Journal of Medicinal Chemistry

ARTICLE

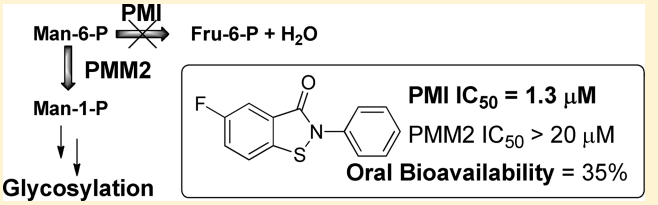
pubs.acs.org/jmc

Potent, Selective, and Orally Available Benzoisothiazolone Phosphomannose Isomerase Inhibitors as Probes for Congenital Disorder of Glycosylation Ia

Russell Dahl,[†,‡] Yalda Bravo,[†,‡] Vandana Sharma,[§] Mie Ichikawa,[§] Raveendra-Panickar Dhanya,[†,‡] Michael Hedrick,[‡] Brock Brown,[‡] Justin Rascon,[‡] Michael Vicchiarelli,[‡] Arianna Mangravita-Novo,[‡] Li Yang,[†,‡] Derek Stonich,[‡] Ying Su,[‡] Layton H. Smith,[‡] Eduard Sergienko,[‡] Hudson H. Freeze,[§] and Nicholas D. P. Cosford*[,†,‡]

[†]Apoptosis and Cell Death Research Program, [‡]Conrad Prebys Center for Chemical Genomics, and [§]Sanford Children's Health Research Center, Sanford-Burnham Medical Research Institute, 10901 North Torrey Pines Road, La Jolla, California 92037, United States

ABSTRACT: We report the discovery and validation of a series of benzoisothiazolones as potent inhibitors of phosphomannose isomerase (PMI), an enzyme that converts mannose-6-phosphate (Man-6-P) into fructose-6-phosphate (Fru-6-P) and, more importantly, competes with phosphomannomutase 2 (PMM2) for Man-6-P, diverting this substrate from critical protein glycosylation events. In congenital disorder of glycosylation type Ia, PMM2 activity is compromised; thus, PMI inhibition is a potential strategy for the development of therapeutics. High-throughput screening (HTS) and subsequent chemical optimization led to the identification of a novel class of benzoisothiazolones as potent PMI inhibitors having little or no PMM2 inhibition. Two complementary synthetic routes were developed, enabling the critical structural requirements for activity to be determined, and the compounds were subsequently profiled in biochemical and cellular assays to assess efficacy. The most promising compounds were also profiled for bioavailability parameters, including metabolic stability, plasma stability, and permeability. The pharmacokinetic profile of a representative of this series (compound **19**; **ML089**) was also assessed, demonstrating the potential of this series for in vivo efficacy when dosed orally in disease models.



INTRODUCTION

Congenital disorder of glycosylation type Ia (CDG-Ia) is a genetic disorder caused by mutations in the pmm2 gene that leads to reduced phosphomannomutase 2 (PMM2) enzyme activity. PMM2 plays an important role in N-glycosylation by competing with phosphomannose isomerase (PMI) for a common substrate, mannose-6-phosphate (Man-6-P), and converting it to mannose-1-phosphate (Man-1-P), which ultimately enters the N-glycosylation pathway (Figure 1). Any aberration in PMM2 results in underglycosylation of proteins in CDG-Ia patients, and this leads to multiorgan symptoms including neurological problems. There is currently no therapy for CDG-Ia patients, and the prognosis is extremely poor.[1]

Unfortunately, mannose therapy alone does not benefit CDG-Ia patients since most of the supplied mannose is catabolized by PMI and is therefore unavailable for glycosylation. We hypothesized that CDG-Ia patients might benefit from dietary mannose supplementation combined with inhibition of PMI using small molecule inhibitors selective for PMI over PMM2, thus diverting metabolic flux toward the glycosylation pathway (Figure 1).[1a,2] Small molecule inhibitors of PMI are scarce. The only inhibitors reported are either substrate based or show very weak inhibition (Figure 2).[3] In contrast, many of our newly discovered inhibitors have more than a 10-fold increase in potency over the most potent of the previously reported inhibitors. In addition, no cell-based efficacy or selectivity over PMM2 has been reported for any PMI inhibitors prior to this work. Herein, we disclose the discovery and validation of benzoisothiazolone derivatives that are selective inhibitors of human PMI. This class shows favorable absorption, distribution, metabolism, excretion (ADME) profiles, and a representative compound is orally available when dosed in mice. These compounds are viable leads for the development of novel therapeutics to treat CDG-Ia.

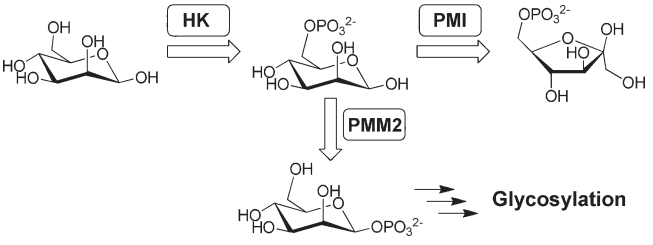


Figure 1. PMI and phosphomannomutase (PMM2) are important regulators of glycosylation. Inhibitors were designed to inhibit PMI but not PMM2, facilitating the accumulation of Man-6-P to drive glycosylation (HK = hexokinase).

Received: April 26, 2010
Published: May 03, 2011

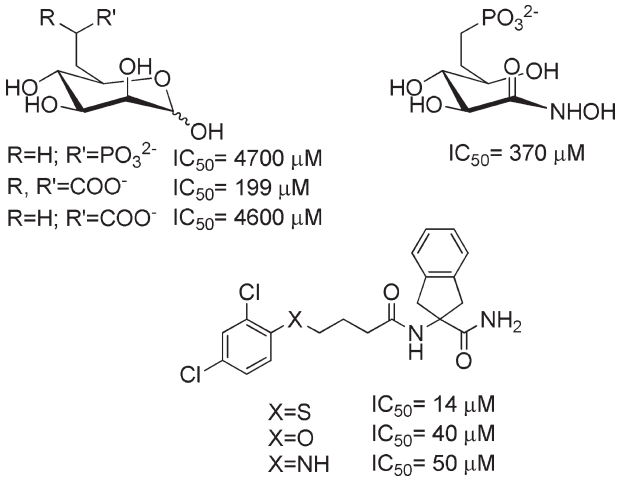


Figure 2. Structures and activities of previously reported PMI inhibitors.[3]

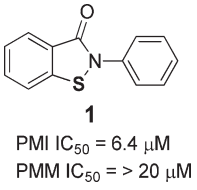
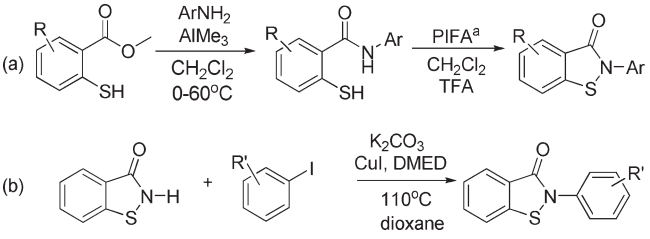


Figure 3. Benzoisothiazolone PMI inhibitor HTS hit.

RESULTS AND DISCUSSION

High-throughput screening (HTS) of 196,000 compounds from the NIH Molecular Libraries Small Molecule Repository (MLSMR) was conducted to identify small molecule inhibitors of PMI. Because compounds of interest were expected to show efficacy in an environment rich in Man-6-P content resulting from PMM2 deficiency and a supply of external mannose, we were specifically interested in identifying compounds with non- or uncompetitive modes of inhibition. To help select the most promising scaffolds, parallel primary screening was performed in the presence of $2 \times K_M$ and $10 \times K_M$ concentrations of Man-6-P. Compounds of interest were expected to be efficacious in both assays. Among the confirmed hits that inhibited in both assays was a novel class of benzoisothiazolones showing robust PMI inhibition, PMI/PMM2 selectivity, and a structure amenable to optimization of both biological activity and drug properties. This series is exemplified by compound **1** (Figure 3), a PMI inhibitor that was among the HTS hits chosen for follow up. While this compound was selective for PMI over PMM2 when tested up to 20 μM, it was recognized as a relatively weak inhibitor of PMI (IC_{50} = 6.4 μM). Thus, because of the observed PMI/PMM2 selectivity and the potential for parallel synthesis, we initiated structure−activity relationship (SAR) studies around this scaffold to optimize potency, selectivity, and cellular PMI inhibitory activity. In addition, the most promising compounds were evaluated for their ADME and pharmacokinetic (PK) properties with the goal of developing a compound with a profile suitable for in vivo proof-of-concept studies.

Scheme 1. Synthesis of Benzisothiazolones via (a) Acylnitrenium Ion-Mediated Cyclization or (b) Copper-Catalyzed *N*-Arylation[a]



[a] PIFA = phenyliodine bis(trifluoroacetate).

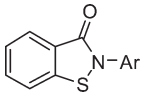
Chemical Synthesis. To facilitate the generation of analogues, two chemical routes were developed to enable parallel synthesis of chemical libraries around the benzisothiazolone scaffold (Scheme 1). The first route utilized chemistry developed by Correa et al.[4] and involved a key cyclization step using phenyliodine bis(trifluoroacetate) (PIFA) to generate a *N*-acylnitrenium ion followed by intramolecular trapping by sulfur (Scheme 1a). This synthetic methodology allowed for substitution of both aromatic portions of the molecule and was utilized particularly to probe the effects of substituents on the core benzisothiazolone ring.

To efficiently assess the effects of substituents on the *N*-phenyl ring, we developed a copper-mediated Ullman type *N*-arylation reaction, based on a variation of methods reported by Buchwald and others.[5] Beginning from commercially available benzoisothiazolone, this reaction employs catalytic amounts of copper iodide and *N*,*N*′-dimethylethylenediamine as the ligand (Scheme 1b). These conditions allowed rapid access to a diverse set of analogues in one step. It should also be noted that other ligands and copper sources were also screened. Generally, diamines and CuI gave the best overall yields (yield range 6−42%), although CuCl and Cu_2O were almost as effective. This reaction was also performed using microwave irradiation with acceptable product recovery in minutes. This is the first example of *N*-arylation chemistry of this heterocycle type and allowed rapid interrogation of the *N*-aryl species. Using a combination of both routes, the relevant sites of SAR were investigated to afford optimal substitutions with respect to PMI potency, PMM2 selectivity, and cellular efficacy.

Biochemical Evaluation. The potency and selectivity of the synthesized compounds were assessed by in vitro enzymatic assays using purified human PMI and PMM2. The effects of substitution on the pendant *N*-phenyl ring on PMI and PMM2 inhibition are shown in Table 1. Like the lead compound **1**, all of the synthesized derivatives showed selectivity for PMI over PMM2, with the exception of the 4-trifluoromethylphenyl derivative **6**, which was inactive against both enzymes. In general, para substitution was favored over meta, with a 2−3-fold increase in PMI inhibition seen. The exceptions to this trend included trifluoromethyl substitution (compounds **5** and **6**) and ester substitution (**9** and **10**). The effect of ortho substitution was explored with compound **16**. In comparison to compound **3**, the des-methyl variant of this compound, little effect on PMI activity was seen. However, a measurable inhibition of PMM2 was observed (Table 1). Some notable examples for overall potency and selectivity include compound **8** with a PMI IC_{50} of 1.8 μM and compound **12**, which showed comparable activity. These

dx.doi.org/10.1021/jm101401a |J. Med. Chem. 2011, 54, 3661–3668

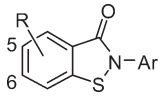
Table 1. SAR of *N*-Phenyl Ring Substituents[a]



compd	Ar	IC$_{50}$ (μM) PMI	PMM2
1	Ph	6.4	>20
2	2-naphthyl	9.4	>20
3	3-Me-Ph	6.0	>20
4	4-Me-Ph	5.2	>20
5	3-CF$_3$—Ph	3.4	13.9
6	4-CF$_3$—Ph	>20	>20
7	3-Cl-Ph	4.8	>20
8	4-Cl-Ph	1.9	>20
9	3-COOMe-Ph	4.9	15.4
10	4-COOEt-Ph	7.2	>20
11	3-N(Me)$_2$-Ph	8.5	>20
12	4-N(Me)$_2$-Ph	1.9	13.3
13	3-I-Ph	4.3	>20
14	4-*t*Bu-Ph	5.0	>20
15	3-OMe-Ph	3.7	>10
16	2,5-di-Me-Ph	6.6	17.9

[a] PMI and PMM assay data are the mean of at least three determinations. See the Experimental Section for assay details.

Table 2. SAR of Fluorine-Substituted Benzisothiazolones[a]



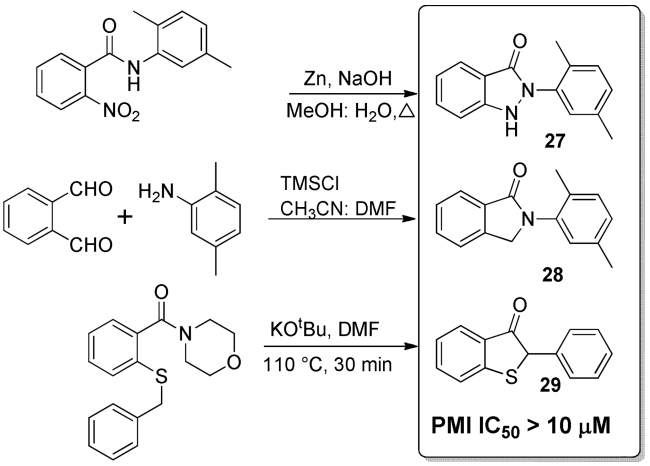
compd	R	Ar	IC$_{50}$ (μM) PMI	PMM2
17	6-F	2,5-di-Me-Ph	1.0	7.3
18	6-F	4-OMe-Ph	3.1	12.9
19	5-F	Ph	1.3	>80
20	5-F	2,5-di-Me-Ph	1.9	31.3
21	5-F	4-F-Ph	3.6	>80
22	5-F	4-OMe-Ph	1.0	9.1
23	5-F	2-F-Ph	4.3	>20
24	5-F	4-Cl-Ph	1.8	>20
25	5-F	3-F-Ph	8.3	>20
26	6-F	2-Me-Ph	2.9	>20

[a] PMI and PMM2 assay data are the mean of at least three determinations. See the Experimental Section for assay details.

compounds were identified as promising leads for further optimization.

Next, the effects of fluorine substitution at positions 5 and 6 on the core benzisothiazolone aryl ring were assessed with respect to PMI potency and PMM2 selectivity (Table 2). Generally, fluorine substitution at these positions afforded an overall increase in PMI potency with all of the examples maintaining selectivity over

Scheme 2. Replacement of Heteroatoms of the Benzisothiazolone Abolished All PMI Inhibition



PMM2. For example, compound **19**, which has an unsubstituted phenyl ring and fluorine at the 5-position of the fused aryl ring, had a PMI IC$_{50}$ of 1.3 μM, as compared to the analogous des-fluoro derivative **1**, which had a PMI IC$_{50}$ of 6.4 μM. In addition, both of these compounds were inactive against PMM2 when tested up to 20 μM. An exception to this trend was seen with the 4-chloro-substituted compounds **24** and **8**, with PMI IC$_{50}$ values of 1.8 and 1.9 μM, respectively. The most potent compounds were in this series, specifically the dimethyl substituted **17** with fluorine in the 6-position and the 4-methoxy derivative **22** with fluorine in the 5-position. These derivatives both showed PMI inhibition of 1.0 μM, representing a full fold-better potency than the most potent derivatives from the previous des-methyl series. While inhibition of PMM2 was seen in these most potent examples, they still maintained a 7—9-fold selectivity for PMI.

Finally, the importance of heteroatoms in the benzisothiazolone ring was investigated. To accomplish this, selected analogues were synthesized with the nitrogen and sulfur atoms in the benzisothiazolone ring either removed or replaced (compounds **27**—**29**, Scheme 2). As can be seen, all of these modifications abrogated all PMI activity when tested up to 10 μM in the enzyme assay. These data support the importance of the benzisothiazolone ring system for PMI inhibition.

Cellular Evaluation. A cellular assay was developed to evaluate the biological and functional efficacy of the PMI inhibitors. [2-^{3}H]-Mannose is a specific label, which is used to measure the amount of mannose that is incorporated into glycoproteins versus that which is catabolized (Figure 1). In the presence of PMI inhibitors, more ^{3}H-mannose will be diverted toward PMM2, resulting in more of the ^{3}H radiolabel present in glycoproteins as compared with untreated cells. Representative PMI inhibitors that display reproducible cellular activity are shown in Figure 4. Briefly, cells were incubated with inhibitor and labeled with ^{3}H-mannose and ^{35}S-Met/Cys. The amounts of ^{3}H- and ^{35}S radiolabel were then determined in the precipitated proteins. The ^{35}S label was included to determine the protein synthesis capacity of the cells as well as the toxicity. Decreased ^{35}S-Met/Cys incorporation at high concentration was observed for some compounds. This was attributed to off-target activity because similar effects were observed in PMI null cells (data not shown). Similarly, the toxicity was unlikely to be due to PMM2 inhibition

dx.doi.org/10.1021/jm101401a |*J. Med. Chem.* 2011, 54, 3661–3668

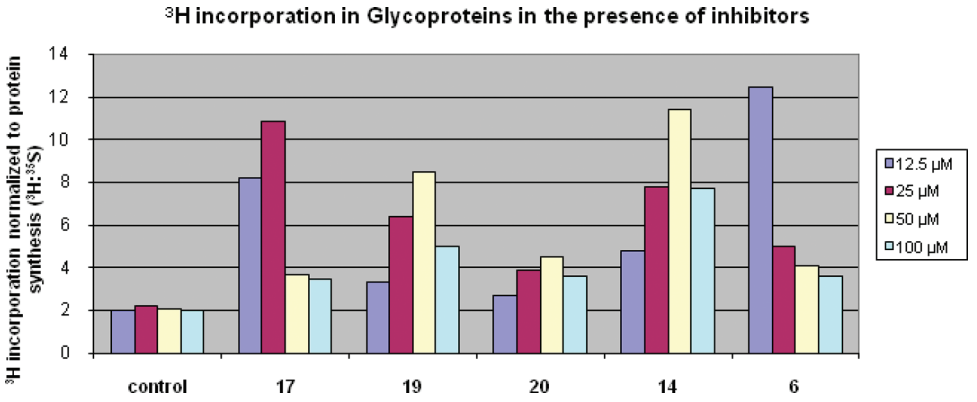


Figure 4. Cell-based PMI inhibitor data. Shown is the ^{3}H-mannose incorporation normalized to ^{35}S protein synthesis in the presence of increasing inhibitor concentrations.

Table 3. ADME Profiles of Selected PMI Inhibitors

compd	solubility pH 7.4 (μg/mL)	Log P_e[a]	plasma stability[b] (mouse)	microsomal stability[b] (mouse)
14	2.5	−2.8	10	62
17	22.5	−2.9	48	82.3
19	3.8	−3.2	99	85
20	11	−2.9	ND	81

[a] Permeability determined by PAMPA assay. [b] Percent of compound remaining after incubation for 3 (plasma) or 1 h (microsomes) at 37 °C.

because PMM2-deficient CDG-Ia patient fibroblasts with 20% or less residual PMM2 activity grow slowly but normally and do not show less ^{35}S-incorporation into the glycoproteins. Compound **6**, which was inactive against both PMI and PMM2, was included as an example of a compound that exhibits cellular toxicity in the assay. While all of the PMI inhibitors display enhanced glycosylation as indicated by increased ^{3}H-mannose incorporation, compounds **14** and **19** both show a favorable combination of cellular efficacy with lessened toxicity. While compound **19** is the most potent compound in vitro, the maximal efficacy in cells appeared to be slightly lower than other derivatives (Figure 4). This may be due to the membrane permeability of compound **19**, as it is predicted to be moderately permeable when assessed in vitro (Table 3). However, on the basis of its overall profile, compound **19** was selected for evaluation at an extended range of inhibitor concentrations and displayed a dose-dependent increase in mannose incorporation (Figure 5).

In Vitro ADME Profiling. The PMI inhibitors with the most favorable cellular efficacy were also profiled in in vitro ADME assays to assess their drug-likeness[6] and potential for systemic activity in animal models. Many of the benzisothiazolones were shown to have suitable properties for oral administration including acceptable metabolic and plasma stabilities, good permeability across artificial lipid membranes, and good solubility. The results of the specific in vitro ADME profiling assays are shown in Table 3. Although the profiles of many of the synthesized analogues had druglike ADME profiles, it was clear that in addition to showing acceptable aqueous solubility at physiological pH, the mouse plasma and microsomal stabilities of **19** were superior (Table 3). Compound **19** was therefore selected for additional studies, including its propensity to generate glutathione (GSH)-

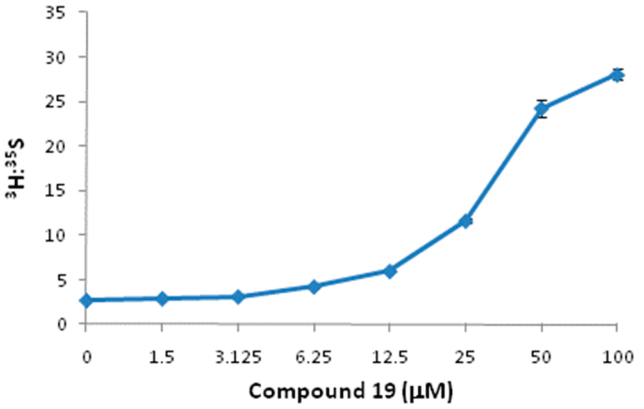


Figure 5. Cell-based efficacy of compound **19**. Shown is the ^{3}H-mannose incorporation normalized to ^{35}S protein synthesis in the presence of increasing concentrations of **19**.

Table 4. PK Parameters of Compound 19

compd	oral dose (mg/kg)	C_{max} (nM)	T_{max} (min)	AUC (0−t) (nM h)	$t_{1/2}$ (h)	F (%)
19	20	162 ± 32	45 ± 21	305 ± 34	1.3 ± 0.64	35

trapped reactive metabolites. The S9-based GSH transferase assay has been shown to be a reliable means of identifying compounds known to react with GSH in vivo. When tested in the presence of rat liver S9 fraction and GSH for 2 h, the quantity of **19** was not significantly reduced, and no GSH adducts were observed by liquid chromatography−mass spectrometry (LCMS) analysis.

In Vivo PK. On the basis of the promising properties displayed by compound **19**, i.e. potent PMI inhibition, PMI/PMM2 selectivity, cellular efficacy with low toxicity, and a favorable ADME profile, the PK and oral availability of this compound were evaluated in mice to enable future advanced studies in CDG-Ia disease models. To this end, compound **19** was dosed at 5 mg/kg i.v. and 20 mg/kg p.o. in mice. As predicted by the favorable ADME profile, the bioavailability of **19** (F = 35%) was satisfactory. The various PK parameters are shown in Table 4. Overall, the compound has a good PK profile with acceptable parameters to enable future in vivo efficacy experiments.

dx.doi.org/10.1021/jm101401a |*J. Med. Chem.* 2011, 54, 3661–3668

CONCLUSION

A series of novel, druglike benzisothiazolone inhibitors of PMI were synthesized, and their ability to modulate flux toward glycosylation has been demonstrated. Two synthetic routes for this scaffold were optimized, including a novel copper-catalyzed *N*-arylation reaction amenable to parallel derivitization. These compounds are the most potent inhibitors of PMI to date, and their dose-dependent efficacy in cells has been demonstrated. In addition, they are selective over PMM2 at all concentrations tested. The compounds possess favorable ADME and PK profiles, including good oral bioavailability, which should enable the further therapeutic development of these compounds.

EXPERIMENTAL SECTION

Compound Collection Utilized in HTS. The compound library was supplied by the MLSMR (http://www.mli.nih.gov/mlsmr). The MLSMR, funded by the NIH, is responsible for the selection of small molecules for HTS screening, their purchase and QC analysis, library maintenance, and distribution within the NIH Molecular Libraries Screening Center Network (MLPCN, http://www.mli.nih.gov/mlpcn). Both MLSMR and MLPCN are parts of the Molecular Libraries Initiatives (MLI, http://nihroadmap.nih.gov/molecularlibraries) under the NIH Roadmap Initiative (www.nihroadmap.nih.gov). MLSMR compounds are acquired from commercial and in part from academic and government sources and are selected based on the following criteria: Samples are available for resupply in 10 mg quantities, are at least 90% pure, have acceptable physicochemical properties, and contain no functional groups or moieties, which are known to generate artifacts in HTS (http://mlsmr.glpg.com/). Compounds are selected to represent diversified chemical space with clusters of closely related analogues around them to aid in the HTS-based SAR analysis.

In Vitro Assays. For concentration−response assays, 9 μL of the substrate solution was dispensed into 384-well black plates (Greiner 784076), and either the compound (2 μL in 10% DMSO) or the 10% DMSO alone was added for the test and control wells, respectively. The reaction was initiated with 9 μL of the enzyme solution. Fluorescence in the rhodamine channel (excitation, 544 nm; emission, 590 nm; dichroic mirror, 561 nm) was measured on a Molecular Devices Analyst HT after a 20 min incubation at room temperature.

The PMI substrate solution contained 50 mM Hepes, pH 7.4, 0.4 mM Man-6-P, 1.6 IU/mL diaphorase, and 0.2 mM resazurin. The PMI enzyme solution contained 50 mM Hepes, pH 7.4, 0.5 mM $NADP^+$, 10 mM $MgCl_2$, 4.6 μg/mL phosphoglucose isomerase, 2 μg/mL glucose-6-phosphate dehydrogenase, 30 ng/mL PMI, and 0.011% Tween 20 (PubChem AID 1209). In the primary screening, the same assay was also performed in the presence of saturating PMI substrate concentrations. Both working solutions had the composition as above, except for 2 mM Man-6-P in the substrate solution (PubChem AID 1220).

The PMM2 substrate solution contained 50 mM Hepes, pH 7.4, 0.17 mM Man-1-P, 0.4 IU/mL diaphorase, and 56 μM resazurin. The PMM2 enzyme solution contained 50 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 1.1 mM $NADP^+$, 0.11 mM α-D-glucose 1,6-bisphosphate, 5 μg/mL phosphoglucose isomerase, 3.33 μg/mL PMI, 2 μg/mL glucose-6-phosphate dehydrogenase, 1.67 μg/mL PMM2, and 0.011% Tween 20 (PubChem AID 1655). Man-6-P and Man-1-P were omitted from the positive control wells for PMI and PMM2, respectively. The IC_{50} values were determined using nonlinear regression data analysis according to the Hill equation.

General Synthetic Procedures. All solvents and chemicals used were purchased from Sigma-Aldrich, Acros, or Chembridge and were used as received without further purification. Purity and characterization of compounds were established by a combination of LCMS and NMR analytical techniques and was >95% for all tested compounds. Silica gel column chromatography was carried out using prepacked silica cartridges from RediSep (ISCO Ltd.) and eluted using an Isco Companion system. ^{1}H NMR spectra were acquired on a Varian Inova 300 MHz instrument. Chemical shifts are reported in ppm from residual solvent peaks (δ 7.27 for $CDCl_3$ ^{1}H NMR). HPLC-MS analyses were performed on a Shimadzu 2010EV LCMS using the following conditions: Kromisil C18 column (reverse phase, 4.6 mm × 50 mm); a linear gradient from 10% acetonitrile and 90% water to 95% acetonitrile and 5% water over 4.5 min; flow rate of 1 mL/min; and UV photodiode array detection from 200 to 300 nm. High-resolution ESI-TOF mass spectra were acquired at the Center for Mass Spectrometry at The Scripps Research Institute (La Jolla, CA).

General Methods for the Synthesis of Benzoisothiazolone PMI Inhibitors. *General Method A.* To a stirred solution of the amine (900 mg, 5.95 mmol) in CH_2Cl_2 at 0 °C under nitrogen, $AlMe_3$ (6 mL, 2 M in THF) was added dropwise, and the reaction was slowly warmed to room temperature. The mixture was stirred continuously for an additional 30 min. Methyl thiosalicylate (500 mg, 2.97 mmol) was added, and the reaction was heated to 60 °C and heated under reflux overnight. The reaction was quenched with HCl (5% aq.), and CH_2Cl_2 was added (50 mL). The organic layer was separated and washed with saturated $NaHCO_3$ solution, then brine, and dried over Na_2SO_4. The solvents were removed by rotary evaporation, and the products were isolated by flash chromatography or reverse phase HPLC and lyophilized to provide the final compounds, which were determined to be >95% pure by HPLC-UV, HPLC-MS, and ^{1}H NMR.

General Method B. To a crimp top microwave vial was added the benzoisothiazolone (76 mg, 0.5 mmol), aryl-X (1.05 mmol), K_2CO_3 (138 mg, 1.0 mmol), CuI (20 mol %), and DMEDA (20 mol %) in dioxane (5 mL). The reaction mixture was heated in the microwave at 195 °C for 7 min. Following filtration and evaporation of solvents, the products were isolated by flash chromatography or reverse phase HPLC and lyophilized to provide the final compounds, which were determined to be >95% pure by HPLC-UV, HPLC-MS, and ^{1}H NMR.

2-Phenyl-2-hydrobenzo[*d*]isothiazol-3-one (1). Compound **1** was prepared according to general procedure A (67%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.32 (m, 1H), 7.51 (m, 3H), 7.57 (m, 1H), 7.67 (m, 3H), 8.09 (m, J = 7.9, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): 120.0, 124.6, 125.8. 127.0, 127.2, 129.3, 132.3, 137.2, 139.9, 164.1. ESI-MS m/z 227 $[M + H]^+$. HRMS m/z calcd for $C_{13}H_9NOS$ $[M + H]^+$, 228.0405; found, 228.0522.

2-(3-Methylphenyl)-2-hydrobenzo[*d*]isothiazol-3-one (3). Compound **3** was prepared according to general procedure B (22%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.40 (s, 3H), 7.12 (d, J = 7.3, 1H), 7.33 (t, J = 7.9, 1H), 7.49 (m, 4H), 7.62 (m, 1H), 8.08 (d, J = 7.9, 1H). ESI-MS m/z 241 $[M + H]^+$. HRMS m/z calcd for $C_{14}H_{11}NOS$ $[M + H]^+$, 242.0561; found, 242.0672.

2-(4-Methylphenyl)-2-hydrobenzo[*d*]isothiazol-3-one (4). Compound **4** was prepared according to general procedure B (6%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.37 (s, 3H). 7.26 (m, 3H), 7.42 (m, 1H), 7.55 (m, 2H), 7.64 (m, 1H), 8.08 (m, 1H). ESI-MS m/z 241 $[M + H]^+$. HRMS m/z calcd for $C_{14}H_{11}NOS$ $[M + H]^+$, 242.0561; found, 242.0673.

2-[3-(Trifluoromethyl)phenyl]-2-hydrobenzo[*d*]isothiazol-3-one (5). Compound **5** was prepared according to general procedure B (15%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.46 (t, J = 7.9, 1H), 7.58 (m, 3H), 7.68 (m, 1H), 7.93 (d, J = 7.9, 1H), 8.01 (s, 1H), 8.10 (d, J = 7.9, 1H). ESI-MS m/z 295 $[M + H]^+$. HRMS m/z calcd for $C_{14}H_8F_3NOS$ $[M + H]^+$, 296.0279; found, 296.0391.

2-[4-(Trifluoromethyl)phenyl]-2-hydrobenzo[*d*]isothiazol-3-one (6). Compound **6** was prepared according to general procedure B (27%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.59 (m, 5H), 7.90 (m, 2H), 8.10 (d, J = 7.3, 1H). ESI-MS m/z 295 $[M + H]^+$. HRMS m/z calcd for $C_{14}H_8F_3NOS$ $[M + H]^+$, 296.0279; found, 296.0390.

2-(3-Chlorophenyl)-2-hydrobenzo[*d*]isothiazol-3-one (7). Compound **7** was prepared according to general procedure B (15%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.26 (m, 1H), 7.42 (m, 2H), 7.62 (m, 3H), 7.78 (m, 1H), 8.09 (m, 1H). ESI-MS m/z 261 $[M + H]^+$. HRMS m/z calcd for $C_{13}H_8ClNOS$ $[M + H]^+$, 262.0015; found, 262.0092.

2-(4-Chlorophenyl)-2-hydrobenzo[*d*]isothiazol-3-one (8). Compound **8** was prepared according to general procedure B (12%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.43 (m, 3H), 7.57 (d, J = 7.9, 1H), 7.65 (m, 3H), 8.05 (d, J = 7.9, 1H). ESI-MS m/z 261 $[M + H]^+$. HRMS m/z calcd for $C_{13}H_8ClNOS$ $[M + H]^+$, 262.0015; found, 262.0098.

2-[4-(Dimethylamino)phenyl]-2-hydrobenzo[*d*]isothiazol-3-one (12). Compound **12** was prepared according to general procedure B (38%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.97 (s, 6H), 6.75 (m, 2H), 7.42 (m, 3H), 7.54 (m, 1H), 7.60 (m, 1H), 8.07 (d, J = 7.3, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): 40.5, 112.5, 120.0, 124.8, 125.4, 125.5, 126.8, 127.0, 131.9, 140.2, 149.9, 164.4. ESI-MS m/z 270 $[M + H]^+$. HRMS m/z calcd for $C_{15}H_{14}N_2OS$ $[M + H]^+$, 271.0827; found, 271.078.

2-(3-Iodophenyl)-2-hydrobenzo[*d*]isothiazol-3-one (13). Compound **13** was prepared according to general procedure B (18%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.17 (t, J = 7.9, 1H), 7.44 (m, 1H), 7.66 (m, 4H), 8.08 (m, 2H). ^{13}C NMR (300 MHz, CDCl3): 94.1, 120.1, 123.6, 125.0, 126.0, 127.3, 130.7, 132.6, 133.0, 136.0, 138.3, 139.6, 164.0. ESI-MS m/z 352 $[M + H]^+$. HRMS m/z calcd for $C_{13}H_8INOS$ $[M + H]^+$, 353.9371; found, 353.9492.

2-[4-(*tert*-Butyl)phenyl]-2-hydrobenzo[*d*]isothiazol-3-one (14). Compound **14** was prepared according to general procedure B (42%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.33 (s, 9H), 7.46 (m, 3H), 7.61 (m, 4H), 8.10 (d, J = 7.9, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): 31.3, 34.7, 120.0, 124.4, 125.7, 126.3, 127.1, 132.2, 134.4, 150.3, 164.2. ESI-MS m/z 283 $[M + H]^+$. HRMS m/z calcd for $C_{17}H_{17}NOS$ $[M + H]^+$, 284.1031; found, 284.1131.

2-(3-Methoxyphenyl)-2-hydrobenzo[*d*]isothiazol-3-one (15). Compound **15** was prepared according to general procedure B (12%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.84 (s, 3H), 6.81 (m, 1H), 7.25 (m,1H), 7.36 (m, 2H), 7.43 (m, 1H), 7.57 (m, 1H), 7.65 (m, 1H), 8.09 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): 55.5, 110.1, 113.1, 116.5, 120.0, 125.0, 125.8, 127.1, 130.0, 132.4, 138.3, 140.0, 160.2, 164.1. ESI-MS m/z 257 $[M + H]^+$. HRMS m/z calcd for $C_{17}H_{17}NO_2S$ $[M + H]^+$, 258.0510; found, 258.0622.

2-(2,5-Dimethylphenyl)benzo[*d*]isothiazol-3(2*H*)-one (16). Compound **16** was prepared according to general procedure B (18%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.18 (s, 3H), 2.35 (s, 1H), 7.19 (m, 3H), 7.43 (m, 1H), 7.56 (m, 1H), 7.65 (m, 1H), 8.09 (d, J = 7.93, 1H). ESI-MS m/z 256 $[M + H]^+$.

2-(2,5-Dimethylphenyl)-6-fluorobenzo[*d*]isothiazol-3(2*H*)-one (17). To a stirred solution of the 4-fluoro-2-mercaptobenzoic acid (10 mmol) in ethyl acetate was added 2-propanephosphonic acid anhydride (30 mmol), and this mixture was stirred at room temperature for 15 min. 2,5-Dimethylaniline (10 mmol) and *N*-methylmorpholine (120 mmmol) were added, and the reaction was stirred at room temperature for 3 h, quenched with ethyl acetate, and washed with 5% potassium sulfate solution, saturated sodium bicarbonate solution, and brine, and dried over sodium sulfate. The organic phase was concentrated in vacuo and purified by reverse-phase preparative liquid chromatography to afford the product in 40% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.19 (s, 3H), 2.35 (s, 3H), 7.18 (m, 5H), 8.09 (m, 1H). ESI-MS m/z 274 $[M + H]^+$.

6-Fluoro-2-(4-methoxyphenyl)benzo[*d*]isothiazol-3(2*H*)-one (18). ^{1}H NMR (500 MHz, $(CD_3)_2SO$): δ 2.18 (s, 3H), 2.35 (s, 1H), 7.19 (m, 3H), 7.43 (m, 1H), 7.56 (m, 1H), 7.65 (m, 1H), 8.09 (d, J = 7.9, 1H). ESI-MS m/z 276 $[M + H]^+$.

5-Fluoro-2-phenylbenzo[*d*]isothiazol-3(2H)-one (19). Compound **19** was prepared according to general procedure A (51%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.33 (m, 1H), 7.45 (m, 3H), 7.54 (m, 1H), 7.67 (m, 2H), 7.77 (dd, J = 2.4, 7.9, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): 113.0, 113.3, 121.0, 121.3, 121.6, 121.7, 124.6, 127.3, 129.4, 135.1, 137.0, 160.1, 162.5. ESI-MS m/z 273 $[M + H]^+$. HRMS m/z calcd for $C_{15}H_{12}FNOS$ $[M + H]^+$, 274.0624; found, 274.0739.

5-Fluoro-2-(4-fluorophenyl)-2-hydrobenzo[*d*]isothiazol-3-one (20). Compound **20** was prepared according to general procedure A (65%). ^{1}H NMR (300 MHz, $CDCl_3$): 7.76 (dd, J = 2.4, 7.9, 1H), 7.67 (m, 2H), 7.54 (m, 1H), 7.44 (m, 3H), 7.31 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): 40.5, 112.5, 120.0, 124.8, 125.4, 125.5, 126.8, 127.0, 131.9, 140.2, 149.9, 164.3. ESI-MS m/z 245 $[M + H]^+$. HRMS m/z calcd for $C_{13}H_8FNOS$ $[M + H]^+$, 246.0311; found, 246.0395.

5-Fluoro-2-(4-fluorophenyl)-2-hydrobenzo[*d*]isothiazol-3-one (21). Compound **21** was prepared according to general procedure A (33%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.15 (m, 2H), 7.42 (m, 1H), 7.54 (m, 1H), 7.62 (m, 2H), 7.76 (dd, J = 2.4, 7.9, 1H). ESI-MS m/z 263 $[M + H]^+$. HRMS m/z calcd for $C_{13}H_7F_2NOS$ $[M + H]^+$, 264.0216; found, 264.0299.

5-Fluoro-2-(4-methoxyphenyl)-2-hydrobenzo[*d*]isothiazol-3-one (22). Compound **22** was prepared according to general procedure A (80%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.83 (s, 3H), 6.97 (m, 2H), 7.41 (m, 1H), 7.52 (m, 3H), 7.76 (dd, J = 2.4, 7.9, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): 55.6, 113.0, 113.2, 114.6, 126.8, 129.4, 135.2, 159.0, 162.4. ESI-MS m/z 275 $[M + H]^+$. HRMS m/z calcd for $C_{14}H_{10}FNO_2S$ $[M + H]^+$, 276.0416; found, 276.0499.

5-Fluoro-2-(4-fluorophenyl)-2-hydrobenzo[*d*]isothiazol-3-one (23). Compound **23** was prepared according to general procedure A (47%). ^{1}H NMR (300 MHz, $CDCl_3$): 8.12 (m, 1H), 7.67 (m, 1H), 7.58 (m, 1H), 7.44 (m, 3H), 7.22 (m, 1H). ESI-MS m/z 263 $[M + H]^+$. HRMS m/z calcd for $C_{13}H_7F_2NOS$ $[M + H]^+$, 264.0216; found, 264.0404.

2-(4-Chlorophenyl)-5-fluoro-2-hydrobenzo[*d*]isothiazol-3-one (24). Compound **24** was prepared according to general procedure A (55%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.42 (m, 3H), 7.54 (m, 1H), 7.63 (m, 2H), 7.76 (dd, J = 2.4, 7.9, 1H). ESI-MS m/z 278 $[M + H]^+$. HRMS m/z calcd for $C_{13}H_7ClFNOS$ $[M + H]^+$, 279.9921; found, 280.0017.

5-Fluoro-2-(3-fluorophenyl)-2-hydrobenzo[*d*]isothiazol-3-one (25). Compound **25** was prepared according to general procedure A (60%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.02 (m, 1H), 7.43 (m, 3H), 7.54 (m, 2H), 7.75 (dd, J = 2.4, 7.9, 1H). ESI-MS m/z 263 $[M + H]^+$. HRMS m/z calcd for $C_{13}H_7F_2NOS$ $[M + H]^+$, 264.0216; found, 264.0305.

6-Fluoro-2-*o*-tolylbenzo[*d*]isothiazol-3(2*H*)-one (26). Compound **26** was prepared according to general procedure A (55%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.17 (s, 3H), 2.33 (s, 3H), 7.18 (m, 3H), 7.41 (m, 1H), 7.53 (m, 1H), 7.78 (dd, J = 2.4, 7.9, 1H). ESI-MS m/z 273 $[M + H]^+$. HRMS m/z calcd for $C_{15}H_{12}FNOS$ $[M + H]^+$, 274.0624; found, 274.0734.

2-Phenyl-1*H*-2-hydroindazol-3-one (27). A solution of *o*-nitrobenzaldehyde (242 mg, 1 mmol) in methanol (3 mL) was added to sodium hydroxide in water (4 mL) together with zinc dust. The resulting reaction mixture was then heated under reflux for 15 h and filtered while hot. The filtrate was concentrated to half volume and cooled. Any unreacted material was removed by filtration. The filtrate was diluted with water and acidified with dilute HCl. The crude product precipitated was collected by filtration and further purified by column chromatography using hexanes:ethyl acetate to afford 0.076 g (36%) of indazolone as a pale yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.24 (s, 3H), 2.31 (s, 3H), 7.19 (m, 7H), 7.55 (m, 1H), 7.89 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): 17.6, 20.7, 112.3, 118.5, 122.6, 124.2, 127.8, 129.8, 130.9, 132.1, 132.9, 134.8, 136.4, 147.1, 161.7. ESI-MS m/z 238 $[M + H]^+$. HRMS m/z calcd for $C_{15}H_{14}N_2O$ $[M + H]^+$, 239.1106; found, 239.1205.

2-(2,5-Dimethylphenyl)isoindolin-1-one (28). To a stirred solution of the phthalaldehyde (250 mg, 1.85 mmol) in CH_3CN:DMF

was added the amine (230 μL, 1.85 mmol) followed by TMSCl (188 μL, 1.48 mmol) and was stirred at room temperature overnight (62%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.18 (s, 3H), 2.31 (s, 3H), 4.70 (s, 2H), 7.18 (m, 2H), 7.52 (m, 3H), 7.93 (m, 1H). ESI-MS *m/z* 237 $[M + H]^+$. HRMS *m/z* calcd for $C_{16}H_{15}NO$ $[M + H]^+$, 238.1154; found, 238.1276.

Permeability Assay. The capacity of compounds to cross cellular membranes was determined using a parallel artificial membrane permeation assay (PAMPA).[7] Briefly, the effective permeability of the compounds was measured at an initial concentration of 25 μM. The buffer solution (pH 7.4) was prepared from a concentrated stock available from pION and according to the manufacturer's protocol. The compound of interest was dissolved in buffer solution or water (in the case of cargos alone, evaluated by HPLC-MS) and acetonitrile (20%, cosolvent) to the desired concentration. The sandwich plate was separated, and the donor well was filled with 200 μL of the compound solution of interest. The acceptor plate was placed into the donor plate, ensuring that the underside of the membrane was in contact with buffer. Four microliters of the mixture of phospholipids (20 mg/mL) in dodecane was added to the filter of each well, followed by 200 μL of buffer solution. The plate was covered and incubated at room temperature in a saturated humidity atmosphere for 4 h under orbital agitation at 100 rpm. The concentrations of the compound remaining in the donor well, diffused through the membrane and into the acceptor well, and reference compounds were measured by LC/MS/MS. Results were classified as high, medium, or low predicted absorption. Test articles were run in triplicate at one concentration (25 μM), and permeability was assessed at one time point (4 h) and pH (7.4). The membrane consisted of phosphatidylcholine in dodecane on a Multiscreen PVDF membrane (0.45 μm). The positive control was verapamil (high permeability), and the negative control was theophylline (low permeability).

Plasma and Microsomal Stability Assays. Test articles were run in duplicate in the presence of either fresh plasma (mouse) or 0.5 mg/mL liver microsomes (mouse) at one concentration of test compound (10 μM). Aliquots were taken at two time points, *t* = 0 h and *t* = 1 h (microsomes) or *t* = 3 h (plasma). LC/MS/MS measurement of remaining parent compound as % parent remaining at a specific time point was assessed. For microsomal stability, the positive control was formation of acetaminophen from phenacetin, and the negative control was an NADPH-deficient test mixture.

GSH Conjugation Assay. To assess the potential of compound **19** for covalent modification, the GSH transferase activity in rat hepatic S9 fraction was measured.[8] Compound **19** was incubated at 10 μM in PBS buffer with 2 mg/mL rat hepatic S9 fraction and 10 mM GSH at 37 °C for 2 h. The incubation was stopped by protein precipitation using acetonitrile. After the supernatant was dried down, the residues were reconstituted and analyzed by positive-ion electrospray LCMS and analyzed for the presence of any GSH conjugates. Compound **19** was tested in triplicate along with the positive control diclofenac, a known GSH conjugator, which showed the expected conjugate masses.

Cellular Assay. Hela cells were grown to 70% confluency in 24 well plates. The cells were incubated with compounds for 2 h at 37 °C followed by the addition of 50 μCi/mL ^{3}H-mannose and 5 μCi/mL ^{35}S-Met/Cys trans label and further incubation at 37 °C for 1 h. The cells were then washed twice with PBS and lysed in 10 mM Tris (pH 7.4) containing 1% NP-40. The proteins were precipitated with 10% trichloroacetic acid. ^{3}H and ^{35}S radiolabel were measured in precipitated proteins, and the ^{3}H:^{35}S ratio was calculated. [^{3}H]-Mannose incorporation in the glycoproteins of inhibitor treated cells was compared to cells without inhibitor (treated only with DMSO and having maximal PMI activity), which served as the negative control. No cell permeable positive control compound, which increases mannose flux, was available other than the new compounds tested in the present study.

In Vivo PK. C57BL/6 mice (*n* = 3 per dose) were treated with **19** at doses of 5 mg/kg IV and 20 mg/kg po. The mice were sacrificed, and blood samples were collected at several time points (po: 0.5, 1, 2, 4, and 6 h; IV: 10 min and 0.5, 1, 2, 4, and 6 h). Plasma was separated from the red cells by centrifugation and frozen at −20 °C until assayed. Compound **19** was extracted from the plasma samples using a 2-fold excess of acetonitrile, and the extracts were vortexed for 3 min and then centrifuged for 15 min (7500 g) after which the supernatants were collected to be assayed by LCMS. PK data were analyzed using Prism and Excel.

AUTHOR INFORMATION

Corresponding Author

*Tel: 858-646-3100. Fax: 858-795-5225. E-mail: ncosford@sanfordburnham.org.

ACKNOWLEDGMENT

This work was supported by NIH Grant HG003916, by The Rocket Williams Fund, and by NIH Grants R01DK55615 and R21HD062914 to H.H.F., who is a Sanford Center Professor.

ABBREVIATIONS USED

PMI, phosphomannose isomerase; PMM2, phosphomannomutase 2; CDG, congenital disorders of glycosylation; HTS, high-throughput screening; ADME, absorption, distribution, metabolism, excretion; PK, pharmacokinetic; i.v., intravenous; p.o., per os; HK, hexokinase; Man, mannose; Man-6-P, mannose-6-phosphate; Man-1-P, mannose-1-phosphate; Fru-6-P, fructose-6-phosphate; MLSMR, NIH Molecular Libraries Small Molecule Repository; PIFA, phenyliodine bis(trifluoroacetate); MLPCN, NIH Molecular Libraries Probe Production Centers Network; GSH, glutathione; LCMS, liquid chromatography−mass spectrometry

REFERENCES

(1) (a) Freeze, H. H. Towards a therapy for phosphomannomutase 2 deficiency, the defect in CDG-Ia patients. *Biochim. Biophys. Acta* **2009**, *1792*, 835–840. (b) Jaeken, J.; Matthijs, G. Congenital disorders of glycosylation: A rapidly expanding disease family. *Annu. Rev. Genomics Hum. Genet.* **2007**, *8*, 261–278. (c) Freeze, H. H. Genetic defects in the human glycome. *Nat. Rev. Genet.* **2006**, *7*, 537–551. (d) Eklund, E. A.; Freeze, H. H. Essentials of glycosylation. *Semin. Pediatr. Neurol.* **2005**, *12*, 134–143. (e) Eklund, E. A.; Merbouh, N.; Ichikawa, M.; Nishikawa, A.; Clima, J. M.; Dorman, J. A.; Norberg, T.; Freeze, H. H. Hydrophobic Man-1-P derivatives correct abnormal glycosylation in Type I congenital disorder of glycosylation fibroblasts. *Glycobiology* **2005**, *15*, 1084–1093.

(2) (a) Freeze, H. H.; Sharma, V. Metabolic manipulation of glycosylation disorders in humans and animal models. *Semin. Cell Dev. Biol.* **2010**, *21*, 655–662. (b) Fujita, N.; Tamura, A.; Higashidani, A.; Tonozuka, T.; Freeze, H. H.; Nishikawa, A. The relative contribution of mannose salvage pathways to glycosylation in PMI-deficient mouse embryonic fibroblast cells. *FEBS J.* **2008**, *275*, 788–798. (c) Niehues, R.; Hasilik, M.; Alton, G.; Körner, C.; Schiebe-Sukumar, M.; Koch, H. G.; Zimmer, K. P.; Wu, R.; Harms, E.; Reiter, K.; von Figura, K.; Freeze, H. H.; Harms, H. K.; Marquardt, T. Carbohydrate-deficient glycoprotein syndrome type Ib. Phosphomannose isomerase deficiency and mannose therapy. *J. Clin. Invest.* **1998**, *101*, 1414–1420.

(3) (a) Foret, J.; de Courcy, B.; Gresh, N.; Piquemal, J. P.; Salmon, L. Synthesis and evaluation of non-hydrolyzable D-mannose 6-phosphate surrogates reveal 6-deoxy-6-dicarboxymethyl-D-mannose as a new strong inhibitor of phosphomannose isomerases. *Bioorg. Med. Chem.* **2009**, *17*, 7100–7107. (b) Bhandari, A.; Jones, D. G.; Schullek, J. R.; Vo, K.; Schunk, C. A.; Tamanaha, L. L.; Chen, D.; Yuan, Z.; Needels, M. C.; Gallop, M. A. Exploring structure-activity relationships around the phosphomannose isomerase inhibitor AF14049 via combinatorial

synthesis. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2303–2308. (c) Roux, C.; Lee, J. H.; Jeffery, C. J.; Salmon, L. Inhibition of type I and type II phosphomannose isomerases by the reaction intermediate analogue 5-phospho-D-arabinonohydroxamic acid supports a catalytic role for the metal cofactor. *Biochemistry* **2004**, *43*, 2926–2934.

(4) Correa, A.; Tellitu, I.; Domínguez, E.; SanMartin, R. Novel alternative for the N-S bond formation and its application to the synthesis of benzisothiazol-3-ones. *Org. Lett.* **2006**, *8*, 4811–4813.

(5) Klapars, A.; Huang, X.; Buchwald, S. L. A general and efficient copper catalyst for the amidation of aryl halides. *J. Am. Chem. Soc.* **2002**, *124*, 7421–7428.

(6) Lipinski, C. A. Drug-like properties and the causes of poor solubility and poor permeability. *J. Pharmacol. Toxicol. Methods* **2000**, *44*, 235–249.

(7) Kansy, M.; Senner, F.; Gubernator, K. Physicochemical high throughput screening: Parallel artificial membrane permeation assay in the description of passive absorption processes. *J. Med. Chem.* **1998**, *41*, 1007–1010.

(8) Baillie, T. A.; Davis, M. R. Mass spectrometry in the analysis of glutathione conjugates. *Biol. Mass Spectrom.* **1993**, *22*, 319–325.