Dual Effects of Bisphosphonates on Ectopic Skin and Vascular Soft Tissue Mineralization versus Bone Microarchitecture in a Mouse Model of Generalized Arterial Calcification of Infancy

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Generalized arterial calcification of infancy is an intractable ectopic mineralization disorder caused by mutations in the *ENPP1* gene, resulting in reduced plasma inorganic pyrophosphate (PPi) levels. We previously characterized the *Enpp1*^{asj} mutant mouse as a model of generalized arterial calcification of infancy, and we have now explored the potential efficacy of bisphosphonates, nonhydrolyzable PPi analogs, in preventing ectopic mineralization in these mice. The mice were maintained on either basic diet (control) or diets containing etidronate or alendronate in three different concentrations (experimental). Considering low bioavailability of bisphosphonates when administered orally, subsequent studies tested the mice with subcutaneous injections of etidronate. The treatments were initiated at 4 weeks of age, and the degree of mineralization was assessed at 12 weeks of age by quantitation of calcium deposits in the muzzle skin containing dermal sheath of vibrissae and in aorta. We found that bisphosphonate treatments significantly reduced mineralization in skin and aorta. These changes in treated mice were accompanied with restoration of their bone microarchitecture, determined by microcomputed tomography. The inhibitory capacity of bisphosphonates, with mechanistic implications, was confirmed in a cell-based mineralization assay in vitro. Collectively, these results suggest that bisphosphonate treatment may be beneficial by a dual effect for preventing ectopic soft tissue mineralization while correcting decreased bone mineralization in generalized arterial calcification of infancy caused by *ENPP1* mutations.

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INTRODUCTION

Generalized arterial calcification of infancy (GACI) (OMIM 20800) is an autosomal recessive disorder characterized by ectopic mineralization of the cardiovascular system (Rutsch et al., 2011). The deposition of calcium hydroxyapatite initially on arterial blood vessels, but also on other soft connective tissues, including skin, starts during fetal development, and the disorder is often diagnosed by prenatal ultrasound. The children are born with extensive calcification of arteries, and as a consequence, in most cases they die during the first 12 months of life from cardiovascular complications. The classic form of this disease, GACI type 1, is caused by mutations in the *ENPP1* gene (Ruf et al., 2005; Rutsch et al., 2003), which encodes ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), an enzyme that hydrolyzes adenosine triphosphate to adenosine monophosphate and inorganic pyrophosphate (PPi). Under normal physiologic conditions, PPi is a powerful antimineralization factor and the physiological ratio of PPi to inorganic phosphate (Pi) is required to prevent spontaneous precipitation of calcium phosphate complexes on soft connective tissues. Thus, as a result of loss-of-function mutations in the *ENPP1* gene, the synthesis of PPi is reduced, resulting in a low PPi/Pi ratio that then allows the ectopic mineralization processes to ensue. Loss-of-function *ENPP1* mutations can also cause autosomal recessive hypophosphatemic rickets (Lorenz-Depiereux et al., 2010), suggesting an as-yet-elusive mechanism that balances arterial calcification with bone mineralization. In addition, *ENPP1* mutations have been identified in some patients with pseudoxanthoma elasticum (PXE), another ectopic mineralization disorder, but most cases with this disorder harbor mutations in the *ABCC6* gene (Li et al., 2012; Nitschke et al., 2012).

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Abbreviations: AST, alendronate sodium trihydrate; ETD, etidronate disodium; GACI, generalized arterial calcification of infancy; PXE, pseudoxanthoma elasticum; WT, wild-type

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There is no effective or specific treatment for GACI. A few studies have suggested that administration of bisphosphonates might be helpful in counteracting the ectopic mineralization in GACI; however, there is no consensus about the efficacy of these compounds. In some studies, an apparent improvement has been reported, whereas in other cases there has been very little, if any, effect (Edouard et al., 2011; Galletti et al., 2011; Ramjan et al., 2009; Rutsch et al., 2008). Bisphosphonates have also been reported to be accompanied by severe side effects, particularly on the development of bones (Otero et al., 2013; Silverman et al., 1994; Thomas et al., 1995). These differences can possibly be explained, in part, by the types and doses of bisphosphonates used and the route of administration that vary considerably in different studies.

Bisphosphonates are structural analogs of PPi in which the oxygen linkage to phosphonate (PO3) groups is replaced by a carbon molecule making them stable. The bisphosphonates have two side groups (R1, R2) that determine their potency as well as their pharmacologic characteristics. There are two classes of bisphosphonates, that is, those containing nitrogen in the side groups and non—nitrogen-containing forms. Bisphosphonates have two principal activities relating to mineralization: (a) they can serve as an antimineralization factor, and (b) they are inhibitors of osteoclast activity. The latter property is the basis for their use in the treatment of bone diseases, particularly osteoporosis, but also for Paget's disease and bone metastases (Rodan and Fleisch, 1996; Russell, 2006; Uludag, 2002). The first-generation bisphosphonates, as exemplified by etidronate (ETD), a non—nitrogen-containing bisphosphonate, display considerable antimineralization activities but are less potent inhibitors of osteoclasts than currently used third-generation nitrogen-containing bisphosphonates, such as alendronate (AST). In this study, we have tested the potential use of these prototypic bisphosphonates, ETD and AST, for the treatment of GACI. Specifically, these bisphosphonates were administered orally or by subcutaneous injections to *Enpp1*^{asj} mice (hereafter referred to as *asj*), a mouse model of GACI (Li et al., 2013). These mice harbor a homozygous missense mutation in the *Enpp1* gene that results in markedly reduced ENPP1 enzymatic activity and lowered plasma PPi concentration that subsequently allows for ectopic mineralization of soft connective tissues in the skin and arterial blood vessels to ensue (Li et al., 2013). In this study, we investigated the effects of bisphosphonates in *asj* mice on ectopic mineralization in skin and vascular tissues as well as on bone microarchitecture and mineralization.

RESULTS

GACI is a devastating ectopic mineralization disorder with the demise of affected individuals usually during the first year of life. There is no effective or specific treatment for this disorder. In this study, we tested the hypothesis that bisphosphonates might counteract the ectopic mineralization in skin and vascular tissues, while enhancing bone mineralization, using *asj* mice as a preclinical platform.

Oral administration of bisphosphonates to *asj* mice

In the first set of experiments (set 1), two different prototypic bisphosphonates, ETD or AST, in three different concentrations that were calculated to correspond to 1×, 5×, and 12× of the corresponding human dose used for the treatment of osteoporosis, respectively, were tested by oral administration. Groups of *asj* mice and wild-type (WT) mice were kept on "acceleration diet," which facilitates the mineralization process in these mice (Li et al., 2013) without bisphosphonates serving as positive and negative controls of mineralization. For the experimental groups, all mice were kept on the acceleration diet with or without bisphosphonates (see Table 1). The degree of mineralization was first assessed by histopathologic examination of the dermal sheath of vibrissae, a connective tissue capsule surrounding the bulb of vibrissae in the muzzle skin, which serves as an early progressive biomarker of overall mineralization in these mice (Li et al., 2013).

Table 1. Experimental groups of *Enpp1*^{asj} mice by genotype and treatment[1]

Group	Genotype	No. of mice examined (M+F)	Treatment
Set 1 (p.o.)			
A	*asj*	9 (6 + 3)	Control diet
B	*asj*	9 (6 + 3)	Diet containing 1× ETD
C	*asj*	6 (1 + 5)	Diet containing 5× ETD
D	*asj*	8 (5 + 3)	Diet containing 12× ETD
E	*asj*	6 (4 + 2)	Diet containing 1× AST
F	*asj*	7 (3 + 4)	Diet containing 5× AST
G	*asj*	8 (4 + 4)	Diet containing 12× AST
H	*WT*	9 (6 + 3)	Control diet
Set 2 (s.c.)			
I	*asj*	7 (3 + 4)	Saline
J	*asj*	8 (3 + 5)	0.01× ETD
K	*asj*	11 (8 + 3)	0.12× ETD

Abbreviations: AST, alendronate sodium trihydrate; ETD, etidronate disodium; F, female; M, male; p.o., perioral; s.c., subcutaneous.
[1]The mice were placed on either bisphosphonate-containing diets (set 1) or injected with ETD subcutaneously (set 2) at 4 weeks of age and followed for another 8 weeks. The mice were killed at the age of 12 weeks for histopathological analysis. Control diet: acceleration diet TD.00442.

Histopathologic examination of the *asj* mice on acceleration diet revealed extensive mineralization, whereas no evidence of mineralization was noted in WT mice on the same diet (Figure 1). Evidence of mineralization was also noted in the vibrissae and aorta of *asj* mice treated with various doses of ETD or AST, but histopathologic examination suggested a lesser extent of mineral deposits. The presence of tissue mineralization in *asj* mice was also examined semiquantitatively by histopathology of kidneys, heart, descending thoracic aorta, and eyes of the *asj* mice. The majority of *asj* mice treated with either ETD or AST demonstrated mineralization, and no statistical difference in the proportional mineralization in the kidney, heart, and the eyes was noted (Supplementary Table S1 online). It should be noted that the values in Supplementary Table S1 report the presence of any degree of mineralization. On the other hand, as shown in Figure 1, the degree of mineralization was reduced by the bisphosphonate treatments, although this treatment did not result in the complete absence of mineralization in most cases. Therefore, the values in Supplementary Table S1, which reflect semiquantitative