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Lansoprazole is an uncompetitive inhibitor of tissue-nonspecific alkaline phosphatase

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Lansoprazole, a known H^+/K^+-ATPase inhibitor, is currently used as a therapeutical option for the initial treatment of gastroesophageal reflux disease. Recently, lansoprazole has been found to be an inhibitor of cytosolic PHOSPHO1 (a phosphatase which hydrolyses phosphocholine and phosphoethanolamine), providing a possible therapeutical target to cure pathological mineralization. Since PHOSPHO1 is present inside matrix vesicles, we tested the effect of lansoprazole on matrix vesicles containing several key enzymes for the mineralization process including tissue-nonspecific alkaline phosphatase. We found that lansoprazole can inhibit in an uncompetitive manner tissue-nonspecific alkaline phosphatase. A K_i value of 1.74±0.12 mM has been determined for the inhibition of tissue-nonspecific alkaline phosphatase by lansoprazole. Lansoprazole, currently used for treating gastroesophageal disease, by inhibiting PHOSPHO1 and tissue-nonspecific alkaline phosphatase could prevent hydroxyapatite-deposition disease and could serve as an adjunct treatment for osteoarthritis.

Keywords: alkaline phosphatase, inhibition, lansoprazole, osteoarthritis, PHOSPHO1, phosphocholine

INTRODUCTION

Lansoprazole is a selective H^+/K^+-ATPase inhibitor (Gremse, 2001). Acid suppression with proton pump inhibitors is now the preferred treatment option for the initial treatment of gastroesophageal reflux disease (DeVault & Castel, 1999; Der, 2003; Kivioja *et al*., 2004; Shi & Klotz, 2008). Lansoprazole is used for the short term treatment of erosive reflux oesophagitis, active gastric ulcer, active duodenal ulcer and the treatment of non-steroidal anti-inflammatory drug-induced gastric and duodenal ulcers. It has been also approved for the long-term treatment of healed reflux oesophagitis, healed duodenal ulcer, the treatment of hypersecretory conditions as Zollinger-Ellison syndrome (Gremse, 2001). Lansoprazole, as other substituted 2-pyridylmethylsulfinyl benzimidazoles such as omeprazole, pantoprazole and rabeprazole are weak bases, accumulating inside the acidic environment of H^+/K^+-ATPase. At acidic pH they undergo an acid catalyzed rearrangement to a cationic sulfenamide, forming disulfides with cysteines of the H^+/K^+-ATPase proton pump (Besancon *et al*., 1997). Although no use of lansoprazole has been reported to cure bone diseases, it was recently reported that lansoprazole has the ability to modulate *in vitro* mineralization induced by matrix vesicles (MVs), isolated from calvaria of tissue-nonspecific alkaline phosphatase (TNAP) null mice, by inhibiting PHOSPHO1 (Narisawa *et al*., 2007). The phosphatase PHOSPHO1 is a soluble enzyme which hydrolyzes specifically phosphoethanolamine and phosphocholine (Pchol) (Roberts *et al*., 2005) and contributes to increase P_i inside MVs thereby leading to mineralization (Roberts *et al*., 2006). Osteoblasts and hypertrophic chondrocytes can initiate mineralization by releasing MVs (Anderson, 2007; Kirsch, 2007). MVs are involved in the initial step of mineralization by promoting the formation of hydroxyapatite (HA) inside MVs. Once the crystals have reached a certain size, they rupture the MV membranes and migrate into the extracellular matrix. This is followed by HA

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Abbreviations: HA, hydroxyapatite; MVs, matrix vesicles; Pchol, phosphocholine; PI-PLC, phosphatidylinositol phosphatase C; *p*NPP, *p*-nitrophenylphosphate; P_i, inorganic phospahte; PP_i, inorganic pyrophosphate; TNAP, tissue-nonspecific alkaline phosphatase.

propagation outside the MVs, supplemented by the Ca^{2+} and P_i through circulation, and ultimately HA fills up the space between collagen fibers of the skeletal matrix (Anderson, 2007; Kirsch, 2007). Although P_i is continuously supplied by circulation, other sources of P_i sustain the mineralization process. One likely source of P_i could come from the hydrolysis of phosphomonoesters by 5′-AMPase (Einhorn *et al*., 1985), ATPases (Hsu & Anderson, 1996; Hsu *et al*., 1999), PHOSPHO1 (Roberts *et al*., 2004; 2005; 2007) and tissue-nonspecific alkaline phosphatase (TNAP) (Register *et al*., 1986; Balcerzak *et al.* 2003; Anderson *et al*., 2004). Inhibitors of any phosphomonoesterase enzymes should affect the mineralization process, providing a possible therapeutical target to cure pathological mineralization, such as osteoarthritis, crystal-deposition arthritis, atherosclerosis and tumor calcification (in breast cancer) (Andersen, 2007; Kirsch, 2007). Since PHOSPHO1, is also entrapped in MVs, we tested the effect of lansoprazole on MVs, containing several key enzymes implicated in the mineralization process, including TNAP. We confirmed the therapeutical potential of lansoprazole, as inhibitor of PHOSPHO1 (Roberts *et al*., 2007) and in addition we found that lansoprazole can also inhibit in an uncompetitive manner TNAP. Therefore the inhibition properties of lansoprazole toward PHOSPHO1 and TNAP, implicated both in the mineralization process could boost the efficiency of lansoprazole to inhibit mineral formation.

MATERIALS AND METHODS

Isolation of matrix vesicles. MVs were isolated from 17-day-old chicken embryos (Balcerzak *et al*., 2007). They were stored in ice cold synthetic cartilage lymph (SCL) containing 2 mM $CaCl_2$ and 1.42 mM NaH_2PO_4 in addition to 100 mM NaCl, 1.83 mM $NaHCO_3$, 12.70 mM KCl, 0.57 mM $MgCl{\cdot}6H_2O$, 0.57 mM Na_2SO_4, 63.50 mM sucrose, 5.55 mM D-glucose, 16.5 mM TES pH 7.4 (Wu *et al*., 1997). Protein concentration in the vesicles was determined by the method of Bradford (Bradford, 1976).

Isolation of tissue-nonspecific alkaline phosphatase by phosphatidylinositol-phospholipase C treatment. MVs were incubated in SCL medium containing 6 μM $ZnCl_2$, 20 mM $MgCl_2$ and 1.5×10^{-3} units phosphatidylinositol phospholipase C (PI-PLC) /μg MVs proteins for 6 h at 37°C under gentle vortexing (Zhang *et al*., 2005). The supernatant and the pellet were separated by centrifugation at $100\,000\times g$ for 30 min. The supernatant containing tissue-nonspecific alkaline phosphatase was collected. For comparison, commercial tissue-nonspecific alkaline phosphatase from porcine kindey was purchassed from Sigma.

Fluorescence determination of phosphocholine hydrolysis. The fluorescence of resorufin induced by the reaction of 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red PLD Kit, Molecular Probes) and H_2O_2 produced by the oxidation of Chol to betaine was monitored at 585 nm after sample excitation at 571 nm on a F-4500 Hitachi Fluorescence Spectrophotometer. Slits for excitation and emission were set at 2.5 and 5 nm, respectively. MVs (20 μg protein/ml) in 50 mM Tris/HCl, pH 7.4, 140 mM NaCl without any additions or in the presence of inhibitors such as 1–5 mM levamisole , 0.1 mM vanadate or 1 mM lansoprazole were mixed with 500 μl of reaction buffer containing 50 μM Amplex reagent, 1 U/ml of horseradish peroxidase (HRP) and 1 U/ml of choline oxidase from *Alcaligenes* sp. (Balcerzak *et al*., 2006).

Phosphomonoesterase activity assays. The phosphomonoesterase (PME) activity of TNAP was measured (at 420 nm) with 10 mM *p*-nitrophenyl phosphate (*p*NPP) as substrate (Engstrom, 1961) in the reaction solution containing 25 mM piperazine and 25 mM glycylglycine at pH 10.4 and at 37°C. One unit of the activity was defined as the amount of enzyme hydrolyzing 1 μmol of substrate per min.

RESULTS

Lansoprazole inhibition of phosphocholine hydrolysis by tissue-nonspecific alkaline phosphatase

To determine PHOSPHO1 activity in MVs, addition of exogenous Pchol (a substrate for PHOSPHO1 as well as for other phosphomonesterase enzymes such as TNAP), we measured the hydrolysis of Pchol, using the fluorescence of resorufin. MVs (20 μg protein/mL) were able to hydrolyze completely within 20 min at pH 7.4, 1 μM exogenous-Pchol by forming choline and P_i (Fig. 1). Since PHOSPHO1 is a cytosolic enzyme entrapped inside MVs, this suggested either that phosphocholine was transported inside MVs and hydrolyzed inside by PHOSPHO1 and/or that phosphocholine was hydrolyzed by MV-phosphomonoesterase enzymes such as TNAP pointing to the extracellular side. Since MVs are enriched in GPI-anchored TNAP, we checked the phosphocholine hydrolysis by using TNAP inhibitors. Addition of TNAP inhibitor (either 5 mM levamisole or 0.1 mM vanadate) prevented almost completely the Pchol hydrolysis, indicating that TNAP was implicated in the hydrolysis of Pchol. Addition of 1 mM lansoprazole, a PHOSPHO1 inhibitor (Roberts *et al*., 2007) inhibited partially the Pchol

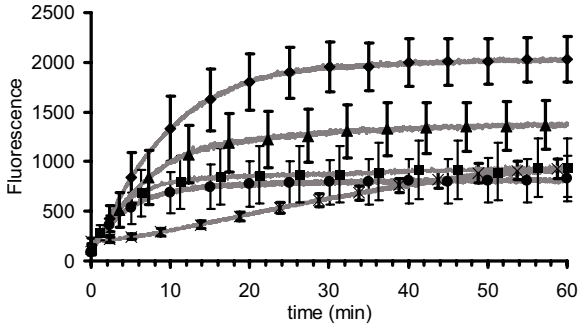

Figure 1. Pchosphocholine hydrolysis at pH 7.4 by matrix vesicles.
MVs (20 μg protein/μL) in 50 mM Tris/HCl, pH 7.4, 140 mM NaCl at 25°C without any additions (●). MVs (20 μg protein/μL) in the presence of 1 μM Pchol without any inhibitor (◆) or with 5 mM levamisole (×), or with 0.1 mM vanadate (■) or with 1 mM lansoprazole (▲). Values are the mean from three samples.

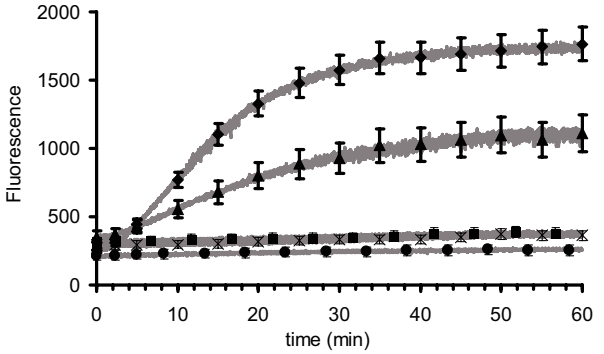

Figure 3. Pchosphocholine hydrolysis by commercial TNAP from porcine kidney.
Conditions: 50 mM Tris/HCl, pH 7.4, 140 mM NaCl, 25°C, 0.25 TNAP unit (●) 0.25 unit TNAP and Pchol 1 μM (◆) and in the presence of inhibitors: levamisole 5 mM (×), vanadate 0.1 mM (■) or lansoprazole 1 mM (▲). Values are the mean from three samples taken from all of the time points.

hydrolysis by MVs, suggesting that PHOSPHO1 was implicated in the Pchol hydrolysis and that the remaining hydrolysis activity was due to other phosphomonesterase enzymes. To confirm the origin of enzymatic inhibition by lansoprazole, TNAP was isolated from MVs after PI-PLC treatment and centrifugation. The released TNAP from MVs was in the supernatant, while the PI-PLC treated MVs without TNAP was pelleted down. We observed that 1 mM lansoprazole moderately inhibited the Pchol hydrolysis by isolated TNAP in the supernatant (Fig. 2), while 0.1 mM vanadate and 5 mM levamisole (Fig. 2) completely inhibited its activity. Lansoprazole also inhibited the Pchol hydrolysis at pH 7.4 (Fig. 3) as well as *p*NPP hydrolysis at pH 10.4 (Fig. 4) by commercial TNAP from porcine kidney, confirming its inhibitory activity to TNAP.

Determination of K_i value of lansoprazole

The *p*NPP-hydrolysis inhibition of lansoprazole on TNAP at pH 10.4 was determined by using Lineweaver-Burk plots. Four distinct lansoprazole concentrations from 0, 0.5, 1, and 1.5 mM and *p*NPP concentrations ranging from 15 mM to 250 mM served to determine the kinetic parameters (Table 1). An apparent K_i value of 1.74±0.12 mM was determined and our findings indicated that lansoprazole inhibited the hydrolysis by TNAP in an uncompetitive manner.

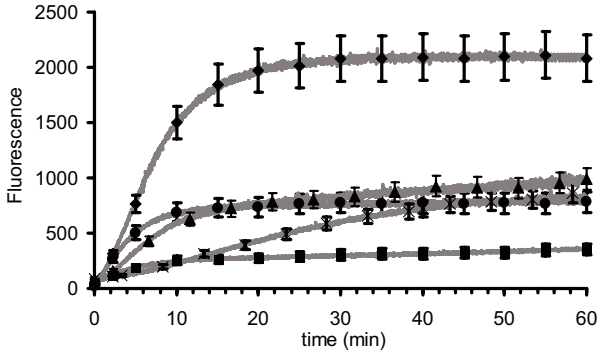

Figure 2. Pchosphocholine hydrolysis at pH 7.4 by TNAP released from matrix vesicles after PI-PLC treatment.
20 μg protein/mL TNAP extracted from MVs in 50 mM Tris/HCl, pH 7.4, 140 mM NaCl at 25°C: control (●). 20 μg protein/mL TNAP extracted from MVs with 1 μM Pchol (◆); with 1 μM Pchol and levamisole 5 mM (×); with 1 μM Pchol and vanadate 0.1 mM (■) or with 1 μM Pchol and lansoprazole 1 mM (▲). Values are the mean from three samples taken from all of the time points.

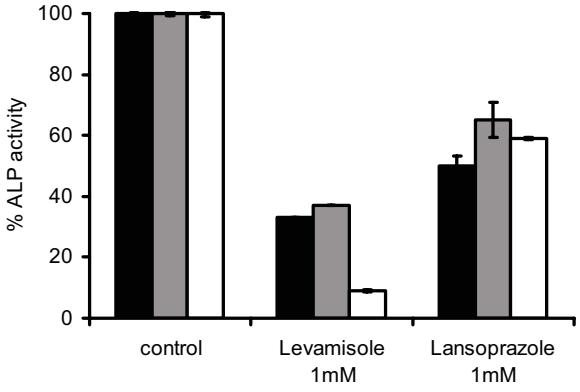

Figure 4. *p*NPP hydrolysis by matrix-vesicle TNAP.
Conditions: 25 mM piperazine and 25 mM glycylglycine at pH 10.4, 37°C. Matrix vesicles 20 μg protein/mL (dark). TNAP from matrix vesicles after PI-PLC treatment 20 μg protein/mL (grey) and commercial TNAP from porcine kidney 0.25 unit (white). As indicated in the figure, with or without inhibitors, 1 mM levamisole or 1 mM lansoprazole. Values are the mean from three samples.

Table 1. Apparent K_i and K_m values for lansoprazole concentrations ranging from 0 to 1.5 mM, obtained from Lineweaver-Burk plot with porcine TNAP and 10 mM *p*NPP pH 10.4.

	Apparent K_m	K_i
TNAP	0.70±0.10 mM	
TNAP + 500 μM lansoprasole	0.54±0.05 mM	1.83 mM
TNAP + 1 mM lansoprasole	0.43±0.11 mM	1.60 mM
TNAP + 1.5 mM lansoprasole	0.38±0.02 mM	1.78 mM
Mean		1.74±0.12 mM

DISCUSSIONS

In this work, we found that lansoprazole, a potent inhibitor of PHOSPHO1 (Roberts *et al.*, 2006) inhibited the Pchol hydrolysis in an uncompetitive manner by TNAP within MVs or by isolated TNAP at pH 7.4. A K_i value of 1.74±0.12 mM for the uncompetitive inhibition of *p*NPP at pH 10.4 by alkaline phosphatase with lansoprazole was found while a K_i value of 28±9.21 μM was obtained for lansoprazole inhibiting PHOSPHO1 (Roberts *et al.*, 2006). Due to the fact that PHOSPHO1 is a cytosolic enzyme entrapped inside MVs, while TNAP active site is located toward the extracellular matrix, lansoprazole could inhibit not only PHOSPHO1 but also TNAP despite its relatively higher K_i value than that of PHOSPHO1. Although lansoprazole may not be the best therapeutical option for curing osteoarthritis diseases characterized by HA deposits, it may contribute as an adjunct therapy to cure osteoarthritis. This is especially true for patients being treated for gastroesophageal reflux disease with lansoprazole and having osteoarthritis. Lansoprazole, used for the treatment of gastroesophageal reflux disease, under acidic conditions undergoes to an acid catalyzed rearrangement to a cationic sulfenamide, forming disulfides with cysteines of the H^+/K^+-ATPase proton pump and inhibiting it. However, in the case of TNAP and PHOSPHO1, the inhibition of phosphomonoesterase activity can occur at pH 7.4 and therefore the mode of action is different. The rationale to cure pathological calcification by targeting TNAP and PHOSPHO1 within MVs is supported by the fact that not only MVs appear to initiate skeletal mineralization, but MVs are also involved in a variety of pathological calcifications (Anderson 1983; 1988; 2007; Ali, 1985; Kirsch, 2007). Calcified diseases associated with osteoarthritis are correlated with the deposition of calcium pyrophosphate dihydrate (CPPD) crystals (25–55% of the time) and/or of the deposition of basic calcium phosphate (BCP) crystals (35–70% of the time) consisting of carbonate-substituted HA and octacalcium phosphate (Gordon *et al.*, 1984; Carrol *et al.*, 1991; Derfus *et al.*, 2002; Nalbant *et al.*, 2003). It has been proposed that MVs could be the target of a potential therapy for pathologic calcifications (Anderson, 2007). MVs of affected cartilage in osteoarthritis increases TNAP activity as much as 30-fold and induce HA deposition (Ali, 1985) indicating that TNAP (Narisawa *et al.*, 2007; Sidique *et al.*, 2009) could be a likely drug target, among other enzymes implicated in the mineral formation. Indeed, one likely source of P_i in the extracellular matrix to sustain HA nucleation and growth is obtained through the activity of TNAP (either within cells or within MVs) (Register *et al.*, 1986; Balcerzak *et al.*, 2003; Anderson *et al.*, 2004) which can produce P_i but can also hydrolyze PP_i, an inhibitor of HA formation (Register & Wuthier, 1985). A complete inhibition of TNAP may decrease HA deposition; however, it will increase calcium pyrophosphate dihydrate (CPPD) deposition, since accumulation of PP_i in the presence of Ca^{2+} leads to CPPD. Therefore, a partial inhibition of TNAP could be more effective than a complete inhibition to prevent calcified diseases. Levamisole, a known TNAP inhibitor, has been used for curing rheumatoid arthritis (McGill, 1976; Miller *et al.*, 1980) suggesting that TNAP could serve as drug target for preventing HA deposition in cartilage.

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