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# Improvement of the skeletal and dental hypophosphatasia phenotype in *Alpl*$^{-/-}$ mice by administration of soluble (non-targeted) chimeric alkaline phosphatase

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ABSTRACT

Hypophosphatasia (HPP) results from *ALPL* gene mutations, which lead to a deficiency of tissue-nonspecific alkaline phosphatase (TNAP), and accumulation of inorganic pyrophosphate, a potent inhibitor of mineralization that is also a natural substrate of TNAP, in the extracellular space. HPP causes mineralization disorders including soft bones (rickets or osteomalacia) and defects in teeth and periodontal tissues. Enzyme replacement therapy using mineral-targeting recombinant TNAP has proven effective in preventing skeletal and dental defects in TNAP knockout (*Alpl*$^{-/-}$) mice, a model for life-threatening HPP. Here, we show that the administration of a soluble, intestinal-like chimeric alkaline phosphatase (ChimAP) improves the manifestations of HPP in *Alpl*$^{-/-}$ mice. Mice received daily subcutaneous injections of ChimAP at doses of 1, 8 or 16 mg/kg, from birth for up to 53 days. Lifespan and body weight of *Alpl*$^{-/-}$ mice were normalized, and vitamin B6-associated seizures were absent with 16 mg/kg/day of ChimAP. Radiographs, μCT and histological analyses documented improved mineralization in cortical and trabecular bone and secondary ossification centers in long bones of ChimAP16-treated mice. There was no evidence of craniosynostosis in the ChimAP16-treated mice and we did not detect ectopic calcification by radiography and histology in the aortas, stomachs, kidneys or lungs in any of the treatment groups. Molar tooth development and function improved with the highest ChimAP dose, including enamel, dentin, and tooth morphology. Cementum remained deficient and alveolar bone mineralization was reduced compared to controls, though ChimAP-treated *Alpl*$^{-/-}$ mice featured periodontal attachment and retained teeth. This study provides the first evidence for the pharmacological efficacy of ChimAP for use in the treatment of skeletal and dental manifestations of HPP.

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## Introduction

Hypophosphatasia (HPP) is an inborn-error-of-metabolism caused by loss-of-function mutations in the *ALPL* gene located on chromosome 1 [16,39,57], which encodes the tissue-nonspecific of alkaline phosphatase (TNAP), also known as liver–bone–kidney type alkaline phosphatase (AP) [27,41]. The other three human APs are the placental (PLAP), germ cell (GCAP) and intestinal (IAP) isozymes encoded by syntenic genes (*ALPP*, *ALPPL2* and *ALPI*, respectively) found clustered on chromosome 2 [27]. HPP is characterized by subnormal plasma TNAP activity, leading to accumulation of the physiological substrates inorganic pyrophosphate ($PP_i$), a potent mineralization inhibitor [24], and pyridoxal-5′-phosphate (PLP), the major circulating form of vitamin B6 [31,38,41]. These biochemical changes underlie the most significant symptomatology in individuals with HPP: accumulation of $PP_i$ resulting in rickets in children or osteomalacia in adults [41,58,59], as well as dental defects [14,15,34]. The inability to produce pyridoxal (PL) from PLP leads to defective production of neurotransmitters such as γ-aminobutyric acid (GABA) and serotonin, leading to seizures in the most severely affected patients [3,30,38].

The clinical severity of HPP is broad-ranging, encompassing stillbirth with complete absence of mineralized bones to defects limited to teeth and supporting structures, as in the odonto-HPP form [41,58,59]. To-date, 280 different *ALPL* mutations have been found associated with HPP, about 70% of which are missense mutations (http://www.sesep.

*Abbreviations:* ChimAP, chimeric alkaline phosphatase; PL, pyridoxal; PLP, pyridoxal phosphate; TNAP, tissue-nonspecific alkaline phosphatase; PDL, periodontal ligament; $PP_i$, inorganic pyrophosphate

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uvsq.fr/03_hypo_mutations.php). Compound heterozygosity of severe recessive *ALPL* alleles are most often associated with life-threatening HPP disease [16,30,50], while the mild forms of HPP mostly result from dominant negative effects of severe alleles or from compound heterozygosity for severe and moderate alleles [8]. TNAP knockout mice (*Alpl*$^{-/-}$) phenocopy the infantile form of HPP extremely well [9,32]. *Alpl*$^{-/-}$ mice are born with a normal skeleton, but develop rickets starting from postnatal day 6 and die before weaning with severe skeletal disease, following acute episodes of apnea and seizures [1,2,9,17,31,32].

To date, there is no established medical treatment for HPP [41,58,59]. Attempted enzyme replacement therapy (ERT) using intravenous (IV) infusions of ALP-rich plasma from Paget's bone disease patients, purified human liver ALP, or purified human placental ALP failed to rescue severely affected infants [40,43–45]. From these studies, it was concluded that TNAP activity might need to be directed to the skeleton rather than simply enhanced in the circulation in order to prevent or reverse the pathophysiology of HPP [5,42]. This hypothesis was supported by improvement in two unrelated girls with infantile HPP, following transplantation of healthy mesenchyme-derived cells in the skeleton [5,42]. Thus, scientists from 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 group administered it subcutaneously to *Alpl*$^{-/-}$ mice from birth [28]. The treated *Alpl*$^{-/-}$ mice grew normally, appeared well and showed no evidence of epilepsy or skeletal mineralization defects [28,47]. The first clinical study using asfotase alfa in patients with life-threatening HPP disease has also reported skeletal improvement and prevention of seizures in patients diagnosed with life-threatening HPP [46]. This treatment also prevented dental defects in *Alpl*$^{-/-}$ mice, preserving acellular cementum [25], dentin [13] and enamel mineralization [48], demonstrating the robust nature of this mineral-targeting enzyme replacement therapy.

However, we and others have also shown that the sustained availability of soluble (non-targeted) TNAP, introduced either via an adeno-associated viral vector [23] or via direct injection of recombinant soluble TNAP [52] can prolong life, prevent seizures and improve the skeletal phenotype of *Alpl*$^{-/-}$ mice. In this paper, we report studies using a human chimeric recombinant alkaline phosphatase, ChimAP, generated by substituting the flexible crown domain of a human IAP with that of a human PLAP isozyme, a substitution that preserves the three-dimensional structure and dimeric configuration of the recombinant enzyme [19]. ChimAP has catalytic properties distinct from those of recombinant TNAP, and has been developed for therapeutic use in acute kidney injury due to its increased stability, increased $Zn^{2+}$ binding affinity, increased transphosphorylation, a higher turnover number and narrower substrate specificity with selectivity for bacterial-derived lipopolysaccharides (LPS) compared to the parent IAP isozyme or to TNAP [19]. Here we show remarkable efficacy of daily administration of soluble (non-targeted) ChimAP in prolonging life, preventing seizures and ameliorating skeletal and dental disease in *Alpl*$^{-/-}$ mice. We discuss the potential therapeutic applications of this ChimAP in HPP.

## Materials and methods

### *Mice*

The generation and characterization of the *Alpl*$^{-/-}$ mice has been reported previously [32]. *Alpl*$^{-/-}$ mice phenocopy infantile HPP, including global deficiency of TNAP, $PP_i$ accumulation and mineralization defects [1,9,28,31]. Dietary supplementation with vitamin B6 briefly suppresses seizures and extends lifespan until postnatal days 18–22 but hypomineralization and accumulation of osteoid continued to worsen with age [9,28,31,32]. Therefore, all animals (breeders, nursing mothers, pups, and weanlings) in this study were given free access to modified laboratory rodent diet 5001 containing increased levels (325 ppm) of pyridoxine. Genotyping was performed by PCR on genomic DNA as previously described [47]. The Institutional Animal Care and Use Committee (IACUC) approved all animal studies.

### *Soluble chimeric human alkaline phosphatase (ChimAP)*

The chimeric enzyme was generated by substituting the flexible crown domain of a human intestinal alkaline phosphatase (IAP) with that of a human placental alkaline phosphatase (PLAP) isozyme [19]. ChimAP retains the structural folding and dimeric configuration of IAP, but displays greatly increased stability, active site $Zn^{2+}$ binding, increased transphosphorylation, a higher turnover number and narrower substrate specificity, with comparable selectivity for bacterial LPS. Regarding $PP_i$ hydrolysis, ChimAP has a ten-fold higher $K_m$ and similar $k_{cat}$ values compared to TNAP [19]. A solution of ChimAP at 10.1 mg/ml was provided by AM-Pharma (Bunnik, The Netherlands) in 25% glycerol w/v, 5 mM Tris/HCl, 2 mM $MgCl_2$, 50 μM $ZnCl_2$, and at pH 8.0. The enzyme had a purity of >99.99% as determined by high-pressure liquid chromatography.

### *Dose–response study with ChimAP*

*Alpl*$^{-/-}$ mice were divided into 5 cohorts: Vehicle-treated: *Alpl*$^{-/-}$ mice treated with vehicle (n = 14) only; ChimAP1: *Alpl*$^{-/-}$ mice treated with ChimAP at 1 mg/kg/day (n = 14); ChimAP8: *Alpl*$^{-/-}$ mice treated with ChimAP at 8 mg/kg/day (n = 12); and ChimAP16: *Alpl*$^{-/-}$ mice treated with ChimAP at 16 mg/kg/day (n = 10). Wild-type littermates of *Alpl*$^{-/-}$ mice served as reference animals and did not receive injections (n = 14). The vehicle or ChimAP cohorts were injected daily SC into the scapular region. Injections were administered between 8:00 and 11:00 AM. Volumes administered were calculated based on body weight measured prior to injection. All treatments began on postnatal day 1, and were repeated daily for up to 53 days or until the time of necropsy.

### *Sample collection*

Necropsy was performed on postnatal day 53 (p53), 24 h after the final injection of ChimAP for those animals that completed the experimental protocol, or sooner for those animals that appeared terminally ill. Avertin was administered intraperitoneally prior to euthanasia. Blood was collected into lithium heparin tubes by cardiac puncture. Necropsy consisted of a gross pathology examination and radiographic assessment.

### *Radiography and microcomputed tomography (μCT)*

Radiographic images of the entire skeletons and of partially dissected forelimbs, hindlimbs, paws and rib cage were obtained with a Faxitron MX-20DC4 (Chicago, IL, USA), using an energy of 20 kV as previously described [47,49]. Whole dissected skulls from P21 mice were fixed, then scanned at an 18 μm isotropic voxel resolution using the eXplore Locus SP μCT imaging system (GE Healthcare Pre-Clinical Imaging, London, ON, Canada). Measurements were taken at an operating voltage of 80 kV and 80 mA of current, with an exposure time of 1600 ms using the Parker method scan technique, which rotates the sample 180° plus a fan angle of 20°. Scans were calibrated to a hydroxyapatite phantom and 3D images were reconstructed at an effective voxel size of 18 $\mu m^3$. A fixed threshold of 1400 Hounsfield Units was used to discriminate mineralized tissue. Regions of interest (ROIs) for parietal and frontal bones were established as 1 mm in length, 1 mm in width, depth equivalent to thickness of bone and position starting at a 0.75 mm distance from sagittal and coronal sutures, as previously described [20]. Parameters of bone volume, density and structure were measured using Microview version 2.2 software (GE Healthcare Pre-Clinical Imaging, London, ON) and established algorithms [26,37]. Student's t-tests comparing quantitative results were performed to establish statistically significant differences

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