HHS Public Access

Author manuscript

J Bone Miner Res. Author manuscript; available in PMC 2021 August 24.

Published in final edited form as:

J Bone Miner Res. 2020 September ; 35(9): 1765–1771. doi:10.1002/jbmr.4038.

Visualization of mineral-targeted alkaline phosphatase binding to sites of calcification *in vivo*

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Abstract

A mineral-targeted form of recombinant tissue-nonspecific alkaline phosphatase (TNAP), asfotase alfa, was approved multi-nationally as an enzyme replacement therapy for hypophosphatasia in 2015. Two reports to-date have shown evidence of binding of this drug to mineralizing tissues using histochemistry and immunohistochemistry. Here, we sought to expand on those earlier studies by directly visualizing the *in vivo* binding of asfotase alfa conjugated with AnaTag HiLyte Fluor 750 or Alexa Fluor 647 fluorescent dye to sites of skeletal/dental mineralization and ectopic calcification. We utilized 40-day-old *Tagln-Cre; Hprt*$^{ALPL/Y}$ mice, a model of severe medial vascular calcification, *Tie2-Cre; Hprt*$^{ALPL/Y}$ mice, a model of severe intimal calcification, and sibling WT *Hprt*^{ALPL} mice devoid of soft-tissue calcification. A single dose of 8 mg/Kg labeled asfotase alfa was injected via the retro-orbital route. Skeletal tissues and soft organs were imaged *ex-vivo* two days after the injection. Strong fluorescence signal was observed in all skeletal tissues (calvaria, vertebra, long bones, jaw and mandibles) from mutants and WT. Fluorescence analysis of histological sections from bones revealed strong binding of asfotase alfa. Asfotase alfa binding to sites of ectopic calcification in the heart, aorta and renal artery were found in both the *Tagln-Cre; Hprt*$^{ALPL/Y}$ and *Tie2-Cre; Hprt*$^{ALPL/}$ mice but not in WT mice. In addition, asfotase alfa binding was also seen in the kidney stroma and brain of the *Tie-Cre; Hprt*$^{ALPL/Y}$ mice. Our results show that fluorescence-labeled asfotase alfa administered *in vivo* binds not only to sites of skeletal and dental mineralization but also to sites of ectopic calcification in these animal models.

INTRODUCTION

Hypophosphatasia (HPP) is the rare heritable form of rickets or osteomalacia[1] caused by loss-of-function mutation(s) in the *ALPL* gene[2,3] that encodes tissue-nonspecific alkaline phosphatase (TNAP; a.k.a. liver/bone/kidney type AP). The disease can be classified according to patient age when the first signs and symptoms manifest; i.e., perinatal, infantile, childhood, adult and odonto HPP.[4] In 2005, Enobia Pharma (Montreal, Canada) developed a mineral-targeted form of recombinant TNAP (sALP-Fc-D_{10}, aka as ENB-0040 or asfotase alfa) and our laboratory administered it subcutaneously to *Alpl*$^{-/-}$ mice, a model of infantile HPP, from birth.[5,6] The treated *Alpl*$^{-/-}$ mice grew normally and showed no evidence of

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DISCLOSURES

The authors report no conflicts of interest

seizures or skeletal abnormalities, that were characteristic and rapidly lethal in the untreated mice. This treatment also prevented the dental defects preserving acellular cementum,[7] dentin[8] and enamel mineralization,[9] demonstrating the robustness of mineral-targeted enzyme replacement therapy for the management of severe HPP disease. Subsequent clinical trials using asfotase alfa in patients with life-threatening HPP reported skeletal improvement and prevention of seizures and this biologic, under the name Strensiq (Alexion Inc), was approved in 2015 typically for the treatment of pediatric-onset HPP.[10]

Despite the pre-clinical and clinical efficacy of asfotase alfa, studies showing homing of asfotase alfa to mineralizing tissues are scarce. Asfotase alfa was shown to bind 32-fold better than the native enzyme to synthetic hydroxyapatite *in vitro*.[5] Direct binding of immune-gold labeled asfotase alfa to synthetic hydroxyapatite was also reported with an estimated binding energy of −21.3 kcal/mol using RosettaSurface adsorption energy calculations.[7] One report[5] showed an image of histochemical staining for alkaline phosphatase activity in the proximal tibia of asfotase alfa–treated $Alpl^{-/-}$ mice and another detected bound TNAP by immunohistochemistry in the incisor enamel matrix of $Alpl^{-/-}$ mice.[9] Given that asfotase alfa was designed to bind to hydroxyapatite, the possibility that this drug could home to sites of ectopic calcification has been implicit but our laboratory has had difficulties obtaining evidence of this using immunohistochemical techniques. We have recently demonstrated that overexpression of TNAP to either the medial[11] or the endothelial[12] layer of the vasculature leads to cardiovascular compromise. These data prompted us to renew efforts to visualize if asfotase alfa could bind to sites of vascular calcification. This is relevant to HPP because adult HPP patients or patients with pediatric-onset HPP undergoing life-long treatments may develop co-morbidities associated with arterial calcification, such as diabetes and chronic kidney disease.[3] Physicians who treat HPP using asfotase alfa are aware of the potential for adverse effects on the vasculature due to the mineral-targeted nature of asfotase alfa and at least one clinical study has reported absence of ectopic calcification in the heart of an elderly woman with HPP before and after 8 months of treatment.[13] In the present study, we administered a single dose of fluorescent labeled asfotase alfa to mouse models of medial and intimal vascular calcification as well as WT control mice to examine in detail the binding of asfotase alfa to the skeleton, dental tissues and sites of soft-tissue calcification *in vivo*, *ex vivo* and *in vitro*.

MATERIALS AND METHODS

Animals

Transgenic mice expressing Cre-recombinase driven by the *Tagln* or *Tie2* gene promoters (*Tagln-Cre* and *Tie2-Cre* respectively) were obtained from the The Jackson Laboratory (Bar Harbor, ME).

Tagln-Cre mice (aka SM22-Cre), express Cre recombinase driven by a smooth muscle cell-specific promoter. Strong expression of Cre in the vascular media was observed, but not in the endothelium during embryogenesis.[11,14,15] The *Tie2-Cre* mouse line expresses Cre recombinase driven by an endothelial cell (EC)-specific promoter/enhancer.[16] $Hprt^{ALPL}$ knock-in mice were generated by GenOway (Lyon, France) using their proprietary “Quick Knock-in™” technology.[16]

The crossbreeding of the Cre-expressing animals with the *Hprt*^{ALPL} mice results in excision of the stop cassette and transgene expression. Homozygous *Tagln-Cre* or heterozygous *Tie2-Cre* male mice were bred with homozygous female *Hprt*^{ALPL} mice to produce mice expressing TNAP in VSMCs or ECs respectively.[11]

This work was conducted with approval of the Institutional Animal Care and Use Committees of Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA, for all animal studies.

Asfotase alfa labeling

Asfotase alfa is a chimeric molecule consisting of the polypeptide chain of human TNAP but with the C-terminal hydrophobic glycosylphosphatdylinositol-anchoring sequence replaced by the Fc region of immunoglobulin to enable rapid purification and a deca-aspartate motif to confer binding to hydroxyapatite.[5] To fluorescence-label asfotase alfa, we used 1 mg of asfotase alfa (Alexion Pharmaceuticals, Inc., Boston, MA - Lot W000783 - 80 mg/0.8 ml) for the AnaTag HiLyte Fluor 750 (MW: 1328.75) protein labeling kit (AnaSpec, Freemont, CA, USA) and 1 mg of asfotase alfa for the Alexa Fluor 647 (MW: 1250) protein labeling kit (Molecular Probes, Eugene, OR, USA). The conjugation was done by the dye/protein molar ratio of 10:1. After that, dye-protein conjugates were purified by using a desalting column and the characterization of the dye-labeled enzyme was determined according to the degree of substitution (DOS) by reading absorbance at 280 nm and 754 nm for HiLyte Fluor 750 or at 280 nm and 650 nm for Alexa Fluor 647 dye. Labeled and unlabeled asfotase alfa preparations were electrophoresed on SDS-PAGE gels and stained with Coomassie blue to verify the integrity of the polypeptide chains after labeling and kinetic assays, using 1 mM p-nitrophenylphosphate as substrate[5], were conducted to verify preservation of enzymatic activity after labeling.

In vivo and *ex vivo* fluorescence imaging

To detect fluorescence in live mice, a single dose of asfotase alfa labeled with HiLyte Fluor 750 or Alexa Fluor 647 (8 mg/kg body weight (bw)) was intravenously administered via retro-orbital injection. Mice were subjected to live imaging using the Xenogen IVIS 200 Imaging System (Perkin Elmer, Waltham, MA, USA). Fluorescence intensity (photons/second (p/s)) in the whole body and blood was monitored for up to 2 days or up to 14 days after administration for the time course experiment. Blood droplets were collected from the tails of anesthetized animals at time 0 and within 15 minutes, 1 hour, 4 hours, 1 and 2 days after labeled asfotase alfa administration and imaged. Organs were imaged *ex vivo* immediately after dissection 2 days post-administration of labeled asfotase alfa. All images were acquired using the Xenogen IVIS 200 Imaging System (Perkin Elmer, Waltham, MA, USA).

Tissue collection and processing for histological studies

Mice were euthanized and skeletal/soft organs were collected, fixed in 4% paraformaldehyde/PBS solution and processed for histological analysis. Bone samples were cryo-embedded in hexane dry-ice bath and the undecalcified samples were sectioned by

following the Kawamoto method.[16] Soft organs were incubated after fixation in 10%, 15% and 20 % sucrose/PBS solution prior to cryo-embedding in ethanol dry-ice bath.

For immunostaining, bone and soft tissue sections were air dried, and 0.3% Triton X-100 was used for permeabilization. Samples were blocked in 5% donkey serum and probed with primary antibody for CD31 (1:150) diluted in 5% donkey serum in PBS for 3 hours at room temperature (RT). After primary antibody incubation, sections were washed out with PBS and incubated with Alexa Fluor 488-coupled secondary antibody (1:300) for 90 minutes at RT. Sections were washed, and the nuclei were counterstained with DAPI solution for 5 minutes and mounted for imaging by using Zeiss LSM 710 NLO Confocal Microscope or EVOS FL Auto Imaging system.

To visualize mineralization, sections were incubated with 1% alizarin red (pH 6.4) (Sigma, Saint Louis, MO, USA) for 10 minutes, washed briefly with PBS, and then 50% ethanol/50% PBS solution, and immersed in 100% ethanol for 2 minutes. Sections were mounted and scanned by Aperio AT2 system (Leica Biosystems) and images were analyzed by Aperio Color Deconvolution Algorithm software (Leica Biosystems).

Statistical analysis

Values for alizarin red staining quantification were expressed as the mean ± SD. Statistical analysis were performed using One-way ANOVA followed by Tukey's multiple comparisons test. Differences were statistically significant at $^{*}P < 0.05$.

RESULTS

In vivo distribution of labeled asfotase alfa in WT mice

To identify the binding sites of asfotase alfa, a single bolus injection of labeled asfotase alfa (asfotase alfa + AnaTag HiLyte Fluor 750 conjugated) at a dose of 8 mg/Kg (bw) that has consistently shown the best efficacy in preventing HPP disease in mice[5-9] was administered via retro-orbital intravenous injection to wild-type (WT) mice. Whole-body *in vivo* imaging demonstrated that 15 minutes after injection, fluorescence in the whole body was observable and increased up to 4 hours and then gradually decreased. In mice injected with the free dye (HiLyte Fluor 750) alone, fluorescence decreased after 1 hour of injection (Supplemental Figure 1 A). Fluorescence imaging analysis of blood drops collected from labeled asfotase alfa and free dye mice groups, also showed stronger fluorescence intensity 15 minutes post-injection in the both groups which gradually decreased with no fluorescence after 4 hours in the free dye mice, whereas in the labeled asfotase alfa injected mice fluorescence was still observed 1 day after injection (Supplemental Figure 1 B). Extending the period of observation up to 14 days post-injection indicated that the fluorescence signal in calvaria and long bones was found at all time points after the single dose of labeled asfotase alfa injection (Supplemental Figure 1 E) with gradually decreasing fluorescence levels observable after day 2 (Supplemental Figure 1 F).

Thus, to examine fluorescence labeling of the mineralizing tissues we selected two days after a single dose injection of labeled asfotase alfa to dissect calvaria and long bones from WT mice and performed *ex vivo* fluorescence imaging analysis and prepared histological

sections for fluorescence microscopy. Strong fluorescence was observed only in skeletal samples from labeled asfotase alfa injected mice group but not in the free dye-injected mice (Supplemental Figure 1 C & D).

Homing of labeled asfotase alfa to sites of normal and pathological mineralization

We used 40 day-old *Tagln-Cre; Hprt*$^{ALPL/Y}$ mice (a model of severe medial vascular calcification), *Tie2-Cre; Hprt*$^{ALPL/Y}$ (a model of severe intimal calcification) and WT *Hprt*$^{ALPL/Y}$ mice (devoid of soft-tissue calcification) as controls, to examine in detail binding of asfotase alfa to the skeleton, dental tissues and sites of soft-tissue calcification. As before, a single dose of the labeled drug (8 mg/Kg bw) showed higher fluorescence intensity up to 4 hours in the whole body of the mutant and control mice and it gradually decreased 2 days after injection (Supplemental Figure 2 A). The same profile was observed in the blood of the mutants, showing higher fluorescence intensity up to 4 hours in all the mice (Supplemental Figure 2 B). Next, bone samples such as calvaria, mandibles, jaw, long bones and vertebra and soft tissues such as, heart and aorta from these mice were dissected and examined for asfotase alfa binding prior to tissue sectioning. Strong fluorescence was observed in all the bone samples from all the animals and in the heart and aorta from *Tagln-Cre; Hprt*$^{ALPL/Y}$ (Supplemental Figure 2C). Fluorescence imaging analysis of skeletal and dental histological sections (calvaria, vertebra, femur and alveolar bone/dental tissues) showed asfotase alfa binding to sites of skeletal/dental mineralization in all three mouse models (Figure 1 A-D).

Next, we performed alizarin red staining of tissue sections to examine the degree of calcification on the soft tissues in these models. Ectopic calcification is seen in the heart and aorta of *Tagln-Cre; Hprt*$^{ALPL/Y}$ and *Tie2-Cre; Hprt*$^{ALPL/Y}$ mice but absent in WT mice with a higher percentage of positive staining in the *Tagln-Cre; Hprt*$^{ALPL/Y}$ mice (Figure 2A & 2B). The quantification of calcified spots in the kidneys was higher in *Tie2-Cre; Hprt*$^{ALPL/Y}$ mice compared than the other mouse models (Figure 2C). Massive calcification was found in the renal arteries of *Tagln-Cre; Hprt*$^{ALPL/Y}$ and *Tie2-Cre; Hprt*$^{ALPL/Y}$ mice models, but not in WT mice (Figure 2D). There were also calcified sites in the brain of *Tie2-Cre; Hprt*$^{ALPL/Y}$ mice near the thalamus (Figure 2E). Fluorescence imaging analysis on the histological sections of soft tissues showed binding of asfotase alfa to sites of calcification in the heart from *Tagln-Cre; Hprt*$^{ALPL/Y}$ and *Tie2-Cre; Hprt*$^{ALPL/Y}$ mice but not from WT mice (Figure 1E and Figure 3A). Strong fluorescence intensity was observed in the calcified aorta from *Tagln-Cre; Hprt*$^{ALPL/Y}$ and in areas of spotty calcification in the *Tie2-Cre; HprtALPL/Y* mice (Figure 1F). In agreement with the alizarin red data, we observed more pronounced asfotase alfa binding to the kidneys from *Tie2-Cre; Hprt* $^{ALPL/Y}$ mice than to *Tagln-Cre; Hprt*$^{ALPL/Y}$ kidneys (Figure 3A). Coronal sections of the brain showed alizarin red staining (Figure 2E) and asfotase alfa binding (Figure 3B) near the thalamus in the *Tie2-Cre; Hprt* $^{ALPL/Y}$ mouse model but not in the brains of the *Tagln-Cre; Hprt*$^{ALPL/Y}$ or WT mice.

To complement these *in vivo* studies, we assessed the binding capability of asfotase alfa to to histological sections of the humerus and aorta from WT and mutants *Tagln-Cre; Hprt*$^{ALPL/Y}$ and *Tie2-Cre; Hprt* $^{ALPL/Y}$. We incubated the tissue sections with fluorescence-tagged

asfotase alfa *in vitro* under two conditions: a) with prior incubation with high concentration of unlabeled asfotase alfa as a blocking solution; and b) without prior incubation with unlabeled enzyme. The results showed strong fluorescence intensity in the group without prior enzyme incubation but only a weak fluorescence signal with prior pre-incubation with unlabeled enzyme, indicating specific binding of asfotase alfa (Figure 4A & 4B).

DISCUSSION

Several early attempts of enzyme replacement therapy using TNAP-rich plasma from Paget's bone disease patients, purified human liver alkaline phosphatase or purified human placental alkaline phosphatase failed to rescue infants with life-threatening HPP.[18-21] This, it became clear that in order to normalize local inorganic pyrophosphate concentrations at sites of calcification, a targeted strategy would need to be adopted to successfully treat HPP.[22] This gave rise to the development of a mineral-targeted form of recombinant TNAP (sALP-Fc-D_{10}, aka as ENB-0040 or asfotase alfa) by Enobia Pharma (Montreal, Canada). A deca-aspartate sequence (D10) to target mineralized tissues was introduced at the C-terminal of human TNAP and an Fc region of human IgG was added to allow a one-step purification and to increase *in vivo* half-life. [5,23] Daily injections of asfotase alfa in the *Alpl*$^{-/-}$ mouse model of infantile HPP prevented the skeletal and dental defects as well as the seizures characteristic of this experimental model.[5] Following successful clinical trials,[10] asfotase alfa was approved for the treatment of pediatric-onset HPP in 2015.

We have shown binding of asfotase alfa to bone and dental tissues using AP activity staining or immunohistochemistry,[5,9] however, those signals were far lower than we had expected considering the actual efficacy of asfotase alfa. We surmise that standard protocols for tissue fixation/processing and staining require multiple steps of incubation in buffers and solutions which are likely to wash away the bound asfotase alfa.[7] In the present study, we have detected fluorescent-labeled asfotase alfa binding to freshly frozen, unfixed mouse tissues sectioned by the Kawamoto method and were able to observe fluorescent signals directly, omitting steps that would possibly wash out the bound enzyme.

Because this drug was designed to specifically bind to sites of mineralization, it is inescapable that it also has the potential to bind to sites of ectopic calcification. Our current results clearly showed binding of fluorescent labeled asfotase alfa to mineralized skeletal and dental tissues in WT mice in agreement with our earlier reports.[5,9] Furthermore, we were able to show that asfotase alfa can bind to sites of arterial calcification in two different mouse models. Given that HPP patients undergoing life-long treatment with afotase alfa may develop co-morbidities associated with vascular calcification, such as diabetes or chronic kidney disease, evaluating the consequences of such binding may be clinically relevant. In 2015, we demonstrated that the upregulation of TNAP activity in vascular smooth muscle (*Tagln-Cre; Hprt*$^{ALPL/Y}$) leads to severe medial vascular calcification, hypertension, cardiac hypertrophy and death[11] and that the pan-endothelial overexpression of TNAP (*Tie2-Cre; Hprt*$^{ALPL/Y}$) induces systemic arterial calcification that leads to severe atherosclerosis and accelerated mortality when combined with an atherogenic diet.[12] Therefore, in this study, we used these mouse models; *Tagln-Cre; Hprt*$^{ALPL/Y}$ mice - a model of severe medial vascular calcification, *Tie2-Cre; Hprt*$^{ALPL/Y}$ mice - a model of severe intimal calcification

and WT *Hprt*^{ALPL} mice as controls to study the binding sites of labeled asfotase alfa in the skeleton and the soft tissues with vascular calcification. Alizarin red staining data showed increased mineralization in the heart, aorta, kidney, renal artery and brain of these medial and intimal vascular calcification mouse models compared to WT mice. Mineralization was higher in heart and aorta of *Tagln-Cre; Hprt*$^{ALPL/Y}$ compared to the *Tie-Cre; Hprt*$^{ALPL/Y}$ mice, whereas in kidney and brain the mineralization was higher in *Tie-Cre; Hprt*$^{ALPL/Y}$. Importantly, we found asfotase alfa binding to all of these sites of soft organ mineralization in the two hypercalcification mouse models *in vivo*, whereas no asfotase alfa binding was detectable in the uncalcified soft organs of the WT mice. Our data clearly indicates that due to its mineral-seeking properties, asfotase alfa binds not only to the skeleton and dental tissues but also to sites of calcification in soft organs. This suggests that patients undergoing life-long treatment with asfotase alfa would benefit if they were evaluated early for the presence of ectopic calcification and followed for any pathophysiological changes that might be considered associated with the treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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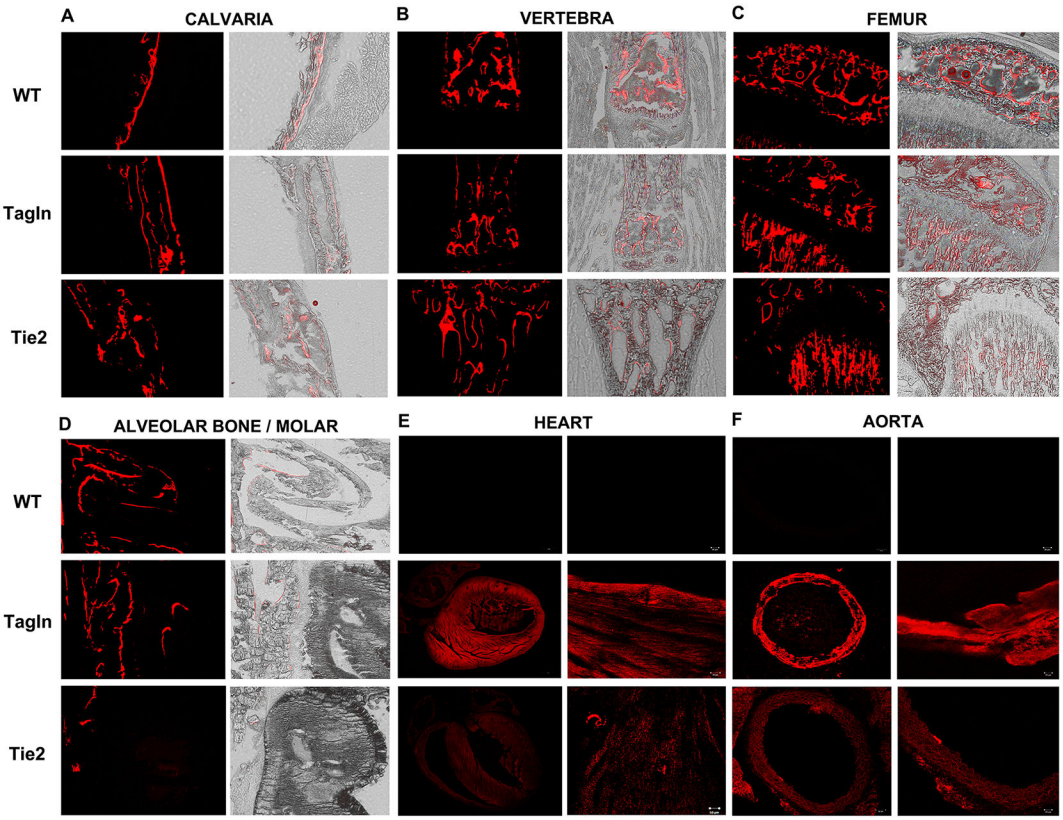

Figure 1. Distribution of fluorescence-tagged asfotase alfa in bones and calcified soft organs.

Single dose of fluorescence-tagged asfotase alfa (8 mg/Kg bw) was IV administered in *TagIn-Cre; Hprt* $^{ALPL/Y}$ and *Tie2-Cre; Hprt* $^{ALPL/Y}$. *Hprt*$^{ALPL/Y}$ mice were used as control. Samples were collected within 2 days after injection. Histological sections of (A) calvaria, (B) vertebra, (C) femur, (D) alveolar bone with first molar, from mandible, (E) heart and (F) aorta were processed and tiles (1 mm) and single images (50 μm) acquired by EVOS FL Auto Imaging System and Zeiss LSM 710 confocal microscope system. Asfotase alfa is shown in red using the 750 nm (Cy7) or 647 nm (Cy5) filters for bones and soft tissues, respectively. This fluorescence imaging experiment showed asfotase alfa binding mineralized tissues. Due to its mineral targeting properties, this biologic is able to bind to mineralizing skeletal and dental tissues as well as to sites of calcification in soft organs.

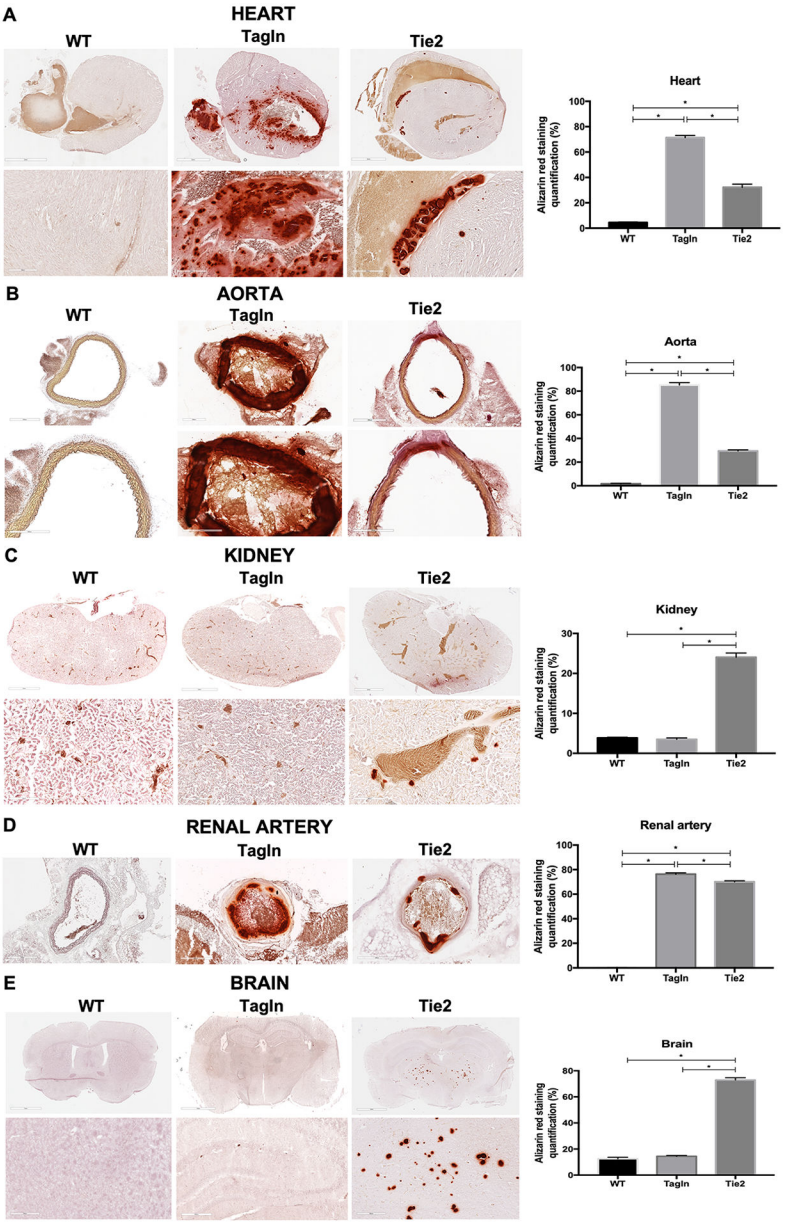

Figure 2: Presence of ectopic calcification in soft tissues.

Alizarin red staining was performed to show the ectopic calcification in some soft organs and vasculature of the *TagIn-Cre; Hprt*$^{ALPL/Y}$ and *Tie2-Cre; Hprt*$^{ALPL/Y}$ mouse models. *Hprt*$^{ALPL/Y}$ mice were used as control. Histological sections of (A) heart, (B) aorta, (C) kidney, (D) renal artery and (E) brain. The quantification of calcified sites is shown as a percentage strong positive alizarin red staining.

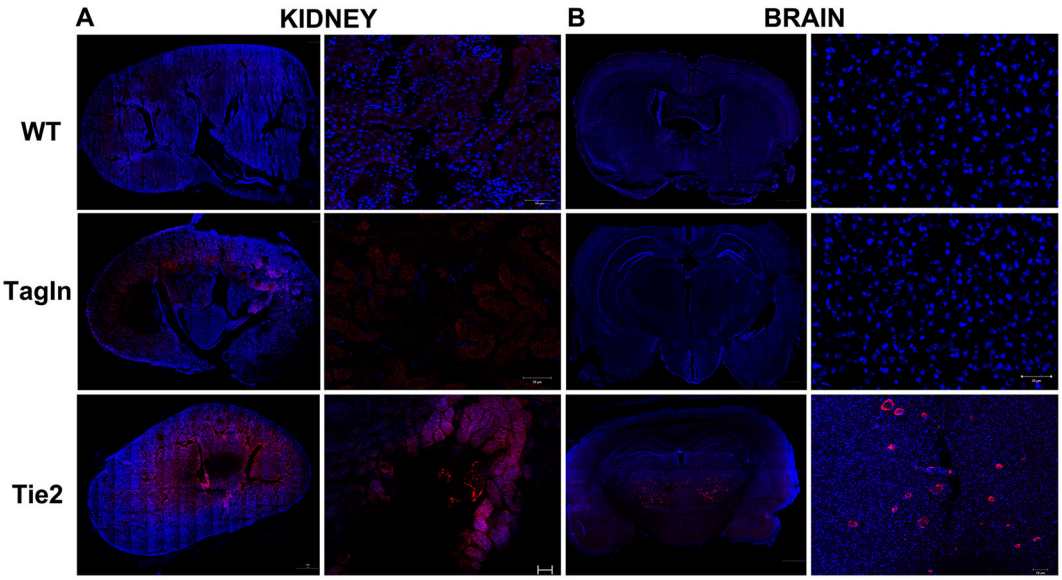

Figure 3: Binding of fluoresecence-labeled asfotase alfa to kidney and brain.

Fluorescence analysis demonstrated asfotase alfa binding to sites of ectopic calcification. Single dose of labeled asfotase alfa (8 mg/Kg bw) was IV administered in *TagIn-Cre;* $Hprt^{ALPL/Y}$ and *Tie2-Cre;* $Hprt^{ALPL/Y}$. $Hprt^{ALPL/Y}$ mice were used as control. Organs were collected within 2 days after injection. Histological sections of (A) kidney and (B) brain were processed and tiles/single images acquired by Zeiss LSM 710 confocal microscope system. Asfotase alfa is shown in red and DAPI (blue) as counterstain.

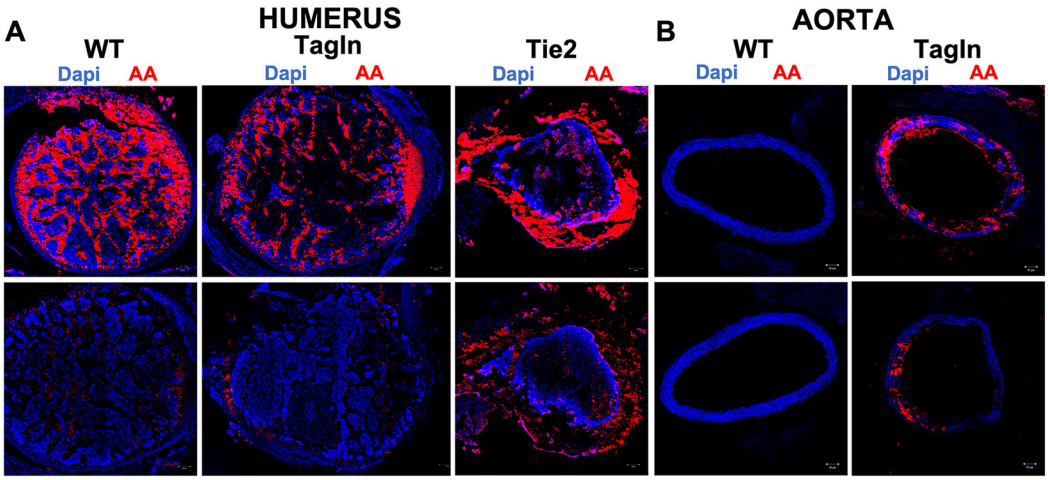

Figure 4: Binding of fluorescence-tagged asfotase alfa to histological sections *in vitro*.

Histological sections of (A) humerus and (B) aorta were incubated with fluorescence-labeled asfotase alfa (45 ug/mL) for 2 hours after pre-incubation with unlabeled enzyme (lower panel) or PBS (upper panel). Images were acquired using Zeiss LSM 710 confocal microscope system (fluorescence-labeled asfotase alfa shown in red and DAPI in blue). The experiment showed the substantial blocking of binding of fluorescence-labeled asfotase alfa by unlabeled enzyme.