Probe Report

Title: Therapeutic Inhibitors of Phosphomannose Isomerase
Authors: Michael Hedrick, Brock Brown, Justin Rascon, Eduard Sergienko, Vandana Sharma, Bobby Ng, Mie Ichikawa, Hudson Freeze, Derek Stonich, Ying Su, Shakeela Dad, Thomas D.Y. Chung, Russell Dahl, Nick Cosford
**Assigned Assay Grant #:** R03 MH082386-01
Screening Center Name & PI: *Conrad Prebys* Center for Chemical Genomics (*formerly Burnham Center for Chemical Genomics*) & Dr. John C. Reed
Chemistry Center Name & PI: *Conrad Prebys* Center for Chemical Genomics (*formerly Burnham Center for Chemical Genomics)* & Dr. John C. Reed
Assay Submitter & Institution: Dr. Hudson Freeze, Sanford-Burnham Medical Research Institute (*formerly Burnham Institute for Medical Research*)

PubChem Summary Bioassay Identifier (AID): 1545

Probe Structure & Characteristics:
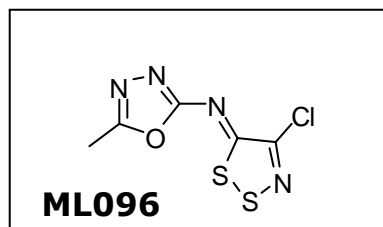
ML096

CID/ML	Target Name	IC50/EC50 (nM) [SID, AID]	Anti-target Name(s)	IC50/EC50 (µM) [SID, AID]	Select-ivity	Secondary Assay(s) Name: IC50/EC50 (nM) [SID, AID]
25199533 ML096	Phospho-mannose Isomerase (PMI)	1,070 nM IC50 SID-57309177 AID-1535	PMM2	>100 uM IC50 SID-57309177 AID-1655	>93 X	

Recommendations for the scientific use of this probe:

The probe can be used to inhibit human phosphomannose isomerase and due to the high homology between other eukaryotic orthologs, it will likely inhibit them as well. The probe can also be used to inhibit this enzyme in living cells since it is membrane permeable. One use of the probe would be to block catabolism of mannose-6-P in cells and direct it toward protein glycosylation. It may have therapeutic potential in phosphomannomutase 2-deficient CDG-Ia patients who are given mannose dietary supplements.

1. Scientific Rationale for Project

Congenital Disorders of Glycosylation (CDG) are autosomal recessive defects in the synthesis of N-linked oligosaccharide chains (1). CDG group I (CDG-I) defects are defined as those caused by mutations in genes encoding enzymes used for the synthesis and transfer of lipid linked oligosaccharide (LLO) to newly synthesized proteins in the lumen of the ER. The steps in this pathway and the genes encoding them are very similar from yeast to human. It requires 30-40 single gene products, each dependent on the previous step in the linear sequence to produce and transfer the LLO to protein. Therefore, mutations in any step may cause a type of CDG. There is considerable overlap in the clinical presentations between different types of CDG and a broad diversity within each type. The most common form of CDG, called Type Ia (CDG-Ia), is caused by defects in PMM2 (Man-6-P to Man-1-P), the gene that encodes phosphomannomutase. Mortality is 20% in the first 5 years, but then patients stabilize. Currently, there is no treatment for the CDG-Ia.

CDG-Ib patients, who are deficient in phosphomannose isomerase (PMI) catalyzing conversion of Man-6-P to Fru-6-P, are successfully treated with free mannose (2 – 6). Unfortunately, mannose therapy is not effective for CDG-Ia patients, most likely due to efficient Man-6-P consumption in the PMI reaction (7,8). It is believed that patients with Congenital Disorder of Glycosylation Type Ia (CDG-Ia) will benefit from dietary mannose if there is a simultaneous reduction of phosphomannose isomerase (PMI) activity. This would allow a modest intracellular accumulation of Man-6-P and drive metabolic flux into the glycosylation pathway using the residual PMM2 activity (9). It is assumed that a non-competitive inhibitor would work best in this setting; however, identification of chemical probes with diverse modes of action (MOA) would be advantageous for further characterization of PMI and PMM variants.

We proposed that CDG-Ia patients would benefit from dietary mannose if we simultaneously reduce PMI activity with a non-competitive or un-competitive inhibitor. This would allow a modest intracellular accumulation of Man-6-P and drive metabolic flux into the glycosylation pathway using the residual PMM2 activity.

Novel chemical probes elucidated in this way are invaluable tools to help explain the role of PMI in various biochemical pathways, and will ultimately lead to the first therapy for the growing number of CDG-Ia patients.

2. Project Description

a. The original goal for probe characteristics.

The original overall goal for this project was to identify novel non-competitive inhibitors of phosphomannose isomerase (PMI) that can be used as a therapeutic for treating patients with Congenital Disorder of Glycosylation Type Ia (CDG-Ia). A non-competitive inhibitor is preferred (yet not required) to avoid competition by the increased Man-6-P level. The main aims undertaken were: 1) Identify small molecule compounds in the MLSMR collection screening 2) Validated the specificity, activity, stability and kinetic behavior of the selected compound(s) 3) Test selected inhibitors of PMI for in cell based assay to determine their effects on glycosylation in intact cells.

b.

i. PubChem Bioassay Name(s), AID(s), Assay-Type (Primary, DR, Counterscreen, Secondary)

PubChemBioAssay Name	AIDs	Probe Type	Assay Type	Assay Format	Assay Detection & well format
HTS identification of compounds inhibiting phosphomannose isomerase (PMI) via a fluorescence intensity assay	1209	Inhibitor	Primary/ Confirmatory	Biochemical	Fluorescence 1536 well
HTS identification of compounds inhibiting phosphomannose isomerase (PMI) via a fluorescence intensity assay using a high concentration of mannose 6-phosphate [Confirmatory]	1220	Inhibitor	Primary/ Confirmatory/ Mechanistic	Biochemical	Fluorescence 1536 well
Confirmation of compounds inhibiting phosphomannose isomerase (PMI) via a fluorescence intensity assay. [Confirmatory]	1535	Inhibitor	SAR	Biochemical	Fluorescence 384 well
Confirmation of compounds inhibiting phosphomannose isomerase (PMI) via a fluorescence intensity assay using a high concentration of mannose 6-phosphate. [Confirmatory]	1536	Inhibitor	SAR/ mechanistic	Biochemical	Fluorescence 384 well
uHTS Identification of Diaphorase Inhibitors and Chemical Oxidizers: Counter Screen for Diaphorase-based Primary Assays [Primary Screening]	1217	Inhibitor	Primary/ Counter screen	Biochemical	Fluorescence 1536 well
Counter Screen for Glucose-6-Phosphate Dehydrogenase-based Primary Assay	1020	Inhibitor	Primary/ Counter Screen	Biochemical	Fluorescence 1536 well
Phosophomannose Mutase 2 (PMM2)	1655	Inhibitor	Confirmatory/ Counter screen	Biochemical	Fluorescence 1536 well

ii. Assay Rationale & Description.

The purpose of this assay was to identify inhibitors of human PMI. This was accomplished by using a G6PD- NADPH-coupled assay. In the assay PMI activity was detected through conversion of its product, fructose-6-phosphate, to glucose-6-phosphate catalyzed by phosphoglucose isomerase (PGI) and subsequent oxidation of glucose-6-phosphate to 6-phosphogluconolactone concomitant with NADP-to-NADPH conversion catalyzed by glucose-6-phosphate dehydrogenase (G6PDH). The NADPH was then detected via a resazurin-diaphorase fluorogenic reaction.

This assay was performed in the presence of near-Km concentrations of the PMI substrate, mannose-6-phosphate, to ensure the identification of inhibitors with diverse Mechanism of Actions (MOAs). Keeping in mind that non- or un-competitive inhibitors might provide a better probe, we also developed and utilized for the full-deck screening the assay performed in the presence of 10xKm of the PMI substrate be better for to help with prioritization of hits for follow-up work. The idea being, that the competitive inhibitors would show much less inhibition in these conditions comparing to the normal screening. Thus, we selected scaffolds that inhibit in both assays with similar potency for further work; the described herein probe originated from this type of scaffold. In addition, we also developed and implemented the following assays for counter screening: G6PDH (primary HTS), diaphorase (primary HTS). Compounds inhibitory in any of these counter-screen assays were excluded from further consideration.

Protocol

PMI assay materials
1) Human PMI protein was provided by Dr. Hudson Freeze (Burnham Institute for Medical Research, San Diego, CA). The target protein, recombinant Phosphomannose Isomerase (PMI), was made in 1 L batches from high expressing baculovirus systems in SF9 insect cells in Dr Hudson Freeze laboratory.
2) Substrate working solution: 50 mM HEPES, pH 7.4, 0.4 mM Mannose-6-phosphate, 1.6 U/ml Diaphorase, 0.2 mM Resazurin.
3) Enzyme working solution: 50 mM HEPES, pH 7.4, 0.44 mM NADP+, 9.048 mM MgCl2, 0.01% Tween 20, 4.6 ug/ml phosphoglucose isomerase, 30 ng/ml PMI, 1.8 ug/ml G6PDH.

Table of Reagents and source used in experiments	
Reagents name	Vendors name
PhosphoGlucose Isomerase (PGI)	Roche Diagnostics
Glucose-6 phosphate Dehydrogenase (G6PD)	USB
Mannose-6 Phosphate	Sigma-Aldrich
NADP	ISC/Bioexpress
Hepes Buffer	Sigma-Aldrich
Magnesium Chloride	Sigma-Aldrich

PMI HTS protocol:
1) 2 uL of Substrate working solution was added to columns 3-48 of a Costar 1536-well black plate (cat #3724) using a Thermo Multidrop Combi dispenser
2) 2 ul of Substrate working solution without mannose-6-p was added to columns 1 and 2 (positive control) of a Costar 1536-well black plate (cat #3724) using a Thermo Multidrop Combi dispenser
3) 40 nL of 100% DMSO was added to columns 1-4 using a HighRes biosolutions pintool and V&P Scientific pins
3) 40 nL of 2 mM compounds in 100% DMSO were dispensed in columns 5-48 using a HighRes biosolutions pintool and V&P Scientific pins
4) 2 uL of Enzyme working solution was added to the whole plate using a Thermo Multidrop Combi dispenser.
5) Plates were incubated at room temperature for 20 min.
6) After 20 minutes the plates were read on a ViewLux plate reader (Perkin Elmer), Ex544, Em590.
7) The screening was performed using a HighRes biosolution fully integrated HTS POD-based system
8) Data analysis was performed using CBIS software (ChemInnovations, Inc).
Compounds showing more than 50% inhibition were requested from MLSMR for dose-response confirmation.

PMI dose-response assay protocol:
1) 9 uL of Substrate working solution was added to columns 3-24 of a Greiner 384-well black plates (cat #784076) using a Thermo Multidrop Combi dispenser
2) 9 ul of Substrate working solution without mannose-6-p was added to columns 1 and 2 (positive control) using a Thermo Multidrop Combi dispenser
3) 2 uL of serially diluted compounds in 10% DMSO were added to columns 3-22 using Biomek FX
3) 2 uL of 10% DMSO were added to columns 1-2, 23-24
4) 9 uL of Enzyme working solution was added to the whole plate using a Thermo Multidrop Combi dispenser.
5) Plates were incubated at room temperature for 20 min.
6) After 20 minutes the plates were read on an Analyst plate reader (Molecular Devices), Ex544, Em590.

7) Data analysis was performed using CBIS software (ChemInnovations, Inc).

iii. Center Summary of Results

A total of 194,158 compounds were tested at 20uM and 926 had an activity ≥ 50% in the assay. The average Z' for the screen was 0.81, signal to background was 3.9, signal to noise was 52.4 and signal window was 19.1. Analog-by-catalog (ABC) SAR studies on the **dithiazolimine series** are summarized in Table 1 below.

b. Probe Optimization

SAR & chemistry strategy (including structure and data) that led to the probe.
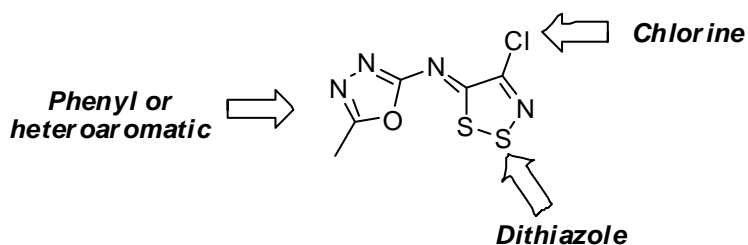

Figure 1. Summary of required features for CID-25199533 and analogues.

Development of initial SAR around the dithiazolimine series of PMI inhibitors via purchased and synthesized analogues established 3 structural features critical for the inhibitory activity of CID-25199533 (MLS-0390940) and its derivatives: (a) a dithiazole core ring, (b) a pendant aromatic or heteroaromatic ring, and (c) a chlorine in the 5 position of the dithiazole ring. These required features are exemplified in Figure 1. Systematic replacement of each of these key moieties resulted in derivates having significantly diminished inhibitory activity (examples not shown). Thus, having established these required features, further SAR optimization efforts focused on two main goals: (1)

Table 1. *In vitro* enzyme activity & SAR for dithiazolimine PMI inhibitors.

Structure	Compound ID	PubChem CID	PubChem SID	PMI IC$_{50}$ (uM) near Km	PMM IC$_{50}$ (uM)
	MLS-0390940	25199533	57309177	1.07	>100
	MLS-0390932	25181240	57287676	7.37	4.8
	MLS-0053966	715715	56373582	2.91	>100
	MLS-0390826	602897	57287569	3.12	97.3
	MLS-0390818	6114286	57287561	2.30	>100
	MLS-0390820	3919997	57287563	3.52	58.4
	MLS-0390829	715698	57287572	4.14	>100
	MLS-0390816	5527821	57287559	4.34	>100
	MLS-0390822	483894	57287565	4.35	>100

development of inhibitors with increased potency *in vitro* against PMI and (2) optimization of the selectivity of these potent inhibitors over PMM. The *in vitro* enzyme inhibition and cell-based efficacy data for selected analogues of CID-25199533 (MLS-0390940) are shown in Table 1. Compound CID-715715 (MLS-0053966) was the primary lead compound based on the initial *in vitro* HTS data. This compound showed acceptable PMI inhibition (IC$_{50}$ = 2.91 uM) and selectivity over PMM (IC$_{50}$ >100 uM), thus analogue synthesis was executed

around this hit. A variety of analogues were prepared to elucidate the optimal substitutions of the aryl ring. As can be seen in Table 1, methoxy substituents as in CID-602897 (MLS-0390826) and CID-483894 (MLS-0390822) suggested that the presence of hydrogen-bonding moieties were essential for activity. A key discovery was made when *ortho* nitro substituents were incorporated, yielding potent analogues such as CID-3919997 (MLS-0390820), which had acceptable potency (PMI IC$_{50}$ = 3.52 uM). However, this analogue showed slight inhibition of PMM (IC$_{50}$ = 58.4 uM). A companion series having a heteroaromatic substituent in place of the phenyl ring was showing potent PMI inhibition and PMM selectivity, as exemplified by the thiadiazole-containing analogue CID-6114286 (MLS-0390818; PMI IC$_{50}$ = 2.3 uM; PMM IC$_{50}$ > 100 uM). It was envisioned that this replacement represented an opportunity to maintain PMI potency while improving selectivity over PMM. Thus, selected analogues were synthesized incorporating small heteroaryl rings at this position, yielding potent and selective derivatives such as CID-5527821 (MLS-0390816) (PMI IC$_{50}$ = 4.34 uM; PMM IC$_{50}$ > 100 uM) and the probe compound CID-25199533 (MLS-0390940), a potent and selective PMI inhibitor with IC$_{50}$ = 1.07 uM and completely inactive at PMM when tested at concentrations up to 100 uM.

3. Probe

a. Chemical name
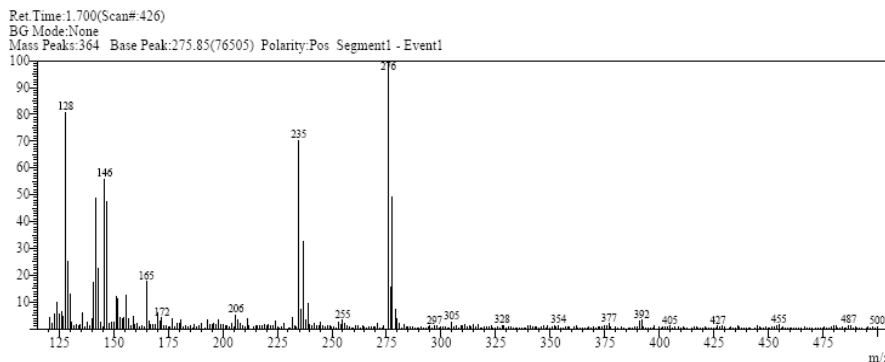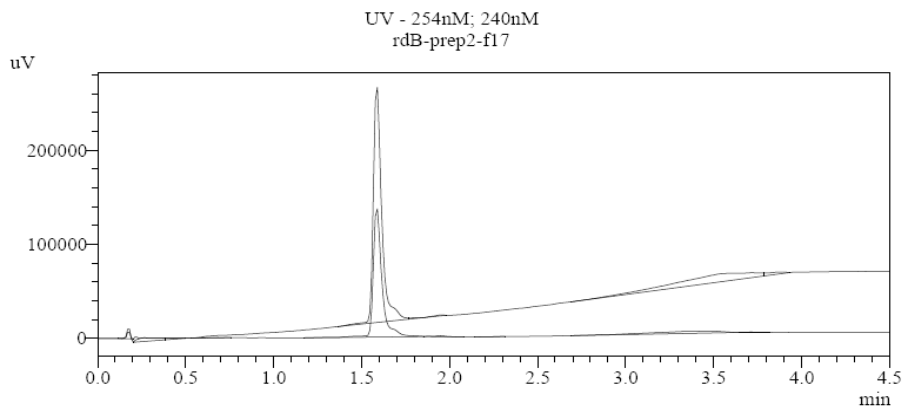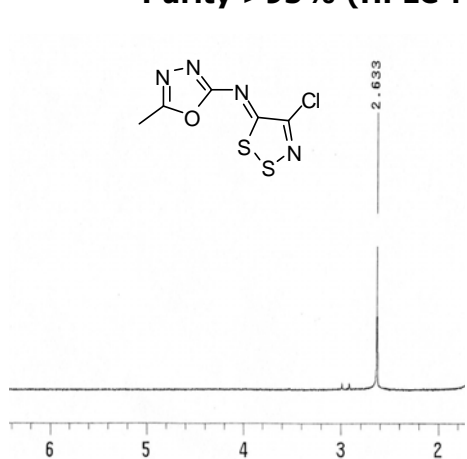4-chloro-N-(5-methyl-1,3,4-oxadiazol-2-yl)dithiazol-5-imine **[ML096]**

b. Probe chemical structure
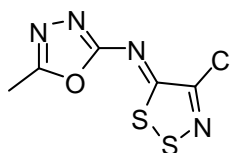
c. Structural Verification Information of probe SID
SID-57309177

Purity >95% (HPLC-MS)

d. PubChem CID (corresponding to the SID)
25199533

e. Availability from a vendor.
This probe is not commercially available.

f. MLS# that verifies the submission of probe molecule and five related samples that were submitted to the SMR collection:

Probe /Analog	MLS-# (BCCG#)	CID	SID	Source (vendor or BCCG syn)	Amt (mg)	Date ordered/ submitted
Probe	MLS-0390940	25199533	57309177	BCCG syn	15	5/18/09
Analog 1	MLS-0390932	25181240	57287676	BCCG syn	15	5/18/09
Analog 2	MLS-0053966	715715	4245711	Chemical Block	15	5/18/09
Analog 3	MLS-0390826	602897	57287569	Chemical Block	15	5/18/09
Analog 4	MLS-0390818	6114286	57287561	BCCG syn	15	5/18/09
Analog 5	MLS-0390829	715698	57287572	BCCG syn	15	5/18/09

g. Mode of action for biological activity of probe
The mode of action is not yet fully characterized. The dithiazolimine scaffold with all its primary hits was identified as non- or un-competitive since it inhibited irrespective of substrate concentration. As alterations to the structure of the primary hits that were made to arrive at the probe are limited, the probe is very likely to have the same MOA.

h. Detailed synthetic pathway for making probe
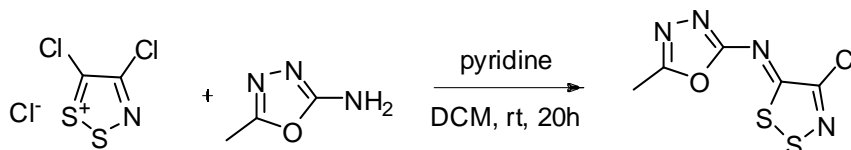
Scheme 1. Synthesis of CID-25199533.

i. Probe properties (solubility, absorbance/fluorescence, reactivity, toxicity, etc.)
CID-25199533 (MLS-0390940) demonstrated potent inhibition and also demonstrated selectivity within the class.

j. Properties Computed from Structure

Molecular Weight	234.68652
Molecular Formula	$C_5H_3ClN_4OS_2$
XLogP3-AA	2.1
H-Bond Donor	0
H-Bond Acceptor	5
Rotatable Bond Count	1
Exact Mass	233.94368
Topological Polar Surface Area	114
Heavy Atom Count	13
Formal Charge	0

3.

   b. Comparative data on (1) probe, (2) similar compound structures (establishing SAR) and (3) prior probes
   See list of compounds ordered for SAR by catalog attached separately to end of this probe report.

   c. Comparative data showing probe specificity for target
   See **Table 1** above for selectivity

4. Bibliography

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