Center Probe Report

Project Title: Pharmacological Inhibitors of Tissue-nonspecific Alkaline Phosphatase (TNAP)
Grant Number: MH077602-01

Screening Center Name: San Diego Center for Chemical Genomics (SDCCG)
Principal Investigator of Screening Center: Dr. John Reed

Assay Provider & Institution: Dr. Jose Luis Millan, Burnham Institute for Medical Research

Assay or Pathway Target: Tissue-nonspecific Alkaline Phosphatase (TNAP)

Probe PubChem Compound Identifiers (CID/SID): MLS-0146291 (probe submission to PubChem in progress); optimized from lead CID-646303 (pyrazole series).

A. Assay provider information

Alkaline phosphatases (EC 3.1.3.1) (APs) catalyze the hydrolysis of phosphomonoesters, releasing phosphate and alcohol (Hessle *et al*. 2002; Hui *et al.* 1998; Fishman *et al*. 1971). APs are dimeric enzymes found in most organisms. In humans, four isozymes of APs have been identified. Three isozymes are tissue-specific and the fourth one is designated tissue-nonspecific alkaline phosphatase (TNAP). TNAP is implicated in disorders associated with defective bone mineralization such as rickets and osteomalacia, and also in the calcification of blood vessels. Small molecule inhibitors of TNAP would be of great value to investigate the potential of TNAP as a therapeutic point of intervention. The aim of this project, therefore, was to identify novel highly potent and selective inhibitors of TNAP which to date have not been available.

B. Screening Center information

B1. Assay Implementation and Screening

B1.1. PubChem Bioassay Name: TNAP luminescent HTS assay
B1*.2. List of PubChem bioassay identifiers generated for this screening project (AIDS)*: 518, 614, 615, and 1012.
B1.2a. List of relevant AIDs that may be used as counterscreen information: 690, 696, 813, 1001, and 1016.

B1.3. Primary Assay Description as defined in PubChem:

TNAP luminescent HTS assay (AID 518):

TNAP screening was developed and performed at the San Diego Center for Chemical Genomics (SDCCG) as part of the Molecular Library Screening Center Network (MLSCN). XO1 submission, MH077602-01, Pharmacological inhibitors of tissue-nonspecific alkaline phosphatase (TNAP), Assay Provider Dr. Jose Luis Millan, Burnham Institute for Medical Research, La Jolla, CA.

TNAP assay protocol:

Materials:

1) TNAP protein was provided by Dr. Jose Luis Millan (Burnham Institute for Medical Research, San Diego, CA). The CDP-star was obtained from Applied Biosystems.
2) Assay Buffer: 250 mM DEA, pH 9.8, 2.5 mM $MgCl_2$, and 0.05 mM $ZnCl_2$.
3) TNAP working solution contained a 1/800 dilution in assay buffer. Solution was prepared fresh prior to use.
4) CDP-star working solution contained 125 uM CDP-star in MQ water.
5) Levamisole working solution - 5 mM in 10% DMSO.

HTS protocol:

1) 4 uL of 100 uM compounds in 10% DMSO were dispensed in columns 3-24 of Greiner 384-well white small volume plates (784075).
2) Using the Thermo wellmate dispenser 4 uL the following solutions were added:
   a. 10% DMSO - column 1 (negative control).
   b. Levamisole working solution - columns 2 (positive control).
3) 8 uL of TNAP working solution was added to the whole plate using WellMate bulk dispenser (Matrix).
4) 8 uL of CDP-star working solution was added to the whole plate using WellMate bulk dispenser (Matrix).
5) Final concentrations of the components in the assay were as follows:
   a. 100 mM DEA, pH 9.8, 1.0 mM $MgCl_2$, 0.02 mM $ZnCl_2$ (columns 1-24)
   b. 1/2000 dilution TNAP (columns 1-24)
   c. 50 uM CDP-star (columns 1-24)
   d. 1 mM Levamisole (columns 2)
   e. 2 % DMSO (columns 1-24)
   f. 20 uM compounds (columns 3-24)
6) Plates were incubated for 30 mins at room temperature.
7) Luminescence was measured on the Envision plate reader (Perkin Elmer).
8) Data analysis was performed using CBIS software (ChemInnovations, Inc).
9) Compounds with greater than 50% inhibition of TNAP at 20-uM concentration are defined as actives of the primary screening and proceed to the dose-response confirmation stage.

Dose-response confirmation screening protocol:

1) Dose-response curves contained 10 concentrations of compounds obtained using 2-fold serial dilution. Compounds were serially diluted in 100% DMSO, and then diluted with water to 10% final DMSO concentration. 4 uL compounds in 10% DMSO were transferred into columns 3-22 of Greiner 384-well white small-volume plates (784075). Columns 1-2 and 23-24 contained 4 uL of levamisole working solution and 10% DMSO, respectively.
2) 8 uL of TNAP working solution was added to the whole plate using WellMate bulk dispenser (Matrix).
3) 8 uL of CDP-star working solution was added to the whole plate using WellMate bulk dispenser (Matrix).
4) Plates were incubated for 30 mins at room temperature.
5) Luminescence was measured on the EnVision plate reader (Perkin Elmer).

6) Data analysis was performed using CBIS software (ChemInnovations, Inc) using sigmoidal dose-response equation through non-linear regression.

B1.4. Center Summary of the Primary and Confirmatory Screen:

The MLSMR collection containing 64394 compounds was screened for inhibitors of TNAP in the luminescent assay at a concentration of 20 uM. The concentration of CDP-star substrate was kept equal to its K_m value to ensure optimal sensitivity for inhibitors with various mode of actions. During the primary screen 73 primary positives were identified resulting in a 0.11% hit rate. All the primary positives were tested in dose-response mode. Of those, 53 compounds were confirmed to have apparent inhibition and IC_{50} values in the range of tested concentrations. This represents a confirmation rate of 73%.

All positives were also tested in an assay panel containing 1) placental alkaline phosphatase (PLAP) luminescent assay (substrate: CDP-star), 2) solubility and 3) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) assay. The latter two assays are designed to identify compounds that are likely to interfere with the assay components and would appear as unspecific inhibitors.

B1.5. Comparative data on probe, similar compound structures and information on existing probes available to the public:

Levamisole was previously reported by the assay provider to act as an inhibitor of TNAP with a Ki = 21.4 uM (Narisawa et al. 2007). In the same study a high throughput screening approach was used to identify three additional small molecule inhibitors of TNAP, ChemBridge DIVERSet compounds 5361418, 5923412 and 5804079 (Table 1).

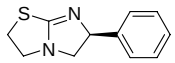
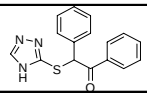
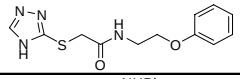
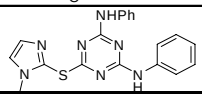
		TNAP-C
Compound #	**Structure**	**Ki (uM)**
Levamisole		21.4
5361418		5.6
5923412		5.6
5804079		6.5

Table 1. In vitro data on existing inhibitors of TNAP.

B2. Probe Optimization

B2.1 Chemical name of probe compound: N-(2-hydroxyethyl)-3-(2,3,4-trichlorophenyl)-1H-pyrazole-5-carboxamide

B2.2. Draw probe chemical structure and show stereochemistry if known: see Figure 1.

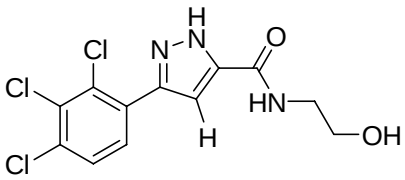


Figure 1. Structure of MLS-0146291

B2.3. Describe mode of action for biological activity of probe:

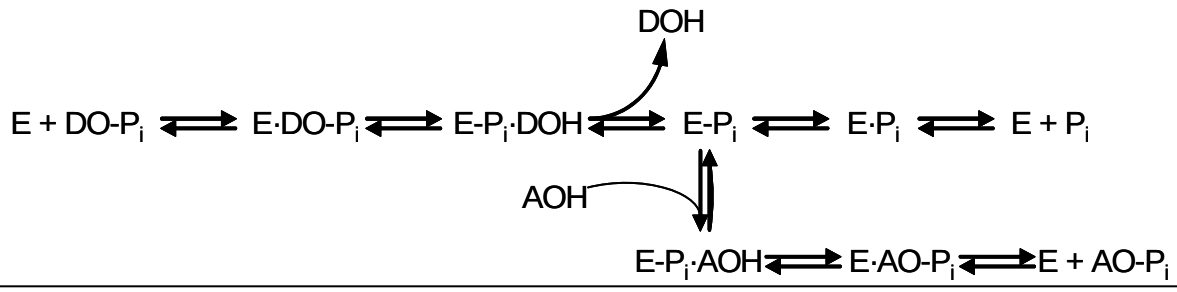
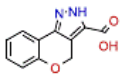
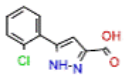
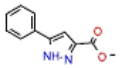
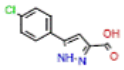
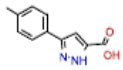
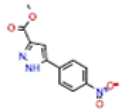
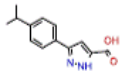
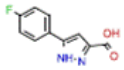
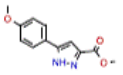
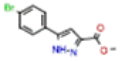
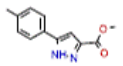
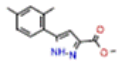
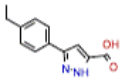
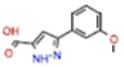
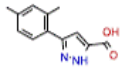
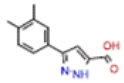
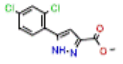
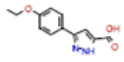
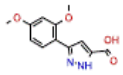
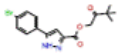
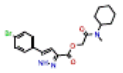
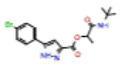
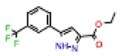


Figure 2: The catalytic mechanism of the alkaline phosphatase reaction (Holtz, K.M. (1999). The initial alkaline phosphatase (E) catalyzed reaction consists of a substrate (DO-Pi) binding step, phosphate-moiety transfer to Ser-93 (in the TNAP sequence of its active site) and product alcohol (DOH) release. In the second step of the reaction, phosphate is released through hydrolysis of the covalent intermediate (E-Pi) and the non-covalent complex (E·Pi) of the inorganic phosphate in the active site. In the presence of nitrogen-containing alcohol molecules (AOH), such as the buffer diethanolamine (DEA), phosphate is also released via a transphosphorylation reaction.

TNAP catalyzes a dephosphorylation reaction that proceeds through the formation of a stable covalent intermediate. The majority of mechanistic studies on alkaline phosphatases have been performed on *E.coli* alkaline phosphatase. This information is directly applicable to the mammalian alkaline phosphatases due to a high degree of sequence and structural homology. All alkaline phosphatases exist as homodimers, and oligomerization is required for their catalytic activity. Alkaline phosphatases catalyze the hydrolysis of phosphate monoesters via a covalently bonded phosphoserine intermediate. The detailed mechanism of a general alkaline phosphatase reaction is outlined in Figure 2.
Inorganic pyrophosphate (PP_i) and pyridoxal-5'-phosphate (PLP), a form of vitamin B6, are the endogenous substrates for TNAP. As with other alkaline phosphatases, the hydrolysis of the phosphoserine intermediate is the rate limiting step of the TNAP overall reaction and consequently in the steady state the majority of the enzyme exists in the covalent intermediate form. The new small molecule probes inhibit TNAP catalytic activity through reversible equilibrium binding. No difference was noticed in compound potency whether TNAP was pre-incubated with the compound or not. The binding of TNAP inhibitors is uncompetitive versus the phosphate donating substrate and competitive versus the phosphate accepting substrate, such as DEA.

B2.4. Has this compound been provided to the MLSMR: Submission to the MLSMR is in progress.

B2.4a. Commercial vendor information if available for probe or analogs that have been purchased: See Table 2 below for Vendor information on purchased pyrazole series analogues.

Structure	Source	Cat #	PubChem SID	PubChem CID	Structure	Source	Cat #	PubChem SID	PubChem CID
	ChemBridge	7694322	26513937	776480		Asinex	BAS 06804308	26514187	646303
	Asinex	BAS 06491662	26514188	644585		ChemBridge	7226006	26513923	738819
	ChemBridge	7278181	26513823	818267		ChemBridge	7510433	26513928	891992
	ChemBridge	7680660	26513936	975396		ChemBridge	7928141	26513950	940540
	ChemBridge	7942049	26513951	1243311		ChemBridge	9033609	26513966	3239557
	ChemBridge	9035017	26513968	2060300		ChemBridge	9056965	26513969	4737229
	ChemBridge	9058573	26513970	3524248		ChemBridge	9059707	26513971	1399024
	ChemBridge	9060908	26513972	4274816		ChemBridge	9061454	26513973	4598704
	ChemBridge	9061903	26513974	6472157		ChemBridge	9066340	26513978	3159737
	ChemBridge	9066402	26513979	4737220		Enamine	T5686634	26514041	16384437
	Enamine	T5687507	26514042	16381787		Enamine	T5687508	26514043	16382054
	Enamine	T0517-4276	26514027	2422649		ChemBridge	9107129	26513824	4736813

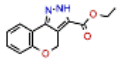
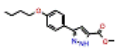
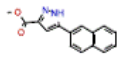
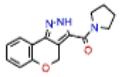
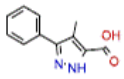
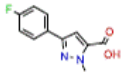
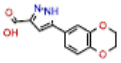
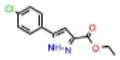
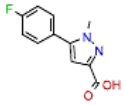
	ChemDiv	C066-3808	26513991	6621083		ChemDiv	C796-0421	26514004	16654604
	ChemDiv	C796-0480	26514005	2771808		ChemDiv	G365-0418	26514018	16654607
	Asinex	BAS 07750312	26513862	3156991		Asinex	BAS 14036298	26513836	6498679
	Asinex	BAS 01543594	26513852	847161		Asinex	BAS 07800681	26513863	619485
	Asinex	BAS 10150934	26513864	6484191					

Table 2. Commercial vendor description for purchased pyrazole series analogues.

B2.5. Description of any secondary screens used to optimize probe structure:

We confirmed the selectivity of the probe in PLAP assay, and optimized its physicochemical properties using GAPDH and solubility assays, mentioned above.

B2.6. Center comments on chemistry strategy leading to probe identification:

Pyrazole Series

As with the other TNAP hits, initial follow up on the pyrazole derivative CID-646303 was accomplished through analogue by catalogue. During the initial phase of testing analogues we discovered some important features of the SAR. Thus, we improved potency in this series from IC_{50} = 0.98 uM for the lead to IC_{50} = 0.50 uM for the 2,4-dichlorophenyl ester derivative MLS-0091995 (see Figure 3). Furthermore, conversion of the tricyclic derivative MLS-0004120, with an IC_{50} value of 1.33 uM, to the pyrrolidine amide analogue MLS-0111547 leads to a 3-fold improvement in potency (IC_{50} = 0.48 uM). We used this information to design a library of amide analogues as shown in Figure 3 and the Schemes below. Through **2** iterations of synthesis a total of **54** analogues (two libraries of 26 and 28 analogs) were prepared and tested *in vitro*. This led to the identifcation of five analogues with potency values in the range of 20-70 nM (see Table 3).

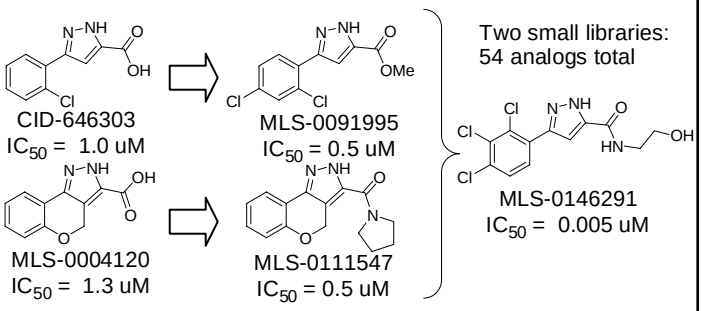
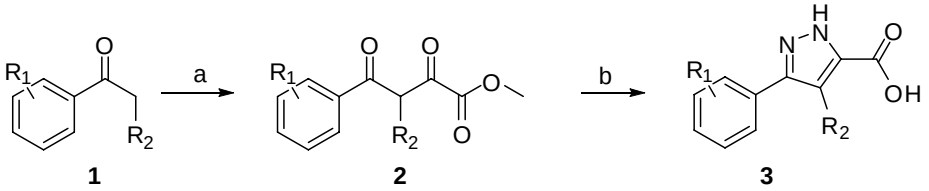
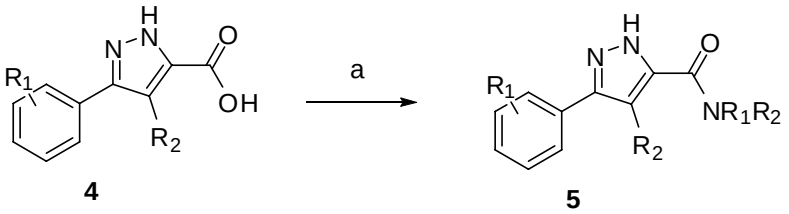


Figure 3: Summary of CID-646303 series optimization.

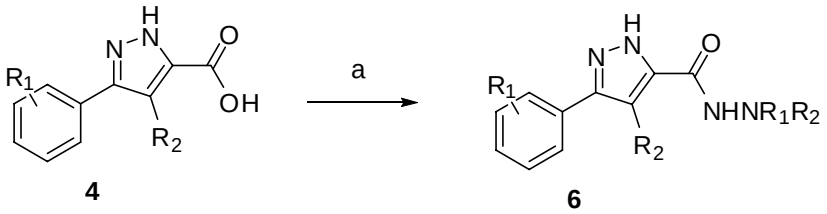
The synthetic chemistry we used for the preparation of the pyrazole acids is shown in Scheme 1. Thus, reaction of acetophenone derivatives **1** with sodium methoxide and dimethyl oxalate gives the 1,3-diketone derivative **2** in excellent yields (75 – 90 %). Compound **2** is reacted with hydrazine to give the corresponding pyrazole. Saponification of the methyl ester allows access to the pyrazoleacids **3**. The synthetic chemistry we used for hit optimization is shown in Scheme 2 and 3. In Scheme 2 the pyrazole acid **4** is treated with HOBT, EDC and DIEA followed by addition of the desired amine producing the amides **5**. In Scheme 3 the pyrazole acid **4** is treated with HOBT, EDC and DIEA followed by addition of the desired hydrazine producing the hydrazide derivative **6**.



Scheme 1. Reagents and conditions: (a) (i) NaOMe, Et_2O, Dimethyl Oxylate, 25^oC, 4 - 12 hours, (ii) HOAc, (75- 90 %) (b) (i) N_2H_4, HOAc, 100 ^{o} C, 12h (50 - 85 %) (ii) LiOH, THF, MeOH, reflux, (iii) HCl (90 - 95 %).



Scheme 2. Reagents and conditions: (a) EDC, HOBT, DMF, DIEA, NHR_1R_2(85-95 %)



Scheme 3. Reagents and conditions: (a) EDC, HOBT, DMF, DIEA, $NH_2NR_1R_2$ (85 - 95 %)

The first set of 26 analogues we synthesized were tested *in vitro* and we observed that amides with chain lengths of 3 carbons or less were the most active (see Table 3). The incorporation of a hydroxyl group on the amide generally increased potency (MLS-0146161 and MLS-0146165). Branching of the amides generally decreased potency in our assay especially when the chain length was greater than three carbon atoms (data not shown). We synthesized one hydrazide analog (MLS-0146145) and it was very potent. Substitution on the phenyl ring was limited to the 2,4-dichloro and 2,4-dichloro-5-fluoro substituent

pattern. In all cases the 2,4-dichloro analogues were more potent than the corresponding 2,4-dichloro-5-fluoro analogues. In light of these results we synthesized a new set of 28 pyrazole analogues that served to investigate several portions of the pyrazole structure.

One goal was to explore the SAR when the substituent pattern on the aromatic ring was changed while fixing the amide as the 2-hydroxy ethyl moiety of MLS-0146161. The best compound in this series is the 2,3,4-tri-Cl-phenyl anologue **MLS-0146291**. It shows exceptional activity with an IC_{50} of 5.19nM. This is the compound that we nominate as our probe. Other analogues synthesized increase diversity of the hydrazides and introduce more hydroxy groups on the amide tails while fixing the 2,4-di-chloro substituents on the aromatic ring. In this series, when the hydroxyl ethyl chain was increased by one or two additional carbon atoms the activity did not decrease. When a bis-hydroxyl ethyl side chain was introduced the activity remained the same as MLS-0146161.

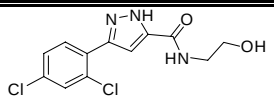
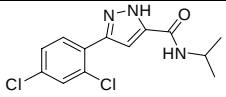
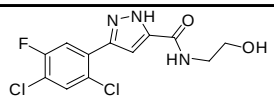
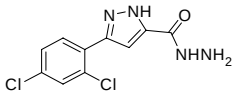
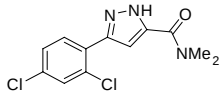
Compound #	Structure	TNAP-L IC_{50} (uM)
MLS-0146161		0.020
MLS-0146149		0.024
MLS-0146165		0.042
MLS-0146145		0.052
MLS-0146148		0.075

Table 3. Data on selected analogues of TNAP hit CID-646303.

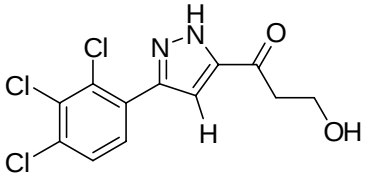
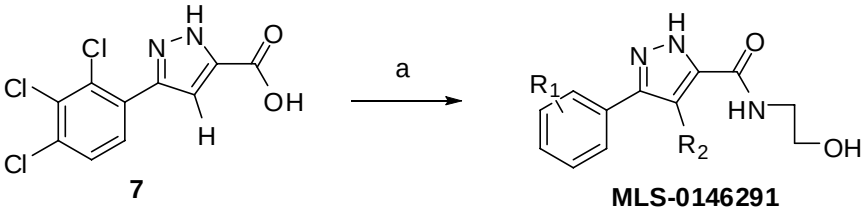


Figure 4. Highly potent TNAP inhibitor probe MLS-0146291 IC_{50} = 5.19 nM

B2.7. Detailed synthetic pathway for making the probe:



Schem 4. Reagents and conditions: (a) EDC, HOBT, DMF, DIEA, $NH_2CH_2CH_2OH$ (85%)

B2.8. Known probe properties:

MLS-0146291 demonstrated predictable behavior in the *in vitro* assay. Its inhibition curves were characterized with a Hill Coefficient nearing unity indicating no issues with the compound solution behavior. This compound also demonstrated high solubility at all the concentrations tested.

B2.9. *Center summary of probe properties (solubility, absorbance/fluorescence, reactivity, toxicity, etc.) and recommendations for the scientific use of probe as research tool:*
The probe has favorable calculated properties, low molecular weight (334.59) and structural features that are similar to known drugs, and can thus be considered a “drug-like” molecule. It has a log P value of 2.682, with 1-3 being the range of most oral drugs. The total polar surface area (TPSA) is 78.96, also indicative of good oral bioavailability. It is also a rigid molecule with 4 rotatable bonds, 3 H-bond donors and 5 H-bond acceptors. Thus, this probe’s physicochemical properties make it useful for continued study, including *in vivo* models of efficacy and tolerance.

C. Appendices

C1. Comparative data on probe, similar compound structures and prior probes
Comparative analysis of the probe MLS-0146291 in relation to prior existing probes is provided in several sections above.

C2. Comparative data showing probe specificity for target or for cell-based/phenotypic assays data should be shown in orthogonal cell-based assay systems that address the pathway of interest.

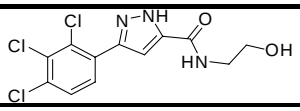
Compound ID	Structure	SDCCG panel (IC_{50}, uM)			PubChem (# of assays)			Total # of assays
		TNAP	PLAP	GAPDH	Active	Inactive	Incon-clusive	
MLS-0146291		0.005	>10	>10	TNAP	199	2	**203**

Table 4. 1. Characterization of MLS-0146291 SDCCG panel of assays.
2. Selectivity of pyrazole hit CID-646303 in PubChem assays.

Compound MLS-0146291 was tested in assays against another alkaline phosphatase (PLAP) which shares 50% sequence identity with TNAP, and a housekeeping enzyme (GAPDH). The compound did not show any inhibition at or below 10 uM in these assays (Table 4), providing a >2000-fold selectivity index against the closest counter-targets. In addition, the lead pyrazole CID-646303 has been extensively profiled in assays deposited in PubChem. Out of 201 assays tested the compound was inactive in 199 and inconclusive in 2 assays.

C3. Bibliography

Fishman, W. H. and Sie, H.-G. (1971) Organ-specific inhibition of human alkaline phosphatase isoenzymes of liver, bone, intestine and placenta; L-phenylalanine, L-tryptophan and L-homoarginine. *Enzymologia* 41: 140-167.

Hessle, L., Johnson, K. A., Anderson, H. C., Narisawa, S., Sali, A., Goding, J.W., Terkeltaub, R., and Millán, J. L. (2002) Tissue-nonspecific alkaline phosphatase and plasma cell membrane glycoprotein-1 are central antagonistic regulators of bone mineralization. Proc. Natl. Acad. Sci. USA 99:9445-9449.

Hui, M., and Tenenbaum, H.C. (1998) New face of an old enzyme: alkaline phosphatase may contribute to human tissue aging by inducing tissue hardening and calcification. Anat. Rec. 253**:**91-4.

Narisawa S, Harmey D, Yadav MC, O'neill WC, Hoylaerts MF, Millán JL. (2007) Novel inhibitors of alkaline phosphatase suppress vascular smooth muscle cell calcification. J Bone Miner Res. Nov; 22(11):1700-10.