and were refractory or unlikely to respond to ESA.

The MEDALIST (NCT02631070) is a multicenter randomized, double-blind, placebo-controlled trial that enrolled 229 patients randomly assigned in a 2:1 ratio to receive luspatercept or placebo, administered subcutaneously every three weeks for 24 weeks. For at least eight weeks, transfusion independence was observed in 38% of the luspatercept group patients, compared with 13% of the placebo group (P<0.001). During the first 24 weeks, 28% in the luspatercept group had transfusion independence for 12 weeks or longer, as compared with 8% in the placebo group, and the corresponding values during weeks 1 through 48 were 33% and 12% (P<0.001). Also, a higher percentage of patients in the luspatercept group than in the placebo group had transfusion independence for 16 weeks or longer. The most common luspatercept-associated adverse events (of any grade) included fatigue, diarrhea, asthenia, nausea, and dizziness. The incidence of adverse events decreased over time.

Conclusions. Activin receptor ligand traps are the first pharmacological treatment approved for TDT. Its introduction in clinical practice has the potential to

dramatically impact on TDT management; however, further studies are needed to elucidate its mechanism of action.

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References:

1. Galanello R, Origa R. β-thalassemia. Orphanet J Rare Dis. 2010;5:11. https://doi.org/10.1186/1750-1172-5-11 PMid:20492708 PMCid:PMC2893117
2. Cappellini MD, Porter J, Origa R, Forni GL, Voskaridou E, Galacteros F, et al. Sotatercept, a novel transforming growth factor β ligand trap, improves anemia in β-thalassemia: a phase II, open-label, dose-finding study. Haematologica. 2019;104(3):477-84. https://doi.org/10.3324/haematol.2018.198887 PMid:30337358 PMCid:PMC6395345
3. Verma A, Suragani RN, Aluri S, Shah N, Bhagat TD, Alexander MJ, et al. Biological basis for efficacy of activin receptor ligand traps in myelodysplastic syndromes. J Clin Invest. 2020;130(2):582-9. https://doi.org/10.1172/JCI133678 PMid:31961337
4. Aleman-Muench GR, Soldevila G. When versatility matters: activins/inhibins as key regulators of immunity. Immunol Cell Biol. 2012;90(2):137-48. https://doi.org/10.1038/icb.2011.32 PMid:21537340
5. Chen YG, Wang Q, Lin SL, Chang CD, Chuang J, Chung J, et al. Activin signaling and its role in regulation of cell proliferation, apoptosis, and carcinogenesis. Exp Biol Med (Maywood). 2006;231(5):534-44. https://doi.org/10.1177/153537020623100507 PMid:16636301
6. Shiozaki M, Sakai R, Tabuchi M, Nakamura T, Sugino K, Sugino H, et al. Evidence for the participation of endogenous activin A/erythroid differentiation factor in the regulation of erythropoiesis. Proc Natl Acad Sci U S A. 1992;89(5):1553-6. https://doi.org/10.1073/pnas.89.5.1553 PMid:1542647 PMCid:PMC48490
7. Greenwald J, Vega ME, Allendorph GP, Fischer WH, Vale W, Choe S. A flexible activin explains the membrane-dependent cooperative assembly of TGF-beta family receptors. Mol Cell. 2004;15(3):485-9. https://doi.org/10.1016/j.molcel.2004.07.011 PMid:15304227
8. Walton KL, Makanji Y, Harrison CA. New insights into the mechanisms of activin action and inhibition. Mol Cell Endocrinol. 2012;359(1-2):2-12. https://doi.org/10.1016/j.mce.2011.06.030 PMid:21763751
9. Abe Y, Minegishi T, Leung PC. Activin receptor signaling. Growth Factors. 2004;22(2):105-10. https://doi.org/10.1080/08977190410001704688 PMid:15253386
10. Keutmann HT, Schneyer AL, Sidis Y. The role of follistatin domains in follistatin biological action. Mol Endocrinol. 2004;18(1):228-40. https://doi.org/10.1210/me.2003-0112 PMid:14563935
11. Welt C, Sidis Y, Keutmann H, Schneyer A. Activins, inhibins, and follistatins: from endocrinology to signaling. A paradigm for the new millennium. Exp Biol Med (Maywood). 2002;227(9):724-52. https://doi.org/10.1177/153537020222700905 PMid:12324653
12. Wu J, Dong Y, Teng X, Cheng M, Shen Z, Chen W. Follistatin-like 1 attenuates differentiation and survival of erythroid cells through Smad2/3 signaling. Biochem Biophys Res Commun. 2015;466(4):711-6. https://doi.org/10.1016/j.bbrc.2015.09.044 PMid:26365350
13. Hill JJ, Davies MV, Pearson AA, Wang JH, Hewick RM, Wolfman NM, et al. The myostatin propeptide and the follistatin-related gene are inhibitory binding proteins of myostatin in normal serum. J Biol Chem. 2002;277(43):40735-41. https://doi.org/10.1074/jbc.M206379200 PMid:12194980
14. Lewis KA, Gray PC, Blount AL, MacConell LA, Wiater E, Bilezikjian LM, et al. Betaglycan binds inhibin and can mediate functional antagonism of activin signalling. Nature. 2000;404(6776):411-4. https://doi.org/10.1038/35006129 PMid:10746731
15. MacConell LA, Leal AM, Vale WW. The distribution of betaglycan protein and mRNA in rat brain, pituitary, and gonads: implications for a role for betaglycan in inhibin-mediated reproductive functions. Endocrinology. 2002;143(3):1066-75. https://doi.org/10.1210/endo.143.3.8707 PMid:11861534
16. Chapman SC, Woodruff TK. Betaglycan localization in the female rat pituitary: implications for the regulation of follicle-stimulating hormone by inhibin. Endocrinology. 2003;144(12):5640-9. https://doi.org/10.1210/en.2003-0670 PMid:14500575
17. Miyazono K. Positive and negative regulation of TGF-beta signaling. J Cell Sci. 2000;113 (Pt 7):1101-9.
18. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-b family signalling. Nature. 2003;425(6958):577-84. https://doi.org/10.1038/nature02006 PMid:14534577
19. Fernandez-Nocelo S, Gallego R, Costoya JA, Arce VM. Expression of myostatin in human hematopoietic cells unveils novel autocrine/paracrine actions for the hormone. J Cell Physiol. 2019;234(5):7236-46. https://doi.org/10.1002/jcp.27494 PMid:30370618
20. Rochette L, Zeller M, Cottin Y, Vergely C. Growth and differentiation factor 11 (GDF11): Functions in the regulation of erythropoiesis and cardiac regeneration. Pharmacology and Therapeutics. 2015;156:26-33. https://doi.org/10.1016/j.pharmthera.2015.10.006 PMid:26523637
21. Worthington JJ, Klementowicz JE, Travis MA. TGF-β: a sleeping giant awoken by integrins. Trends Biochem Sci. 2011;36(1):47-54. https://doi.org/10.1016/j.tibs.2010.08.002 PMid:20870411
22. Soderberg SS, Karlsson G, Karlsson S. Complex and context dependent regulation of hematopoiesis by TGF-beta superfamily signaling. Ann N Y Acad Sci. 2009;1176:55-69. https://doi.org/10.1111/j.1749-6632.2009.04569.x PMid:19796233
23. He W, Dorn DC, Erdjument-Bromage H, Tempst P, Moore MA, Massague J. Hematopoiesis controlled by distinct TIF-1γ and Smad4 branches of the TGF-β pathway. Cell. 2006;125(5):929-41. https://doi.org/10.1016/j.cell.2006.03.045 PMid:16751102
24. Blank U, Karlsson S. TGF-β signaling in the control of hematopoietic stem cells. Blood. 2015;125(23):3542-50. https://doi.org/10.1182/blood-2014-12-618090 PMid:25833962
25. Fields SZ, Parshad S, Anne M, Raftopoulos H, Alexander MJ, Sherman ML, et al. Activin receptor antagonists for cancer-related anemia and bone disease. Expert Opinion on Investigational Drugs. 2013;22(1):87-101. https://doi.org/10.1517/13543784.2013.738666 PMid:23127248
26. Tsuchida K, Nakatani M, Hitachi K, Uezumi A, Sunada Y, Ageta H, et al. Activin signaling as an emerging target for therapeutic interventions. Cell Commun Signal. 2009;7:15. doi: 10.1186/1478-811X-7-15. https://doi.org/10.1186/1478-811X-7-15 PMid:19538713 PMCid:PMC2713245
27. Aykul S, Martinez-Hackert E. Transforming Growth Factor-β Family Ligands Can Function as Antagonists by Competing for Type II Receptor Binding. J Biol Chem. 2016;291(20):10792-804.

https://doi.org/10.1074/jbc.M115.713487
PMid:26961869 PMCid:PMC4865925
28. Sako D, Grinberg AV, Liu J, Davies MV, Castonguay R, Maniatis S, et al. Characterization of the ligand binding functionality of the extracellular domain of activin receptor type IIB. J Biol Chem. 2010;285(27):21037-48.
https://doi.org/10.1074/jbc.M110.114959
PMid:20385559 PMCid:PMC2898293
29. Pearsall R, Canalis E, Cornwall-Brady M, Underwood K, Haigis B, Ucran J, et al. A soluble activin type IIA receptor induces bone formation and improves skeletal integrity. Proceedings of the National Academy of Sciences of the United States of America. 2008;105(19):7082-7.
https://doi.org/10.1073/pnas.0711263105
PMid:18460605 PMCid:PMC2383948
30. Ruckle J, Jacobs M, Kramer W, Pearsall AE, Kumar R, Underwood KW, et al. Single-dose, randomized, double-blind, placebo-controlled study of ACE-011 (ActRIIA-IgG1) in post-menopausal women. J Bone Miner Res. 2009;24(4):744-52.
https://doi.org/10.1359/jbmr.081208
PMid:19049340
31. Sherman ML, Borgstein NG, Mook L, Wilson D, Yang Y, Chen N, et al. Multiple-dose, safety, pharmacokinetic, and pharmacodynamic study of sotatercept (ActRIIA-IgG1), a Novel erythropoietic agent, in healthy post-menopausal women. Journal of Clinical Pharmacology. 2013;53(11):1121-30.
https://doi.org/10.1002/jcph.160
PMid:23939631
32. Suragani RNVS, Cadena SM, Cawley SM, Sako D, Mitchell D, Li R, et al. Transforming growth factor-β superfamily ligand trap ACE-536 corrects anemia by promoting late-stage erythropoiesis. Nature Medicine. 2014;20(4):408-14.
https://doi.org/10.1038/nm.3512
PMid:24658078
33. Carrancio S, Markovics J, Wong P, Leisten J, Castiglioni P, Groza MC, et al. An activin receptor IIA ligand trap promotes erythropoiesis resulting in a rapid induction of red blood cells and haemoglobin. British Journal of Haematology. 2014;165(6):870-82.
https://doi.org/10.1111/bjh.12838
PMid:24635723 PMCid:PMC4282119
34. Suragani RN, Cawley SM, Li R, Wallner S, Alexander MJ, Mulivor AW, et al. Modified activin receptor IIB ligand trap mitigates ineffective erythropoiesis and disease complications in murine β-thalassemia. Blood. 2014;123(25):3864-72.
https://doi.org/10.1182/blood-2013-06-511238
PMid:24795345 PMCid:PMC4064330
35. Dussiot M, Maciel TT, Fricot A, Chartier C, Negre O, Veiga J, et al. An activin receptor IIA ligand trap corrects ineffective erythropoiesis in β-thalassemia. Nature Medicine. 2014;20(4):398-407.
https://doi.org/10.1038/nm.3468
PMid:24658077
36. Iancu-Rubin C, Mosoyan G, Wang J, Kraus T, Sung V, Hoffman R. Stromal cell-mediated inhibition of erythropoiesis can be attenuated by Sotatercept (ACE-011), an activin receptor type II ligand trap. Experimental Hematology. 2013;41(2):155-66.
https://doi.org/10.1016/j.exphem.2012.12.002
PMid:23261964
37. Flotta S, Delbini P, Graziadei G, Marcon A, Sung V, Cappellini MD. Erythropoietic response to a ligand trap of activin receptor II in cultures from β-thalassemia patients. Haematologica. 2015;100:766.
38. Attie KM, Allison MJ, McClure T, Boyd IE, Wilson DM, Pearsall AE, et al. A phase 1 study of ACE-536, a regulator of erythroid differentiation, in healthy volunteers. Am J Hematol. 2014;89(7):766-70.
https://doi.org/10.1002/ajh.23732
PMid:24715706 PMCid:PMC4173124
39. Guerra A, Oikonomidou PR, Sinha S, Zhang J, Presti VL, Hamilton CR, et al. Lack of GDF11 does not improve anemia or prevent the activity of RAP-536 in a mouse model of β-thalassemia. Blood. 2019;134(6):568-72.
https://doi.org/10.1182/blood.2019001057
PMid:31151988 PMCid:PMC6688431
40. Martinez PA, Li R, Ramanathan HN, Bhasin M, Pearsall RS, Kumar R, et al. Smad2/3-pathway ligand trap luspatercept enhances erythroid differentiation in murine β-thalassaemia by increasing GATA-1 availability. Journal of Cellular and Molecular Medicine. 2020.
https://doi.org/10.1111/jcmm.15243
PMid:32351032 PMCid:PMC7294138
41. Sherman ML, Borgstein NG, Mook L, Wilson D, Yang Y, Chen N, et al. Multiple-dose, safety, pharmacokinetic, and pharmacodynamic study of sotatercept (ActRIIA-IgG1), a novel erythropoietic agent, in healthy post-menopausal women. J Clin Pharmacol. 2013;53(11):1121-30.
https://doi.org/10.1002/jcph.160
PMid:23939631
42. Abdulkadyrov KM, Salogub GN, Khuazheva NK, Sherman ML, Laadem A, Barger R, et al. Sotatercept in patients with osteolytic lesions of multiple myeloma. Br J Haematol. 2014;165(6):814-23.
https://doi.org/10.1111/bjh.12835
PMid:24650009 PMCid:PMC4312883
43. Raftopoulos H, Laadem A, Hesketh PJ, Goldschmidt J, Gabrail N, Osborne C, et al. Sotatercept (ACE-011) for the treatment of chemotherapy-induced anemia in patients with metastatic breast cancer or advanced or metastatic solid tumors treated with platinum-based chemotherapeutic regimens: results from two phase 2 studies. Support Care Cancer. 2016;24(4):1517-25.
https://doi.org/10.1007/s00520-015-2929-9
PMid:26370220 PMCid:PMC4766217
44. Reblozyl.
https://www.accessdata.fda.gov/drugsatfda_docs/label/2020/761136orig2lbl.pdf Accessed June 06, 2020.
45. Piga A, Perrotta S, Gamberini MR, Voskaridou E, Melpignano A, Filosa A, et al. Luspatercept improves hemoglobin levels and blood transfusion requirements in a study of patients with β-thalassemia. Blood. 2019.
https://doi.org/10.1182/blood-2018-10-879247
PMid:30617198 PMCid:PMC6440118
46. Cappellini MD, Cohen A, Piga A, Bejaoui M, Perrotta S, Agaoglu L, et al. A phase 3 study of deferasirox (ICL670), a once-daily oral iron chelator, in patients with b-thalassemia. Blood. 2006;107(9):3455-62.
https://doi.org/10.1182/blood-2005-08-3430
PMid:16352812
47. Komrokji R, Garcia-Manero G, Ades L, Prebet T, Steensma DP, Jurcic JG, et al. Sotatercept with long-term extension for the treatment of anaemia in patients with lower-risk myelodysplastic syndromes: a phase 2, dose-ranging trial. Lancet Haematol. 2018;5(2):e63-e72.
https://doi.org/10.1016/S2352-3026(18)30002-4
48. Platzbecker U, Germing U, Gotze KS, Kiewe P, Mayer K, Chromik J, et al. Luspatercept for the treatment of anaemia in patients with lower-risk myelodysplastic syndromes (PACE-MDS): a multicentre, open-label phase 2 dose-finding study with long-term extension study. Lancet Oncol. 2017;18(10):1338-47.
https://doi.org/10.1016/S1470-2045(17)30615-0