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[Continued on next page]

(54) Title: BONE TARGETED ALKALINE PHOSPHATASE, KITS AND METHODS OF USE THEREOF

Figure 1

(57) Abstract: A bone targeted alkaline phosphatase comprising a polypeptide having the structure : Z-sALP-Y-spacer-X-W_n-V, wherein sALP is the extracellular domain of the alkaline phosphatase; wherein V is absent or is an amino acid sequence of at least one amino acid; X is absent or is an amino acid sequence of at least one amino acid; Y is absent or is an amino acid sequence of at least one amino acid; Z is absent or is an amino acid sequence of at least one amino acid; and W_n is a polyaspartate or a polyglutamate wherein n = 10 to 16. Kits and methods of use thereof.

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TITLE OF THE INVENTION

BONE TARGETED ALKALINE PHOSPHATASE, KITS AND METHODS OF USE THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority on U.S. provisional application Serial No. 60/917,589, filed on May 11, 2007. All documents above are incorporated herein in their entirety by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] N/A.

FIELD OF THE INVENTION

[0003] The present invention relates to bone targeted alkaline phosphatase, kits and methods of use thereof.

BACKGROUND OF THE INVENTION

[0004] Hypophosphatasia (HPP) is a rare, heritable form of rickets or osteomalacia (Whyte 2001) with an incidence as great as 1 per 2,500 births in Canadian Mennonites (Greenberg, 1993) and of 1 per 100,000 births in the general population for the more severe form of the disease. Milder forms are more prevalent. This "inborn error of metabolism" is caused by loss-of-function mutation(s) in the gene (*ALPL*) that encodes the tissue-nonspecific isozyme of alkaline phosphatase (TNALP; a.k.a liver/bone/kidney type ALP) (Weiss et al. 1988; Henthorn et al. 1992a; Henthorn et al. 1992b; Zurutuza et al. 1999; Millán 1995). The biochemical hallmark is subnormal ALP activity in serum (hypophosphatasemia), which leads to elevated blood and/or urine levels of three phosphocompound substrates: inorganic pyrophosphate (PP_i)

phosphoethanolamine (PEA), and pyridoxal 5'-phosphate (PLP) (Whyte 1994).

[0005] HPP features a remarkable range of severity ranging from (most severe to mildest) perinatal, infantile, childhood, adult, and odontohypophosphatasia forms, classified historically according to age at diagnosis (Whyte 2001). There may be almost complete absence of bone mineralization *in utero* with stillbirth, or spontaneous fractures and dental disease occurring first in adult life. Perinatal (lethal) Hypophosphatasia is expressed *in utero* and can cause stillbirth. Some neonates may survive several days but suffer increased respiratory compromise due to the hypoplastic and rachitic disease of the chest. In infantile HPP, diagnosed before 6 months-of-age, postnatal development seems normal until onset of poor feeding, inadequate weight gain, and appearance of rickets. Radiological features are characteristic and show impaired skeletal mineralization, sometimes with progressive skeletal demineralization leading to rib fractures and chest deformity. Childhood Hypophosphatasia has also highly variable clinical expression. Premature loss of deciduous teeth results from aplasia, hypoplasia or dysplasia of dental cementum that connects the tooth root with the periodontal ligament. Rickets causes short stature and the skeletal deformities may include bowed legs, enlargement of the wrists, knees and ankles as a result of flared metaphysis. Adult HPP usually presents during middle age, although frequently there is a history of rickets and/or early loss of teeth followed by good health during adolescence and young adult life. Recurrent metatarsal stress fractures are common and calcium pyrophosphate dihydrate deposition causes attacks of arthritis and pyrophosphate arthropathy. Odontohypophosphatasia is diagnosed when the only clinical abnormality is dental disease and radiological studies and even bone biopsies reveal no signs of rickets or osteomalacia.

[0006] The severe clinical forms of Hypophosphatasia are usually inherited as autosomal recessive traits with parents of such patients showing

subnormal levels of serum AP activity (Whyte 2001). For the milder forms of hypophosphatasia, i.e., adult and odontohypophosphatasia, an autosomal dominant pattern of inheritance has also been documented (Whyte 2001).

[0007] In the healthy skeleton, TNALP is an ectoenzyme present on the surface of the plasma membrane of osteoblasts and chondrocytes, including on the membranes of their shed matrix vesicles (MVs) (Ali et al. 1970; Bernard 1978) where the enzyme is particularly enriched (Morris et al. 1992). Deposition of hydroxyapatite during bone mineralization normally initiates within the lumen of these MVs (Anderson et al. 2005a). Electron microscopy has shown that TNALP-deficient MVs from severely affected HPP patients and *Akp2*$^{-/-}$ mice (a TNALP null mouse model, see below) contain hydroxyapatite crystals, but that extravesicular crystal propagation appears retarded (Anderson 1997; Anderson 2004). This defect is attributed to the extracellular accumulation of PP_i, a potent inhibitor of calcification (Meyer 1984) due to deficiency of TNALP activity (Hessle et al. 2002; Harmey et al. 2004; Harmey et al. 2006.).

[0008] When PPi is present at near physiological concentrations, in the range of 0.01-0.1 mM, PPi has the ability to stimulate mineralization in organ-cultured chick femurs (Anderson & Reynolds 1973) and also by isolated rat MVs (Anderson et al. 2005b), while at concentrations above 1 mM, PPi inhibits calcium phosphate mineral formation by coating hydroxyapatite crystals, thus preventing mineral crystal growth and proliferative self-nucleation. Thus, PPi has a dual physiological role; it can function as a promoter of mineralization at low concentrations but as an inhibitor of mineralization at higher concentrations. TNALP has been shown to hydrolyze the mineralization inhibitor PPi to facilitate mineral precipitation and growth (Rezende et al. 1998). Recent studies using the Akp2$^{-/-}$ mice have indicated that the primary role of TNALP *in vivo* is to restrict the size of the extracellular PPi pool to allow proper skeletal mineralization (Hessle et al. 2002; Harmey et al. 2004).

[0009] The severity of Hypophosphatasia is variable and modulated by the nature of the TNALP mutation. Missense mutations in the enzyme's active site vicinity, homodimer interface, crown domain, amino-terminal arm and calcium-binding site have all been found to affect the catalytic activity of TNALP (Zurutuza et al. 1999). Additionally, other missense, nonsense, frame-shift and splice site mutations have been shown to lead to aberrant mutant proteins or intracellular trafficking defects that lead to subnormal activity on the cell surface. The multitude of mutations and the fact that compound heterozygocity is a common occurrence in Hypophosphatasia also explains the variable expressivity and incomplete penetrance often observed in this disease (Whyte 2001).

[0010] Progress on the human form of the disease benefits greatly from the existence of the TNALP null mice (*Akp2*$^{-/-}$) as an animal model. These *Akp2*$^{-/-}$ mice phenocopy infantile HPP remarkably well, as they are born with a normally mineralized skeleton, but develop radiographically apparent rickets at about 6 days of age, and die between day 12-16 suffering severe skeletal hypomineralization and episodes of apnea and epileptic seizures attributable to disturbances in PLP (vitamin B_6) metabolism (Waymire et al. 1995; Narisawa et al. 1997; Fedde et al. 1999; Narisawa et al. 2001).

[0011] Some TNALP active site mutations have been shown to affect the ability of the enzyme to metabolize PPi or PLP differently (Di Mauro et al. 2002). Both PLP and PPi are confirmed natural substrates of TNALP and abnormalities in PLP metabolism explain the epileptic seizures observed in Akp2$^{-/-}$ mice (Waymire et al. 1995; Narisawa et al. 2001), while abnormalities in PPi metabolism explain the skeletal phenotype in this mouse model of Hypophosphatasia (Hessle et al. 2002; Anderson et al. 2004; Harmey et al. 2004; Harmey et al. 2006; Anderson et al. 2005a).

[0012] There is no established medical therapy for HPP. Case reports

of enzyme replacement therapy (ERT) using intravenous (i.v.) infusions of TNALP-rich plasma from Paget bone disease patients and purified placental ALP have described failure to rescue affected infants (Whyte et al. 1982; Whyte et al. 1984). In another similar study, Weninger et al. (Weninger et al. 1989) attempted ERT for a severely affected premature boy with Hypophosphatasia by infusions of purified human liver TNALP. Treatment (1.2 IU/kg/min) started at age three weeks and was repeated in weekly intervals until age 10 weeks, when the child died. Samples of TNALP were diluted with 10 ml of physiological saline and infused over 30 min via an umbilical arterial catheter. No toxic or allergic side effects were observed. Serum TNALP activity increased from 3 IU/L before treatment to a maximum level of 195 IU/L with a half-life time between 37 and 62 hours. Sequential radiographic studies however showed no improvement of bone mineralization (Weninger et al. 1989).

[0013] It seems that ALP activity must be increased not in the circulation, but in the skeleton itself. This hypothesis is supported by seemingly beneficial responses of two girls with infantile HPP following marrow cell transplantation where TNALP-containing cells were introduced throughout the skeleton (Whyte et al. 2003). Thus there seems to be a need to provide active TNALP to the skeleton of these patients. Recent reports have indicated that poly-aspartate sequences confer bone homing properties to recombinant TNALP (WO 2005/103263 to Crine et al.; Nishioka et al. 2006).

[0014] A recent report showed that the mutated form of TNALP R450C (although Nasu et al. refers to a R433C mutation, his numbering applies to the mature protein and not to one comprising the signal peptide) produces a protein having a dimeric structure joined by a disulfide bridge between the cysteine residues at position 450 of each subunit which strongly inhibited its alkaline phosphatase activity. Nasu et al. concluded that the loss of function results from the interchain disulfide bridge and is the molecular basis for the lethal

hypophosphatasia associated with R450C (Nasu et al. 2006).

[0015] The present description refers to a number of documents, the content of which is herein incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

[0016] Given the current limitations in the clinical management and treatment of patients with HPP, an alternative and efficient treatment was needed. Accordingly, the present invention provides an efficient enzyme replacement therapy for the treatment of HPP.

[0017] To the Applicant's knowledge, and as opposed to previous enzyme replacement therapy efforts in either TNALP null mice or HPP infants in which TNALP or other ALP isozymes were delivered intravenously, the present invention marks the first time where near complete resolution of clinical radiographic and biochemical changes has been documented to occur with enzyme replacement alone.

Bone targeted sALP

[0018] The bone targeted composition of the present invention comprise a fusion protein including in order from the amino side to the carboxylic side a sALP, a spacer, and a bone targeting negatively charged peptide.

ALPs

[0019] There are four known isozymes of ALP, namely tissue non specific alkaline phosphatase further described below, placental alkaline phosphatase (PALP) (e.g., [NP_112603], [NP_001623]), germ cell alkaline phosphatase (GCALP) (e.g., [P10696]) and intestinal alkaline phosphatase (e.g.,

[NP_001622]). These enzymes possess very similar three dimensional structure. Each of their catalytic sites contains four metal binding domains for metal ions necessary for enzymatic activity including two Zn and one Mg. These enzymes catalyze the hydrolysis of monoesters of phosphoric acid and also catalyze a transphosphorylation reaction in the presence of high concentrations of phosphate acceptors. It has been shown in particular that PALP is physiologically active toward phosphoethanolamine (PEA), inorganic pyrophosphate (PPi) and pyridoxal 5'-phosphate (PLP), all three being known natural substrate for TNALP (Whyte, 1995). An alignment between these isozymes is presented in Figure 30.

TNALP

[0020] As indicated above, TNALP is a membrane-bound protein anchored through a glycolipid to its C-terminal (Swiss-Prot, P05186). This glycolipid anchor (GPI) is added post translationally after removal of a hydrophobic C-terminal end which serves both as a temporary membrane anchor and as a signal for the addition of the GPI. Hence the soluble human TNALP used in all Examples below is comprised of a TNALP wherein the first amino acid of the hydrophobic C-terminal sequence, namely alanine, is replaced by a stop codon. The soluble TNALP (herein called sTNALP) so formed contains all amino acids of the native anchored form of TNALP necessary for the formation of the catalytic site but lacks the GPI membrane anchor. Known TNALP include human TNALP [NP-000469, AAI10910, AAH90861, AAH66116, AAH21289, AAI26166]; rhesus TNALP [XP-001109717];rat TNALP [NP_037191]; dog TNALP [AAF64516]; pig TNALP [AAN64273], mouse [NP_031457], bovine [NP_789828, NP_776412, AAI18209, AAC33858], and cat [NP_001036028].

[0021] The bone targeted composition of the present invention encompasses sequences satisfying a consensus sequence derived from the ALP extracellular domain of human ALP isozymes and of known functional TNALPs (human, mouse, rat, bovine, cat and dog). As used herein the terminology

"extracellular domain" is meant to refer to any functional extracellular portion of the native protein (i.e. without the peptide signal). It has been shown that recombinant sTNALP retaining original amino acids 1 to 501 (18 to 501 when secreted) (see Oda et al., J. Biochem 126: 694-699, 1999), amino acids 1 to 504 (18 to 504 when secreted) (US Patent 6,905,689 to Bernd et al.) and amino acids 1 to 505 (18-505 when secreted) (US 2007/0081984 to Tomatsu et al.), are enzymatically active. Examples presented herein also show that a recombinant sTNALP retaining amino acids 1 to 502 (18 to 502 when secreted) (Figure 3) of the original TNALP is enzymatically active. This indicates that amino acid residues can be removed from the C-terminal end of the native protein without affecting its enzymatic activity.

[0022] Table 1 below provides a list of 194 mutations known to cause HPP. In specific embodiments of the bone targeted polypeptides of present invention, the ALP sequence does not include any of these mutations.

[0023] Hence, in sALPs of the present invention, using the numbering of a consensus sequence derived from an alignment of various TNALPs and of human ALP isozymes, the amino acid at position 22 is not a phenylalanine residue; the amino acid at position 33 (position 11 in the sequence without signal peptide) is not a cysteine residue; the amino acid at position 38 (position 16 in the sequence without signal peptide) is not a valine residue; the amino acid at position 42 (position 20 in the sequence without signal peptide) is not a proline residue; the amino acid at position 45 (position 23 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 56 (position 34 in the sequence without signal peptide) is not a serine or a valine residue; the amino acid residue at position 67 (position 45 in the sequence without signal peptide) is not a leucine, an isoleucine or a valine residue; the amino acid residue at position 68 (position 46 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 73 (position 51 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 76 (position 54 in the

sequence without signal peptide) is not a cysteine, a serine, a proline or a histidine residue; the amino acid residue at position 77 (position 55 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 80 (position 58 in the sequence without signal peptide) is not a serine residue; the amino acid residue at position 81 (position 59 in the sequence without signal peptide) is not an asparagine residue; the amino acid residue at position 105 (position 83 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 113 (position 89 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 116 (position 94 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 117 (position 95 in the sequence without signal peptide) is not a serine residue; the amino acid residue at position 119 (position 97 in the sequence without signal peptide) is not a glycine residue; the amino acid residue at position 121 (position 99 in the sequence without signal peptide) is not a serine or a threonine residue; the amino acid residue at position 125 (position 103 in the sequence without signal peptide) is not an arginine residue; the amino acid residue at position 128 (position 106 in the sequence without signal peptide) is not a aspartate residue; the amino acid residue at position 133 (position 111 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 134 (position 112 in the sequence without signal peptide) is not an arginine residue; the amino acid residue at position 137 (position 115 in the sequence without signal peptide) is not a threonine or a valine residue; the amino acid residue at position 139 (position 117 in the sequence without signal peptide) is not a histidine or an asparagine residue; the amino acid residue at position 141 (position 119 in the sequence without signal peptide) is not a histidine residue; the amino acid residue at position 153 (position 131 in the sequence without signal peptide) is not an alanine or an isoleucine residue; the amino acid residue at position 167 (position 145 in the sequence without signal peptide) is not a serine or a valine residue; the amino acid residue at position 172 (position 150 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 175 (position 153 in the sequence without signal peptide) is not

an aspartate residue; the amino acid residue at position 176 (position 154 in the sequence without signal peptide) is not a tyrosine or an arginine residue; the amino acid residue at position 181 (position 159 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 182 (position 160 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 184 (position 162 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 186 (position 164 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 189 (position 167 in the sequence without signal peptide) is not a tryptophan residue; the amino acid residue at position 194 (position 172 in the sequence without signal peptide) is not a glutamate residue; the amino acid residue at position 196 (position 174 in the sequence without signal peptide) is not a lysine or a glycine residue; the amino acid residue at position 197 (position 175 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 198 (position 176 in the sequence without signal peptide) is not an alanine residue; the amino acid residue at position 206 (position 184 in the sequence without signal peptide) is not a tyrosine residue; the amino acid residue at position 208 (position 186 in the sequence without signal peptide) is not a glutamate residue; the amino acid residue at position 207 (position 190 in the sequence without signal peptide) is not a proline residue; the amino acid residue at position 216 (position 194 in the sequence without signal peptide) is not a aspartate residue; the amino acid residue at position 217 (position 195 in the sequence without signal peptide) is not a phenylalanine residue; the amino acid residue at position 223 (position 201 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 225 (position 203 in the sequence without signal peptide) is not a valine or an alanine residue; the amino acid residue at position 226 (position 204 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 228 (position 206 in the sequence without signal peptide) is not a tryptophan or a glutamine residue; the amino acid residue at position 229 (position 207 in the sequence without signal peptide) is not a glutamate residue; the amino acid residue at position 231

(position 209 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 240 (position 218 in the sequence without signal peptide) is not a glycine residue; the amino acid residue at position 251 (position 229 in the sequence without signal peptide) is not a serine residue; the amino acid residue at position 254 (position 232 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 269 (position 247 in the sequence without signal peptide) is not an arginine residue; the amino acid residue at position 277 (position 255 in the sequence without signal peptide) is not a cysteine, a leucine or a histidine residue; the amino acid residue at position 280 (position 258 in the sequence without signal peptide) is not a proline residue; the amino acid residue at position 295 (position 273 in the sequence without signal peptide) is not a phenylalanine residue; the amino acid residue at position 297 (position 275 in the sequence without signal peptide) is not a lysine residue; the amino acid residue at position 298 (position 276 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 300 (position 278 in the sequence without signal peptide) is not a tyrosine or an alanine residue; the amino acid residue at position 301 (position 279 in the sequence without signal peptide) is not a valine, a threonine or an isoleucine residue; the amino acid residue at position 303 (position 281 in the sequence without signal peptide) is not an aspirate residue; the amino acid residue at position 304 (position 282 in the sequence without signal peptide) is not a lysine residue; the amino acid residue at position 305 (position 283 in the sequence without signal peptide) is not a proline residue; the amino acid residue at position 312 (position 290 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 313 (position 291 in the sequence without signal peptide) is not a serine or a leucine residue; the amino acid residue at position 317 (position 295 in the sequence without signal peptide) is not a lysine residue; the amino acid residue at position 332 (position 310 in the sequence without signal peptide) is not an arginine residue; the amino acid residue at position 333 (position 311 in the sequence without signal peptide) is not a cysteine, a glycine or a leucine residue; the amino acid residue at position 334 (position 312 in the sequence without signal

peptide) is not a leucine residue; the amino acid residue at position 340 (position 318 in the sequence without signal peptide) is not an aspartate residue; the amino acid residue at position 345 (position 323 in the sequence without signal peptide) is not an arginine or a glutamate residue; the amino acid residue at position 354 (position 332 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 360 (position 338 in the sequence without signal peptide) is not an aspartate residue; the amino acid residue at position 361 (position 339 in the sequence without signal peptide) is not a threonine or an isoleucine residue; the amino acid residue at position 377 (position 355 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 380 (position 358 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 383 (position 361 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 384 (position 362 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 387 (position 365 in the sequence without signal peptide) is not an arginine residue; the amino acid residue at position 388 (position 366 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 395 (position 373 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 397 (position 375 in the sequence without signal peptide) is not a cysteine or a histidine residue; the amino acid residue at position 398 (position 376 in the sequence without signal peptide) is not an alanine residue; the amino acid residue at position 401 (position 379 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 405 (position 383 in the sequence without signal peptide) is not a serine or a valine residue; the amino acid residue at position 406 (position 384 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 412 (position 390 in the sequence without signal peptide) is not a glycine residue; the amino acid residue at position 416 (position 394 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 417 (position 395 in the sequence without signal peptide) is not an alanine residue; the amino acid residue at position

420 (position 398 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 423 (position 401 in the sequence without signal peptide) is not a serine residue; the amino acid residue at position 426 (position 404 in the sequence without signal peptide) is not a serine residue; the amino acid residue at position 429 (position 407 in the sequence without signal peptide) is not an alanine residue; the amino acid residue at position 430 (position 408 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 432 (position 410 in the sequence without signal peptide) is not a cysteine or an aspartate residue; amino acid residue at position 434 (position 412 in the sequence without signal peptide) is not a proline residue; amino acid residue at position 435 (position 413 in the sequence without signal peptide) is not a lysine residue; amino acid residue at position 442 (position 420 in the sequence without signal peptide) is not a histidine residue; amino acid residue at position 451 (position 429 in the sequence without signal peptide) is not a proline residue; amino acid residue at position 456 (position 434 in the sequence without signal peptide) is not a histidine or a cysteine residue; amino acid residue at position 458 (position 436 in the sequence without signal peptide) is not a lysine residue; amino acid residue at position 460 (position 438 in the sequence without signal peptide) is not an arginine residue; amino acid residue at position 461 (position 439 in the sequence without signal peptide) is not a serine or an aspartate residue; amino acid residue at position 462 (position 440 in the sequence without signal peptide) is not a tryptophan or an arginine residue; amino acid residue at position 465 (position 443 in the sequence without signal peptide) is not a methionine or a leucine residue; amino acid residue at position 472 (position 450 in the sequence without signal peptide) is not a leucine residue; amino acid residue at position 473 (position 451 in the sequence without signal peptide) is not a threonine residue; amino acid residue at position 474 (position 452 in the sequence without signal peptide) is not a threonine residue; amino acid residue at position 479 (position 457 in the sequence without signal peptide) is not a serine residue; amino acid residue at position 482 (position 460 in the sequence without signal peptide) is not a lysine or a glycine residue; amino acid residue at position 484 (position 462 in

the sequence without signal peptide) is not a leucine residue; amino acid residue at position 495 (position 473 in the sequence without signal peptide) is not a serine residue; amino acid residue at position 496 (position 474 in the sequence without signal peptide) is not a phenylalanine residue; and amino acid residue at position 497 (position 475 in the sequence without signal peptide) is not an arginine residue.

[0024] Also more specifically, when a sTNALP is used in the bone targeted sALPs of the present invention, using the numbering of the human TNALP sequence, the amino acid at position 17 is not a phenylalanine residue; the amino acid at position 28 (position 11 in the sequence without signal peptide) is not a cysteine residue; the amino acid at position 33 (position 16 in the sequence without signal peptide) is not a valine residue; the amino acid at position 37 (position 20 in the sequence without signal peptide) is not a proline residue; the amino acid at position 40 (position 23 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 51 (position 34 in the sequence without signal peptide) is not a serine or a valine residue; the amino acid residue at position 62 (position 45 in the sequence without signal peptide) is not a leucine, an isoleucine or a valine residue; the amino acid residue at position 63 (position 46 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 68 (position 51 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 71 (position 54 in the sequence without signal peptide) is not a cysteine, a serine, a proline or a histidine residue; the amino acid residue at position 72 (position 55 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 75 (position 58 in the sequence without signal peptide) is not a serine residue; the amino acid residue at position 76 (position 59 in the sequence without signal peptide) is not an asparagine residue; the amino acid residue at position 100 (position 83 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 108 (position 89 in the sequence without signal

peptide) is not a leucine residue; the amino acid residue at position 111 (position 94 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 112 (position 95 in the sequence without signal peptide) is not a serine residue; the amino acid residue at position 114 (position 97 in the sequence without signal peptide) is not a glycine residue; the amino acid residue at position 116 (position 99 in the sequence without signal peptide) is not a serine or a threonine residue; the amino acid residue at position 120 (position 103 in the sequence without signal peptide) is not an arginine residue; the amino acid residue at position 123 (position 106 in the sequence without signal peptide) is not a aspartate residue; the amino acid residue at position 128 (position 111 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 129 (position 112 in the sequence without signal peptide) is not an arginine residue; the amino acid residue at position 132 (position 115 in the sequence without signal peptide) is not a threonine or a valine residue; the amino acid residue at position 134 (position 117 in the sequence without signal peptide) is not a histidine or an asparagine residue; the amino acid residue at position 136 (position 119 in the sequence without signal peptide) is not a histidine residue; the amino acid residue at position 148 (position 131 in the sequence without signal peptide) is not an alanine or an isoleucine residue; the amino acid residue at position 162 (position 145 in the sequence without signal peptide) is not a serine or a valine residue; the amino acid residue at position 167 (position 150 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 170 (position 153 in the sequence without signal peptide) is not an aspartate residue; the amino acid residue at position 171 (position 154 in the sequence without signal peptide) is not a tyrosine or an arginine residue; the amino acid residue at position 176 (position 159 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 177 (position 160 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 179 (position 162 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 181 (position 164 in the sequence without signal peptide) is not a leucine residue; the amino acid residue

at position 184 (position 167 in the sequence without signal peptide) is not a tryptophane residue; the amino acid residue at position 189 (position 172 in the sequence without signal peptide) is not a glutamate residue; the amino acid residue at position 191 (position 174 in the sequence without signal peptide) is not a lysine or a glycine residue; the amino acid residue at position 192 (position 175 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 193 (position 176 in the sequence without signal peptide) is not an alanine residue; the amino acid residue at position 201 (position 184 in the sequence without signal peptide) is not a tyrosine residue; the amino acid residue at position 203 (position 186 in the sequence without signal peptide) is not a glutamate residue; the amino acid residue at position 207 (position 190 in the sequence without signal peptide) is not a proline residue; the amino acid residue at position 211 (position 194 in the sequence without signal peptide) is not a aspartate residue; the amino acid residue at position 212 (position 195 in the sequence without signal peptide) is not a phenylalanine residue; the amino acid residue at position 218 (position 201 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 220 (position 203 in the sequence without signal peptide) is not a valine or an alanine residue; the amino acid residue at position 221 (position 204 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 223 (position 206 in the sequence without signal peptide) is not a tryptophane or a glutamine residue; the amino acid residue at position 224 (position 207 in the sequence without signal peptide) is not a glutamate residue; the amino acid residue at position 226 (position 209 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 235 (position 218 in the sequence without signal peptide) is not a glycine residue; the amino acid residue at position 246 (position 229 in the sequence without signal peptide) is not a serine residue; the amino acid residue at position 249(position 232 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 264 (position 247 in the sequence without signal peptide) is not an arginine residue; the amino acid residue at position 272 (position 255 in the sequence without signal peptide) is not a

cysteine, a leucine or a histidine residue; the amino acid residue at position 275 (position 258 in the sequence without signal peptide) is not a proline residue; the amino acid residue at position 289 (position 272 in the sequence without signal peptide) is not a phenylalanine residue; the amino acid residue at position 291(position 274 in the sequence without signal peptide) is not a lysine residue; the amino acid residue at position 292 (position 275 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 294 (position 277 in the sequence without signal peptide) is not a tyrosine or an alanine residue; the amino acid residue at position 295 (position 278 in the sequence without signal peptide) is not a valine, a threonine or an isoleucine residue; the amino acid residue at position 297 (position 280 in the sequence without signal peptide) is not an aspirate residue; the amino acid residue at position 298 (position 281 in the sequence without signal peptide) is not a lysine residue; the amino acid residue at position 299 (position 282 in the sequence without signal peptide) is not a proline residue; the amino acid residue at position 306 (position 289 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 307 (position 290 in the sequence without signal peptide) is not a serine or a leucine residue; the amino acid residue at position 311 (position 294 in the sequence without signal peptide) is not a lysine residue; the amino acid residue at position 326 (position 309 in the sequence without signal peptide) is not an arginine residue; the amino acid residue at position 327 (position 310 in the sequence without signal peptide) is not a cysteine, a glycine or a leucine residue; the amino acid residue at position 328 (position 311 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 334 (position 317 in the sequence without signal peptide) is not an aspartate residue; the amino acid residue at position 339 (position 322 in the sequence without signal peptide) is not an arginine or a glutamate residue; the amino acid residue at position 348 (position 331 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 354 (position 337 in the sequence without signal peptide) is not an aspartate residue; the amino acid residue at position 355 (position 338 in the sequence without signal peptide) is not a threonine or an

isoleucine residue; the amino acid residue at position 371 (position 354 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 374 (position 357 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 377 (position 360 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 378 (position 361 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 381 (position 364 in the sequence without signal peptide) is not an arginine residue; the amino acid residue at position 382 (position 365 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 389 (position 372 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 391 (position 374 in the sequence without signal peptide) is not a cysteine or a histidine residue; the amino acid residue at position 392 (position 375 in the sequence without signal peptide) is not an alanine residue; the amino acid residue at position 395 (position 378 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 399 (position 382 in the sequence without signal peptide) is not a serine or a valine residue; the amino acid residue at position 400 (position 383 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 406 (position 389 in the sequence without signal peptide) is not a glycine residue; the amino acid residue at position 410 (position 393 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 411 (position 394 in the sequence without signal peptide) is not an alanine residue; the amino acid residue at position 414 (position 397 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 417 (position 400 in the sequence without signal peptide) is not a serine residue; the amino acid residue at position 420 (position 403 in the sequence without signal peptide) is not a serine residue; the amino acid residue at position 423 (position 406 in the sequence without signal peptide) is not an alanine residue; the amino acid residue at position 424 (position 407 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 426 (position 409 in the sequence without signal peptide) is

not a cysteine or an aspartate residue; amino acid residue at position 428 (position 411 in the sequence without signal peptide) is not a proline residue; amino acid residue at position 429 (position 412 in the sequence without signal peptide) is not a lysine residue; amino acid residue at position 436 (position 419 in the sequence without signal peptide) is not a histidine residue; amino acid residue at position 445 (position 428 in the sequence without signal peptide) is not a proline residue; amino acid residue at position 450 (position 433 in the sequence without signal peptide) is not a histidine or a cysteine residue; amino acid residue at position 452 (position 435 in the sequence without signal peptide) is not a lysine residue; amino acid residue at position 454 (position 437 in the sequence without signal peptide) is not an arginine residue; amino acid residue at position 455 (position 438 in the sequence without signal peptide) is not a serine or an aspartate residue; amino acid residue at position 456 (position 439 in the sequence without signal peptide) is not a tryptophane or an arginine residue; amino acid residue at position 459 (position 442 in the sequence without signal peptide) is not a methionine or a leucine residue; amino acid residue at position 466 (position 449 in the sequence without signal peptide) is not a leucine residue; amino acid residue at position 467 (position 450 in the sequence without signal peptide) is not a threonine residue; amino acid residue at position 468 (position 451 in the sequence without signal peptide) is not a threonine residue; amino acid residue at position 473 (position 456 in the sequence without signal peptide) is not a serine residue; amino acid residue at position 476 (position 459 in the sequence without signal peptide) is not a lysine or a glycine residue; amino acid residue at position 478 (position 461 in the sequence without signal peptide) is not a leucine residue; amino acid residue at position 489 (position 472 in the sequence without signal peptide) is not a serine residue; amino acid residue at position 490 (position 473 in the sequence without signal peptide) is not a phenylalanine residue; and amino acid residue at position 491 (position 474 in the sequence without signal peptide) is not an arginine residue. In other specific embodiments, one or more Xs are defined as being any of the amino acids found at that position in the sequences of the alignment or a residue that constitutes a conserved or semi-conserved substitution of any of these

amino acids. In other specific embodiments, Xs are defined as being any of the amino acids found at that position in the sequences of the alignment. For instance, the amino acid residue at position 51 (position 34 in the sequence without signal peptide) is an alanine or a valine residue; the amino acid residue at position 177 (position 160 in the sequence without signal peptide) is an alanine or a serine residue; the amino acid residue at position 212 (position 195 in the sequence without signal peptide) is an isoleucine or a valine residue; the amino acid residue at position 291 (position 274 in the sequence without signal peptide) is a glutamic acid or an aspartic acid residue; and the amino acid residue at position 374 (position 357 in the sequence without signal peptide) is a valine or an isoleucine residue.

[0025] In specific embodiments, the sALP fragment in the bone targeted fusion protein of the present invention consists of any one of the fragments of a consensus sequence derived from an alignment of human ALP isozymes and TNALPs from various mammalian species corresponding to amino acid residues 18-498, 18-499, 18-500, 18-501, 18-502, 18-503, 18-504, or 18 to 505 of human TNALP. These consensus fragments are amino acid residues 23 to 508, 23 to 509, 23 to 510, 23 to 511, 23 to 512, 23 to 513, 23 to 514 and 23 to 515 of SEQ ID NO: 15, respectively. In these consensus fragments, X is any amino acid except an amino acid corresponding to a pathological mutation at that position of human TNALP as reported in Table 1. In other specific embodiments, these consensus fragments are amino acid residues 23 to 508, 23 to 509, 23 to 510, 23 to 511, 23 to 512, 23 to 513, 23 to 514 and 23 to 515 of SEQ ID NO: 18, respectively. In these consensus fragments, X is any amino acid found at that position in the ALP of either one of the species and human ALP isozymes of the alignment from which the consensus is derived but is not an amino acid corresponding to a pathological mutation at that position of human TNALP as reported in Table 1 (See Figure 30).

[0026] In other specific embodiments, the sALP fragment in the bone targeted fusion protein of the present invention consist of any of the fragments of a consensus sequence derived from an alignment of TNALPs from various mammalian species corresponding to amino acid residues 18-498, 18-499, 18-500, 18-501, 18-502, 18-503, 18-504, and 18 to 505 of human TNALP. These consensus fragments are amino acid residues 18-498, 18-499, 18-500, 18-501, 18-502, 18-503, 18-504, and 18 to 505 of SEQ ID NO: 16, respectively. In these consensus fragments, X is any amino acid except an amino acid corresponding to a pathological mutation at that position of human TNALP as reported in Table 1. In other specific embodiments, these consensus fragments are amino acid residues 18-498, 18-499, 18-500, 18-501, 18-502, 18-503, 18-504, and 18 to 505 of SEQ ID NO: 19, respectively. In these consensus fragments, X is any amino acid found at that position in the TNALP of either one of the species of the alignment from which the consensus is derived but is not an amino acid corresponding to a pathological mutation at that position of human TNALP as reported in Table 1 (See Figure 31).

[0027] Table 1 : Pathological mutations in human TNALP

Total number of mutations 188										
Exon	Base change	Amino acid change		Reference	Clinical form in patient	Genotype of patient	% WT	ref.	E.coli	
		Non-standardized nomenclature	Standardized nomenclature							
1	c.-195C>T			Taillandier et al. 2000	perinatal	c.-195C>T/C184Y			na	Affects transcription start site
2	c.17T>A	L-12X	p.L6X	Taillandier et al. 2000	childhood	L-12X/?			na	Nonsense mutation
2	c.50C> T	S-1F	p.S17F	Mornet et al. 1998	infantile	S-1F/G58S	19.0	1	na	
3	c.83A>G	Y11C	p.Y28C	Taillandier et al. 2001	infantile	Y11C/R119H	7.2	2	-	
3	c.98C>T	A16V	p.A33V	Henthorn et al. 1992	childhood	A16V/Y419H			-	
3	c.110T>C	L20P	p.L37P	Versailles lab oct. 2003	perinatal	L20P/L20P			+	
3	c.119C>T	A23V	p.A40V	Mornet et al. 1998	perinatal	A23V/G456S	2.3	1	+	
3	c.132C>T	Q27X	p.Q44X	Mornet E, unpublished	perinatal	Q27X/c.662insG			na	Nonsense mutation
3	c.151G>T	A34S	p.A51S	Mumm et al. 2002	infantile	A34S/T117H			+	
3	c.152G>T	A34V	p.A51V	Taillandier et al. 2001	infantile	A34V/V442M			+	
4	c.184A>T	M45L	p.M62L	Taillandier et al. 1999	infantile	M45L/c.1172delC	27.4	1	+	
4	c.184A>G	M45V	p.M62V	Spentchian et al. 2003	infantile	M45V/M45V			+	
4	c.186G>C	M45I	p.M62I	Taillandier et al.2005	childhood	M45I/E174K	0	16	+	
4	c.187G>C	G46R	p.G63R	Spentchian et al. 2003	infantile	G46R/G46R			+	
4	c.188G>T	G46V	p.G63V	Lia-Baldini et al. 2001	infantile	G46V/N	0.8	3	+	
4	c.203C>T	T51M	p.T68M	Orimo et al. 2002	childhood	T51M/A160T	5.2	4	+	
4	c.211C>T	R54C	p.R71C	Henthorn et al. 1992	infantile	R54C/D277A	0	17	+	
4	c.211C>A	R54S	p.R71S	Orimo et al. 2002	childhood	R54S/?	2.9	4	+	

4	c.212G>C	R54P	p.R71P	Henthorn et al. 1992	perinatal	R54P/Q190P		+	
4	c.212G>A	R54H	p.R71H	Taillandier et al. 2001	perinatal	A23V/R54H		+	
4	c.219T>C	I55T	p.I72T	Versailles lab oct. 2004	odonto	I55T/N		-	
4	c.223G>A	G58S	p.G75S	Mornet et al. 1998	infantile	S-1F/G58S	3.5	+	
4	c.227A>G	Q59R	p.Q76R	Mornet et al. 2001	infantile	Q59R/T117N		-	
IVS4	c.298-2A>G			Taillandier et al. 2000	perinatal	c.298-2A>G/c.997+3A>C		na	This mutation affects splicing and not coding sequence
5	c.299C>T	T83M	p.T100M	Mornet et al. 2001	infantile	T83M/E174K		+	
5	c.303_311del	N85_N87del	p.N102_N104del	Versailles lab Jul 2007	perinatal	c.303_311del/G474R		na	Deletion
5	c.323C>T	P91L	p.P108L	Herasse et al. 2003	odonto	P91L/N	0.4	unp.	-
5	c.331G>A	A94T	p.A111T	Goseki-Sone et al. 1998	odonto	A94T/?		+	
5	c.334G>A	G95S	p.G112S	Witters et al. 2004	infantile	G95S/R374C		-	
5	c.340G>A	A97T	p.A114T	Mumm et al. 2001	infantile	A97T/D277A		+	
5	c.341C>G	A97G	p.A114G	Draguet et al. 2004	perinatal	A97G+c.348_349insACCGTC /G309R		+	
5	c.348_349insACCGTC			Draguet et al. 2004	perinatal	A97G+c.348_349insACCGTC /G309R		na	Two missense mutations and insertion
5	c.346G>T	A99S	p.A116S	Versailles lab Jul 2007	adult	A99S/N400S		+	
5	c.346G>A	A99T	p.A116T	Hu et al. 2000	adult	A99T/N	0.8	3	+
5	c.358G>A	G103R	p.G120R	Mornet et al. 1998	perinatal	G103R/648+1G>A		+	
5	c.368C>A	A106D	p.A123D	Spentchian et al. 2006	perinatal	A106D/S249_H250del		-	

5	c.382G>A	V111M	p.V128M	Mumm et al. 2002	perinatal	V111M/R206W		-		
5	c.385G>A	G112R	p.G129R	Mornet et al. 1998	perinatal	G112R/G474R		+		
5	c.388_391delGTAA			Spentchian et al. 2003	perinatal	E294K/388_391delGTAA		na	Frameshift mutation	
5	c.389delT			Spentchian et al. 2003	perinatal	c.389delT/c.389delT		na	Frameshift mutation	
5	c.392delG			Mumm et al. 2002	perinat/infant	c.392delG/A331T		na	Frameshift mutation	
5	c.394G>A	A115T	p.A132T	Versailles lab Jul 2006	adult	A115T/E174K				
5	c.395C>T	A115V	p.A132V	Watanabe et al. 2001	adult	A115V/?	16.9	14	-	
5	c.400_401AC>CA	T117H	p.T134H	Mumm et al. 2002	perinatal	T117H/F310del		-		
5	c.401C>A	T117N	p.T134N	Taillandier et al. 2000	perinatal	T117N/T117N	20.5	5	-	
5	c.406C>T	R119C	p.R136C	Versailles lab oct. 2003	odonto	R119C/R119H		-		
5	c.407G>A	R119H	p.R136H	Taillandier et al. 1999	infantile	R119H/G145V	33.4	1	-	
5	c.442A>G	T131A	p.T148A	Michigami et al. 2005	perinatal	T131A/?		-		
5	c.443C>T	T131I	p.T148I	Spentchian et al. 2003	infantile	T131I/G145S		-		
6	c.480delT			Versailles lab. Jan. 2008	perinatal	c.480delT/R206W		na	deletion	
6	c.484G>A	G145S	p.G162S	Spentchian et al. 2003	infantile	T131I/G145S		+		
6	c.485G>T	G145V	p.G162V	Taillandier et al. 1999	infantile	R119H/G145V	1.3	1	+	
6	c.500C>T	T150M	p.T167M	Versailles lab oct. 2003	infantile	T150M/E174K	0		+	
6	c.508A>G	N153D	p.N170D	Mornet et al. 1998	perinatal	N153D/N153D	0	13	-	
6	c.511C>T	H154Y	p.H171Y	Taillandier et al. 1999	infantile	H154Y/E174K	2.1	1	-	

6	c.512A>G	H154R	p.H171R	Mornet E, unpublished	adult	H154R/E174K		-		
6	c.526G>A	A159T	p.A176T	Taillandier et al. 2000	childhood	A159T/R229S	45.4	5	+	
6	c.529G>A	A160T	p.A177T	Goseki-Sone et al. 1998	adult	A160T/F310L	83.8	4	-	
6	c.535G>A	A162T	p.A179T	Weiss et al. 1988	perinatal	A162T/A162T	18	6	+	
6	c.542C>T	S164L	p.S181L	Lia-Baldini et al. 2001	infantile	S164L/del(ex12)	1.3	3	-	
6	c.544delG			Taillandier et al. 1999	perinatal	G232V/544delG			na	Frameshift mutation
6	c.550C>T	R167W	p.R184W	Mornet et al. 1998	perinatal	R167W/W253X	0.6	3	+	
6	c.567C>A	D172E	p.D189E	Spentchian et al. 2003	perinatal	D172E/D172E			-	
6	c.568_570delAAC	N173del	p.N190del	Michigami et al. 2005	perinatal	c.1559delT/N173del			-	Deletion of 1 a.a.
6	c.571G>A	E174K	p.E191K	Henthorn et al. 1992	infantile	E174K/D361V	88.0	1	-	
6	c.572A>G	E174G	p.E191G	Goseki-Sone et al. 1998	odonto	E174G/c.1559delT			-	
6	c.575T>C	M175T	p.M192T	Versailles lab Jul 2007	infantile	M175T/E294K			-	
6	c.577C>G	P176A	p.P193A	Mumm et al. 2002	adult	A97T/P176A			+	
6	c.602G>A	C184Y	p.C201Y	Taillandier et al. 1999	perinatal	c.-195C>T/C184Y			-	
6	c.609C>G	D186E	p.D203E	Versailles lab oct. 2004	perinatal	D186E/D186E			-	
6	c.620A>C	Q190P	p.Q207P	Henthorn et al. 1992	perinatal	R54P/Q190P			+	
6	c.631A>G	N194D	p.N211D	Taillandier et al. 2001	infantile	A99T/N194D			+	
6	c.634A>T	I195F	p.I212F	Souka et al. 2002	perinatal	I195F/E337D			-	
IVS6	c.648+1G>T			Brun-Heath et al. 2005	perinatal	c.648+1G>T/D277A				Affects splicing
IVS6	c.648+1G>A			Mornet et al. 1998	perinatal	G103R/c.648+1G>A			na	Affects splicing

IVS6	c.649-1_3delinsAA			Versailles lab Jul 2006	perinatal	c.649-1_3delinsAA/c.649-1_3delinsAA				Frameshift mutation
7	c.653T>C	I201T	p.I218T	Utsch et al., 2005, contact	perinatal	I201T/R374C	3.7	unp.	-	
7	659G>T	G203V	p.G220V	Taillandier et al. 2001	odonto	E174K/G203V			+	
7	659G>C	G203A	p.G220A	Spentchian et al. 2003	perinatal	G203A/G203A			+	
7	662insG			Mornet E, unpublished	perinatal	Q27X/662insG			na	Frameshift mutation
7	c.662delG			Spentchian et al. 2003	perinatal	R255L/c.662delG			na	Frameshift mutation
7	c.662G>T	G204V	p.G221V	Versailles lab oct. 2004	perinatal	G204V/M338T			+	
7	c.667C>T	R206W	p.R223W	Mornet et al. 1998	perinatal	R206W/?	2.8	3	-	
7	c.668G>A	R206Q	p.R223Q	Mumm et al. 2002	perinatal	R206Q/deletion			-	
7	c.670A>G	K207E	p.K224E	Mochizuki et al. 2000	infantile	K207E/G409C	43	15	+	
7	c.677T>C	M209T	p.M226T	Baumgartner-Sigl et al. 2007	infantile	M209T/T354I			-	
7	c.704A>G	E218G	p.E235G	Taillandier et al. 2001	adult	E218G/A382S	3.6	7	+	
7	c.738G>T	R229S	p.R246S	Taillandier et al. 2000	childhood	A159T/R229S	4.4	5	-	
7	c.746G>T	G232V	p.G249V	Fedde et al. 1996	perinatal	G232V/N	34.5	3	+	
7	c.971A>G	K247R	p.K264R	Versailles lab Jan.2007	perinatal	K247R/D361V			-	
8	c.797_802del	S249_H250del	p.S266_H267del	Spentchian et al. 2006	perinatal	A106D/S249_H250del				Deletion of 2 a.a.
8	c.809G>A	W253X	p.W270X	Mornet et al. 1998	perinatal	R167W/W253X			na	Nonsense mutation
8	c.814C>T	R255C	p.R272C	Spentchian et al. 2006	perinatal	R255C/T117H			-	

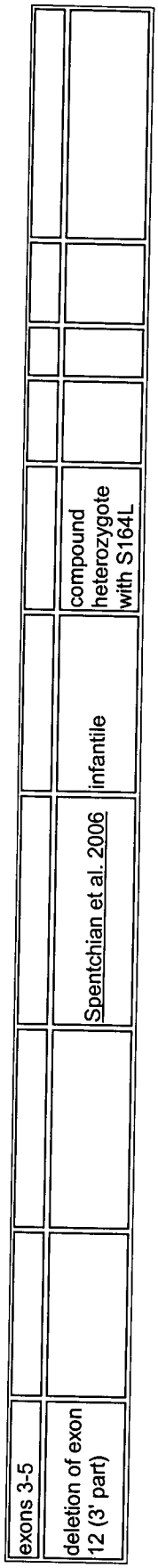
8	c.815G>T	R255L	p.R272L	Spentchian et al. 2003	perinatal	R255L/c.662delG		-		
8	c.815G>A	R255H	p.R272H	Brun-Heath et al. 2005	infantile	R255H/R255H	6.8	16	-	
8	c.824T>C	L258P	p.L275P	Orimo et al. 2002	childhood	L258P/A160T	3.3	4	-	
8	c.853_854insGATC	Y268X	p.Y285X	Michigami et al. 2005	perinatal	c1559delT/Y268X			na	Nonsense mutation
IVS8	c.862+5G>A			Taillandier et al. 1999	infantile	c.862+5G>A/c.862+5G>A			na	Affects splicing
9	c.865C>T	L272F	p.L289F	Sugimoto et al. 1998	infantile	L272F/?	50	8	-	
9	c.871G>A	E274K	p.E291K	Mornet et al. 1998	infantile	E174K/E274K	8.3	1	-	
9	c.871G>T	E274X	p.E291X	Taillandier et al. 2000	perinatal	A94T/E274X			-	Nonsense mutation
9	c.874C>A	P275T	p.P292T	Brun-Heath et al. 2005	infantile	P275T/A16V	4.0	16	+	
9	c.876_881delAGGGGA	G276_D277del		Spentchian et al. 2003	perinatal	G276_D277del/c.962delG			na	
9	c.880G>T	D277Y	p.D294Y	Taillandier et al. 2001	infantile	A159T/D277Y			-	
9	c.881A>C	D277A	p.D294A	Henthorn et al. 1992	infantile	R54C/D277A	0	17	-	
9	c.883A>G	M278V	p.M295V	Mornet et al. 2001	childhood	E174K/M278V			-	
9	c.884T>C	M278T	p.M295T	Brun-Heath et al. 2005	perinatal	M278T/R206W	8.5	16	-	
9	c.885G>A	M278I	p.M295I	Michigami et al. 2005	perinatal	M278I/c.1559delT			-	
9	c.889T>G	Y280D	p.Y297D	Brun-Heath et al. 2005	childhood	R119H/Y280D	1.3	16	-	
9	c.892G>A	E281K	p.E298K	Orimo et al. 1994	infantile	E281K/1559delT			-	
9	c.896T>C	L282P	p.L299P	Versailles lab oct. 2003	infantile	L282P/L282P	9.7	15	-	
9	c.917A>T	D289V	p.D306V	Taillandier et al. 1999	infantile	D289V/D289V	0	12	-	
9	c.919C>T	P290S	p.P307S	Versailles lab oct. 2004	infantile	P290S/M450T			+	
9	c.920C>T	P290L	p.P307L	Versailles lab Jul. 2006	childhood	P290L/S164L				

9	c.928_929delTC		Brun-Heath et al. 2005	perinatal	T394A/c.928_929delTC				Frameshift mutation
9	c.931G>A	E294K	p.E311K	Spentchian et al. 2003	perinatal	E294K/c.388_391delGTAA		-	
9	c.962delG			Spentchian et al. 2003	perinatal	G276_D277del/c.962delG		na	Frameshift mutation
9	c.976G>C	G309R	p.G326R	Litmanovitz et al. 2002	perinatal	G309R/E274K		+	
9	c.981_983delCTT	F310del	p.F327del	Orimo et al. 1997	infantile	F310del/c.1559delT	~10	15 +	Amino acid deletion
9	c.979T>G	F310C	p.F327C	Mornet et al. 2001	perinatal	T117N/F310C		+	
9	c.979_980TT>GG	F310G	p.F327G	Taillandier et al. 2001	adult	E174K/F310G		+	
9	c.979T>C	F310L	p.F327L	Ozono et al. 1996	infantile	F310L/G439R	72	9 +	
9	c.982T>A	F311L	p.F328L	Michigami et al. 2005	perinatal non-lethal	F311L/T83M	~10	15 +	
IVS9	c.997+2T>A			Taillandier et al. 2000	perinatal	c.997+2T>A/C472S		na	Affects splicing
IVS9	c.997+2T>G			Brun-Heath et al. 2005	perinatal	c.997+2T>G/c.997+2T>G			Affects splicing
IVS9	c.997+3A>C			Mornet et al. 1998	perinatal	c.997+3A>C/c.997+3A>C		na	Affects splicing
IVS9	c.998-1G>T			Taillandier et al. 2001	perinatal	E174K/c.998-1G>T		na	Affects splicing
10	c.1001G>A	G317D	p.G334D	Greenberg et al. 1993	perinatal	G317D/G317D	0	10 -	
10	c.1015G>A	G322R	p.G339R	Mumm et al. 2002	perinatal	G322R/A159T		-	
10	c.1016G>A	G322E	p.G339E	Versailles lab oct. 2004	infantile	G322E/V111M		-	
10	c.1042G>A	A331T	p.A348T	Taillandier et al. 2000	infantile	E174K/A331T	33.2	5 -	
10	c.1044_1055del	L332_A335del	p.L349_A352del	Spentchian et al. 2006	perinatal	L332_A335del/G474R			Deletion of 4 a.a.

10	c.1062G>C	E337D	p.E354D	Souka et al. 2002	perinatal	I195F/E337D			+	
10	c.1064A>C	M338T	p.M355T	Versailles lab oct. 2004	perinatal	G204V/M338T			-	
10	c.1065G>A	M338I	p.M355I	Versailles lab. Jan. 2008	infantile	M338I/R374C			–	
10	c.1101_1103delCTC	S351del	p.S368del	Versailles lab oct. 2004	perinatal	c.1101_1103delCTC/T372I				Deletion of 1 a.a.
10	c.1112C>T	T354I	p.T371I	Baumgartner-Sigl et al. 2007	infantile	M209T/T354I			-	
10	c.1120G>A	V357M	p.V374M	Versailles lab oct. 2004	adult	V357M/E281K			+	
10	c.1130C>T	A360V	p.A377V	Mornet et al. 2001	perinatal	A360V/A360V			+	
10	c.1133A>T	D361V	p.D378V	Henthorn et al. 1992	infantile	E174K/D361V	1.2	3	+	
10	c.1142A>G	H364R	p.H381R	Taillandier et al. 2000	infantile	A23V/H364R			+	
10	c.1144G>A	V365I	p.V382I	Goseki-Sone et al. 1998	childhood	F310L/V365I	0	11	+	
10	c.1166C>T	T372I	p.T389I	Versailles lab oct. 2004	perinatal	T372I/S351delI			-	
10	c.1171C>T	R374C	p.R391C	Zurutuza et al. 1999	childhood	E174K/R374C	10.3	1	-	
10	c.1172G>A	R374H	p.R391H	Orimo et al. 2002	childhood	R374H/?	3.7	4	-	
10	c.1172delC			Taillandier et al. 1999	infantile	M45L/c.1172delC			na	Frameshift mutation
10	c.1175G>C	G375A	p.G392A	Versailles lab. Jan. 2008	perinatal	G375A/R119C			-	
10	c.1182T>C	I378T	p.I395T	Versailles lab Jul. 2006	perinatal	I378T/E174K				
11	c.1195G>T	A382S	p.A399S	Taillandier et al. 2001	adult	E218G/A382S			-	
11	c.1196C>T	A382V	p.A399V	Spentchian et al. 2006	adult	A382V/A16V			-	
11	c.1199C>T	P383L	p.P400L	Spentchian et al. 2006	infantile	P383L/P383L			+	
11	c.1214_1215delCA			Versailles lab Jul. 2006	adult	c.1214_1215delCA/E174K				Frameshift mutation
11	c.1216 1219del			Brun-Heath et al. 2005	perinatal	c.1216 1219d				

	IGACA				elGACA/?				
11	c.1217A>G	D389G	p.D406G	Taillandier et al. 2000	odonto.	D389G/R433H	14.9	5	+
11	c.1228T>C	F393L	p.F410L	Versailles lab oct. 2004	infantile	F393L/E174K			-
11	c.1231A>G	T394A	p.T411A	Brun-Heath et al. 2005	perinatal	T394A/c.926_927delTC	0.3	16	-
11	c.1240C>A	L397M	p.L414M	Mumm et al. 2002	perinatal	L397M/D277A			-
11	c.1250A>G	N400S	p.N417S	Sergi et al. 2001	perinatal	N400S/c.648+1G>A	3	unp.	+
11	c.1256delC			Taillandier et al. 2000	perinatal	c.1256delC/?			na
11	c.1258G>A	G403S	p.G420S	Glaser et al. 2004	perinatal	G403S/G403S	0.4	unp.	-
11	c.1268T>C	V406A	p.V423A	Taillandier et al. 2001	perinatal	A99T/V406A	15.7	2	-
11	c.1270G>A	V407M	p.V424M	Versailles lab jan. 2007	adult	V407M/V407M			-
11	c.1276G>T	G409C	p.G426C	Mochizuki et al. 2000	infantile	K207A/G409C	18.5	15	-
11	c.1277G>A	G409D	p.G426D	Mumm et al. 2002	childhood	G409D/E174K			-
11	c.1282C>T	R411X	p.R428X	Taillandier et al. 1999	perinatal	R411X/R411X			na
11	c.1283G>C	R411P	p.R428P	Spentchian et al. 2006	perinatal	R411P/c.997+2T>A			-
11	c.1285G>A	E412K	p.E429K	Versailles lab Jul. 2006	odonto.	E412K/?			
11	c.1306T>C	Y419H	p.Y436H	Henthorn et al. 1992	childhood	A16V/Y419H			na
12	c.1333T>C	S428P	p.S445P	Mornet et al. 1998	infantile	S428P/?	2.1	1	-
12	c.1349G>A	R433H	p.R450H	Taillandier et al. 2000	odonto.	D389G/R433H			-
12	c.1348C>T	R433C	p.R450C	Mornet et al. 1998	infantile	R433C/R433C	4.0	1	-
12	c.1354G>A	E435K	p.E452K	Spentchian et al. 2003	perinatal	A94T/E435K			+
12	c.1361A>G	H437R	p.H454R	Versailles lab oct. 2003	childhood	E174K/H437R			+

12	c.1363G>A	G438S	p.G455S	Draguet et al. 2004	adult	G438S/G474R			-	
12	c.1364G>A	G438D	p.G455D	Versailles lab jan. 2007	perinatal	G438D/G438D			-	
12	c.1366G>T	G439W	p.G456W	Versailles lab oct. 2003	childhood	G439W/?			+	
12	c.1366G>A	G439R	p.G456R	Ozono et al. 1996	infantile	G439R/?	1.5	unp.	+	
12	c.1375G>A	V442M	p.V459M	Taillandier et al. 2000	infantile	A34V/V442M			+	
12	c.1375G>T	V442L	p.V459L	Versailles lab oct. 2004	perinatal	V442L/E435K			-	
12	c.1396C>T	P449L	p.P466L	Versailles lab oct. 2003	perinatal	P449L/?			+	
12	c.1400T>C	M450T	p.M467T	Versailles lab oct. 2004	infantile	M450T/P290S			-	
12	c.1402G>A	A451T	p.A468T	Spentchian et al. 2003	perinatal	A451T/A451T			+	
12	c.1417G>A	G456S	p.G473S	Mornet et al. 1998	perinatal	A23V/G456S			+	
12	c.1426G>A	E459K	p.E476K	Taillandier et al. 1999	perinatal	A94T/E459K			+	
12	c.1427A>G	E459G	p.E476G	Mornet et al. 2001	perinatal	E459G/E459G			+	
12	c.1433A>T	N461I	p.N478I	Taillandier et al. 2000	childhood	N461I/N	1.1	3	-	
12	c.1444_1445insC			Brun-Heath et al. 2005	perinatal	c.1444_1445insC/G317D				Frameshift mutation
12	c.1456G>C	C472S	p.C489S	Taillandier et al. 2000	perinatal	C472S/c.997+2T>A	9.4	5	-	
12	c.1468A>T	I473F	p.I490F	Lia-Baldini et al. 2001	adult	I473F/?	37.1	3	-	
12	c.1471G>A	G474R	p.G491R	Mornet et al. 1998	perinatal	G112R/G474R			-	
12	c.1471delG			Brun-Heath et al. 2005	odonto	c.1471delG/R119H				Frameshift mutation
12	c.1559delT			Orimo et al. 1994	infantile	E281K/c.1559delT	28	18	na	Frameshift mutation
Large deletions										
deletion of				Spentchian et al. 2006	perinatal	homozygote				


exons 3-5
deletion of exon 12 (3' part)
Spentchian et al. 2006
infantile
compound heterozygote with S164L

Spacer

[0028] Without being limited to this theory, it is believed that the Fc fragment used in the bone targeted sALP fusion protein presented in Examples below acts as a spacer which allows the protein to be more efficiently folded since expression of sTNALP-Fc-D10 was higher than that of sTNALP-D10 (see Example 2 below). One possible explanation is that the introduction of the Fc fragment alleviates the repulsive forces caused by the presence of the highly negatively charges D10 sequence added at the C-terminus of the tested sALP sequence.

[0029] Useful spacers for the present invention include polypeptides comprising a Fc, and hydrophilic and flexible polypeptides able to alleviate the repulsive forces caused by the presence of the highly negatively charged D10 sequence added at the C-terminus of the sALP sequence. In specific embodiment the spacer alleviates the steric hindrance preventing two sALP domains from two sALP monomers from interacting with each other to constitute the minimal catalytically active entity.

Fragment crystallizable region (Fc) fragments

[0030] Useful Fc fragments for the present invention include FC fragments of IgG that comprise the hinge, and the CH2 and CH3 domains. IgG-1, IgG-2, IgG-3, IgG-3 and IgG-4 for instance can be used.

Negatively charged peptide

[0031] The negatively charged peptide according to the present invention may be a poly-aspartate or poly-glutamate selected from the group consisting of D10 to D16 or E10 to E16.

[0032] In specific embodiments, the bone targeted sALP fusion proteins of the present invention are associated so as to form dimers or tetramers.

[0033] Without being limited to this particular theory, in specific embodiments of the invention using a polypeptide comprising a Fc as a spacer, dimers are presumably constituted of two bone targeted sALP monomers covalently linked through the two disulfide bonds located in the hinge regions of the two Fc fragments. In this dimeric configuration the steric hindrance imposed by the formation of the interchain disulfide bonds are presumably preventing the association of sALP domains to associate into the dimeric minimal catalytically active entity present in normal cells.

[0034] Without being limited to this particular theory, it is believed that in its tetrameric structure, the association of the fusion proteins would involve one sALP domain from one dimer and another one from another dimer. The steric hindrance presumably preventing two sALP domains from the same Fc-joined dimer from interacting with each other to constitute the minimal catalytically active entity could eventually be relieved by inserting a longer spacer than the Fc described in Examples presented herein between the sALP fragment and the polyaspartate or polyglutamate fragment.

[0035] The bone targeted sALP may further optionally comprise one or more additional amino acids 1) downstream from the poly-aspartate or poly-glutamate; and/or 2) between the poly-aspartate and the Fc fragment; and/or 3) between the spacer such as the Fc fragment and the sALP fragment. This is the case for instance when the cloning strategy used to produce the bone targeting conjugate introduces exogenous amino acids in these locations. However the exogenous amino acids should be selected so as not to provide an additional GPI anchoring signal. The likelihood of a designed sequence being cleaved by the transamidase of the host cell can be predicted as described by Ikezawa (Ikezawa 2002).

[0036] The present invention also encompasses the fusion protein as post-

translationally modified such as by glycolisation including those expressly mentioned herein, acetylation, amidation, blockage, formylation, gamma-carboxyglutamic acid hydroxylation, methylation, phosphorylation, pyrrolidone carboxylic acid, and sulfatation.

[0037] The term "recombinant protein" is used herein to refer to a protein encoded by a genetically manipulated nucleic acid inserted into a prokaryotic or eukaryotic host cell. The nucleic acid is generally placed within a vector, such as a plasmid or virus, as appropriate for the host cell. Although Chinese Hamster Ovary (CHO) cells have been used as a host for expressing the conjugates of the present invention in the Examples presented herein, a person of ordinary skill in the art will understand that a number of other hosts may be used to produce recombinant proteins according to methods that are routine in the art. Representative methods are disclosed in Maniatis, et al. Cold Springs Harbor Laboratory (1989). "Recombinant cleavable protein" as used herein is meant to refer to a recombinant protein that may be cleaved by a host's enzyme so as to produce a secreted/soluble protein. Without being so limited HEK293 cells, PerC6, Baby hamster Kidney cells can also be used.

[0038] As used herein the terminology "conditions suitable to effect expression of the polypeptide" is meant to refer to any culture medium that will enable production of the fusion protein of the present invention. Without being so limited, it includes media prepared with a buffer, bicarbonate and/or HEPES, ions like chloride, phosphate, calcium, sodium, potassium, magnesium, iron, carbon sources like simple sugars, amino acids, potentially lipids, nucleotides, vitamins and growth factors like insulin; regular commercially available media like alpha-MEM, DMEM, Ham's-F12 and IMDM supplemented with 2-4 mM L-glutamine and 5% Fetal bovine serum; regular commercially available animal protein free media like Hyclone™ SFM4CHO, Sigma CHO DHFR-, Cambrex POWER™ CHO CD supplemented with 2-4 mM L-glutamine. These media are desirably prepared

without thymidine, hypoxanthine and L-glycine to maintain selective pressure allowing stable protein-product expression.

[0039] Without being so limited, host cells useful for expressing the fusion of the present invention include L cell, C127 cells, 3T3 cells, CHO cells, BHK cells, COS-7 cells or Chinese Hamster Ovary (CHO) cell. Particular CHO cells of interest for expressing the fusion protein of the present invention include CHO-DG44 and CHO/dhfr$^-$ also referred to as CHO duk$^-$. This latter cell line is available through the American Type Culture Collection (ATCC number CRL-9096).

[0040] The term "bone tissue" is used herein to refer to tissue synthesized by osteoblasts composed of an organic matrix containing mostly collagen and mineralized by the deposition of hydroxyapatite crystals.

[0041] The fusion proteins comprised in the bone delivery conjugates of the present invention are useful for therapeutic treatment of bone defective conditions by providing an effective amount of the fusion protein to the bone. The fusion proteins are provided in the form of pharmaceutical compositions in any standard pharmaceutically acceptable carriers, and are administered by any standard procedure, for example by intravenous injection.

[0042] As used herein the terminology "HPP phenotype" is meant to refer to any one of rickets (defect in growth plate cartilage), osteomalacia, elevated blood and/or urine levels of inorganic pyrophosphate (PP_i), phosphoethanolamine (PEA), or pyridoxal 5'-phosphate (PLP), seizure, bone pains, calcium pyrophosphate dihydrate crystal deposition (CPPD) in joints leading to chondrocalcinosis and premature death. Without being so limited, a HPP phenotype can be documented by growth retardation with a decrease of long bone length (such as femur, tibia, humerus, radius, ulna), a decrease of the mean density of total bone and a decrease of bone mineralization in bones such as

femur, tibia, ribs and metatarsi, and phalange, a decrease in teeth mineralization, a premature loss of deciduous teeth (e.g., aplasia, hypoplasia or dysplasia of dental cementum). Without being so limited, correction or prevention of bone mineralization defect may be observed by one or more of the following: an increase of long bone length, an increase of mineralization in bone and/or teeth, a correction of bowing of the legs, a reduction of bone pain and a reduction of CPPD crystal deposition in joints.

[0043] As used herein the terminology "correct" in the expression" correct a hypophosphatasia phenotype" is meant to refer to any partial or complete reduction of a pre-existing HPP phenotype. Similarly the terminology "prevent" in the expression "prevent a hypophosphatasia phenotype" is meant to refer to any delay or slowing in the development of a HPP phenotype or any partial or complete avoidance of the development of a HPP phenotype.

[0044] As used herein the term "subject" is meant to refer to any mammal including human, mice, rat, dog, cat, pig, cow, monkey, horse, etc. In a particular embodiment, it refers to a human.

[0045] As used herein, the term "subject in need thereof" in a method of administering a compound of the present invention is meant to refer to a subject that would benefit from receiving a compound of the present invention. In specific embodiments, it refers to a subject that already has at least one HPP phenotype or to a subject likely to develop at least one HPP phenotype or at least one more HPP phenotype. In another embodiment it further refers to a subject that has aplasia, hypoplasia or dysplasia of dental cementum or a subject likely to develop aplasia, hypoplasia or dysplasia of dental cementum.

[0046] As used herein "a subject likely to develop at least one HPP phenotype" is a subject having at least one loss-of-function mutation in the gene

(*ALPL*).

[0047] As used herein "a subject likely to develop aplasia, hypoplasia or dysplasia of dental cementum" is a subject having HPP or a periodontal disease due to a bacterial infection. Periodontal disease due to a bacterial infection may induce alteration of cementum which may lead to exfoliation of teeth.

Route of administration

[0048] Bone targeted sALPs of the present invention can be administered by routes such as orally, nasally, intravenously, intramuscularly, subcutaneously, sublingually, intrathecally, or intradermally. The route of administration can depend on a variety of factors, such as the environment and therapeutic goals. As used herein, subjects refer to animals such as humans in which prevention, or correction of bone mineralization defect characterizing HPP or other phenotypes associated with HPP or prevention or correction of defective cementum is desirable.

[0049] By way of example, pharmaceutical composition of the invention can be in the form of a liquid, solution, suspension, pill, capsule, tablet, gelcap, powder, gel, ointment, cream, nebulae, mist, atomized vapor, aerosol, or phytosome. For oral administration, tablets or capsules can be prepared by conventional means with pharmaceutically acceptable excipients such as binding agents, fillers, lubricants, disintegrants, or wetting agents. The tablets can be coated by methods known in the art. Liquid preparations for oral administration can take the form of, for example, solutions, syrups, or suspension, or they can be presented as a dry product for constitution with saline or other suitable liquid vehicle before use. Dietary supplements of the invention also can contain pharmaceutically acceptable additives such as suspending agents, emulsifying agents, non-aqueous vehicles, preservatives, buffer salts, flavoring, coloring, and sweetening agents as appropriate. Preparations for oral administration also can be

suitably formulated to give controlled release of the active ingredients.

[0050] Enteric coatings can further be used on tablets of the present invention to resist prolonged contact with the strongly acidic gastric fluid, but dissolve in the mildly acidic or neutral intestinal environment. Without being so limited, cellulose acetate phthalate, Eudragit™ and hydroxypropyl methylcellulose phthalate (HPMCP) can be used in enteric coatings of pharmaceutical compositions of the present invention. Cellulose acetate phthalate concentrations generally used are 0.5-9.0% of the core weight. The addition of plasticizers improves the water resistance of this coating material, and formulations using such plasticizers are more effective than when cellulose acetate phthalate is used alone. Cellulose acetate phthalate is compatible with many plasticizers, including acetylated monoglyceride; butyl phthalybutyl glycolate; dibutyl tartrate; diethyl phthalate; dimethyl phthalate; ethyl phthalylethyl glycolate; glycerin; propylene glycol; triacetin; triacetin citrate; and tripropionin. It is also used in combination with other coating agents such as ethyl cellulose, in drug controlled-release preparations.

Dosage

[0051] Any amount of a pharmaceutical composition can be administered to a subject. The dosages will depend on many factors including the mode of administration and the age of the subject. Typically, the amount of bone targeted ALP of the invention contained within a single dose will be an amount that effectively prevent, delay or correct bone mineralization defect in HPP without inducing significant toxicity. As used herein the term "therapeutically effective amount" is meant to refer to an amount effective to achieve the desired therapeutic effect while avoiding adverse side effects. Typically, bone targeted sALPs in accordance with the present invention can be administered to subjects in doses ranging from 0.001 to 500 mg/kg/day and, in a more specific embodiment, about 0.1 to about 100 mg/kg/day, and, in a more specific embodiment, about 0.2 to

about 20 mg/kg/day. The allometric scaling method of Mahmood et al. (Mahmood et al. 2003) can be used to extrapolate the dose from mice to human. The dosage will be adapted by the clinician in accordance with conventional factors such as the extent of the disease and different parameters from the patient.

[0052] The therapeutically effective amount of the bone targeted sALP may also be measured directly. The effective amount may be given daily or weekly or fractions thereof. Typically, a pharmaceutical composition of the invention can be administered in an amount from about 0.001 mg up to about 500 mg per kg of body weight per day (e.g., 0.05, 0.01, 0.1, 0.2, 0.3, 0.5, 0.7, 0.8, 1 mg, 2 mg, 3 mg, 4mg, 5 mg, 10 mg, 15 mg, 20 mg, 30 mg, 50 mg, 100 mg, or 250 mg). Dosages may be provided in either a single or multiple dosage regimens. For example, in some embodiments the effective amount is a dose that ranges from about 0.1 to about 100 mg/kg/day, from about 0.2 mg to about 20 mg of the bone targeted sALP per day, about 1 mg to about 10 mg of the bone targeted sALP per day, from about .07 mg to about 210 mg of the bone targeted sALP per week, 1.4 mg to about 140 mg of the bone targeted sALP per week, about 0.3 mg to about 300 mg of the bone targeted sALP every three days, about 0.4 mg to about 40 mg of the bone targeted sALP every other day, and about 2 mg to about 20 mg of the bone targeted sALP every other day.

[0053] These are simply guidelines since the actual dose must be carefully selected and titrated by the attending physician based upon clinical factors unique to each patient or by a nutritionist. The optimal daily dose will be determined by methods known in the art and will be influenced by factors such as the age of the patient as indicated above and other clinically relevant factors. In addition, patients may be taking medications for other diseases or conditions. The other medications may be continued during the time that a bone targeted sALP is given to the patient, but it is particularly advisable in such cases to begin with low doses to determine if adverse side effects are experienced.

Carriers/vehicles

[0054] Preparations containing a bone targeted sALP may be provided to patients in combination with pharmaceutically acceptable sterile aqueous or non-aqueous solvents, suspensions or emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil, fish oil, and injectable organic esters. Aqueous carriers include water, water-alcohol solutions, emulsions or suspensions, including saline and buffered medical parenteral vehicles including sodium chloride solution, Ringer's dextrose solution, dextrose plus sodium chloride solution, Ringer's solution containing lactose, or fixed oils. Intravenous vehicles may include fluid and nutrient replenishers, electrolyte replenishers, such as those based upon Ringer's dextrose, and the like.

[0055] In yet another embodiment, the pharmaceutical compositions of the present invention can be delivered in a controlled release system. In one embodiment polymeric materials including polylactic acid, polyorthoesters, cross-linked amphipathic block copolymers and hydrogels, polyhydroxy butyric acid and polydihydropyrans can be used (see also Smolen and Ball, Controlled Drug Bioavailability, Drug product design and performance, 1984, John Wiley & Sons; Ranade and Hollinger, Drug Delivery Systems, pharmacology and toxicology series, 2003, 2nd edition, CRRC Press), in another embodiment, a pump may be used (Saudek *et al.*, 1989, N. Engl. J. Med. 321: 574).

[0056] The fusion proteins of the present invention could be in the form of a lyophilized powder using appropriate excipient solutions (e.g., sucrose) as diluents.

[0057] Further, the nucleotide segments or proteins according to the present invention can be introduced into individuals in a number of ways. For example, osteoblasts can be isolated from the afflicted individual, transformed with a nucleotide construct according to the invention and reintroduced to the afflicted individual in a number of ways, including intravenous injection. Alternatively, the

nucleotide construct can be administered directly to the afflicted individual, for example, by injection. The nucleotide construct can also be delivered through a vehicle such as a liposome, which can be designed to be targeted to a specific cell type, and engineered to be administered through different routes.

[0058] The fusion proteins of the present invention could also be advantageously delivered through gene therapy. Useful gene therapy methods include those described in WO06060641A2, US7179903 and WO0136620A2 to Genzyme using for instance an adenovirus vector for the therapeutic protein and targeting hepatocytes as protein producing cells.

[0059] A "gene delivery vehicle" is defined as any molecule that can carry inserted polynucleotides into a host cell. Examples of gene delivery vehicles are liposomes, biocompatible polymers, including natural polymers and synthetic polymers; lipoproteins; polypeptides; polysaccharides; lipopolysaccharides; artificial viral envelopes; metal particles; and bacteria, or viruses, such as baculovirus, adenovirus and retrovirus, bacteriophage, cosmid, plasmid, fungal vectors and other recombination vehicles typically used in the art which have been described for expression in a variety of eukaryotic and prokaryotic hosts, and may be used for gene therapy as well as for simple protein expression. "Gene delivery," "gene transfer," and the like as used herein, are terms referring to the introduction of an exogenous polynucleotide (sometimes referred to as a "transgene") into a host cell, irrespective of the method used for the introduction. Such methods include a variety of well-known techniques such as vector-mediated gene transfer (e.g., viral infection/transfection, or various other protein-based or lipid-based gene delivery complexes) as well as techniques facilitating the delivery of "naked" polynucleotides (such as electroporation, "gene gun" delivery and various other techniques used for the introduction of polynucleotides). The introduced polynucleotide may be stably or transiently maintained in the host cell. Stable maintenance typically requires that the introduced polynucleotide either contains

an origin of replication compatible with the host cell or integrates into a replicon of the host cell such as an extrachromosomal replicon (e.g., a plasmid) or a nuclear or mitochondrial chromosome. A number of vectors are known to be capable of mediating transfer of genes to mammalian cells, as is known in the art and described herein.

[0060] A "viral vector" is defined as a recombinantly produced virus or viral; particle that comprises a polynucleotide to be delivered into a host cell, either in viva, ex viva or in vitro. Examples of viral vectors include retroviral vectors, adenovirus vectors, adeno-associated virus vectors such as those described in WO06002203A2, alphavirus vectors and the like. Alphavirus vectors, such as Semliki Forest virus-based vectors and Sindbis virus-based vectors, have also been developed for use in gene therapy and immunotherapy.

[0061] In aspects where gene transfer is mediated by a DNA viral vector, such as an adenovirus (Ad) or adeno-associated virus (MV), a vector construct refers to the polynucleotide comprising the viral genome or part thereof, and a transgene. Adenoviruses (Ads) are a relatively well characterized, homogenous group of viruses, including over 50 serotypes. See, e.g., International PCT Application No. WO 95/27071. Ads are easy to grow and do not require integration into the host cell genome. Recombinant Ad derived vectors, particularly those that reduce the potential for recombination and generation of wild-type virus, have also been constructed. See, International PCT Application Nos. WO 95/00655 and WO 95/11984. Vectors that contain both a promoter and a cloning site into which a polynucleotide can be operatively linked are well known in the art. Such vectors are capable of transcribing RNA in vitro or in vivo, and are commercially available from sources such as Stratagene (La Jolla, CA) and Promega Biotech (Madison, WI). In order to optimize expression and/or in vitro transcription, it may be necessary to remove, add or alter 5' and/or 3' untranslated portions of the clones to eliminate extra, potential inappropriate alternative

translation initiation codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation.

[0062] The bone targeted sALP of the present invention may also be used in combination with at least one other active ingredient to correct a bone mineralization defect or another detrimental symptom of HPP. It may also be used in combination with at least one with at least one other active ingredient to correct cementum defect.

[0063] The term "high stringency conditions" are meant to refer to conditions enabling sequences with a high homology to bind. Without being so limited, examples of such conditions are listed In the handbook "*Molecular cloning, a laboratory manual*, second edition of 1989 from Sambrook *et al.*: 6XSSC or 6XSSPE, Denhardt's reagent or not, 0.5% SDS and the temperature used for obtaining high stringency conditions is most often in around 68°C (see pages 9.47 to 9.55 of Sambrook) for nucleic acid of 300 to 1500 nucleotides. Although the optimal temperature to be used for a specific nucleic acid probe may be empirically calculated, and although there is room for alternatives in the buffer conditions selected, within these very well known condition ranges, the nucleic acid captured will not vary significantly. Indeed, Sambrook clearly indicates that the "choice depends to a large extent on personal preference" (see page 9.47). Sambrook specifies that the formula to calculate the optimal temperature which varies according to the fraction of guanine and cytosine in the nucleic acid probe and the length of the probe (10 to 20°C lower than T_m wherein T_m = 81.5°C + 16.6($\log_{10}[Na^+]$) + 0.41(fraction G+C)-0.63 (% formamide -(600/l)) (see pages 9.50 and 9.51 of Sambrook).

Kits

[0064] The present invention also relates to a kit for correcting or preventing an HPP phenotype or a cementum defect comprising a nucleic acid, a

protein or a ligand in accordance with the present invention. For instance it may comprise a bone targeted composition of the present invention or a vector encoding same, and instructions to administer said composition or vector to a subject to correct or prevent a HPP phenotype. Such kits may further comprise at least one other active agent able to prevent or correct a HPP phenotype. When the kit is used to prevent or correct a HPP phenotype in a HPP subject, the kit may also further comprise at least one other active agent capable of preventing or correcting any other detrimental symptoms of HPP. In addition, a compartmentalized kit in accordance with the present invention includes any kit in which reagents are contained in separate containers. Such containers include small glass containers, plastic containers or strips of plastic or paper. Such containers allow the efficient transfer of reagents from one compartment to another compartment such that the samples and reagents are not cross-contaminated and the agents or solutions of each container can be added in a quantitative fashion from one compartment to another.

[0065] More specifically, in accordance with a first aspect of the present invention, there is provided a bone targeted alkaline phosphatase comprising a polypeptide having the structure : Z-sALP-Y-spacer-X-Wn-V, wherein sALP is the extracellular domain of the alkaline phosphatase; wherein V is absent or is an amino acid sequence of at least one amino acid; X is absent or is an amino acid sequence of at least one amino acid; Y is absent or is an amino acid sequence of at least one amino acid; Z is absent or is an amino acid sequence of at least one amino acid; and Wn is a polyaspartate or a polyglutamate wherein n = 10 to 16.

[0066] In a specific embodiment, the sALP comprises amino acid residues 23-508 of SEQ ID NO: 15. In another specific embodiment, the sALP consists of amino acid residues 23-512 of SEQ ID NO: 15. In another specific embodiment, the sALP comprises amino acid residues 23-508 of SEQ ID NO: 18.

In another specific embodiment, the sALP consists of amino acid residues 23-512 of SEQ ID NO: 18. In another specific embodiment, the sALP comprises amino acid residues 18-498 of SEQ ID NO: 16. In another specific embodiment, the sALP consists of amino acid residues 18-502 of SEQ ID NO: 16. In another specific embodiment, the sALP comprises amino acid residues 18-498 of SEQ ID NO: 19. In another specific embodiment, the sALP consists of amino acid residues 18-502 of SEQ ID NO: 19. In another specific embodiment, the sALP comprises amino acid residues 18-498 of SEQ ID NO: 19. In another specific embodiment, the sALP consists of amino acid residues 18-502 of SEQ ID NO: 19. In another specific embodiment, the sALP comprises amino acid residues 18-498 of SEQ ID NO: 8. In another specific embodiment, the sALP consists of amino acid residues 18-502 of SEQ ID NO: 8.

[0067] In another specific embodiment, the spacer comprises a fragment crystallizable region (Fc). In another specific embodiment, the Fc comprises a CH2 domain, a CH3 domain and a hinge region. In another specific embodiment, the Fc is a constant domain of an immunoglobulin selected from the group consisting of IgG-1, IgG-2, IgG-3, IgG-3 and IgG-4. In another specific embodiment, the Fc is a constant domain of an immunoglobulin IgG-1. In another specific embodiment, the Fc is as set forth in SEQ ID NO: 3. In another specific embodiment, Wn is a polyaspartate. In another specific embodiment, n=10. In another specific embodiment, Z is absent. In another specific embodiment, Y is two amino acid residues. In another specific embodiment, Y is leucine-lysine. In another specific embodiment, X is 2 amino acid residues. In another specific embodiment, X is aspartate-isoleucine. In another specific embodiment, V is absent. In another specific embodiment, the polypeptide is as set forth in SEQ ID NO: 4.

[0068] In another specific embodiment, the bone targeted alkaline phosphatase comprises the polypeptide in a form comprising a dimer. In another specific

embodiment, the bone targeted alkaline phosphatase comprises the polypeptide in a form of a tetramer.

[0069] In another specific embodiment, the bone targeted alkaline phosphatase is in a pharmaceutically acceptable carrier. In another specific embodiment, the pharmaceutically acceptable carrier is a saline. In another specific embodiment, the bone targeted alkaline phosphatase is in a lyophilized form. In another specific embodiment, the bone targeted alkaline phosphatase is in a daily dosage of about 0.2 to about 20 mg/kg. In another specific embodiment, the bone targeted alkaline phosphatase is in a dosage of about 0.6 to about 60 mg/kg for administration every three days. In another specific embodiment, the bone targeted alkaline phosphatase is in a weekly dosage of about 1.4 to about 140 mg/kg. In another specific embodiment, the bone targeted alkaline phosphatase is in a weekly dosage of about 0.5 mg/kg.

[0070] More specifically, in accordance with another aspect of the present invention, there is provided an isolated nucleic acid comprising a sequence that encodes the polypeptide of the present invention.

[0071] In accordance with another aspect of the present invention, there is provided an isolated nucleic acid consisting of a sequence that encodes the polypeptide of the present invention. More specifically, in accordance with another aspect of the present invention, there is provided an isolated nucleic acid comprising a sequence as set forth in SEQ ID NO: 17.

[0072] In accordance with another aspect of the present invention, there is provided a recombinant expression vector comprising the nucleic acid of the present invention. More specifically, in accordance with another aspect of the present invention, there is provided a recombinant adeno-associated virus vector comprising the nucleic acid of the present invention. More specifically, in

accordance with another aspect of the present invention, there is provided an isolated recombinant host cell transformed or transfected with the vector of the present invention.

[0073] In accordance with another aspect of the present invention, there is provided a method of producing the bone targeted alkaline phosphatase of the present invention, comprising culturing the host cell of the present invention, under conditions suitable to effect expression of the bone targeted alkaline phosphatase and recovering the bone targeted alkaline phosphatase from the culture medium.

[0074] In a specific embodiment, the host cell is a L cell, C127 cell, 3T3 cell, CHO cell, BHK cell, COS-7 cell or a Chinese Hamster Ovary (CHO) cell. In another specific embodiment, the host cell is a Chinese Hamster Ovary (CHO) cell. In a specific embodiment, the host cell is a CHO-DG44 cell.

[0075] In accordance with another aspect of the present invention, there is provided a kit comprising the bone targeted alkaline phosphatase of the present invention, and instructions to administer the polypeptide to a subject to correct or prevent a hypophosphatasia (HPP) phenotype.

[0076] In accordance with another aspect of the present invention, there is provided a kit comprising the bone targeted alkaline phosphatase of the present invention, and instructions to administer the polypeptide to a subject to correct or prevent aplasia, hypoplasia or dysplasia of dental cementum.

[0077] In accordance with another aspect of the present invention, there is provided a method of using the bone targeted alkaline phosphatase of the present invention, for correcting or preventing at least one hypophosphatasia (HPP) phenotype, comprising administering a therapeutically effective amount of the bone targeted alkaline phosphatase to a subject in need thereof, whereby the at

least one HPP phenotype is corrected or prevented in the subject.

[0078] In a specific embodiment, the subject has at least one HPP phenotype. In another specific embodiment, the subject is likely to develop at least one HPP phenotype. In another specific embodiment, the at least one HPP phenotype comprises HPP-related seizure. In another specific embodiment, the at least one HPP phenotype comprises premature loss of deciduous teeth. In another specific embodiment, the at least one HPP phenotype comprises incomplete bone mineralization. In another specific embodiment, incomplete bone mineralization is incomplete femoral bone mineralization. In another specific embodiment, incomplete bone mineralization is incomplete tibial bone mineralization. In another specific embodiment, incomplete bone mineralization is incomplete metatarsal bone mineralization. In another specific embodiment, incomplete bone mineralization is incomplete ribs bone mineralization. In another specific embodiment, the at least one HPP phenotype comprises elevated blood and/or urine levels of inorganic pyrophosphate (PPi). In another specific embodiment, the at least one HPP phenotype comprises elevated blood and/or urine levels of phosphoethanolamine (PEA). In another specific embodiment, the at least one HPP phenotype comprises elevated blood and/or urine levels of pyridoxal 5'-phosphate (PLP). In another specific embodiment, the at least one HPP phenotype comprises inadequate weight gain. In another specific embodiment, the at least one HPP phenotype comprises rickets. In another specific embodiment, the at least one HPP phenotype comprises bone pain. In another specific embodiment, the at least one HPP phenotype comprises calcium pyrophosphate dihydrate crystal deposition. In another specific embodiment, the at least one HPP phenotype comprises aplasia, hypoplasia or dysplasia of dental cementum. In another specific embodiment, the subject in need thereof has infantile HPP. In another specific embodiment, the subject in need thereof has childhood HPP. In another specific embodiment, the subject in need thereof has perinatal HPP. In another specific embodiment, the subject in need thereof has adult HPP. In

another specific embodiment, the subject in need thereof has odontohypophosphatasia HPP.

[0079] In accordance with another aspect of the present invention, there is provided a method of using the bone targeted alkaline phosphatase of the present invention, for correcting or preventing aplasia, hypoplasia or dysplasia of dental cementum, comprising administering a therapeutically effective amount of the bone targeted alkaline phosphatase to a subject in need thereof, whereby aplasia, hypoplasia or dysplasia of dental cementum is corrected or prevented in the subject.

[0080] In a specific embodiment, the administering comprises transfecting a cell in the subject with a nucleic acid encoding the alkaline phosphatase. In another specific embodiment, the transfecting the cell is performed *in vitro* such that the bone targeted alkaline phosphatase is expressed and secreted in an active form and administered to the subject with said cell. In another specific embodiment, the administering comprises subcutaneous administration of the bone targeted alkaline phosphatase to the subject. In another specific embodiment, the administering comprises intravenous administration of the bone targeted alkaline phosphatase to the subject.

[0081] In accordance with another aspect of the present invention, there is provided the bone targeted alkaline phosphatase of the present invention, for use in correcting or preventing at least one HPP phenotype.

[0082] In accordance with another aspect of the present invention, there is provided the bone targeted alkaline phosphatase of the present invention, for use in correcting or preventing aplasia, hypoplasia or dysplasia of dental cementum.

[0083] In accordance with another aspect of the present invention, there is

provided a use of the bone targeted alkaline phosphatase of the present invention, in the making of a medicament.

[0084] In accordance with another aspect of the present invention, there is provided a use of the bone targeted alkaline phosphatase of the present invention, for correcting or preventing at least one HPP phenotype.

[0085] In accordance with another aspect of the present invention, there is provided a use of the bone targeted alkaline phosphatase of the present invention, for correcting or preventing aplasia, hypoplasia or dysplasia of dental cementum.

[0086] Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of specific embodiments thereof, given by way of example only with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0087] In the appended drawings:

[0088] Figure 1 presents the design and schematic structure of the bone targeted ALP of the present invention exemplified by hsTNALP-FcD10. Panel A presents a schematic representation of the complete primary translation product of the human tissue non-specific alkaline phosphatase gene (TNALP) including the N-terminal signal peptide and the transient membrane-anchored signal for GPI-addition. Panel B presents the primary translation product of the fusion protein. Panel C presents the primary translation product lacking the cleavable TNALP signal peptide;

[0089] Figure 2 presents the protein sequence for hTNALP-FcD10

((SEQ ID NO: 1), including the N-terminal peptide signal–17 first aa), wherein the hTNALP portion (SEQ ID NO: 2) is italicized including the peptide signal portion shown italicized and underlined, and the Fc fragment is underlined (SEQ ID NO: 3);

[0090] Figure 3 presents the protein sequence for the hsTNALP-FcD10 used in Examples presented herein (SEQ ID NO: 4) (without the N-terminal peptide signal) wherein the hsTNALP portion (SEQ ID NO: 5) is italicized, and the Fc fragment is underlined (SEQ ID NO: 3). Double underlined asparagine (N) residues correspond to putative N-glycosylation sites and bold amino acid residues (LK & DI) correspond to linkers between hsTNALP and Fc, and Fc and D10 domains respectively. These linkers are derived from endonuclease restriction sites introduced during cDNA engineering;

[0091] Figure 4 graphically presents the comparative expression of sTNALP-D10 and sTNALP-FcD10 in CHO-DG44 cells;

[0092] Figure 5 presents sTNALP-FcD10 purification on protein-A Sepharose molecular sieve chromatography on Sephacryl™ 3-300 as well as SDS-PAGE analysis of purified sTNALP-FcD10 under reducing (DTT +) and non reducing (DTT-) conditions. It also presents a schematized version of sTNALP-FcD10.The protein purified by Protein A-Sepharose™ affinity chromatography was analyzed by SDS-PAGE and bands stained with Sypro™ Ruby. Main species of sTNALP-FcD10 migrated with an apparent molecular mass of 90,000 Da under reducing conditions and 200,000 Da under non reducing conditions;

[0093] Figure 6 presents the position of the papain cleavage site in sTNALP-FcD10;

[0094] Figure 7 presents a non denaturing SEC-HPLC analysis of

sTNALP-FcD10 on TSK-Gel G3000WXL column. Plain curve: papain digested sample. -X- curve: identical sample incubated in the same conditions without papain (control);

[0095] Figure 8 presents a SDS-PAGE analysis of sTNALP-FcD10 incubated with or without papain showing which fragment is responsible for which band on the gel. Analysis was performed under reducing (+ DTT) or non reducing (- DTT) conditions;

[0096] Figure 9 presents an *in vitro* binding assay. sTNALP-FcD10 and bovine kidney tissue non specific alkaline phosphatase were compared in the reconstituted mineral binding assay as described in Example 2. Total activity is the sum of the enzymatic activity recovered in the free and bound fractions. Total activity was found to be 84% and 96% of initial amount of enzymatic activity introduced in each set of assays for the bovine and sTNALP-FcD10 forms of enzyme, respectively. Results are the average of two bindings;

[0097] Figure 10 presents pharmacokinetic and distribution profiles of sTNALP-FcD10 in serum, tibia and muscle of adult WT mice. Concentrations of sTNALP-FcD10 in serum, tibia and muscle, is expressed in µg/g tissue (wet weight) after a single bolus intravenous injection of 5 mg/kg in adult WT mice;

[0098] Figure 11 presents pharmacokinetic profile of sTNALP-FcD10 serum concentration in newborn WT mice. Serum concentrations of sTNALP-FcD10 as a function of time after a single i.p. (panel A) or s.c. (panel B) injection of 3.7 mg/kg in (1 day old) newborn WT mice;

[0099] Figure 12 presents the predicted pharmacokinetic profile of sTNALP-FcD10 in serum. Predicted maximal (Cmax) and minimal (Cmin) circulating steady-state levels of sTNALP-FcD10 after repeated (every 24 hrs)

subcutaneous injections of 10 mg/Kg in newborn mice;

[00100] Figure 13 presents the experimentally tested pharmacokinetic profile of sTNALP-FcD10 in the serum of newborn mice. Measured minimal (Cmin) circulating steady-state levels of sTNALP-FcD10 24 h after the last subcutaneous injections of 10 mg/Kg in newborn mice. Homo: homozygous, hetero: heterozygous;

[00101] Figure 14 presents short-term (15 days), low dose (1 mg/Kg), efficacy results in terms of sTNALP-FcD10 serum concentrations in treated $Akp2^{-/-}$ mice. sTNALP-FcD10 serum concentrations at day 16 of mice treated for 15 days with daily s.c. injections of 1 mg/kg sTNALP-FcD10;

[00102] Figure 15 presents short-term (15 days), low dose (1 mg/Kg), efficacy results in terms of serum PPi concentrations in treated $Akp2^{-/-}$ mice. Measurement of serum PPi concentrations. A low dose of 1 mg/kg was sufficient to normalize PPi levels in ERT-treated mice;

[00103] Figure 16 presents short-term (15 days), low dose (1 mg/Kg), efficacy results in terms of physeal morphology in treated $Akp2^{-/-}$ mice. Goldner's trichrome staining of the growth plates of WT, untreated and treated $Akp2^{-/-}$ mice. The proximal tibial growth plates (physes) showed excessive widening of the hypertrophic zone in both sTNALP-FcD10 and vehicle injected in $Akp2^{-/-}$ mice, consistent with early rickets. However, physeal morphology seemed less disturbed in the animals treated with sTNALP-FcD10;

[00104] Figure 17 presents short-term (15 days), low dose (1 mg/Kg), efficacy results in terms of physeal hypertrophic area size of treated $Akp2^{-/-}$ mice. Size of the hypertrophic area of the growth plate is expressed as a percentage of the total growth plate area. Note the normalization of the hypertrophic area in the

treated mice;

[00105] Figure 18 presents short-term (15 days), high dose (8.2 mg/Kg), efficacy results in terms of body weight in treated Akp2-/- mice. Effect of sTNALP-FcD10 on body weight;

[00106] Figure 19 presents short-term (15 days), high dose (8.2 mg/Kg), efficacy results in terms of long bone length in treated $Akp2^{-/-}$ mice. Effect of sTNALP-FcD10 on femur and tibial length (measurements done at day 16);

[00107] Figure 20 presents short-term (15 days), high dose (8.2 mg/Kg), efficacy results in terms of sTNALP-FcD10 serum concentration in treated $Akp2^{-/-}$ mice. sTNALP-FcD10 serum concentrations at day 16 of mice treated for 15 days with daily s.c. injections of 8.2 mg/kg sTNALP-FcD10;

[00108] Figure 21 presents short-term (15 days), high dose (8.2 mg/Kg), efficacy results in terms of mineralization of bones in treated $Akp2^{-/-}$ mice. X-ray analysis of feet, rib cages and hind limbs of $Akp2^{-/-}$ mice (16 days) and a Faxitron™ image distribution table. Feet and rib cages were classified as severe, moderate or healthy to take into account the extent of the bone mineralization defects. Legs were simply classified as abnormal (at least one defect) or healthy (no visible defect);

[00109] Figure 22 presents short-term (15 days), high dose (8.2 mg/Kg), efficacy results in terms of defects in teeth in treated $Akp2^{-/-}$ mice. Histological analysis of teeth of $Akp2^{-/-}$ mice injected vehicle or sTNALP-FcD10 and wild-type mice.. Thin sections were prepared and stained as described in Millan et al. PDL=Peridontal ligament;

[00110] Figure 23 presents long-term (52 days), high dose (8.2 mg/Kg), efficacy results in terms of survival in treated $Akp2^{-/-}$ mice. Long-term survival of $Akp2^{-/-}$ mice treated with sTNALP-FcD10 compared to the early demise of $Akp2^{-/-}$ treated only with control vehicle;

[00111] Figure 24 presents long-term (52 days), high dose (8.2 mg/Kg), efficacy results in terms of size, mobility and appearance in treated $Akp2^{-/-}$ mice. Treatment normalizes size, mobility and appearance of treated $Akp2^{-/-}$ mice. Untreated mouse from the same litter is shown for comparison;

[00112] Figure 25 presents long-term (52 days), high dose (8.2 mg/Kg), efficacy results in terms of mineralization and length of bones in treated $Akp2^{-/-}$ mice. X-ray images of the metatarsal bones of 46 and 53-days old treated $Akp2^{-/-}$ mice in comparison with WT mice;

[00113] Figure 26 presents long-term (52 days), high dose (8.2 mg/Kg), efficacy results in terms of sTNALP-FcD10 serum concentration in treated $Akp2^{-/-}$ mice. sTNALP-FcD10 serum concentrations at day 53 of mice treated for 52 days with daily s.c. injections of 8.2 mg/kg sTNALP-FcD10;

[00114] Figure 27 presents A) survival curves of $Akp2^{-/-}$ mice receiving sTNALP-FcD10 at doses of either 4.3 mg/kg daily (Tx-1) or 15.2 mg/kg every 3 days (Tx-3) or 15.2 mg/kg every week (Tx-7) and B) median survival for each of these regimen . Survival of the treated mice was compared to the survival of mice injected vehicle;

[00115] Figure 28 presents A) survival curves of $Akp2^{-/-}$ mice receiving sTNALP-FcD10 at doses of 8.2 mg/kg daily (RTx) starting at day 15 after birth and B) median survival for treated and vehicle injected mice. Survival of the treated mice is compared to the survival of mice injected vehicle (RVehicle);

[00116] Figure 29 presents the effects on body weight of daily 8.2 mg/kg doses of sTNALP-FcD10 injected to $Akp2^{-/-}$ mice (RTx) starting at day 15 after birth. Daily body weights are compared to that of vehicle-injected $Akp2^{-/-}$ mice (RVehicle) or wild-type littermates (WT);

[00117] Figure 30 presents an alignment of various ALPs established by CLUSTAL™ W (1.82) multiple sequence alignment, namely a bovine TNALP sequence (SEQ ID NO: 6); a cat TNALP sequence (SEQ ID NO: 7), a human TNALP sequence (SEQ ID NO: 8), a mouse TNALP sequence (SEQ ID NO: 9), a rat TNALP sequence (SEQ ID NO: 10) and a partial dog TNALP sequence (SEQ ID NO: 11) wherein the nature of the first 22 amino acid residues are unknown; a human IALP (SEQ ID NO: 12) (Accession no: NP_001622), a human GCALP (SEQ ID NO: 13) (Accession no: P10696), and a human PLALP (SEQ ID NO: 14) (Accession no: NP_112603)."*" denotes that the residues in that column are identical in all sequences of the alignment,":" denotes that conserved substitutions have been observed, and "." denotes that semi-conserved substitutions have been observed. A consensus sequence derived from this alignment (SEQ ID NO: 15) is also presented wherein x is any amino acid;

[00118] Figure 31 presents an alignment of TNALPs from various species established by CLUSTAL™ W (1.82) multiple sequence alignment, namely the bovine sequence (SEQ ID NO: 6); the cat sequence (SEQ ID NO: 7), the human sequence (SEQ ID NO: 8), the mouse sequence (SEQ ID NO: 9), the rat sequence (SEQ ID NO: 10) and a partial dog sequence (SEQ ID NO: 11) wherein the nature of the first 22 amino acid residues are unknown. "*" denotes that the residues in that column are identical in all sequences of the alignment,":" denotes that conserved substitutions have been observed, and "." denotes that semi-conserved substitutions have been observed. A consensus sequence derived from this alignment (SEQ ID NO: 16) is also presented wherein x is any amino acid; and

[00119] Figure 32 presents the nucleic acid sequence (SEQ ID NO:17) encoding the polypeptide sequence described in Figure 1.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[00120] Examples provided below present the first successful treatment of TNALP knockout (Akp2-/-) mice using subcutaneous injections of a recombinant form of ALP. $Akp2^{-/-}$ mice recapitulate the severe, often lethal, infantile form of Hypophosphatasia.

[00121] The well-described TNSALP-homozygous null murine model which mirrors many of the skeletal and biochemical abnormalities associated with infantile HPP was used. Mice were treated with a novel soluble recombinant form of human TNSALP engineered at its carboxy-terminus to contain both a spacer in the form of the crystalline fragment (Fc) region of human IgG-1 fused to a bone targeting sequence composed of ten sequential aspartic acid (D10) residues. It was shown that relative to native TNSALP purified from kidney, the modified recombinant form of the enzyme, binds hydroxyapatite much more avidly, while retaining its enzymatic activity. Treatment with the recombinant TNSALP of the present invention surprisingly normalized plasma PPi levels, and improved mineralization of the feet thoraces, hind limbs and dentition of homozygous null mice when compared to mice who received the vehicle alone. The treatment was also shown to prolong survival, with near radiographic normalization of the skeletal phenotype.

[00122] In addition to its beneficial *in vivo* therapeutic effect, it was surprisingly discovered that the recombinant active form of the modified enzyme which contains a spacer is expressed at higher levels than its recombinant counterpart lacking such spacer. In addition, it was demonstrated that the enzyme functions as a tetramer.

[00123] The present invention is illustrated in further details by the following non-limiting examples.

EXAMPLE 1

Expression and purification of recombinant sTNALP-FcD10

[00124] In order to facilitate the expression and purification of recombinant TNALP, the hydrophobic C-terminal sequence that specifies GPI-anchor attachment in TNALP was eliminated to make it a soluble secreted enzyme (Di Mauro et al. 2002). The coding sequence of the TNALP ectodomain was also extended with the Fc region of the human IgG (γ1 form (IgG1), Swiss-Prot P01857). This allowed rapid purification of the recombinant enzyme on Protein A chromatography and surprisingly, its increased expression. Furthermore, to target the recombinant TNALP to bone tissue, a deca-aspartate (D10) sequence was attached to the C-terminal of the Fc region. This chimeric form of TNALP, designated sTNALP-FcD10, retains full enzymatic activity both when assayed at pH 9.8 using the artificial substrate p-nitrophenylphosphate and when assayed at pH 7.4 using inorganic pyrophosphate (PPi), as the physiological substrate. As in the naturally occurring form of TNALP the N-terminal signal peptide is cleaved off during the cotranslational translocation of the protein across the rough endoplasmic reticulum. Its design and structure is schematically illustrated in Figure 1. The amino acid sequence of the fusion protein (including the signal peptide) is shown in Figure 2. The amino acid sequence of the fusion protein as secreted (i.e. without the signal peptide) is shown in Figure 3.

[00125] The method that was used to construct this fusion protein is as follows. The cDNA encoding the fusion protein (See Figure 32) was inserted in the pIRES vector (Clontech^{TM}) in the first multiple cloning site located upstream of the IRES using NheI and BamHI endonuclease restriction sites. The dihydrofolate reductase (DHFR) gene was inserted in the second multiple cloning site located

downstream of the IRES using SmaI and XbaI endonuclease restriction sites. The resulting vector was transfected into Chinese Hamster Ovary (CHO-DG44) cells lacking both DHFR gene alleles (Urlaub et al. 1983, obtained from Dr Lawrence A. Chasin, Columbia University) using the Lipofectamine™ transfection kit (Invitrogen). Two days after transfection, media was changed and the cells were maintained in a nucleotide free medium (IMDM supplemented with 5% dialyzed FBS) for 15 days to isolate stable transfectants for plaque cloning.

[00126] Cells from the three best clones or the five originally selected growing in the nucleotide-free medium were pooled and further cultivated in media (IMDM + 5% dialyzed FBS) containing increasing concentration of methotrexate (MTX). Cultures resistant to 50 nM MTX were further expanded in Cellstacks™ (Corning) containing IMDM medium supplemented with 5% FBS. Upon reaching confluency, the cell layer was rinsed with Phosphate Buffered Saline (PBS) and cells were incubated for three additional days with IMDM containing 3.5 mM sodium butyrate to increase protein expression. At the end of the culture the concentration of sTNALP-FcD10 in the spent medium was 3.5 mg/l as assessed by measuring TNALP enzymatic activity.

[00127] Levels of sALP-FcD10 in spent medium were quantified using a colorimetric assay for ALP activity where absorbance of released p-nitrophenol is proportional to the reaction products. The reaction occurred in 100 µl of ALP buffer (20 mM Bis Tris Propane (HCl) pH 9, 50 mM NaCl, 0.5 mM $MgCl_2$, and 50 µM $ZnCl_2$) containing 10 µl of diluted spent medium and 1 mM pNPP. The latter compound was added last to initiate the reaction. Absorbance was recorded at 405 nm every 45 seconds over 20 minutes using a spectrophotometric plate reader. sTNALP-FcD10 catalytic activity, expressed as an initial rate, was assessed by fitting the steepest slope for 8 sequential values. Standards were prepared with varying concentrations of sALP-FcD10, and ALP activity was determined as above. The standard curve was generated by plotting Log of the initial rate as a function of the Log of the standard concentrations. sTNALP-FcD10 concentration in the different samples was read from

the standard curve using their respective ALP absorbance. Activity measures were transformed into concentrations of sALP-FcD10 by using a calibration curve obtained by plotting the activity of known concentrations of purified recombinant enzyme.

[00128] Culture supernatant was then concentrated and dialyzed against PBS using tangential flow filtration and loaded on MabSelect SuRe™ column (GE Health Care) equilibrated with 150 mM NaCl, 10 mM sodium PO_4. Bound proteins were eluted with 50 mM Tris pH 11, pH 11.0 buffer. Collected fractions were adjusted to pH 8-9 with 200 mM Tris-HCl pH 5.5. Fractions containing most of the eluted material were dialyzed against 150 mM NaCl, 25 mM sodium PO_4 pH 7.4 buffer containing 0.1 mM $MgCl_2$, 20 µM $ZnCl_2$ and filtered on a 0.22 µm (Millipore, Millex-GP™) membrane under sterile conditions. The overall yield of the purification procedure was 50% with a purity above 95% as assessed by Sypro™ ruby stained SDS-PAGE. Purified sTNALP-FcD10 preparation was stored at 4°C and remained stable for several months.

[00129] The following purification technique was also tested with success. Culture supernatant was concentrated and dialyzed against PBS using tangential flow filtration and loaded on Protein A-Sepharose™ column (Hi-Trap™ 5 ml, GE Health Care) equilibrated with PBS. Bound proteins were eluted with 100 mM citrate pH 4.0 buffer. Collected fractions were immediately adjusted to pH 7.5 with 1 M Tris pH 9.0. Fractions containing most of the eluted material were dialyzed against 150 mM NaCl, 25 mM sodium PO_4 pH 7.4 buffer containing 0.1 mM $MgCl_2$, 20 µM $ZnCl_2$ and filtered on a 0.22 µm (Millipore, Millex-GP™) membrane under sterile conditions. The overall yield of the purification procedure was 50% with a purity above 95% as assessed by Sypro™ ruby stained SDS-PAGE. Purified sTNALP-FcD10 preparation was stored at 4°C and remained stable for several months.

[00130] The number of copies of the sTNALP-FcD10 gene was

increased by culturing transfected CHO-DG44194 cells in the presence of increasing concentration of methotrexate. Clones of cells resistant to 100 nM methotrexate were isolated and evaluated for their capacity to produce sTNALP-FcD10 at a high yield. The best producers were adapted to culture in suspension in Hyclone media™ SFM4CHO™ (cat # SH30549) in absence of fetal bovine serum. Cultures that maintained a high production yield under those conditions were transferred to disposable Wave™ bioreactor bags. The medium (25 L total volume) was seeded at a density of 0.4 x 10^6 cells per ml. Temperature of the culture was maintained at 37 °C until the cell density reached 2 x 10^6 cells/ml. The temperature was then reduced to 30°C and the culture was supplemented with 125 ml of CHO generic feed (Sigma, C1615). Those conditions were found to slow down cell division and increase product secretion in the culture medium. These conditions were maintained for 6 days before harvesting cell culture supernatant containing secreted sTNALP-FcD10.

EXAMPLE 2

Comparative expression of sTNALP-D10 and sTNALP-FcD10

[00131] Plasmid vectors encoding either sTNALP-FcD10 or sTNALP-D10 were transfected in CHO-DG44 cells using Lipofectamine™ and grown in selective media (i.e. devoid of nucleotides) designed to promote survival of cells expressing the DHFR gene as described in Example 1 above. Stable transfectants were isolated by plaque cloning and ranked according to their level of protein expression using the alkaline phosphatase enzymatic assay also described in Example 1 above. Screening allowed the identification of one clone only for sTNALP-D10 (0.120 pg/cell/day) and five clones for sTNALP-FcD10 (0.377, 0.258, 0.203, 0.099 and 0.088 pg/cell/day). Methotrexate (MTX) gene amplification was performed as described in Example 1 above (MTX ranging from 0 to 100mM) and allowed an 8-fold expression increase for sTNALP-FcD10 while no amplification was observed with the sTNALP-D10 cultures (see Figure 4). Using a similar

process for cell line development, it was unexpectedly found that the sTNALP-FcD10 protein was easier to express compared to sTNALP-D10 (see Figure 4).

EXAMPLE 3

sTNALP-FcD10 characterization

[00132] sTNALP-FcD10 was first purified on Protein-A Sepharose™ and was analyzed on SDS-PAGE under reducing and non-reducing conditions.

[00133] Under reducing conditions, it migrated as a broad band with an apparent molecular mass of ~ 90,000 Da (DTT+ in Figure 5). Digestion with peptide N-Glycosidase F (PNGAse F) reduced the apparent molecular mass of the protein to about 80,000 which closely approximates the calculated mass of 80,500 Da for the non-glycosylated sTNALP-FcD10 monomer shown in Figure 1. Soluble TNALP in serum, like TNALP present as a GPI anchored protein on the outer surface of osteoblasts, is a highly glycosylated protein with carbohydrates comprising ≤ 20% of the total mass of the enzyme (Farley & Magnusson 2005). Although the specific carbohydrate structures on TNALP have not been identified, sequence studies indicate that the enzyme possesses five putative sites for N-linked glycosylation, and biochemical studies have shown evidence for both N- and O-linked carbohydrates (Nosjean et al. 1997). In agreement with these earlier observations, the electrophoretic migration of sTNALP-FcD10 and its sensitivity to PNGAse F suggests it is also a heavily N-glycosylated protein. Soluble TNALP in serum, like TNALP present as a GPI anchored protein on the outer surface of osteoblasts, is a highly glycosylated protein with carbohydrates comprising ≤ 20% of the total mass of the enzyme (Farley & Magnusson 2005).

[00134] When SDS-PAGE was repeated under non reducing conditions the apparent molecular mass of sTNALP-FcD10 was found to be 200,000 (DTT- in Figure 5) consistent with that of a homodimer as in native unaltered TNALP (Millán

2006). This homodimer likely results from the formation of two disulfide bridges between two monomeric Fc regions (upper right panel, Figure 5).

[00135] The molecular mass of purified sTNALP-FcD10 under native conditions was next evaluated using size exclusion FPLC chromatography on a column of Sephacryl™ S-300 (GE Health Care) equilibrated in 150 mM NaCl, 20 mM Tris pH 7.5 buffer. The column was previously calibrated with a standard protein kit (HMW calibration kit, GE Health care) (lower left panel, Figure 5).

[00136] Collected chromatography fractions were assayed for alkaline phosphatase enzymatic activity and the material in each peak. Surprisingly, 78% of the material eluted at a position corresponding to proteins of 370kDa (lower left panel, Figure 5) suggesting a tetrameric form for the native sTNALP-FcD10 recombinant enzyme produced in CHO cells. When fractions from the Sephacryl S-300 column were tested for activity, all of the enzymatic activity was associated with the 370 kDa fraction. The remaining material was of a much higher molecular weight indicating the formation of some sTNALP-FcD10 aggregates. Both the tetrameric forms, which may not be observed on the SDS-page because the latter destroys the non covalent binding maintaining the tetramer together, and aggregate forms were resolved as sTNALP-FcD10 monomers with an apparent molecular weight of 90,000 by SDS-PAGE under reducing conditions (DTT+, lower right panel in Figure 5) and as dimers with an apparent molecular weight of 200,000 in non reducing conditions (DTT-, lower right panel in Figure 5). Recombinant sTNALP-FcD10 appears to consist mainly of enzymatically functional homotetramers formed by non covalent association of two sTNALP-FcD10 disulfide-linked dimers.

[00137] The tetrameric structure of sTNALP-FcD10 was further tested by limited papain digestion (Figures 6-8). This protease is known to cleave IgG heavy chains close to the hinge region and on the N-terminal side of the disulfide

bonds, thereby generating whole monomeric Fab fragments and dimeric disulfide-linked Fc dimers. Digestion of sTNALP-FcD10 should thus liberate enzymatically active sTNALP dimers from the intact Fc domains (see Figure 6).

[00138] Aliquots containing 400 µg of sTNALP-FcD10 were digested with 208 mU of papain-agarose (Sigma) in a 20 mM phosphate buffer (pH 7.0) containing 250 µM dithiothreitol. Digestion was left to proceed at 37°C for 1h under gentle agitation. The reaction was stopped by removing the papain-agarose beads by centrifugation. In those conditions, there was no significant loss of sTNALP-FcD10 enzymatic activity during the first 4 h of incubation. sTNALP-FcD10 incubated for one h in the presence or absence of papain-agarose was next analyzed by SEC-HPLC on a TSK-Gel G3000WXL (Tosoh Bioscience) in non denaturing conditions.

[00139] Figure 7 shows that the main product eluting with an apparent Mr of 370 kDa was no longer observed after a 1 h papain digestion. In those conditions papain digestion generates two main fragments of 135 kDa and 62 kDa respectively. A minor peak with Mr of 35 kDa was also observed.

[00140] Under reducing SDS-PAGE conditions (DTT+, Figure 8) the product of the non papain treated sample was resolved into a major band (102 kDa) (DTT+, papain-), which was previously shown to correspond to monomeric sTNALP-FcD10. In Western blots this band can indeed be stained with antibodies for both TNALP and the Fc domain of the IgG_1 molecule (not shown). After papain digestion this band is cleaved into two major fragments: 1) The 32 kDa band, which binds the anti-Fc but not the anti-TNALP antibody and is proposed to correspond to the FcD10 fragment; and 2) The broad and diffuse protein band (66 – 90 kDa) which can be stained with the anti-ALP antibody but not with anti-Fc antibody and is thus thought to correspond to TNALP ectodomain monomers. The heterogeneity of this material is presumably due to its glycosylation as it can be

reduced by digestion with Peptide-N-Glycosidase F, which also decreases its apparent molecular mass to 52 kDa (results not shown).

[00141] Under non-reducing conditions (DTT-, Figure 8), sTNALP-FcD10 incubated without papain was found to migrate in SDS-Page as a 216 kDa protein (DTT-, papain-, Figure 8). Western blotting also demonstrates that this protein contains epitopes for both the TNALP and Fc moieties (results not shown). This molecular species was previously proposed to consist of disulfide-bonded sTNALP-FcD10 dimers. As under reducing conditions, papain cleavage under non-reducing conditions (DTT-, papain +) generates two main fragments. In Western blots, the 55 kDa fragment can be stained with the anti-Fc but not with the anti-TNALP antibody. This fragment is most probably identical to the 62 kDa species observed on SEC-HPLC in native conditions and is proposed to correspond to disulfide-bonded Fc dimers. The other major species comigrates with the major protein band (66-90 kDa) observed under reducing conditions. This is consistent with it being composed of TNALP ectodomain monomers. When analyzed by HPLC in non denaturing conditions these monomers are non-covalently associated in the enzymatically active TNALP dimers eluting from the SEC column as the 135 kDa species.

EXAMPLE 4

Compared affinity for hydroxyapatite of sTNALP-FcD10 protein and bovine kidney sALP

[00142] The affinity of the purified sTNALP-FcD10 protein for hydroxyapatite was also compared to that of bovine kidney (tissue non specific) soluble alkaline phosphatase (Calzyme) using the following procedure. Bovine kidney TNALP was used instead of human bone TNALP because it was commercially available. Hydroxyapatite ceramic beads (Biorad) were first solubilized in 1 M HCl and the mineral was precipitated by bringing back the

solution to pH to 7.4 with 10 N NaOH. Binding to this reconstituted mineral was performed by incubating aliquots of the mineral suspension containing 750 µg of mineral with 5 µg of protein in 100 µl of 150 mM NaCl, 80 mM sodium phosphate pH 7.4, buffer. The samples were kept at 21 ± 2°C for 30 minutes on a rotating wheel. Mineral was spun down by low speed centrifugation and total enzymatic activity recovered in both the mineral pellet and the supernatant was measured. Figure 9 clearly shows that sTNALP-FcD10 binds more efficiently to reconstituted hydroxyapatite mineral than bovine kidney TNALP. Furthermore, most of the recombinant sTNALP-FcD10 protein introduced in the assay was recovered by summing up the enzymatic activity recovered in both the bound and non bound fractions. This indicates that binding of the recombinant protein to the reconstituted mineral phase does not significantly alter its enzymatic activity.

EXAMPLE 5

Mouse model

[00143] The $Akp2^{-/-}$ mice were created by insertion of the Neo cassette into exon VI of the mouse TNALP gene (Akp2) via homologous recombination (Narisawa et al. 1997; Fedde et al. 1999). This mutation caused the functional inactivation of the Akp2 gene and no mRNA or TNALP protein is detectable in these knockout mice (Narisawa et al. 1997). Phenotypically, the $Akp2^{-/-}$ mice mimic severe infantile HPP. These mice have no obvious hypophosphatasia phenotype at birth, skeletal defects usually appearing at or around day 6, and worsen thereafter. They have stunted growth with rickets, develop epileptic seizures and apnea, and were reported to die between postnatal days 12-16. Like HPP patients, $Akp2^{-/-}$ mice feature hypophosphatasemia due to global deficiency of TNALP activity, endogenous accumulation of the ALP substrates, PPi, PLP and PEA and suffer impaired skeletal matrix mineralization leading to rickets or osteomalacia (Fedde et al. 1999).

[00144] To understand how defects in alkaline phosphatase can lead to neurological manifestations of the disease in both human and mice, one has to review the role and metabolism of Vitamin B6 in the CNS. Vitamin B6 is an important nutrient that serves as a cofactor for at least 110 enzymes, including those involved in the biosynthesis of the neurotransmitters γ-aminobutyric acid (GABA), dopamine and serotonin. Vitamin B6 can be found in three free forms (or vitamers), i.e., pyridoxal (PL), pyridoxamine (PM), and pyridoxine (PN), all of which can be phosphorylated to the corresponding 5'-phosphated derivatives, PLP, PMP and PNP (Jansonius 1998). Removal of the phosphate group is a function of ALP, and primarily that of the TNALP isozyme (Whyte 2001). Since only dephosphorylated vitamers can be transported into the cells, decreased TNALP activity in Hypophosphatasia results in marked increases in plasma PLP (Whyte et al. 1985; Whyte 2001) and intracellular deficiency of PLP in peripheral tissues and the central nervous system where it leads to reduced brain levels of GABA. It has also been hypothesized that the epileptic seizures observed in these mice result from glutamic acid decarboxylase dysfunction due to shortage of PLP (Waymire et al. 1995).

[00145] Pyridoxine supplementation suppresses the epileptic seizures of $Akp2^{-/-}$ mice but extends their lifespan only a few days, till postnatal days 18-22 (Narisawa et al. 2001). Therefore, all animals in this study (breeders, nursing moms, pups and weanlings) were given free access to a modified laboratory rodent diet 5001 with increased levels (325 ppm) of pyridoxine.

[00146] $Akp2^{-/-}$ mice (12.5 % C57BL/6 – 87.5%129J hybrid background) were maintained by heterozygote breeding. Animals, breeder pairs or nursing moms with their pups, were housed in a ventilated solid bottom plastic cage equipped with an automatic watering system. All animals had free access to a modified laboratory rodent diet 5001 with 325 ppm pyridoxine (#48057, TestDiet™). Maximum allowable concentrations of contaminants in the diet (e.g.

heavy metals, aflatoxin, organophosphate, chlorinated hydrocarbons, PCBs) were assured by the manufacturers. No known contaminants were present in the dietary material to influence the toxicity of the test article.

EXAMPLE 6

Pharmacokinetics and tissue distribution of injected sTNALP-FcD10 in WT mice

Blood sample collection

[00147] Blood samples were collected into heparin lithium tube (VWR, #CBD365958), put on ice for a maximum of 20 minutes before centrifugation at 2500g for 10 min at room temperature. At least, 15 µl of plasma was transferred into 0.5 ml tube (Sarstedt, #72.699), frozen in liquid nitrogen and kept at -80°C until assayed. If available, another ≤ 50 µl of plasma was transferred into 0.5 ml tube, inactivated at 65°C for 10 minutes, frozen in liquid nitrogen and kept at -80°C until assayed. Any remaining plasma, was pooled with the 15 µl aliquot, frozen in liquid nitrogen and kept at -80°C until assayed.

Determination of plasma sTNALP-FcD10

[00148] Presence of sTNALP-FcD10 in plasma samples was assessed upon completion of treatment using a colorimetric enzymatic assay. Enzymatic activity was determined using a chromogenic substrate where increase of absorbance is proportional to substrate conversion to products. The reaction was carried out in 100 µl of buffer 50 mM NaCl, 20 mM Bis Tris Propane (HCl) pH 9 buffer containing 0.5 mM $MgCl_2$ and 50 µM $ZnCl_2$ to which was added 10 µl of diluted plasma sample. The ALP substrate p-nitrophenyl was added last at a final concentration of 1 mM to initiate the reaction. The absorbance was recorded at 405 nm every 45 seconds over a twenty minutes period using a Spectramax™ 190 (Molecular devices) plate reader. sTNALP-FcD10 enzymatic activity expressed as an initial rate of reaction was assessed by fitting the steepest slope over 8 adjacent

reading values. Standards were prepared with varying concentrations of test article and the enzymatic activity was determined as described above in Example 1. The standard curve was generated by plotting Log of the initial speed rate as a function of the Log of the standard quantities. sTNALP-FcD10 concentration of the different plasma samples was read directly from the standard curve using their respective enzymatic activity.

Determination of plasma PPi

[00149] Circulating levels of PPi were measured in serum obtained from cardiac puncture using differential adsorption on activated charcoal of UDP-D-[6-^{3}H]glucose (Amersham Pharmacia) from the reaction product of 6-phospho[6-^{3}H]gluconate, as previously described (Johnson et al. 1999).

Half-life and tissue distribution of sTNALP-FcD10

[00150] **In adult WT mice,** the half-life and tissue distribution of sTNALP-FcD10 injected into mice were determined. Figure 10 summarizes its pharmacokinetics and tissue distribution after a single, bolus intravenous injection of 5 mg/kg into adult WT mice.

[00151] The half-life was 34 h in blood with an accumulation of the [^{125}I]-labeled sTNALP-FcD10 in bone of up to 1 µg/g of bone (wet weight). This half-life is comparable to that observed previously in unsuccessful reported clinical trials. Levels of bone-targeted material seemed quite stable, as no significant decrease in radiolabeled sTNALP-FcD10 was observed during the experiment. No accumulation of sTNALP-FcD10 was observed in muscle, as the amount of radiolabeled enzyme in that tissue decreased in parallel with that of sTNALP-FcD10 enzymatic activity in blood.

[00152] **In newborn mice.** Because Akp2$^{-/-}$ mice die between days

12-16 and i.v. injection is not feasible in such young mice, the pharmacokinetic analysis of sTNALP-FcD10 in serum was repeated using the i.p. and s.c. routes in WT newborn mice using a dose of 3.7 mg/Kg. The i.p. route was found inadequate due to the high pressure in the abdominal cavity leading to unpredictable losses through the injection site (Figure 11 A). The s.c. route was more reproducible in newborn mice, as seen in the PK experiment of Figure 11 B. The pharmacokinetic parameters of sTNALP-FcD10 in newborn and adult mice is reported in Table 2 below.

[00153] Table 2: Pharmacokinetic parameters of sTNALP-FcD10 in newborn WT mice.

Parameter	Newborn	
	s.c.	i.p.
T1/2 (h)	31	19
Tmax (h)	6	6
Cmax (mg/L)	5	3
AUCinf (mg/L/h)	257	92

[00154] These PK data, analyzed by WinNonlin™ software (Pharsight Corporation, Mountain View, CA), were used to predict circulating blood levels of sTNALP-FcD10 achieved after repeated daily s.c. injections. Circulating sTNALP-FcD10 reached steady state serum concentrations oscillating between Cmin and Cmax values of 26.4 and 36.6 µg/ml, respectively (Figure 12). Steady state was achieved after 5 to 6 daily doses of 10 mg/kg.

[00155] Prediction validity was tested experimentally after 5 daily injections of 10 mg/kg of sTNALP-FcD10. At the day of injection, the mice genotype could not be distinguished. It was later determined which amongst the mice tested were heterozygous or homozygous. There was no difference in the behavior of all the different genotypes. When circulating ALP activity was

measured 24 h after the last injection, namely on day 6, (Cmin), good agreement was observed between experimental and predicted concentrations (Figure 13). In these non treated 5 day old animals, serum TNALP levels were 0.58 µg/ml. These levels will decrease with age. Thus, it was calculated that the injection regimen allowed building up to steady state serum concentrations of sTNALP-FcD10 approximately 50 times higher than normal TNALP concentrations.

EXAMPLE 7

Concentration of sTNALP-FcD10 in adult WT mice bones after bolus intravenous administration

[00156] A 5 mg/kg sTNALP-FcD10 dose was administered i.v. in 129J adult WT mice. The sTNALP-FcD10 concentration in bone at T=25 hours was as follows: 0.64 µg/g calvaria; 1.33 µg/g tibias; and 1.37 µg/g femurs, for a mean concentration of 1.11 µg/g. In rat, bone tissues represent 16.3% of total mass. It is expected that this percentage is also found in mice. The body weight of mice used for this experiment was 18.4 g. The calculated bone tissue weight of these mice was thus about 18.4 g x 0.163 = 3.00 g. The calculated quantity of sTNALP-FcD10 in bone tissues was of 3.33 µg. The percentage of the injected dose in bone tissues was thus of (3.33µg/(5µg/g * 18.4g))*100 = 4%.

[00157] The sTNALP-FcD10 concentration in bone at T=96 hours was as follows: 0.83 µg/g calvaria; 1.33 µg/g tibias and1.63 µg/g femurs, for a mean concentration of 11.26 µg/g. The body weight of mice used for this experiment was 17.8 g. The calculated bone tissue weight of these mice was thus about 17.8 g x 0.163 = 2.90 g. The quantity ofsTNALP-FcD10 in mice bone tissues was thus about 3.66 µg. The percentage of the injected dose in bone tissues was thus of (3.66µg/(5µg/g * 17.8g))*100 = 4%.

EXAMPLE 8

Concentration of sTNALP-FcD10 in newborn WT mice bones after 15 days bolus subcutaneous injection

[00158] A 4.3 mg/kg sTNALP-FcD10 dose was administered subcutaneously in 129J newborn WT mice every day for 15 days for a total administered amount of 65 mg/kg. The sTNALP-FcD10 concentration in bone at T=24 hours was as follows: 6.45 µg/g calvaria; 3.05 µg/g tibias; and 3.71 µg/g femurs, for a mean concentration of 4.40 µg/g. The body weight of mice used for this experiment was 9.83 g. The calculated bone tissue weight of these mice was thus about 9.83 g x 0.163 = 1.60 g. The quantity of sTNALP-FcD10 in mice bone tissues at that time was thus about 7.04 µg. The percentage of the injected dose in bone tissues was thus of (7.04µg/(65µg/g * 9.83g))*100 = 1%.

[00159] The sTNALP-FcD10 concentration in bone at T=168 hours was as follows: 5.33 µg/g calvaria; 1.37 µg/g tibias; and 1.88 µg/g femurs, for a mean concentration of 2.86 µg/g. The body weight of mice used for this experiment was 14.0 g. The calculated bone tissue weight of these mice was thus about 14.0 g x 0.163 = 2.28 g. The quantity of sTNALP-FcD10 in mice bone tissues at that time was thus about 6.52 µg. The percentage of the injected dose in bone tissues was thus of (6.52µg/(65µg/g * 14g))*100 = 0.7%. Table 3 below summarizes results of Examples 7 and 8.

[00160] Table 3: Mean concentration of sTNALP-FcD10 and percentage of injected dose in bones

Experiment	Injection regimen	Mean concentration in bones (µg/g wet tissue)		% of injected dose in bones	
IV Bolus	1 x 5 mg/kg (bolus)	T = 25 h[(1)]	T = 96 h[(1)]	T = 25 h[(1)]	T = 96 h[(1)]
		1.11	1.26	4 %	4 %
SC Bolus over 15 days	15 x 4.3 mg/kg (daily)	T = 24 h[(1)]	T = 168 h[(1)]	T = 24 h[(1)]	T = 168 h[(1)]
		4.40	2.86	1 %	0.7 %

[(1)] Times indicated are from the last injection.

EXAMPLE 9

Short-term (15 days) efficacy of low doses (1 mg/kg) of sTNALP-FcD10 for HPP in Akp2$^{-/-}$ mice

[00161] Daily s.c. injection of sTNALP-FcD$_{10}$ were performed for 15 days in Akp2$^{-/-}$ mice using 1 mg/kg. Treatment groups were constituted from 19 litters. Akp2$^{-/-}$ mice received vehicle (N=13) or sTNALP-FcD10 (N=12).Controls consisted of 15 WT mice (one per litter). Controls were not submitted to injections. Blood was taken 24 h after the last injection as described in Example 6.

[00162] Figure 14 shows that enzyme activities in serum at day 16 were barely above the detection level. Despite low serum values for sTNALP-FcD10, serum PPi levels were corrected (Figure 15). Untreated Akp2$^{-/-}$ mice had serum PPi concentrations of 1.90 ± 0.64 µmol/ml, whereas treated Akp2$^{-/-}$ mice had levels of 1.41 ± 0.30 µmol/ml, comparable to those of WT mice (1.52 ± 0.35 µmol/ml).

[00163] Proximal tibial growth plates showed some widening of the hypertrophic zone in Akp2$^{-/-}$ animals compared WT animals (compare vehicle with wild-type in Figure 16). The same observation made earlier in this strain of Akp2$^{-/-}$ mice (Hessle et al. 2002) is consistent with rickets. A trend toward normalization of the physeal morphology was observed in animals treated with sTNALP-FcD10 for 15 days (Figure 17) compared to vehicle (untreated).

EXAMPLE 10

Short-term (15 days) efficacy of high doses (8.2 mg/kg) of sTNALP-FcD10 for HPP in Akp2$^{-/-}$ mice

[00164] To evaluate 15 days of daily s.c. injections using a significantly higher dose of sTNALP-FcD10 (8.2 mg/kg) on growth and bone mineralization, mice from 20 litters (141 mice total) were used. They were distributed to two groups: 1) Akp2$^{-/-}$ mice given vehicle (N = 19); 2) Akp2$^{-/-}$ mice treated with sTNALP-FcD10 (N = 20); additionally, there was one WT mouse per

litter, non treated (N = 18).

Body weight

[00165] $Akp2^{-/-}$ mice grew more slowly than WT mice. At day 1, no statistical difference in body weights was observed among the vehicle, sTNALP-FcD10, and WT animals. However, daily mean body weights diverged at day 6 (Figure 18). The difference between WT (4.2 ± 0.6 g) and vehicle (3.7 ± 0.7 g) achieved statistical significance (p=0.0217) at day 6; but the difference between vehicle (5.9 ± 1.0 g) and sTNALP-FcD10 treated values (6.7 ± 1.0 g) achieved statistical significance at day 11 (p=0.04), with the treated group paradoxically heavier than WT. At day 16, mean body weight of treated animals (8.2 ± 1.1 g) and WT (8.4 ± 0.8 g) were not statistically different. Animals treated with sTNALP-FcD10 had body weights statistically greater (p=0.026) than those treated with vehicle (6.6 ± 1.4 g). No significant difference between the ERT and WT groups was observed for body weight at any time point.

Bone length

[00166] At the end of this experiment (day 16), tibial length provided an additional measure of skeletal benefit for $Akp2^{-/-}$ mice. The tibia length with ERT was 12.6 ± 0.7 mm and longer (p=0.0135) compared to animals given vehicle (11.7 ± 1.1 mm) (Figure 19). A statistical difference (p=0.0267) was also obtained when femur length was compared between the sTNALP-FcD10 (9.2 ± 0.4 mm) and vehicle (8.6 ± 0.8 mm) groups. No statistical difference was noted for tibia or femur length of the ERT compared to WT mice. A partial preservation (i.e. partial prevention of reduction in bone growth that becomes apparent around two weeks of age) of tibia and femur growth was observed by measures of length at necropsy (Figure 19).

[00167] In all but 5 animals, detectable, but highly variable, levels of sTNALP-FcD10 were found in the plasma of treated $Akp2^{-/-}$ mice at day 16 (Figure

20). Circulating TNALP concentrations in normal animals are given for comparison purposes.

Bone mineralization

[00168] Blinded evaluations of Faxitron™ images of the feet and rib cages distinguished two degrees of severity of mineralization defects in the Akp2-/- mice (Figure 21). Severely affected mice (Severe) had an absence of digital bones (phalanges) and secondary ossification centers. Moderately affected (Moderate) mice had abnormal secondary ossification centers, but all digital bones were present. WT mice (Healthy) had all bony structures present with normal architecture. Radiographic images of the hind limbs were similarly classified as abnormal if evidence of acute or chronic fractures was present, or healthy in the absence of any abnormal findings (Figure 21). ERT minimized mineralization defects in the feet documented by the number of Akp2-/- mice with severe defects, consisting of 5 in the untreated group yet 0 in the ERT group (Table in Figure 21). Chi-Square was significant ($p \leq 0.05$), indicating ERT decreased the severity of the acquired bone defects. Because severely affected infantile HPP patients often die from undermineralized and fractured ribs incapable of supporting respiration, the thoraces were also closely examined. ERT also reduced the incidence of severely dysmorphic rib cages (Table in Figure 21). Chi-Square analysis was significant at $p \leq 0.025$. Similarly, the hind limbs appeared healthy in all treated animals (Table in Figure 21). Chi-Square analysis was significant at $p \leq 0.025$.

Dental defects

[00169] Mandibles from 16-day-old mice were immersion-fixed overnight in sodium cacodylatebuffered aldehyde solution and cut into segments containing the first molar, the underlying incisor, and the surrounding alveolar bone. Samples were dehydrated through a graded ethanolseries and infiltrated with either acrylic (LR White) or epoxy (Epon 812) resin, followed by polymerization of the tissue-containing resin blocks at 55 °C for 2 days. Thin sections (1 μm) were cut on an

ultramicrotome using a diamond knife, and glass slide-mounted sections were stained for mineral using 1% silver nitrate (von Kossa staining, black) and counterstained with 1% toluidine blue. Frontal sections through the mandibles (at the same level of the most mesial root of the first molar) provided longitudinally sectioned molar and cross-sectioned incisor for comparative histological analyses.

[00170] Histological examination of teeth from $Akp2^{-/-}$ mice, shows poorly mineralized dentin tissue and very little cementum between the periodontal ligament and the dentin as compared to wild-type animals (Figure 22, compare $Akp2^{-/-}$ Vehicle and WT-Normal). Restored dentin mineralization and the formation of the cementum is also shown in Figure 22 ($Akp2^{-/-}$ Treated vs. WT-Normal).

EXAMPLE 11

Long-term (52 days) efficacy of high doses (8.2 mg/kg) of sTNALP-FcD10 for HPP in $Akp2^{-/-}$ mice

[00171] Finally, to assess long-term survival and bone mineralization in $Akp2^{-/-}$ mice, either sTNALP-FcD10 (8.2 mg/kg) or vehicle was given daily for 52 days (s.c. injections).

Mice survival, activity and appearance

[00172] Untreated mice had a median survival of 18.5 days (Figure 23) whereas survival was dramatically increased with ERT and this treatment also preserved the normal activity and healthy appearance (Figure 24) of the treated mice.

Bone mineralization

[00173] Radiographs of the feet of 16 day-old $Akp2^{-/-}$ mice showed secondary ossification defects that are a hallmark of the disease (see Figure 25). These defects were prevented in all treated mice by daily doses of sTNALP-FcD10

for 46 or 53 days (Figure 25).

ALP activity

[00174] Plasma ALP activity levels were measured in treated $Akp2^{-/-}$ mice after 53 days. Figure 26 shows that most of the values were between 1 and 4 µg/ml of ALP activity. Three animals, however, had undetectable ALP levels.

[00175] Interestingly, unlike WT mice where a steady-state serum concentration of sTNALP-FcD10 was achievable, great variability in the serum levels of ALP was measured in the treated $Akp2^{-/-}$ mice.

EXAMPLE 12

Long-term efficacy of different dosage intervals of sTNALP-FcD10 in $Akp2^{-/-}$ mice

[00176] Newborn $Akp2^{-/-}$ mice were injected with 4.3 mg/kg daily (Tx-1), 15.2 mg/kg every 3 days (Tx-3) or 15.2 mg/kg every 7 days (Tx-7) of sTNALP-FcD10. Treatment was pursued for 43 days and mice were sacrificed on day 44, namely 24 hours after the last injection. They were monitored to evaluate any improvement of their survival and skeletal mineralization.

Mice survival

[00177] The survival of treated mice was increased compared to the mice that were injected vehicle (Figure 27). This increase was statistically significant ($p<0.0001$).There was no statistically significant difference when the survival curves of treated groups were compared between themselves.

Bone mineralization

[00178] A) For each treatment, the radiographs of the feet were analyzed

and distributed between normal and abnormal. Numbers and percentages (in parentheses) appear in Table 4 below. The bone mineralization defects were evaluated at day 23 and at the end of the study (Day 23-45).

[00179] **Table 4: Distribution of radiographs of feet**

Mid-Study (D23)		
Group	**Abnormal (%)**	**Normal (%)**
Tx-1 (N=18)	6 (33)	12 (67)
Tx-3 (N=19)	4 (21)	15 (79)
Tx-7 (N=20)	10 (50)	10 (50)
WT (N=32)	0 (0)	32 (100)

B)

D23-45		
Group	**Abnormal (%)**	**Normal (%)**
Tx-1 (N=18)	3 (17)	15 (83)
Tx-3 (N=19)	0 (0)	19 (100)
Tx-7 (N=20)	3 (15)	17(85)
WT (N=31)	0 (0)	31 (100)

[00180] At mid study, sTNALP-FcD10 administered at 15.2 mg/kg every 3 days normalized bone mineralization defects in 79% of mice. This rate of normalization approached statistical significance when compared to the 50% rate of normalization evaluated in the mice treated with 15.2 mg/kg every 7 days (Chi Square; p= 0.0596). No other inter treatment comparisons were statistically significant or approached significance.

[00181] At end of study, the percent of normalization improved in all treated groups compared to the percent normalization evaluated at day 23. The Chi Square test comparing the distribution among all sTNALP-FcD10 treatments was not significant (p=0.1844). The 100% rate of normalization observed in the mice treated with every 3 days approached statistical significance when compared to the rate in mice treated daily (83%, p=0.0634) or every 7 days (85%, p=0.0789).

[00182] However, in all treatment groups a significant proportion of the animals classified as abnormal at day 23 improved and became normal at end of the study. In the daily treatment group, 3 out of 6 animals normalized; in the mice treated every 3 days, 4 out of 4 improved, and finally in the weekly treatment group, 7 out of 10 became normal. Although dosage intervals provide satisfying results, the best results were obtained when the resulting daily amount administered was the highest.

EXAMPLE 13

Long-term efficacy of high doses (8.2 mg/kg) of sTNALP-FcD10 in 15 day old $Akp2^{-/-}$ mice

[00183] Efficacy studies as described in Example 11 were conducted in 15 day old mice which have started to manifest skeletal defects as observed on X-ray pictures of feet (see Example 11, Figure 25). sTNALP-FcD10 was administered until the end of the study. The animals were monitored to evaluate any improvement of their survival, body weight and skeletal mineralization.

Mice survival

[00184] Daily injections, starting at day 15, of 8.2 mg/kg sTNALP-FcD10 to $Akp2^{-/-}$ mice increased their survival compared to the mice that were injected vehicle (Figure 28). This increase was statistically significant ($p<0.05$).

Body weight

[00185] At the start of the study, no significant difference in body weight was noticed between groups (Figure 29). At the beginning of treatment (day 15), the body weight of $Akp2^{-/-}$ mice was smaller than that of wild-type animals. While the body weight of animals injected vehicle continued to decrease, $Akp2^{-/-}$ mice treated with sTNALP-FcD10 started to gain weight 4 to 5 days after initiation of treatment and kept gaining weight until the end of the study, without however

reaching the values of the wild-type animals. This weight gain suggests improvement in the well being of the animals treated with sTNALP-FcD10.

Bone mineralization

[00186] For each treatment, the radiographs of the feet were analyzed and distributed between normal and abnormal. Numbers and percentages (in parentheses) appear in Table 5. The radiographs were taken at necropsy.

[00187] Treatment of $Akp2^{-/-}$ mice with 8.2 mg/kg sTNALP-FcD10 daily, starting at day 15 after birth improved mineralization as seen from the radiography of the feet taken at necropsy. The sTNALP-FcD10-treated group showed 41% normal animals compared to 12% in vehicle-injected group of $Akp2^{-/-}$ mice. This difference almost reached statistical significance (p= 0.0645 in Chi square test).

[00188] **Table 5: Distribution of radiographs of feet**

Group	Abnormal	Normal
RVehicle (N=16)	14 (88)	2 (12)
RTx-1 (N=17)	10 (59)	7 (41)
WT (N=30)	0 (0)	30 (100)

EXAMPLE 14

Long-term efficacy of different dosage intervals of sTNALP-FcD10 on the rescue of *Akp2*$^{-/-}$ mice

[00189] Mice were initiated on the treatment at day 12 and injected s.c. with vehicle (RV), 8.2 mg/Kg daily to days 46/47 (RTx-1) or injected with 8.2.mg/Kg daily for 7 days followed by 24.6 mg/Kg every 3 day (RTx-3) or followed by 57.4 mg/Kg every 7 days (RTx-7). The median survival was 19.5 days for the RV mice,

21.0 days for the RTx-7 mice, 30.5 days for the RTx-3 mice and 37.5 days for the RTx-1 mice. In all cases, survival was statistically increased when compared to that of the vehicle-treated group. There is a clear benefit of ERT in *Akp2*$^{-/-}$ mice with well-established hypophosphatasia. Dosing less frequently than daily also appears to statistically increase survival.

EXAMPLE 15

A Maximum Tolerated Dose Intravenous Injection Toxicity Study in Juvenile Sprague-Dawley Rats

[00190] The objective of the study was to determine the maximum tolerated dose (MTD) and toxicity of the test article, sTNALP-FcD10, following repeated administration to juvenile Sprague-Dawley rats by intravenous injection. In Examples 15 to 18, the sALP-FcD10 used is that specifically described in Figure 3.

[00191] sTNALP-FcD10 was administered to juvenile Sprague-Dawley rats (aged at initiation between 22 and 24 days) once weekly for four weeks by intravenous injection as described in Table 6 below:

[00192] **Table 6: Study design**

Group Numbers	Treatment	Dose Level (mg/kg/occasion)	Dose Concentration (mg/mL)	Number of Animals	
				Male	Female
1	Dose 1	10	2.0	3	3
2	Dose 2	30	6.0	3	3
3	Dose 3	90	18.0	3	3
4	Dose 4	180	36.0	3	3

[00193] Throughout the study, the animals were monitored for mortality, body weight, and clinical condition. Hematology, coagulation and clinical chemistry assessments were performed on all animals. Terminally, the rats were euthanized and subjected to necropsy. For each animal, samples of selected tissues were retained and were subjected to histological processing and

microscopic examination.

[00194] There was no mortality in this study and there were no test article-related changes in coagulation parameters or organ weights. The body weights of the High Dose males, in particular, were about 10% below the Low Dose suggesting a treatment-related effect.

[00195] No clinical signs were observed in Groups 1 and 2 animals on the first dosing occasion. In Groups 3 and 4 animals, however, the animals appeared weak immediately following dosing and some Group 4 animals showed slight to moderate decrease in activity. Slight swelling of limbs, pinna and muzzle with skin discoloration (red or blue in appearance) at the extremities were also observed in the two groups. Other clinical signs observed in Group 4 animals included excessive scratching, piloerection and hyperpnea.

[00196] Clinical signs recorded for Groups 1 and 2 animals on the second dosing occasion (Day 8), were swelling of limbs, pinna and muzzle with skin discoloration (red or blue in appearance) at the extremities. Similar clinical signs of skin swelling were also recorded on the third and fourth dosing occasions (Days 15 and 22) for the same groups of animals. On the fourth dosing occasion (Day 22) slight hyperactivity was observed in Group 1 females whereas hypoactivity was observed in Group 2 males. For Group 3 and 4 animals, the clinical signs of reduced motor activity, piloerection, hyperpnea and swelling of limbs, pinna and muzzle with skin coloration became more evident as dosing progressed from the first dosing occasion to the fourth. It is considered that these clinical signs were treatment-related. On Days 16 to 19 and on Day 23, slight swelling and skin coloration of pinna (red in appearance) were observed in one animal (Group 1). Similar clinical signs were observed in another animal (Group 2) on Day 23.

[00197] The clinical signs were acute and the severity increased as dosing progressed but they were transient. All clinical signs appeared within 50 minutes after administration of test article, sTNALP-FcD10, with some animals recovering within, approximately, thirty minutes to 2 hours. For other animals, recovery was complete the next morning (the next scheduled observation time).

[00198] There was a treatment-related decrease in platelet counts (PLT) for males and females from all treatment groups, measured after the last dose, compared to background values. There was an increase in predominantly the percentage but also absolute reticulocytes that was noted generally in animals treated at the three highest dose levels.

[00199] Levels of alkaline phosphatase in serum were higher than could be quantified by the analytical instrument even following dilution. The results that were available for the Low Dose females were dramatically higher than the background range. This was expected as the test article is an active modified ALP.

[00200] Macroscopically, dark focus/area and/or depressed area of the glandular stomach were observed in 3 of 6 Group 3 animals (2 males/1 female) and 4 of 6 Group 4 animals (2 males/2 females).

[00201] Microscopically, minimal to mild erosion/ulcer of the glandular stomach, occasionally associated with submucosal edema was noted in 3 of 6 Group 3 animals (2 males/1 females) and 4 of 6 Group 4 animals (2 males/2 females), correlating gross findings.

[00202] In conclusion, intravenous injection of sTNALP-FcD10 to juvenile Sprague-Dawley rats once weekly for 4 weeks did not cause death at any of the dose levels tested but did cause adverse clinical signs, minor

haematological changes and erosion/ulceration of the glandular stomach, occasionally associated with submucosal edema at dose levels of 90 and 180 mg/kg.

[00203] Changes related to administration of the test article at the two lowest dose levels tested (10 and 30 mg/kg) were limited to transient clinical signs apparent on the day of dosing only. The clinical signs were more severe at the 90 mg/kg dose level but they were also transient. The clinical signs noted in the animals treated with 180 mg/kg were so severe as to prevent this dose level from being used in future studies. Consequently the highest recommended dose level for subsequent longer term studies is 90 mg/kg.

EXAMPLE 16

An Intravenous Injection and Infusion Maximum Tolerated Dose Toxicity Study in Juvenile Cynomolgus Monkeys

[00204] The purpose of this study was to determine the maximum tolerated dose for sTNALP-FcD10, when administered once by intravenous injection or infusion to juvenile Cynomolgus monkeys. The test article dosing formulations were administered once in an incremental fashion, as indicated in Table 7 below.

[00205] **Table 7: Study design**

* Only the Main Study animals were dosed on Day 46.

Study Day	Treatment	Dose Level (mg/kg)	Dose Volume (mL/kg)	Dose Rate (mL/kg/hr)	Dose Conc. (mg/mL)	Number of Animals			
						Main Study		Toxicokinetic	
						Males	Females	Males	Females
1	IV Injection	5	4	N/A	1.25	2	2	1	1
8	IV Injection	15	4	N/A	3.75				
15	IV Infusion	45	4	80	11.25				
22	IV Infusion	90	4	40	22.5				
29	IV Infusion	180	4	20	45				
46*	IV Injection	45	4	N/A	11.25				

[00206] After the last treatment, the animals were released from the

study. Parameters monitored during the study were mortality, clinical observations, body weights, appetence, toxicokinetics, hematology and clinical chemistry.

[00207] No mortality, adverse clinical signs or effect on body weights were observed during the study.

[00208] A marked dose proportional increase in alkaline phosphatase was observed in all animals throughout the study. Since the test article was a synthetic alkaline phosphatase, this increase was principally due to the presence of the drug in the bloodstream of the animals after each dosing.

[00209] Increases in alanine aminotransferase and aspartate aminotransferase were observed in three animals during the study but in the absence of a necropsy, the toxicological significance of this finding is uncertain.

[00210] The pharmacokinetic of sTNALP-FcD10 was well characterized following a single IV administration of 5, 15, 45, 90 and 180 mg/kg to monkeys. For the IV injections mean AUC∞ values ranged from 797 to 2950 mg•h/L and mean Cmax values ranged from 65 to 396 mg/L over the dose range studied. For the infusions, mean AUC∞ ranged from 9410 to 48400 mg•h/L and Cmax ranged from 1230 to 7720 mg/L over the dose range studied.

[00211] Mean t1/2 values of sTNALP-FcD10 appeared to decrease with increasing dose levels of sTNALP-FcD10. Although systemic clearance of sTNALP-FcD10 was relatively consistent across dose levels, the 90 mg/kg dose group appeared to be a pharmacokinetic outlier with a substantially lower clearance when compared to the other dose levels (approximately five fold). No obvious gender related trends were noted.

[00212] In summary, although some reversible blood chemistry changes were observed during the study, the intravenous injection/infusion of sTNALP-FcD10 at up to 180 mg/kg was well tolerated by the juvenile Cynomolgus monkeys. Therefore, under the conditions of this study, the Maximum Tolerated Dose was considered to be at least 180 mg/kg.

EXAMPLE 17

A 4-Week (Once Weekly) Intravenous Injection Toxicity Study of sTNALP-FcD10 in the Juvenile Albino Rat Followed by a 28-Day Recovery Period

[00213] The objective of this study was to investigate the potential toxicity of sTNALP-FcD10 given once weekly by intravenous injection to the juvenile rat for a minimum of 4 consecutive weeks (total of 4 doses) followed by 28 days of recovery. The animals were dosed on study days 1, 8, 15 and 22 and the recovery period began on study day 29. The study design is detailed in Table 8 below.

[00214] **Table 8: Study design**

					Main Study		Recovery Study	
Groups	Target Dose Level (mg/kg/dose)	Actual Dose Level (mg/kg/dose)	Target Concentration (mg/mL)	Actual Concentration (mg/mL)	Males	Females	Males	Females
1- Vehicle Control	0	0	0	0	10	10	5	5
2- Low Dose	3	2.5	0.6	0.5	10	10	5	5
3- Mid Dose	30	26	6	5.1	10	10	5	5
4- High Dose	90	77	18	15.3	10	10	5	5

[00215] The following were evaluated: clinical signs (twice daily), body weight (once during acclimation period and weekly starting on Day 21 *post partum*), food consumption (weekly), ophthalmology (end of treatment and end of recovery period), hematology (at necropsy), serum chemistry (at necropsy), urinalysis (Day 29 and at the end of recovery period), biochemical markers of bone

turnover: osteocalcin (bone formation marker) and C-telopeptide (bone resorption marker) (the morning prior to schedule necropsy), antibody assessment (Day -1 and at necropsy), test article blood concentration evaluation (Day 16 and Day 23), bone densitometry (by DXA *in vivo* Day -1, 28-main and recovery study animals and Day 14 and 56-recovey study animals and pQCT *ex vivo*), radiography and macroscopic observations at necropsy, organ weights and histopathology.

[00216] One male given 90 mg/kg/dose was found dead on study Day 25. As no consequential histological observations (pulmonary and thymic hemorrhages graded slight and minimal, respectively) were made for this rat, its cause of death was undetermined based on pathological investigations. On Day 23, this animal was bled for test article blood concentration evaluation and this procedure may have contributed to its death since there was no evidence of toxicity on Day 22. There were no sTNALP-FcD10-related mortality or effects on ophthalmology, urinalysis, bone formation marker (osteocalcin), organ weights, gross pathology, radiology or microscopy examination.

[00217] sTNALP-FcD10-related clinical signs observed at 3, 30 and/or 90 mg/kg/dose groups are considered to be acute infusion reaction. These included partly closed eyes, decreased muscle tone, lying on the side, hunched posture, cold to touch, uncoordinated movements, decreased activity, abnormal gait and/or blue, red and/or firm swollen hindpaws and/or forepaws during cage-side observations at 5, 15, 30 and/or 60 minutes post dose. These observations were transient and did not occur on nondosing days or during the recovery period.

[00218] Generally, a trend for slightly decreased body weight and body weight gain was noted for males in the 3, 30 and/or 90 mg/kg/dose groups during the recovery period. The effect on bone size on two bones of appendicular skeleton (femur and at tibia) correlated with decreased body weights. Decreases in food consumption were generally consistent with the decreased body weights.

Body weights were comparable to controls for sTNALP-FcD10-treated females.

[00219] sTNALP-FcD10 administered at 90 mg/kg/dose was generally associated with slight decreases in absolute neutrophils, monocytes and/or eosinophils compared to the control group. Additionally, slight increases in lymphocytes, platelets and absolute reticulocytes were observed compared to the control group. At the end of the recovery period, these slight changes were still apparent in the animals treated with 90 mg/kg.

[00220] sTNALP-FcD10 was generally associated with statistically significant dose-related increases in alkaline phosphatase in all treated groups compared to controls. Considering the nature of the test article (alkaline phosphatase), the absence of any changes in other liver enzymes and absence of histopathological correlates, these increases are likely attributed to circulating levels of sTNALP-FcD10. Slight statistically significant increases in phosphorus were observed in males treated with sTNALP-FcD10 at 90 mg/kg/dose during Week 4, associated with a non-significant increases in serum total calcium. At the end of the recovery, these changes, including those statistically significant, returned to control values.

[00221] There were no organ weight, radiological, macroscopic or microscopic changes that were related to sTNALP-FcD10 in juvenile rats treated intravenously once weekly at up to 90 mg/kg/dose for 4 consecutive weeks. There were no delayed effects identified in a subset of these animals allowed a 28-day recovery after completion of the treatment.

[00222] Slightly lower mean CTx values were observed for treated females compared to controls (attaining statistical significance at 90 mg/kg/dose). These lower values were not consistent with the bone density analysis and also with the results obtained for males, therefore the incidental nature for these

decreases cannot be excluded.

[00223] High variability in bone densitometry and bone geometry parameters noted between groups was attributed to the rapid growth phase. At the end of recovery, area and BMC (assessed by DXA and pQCT) were generally lower for treated males, suggesting smaller bones for these animals. The effect on bone size was noted on two bones of appendicular skeleton (femur and at tibia) by two different techniques, however no consistent effect was noted for axial skeleton (suggesting no effect on crown to rump length). Although area and BMC were decreased, the mean BMD values were generally comparable to controls, suggesting the effect on BMC and area was secondary to the effect on growth. Lower body weights and lower food consumption for treated males relative to controls are consistent with these data. However small group size at recovery, lack of consistency with respect to gender as well as the variability confounded these results, therefore an incidental nature for these decreases cannot be completely excluded.

[00224] In conclusion, once weekly intravenous injection to the juvenile rat for a minimum of 4 consecutive weeks followed by 28-day of recovery at doses of 3, 30 and 90 mg/kg/dose resulted in clinical signs associated with transient injection related effects including uncoordinated and reduced activity and paw swelling observed up to 60 minutes post-dose. Males treated at 90 mg/kg/dose showed slight decreases in body weight and food consumption which correlated with slightly smaller tibiae and femurs assessed by densitometry techniques. For females, slightly lower mean values were obtained for C-telopeptide levels compared to controls. Serum phosphorus levels were slightly, although significantly, increased in the 90 mg/kg/dose group. Elevated serum alkaline phosphatase levels were likely attributed to circulating levels of sTNALP-FcD10. sTNALP-FcD10 had no meaningful or consistent effects on bone densitometry and bone geometry for females during treatment and recovery period. For males no

biologically significant effects were noted on bone densitometry or bone geometry during the treatment period. In general, slight decreases in bone densitometry (bone mineral content and/or area assessed by DXA and pQCT) and bone geometry parameters with a corresponding lower mean body weight were noted for males relative to controls at the end of the recovery period. All findings resolved after a 28-day treatment-free period with the exception of the effects on body weight and bone size for high dose males which persisted. There were no evidence of ectopic calcification at the end of treatment or the end of the recovery period. There were no radiological, macroscopic or microscopic findings as well as any organ weight changes associated with sTNALP-FcD10 treatment at any dose level. Because the injection reaction was transient and did not result in any effect on any parameters used to assess toxicity in the 3 and 30 mg/kg/dose groups, it was not considered to be adverse. In the 90 mg/kg/dose group, this reaction was more severe and accompanied by decreases in body weight gain, reduced food consumption, and potentially decrease in bone growth and therefore the effects in this group were considered to be adverse. Consequently, the no observable adverse effect level (NOAEL) was considered to be 30 mg/kg/dose in this study.

EXAMPLE 18

A 4-Week Intravenous Injection Toxicity Study in Juvenile Cynomolgus Monkeys Followed by a 28-Day Recovery Period

[00225] The purpose of this study was to determine the toxicity and toxicokinetics of sTNALP-FcD10 in juvenile Cynomolgus monkeys, when administered once weekly by slow bolus intravenous injection for 4 weeks and to assess reversibility of any changes following a 28-day recovery period.

[00226] The control and test article dosing formulations were administered to juvenile Cynomolgus monkeys by slow intravenous bolus injection once weekly for 4 weeks followed by a 28-day recovery period, as indicated in the Table 9 below:

[00227] **Table 9: Study design**

Group	Dose Level (mg/kg)	Dose Volume (mL/kg)	Dose Conc. (mg/mL)	Number of Animals			
				Main Study		Recovery	
				Males	Females	Males	Females
1 Control *	0	4	0	3	3	2	2
2 Low Dose	5	4	1.25	3	3	2	2
3 Mid Dose	15	4	3.75	3	3	2	2
4 High Dose	45	4	11.25	3	3	2	2

* The Group 1 animals received the vehicle/control article, 25 mM sodium phosphate pH 7.4, 150 mM NaCl.

[00228] After the last treatment (Day 22), the Main Study animals were euthanized on Day 29, while the remaining Recovery animals were observed for an additional 28 days, following which they were euthanized on Day 57. All Main and Recovery animals were subjected to a necropsy examination.

[00229] Evaluations conducted during the study or at its conclusion included mortality, clinical condition, body weight, appetence, body measurements, radiographic assessments of bone development, ophthalmology, electrocardiography, toxicokinetics, immunogenicity, hematology, coagulation, clinical chemistry, urinalysis, biomarkers of bone turnover, organ weights, ex-vivo bone mineral density analyses, and gross and histopathology.

[00230] No mortality or adverse treatment-related clinical observations were noted during the study.

[00231] Based on the body measurements recorded at the end of the treatment and recovery period, there were no noteworthy inter-group differences for cranial circumference, or humerus, forearm, tibia or pelvic limb lengths.

[00232] There were no body weight or food consumption changes related to treatment with the test article at any dose level. There were no ophthalmological or electrocardographic findings related to the test article at any

dose level. There were no haematological, red cell morphological, coagulation or urinalysis changes related to treatment with the test article at any dose level. There were no toxicologically significant changes among clinical biochemistry parameters during the treatment or recovery periods. A slight to pronounced dose related increase in alkaline phosphatase was observed in all test article treated animals at most assessment occasions throughout the treatment period. Alkaline phosphatase levels were generally more comparable to control values by the end of the recovery period. Since the test article is a synthetic alkaline phosphatase, this increase was principally due to the presence of the drug in the bloodstream of the animals after each dose, and thus the increases were considered to be non-adverse.

[00233] At the end of the treatment and recovery periods, there were no noteworthy inter-group differences in absolute or relative organ weights, nor were there any test article-related macroscopic or microscopic findings. Histological changes noted were considered to be either incidental findings, common background findings in this species, or findings related to some aspect of experimental manipulation. Reproductive organs were generally immature but considered normal for this age monkey.

[00234] In conclusion, weekly intravenous injection of sTNALP-FcD10, to male and female Cynomolgus monkeys for 4 weeks, at dose levels of 0, 5, 15 and 45 mg/kg, and followed by a 4-week recovery period, was without evidence of toxicity at any dose level. Therefore the high dose level, 45 mg/kg, was considered to be the No Observed Adverse Effect Level (NOAEL) in this study.

EXAMPLE 19

Determination of Maximum Recommended Starting Dose for Human

[00235] The maximum recommended starting dose (MRSD) for human

is calculated by establishing the No Observed Adverse Effect Level (NOAEL, see Guidance for Industry and Reviewers. December 2002). Various concentrations of the formulation described above have been tested on mice, rat and monkeys including 1 mg/kg, 5 mg/kg, and 8.2 mg/kg daily subcutaneously; 3 mg/kg, 5 mg/kg, 10 mg/kg, 30 mg/kg, 45 mg/kg, 90 mg/kg and 180 mg/kg. The NOAEL for the most sensitive species, namely for rat, was 30 mg/kg.

[00236] This dose was scaled up to a human equivalent dose (HED) using published conversion tables which provide a conversion factor from rat to human of 6. A NOAEL of 30 mg/kg for that species is equivalent to 5 mg/kg in human.

[00237] This value (5 mg/kg) was divided by a security factor of ten. The calculated MRSD is thus 0.5 mg/kg. For an average human weighting 60 kg, a weekly dose of 30 mg or daily dose of 4.28 mg daily could thus be injected to start clinical trials.

[00238] Although the present invention has been described hereinabove by way of specific embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims.

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CLAIMS:

1. A bone targeted alkaline phosphatase comprising a polypeptide having the structure :

Z-sALP-Y-spacer-X-W_n-V,

wherein sALP is the extracellular domain of the alkaline phosphatase;

wherein V is absent or is an amino acid sequence of at least one amino acid;

X is absent or is an amino acid sequence of at least one amino acid;

Y is absent or is an amino acid sequence of at least one amino acid;

Z is absent or is an amino acid sequence of at least one amino acid; and

W_n is a polyaspartate or a polyglutamate wherein n = 10 to 16.

2. The alkaline phosphatase of claim 1, wherein the sALP comprises amino acid residues 23-508 of SEQ ID NO: 15.

3. The alkaline phosphatase of claim 2, wherein the sALP consists of amino acid residues 23-512 of SEQ ID NO: 15.

4. The alkaline phosphatase of claim 2, wherein the sALP comprises amino acid residues 23-508 of SEQ ID NO: 18.

5. The alkaline phosphatase of claim 2, wherein the sALP consists of amino acid residues 23-512 of SEQ ID NO: 18.

6. The alkaline phosphatase of claim 2, wherein the sALP comprises amino acid residues 18-498 of SEQ ID NO: 16.

7. The alkaline phosphatase of claim 6, wherein the sALP consists of amino acid residues 18-502 of SEQ ID NO: 16.

8. The alkaline phosphatase of claim 6, wherein the sALP comprises amino acid residues 18-498 of SEQ ID NO: 19.

9. The alkaline phosphatase of claim 8, wherein the sALP consists of amino acid residues 18-502 of SEQ ID NO: 19.

10. The alkaline phosphatase of claim 8, wherein the sALP comprises amino acid residues 18-498 of SEQ ID NO: 19.

11. The alkaline phosphatase of claim 10, wherein the sALP consists of amino acid residues 18-502 of SEQ ID NO: 19.

12. The alkaline phosphatase of claim 10, wherein the sALP comprises amino acid residues 18-498 of SEQ ID NO: 8.

13. The alkaline phosphatase of claim 12, wherein the sALP consists of amino acid residues 18-502 of SEQ ID NO: 8.

14. The alkaline phosphatase of any one of claims 1 to 13, wherein the spacer comprises a fragment crystallizable region (Fc).

15. The alkaline phosphatase of claim 14, wherein the Fc comprises a CH2 domain, a CH3 domain and a hinge region.

16. The alkaline phosphatase of claim 14 or 15, wherein the Fc is a constant domain of an immunoglobulin selected from the group consisting of IgG-1, IgG-2, IgG-3, IgG-3 and IgG-4.

17. The alkaline phosphatase of claim 16, wherein the Fc is a constant domain of an immunoglobulin IgG-1.

18. The alkaline phosphatase of any one of claims 14 to 17, wherein the Fc is as set forth in SEQ ID NO: 3.

19. The alkaline phosphatase of any one of claims 1 to 18, wherein W_n is a polyaspartate.

20. The alkaline phosphatase of any one of claims 1 to 19, wherein n=10.

21. The alkaline phosphatase of any one of claims 1 to 20, wherein Z is absent.

22. The alkaline phosphatase of any one of claims 1 to 21, wherein Y is two amino acid residues.

23. The alkaline phosphatase of claim 22, wherein Y is leucine-lysine.

24. The alkaline phosphatase of any one of claims 1 to 23, wherein X is 2 amino acid residues.

25. The alkaline phosphatase of claim 24, wherein X is aspartate-isoleucine.

26. The alkaline phosphatase of any one of claims 1 to 24, wherein V is absent.

27. The alkaline phosphatase of claim 1, wherein the polypeptide is as set forth in SEQ ID NO: 4.

28. The alkaline phosphatase of any one of claims 1-27, comprising the polypeptide in a form comprising a dimer.

29. The alkaline phosphatase of any one of claims 1-28, comprising the polypeptide in a form of a tetramer.

30. The alkaline phosphatase of any one of claims 1 to 29, in a pharmaceutically acceptable carrier.

31. The alkaline phosphatase of claim 30, wherein the pharmaceutically acceptable carrier is a saline.

32. The alkaline phosphatase of claim 31, in a lyophilized form.

33. The alkaline phosphatase of any one of claims 1-32, in a daily dosage of about 0.2 to about 20 mg/kg.

34. The alkaline phosphatase of any one of claims 1-32, in a dosage of about 0.6 to about 60 mg/kg for administration every three days.

35. The alkaline phosphatase of any one of claims 1-32, in a weekly dosage of about 1.4 to about 140 mg/kg.

36. The alkaline phosphatase any one of claims 1-32, in a weekly dosage of about 0.5 mg/kg.

37. An isolated nucleic acid comprising a sequence that encodes the polypeptide defined in any one of claims 1-27.

38. An isolated nucleic acid consisting of a sequence that encodes the polypeptide defined in any one of claims 1-27.

39. An isolated nucleic acid comprising a sequence as set forth in SEQ ID NO: 17.

40. A recombinant expression vector comprising the nucleic acid of any one of claims 37-39.

41. A recombinant adeno-associated virus vector comprising the nucleic acid of any one of claims 37-39.

42. An isolated recombinant host cell transformed or transfected with the vector of claim 40 or 41.

43. A method of producing the alkaline phosphatase of any one of claims 1-27, comprising culturing the host cell of claim 42, under conditions suitable to effect expression of the alkaline phosphatase and recovering the alkaline phosphatase from the culture medium.

44. The method of claim 43, wherein the host cell is a L cell, C127 cell, 3T3 cell, CHO cell, BHK cell, COS-7 cell or a Chinese Hamster Ovary (CHO) cell.

45. The method of claim 44, wherein the host cell is a Chinese Hamster Ovary (CHO) cell.

46. The method of claim 45, wherein the host cell is a CHO-DG44 cell.

47. A kit comprising the alkaline phosphatase defined in any one of claims 1 to 27, and instructions to administer the polypeptide to a subject to correct or prevent a hypophosphatasia (HPP) phenotype.

48. A kit comprising the alkaline phosphatase defined in any one of claims 1 to 27, and instructions to administer the polypeptide to a subject to correct or prevent aplasia, hypoplasia or dysplasia of dental cementum.

49. A method of using the alkaline phosphatase of any one of claims 1 to 36, for correcting or preventing at least one hypophosphatasia (HPP) phenotype, comprising administering a therapeutically effective amount of the alkaline phosphatase to a subject in need thereof, whereby the at least one HPP phenotype is corrected or prevented in the subject.

50. The method of claim 49, wherein the subject has at least one HPP phenotype.

51. The method of claim 49, wherein the subject is likely to develop at least one HPP phenotype.

52. The method of any one of claims 49 to 51, wherein the at least one HPP phenotype comprises HPP-related seizure.

53. The method of any one of claims 49 to 51, wherein the at least one HPP phenotype comprises premature loss of deciduous teeth.

54. The method of any one of claims 49 to 51, wherein the at least one HPP phenotype comprises incomplete bone mineralization.

55. The method of any one of claims 49 to 51, wherein incomplete bone mineralization is incomplete femoral bone mineralization.

56. The method of claim 55 wherein incomplete bone mineralization is incomplete tibial bone mineralization.

57. The method of claim 55, wherein incomplete bone mineralization is incomplete metatarsal bone mineralization.

58. The method of claim 55, wherein incomplete bone mineralization is incomplete ribs bone mineralization.

59. The method of any one of claims 49 to 51, wherein the at least one HPP phenotype comprises elevated blood and/or urine levels of inorganic pyrophosphate (PP_i).

60. The method of any one of claims 49 to 51, wherein the at least one HPP phenotype comprises elevated blood and/or urine levels of phosphoethanolamine (PEA).

61. The method of any one of claims 49 to 51, wherein the at least one HPP phenotype comprises elevated blood and/or urine levels of pyridoxal 5'-phosphate (PLP).

62. The method of any one of claims 49 to 51, wherein the at least one HPP phenotype comprises inadequate weight gain.

63. The method of any one of claims 49 to 51, wherein the at least one HPP phenotype comprises rickets.

64. The method of any one of claims 49 to 51, wherein the at least one HPP phenotype comprises bone pain.

65. The method of any one of claims 49 to 51, wherein the at least one HPP phenotype comprises calcium pyrophosphate dihydrate crystal deposition.

66. The method of any one of claims 49 to 51, wherein the at least one HPP phenotype comprises aplasia, hypoplasia or dysplasia of dental cementum.

67. The method of any one of claims 49 to 51, wherein the subject in need thereof has infantile HPP.

68. The method of any one of claims 49 to 66, wherein the subject in need thereof has childhood HPP.

69. The method of any one of claims 49 to 51, wherein the subject in need thereof has perinatal HPP.

70. The method of any one of claims 49 to 66, wherein the subject in need thereof has adult HPP.

71. The method of any one of claims 49 to 51, wherein the subject in need thereof has odontohypophosphatasia HPP.

72. A method of using the alkaline phosphatase of any one of claims 1 to 36, for correcting or preventing aplasia, hypoplasia or dysplasia of dental cementum, comprising administering a therapeutically effective amount of the alkaline phosphatase to a subject in need thereof, whereby aplasia, hypoplasia or dysplasia of dental cementum is corrected or prevented in the subject.

73. The method of any one of claims 49 to 72, wherein the administering comprises transfecting a cell in the subject with a nucleic acid encoding the alkaline phosphatase.

74. The method of claim 73, wherein the transfecting the cell is performed *in vitro* such that the alkaline phosphatase is expressed and secreted in an active form and administered to the subject with said cell.

75. The method of any one of claims 49 to 72, wherein the administering comprises subcutaneous administration of the alkaline phosphatase to the subject.

76. The method of any one of claims 49 to 70, wherein the administering comprises intravenous administration of the alkaline phosphatase to the subject.

77. The alkaline phosphatase of any one of claim 1-27, for use in correcting or preventing at least one HPP phenotype.

78. The alkaline phosphatase of any one of claim 1-27, for use in correcting or preventing aplasia, hypoplasia or dysplasia of dental cementum.

79. A use of the alkaline phosphatase of any one of claim 1-27, in the making of a medicament.

80. A use of the alkaline phosphatase of any one of claim 1-27, for correcting or preventing at least one HPP phenotype.

81. A use of the alkaline phosphatase of any one of claim 1-27, for correcting or preventing aplasia, hypoplasia or dysplasia of dental cementum.

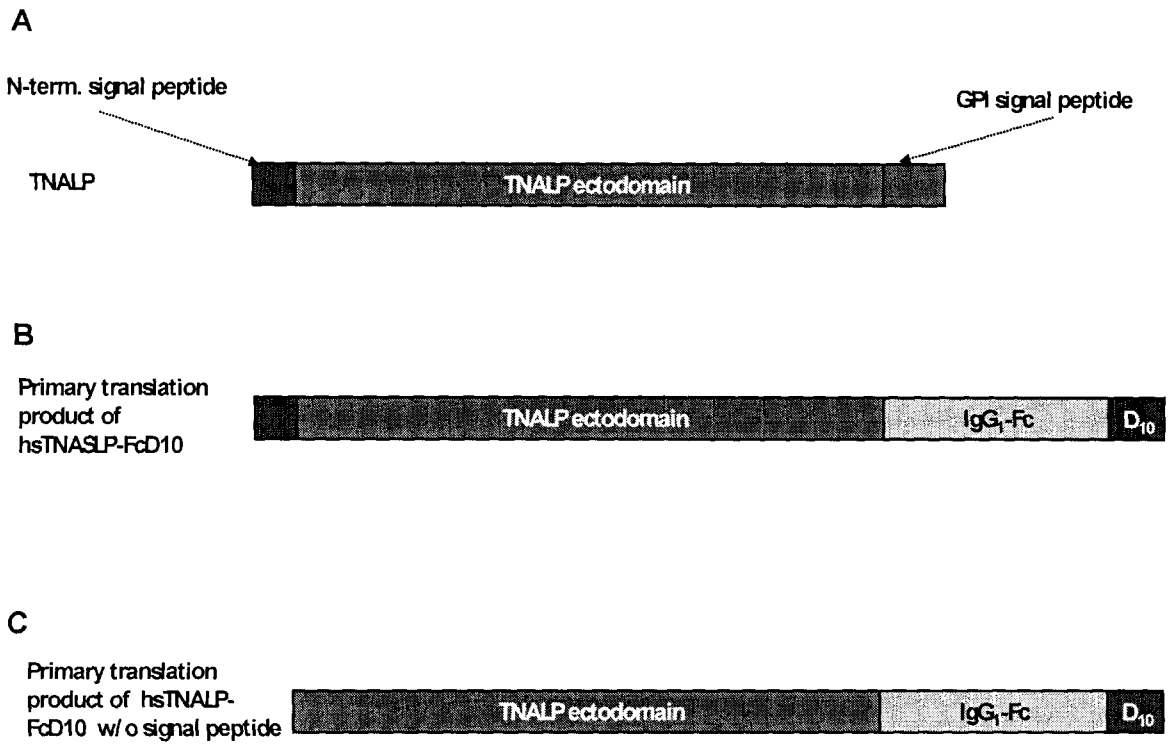

A
N-term. signal peptide
GPI signal peptide
TNALP
TNALP ectodomain
B
Primary translation product of hsTNASLP-FcD10
TNALP ectodomain
IgG1-Fc
D10
C
Primary translation product of hsTNALP-FcD10 w/o signal peptide
TNALP ectodomain
IgG1-Fc
D10

Figure 1

Protein sequence for sTNALP-FcD$_{10}$ with the peptide signal.

MISPFLVLAIGTCLTNSLVPEKEKDPKYWRDQAQETLKYALELQKLNTNVAKNVIMFLGDGMGVS
TVTAARILKGQLHHNPGEETRLEMDKFPFVALSKTYNTNAQVPDSAGTATAYLCGVKANEGTVGV
SAATERSRCNTTQGNEVTSILRWAKDAGKSVGIVTTTRVNHATPSAAYAHSADRDWYSDNEMPPE
ALSQGCKDIAYQLMHNIRDIDVIMGGGRKYMYPKNKTDVEYESDEKARGTRLDGLDLVDTWKSFK
PRYKHSHFIWNRTELLTLDPHNVDYLLGLFEPGDMQYELNRNNVTDPSLSEMVVVAIQILRKNPK
GFFLLVEGGRIDHGHHEGKAKQALHEAVEMDRAIGQAGSLTSSEDTLTVVTADHSHVFTFGGYTP
RGNSIFGLAPMLSDTDKKPFTAILYGNGPGYKVVGGERENVSMVDYAHNNYQAQSAVPLRHETHG
GEDVAVFSKGPMAHLLHGVHEQNYVPHVMAYAACIGANLGHCAPASSLKDKTHTCPPCPAPELLG
GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYR
VVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLT
CLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEA
LHNHYTQKSLSLSPGKDIDDDDDDDDDD

```
        10         20         30         40         50         60
MISPFLVLAI GTCLTNSLVP EKEKDPKYWR DQAQETLKYA LELQKLNTNV AKNVIMFLGD

        70         80         90        100        110        120
GMGVSTVTAA RILKGQLHHN PGEETRLEMD KFPFVALSKT YNTNAQVPDS AGTATAYLCG

       130        140        150        160        170        180
VKANEGTVGV SAATERSRCN TTQGNEVTSI LRWAKDAGKS VGIVTTTRVN HATPSAAYAH

       190        200        210        220        230        240
SADRDWYSDN EMPPEALSQG CKDIAYQLMH NIRDIDVIMG GGRKYMYPKN KTDVEYESDE

       250        260        270        280        290        300
KARGTRLDGL DLVDTWKSFK PRYKHSHFIW NRTELLTLDP HNVDYLLGLF EPGDMQYELN

       310        320        330        340        350        360
RNNVTDPSLS EMVVVAIQIL RKNPKGFFLL VEGGRIDHGH HEGKAKQALH EAVEMDRAIG

       370        380        390        400        410        420
QAGSLTSSED TLTVVTADHS HVFTFGGYTP RGNSIFGLAP MLSDTDKKPF TAILYGNGPG

       430        440        450        460        470        480
YKVVGGEREN VSMVDYAHNN YQAQSAVPLR HETHGGEDVA VFSKGPMAHL LHGVHEQNYV

       490        500        510        520        530        540
PHVMAYAACI GANLGHCAPA SSLKDKTHTC PPCPAPELLG GPSVFLFPPK PKDTLMISRT

       550        560        570        580        590        600
PEVTCVVVDV SHEDPEVKFN WYVDGVEVHN AKTKPREEQY NSTYRVVSVL TVLHQDWLNG

       610        620        630        640        650        660
KEYKCKVSNK ALPAPIEKTI SKAKGQPREP QVYTLPPSRE EMTKNQVSLT CLVKGFYPSD

       670        680        690        700        710        720
IAVEWESNGQ PENNYKTTPP VLDSDGSFFL YSKLTVDKSR WQQGNVFSCS VMHEALHNHY

       730        740
TQKSLSLSPG KDIDDDDDDD DDD
```

Figure 2

Protein sequence for sTNALP-FcD_{10} without the peptide signal.

LVPEKEKDPKYWRDQAQETLKYALELQKLNTNVAKNVIMFL
GDGMGVSTVTAARILKGQLHHNPGEETRLEMDKFPFVALSKTYNTNAQVPDSAGTATAYL
CGVKANEGTVGVSAATERSRCNTTQGNEVTSILRWAKDAGKSVGIVTTTRVNHATPSAAY
AHSADRDWYSDNEMPPEALSQGCKDIAYQLMHNIRDIDVIMGGGRKYMYPKNKTDVEYES
DEKARGTRLDGLDLVDTWKSFKPRYKHSHFIWNRTELLTLDPHNVDYLLGLFEPGDMQYE
LNRNNVTDPSLSEMVVVAIQILRKNPKGFFLLVEGGRIDHGHHEGKAKQALHEAVEMDRA
IGQAGSLTSSEDTLTVVTADHSHVFTFGGYTPRGNSIFGLAPMLSDTDKKPFTAILYGNG
PGYKVVGGERENVSMVDYAHNNYQAQSAVPLRHETHGGEDVAVFSKGPMAHLLHGVHEQN
*YVPHVMAYAACIGANLGHCAPASSLK*DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMIS
RTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWL
NGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYP
SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN
HYTQKSLSLSPGKDIDDDDDDDDDD

10	20	30	40	50	60
LVPEKEKDPK	YWRDQAQETL	KYALELQKLN	TNVAKNVIMF	LGDGMGVSTV	TAARILKGQL
70	80	90	100	110	120
HHNPGEETRL	EMDKFPFVAL	SKTYNTNAQV	PDSAGTATAY	LCGVKANEGT	VGVSAATERS
130	140	150	160	170	180
RCNTTQGNEV	TSILRWAKDA	GKSVGIVTTT	RVNHATPSAA	YAHSADRDWY	SDNEMPPEAL
190	200	210	220	230	240
SQGCKDIAYQ	LMHNIRDIDV	IMGGGRKYMY	PKNKTDVEYE	SDEKARGTRL	DGLDLVDTWK
250	260	270	280	290	300
SFKPRYKHSH	FIWNRTELLT	LDPHNVDYLL	GLFEPGDMQY	ELNRNNVTDP	SLSEMVVVAI
310	320	330	340	350	360
QILRKNPKGF	FLLVEGGRID	HGHHEGKAKQ	ALHEAVEMDR	AIGQAGSLTS	SEDTLTVVTA
370	380	390	400	410	420
DHSHVFTFGG	YTPRGNSIFG	LAPMLSDTDK	KPFTAILYGN	GPGYKVVGGE	RENVSMVDYA
430	440	450	460	470	480
HNNYQAQSAV	PLRHETHGGE	DVAVFSKGPM	AHLLHGVHEQ	NYVPHVMAYA	ACIGANLGHC
490	500	510	520	530	540
APASSLKDKT	HTCPPCPAPE	LLGGPSVFLF	PPKPKDTLMI	SRTPEVTCVV	VDVSHEDPEV
550	560	570	580	590	600
KFNWYVDGVE	VHNAKTKPRE	EQYNSTYRVV	SVLTVLHQDW	LNGKEYKCKV	SNKALPAPIE
610	620	630	640	650	660
KTISKAKGQP	REPQVYTLPP	SREEMTKNQV	SLTCLVKGFY	PSDIAVEWES	NGQPENNYKT
670	680	690	700	710	720
TPPVLDSDGS	FFLYSKLTVD	KSRWQQGNVF	SCSVMHEALH	NHYTQKSLSL	SPGKDIDDDD

DDDDDD

Figure 3

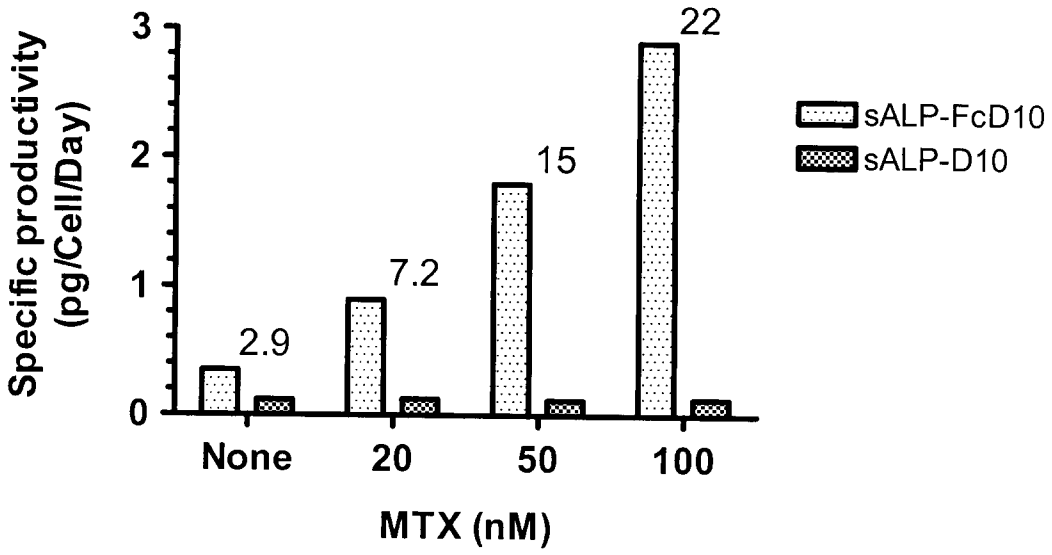

Specific productivity (pg/Cell/Day)
3
2
1
0
2.9
7.2
15
22
None
20
50
100
MTX (nM)
sALP-FcD10
sALP-D10

Figure 4

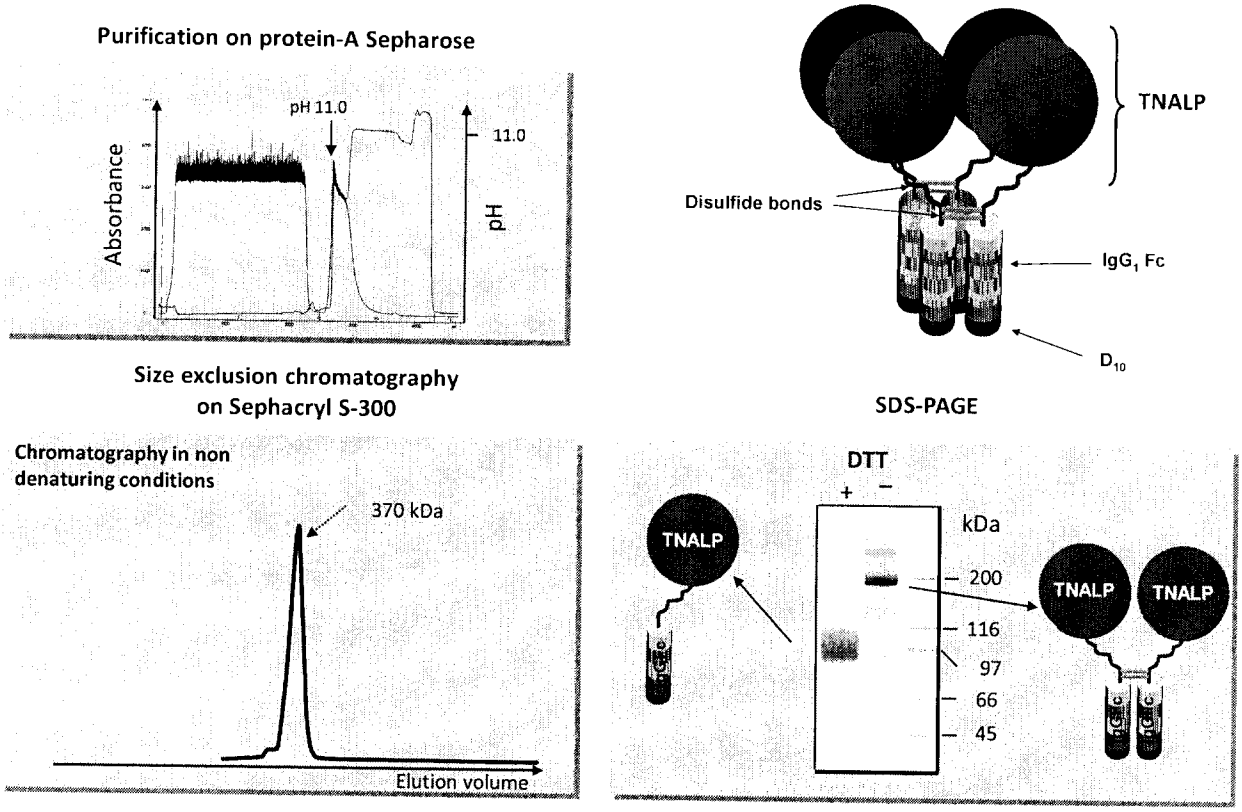

Purification on protein-A Sepharose
pH 11.0
11.0
Absorbance
pH
TNALP
Disulfide bonds
IgG1 Fc
D10
Size exclusion chromatography
on Sephacryl S-300
SDS-PAGE
Chromatography in non
denaturing conditions
370 kDa
Elution volume
DTT
+
−
kDa
200
116
97
66
45
TNALP
TNALP
TNALP

Figure 5

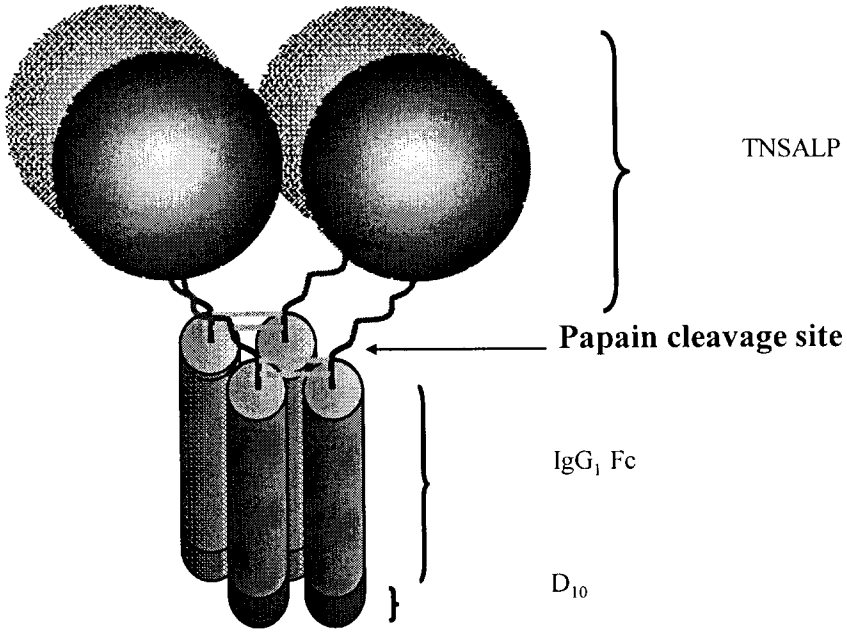


Figure 6

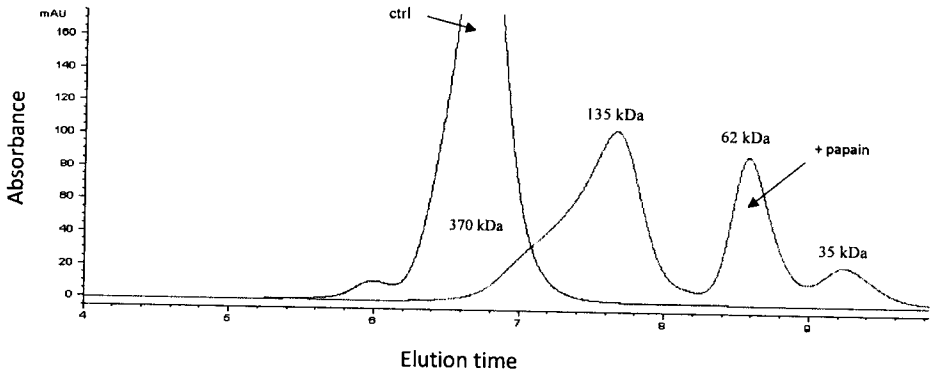

mAU
160
140
120
100
80
60
40
20
0
Absorbance
ctrl
135 kDa
62 kDa
+ papain
370 kDa
35 kDa
4
5
6
7
8
9
Elution time

Figure 7

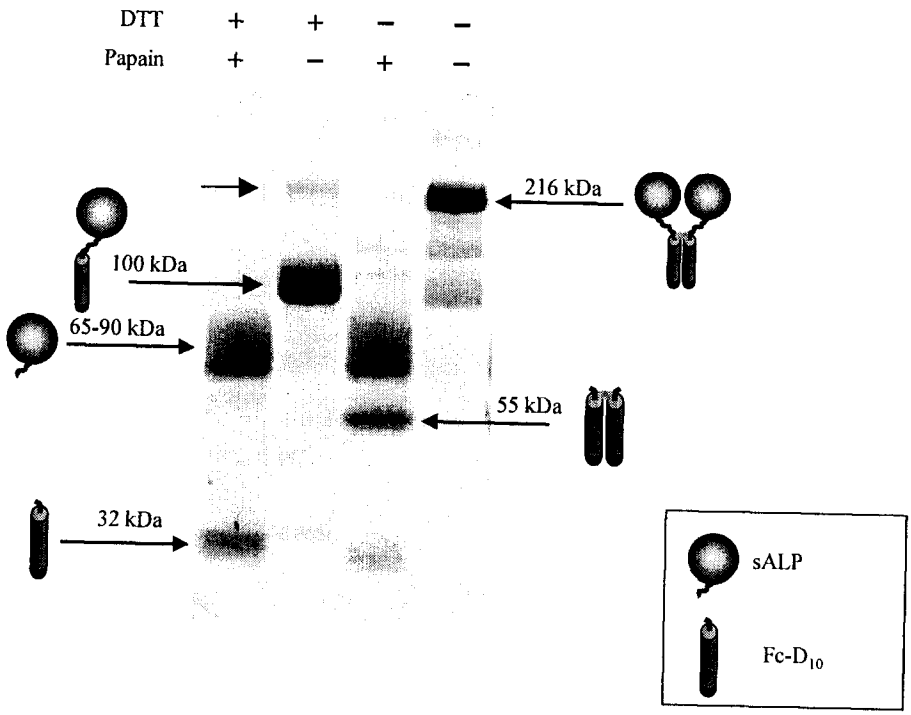


Figure 8

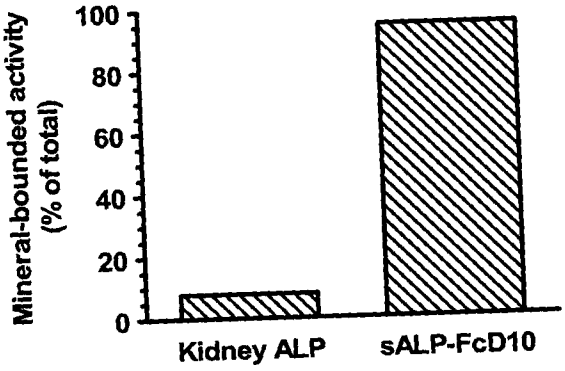

Mineral-bounded activity
(% of total)
100
80
60
40
20
0
Kidney ALP
sALP-FcD10

Figure 9

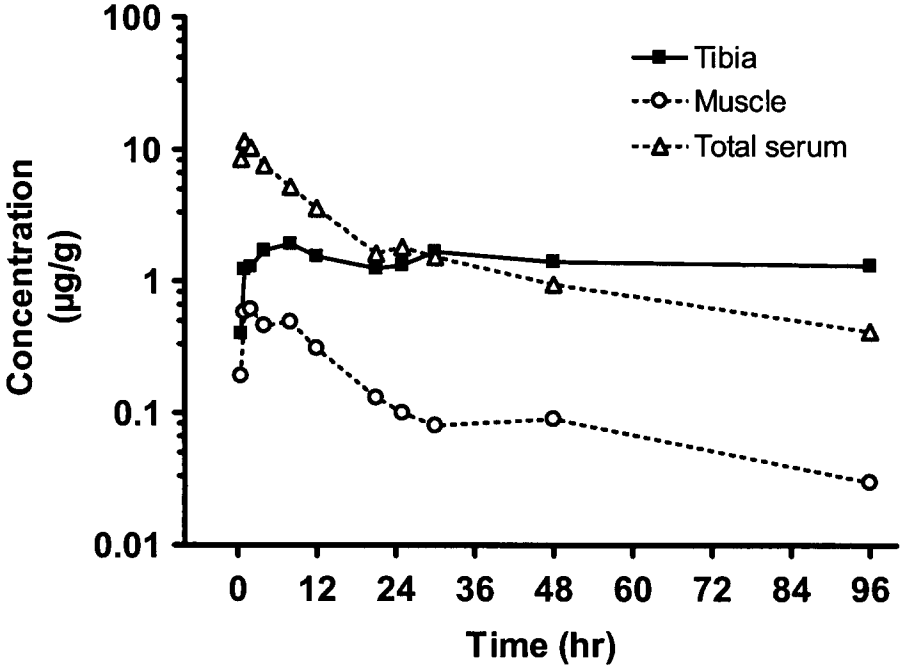

100
10
1
0.1
0.01
Concentration (µg/g)
0
12
24
36
48
60
72
84
96
Time (hr)
Tibia
Muscle
Total serum

Figure 10

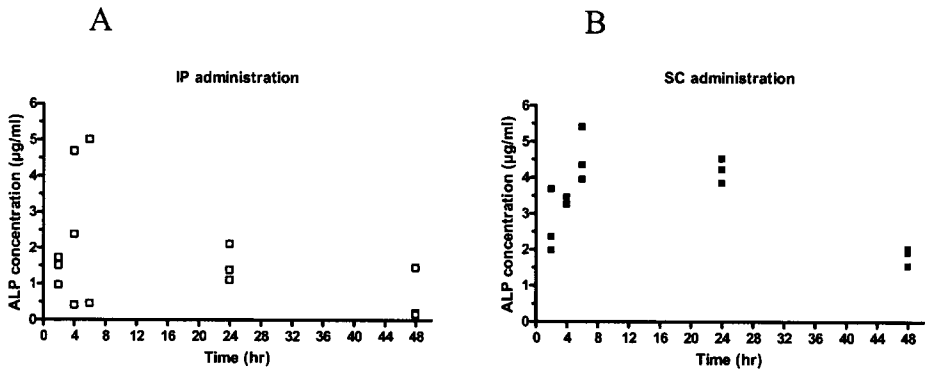

A
IP administration
ALP concentration (µg/ml)
Time (hr)
B
SC administration
ALP concentration (µg/ml)
Time (hr)

Figure 11

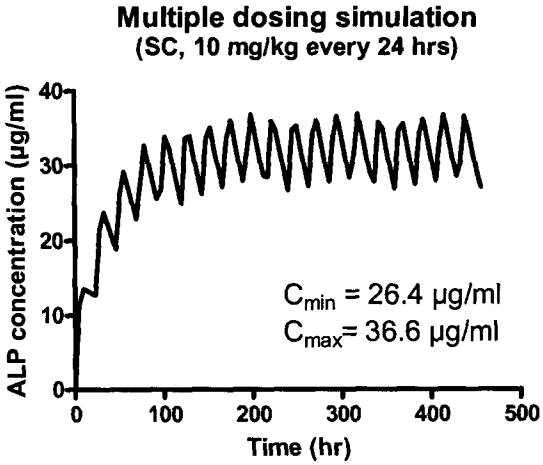

Multiple dosing simulation
(SC, 10 mg/kg every 24 hrs)
ALP concentration (µg/ml)
40
30
20
10
0
0
100
200
300
400
500
Time (hr)
C_{min} = 26.4 µg/ml
C_{max}= 36.6 µg/ml

Figure 12

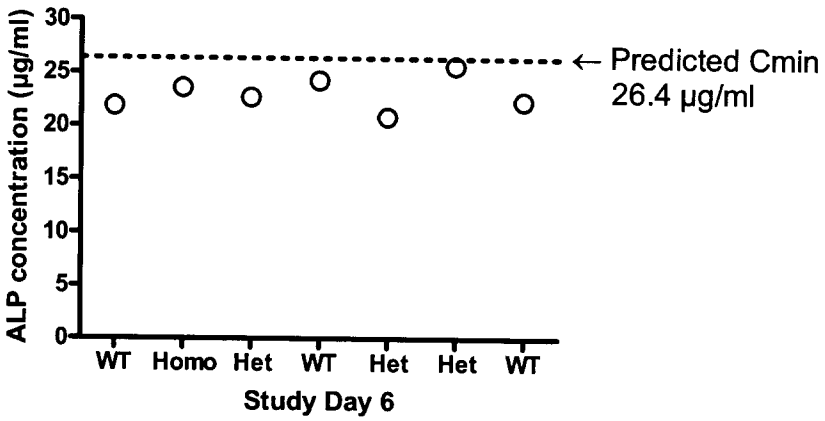

ALP concentration (µg/ml)
30
25
20
15
10
5
0
WT
Homo
Het
WT
Het
Het
WT
Study Day 6
← Predicted Cmin
26.4 µg/ml

Figure 13

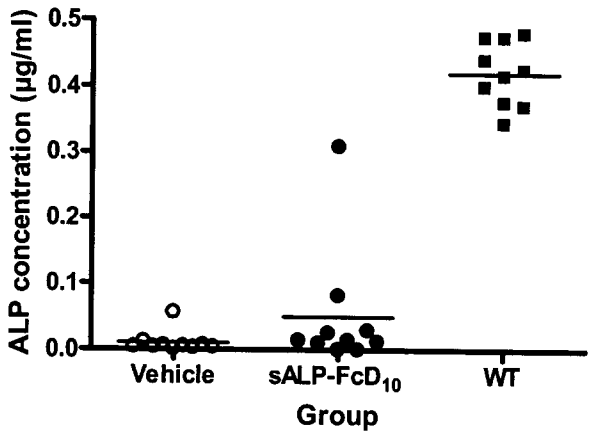

ALP concentration (µg/ml)
0.5
0.4
0.3
0.2
0.1
0.0
Vehicle
sALP-FcD10
WT
Group

Figure 14

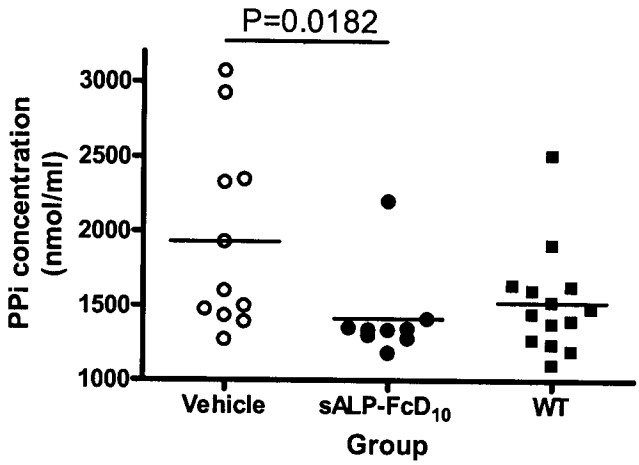

P=0.0182
PPi concentration (nmol/ml)
3000
2500
2000
1500
1000
Vehicle
sALP-FcD10
WT
Group

Figure 15

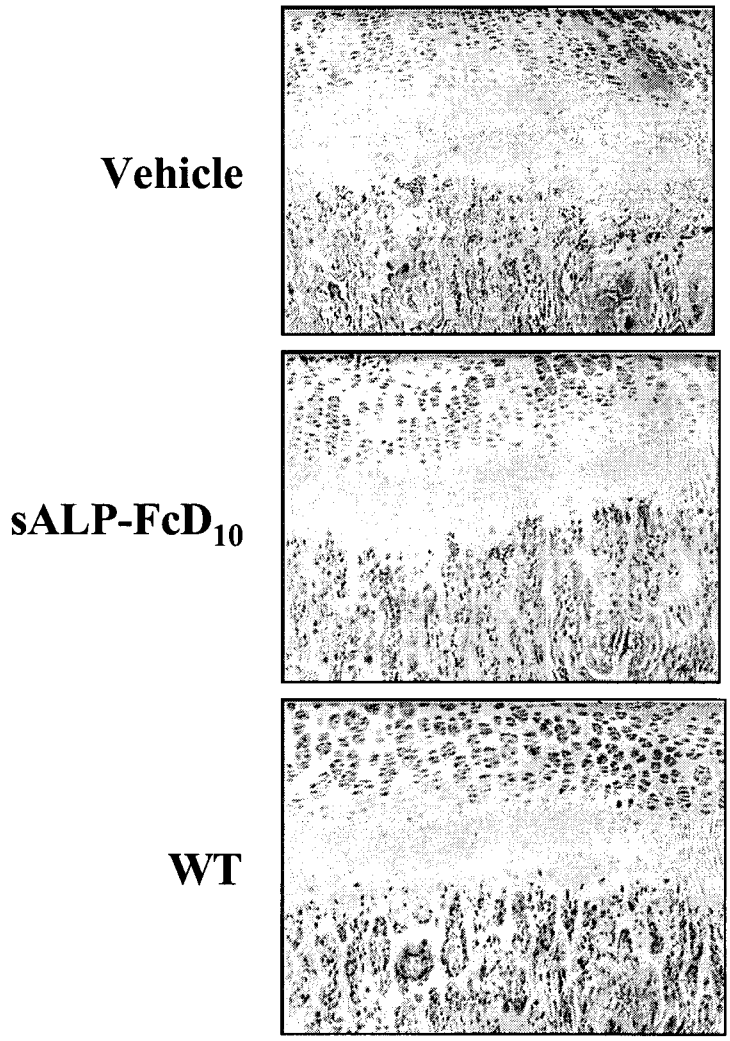


Figure 16

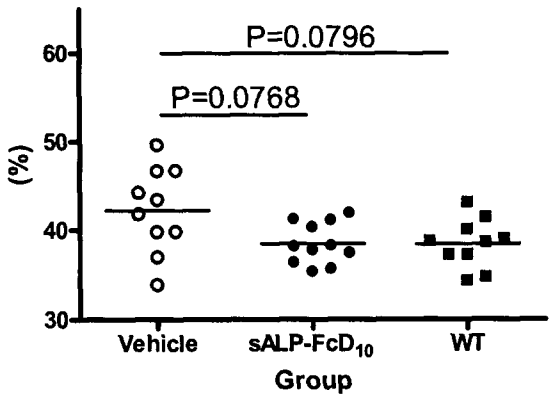


Figure 17

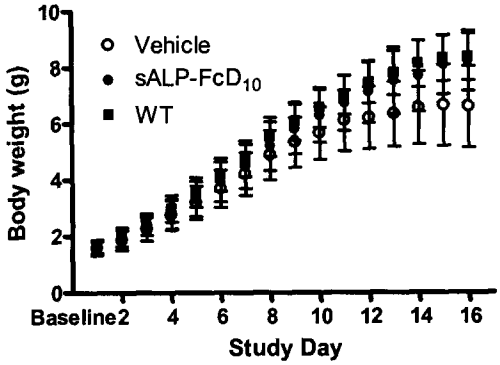

Vehicle
sALP-FcD10
WT
Body weight (g)
0
2
4
6
8
10
Baseline
2
4
6
8
10
12
14
16
Study Day

Figure 18

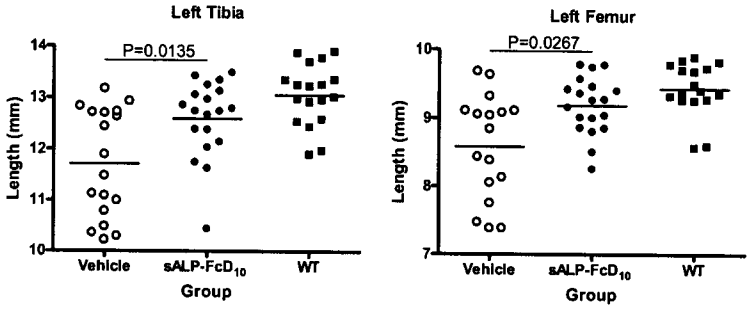

Left Tibia
P=0.0135
Length (mm)
14
13
12
11
10
Vehicle
sALP-FcD10
WT
Group
Left Femur
P=0.0267
Length (mm)
10
9
8
7
Vehicle
sALP-FcD10
WT
Group

Figure 19

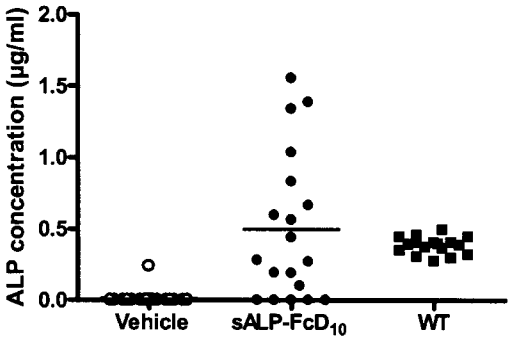
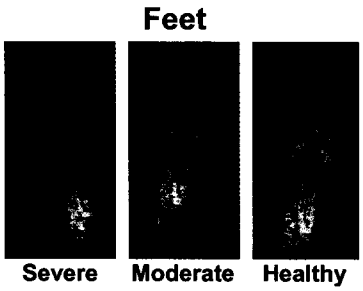
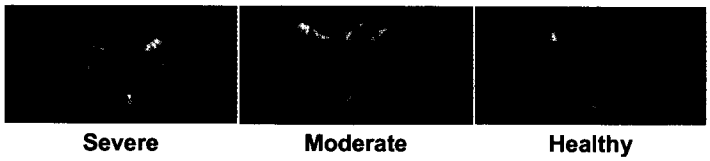


Figure 20



		Vehicle	sALP-FcD$_{10}$	WT
Feet	Healthy	3	2	16
	Moderate	10	17	2
	Severe	5	0	0
Rib Cages	Healthy	1	2	17
	Moderate	11	17	1
	Severe	6	0	0
Legs	Healthy	9	16	18
	Abnormal	9	0	0



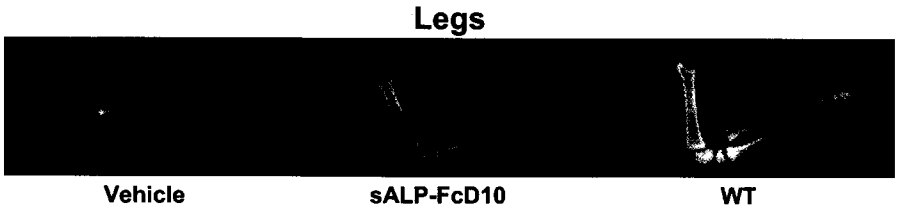


Figure 21

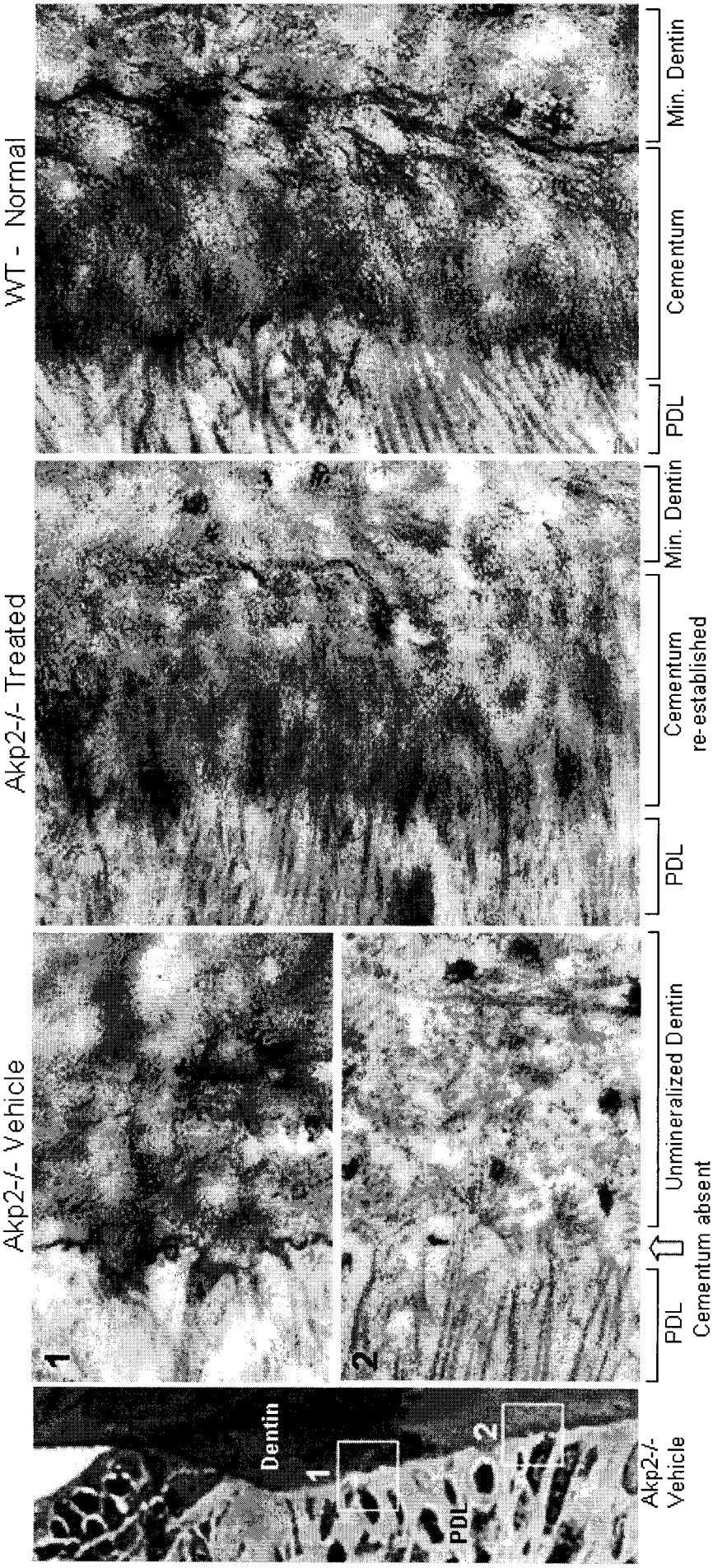

Akp2-/- Vehicle
Akp2-/- Treated
WT - Normal
Dentin
PDL
1
2
Akp2-/- Vehicle
PDL
Unmineralized Dentin
Cementum absent
PDL
Cementum re-established
Min. Dentin
PDL
Cementum
Min. Dentin

Figure 22

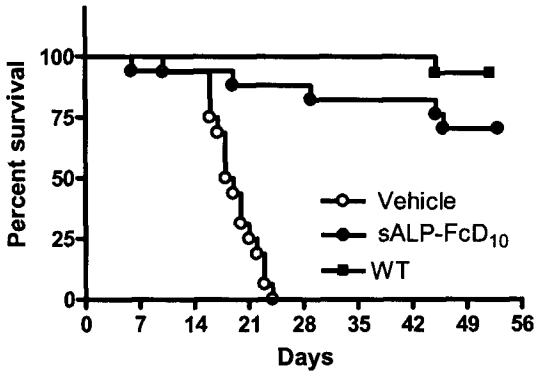

Percent survival
100
75
50
25
0
0
7
14
21
28
35
42
49
56
Days
Vehicle
sALP-FcD10
WT

Figure 23

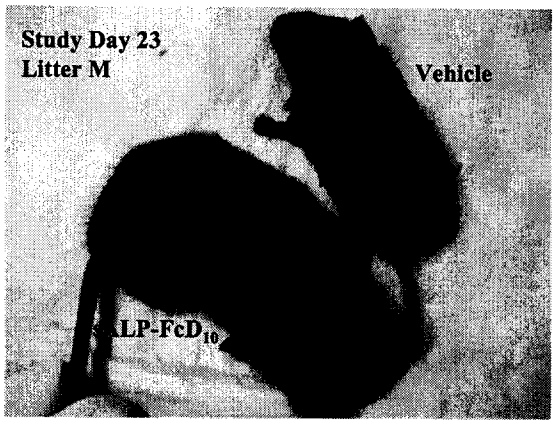


Figure 24

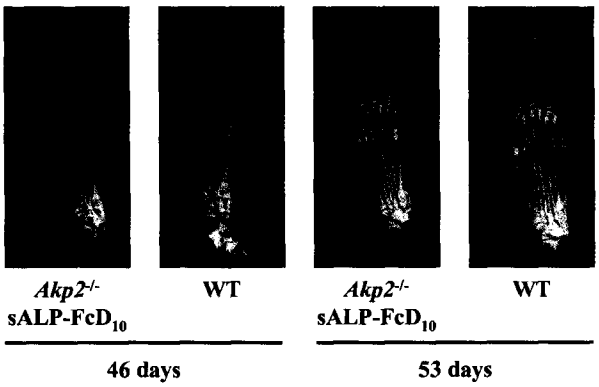


Figure 25

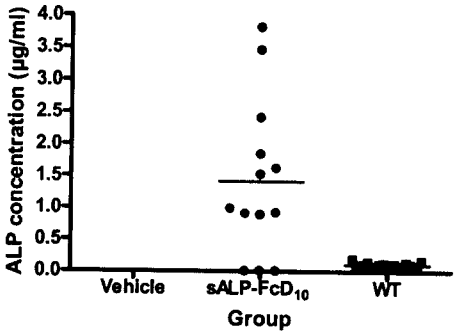
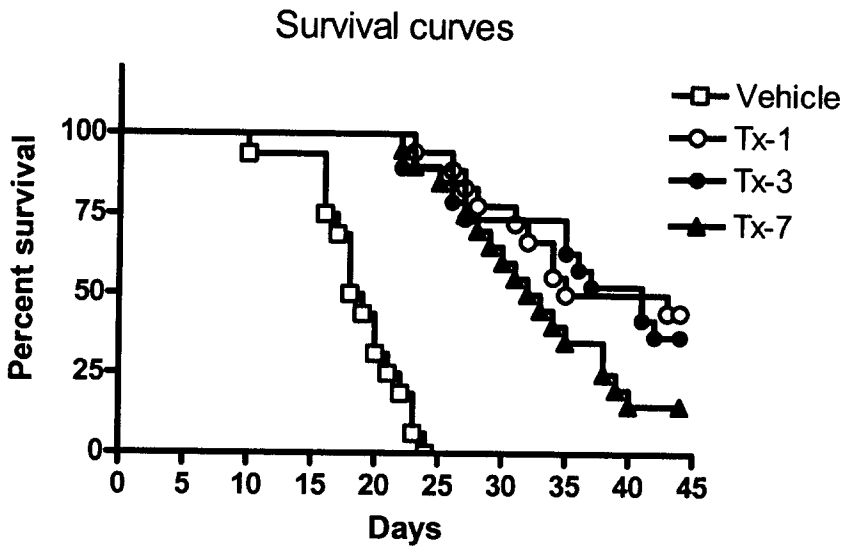

ALP concentration (µg/ml)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Vehicle
sALP-FcD10
WT
Group

Figure 26

A

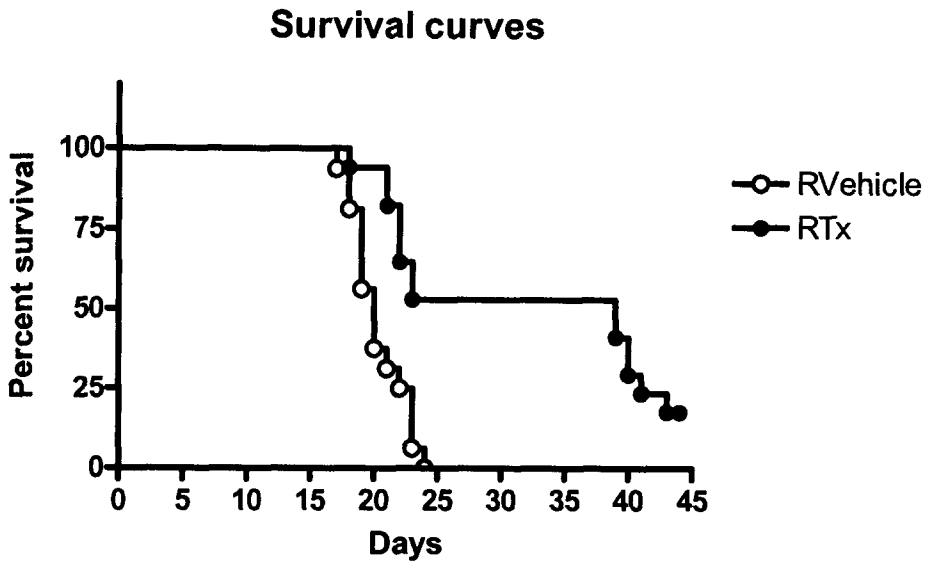


B

Group	Median Survival (Day)
Vehicle	18.5
Tx-1	39
Tx-3	41
Tx-7	32.5

Figure 27

A



B

Group	Median Survival (Day)
Rvehicle	20
RTx	39

Figure 28

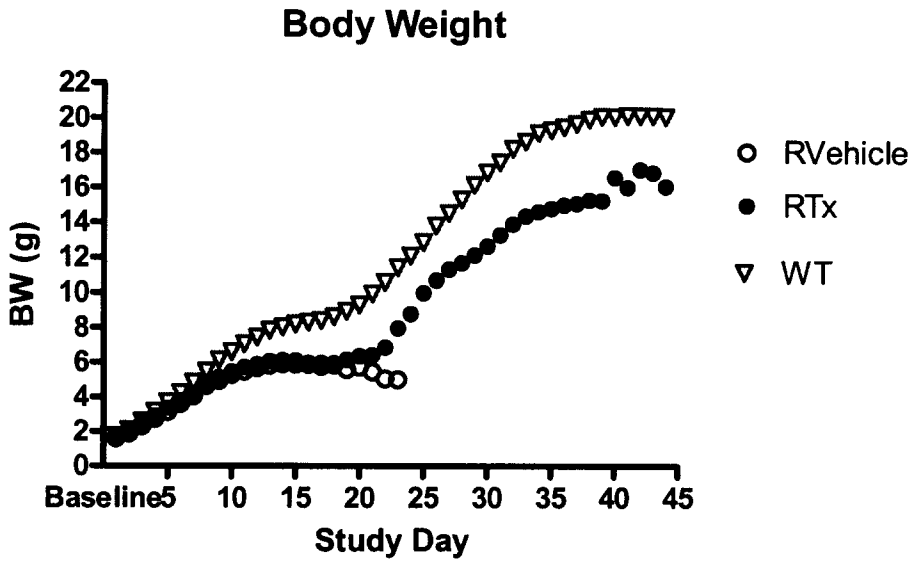

Body Weight
BW (g)
22
20
18
16
14
12
10
8
6
4
2
0
Baseline5
10
15
20
25
30
35
40
45
Study Day
RVehicle
RTx
WT

Figure 29

```
CLUSTAL 2.0.5 multiple sequence alignment

TNALPrn         MILP-----FLVLAIGTCLTNSFVPEKEKDPSYWRQQAQETLKNALKLQKLNTNVAKNII 55
TNALPmm         MISP-----FLVLAIGTCLTNSFVPEKERDPSYWRQQAQETLKNALKLQKLNTNVAKNVI 55
TNALPhs         MISP-----FLVLAIGTCLTNSLVPEKEKDPKYWRDQAQETLKYALELQKLNTNVAKNVI 55
TNALPcf         -------------------------EKDPKYWRDQAQQTLKYALRLQNLNTNVAKNVI 33
TNALPfc         MISP-----FLVLAIGTCLTNSLVPEKEKDPKYWRDQAQQTLKNALRLQKLNTNVVKNVI 55
TNALPbt         MISP-----FLLLAIGTCFASSLVPEKEKDPKYWRDQAQQTLKNALRLQTLNTNVAKNVI 55
GALPhs          MQGPWV---LLLLGLRLQLSLGIIPVEEENPDFWNRQAAEALGAAKKLQPAQT-AAKNLI 56
PLALPhs         MLGPCMLLLLLLLLGLRLQLSLGIIPVEEENPDFWNREAAEALGAAKKLQPAQT-AAKNLI 59
IALPhs          MQGPWV---LLLLGLRLQLSLGVIPAEEENPAFWNRQAAEALDAAKKLQPIQK-VAKNLI 56
                                          *.:* :*. :* ::*  * .**  :. ..**:*
Consensus       XXXXXXXXXXXXXXXXXXXXXXXXXXXEXXPXXWXXXAXXXLXXAXXLQXXXXXXXKNXI


TNALPrn         MFLGDGMGVSTVTAARILKGQLHHNTGEETRLEMDKFPFVALSKTYNTNAQVPDSAGTAT 115
TNALPmm         MFLGDGMGVSTVTAARILKGQLHHNTGEETRLEMDKFPFVALSKTYNTNAQVPDSAGTAT 115
TNALPhs         MFLGDGMGVSTVTAARILKGQLHHNPGEETRLEMDKFPFVALSKTYNTNAQVPDSAGTAT 115
TNALPcf         MFLGDGMGVSTVTATRILKGQLHHNPGEETRLEMDKFPYVALSKTYNTNAQVPDSAGTAT 93
TNALPfc         MFLGDGMGVSTVTAARILKGQLHHNPGEETRLEMDKFPYVALSKTYNTNAQVPDSAGTAT 115
TNALPbt         MFLGDGMGVSTVTAARILKGQLHHSPGEETKLEMDKFPYVALSKTYNTNAQVPDSAGTAT 115
GALPhs          IFLGDGMGVSTVTAARILKGQKKDKLGPETFLAMDRFPYVALSKTYSVDKHVPDSGATAT 116
PLALPhs         IFLGDGMGVSTVTAARILKGQKKDKLGPEIPLAMDRFPYVALSKTYNVDKHVPDSGATAT 119
IALPhs          LFLGDGLGVPTVTATRILKGQKNGKLGPETPLAMDRFPYLALSKTYNVDRQVPDSAATAT 116
                :*****:**.****:****** : . * *  * **:**::******..: :****..***
Consensus       XFLGDGXGVXTVTAXRILKGQXXXXXGXEXXLXMDXFPXXALSKTYXXXXXVPDSXXTAT


TNALPrn         AYLCGVKANEGTVGVSAATERTRCNTTQGNEVTSILRWAKDAGKSVGIVTTTRVNHATPS 175
TNALPmm         AYLCGVKANEGTVGVSAATERTRCNTTQGNEVTSILRWAKDAGKSVGIVTTTRVNHATPS 175
TNALPhs         AYLCGVKANEGTVGVSAATERSRCNTTQGNEVTSILRWAKDAGKSVGIVTTTRVNHATPS 175
TNALPcf         AYLCGVKANEGTVGVSAATQRTHCNTTQGNEVTSILRWAKDAGKSVGIVTTTRVNHATPS 153
TNALPfc         AYLCGVKANEGTVGVSAATQRTQCNTTQGNEVTSILRWAKDSGKSVGIVTTTRVNHATPS 175
TNALPbt         AYLCGVKANEGTVGVSAATQRSQCNTTQGNEVTSILRWAKDAGKSVGIVTTTRVNHATPS 175
GALPhs          AYLCGVKGNFQTIGLSAAARFNQCNTTRGNEVISVMNRAKKAGKSVGVVTTTRVQHASPA 176
PLALPhs         AYLCGVKGNFQTIGLSAAARFNQCNTTRGNEVISVMNRAKKAGKSVGVVTTTRVQHASPA 179
IALPhs          AYLCGVKANFQTIGLSAAARFNQCNTTRGNEVISVMNRAKQAGKSVGVVTTTRVQHASPA 176
                *******.*  *:*:***:. .:****:**** *::. **.:*****:******:**:*:
Consensus       AYLCGVKXNXXTXGXSAAXXXXXCNTTXGNEVXSXXXXAKXXGKSVGXVTTTRVXHAXPX


TNALPrn         AAYAHSADRDWYSDNEMPPEALSQGCKDIAYQLMHNIKDIDVIMGGGRKYMYPKNRTDVE 235
TNALPmm         AAYAHSADRDWYSDNEMPPEALSQGCKDIAYQLMHNIKDIDVIMGGGRKYMYPKNRTDVE 235
TNALPhs         AAYAHSADRDWYSDNEMPPEALSQGCKDIAYQLMHNIRDIDVIMGGGRKYMYPKNKTDVE 235
TNALPcf         AAYAHSADRDWYSDNEMPPEALSQGCKDIAYQLMHNVKDIEVIMGGGRKYMFPKNRTDVE 213
TNALPfc         AAYAHSADRDWYSDNEMPPEALSQGCKDIAYQLMHNVRDIEVIMGGGRKYMFPKNRTDVE 235
TNALPbt         ASYAHSADRDWYSDNEMPPEALSQGCKDIAYQLMYNIKDIEVIMGGGRKYMFPKNRTDVE 235
GALPhs          GAYAHTVNRNWYSDADVPASARQEGCQDIATQLISNM-DIDVILGGGRKYMFPMGTPDPE 235
PLALPhs         GTYAHTVNRNWYSDADVPASARQEGCQDIATQLISNM-DIDVILGGGRKYMFRMGTPDPE 238
IALPhs          GTYAHTVNRNWYSDADMPASARQEGCQDIATQLISNM-DIDVILGGGRKYMFPMGTPDPE 235
                .:***:.:*:**** ::*..* .:**:*** **: *: **:**:*******:  . .* *
Consensus       XXYAHXXXRXWYSDXXXPXXAXXXGCXDIAXQLXXNXXDIXVIXGGGRKYMXXXXXXDXE


TNALPrn         YELDEKARGTRLDGLDLISIWKSFKPRHKHSHYVWNRTELLALDPS-RVDYLLGLFEPGD 294
TNALPmm         YELDEKARGTRLDGLDLISIWKSFKPRHKHSHYVWNRTELLALDPS-RVDYLLGLFEPGD 294
TNALPhs         YESDEKARGTRLDGLDLVDTWKSFKPRYKHSHFIWNRTELLTLDPH-NVDYLLGLFEPGD 294
TNALPcf         YEMDEKSTGARLDGLNLIDIWKNFKPRHKHSHYVWNRTELLALDPY-TVDYLLGLFDPGD 272
TNALPfc         YEMDEKARGTRLDGLNLVDIWKSFKPRHKHSHYVWNRTELLTLDPY-GVDYLLGLFEPGD 294
TNALPbt         YELDEKARGTRLDGLNLIDIWKSFKPKHKHSHYVWNRTDLLALDPH-SVDYLLGLFEPGD 294
GALPhs          YPDDYSQGGTRLDGKNLVQEWL---AKHQGARYVWNRTELLQASLDPSVTHLMGLFEPGD 292
PLALPhs         YPDDYSQGGTRLDGKNLVQEWL---AKRQGARYVWNRTELMQASLDPSVTHLMGLFEPGD 295
IALPhs          YPADASQNGIRLDGKNLVQEWL---AKHQGAWYVWNRTELMQASLDQSVTHLMGLFEPGD 292
                *  * .  * **** :*:. *     .: : : ::****:*:  .     * :*:***:***
Consensus       YXXDXXXXGXRLDGXXLXXXWXXXXXXXXXXXXXWNRTXLXXXXXXXXVXXLXGLFXPGD
```

Figure 30

```
TNALPrn         MQYELNRNNLTDPSLSEMVEVALRILTKNPKGFFLLVEGGRIDHGHHEGKAKQALHEAVE 354
TNALPmm         MQYELNRNNLTDPSLSEMVEVALRILTKNLKGFFLLVEGGRIDHGHHEGKAKQALHEAVE 354
TNALPhs         MQYELNRNNVTDPSLSEMVVVAIQILRKNPKGFFLLVEGGRIDHGHHEGKAKQALHEAVE 354
TNALPcf         MQYELNRNNVTDPSLSEMVEIAIKILSKKPRGFFLLVEGGRIDHGHHEGKAKQALHEAVE 332
TNALPfc         MQYELNRNSTTDPSLSEMVEIAIKILSKNPKGFFLLVEGGRIDHGHHEGKAKQALHEAVE 354
TNALPbt         MQYELNRNNATDPSLSEMVEMAIRILNKNPKGFFLLVEGGRIDHGHHEGKAKQALHEAVE 354
GALPhs          MKYEIHRDSTLDPSLMEMTEAALLLLSRNPRGFFLFVEGGRIDHGHHESRAYRALTETIM 352
PLALPhs         MKYEIHRDSTLDPSLMEMTEAALRLLSRNPRGFFLFVEGGRIDHGHHESRAYRALTETIM 355
IALPhs          TKYEIHRDPTLDPSLMEMTEAALRLLSRNPRGFYLFVEGGRIDHGHHEGVAYQALTEAVM 352
                 :**::*:   **** **.  *: :* :: :**:*:************. * :** *::
Consensus       XXYEXXRXXXXDPSLXEMXXXAXXXLXXXXXGFXLXVEGGRIDHGHHEXXAXXALXEXXX


TNALPrn         MDEAIGKAGTMTSQKDTLTVVTADHSHVFTFGGYTPRGNSIFGLAPMVSDTDKKPFTAIL 414
TNALPmm         MDQAIGKAGAMTSQKDTLTVVTADHSHVFTFGGYTPRGNSIFGLAPMVSDTDKKPFTAIL 414
TNALPhs         MDRAIGQAGSLTSSEDTLTVVTADHSHVFTFGGYTPRGNSIFGLAPMLSDTDKKPFTAIL 414
TNALPcf         MDRAIGKAGVMTSLEDTLTVVTADHSHVFTFGGYTPRGNSIFGLAPMVSDTDKKPFTAIL 392
TNALPfc         MDQAIGRAGAMTSVEDTLTIVTADHSHVFTFGGYTPRGNSIFGLAPMVSDTDKKPFTSIL 414
TNALPbt         MDQAIGQAGAMTSVEDTLTVVTADHSHVFTFGGYTPRGNSIFGLAPMVSDTDKKPFTAIL 414
GALPhs          FDDAIERAGQLTSEEDTLSLVTADHSHVFSFGGYPLRGSSIFGLAPGKAR-DRKAYTVLL 411
PLALPhs         FDDAIERAGQLTSEEDTLSLVTADHSHVFSFGGYPLRGSSIFGLAPGKAR-DRKAYTVLL 414
IALPhs          FDDAIERAGQLTSEEDTLTLVTADHSHVFSFGGYTLRGSSIFGLAPSKAQ-DSKAYTSIL 411
                :* ** :** :** :***::*********:****. **.*******  :  * *.:* :*
Consensus       XDXAIXXAGXXTSXXDTLXXVTADHSHVFXFGGYXXRGXSIFGLAPXXXXXDXKXXTXXL


TNALPrn         YGNGPGYKVVDGERENVSMVDYAHNNYQAQSAVPLRHETHGGEDVAVFAKGPMAHLLHGV 474
TNALPmm         YGNGPGYKVVDGERENVSMVDYAHNNYQAQSAVPLRHETHGGEDVAVFAKGPMAHLLHGV 474
TNALPhs         YGNGPGYKVVGGERENVSMVDYAHNNYQAQSAVPLRHETHGGEDVAVFSKGPMAHLLHGV 474
TNALPcf         YGNGPGYKVVGGERENVSMVDYAHNNYQAQSAVPLRHETHGGEDVAVFAKGPMAHLLHGV 452
TNALPfc         YGNGPGYKVVGGERENVSMVDYAHNNYQAQSAVPLRHETHGGEDVAVFAKGPMAHLLHGV 474
TNALPbt         YGNGPGYKVVGGERENVSMVDYAHNNYQAQSAVPLRHETHGGEDVAVFAKGPMAHLLHGV 474
GALPhs          YGNGPGYVLKDGARPDVTESESGSPEYRQQSAVPLDGETHAGEDVAVFARGPQAHLVHGV 471
PLALPhs         YGNGPGYVLKDGARPDVTESESGSPEYRQQSAVPLDEETHAGEDVAVFARGPQAHLVHGV 474
IALPhs          YGNGPGYVFNSGVRPDVNESESGSPDYQQQAAVPLSSETHGGEDVAVFARGPQAHLVHGV 471
                ******* . .* * :*.  : .  :*: *:****  ***.*******::** ***:***
Consensus       YGNGPGYXXXXGXRXXVXXXXXXXXXYXXQXAVPLXXETHXGEDVAVFXXGPXAHLXHGV


TNALPrn         HEQNYIPHVMAYASCIG----ANLDHCAWASSASSPSPGALLLPLALFPLRTLF------ 524
TNALPmm         HEQNYIPHVMAYASCIG----ANLDHCAWAGSGSAPSPGALLLPLAVLSLPTLF------ 524
TNALPhs         HEQNYVPHVMAYAACIG----ANLGHCAPASSAGSLAAGPLLLALALYPLSVLF------ 524
TNALPcf         HEQNYIPHVMAYAACIG----ANQDHCASASSAGGPSPGPLLLLLALLPVGILF------ 502
TNALPfc         HEQNYIPHVMAYAACIG----ANLDHCASASSAGGPSPGPLFLLLALPSLGILF------ 524
TNALPbt         QEQNYIPHVMAYAACIG----ANRDHCASASSSGSPSPGPLLLLLALLPLGSLF------ 524
GALPhs          QEQTFIAHVMAFAACLEPYTACDLAPRAGTTDAAHPGPSVVPALLPLLAGTLLLLGTATA 531
PLALPhs         QEQTFIAHVMAFAACLEPYTACDLAPPAGTTDAAHPGRSVVPALLPLLAGTLLLLETATA 534
IALPhs          QEQSFVAHVMAFAACLEPYTACDLAPPACTTDAAHP----VAASLPLLAGTLLLLGASAA 527
                :**.::.****:*:*:      .:    * : ...       :   *.: .   *:
Consensus       XEQXXXXHVMAXAXCXXXXXXXXXXXXXAXXXXXXXXXXXXXXXXXXLXXXXXXXLXXXXXXX

TNALPrn         -
TNALPmm         -
TNALPhs         -
TNALPcf         -
TNALPfc         -
TNALPbt         -
GALPhs          P 532
PLALPhs         P 535
IALPhs          P 528

Consensus       X
```

Figure 30 (Continued)

```
CLUSTAL W (1.82) multiple sequence alignment: Tissue Nonspecific Alkaline Phosphatase

                              1
P09487|PPBT_BOVIN             MISPFLLLAI GTCFASSLVP EKEKDPKYWR DQAQQTLKNA LRLQTLNTNV AKNVIMFLGD
Q29486|PPBT_FELCA             MISPFLVLAI GTCLTNSLVP EKEKDPKYWR DQAQQTLKNA LRLQKLNTNV VKNVIMFLGD
P05186|PPBT_HUMAN             MISPFLVLAI GTCLTNSLVP EKEKDPKYWR DQAQETLKYA LELQKLNTNV AKNVIMFLGD
P09242|PPBT_MOUSE             MISPFLVLAI GTCLTNSFVP EKERDPSYWR QQAQETLKNA LKLQKLNTNV AKNVIMFLGD
P08289|PPBT_RAT               MILPFLVLAI GTCLTNSFVP EKEKDPSYWR QQAQETLKNA LKLQKLNTNV AKNIIMFLGD
Q9N0V0|Q9N0V0_CANFA           ---------- ---------- --EKDPKYWR DQAQQTLKYA LRLQNLNTNV AKNVIMFLGD
                                                     *:**.*** :***:*** * *.**.***** .**:******
Consensus                     XXXXXXXXXX XXXXXXXXXX XXEXDPXYWR XQAQXTLKXA LXLQXLNTNV XKNXIMFLGD

                             61
P09487|PPBT_BOVIN              GMGVSTVTAA RILKGQLHHS PGEETKLEMD KFPYVALSKT YNTNAQVPDS AGTATAYLCG
Q29486|PPBT_FELCA              GMGVSTVTAA RILKGQLHHN PGEETRLEMD KFPYVALSKT YNTNAQVPDS AGTATAYLCG
P05186|PPBT_HUMAN              GMGVSTVTAA RILKGQLHHN PGEETRLEMD KFPFVALSKT YNTNAQVPDS AGTATAYLCG
P09242|PPBT_MOUSE              GMGVSTVTAA RILKGQLHHN TGEETRLEMD KFPFVALSKT YNTNAQVPDS AGTATAYLCG
P08289|PPBT_RAT                GMGVSTVTAA RILKGQLHHN TGEETRLEMD KFPFVALSKT YNTNAQVPDS AGTATAYLCG
Q9N0V0|Q9N0V0_CANFA            GMGVSTVTAT RILKGQLHHN PGEETRLEMD KFPYVALSKT YNTNAQVPDS AGTATAYLCG
                               *********: *********. .****:**** ***:****** ********** **********
Consensus                      GMGVSTVTAX RILKGQLHHX XGEETXLEMD KFPXVALSKT YNTNAQVPDS AGTATAYLCG

                            121
P09487|PPBT_BOVIN               VKANEGTVGV SAATQRSQCN TTQGNEVTSI LRWAKDAGKS VGIVTTTRVN HATPSASYAH
Q29486|PPBT_FELCA               VKANEGTVGV SAATQRTQCN TTQGNEVTSI LRWAKDSGKS VGIVTTTRVN HATPSAAYAH
P05186|PPBT_HUMAN               VKANEGTVGV SAATERSRCN TTQGNEVTSI LRWAKDAGKS VGIVTTTRVN HATPSAAYAH
P09242|PPBT_MOUSE               VKANEGTVGV SAATERTRCN TTQGNEVTSI LRWAKDAGKS VGIVTTTRVN HATPSAAYAH
P08289|PPBT_RAT                 VKANEGTVGV SAATERTRCN TTQGNEVTSI LRWAKDAGKS VGIVTTTRVN HATPSAAYAH
Q9N0V0|Q9N0V0_CANFA             VKANEGTVGV SAATQRTHCN TTQGNEVTSI LRWAKDAGKS VGIVTTTRVN HATPSAAYAH
                                ********** ****:*::** ********** ******:*** ********** ******:***
Consensus                       VKANEGTVGV SAATXRXXCN TTQGNEVTSI LRWAKDXGKS VGIVTTTRVN HATPSAXYAH
```

Figure 31

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                          181
P09487|PPBT_BOVIN           SADRDWYSDN EMPPEALSQG CKDIAYQLMY NIKDIEVIMG GGRKYMFPKN RTDVEYELDE
Q29486|PPBT_FELCA           SADRDWYSDN EMPPEALSQG CKDIAYQLMH NVRDIEVIMG GGRKYMFPKN RTDVEYEMDE
P05186|PPBT_HUMAN           SADRDWYSDN EMPPEALSQG CKDIAYQLMH NIRDIDVIMG GGRKYMYPKN KTDVEYESDE
P09242|PPBT_MOUSE           SADRDWYSDN EMPPEALSQG CKDIAYQLMH NIKDIDVIMG GGRKYMYPKN RTDVEYELDE
P08289|PPBT_RAT             SADRDWYSDN EMPPEALSQG CKDIAYQLMH NIKDIDVIMG GGRKYMYPKN RTDVEYELDE
Q9N0V0|Q9N0V0_CANFA         SADRDWYSDN EMPPEALSQG CKDIAYQLMH NVKDIEVIMG GGRKYMFPKN RTDVEYEMDE
                            ********** ********** *********: *:::**:**** ******:*** :****** **
Consensus                   SADRDWYSDN EMPPEALSQG CKDIAYQLMX NXXDIXVIMG GGRKYMXPKN XTDVEYEXDE


                          241
P09487|PPBT_BOVIN           KARGTRLDGL NLIDIWKSFK PKHKHSHYVW NRTDLLALDP HSVDYLLGLF EPGDMQYELN
Q29486|PPBT_FELCA           KARGTRLDGL NLVDIWKSFK PRHKHSHYVW NRTELLTLDP YGVDYLLGLF EPGDMQYELN
P05186|PPBT_HUMAN           KARGTRLDGL DLVDTWKSFK PRYKHSHFIW NRTELLTLDP HNVDYLLGLF EPGDMQYELN
P09242|PPBT_MOUSE           KARGTRLDGL DLISIWKSFK PRHKHSHYVW NRTELLALDP SRVDYLLGLF EPGDMQYELN
P08289|PPBT_RAT             KARGTRLDGL DLISIWKSFK PRHKHSHYVW NRTELLALDP SRVDYLLGLF EPGDMQYELN
Q9N0V0|Q9N0V0_CANFA         KSTGARLDGL NLIDIWKNFK PRHKHSHYVW NRTELLALDP YTVDYLLGLF DPGDMQYELN
                            *: *:***** :*:. **.** *::****::* ***:**:***   ******** :*********
Consensus                   KXXGXRLDGL XLXXXWKXFK PXXKHSHXXW NRTXLLXLDP XXVDYLLGLF XPGDMQYELN


                          301
P09487|PPBT_BOVIN           RNNATDPSLS EMVEMAIRIL NKNPKGFFLL VEGGRIDHGH HEGKAKQALH EAVEMDQAIG
Q29486|PPBT_FELCA           RNSTTDPSLS EMVEIAIKIL SKNPKGFFLL VEGGRIDHGH HEGKAKQALH EAVEMDQAIG
P05186|PPBT_HUMAN           RNNVTDPSLS EMVVVAIQIL RKNPKGFFLL VEGGRIDHGH HEGKAKQALH EAVEMDRAIG
P09242|PPBT_MOUSE           RNNLTDPSLS EMVEVALRIL TKNLKGFFLL VEGGRIDHGH HEGKAKQALH EAVEMDQAIG
P08289|PPBT_RAT             RNNLTDPSLS EMVEVALRIL TKNPKGFFLL VEGGRIDHGH HEGKAKQALH EAVEMDEAIG
Q9N0V0|Q9N0V0_CANFA         RNNVTDPSLS EMVEIAIKIL SKKPRGFFLL VEGGRIDHGH HEGKAKQALH EAVEMDRAIG
                            **. ****** *** :*::**  *: :***** ********** ********** ******.***
Consensus                   RNXXTDPSLS EMVXXAXXIL XKXXXGFFLL VEGGRIDHGH HEGKAKQALH EAVEMDXAIG
```

Figure 31 (Continued)

```
361
P09487|PPBT_BOVIN    QAGAMTSVED TLTVVTADHS HVFTFGGYTP RGNSIFGLAP MVSDTDKKPF TAILYGNGPG
Q29486|PPBT_FELCA    RAGAMTSVED TLTIVTADHS HVFTFGGYTP RGNSIFGLAP MVSDTDKKPF TSILYGNGPG
P05186|PPBT_HUMAN    QAGSLTSSED TLTVVTADHS HVFTFGGYTP RGNSIFGLAP MLSDTDKKPF TAILYGNGPG
P09242|PPBT_MOUSE    KAGAMTSQKD TLTVVTADHS HVFTFGGYTP RGNSIFGLAP MVSDTDKKPF TAILYGNGPG
P08289|PPBT_RAT      KAGTMTSQKD TLTVVTADHS HVFTFGGYTP RGNSIFGLAP MVSDTDKKPF TAILYGNGPG
Q9N0V0|Q9N0V0_CANFA  KAGVMTSLED TLTVVTADHS HVFTFGGYTP RGNSIFGLAP MVSDTDKKPF TAILYGNGPG
                     :** :** :* ***:******* ********** ********** *:********** *:*********
Consensus            XAGXXTSXXD TLTXVTADHS HVFTFGGYTP RGNSIFGLAP MXSDTDKKPF TXILYGNGPG

421
P09487|PPBT_BOVIN    YKVVGGEREN VSMVDYAHNN YQAQSAVPLR HETHGGEDVA VFAKGPMAHL LHGVQEQNYI
Q29486|PPBT_FELCA    YKVVGGEREN VSMVDYAHNN YQAQSAVPLR HETHGGEDVA VFAKGPMAHL LHGVHEQNYI
P05186|PPBT_HUMAN    YKVVGGEREN VSMVDYAHNN YQAQSAVPLR HETHGGEDVA VFSKGPMAHL LHGVHEQNYV
P09242|PPBT_MOUSE    YKVVDGEREN VSMVDYAHNN YQAQSAVPLR HETHGGEDVA VFAKGPMAHL LHGVHEQNYI
P08289|PPBT_RAT      YKVVDGEREN VSMVDYAHNN YQAQSAVPLR HETHGGEDVA VFAKGPMAHL LHGVHEQNYI
Q9N0V0|Q9N0V0_CANFA  YKVVGGEREN VSMVDYAHNN YQAQSAVPLR HETHGGEDVA VFAKGPMAHL LHGVHEQNYI
                     ****.***** ********** ********** ********** **:******** ****:****:
Consensus            YKVVXGEREN VSMVDYAHNN YQAQSAVPLR HETHGGEDVA VFXKGPMAHL LHGVXEQNYX

481
P09487|PPBT_BOVIN    PHVMAYAACI GANRDHCASA SSSGSPSPGP LLLLLALLPL GSLF
Q29486|PPBT_FELCA    PHVMAYAACI GANLDHCASA SSAGGPSPGP LFLLLALPSL GILF
P05186|PPBT_HUMAN    PHVMAYAACI GANLGHCAPA SSAGSLAAGP LLLALALYPL SVLF
P09242|PPBT_MOUSE    PHVMAYASCI GANLDHCAWA GSGSAPSPGA LLLPLAVLSL PTLF
P08289|PPBT_RAT      PHVMAYASCI GANLDHCAWA SSASSPSPGA LLLPLALFPL RTLF
Q9N0V0|Q9N0V0_CANFA  PHVMAYAACI GANQDHCASA SSAGGPSPGP LLLLLALLPV GILF
                     ********:** *** .*** * .*... :.*. *:* **: .:   **
Consensus            PHVMAYAXCI GANXXHCAXA XSXXXXXXGX LXLXLAXXXX XXLF
```

Figure 31(Continued)

```
   1 atggtttcac cattcttagt actggccatt ggcacctgcc ttactaactc cttagtgcca
  61 gagaaagaga aagaccccaa gtactggcga gaccaagcgc aagagacact gaaatatgcc
 121 ctggagcttc agaagctcaa caccaacgtg gctaagaatg tcatcatgtt cctgggagat
 181 gggatgggtg tctccacagt gacggctgcc cgcatcctca agggtcagct ccaccacaac
 241 cctggggagg agaccaggct ggagatggac aagttccgct tcgtggccct ctccaagacg
 301 tacaacacca atgcccaggt ccctgacagc gccggcaccg ccaccgccta cctgtgtggg
 361 gtgaaggcca atgagggcac cgtgggggta agcgcagcca ctgagcgttc ccggtgcaac
 421 accacccagg ggaacgaggt cacctccatc ctgcgctggg ccaaggacgc tgggaaatct
 481 gtgggcattg tgaccaccac gagagtgaac catgccaccc ccagcgccgc ctacgcccac
 541 tcggctgacc gggactggta ctcagacaac gagatgcccc ctgaggcctt gagccagggc
 601 tgtaaggaca tcgcctacca gctcatgcat aacatcaggg acattgacgt gatcatgggg
 661 ggtggccgga aatacatgta ccccaagaat aaaactgatg tggagtatga gagtgacgag
 721 aaagccaggg gcacgaggct ggacggcctg gacctcgttg acacctggaa gagcttcaaa
 781 ccgagataca agcactccca cttcatctgg aaccgcacgg aactcctgac ccttgacccc
 841 cacaatgtgg actacctatt gggtctcttc gagccagggg acatgcagta cgagctgaac
 901 aggaacaacg tgacggaccc gtcactctcc gagatggtgg tggtggccat ccagatcctg
 961 cggaagaacc ccaaaggctt cttcttgctg gtggaaggag gcagaattga ccacgggcac
1021 catgaaggaa aagccaagca ggccctgcat gaggcggtgg agatggaccg ggccatcggg
1081 caggcaggca gcttgacctc ctcggaagac actctgaccg tggtcactgc ggaccattcc
1141 cacgtcttca catttggtgg atacaccccc cgtggcaact ctatctttgg tctggccccc
1201 atgctgagtg acacagacaa gaagcccttc actgccatcc tgtatggcaa tgggcctggc
1261 tacaaggtgg tgggcggtga acgagagaat gtctccatgg tggactatgc tcacaacaac
1321 taccaggcgc agtctgctgt gcccctgcgc cacgagaccc acggcgggga ggacgtggcc
1381 gtcttctcca agggccccat ggcgcacctg ctgcacggcg tccacgagca gaactacgtc
1441 ccccacgtga tggcgtatgc agcctgcatc ggggccaacc tcggccactg tgctcctgcc
1501 agctcgCTTA AGgacaaaac tcacacatgc ccaccgtgcc cagcacctga actcctgggg
1561 ggaccgtcag tcttcctctt ccccccaaaa cccaaggaca ccctcatgat ctcccggacc
1621 cctgaggtca catgcgtggt ggtggacgtg agccacgaag accctgaggt caagttcaac
1681 tggtacgtgg acggcgtgga ggtgcataat gccaagacaa agccgcggga ggagcagtac
1741 aacagcacgt accgtgtggt cagcgtcctc accgtcctgc accaggactg gctgaatggc
1801 aaggagtaca agtgcaaggt ctccaacaaa gccctcccag ccccccatcga gaaaaccatc
1861 tccaaagcca aagggcagcc ccgagaacca caggtgtaca ccctgccccc atcccgggag
1921 gagatgacca agaaccaggt cagcctgacc tgcctggtca aaggcttcta tcccagcgac
1981 atcgccgtgg agtgggagag caatgggcag ccggagaaca actacaagac cacgcctccc
2041 gtgctggact ccgacggctc cttcttcctc tacagcaagc tcaccgtgga caagagcagg
2101 tggcagcagg ggaacgtctt ctcatgctcc gtgatgcatg aggctctgca caaccactac
2161 acgcagaaga gcctctccct gtctccgggt aaaGATATCG ATGACGATGA CGATGACGAT
2221 GACGATGACT AG
```

Figure 32

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2008/000923

A. CLASSIFICATION OF SUBJECT MATTER
IPC: ***C12N 15/55*** (2006.01), ***A61K 38/46*** (2006.01), ***A61P 19/08*** (2006.01), ***C12N 15/85*** (2006.01), ***C12N 5/10*** (2006.01), ***C12N 9/16*** (2006.01)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N 15/55 (2006.01), ***A61K 38/46*** (2006.01), ***A61P 19/08*** (2006.01), ***C12N 15/85*** (2006.01), ***C12N 5/10*** (2006.01), ***C12N 9/16*** (2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
GenomeQuest Sequence Database, CAPLUS, BIOSIS, Pubmed, Google, Canadian Patent Database
SEQ ID NOs: 4 and 17 and Keywords: alkaline phosphatase, hypophosphatasia, rickets, osteomalacia, bone, aspartic or aspartate, glutamic or glutamate, fusion, spacer or linker

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2007/0081984 A1 (TOMATSU, S. et al.) 12 April 2007. See whole document, and in particular abstract and paragraphs 7-9, 23, and 28-30	1, 14-17, 19-21, 26, 30-38, and 40-81
X	NISHIOKA, T. et al. "Enhancement of drug delivery to bone: Characterization of human tissue-nonspecific alkaline phosphatase tagged with an acidic oligopeptide". Mol. Genet. Metab. July 2006. Vol. 88, No. 3, pp. 244-255, ISSN: 1096-7192. See whole document	1, 14-17, 19-21, 26, 30-38, and 40-81
A	WO 2006/039480 A2 (MILLAN, J. L.) 13 April 2006. See whole document	1-81

[ ] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 August 2008 (15-08-2008)	12 September 2008 (12-09-2008)

Name and mailing address of the ISA/CA	Authorized officer
Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Sandra Babin 819- 934-4189

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2008/000923

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. [X] Claim Nos. : 49-76

because they relate to subject matter not required to be searched by this Authority, namely :

Claims 49-76 are directed to a method for treatment of the human or animal body by surgery or therapy which the International Search Authority is not required to search under Rule 39.1(iv) of the PCT. However, this Authority has carried out a search based on the alleged effect or purpose/use of the product defined in these claims.

2. [X] Claim Nos. : 1-26, 28-38, and 40-81

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

See Continuation of Box II, page 6

3. [ ] Claim Nos. :

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows :

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.

Continuation of Box II:

Claims 1–26, 28-38, and 40-81 do not comply with Article 6 of the PCT. The subject matter of these claims is not adequately supported by the present specification. The structure provided in claim 1 for the claimed bone targeted alkaline phosphatase encompasses an extremely large number of possible products due to the fact that the polypeptide at a minimum may contain any extracellular domain of an alkaline phosphatase combined with any spacer and 10 to 16 aspartic acid or glutamic acid residues; and at a maximum the polypeptide may comprise any extracellular domain of an alkaline phosphatase, any spacer, 10 to 16 aspartic acid or glutamic acid residues, and an unlimited number of amino acid residues at the positions represented by the terms V, X, Y, and Z. Furthermore, the specification does not disclose and characterize **all** bone targeted alkaline phosphatases, which may be used in correction and prevention of at least one hypophosphatasia phenotype. Only the bone targeted alkaline phosphatase hsTNALP-Fc-D10, as represented by SEQ ID NO: 4, and the nucleic acid sequence encoding a bone targeted alkaline phosphatase, as represented by SEQ ID NO: 17, has been disclosed and characterized in the present specification. Therefore, because the claimed subject matter lacks support and the entire application lacks disclosure, a meaningful search over the whole of the claimed scope is impossible. Consequently, a structural search has been established for the parts of the application which appear to be clear and supported, namely the bone targeted alkaline phosphatase, hsTNALP-Fc-D10 as represented by SEQ ID NO: 4 and the nucleic acid sequence encoding a bone targeted alkaline phosphatase, as represented by SEQ ID NO: 17. In addition, a keyword search has been established with regards to the broadly defined bone targeted alkaline phosphatase of claim 1 (ie. a bone targeted alkaline phosphatase comprising the extracellular domain of an alkaline phosphatase; a spacer; and a polyaspartate or polyglutamate).

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
US2007081984 A1	12-04-2007	NONE	
WO2006039480 A2	13-04-2006	WO2006039480 A3	24-08-2006