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): CID-2931238; SID-4637691; SID-4261842; SID-3558110; SID-26514529; SID-26514225; SID-26514170; SID-11358621; SID-8929293 (quinolinesulfonamide 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, and 615.
B1.2a. List of relevant AIDs that may be used as counterscreen information: 690 and 696.

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.

<u>TNAP assay protocol:</u>

<u>Materials:</u>

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) TNAP colorimetric assay (substrate: p-nitrophenol phosphate), 2) placental alkaline phosphatase (PLAP) luminescent assay (substrate: CDP-star), 3) intestinal alkaline phosphatase (IAP) luminescent assay (substrate: CDP-star), 4) solubility and 5) 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). Side-by-side comparison of the mentioned compounds with the chemical probe identified in this study is described in more detail below.

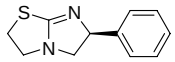
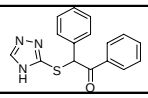
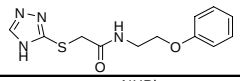
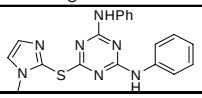
		TNAP-C
Compound #	**Structure**	**Ki (uM)**
Levamisole		21.4
5361418		5.6
5923412		5.6
5804079		6.5

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

B2. Probe Optimization

B2.1 Chemical name of probe compound: 2,5-dimethoxy-N-(quinolin-3-yl)benzenesulfonamide.

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

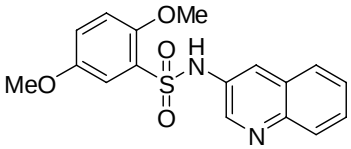


Figure 1. Structure of CID-2931238

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

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.

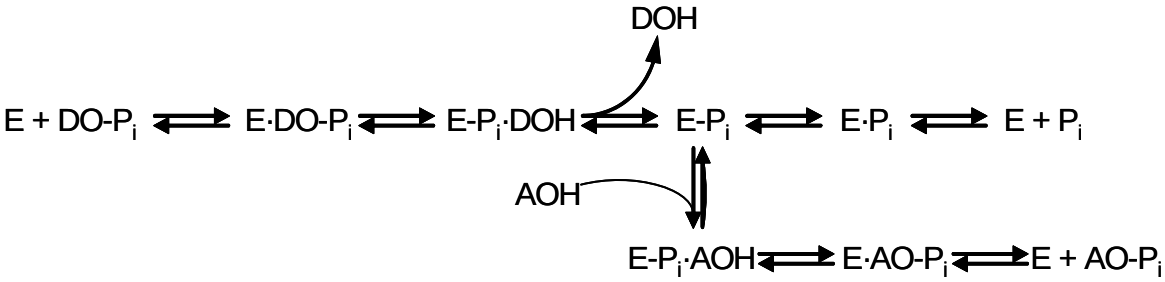
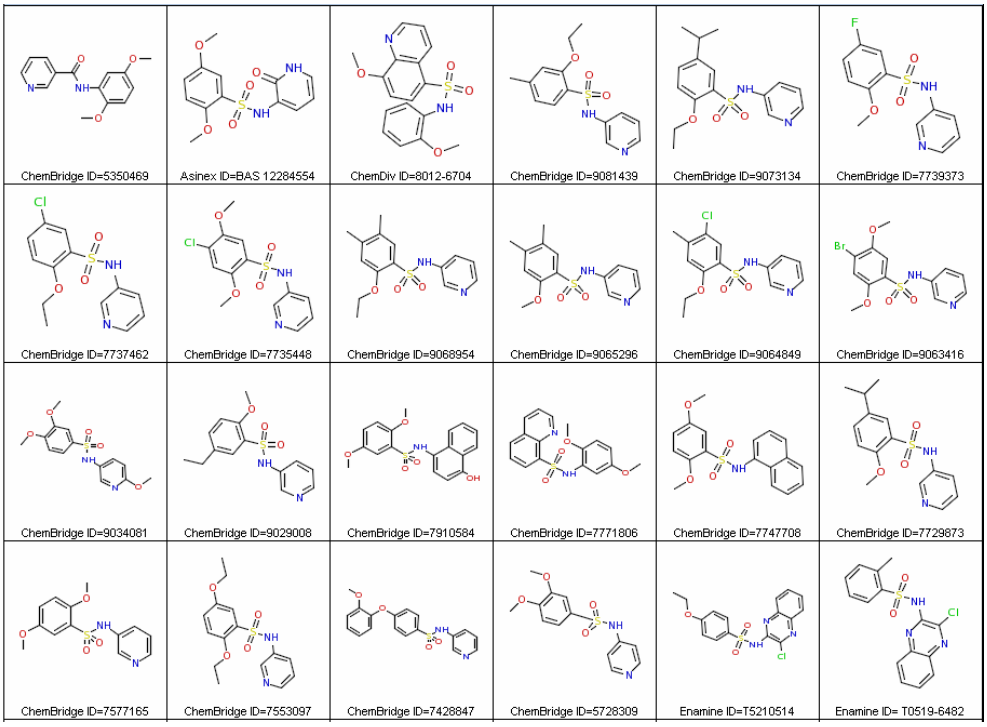


Figure 2: The catalytic mechanism of the alkaline phosphatase reaction (Holtz, K.M. (1999). The initial alkaline phosphatase (E) catalyzed reaction consists of a substrate (DO-Pi) binding step, phosphate-moiety transfer to Ser-93 (in the TNAP sequence of its active site) and product alcohol (DOH) release. 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.

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 compound CID-2931238 inhibits 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 CID-2931238 is uncompetitive versus the phosphate donating substrate (CDP-star or pNPP) and non-competitive versus the phosphate accepting substrate, such as DEA.

B2.4. Has this compound been provided to the MLSMR: The compound was contained within the MLSMR as part of the standard collection.

B2.4a. Commercial vendor information if available for probe or analogs that have been purchased:
The Probe is available commercially from ChemBridge under catalog number of 7234072.
Table 2 below lists the commercial analogs purchased and tested in dose-response mode in the primary assay.



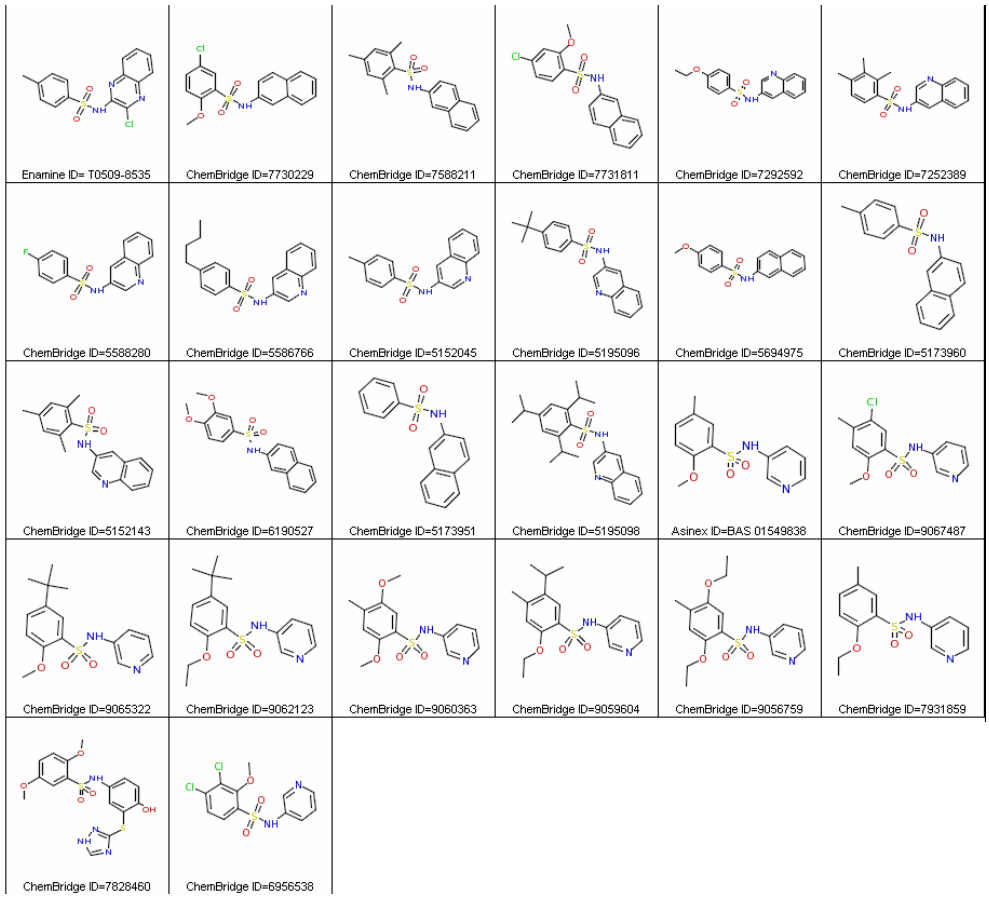


Table 2. Commercial analogs of CID-2931238 purchased and tested in the primary assay.

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

The assay submitted by the PI is based on color change due to dephosphorylation of p-nitrophenol phosphate and concomitant release of p-nitrophenol. It was designed to run in the presence of a saturating concentration of DEA buffer participating in the reaction as a phosphate-acceptor substrate. The high ionic strength of the reaction buffer and potential color interference of yellow-colored compounds caused some concern over the assay performance and sensitivity. To address those issues, two additional assays were developed at the SDCCG. One of them is based on a colorimetric detection of phosphate released from pNPP using a malachite green-molybdate reagent. Due to the high buffer capacity of 1M DEA, pH 9.8 present in the colorimetric assay, a specially formulated detection reagent was utilized.

The second assay is based on CDP-star, a luminescent substrate developed and optimized for the detection of alkaline phosphatases in blotting techniques. The luminescent assay is several orders of magnitude more sensitive than the colorimetric assay. It was optimized to work at a sub-K_m concentration of DEA and lower concentration of the enzyme. The signal output is stable over several hours, requires no quenching and, therefore, provides an automation-friendly approach. Taking this into consideration, the luminescent assay was utilized for primary screening and the colorimetric assay was reserved for secondary confirmatory screening. To assess the selectivity of compounds across the alkaline phosphatase family luminescent assays were developed for human placental alkaline phosphatase (PLAP) and human intestinal alkaline phosphatase (IAP).

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

Our approach to investigating the SAR around CID-2931238 is shown graphically in Figure 3. We defined four structural elements in the molecule to investigate in the initial phase: (a) the quinoline fused aryl ring, (b) the aryl substituents, (c) the sulfonamide linker, and (d) the quinoline heteroatom. Analogues of hits were characterized in the luminescence-based assay (TNAP-L, see Table 3) to determine potency against TNAP. The potency data for selected analogues of CID-2931238 are shown in Table 3. Preliminary *in vitro* data for this series suggests that despite the fact that the SAR around the lead structure is tight, there is a tractable SAR emerging nonetheless. To date, 55 analogues of CID-2931238 have been purchased and tested, while a further 61 analogues have been synthesized. In order to evaluate the importance of the fused aryl ring, an analogue of CID-2931238 was synthesized and tested in which the quinoline moiety was replaced with pyridine (MLS-0068486). This led to a reduction in potency of roughly 4-fold compared with the lead structure (see Table 3). Preliminary data also suggest that the substituents on the phenyl ring are critical for potency. For example, the analogue MLS-0090845 in which both methoxy substituents were removed, is inactive in the assay up to 100 uM. Compound MLS-0090844 is an exact analogue of the lead structure in which the 2-methoxy substituent is removed, resulting in a 100-fold reduction in potency compared with the lead structure. Similarly, the 3,4-dimethoxy (MLS-0090842) and 2,4-dimethoxy (MLS-0111875) derivatives were inactive and 19.5 uM, respectively. We have also synthesized analogues of the lead structure in which the sulfonamide linker is reversed or replaced with an amide. This includes the $-SO_2NH-$ to $-NHCH_2-$ replacement analogues MLS-0111667 and

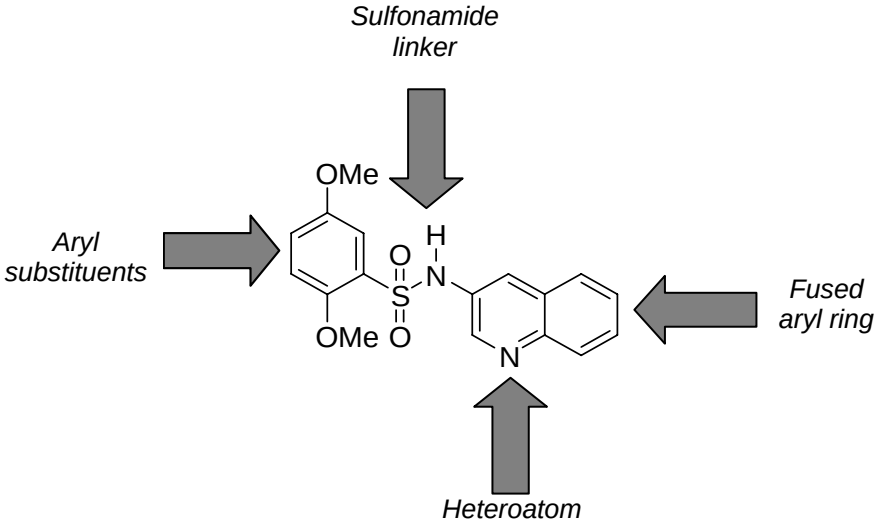


Figure 3. Summary of approach to SAR for CID-2931238

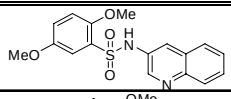
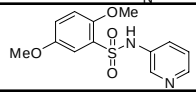
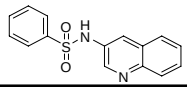
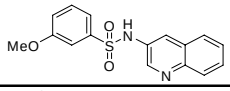
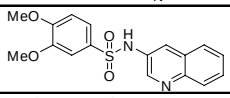
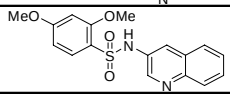
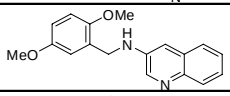
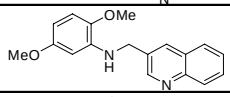
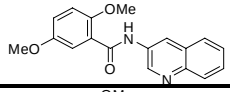
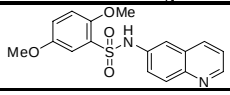
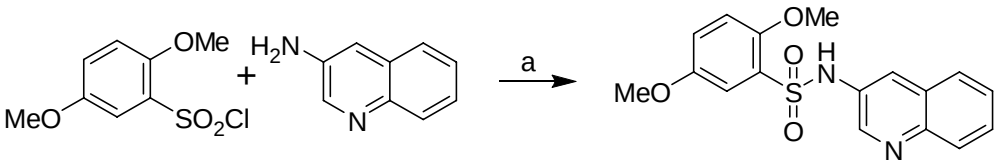
Compound #	Structure	TNAP-L IC_{50} (uM)
CID-2931238		0.25
MLS-0068486		0.916
MLS-0090845		no fit
MLS-0090844		25.7
MLS-0090842		no fit
MLS-0111875		19.5
MLS-0111667		no fit
MLS-0111668		no fit
MLS-0111666		no fit
MLS-0111639		no fit

Table 3. *In vitro* SAR data on inhibitors of TNAP

MLS-0111666, and the amide derivative MLS-0111666, all of which were inactive *in vitro.* Finally, the presence and/or position of the quinoline heteroatom is important for the potency of the scaffold. Several analogues were tested in which the quinoline moiety was replaced with naphthyl *i.e.* substitution of N for C in the molecular framework, and this modification results in complete ablation of *in vitro* activity (data not shown). Similarly, transposition of the quinoline heteroatom, as in MLS-0111639, eliminates activity.

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



Scheme 1. Reagents and conditions: (a) Et_3N, CH_2Cl_2, rt, 2h, 84%.

B2.8. Known probe properties:

The probe is active in 2 assays deposited in PubChem: in addition to TNAP, the only other assay where it shows inhibition (73% at 5 uM) is the CYP2C9 assay. The compound is inactive in 131 other assays as reported in PubChem.

CID-2931238 was also evaluated in an *in vitro* assay measuring stability in rat liver microsomes (RLM) to provide an assessment of the suitability of the compound for *in vivo* experiments. After a 20 minute incubation virtually all of the compound remained (see Figure 4), suggesting that CID-2931238 may be suitable for in vivo proof-of-concept studies.

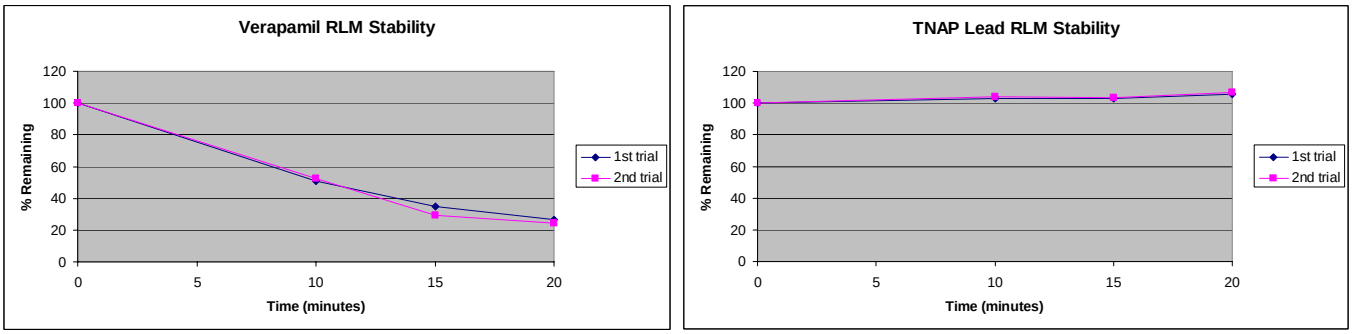


Figure 4. *In vitro* rat liver microsome (RLM) stability data. The graph on left shows data for the control compound verapamil, the graph on right data for the lead quinolinesulfonamide CID-2931238.

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 compound CID-2931238 demonstrated predictable behavior in the performed TNAP assays. Its average IC_{50} values were equal to 193, 158 and 192 nM in the luminescent, colorimetric p-nitrophenol-release and colorimetric phosphate-release assays, respectively. The average Hill coefficient values observed in these assays were equal to 1.12, 1.02, and 0.99, respectively. The CID-2931238 compound was soluble in aqueous solutions at the concentrations below 25 uM. It had no effect on GAPDH activity and demonstrated no cytotoxicity in multiple assays deposited in PubChem. The compound CID-2931238 is a sensitive and novel tool for the characterization of TNAP activity in various biological systems. Due to the extracellular localization of TNAP, the compound could presumably serve as a useful TNAP chemical probe in animal studies.

TNAP inhibition abrogates calcification in* ex vivo *experiments.

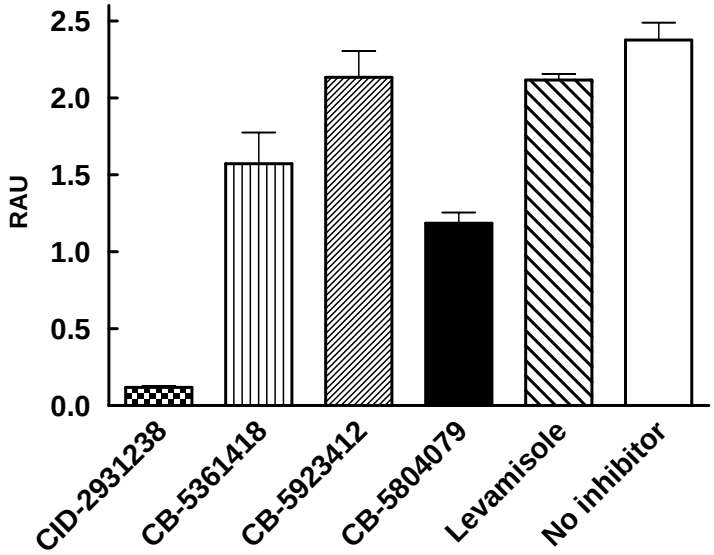


Figure 5. Suppression of calcification by CID-2931238 in calvarial osteoblast cultures. Osteoblasts from calvaria bones of WT mice were cultured for 19 days. Media supplemented with 50 ug/ml ascorbic acid and 10 mM β-glycerophosphate were renewed every second or third day (total 7 medium changes). The concentration of each compound was maintained at 30 uM.

The ability of CID-2931238 to inhibit calcification in vascular smooth muscle cultured cells (VSMCs) was tested in the Assay provider's lab. Levamisole and the three compounds previously identified through the screening of ChemBridge DIVERSet collection and mentioned above were utilized as controls. As shown in Figure 5, CID-2931238 completely inhibited mineralization in this culture system at the concentration tested. It appeared much more potent than either levamisole or the ChemBridge compounds. In summary, compound CID-2931238 exceeds in potency all existing TNAP probes in both *in vitro* and *ex vivo* assays.

C. Appendices

C1. Comparative data on probe, similar compound structures and prior probes.

Comparative analysis of the CID-2931238 probe 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 CID-2931238 was tested in assays against two other alkaline phosphatases, PLAP and IAP which share 50% and 52% sequence identity with TNAP, respectively. The compound did not show any inhibition at or below 100 uM in these assays (Table 4), providing a >500 selectivity index against the closest counter-targets.

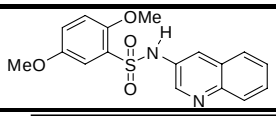
PubChem CID	Structure	SDCCG panel (IC_{50}, uM)			PubChem (# of assays)			Total # of assays
		TNAP	PLAP	IAP	Active	Inactive	Incon-clusive	
2931238		0.193	>100	>100	TNAP; CYP2C19	131	2	**136**

Table 4. Characterization of CID-2931238 in a panel of 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.