Center Probe Report

Project Title: Pharmacological Inhibitors of Human Intestinal Alkaline Phosphatase (IAP)
Grant Number: MH077602-01
(This probe project was initiated by the Center to take advantage of a secondary assay developed at the Center for the TNAP probe project).

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

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

Assay or Pathway Target: Intestinal Alkaline Phosphatase (IAP)

Probe PubChem Compound Identifiers (CID/SID): CID-2946732, SID-14726337, SID-26513934, SID-3604346.

Please provide the following descriptions:

A. Assay provider information

A1. Specific Aim:

The aim of this work is to identify inhibitors of intestinal alkaline phosphatase (IAP) that show selectivity for the target over the other alkaline phosphatase isozymes (PLAP, TNAP). The isozymes of alkaline phosphatase (AP; orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) are ectoenzymes anchored onto the cytoplasmic membrane *via* a phosphatidylinositol glycan moiety (Low and Prasad, 1988). They are capable of catalyzing dephosphorylation and transphosphorylation reactions of a wide spectrum of substrates *in vitro*. The presence of alkaline phosphatase isozymes in a range of species from bacteria to humans and their localization in proximity to the cell surface suggest that they play a role in facilitating the movement of substances across the cell membrane. In humans, four isozymes of APs have been identified. One isozyme is tissue-nonspecific (designated TNAP) and three other isozymes are tissue-specific and named according to the tissue of their predominant expression: intestinal (IAP), placental (PLAP) and germ cell (GCAP) alkaline phosphatases. Exact functions of the alkaline phosphatase isozymes are yet to be elucidated. Finding specific inhibitors for each isozyme would enable their detailed characterization.

A2, A3. Significance and Rationale:

IAP expression is largely restricted to the gut, especially to the epithelial cells (enterocytes) of the small intestinal mucosa. IAP has long been known to be associated with lipid absorption on the basis of the following evidence: 1) during fat absorption, parallel increases in IAP activity and triacylglycerol concentration are observed in the thoracic duct lymph (Glickman *et al.*, 1970); 2) IAP is associated with chylomicron secretion (Mahmood *et al.*, 1994 and 2003; Zhang *et al.*, 1996) but not with chylomicron formation (Nauli *et al.*, 2003), and serum IAP levels are

correlated with the levels of apolipoprotein B-48, a protein exclusive to intestinal chylomicrons in humans (Nakano *et al.*, 2006); 3) IAP is found in the membrane surrounding the neutral fat droplets in the villi of the intestinal mucosa during fat absorption (Mahmood *et al.*, 1994 and 2003) and is thought to transport dietary lipids from the intestinal tract into the circulation as a component of unilamellar membranes called surfactant-like particles (Zhang *et al.*, 1996); 4) L-phenylalanine (Linscheer *et al.*, 1971) and somatostatin (Bayer and Pointner, 1980; Hengl *et al*., 1979), are both inhibitors of IAP activity, attenuate fat absorption in the small intestine. However, the exact biological functions and the mechanism for the involvement of IAP in lipid absorption are still unknown.

IAP, as with all mammalian APs, is inhibited uncompetitively by a number of small molecules inhibitors (Fishman and Sie, 1971). Compounds which inhibit at milimolar concentrations include L-phenylalanine, (Ghosh and Fishman, 1966; Rufo and Fishman, 1972), L-tryptophan (Chi-Wei *et al*., 1971), L-leucine and phenylalanine-glycylglycine (Goldstein *et al*., 1982). While the biological implications of this inhibition are not known, these inhibitors have proven to be useful in the differential determination of AP isozymes as important diagnostic markers in many diseases. However, these known inhibitors of IAP are not specific for this AP isozyme and have very low affinity. In addition, they are common aminoacids that are ubiquitously present in tissues and involved in diverse metabolic pathways, and therefore, are not appropriate tools for biological studies. In this Probe Report we describe the identification and characterization of the first small molecules that inhibit IAP at sub-micromolar levels and which show selectivity for IAP vs. other AP isozymes.

B. Screening center information

B1. Assay Implementation and Screening

B1.1. *PubChem Bioassay Name:* Luminescent assay for identification of inhibitors of human intestinal alkaline phosphatase.
B1.2. *List of PubChem bioassay identifiers generated for this screening project (AIDS):* Data deposited to PubChem, AID 1017.
B1.2a. *List of relevant AIDs that may be used as counterscreen information*: 518, 614, 615, 690, 696, 1001, 1012, and 1016.

B1.3. *Primary Assay Description as defined in PubChem:*
Luminescent assay for identification of inhibitors of human intestinal alkaline phosphatase Data deposited to PubChem, AID number not yet available.

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

IAP luminescent assay protocol:

Materials:

1) Human IAP 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) IAP working solution contained a 1/80 dilution in assay buffer. Solution was prepared 1h prior to use and incubated at room temperature.
4) CDP-star working solution contained 442.5 uM CDP-star in MQ water.
5) L-phenylalanine working solution - 100 mM in 10% DMSO.

<u>IAP dose-response confirmation screening protocol:</u>

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).
2) Columns 1-2 and 23-24 contained 4 uL of L-phenylalanine working solution and 10% DMSO, respectively.
3) 8 uL of IAP 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) Plates were incubated for 30 mins at room temperature.
6) Luminescence was measured on the Envision plate reader (Perkin Elmer).
7) 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*

We originally developed the luminescence-based IAP assay for the selectivity characterization of potential TNAP inhibitor probes. A similar assay developed at the SDCCG to screen for TNAP HTS led to identification of several potent scaffolds that were completely inactive in the TNAP colorimetric assay provided by the assay provider. One of these scaffolds is characterized in detail below as a selective IAP inhibitor.

Representatives of the leading TNAP inhibitor structural series were tested in the IAP assay. It was observed that unlike the other scaffolds, some of the triazole deratives potently inhibit the IAP isozyme. Expanded screening (77 compounds total) led to identification of a compound that is a selective nM-inhibitor of IAP.

An additional sub-micromolar IAP assay using p-nitrophenol phosphate (pNPP) as substrate was developed at the SDCCG and was utilized for hit confirmation. Lead compounds were tested in a panel of assays consisting of 1) TNAP luminescent assay (substrate: CDP-star), 2) TNAP colorimetric assay (substrate: pNPP), 3) PLAP luminescent assay (substrate: CDP-star), 4) IAP luminescent assay (substrate: CDP-star), 5) IAP colorimetric assay (substrate: pNPP), 6) solubility and 7) 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:*

As mentioned above, several aminoacids were previously reported in literature to act as uncompetitive inhibitors of IAP with IC_{50} values in **mM** range (Table 1).

Name	IC_{50} (mM)	Literature Source
L-phenylalanine	0.8-3*	Goldstein et al., 1982; Rufo and Fishman, 1972
L-homoarginine	13-40	Suzuki et al, 1994; Goldstein et al., 1982
L-leucine	3.6	Goldstein et al., 1982
Levamisole	6.8	Goldstein et al., 1982
Phe-Gly-Gly	3.7	Goldstein et al., 1982
L-tryptophane	~2*	Chi-Wei et al, 1971

*calculated from % inhibition reported in the paper

Table 1. Existing IAP small molecule tools available to public.

B2. Probe Optimization

B2.1 *Chemical name of probe compound:* 2-(3-phenyl-1H-1,2,4-triazol-5-yl)-3a,4,7,7a-tetrahydro-1H-isoindole-1,3(2H)-dione.

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

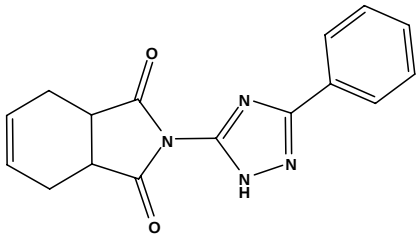


Figure 1. Structure of CID-2946732

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

Mechanistic studies performed on the CID-2946732 demonstrated that it inhibits IAP non-competitive to the CDP-star substrate. This is a novel mode of inhibition, since all known Probes mentioned above are uncompetitive vs the phosphate-donor substrate.

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 compound is available from ChemBridge (cat# 7670562).

B2.5. *Description of any secondary screens used to optimize probe structure:*
We developed two different IAP assays, the luminescent and a colorimetric assay. The colorimetric assay utilized pNPP substrate and monitored activity via changes in absorbance. The selectivity of compounds was tested in an assay panel with two other alkaline phosphatases, human placental alkaline phosphatase (PLAP) and human tissue-nonspecific alkaline phosphatase (TNAP).

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

Our approach to exploring the SAR for the IAP inhibitor series is shown graphically in Figure 2. For initial SAR studies we focused on three structural elements in the molecule to investigate: (a) the tetrahydrophthalimide ring, (b) the aryl ring, (c) the hydrocarbon linker between the aryl ring and the triazole unit. To date, 61 analogues in the triazole series have been purchased and tested, while a further 11 analogues have been synthesized. Compounds were characterized in the luminescence-based assay (IAP-L, see Table 2) to determine their potency against IAP. Inhibition of TNAP (TNAP-L) by analogues was determined in parallel for selectivity profiling. The *in vitro* data for this series suggest that there is a tractable SAR emerging for the IAP inhibitor triazole derivatives.

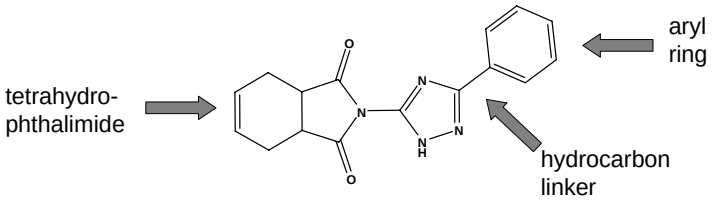


Figure 2. Summary of approach to SAR for CID-2946732

The original hit for this series (CID-2975791) was identified from HTS as a potent inhibitor of TNAP. Selectivity profiling revealed this compound also to be a submicromolar inhibitor of IAP (Table 2). The phthalimide analogue (MLS-0091978) was less potent against IAP (8.9 uM) and more potent against TNAP (0.67 uM), while the hexahydro phthalimide was equipotent against both enzymes. However, extension of the linker chain length to ethyl gave a compound (MLS-0091987) with good potency against IAP (0.72 uM) and 40-fold selectivity over TNAP. Two other analogues with ethyl linkers (MLS-0091984 and 0091982) show similarly high selectivity for IAP but are not as potent. Removal of the benzyl moiety in the hit, as in MLS-0091980, leads to elimination of activity against both isozymes, as does amine substitution onto the triazole ring (MLS-0010757). On the other hand, removal of just the methylene linker in the hit to the phenyl triazole derivative gives a compound (CID-2946732) that has an IC_{50} = 0.12 uM vs. IAP and is 7-fold selective for IAP over TNAP. Furthermore, replacement of the phenyl moiety with pyridine (MLS-0091977) or furan (MLS-0091966) gives derivatives with 6- and 10-fold

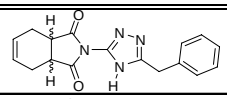
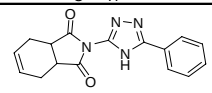
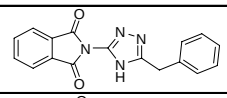
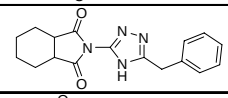
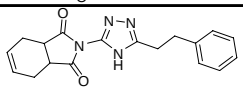
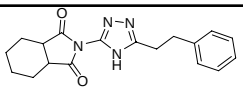
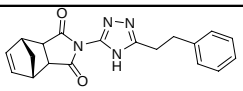
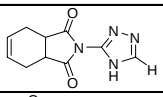
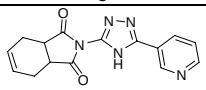
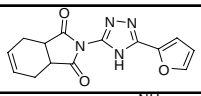
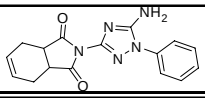
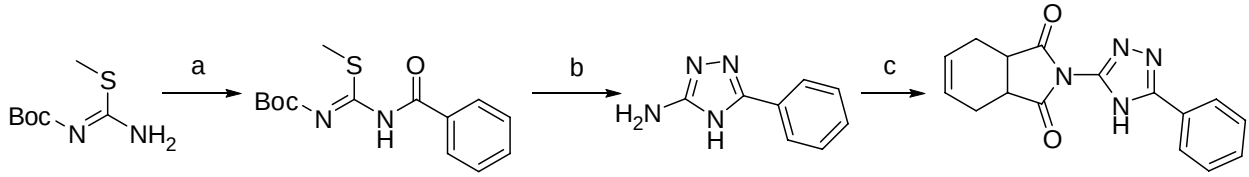
		TNAP-L	IAP-L
Compound #	**Structure**	**IC_{50} (uM)**	**IC_{50} (uM)**
CID-2975791 Hit		0.21	0.54
CID-2946732 Probe		0.80	0.12
MLS-0091978		0.67	8.87
MLS-0091981		1.57	1.10
MLS-0091987		27.90	0.72
MLS-0091984		> 100	1.75
MLS-0091982		30.60	4.75
MLS-0091980		> 100	>100
MLS-0091977		10.50	1.77
MLS-0091966		4.68	0.41
MLS-0010757		> 100	> 100

Table 2. SAR data for selected IAP inhibitors.

selectivity, respectively, for IAP vs. TNAP, and in the case of the furan derivative MLS-0091966 IC_{50} = 0.41 uM. Taking all the data into consideration the phenyl triazole derivative CID-2946732 was selected as a probe for IAP. It should be noted that other analogues in the series, such as MLS-0091987 and MLS-0091966 are also strong probe candidates, and work is ongoing to perform additional profiling studies to characterize these and other compounds in the series.

B2.7. *Reduced to practice detailed synthetic pathway for making the probe*:



Scheme 1. Reagents and conditions: (a) PhCOCl, DIEA, CH_2Cl_2, rt, 4h (70-85%); (b) (i) N_2H_4, DMSO, rt, 12-24h (75-85%); (ii) TFA, CH_2Cl_2, rt, 1h; (c) tetrahydrophthalicanhydride, AcOH,100º C, 6h (72%).

B2.8. *Known probe properties:*

CID-2946732 demonstrated potent inhibition for IAP and selectivity vs. other alkaline phosphatase isozymes TNAP and PLAP (Table 3). *PubChem* searches revealed that CID-2946732 was inactive in 68 other assays and demonstrated activity only in two additional assays, both for CYP isozymes: CYP2C9 and CYP2C19. These data suggest that CID-2946732 could serve as a valuable tool for the characterization of IAP biological function, especially in the gut tissue where alkaline phosphatase activity is predominantly accounted for by the IAP isozyme.

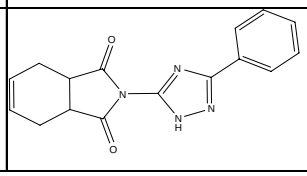
	STRUCTURE	Compound ID	IAP-L (IC_{50}, uM)	IAP-C (IC_{50}, uM)	TNAP-L (IC_{50}, uM)	TNAP-C (IC_{50}, uM)	PLAP-L (IC_{50}, uM)	2C19 (AID_778)	2C9 (AID_777)
CID-2946732	 Structure4	MLS-0091968	0.122 (n=7)	1.38 (n=7)	0.795 (n=7)	>100 (n=6)	4.81 (n=3)	79.6% inhibition at 5 uM	92.5% inhibition at 5 uM

Table 3. Selectivity profile for IAP probe CID-2946732.

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 (294.3) 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 2.096, 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 2 rotatable bonds, 1 H-bond donor and 6 H-bond acceptors. Thus, this probe’s physicochemical properties make it useful for continued study, including *in vivo* models of efficacy and tolerance.

The compound CID-2946732 inhibited IAP in two assays that utilize different substrates and modes of detection. The compound is soluble below 25 uM and demonstrated solubility issues above 25 uM. It had no effect on GAPDH activity and demonstrated no cytotoxicity in multiple assays deposited in *PubChem*.

CID-2946732 is a versatile, novel Probe for the characterization of IAP biological activity in various biological systems. Its potency by far (>6500-fold) and selectivity exceeds all other existing small-molecule inhibitors of IAP.

C3. Bibliography

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