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Discovery and development of small-molecule inhibitors of glycogen synthase

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ABSTRACT

The over-accumulation of glycogen appears as a hallmark in various glycogen storage diseases (GSDs), including Pompe, Cori, Andersen and Lafora disease. Accumulating evidence suggests that suppression of glycogen accumulation represents a potential therapeutic approach for treating these GSDs. Using a fluorescence polarization assay designed to screen for inhibitors of the key glycogen synthetic enzyme, glycogen synthase (GS), we identified a substituted imidazole, (*rac*)-2-methoxy-4-(1-(2-(1-methylpyrrolidin-2-yl)ethyl)-4-phenyl-1*H*-imidazol-5-yl)phenol (**H23**), as a first-in-class inhibitor for yeast glycogen synthase 2 (yGsy2p). Data from X-ray crystallography at 2.85 Å, as well as kinetic data, revealed that **H23** bound within the UDP-glucose binding pocket of yGsy2p. The high conservation of residues between human and yeast GS in direct contact with **H23** informed the development of around 500 **H23** analogs. These analogs produced a structure-activity relationship (SAR) profile that led to the identification of a substituted pyrazole, 4-(4-(4-hydroxyphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-5-yl)pyrogallol, with 300-fold improved potency against human GS. These substituted pyrazoles possess a promising scaffold for drug development efforts targeting GS activity in GSDs associated with excess glycogen accumulation.

INTRODUCTION

Glycogen is a branched polymer of glucose, composed of chains thirteen residues long on average, arranged in layers to form a molecule of up 10^7 daltons, corresponding to 55,000 glucose units. The biosynthesis of glycogen is a highly conserved, complex and coordinated process that involves two key events. Glycogen synthase (GS) lengthens linear chains through the progressive addition of α-1,4-linked glucose residues to the non-reducing end of the polymer, and glycogen-branching enzyme (GBE) catalyzes the intramolecular transfer of seven glucose residues from the end of a linear chain to a C-6 hydroxyl group to produce α-1,6 branch points for further elongation[1,2,3,4].

GS is the rate-limiting enzyme for glycogen biosynthesis[5]. Higher eukaryotes have two isoforms, *GYS1* and *GYS2*[6,7], that share approximately 70% sequence identity at the amino acid level and display the greatest variation within the N- and C-terminal sequence extensions where regulatory sites are located. The two GS isoenzymes are differentially expressed: *GYS2* is exclusively expressed in the liver, while expression of *GYS1* is highest in skeletal muscle but is also expressed in most other tissues. These two principal storage tissues for glycogen serve distinct physiological roles. Glycogen in the liver plays an essential, regulatory role in glucose homoeostasis, but glycogen in other tissues acts as an intracellular energy reserve such as during muscle contraction[8]. Similar to higher eukaryotes, *Saccharomyces cerevisiae* has two genes encoding GS, *GSY1* and *GSY2*, with Gsy2p as the predominant isoform[9]. GS undergoes activation allosterically through glucose-6-phosphate (G6P) binding and inactivation by phosphorylation at a number of sites through the action of multiple protein kinases, including glycogen synthase kinase-3 (GSK-3) in mammals[10,11]. After transport into cells, glucose undergoes phosphorylation to G6P and enters one of several metabolic pathways. Increased

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levels of intracellular G6P stimulates GS activity that in turn drives the incorporation of glucose into glycogen in a feed-forward mechanism[12]. The dephosphorylation of GS is mediated by forms of type 1 protein phosphatase (PP1), the catalytic subunit of which is held in close proximity to the glycogen granule and GS by a family of glycogen targeting subunits, including protein-targeting-to-glycogen (PTG), an indirect activator of GS[13].

Glycogen metabolism constitutes a key pathway in living cells that regulates systemic carbon or energy allocation[14]. A number of diseases are associated with abnormal glycogen metabolism including type 2 diabetes (T2D) and glycogen storage diseases (GSDs)[15,16,17]. Defects in enzymes directly involved in either glycogen synthesis or degradation[18] are the basis for more than 12 different GSDs, and glycogen over-accumulation is characteristic of most GSDs. In Pompe disease (GSD2), a deficiency of the enzyme, acid-α-glucosidase (GAA), leads to lysosomal glycogen accumulation in many tissues including skeletal, cardiac, and smooth muscle[19]. In Cori disease (GSD3) and Andersen disease (GSD4), defects in the glycogen debranching enzyme (AGL) and glycogen branching enzyme (GBE1), respectively, result in the accumulation of glycogen with abnormal structure[20,21]. Lafora disease is a fatal progressive myoclonus epilepsy accompanied by neurodegeneration for which the presence of abnormal glycogen inclusions known as Lafora bodies[22] are the hallmark. Lafora bodies consist of poorly branched, hyperphosphorylated, and insoluble forms of glycogen that occur in neuronal, muscle and other tissues[23,24,25]. Notably, impaired glycogen metabolism and consequent glycogen accumulation was also found to be pathological for β-cell dysfunction in T2D[16,17].

A common finding with excess glycogen accumulation is an association with impaired autophagy and dysregulated mitochondrial metabolism. These derangements often lead to cell death and early-onset lethal, disease progression in GSD-affected patients[16,26,27]. Currently, there

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are no effective treatments for GSDs that ameliorate all cellular and organ dysfunction. For

example, in Pompe disease, the administration of a genetically engineered enzyme (*i.e.*, Enzyme

Replacement Therapy, ERT) that rescues the defective GAA in peripheral tissues fails to reverse

the neurological defects. Partial suppression of glycogen synthesis through inhibition of GS

activity represents an alternative, potentially effective strategy for treating various types of GSDs

where ERT may not provide a complete solution. For instance, mouse models of Lafora disease

that lack either of the causative genes, *EPM2A* or *EPM2B,* recapitulate aspects of the patient

phenotype, in that they accumulate polyglucosan bodies and misfolded proteins, display

increased endoplasmic reticulum stress, and show signs of neurodegeneration[23,27]. Such animal

models provided a venue for disrupting either *PTG* or *GYS1* in these *EPM2A*[(-/-)] and *EPM2B*[(-/-)]

knock-out mice as a mechanism for diminishing glycogen accumulation, Lafora body formation

and the associated neurological and epileptic symptoms[27,28,29,30]. Inhibition of GS activity *via*

suppression of mTOR signaling also increases the effectiveness of treatments for Pompe disease

in conjunction with ERT[31]. Moreover, a recent report has shown that GYS2 inhibition with

RNAi prevents liver injury in mouse models of GSDs[32]. Although human clinical characteristics

may vary from animal phenotypes, the availability of mouse models and the appearance of

promising therapeutic approaches suggest the feasibility of using small-molecule interventions to

treat GSDs. We now report efforts to develop small-molecule inhibitors of human GS based on a

multicomponent study involving high-throughput screening (HTS), X-ray crystallography and

extensive SAR development work that led to substituted pyrazoles with low micromolar,

inhibitory IC_{50} values against human glycogen synthase 1 (hGYS1).

RESULTS

Development of fluorescence polarization assay for HTS

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The standard radiochemical assay for GS activity utilizes a time-consuming ^{14}C-glucose incorporation assay (*i.e.*, UDP-[U-^{14}C]glucose and glycogen$_{(n)}$ affords [^{14}C]glycogen$_{(n+1)}$ and UDP) that involves appropriate safety measures for handling radioisotopes as well as multiple transfer and washing steps to remove residual substrates[33]. The combination of laboratory effort, cost, safety and waste disposal favored the development of a fluorescence polarization (FP) assay for HTS as a sensitive, inexpensive, and rapid alternative[34]. We reserved the classical radiochemical assay for subsequent studies to confirm the hits emerging from the FP assay. An FP assay utilizes a fluorescently-labeled tracer molecule that binds to GS to form a slower rotating complex than fluorophore alone and that leads to enhanced polarization of the emitted light. Incubation of the GS-fluorophore complex with small-molecules that displace the fluorescent tracer produces greater rotational motion of fluorophore than that of the GS-fluorophore complex and causes relative depolarization of the emitted light. Using this assay system as a vehicle for HTS, we identified small-molecules that reduce the fluorescence polarization signal as GS-interacting agents.

The development of any FP assay involves the design and synthesis of a functional, fluorescent probe. Although G6P is a well-known, allosteric activator of GS, the regioselective modification of similarly reactive hydroxyl groups in G6P presented an unwelcome challenge. Because glucosamine-6-phosphate (GlcN6P) also activated GS[35] and has a single, reactive amine group, its selection as a platform on which to incorporate a fluorophore was an attractive alternative to G6P. Using the ^{14}C-glucose incorporation assay, we confirmed that either G6P or GlcN6P activated hGYS1 with AC$_{50}$ values of 1.6 $\pm$ 0.1 mM or 5.9 $\pm$ 0.1 mM, respectively (**Supplemental Figure 1A**). With direct evidence in hand that GlcN6P activated hGS, we synthesized a fluorophore-modified GlcN6P (aka GlcN6P-fluorescein-5-Ex) using GlcN6P and

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the N-hydroxysuccinimidyl ester of fluorescein-5-Ex (**Figure 1A**), purified the fluorophore by

high-performance liquid chromatography (HPLC) and confirmed the expected molecular mass

by mass spectrometry in which it displayed a M+H$^+$ peak at *m/z* 735.1267 consistent with

$[C_{31}H_{31}N_2O_{15}PS + H]^+$ with calculated exact mass within acceptable limits (*i.e.*, calculated *m/z*

735.1256) (**Supplemental Figure 1B**).

We examined the binding of the fluorophore, GlcN6P-fluorescein-5-Ex, to GS by adding the

tracer to varying concentrations of yGsy2p to generate a saturation binding curve and calculate a

dissociation constant of 7.6 $\pm$ 0.7 μM (**Figure 1B**). Furthermore, a competitive displacement by

G6P (K_d = 70.9 $\pm$ 3.6 μM) demonstrated that the tracer molecule bound to the G6P allosteric

site (**Figure 1C**). For comparison, we determined yGsy2p activation by G6P using the standard

^{14}C-glucose incorporation assay which yielded an AC_{50} of 98.1 $\pm$ 3.1 μM (**Supplemental**

Figure 1C). We also tested the binding of GlcN6P-fluorescein-5-Ex and displacement by G6P

using hGYS1. While the GlcN6P-coupled fluorophore and the fluorophore alone bound to

hGYS1, G6P was unable to displace either compound, a finding that suggested nonspecific

binding of the fluorophore to hGYS1. This reason, combined with the fact that production of

human GYS1 in insect cells results in a heavily phosphorylated enzyme that requires very high

concentrations of G6P for activity measurements, makes the full-length recombinant human

enzyme unsuitable for an assay designed to displace G6P. However, since the fluorescent probe

could be fully displaced from yGsy2p through competition with G6P, we used yGsy2p and not

hGYS1 for HTS. The high conservation of residues within the G6P allosteric site, the active site,

and overall protein sequence identity (~55%) between yGsy2p and hGYS1 further supported the

decision to use the yeast enzyme as a screening surrogate for the human enzyme (**Supplemental**

Figure 2). The robustness of the FP assay for HTS was assessed by determining the Z′-factor, a

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parameter reflective of both the signal dynamic range and data variability[36]. While the ideal Z′-factor is 1, a Z′-factor between 0.5 and 1 is considered excellent and suitable for screening assays. The FP values for the positive controls (maximal binding, without G6P) and negative controls (fully displaced tracer, with G6P) were 109.23 ± 1.97 and 26.48 ± 1.93 (mean $\pm$ SD) from the 384-wells, respectively. The Z′-factor for this assay was determined as 0.86, indicating that this assay is well suited for HTS (**Figure 1D**).
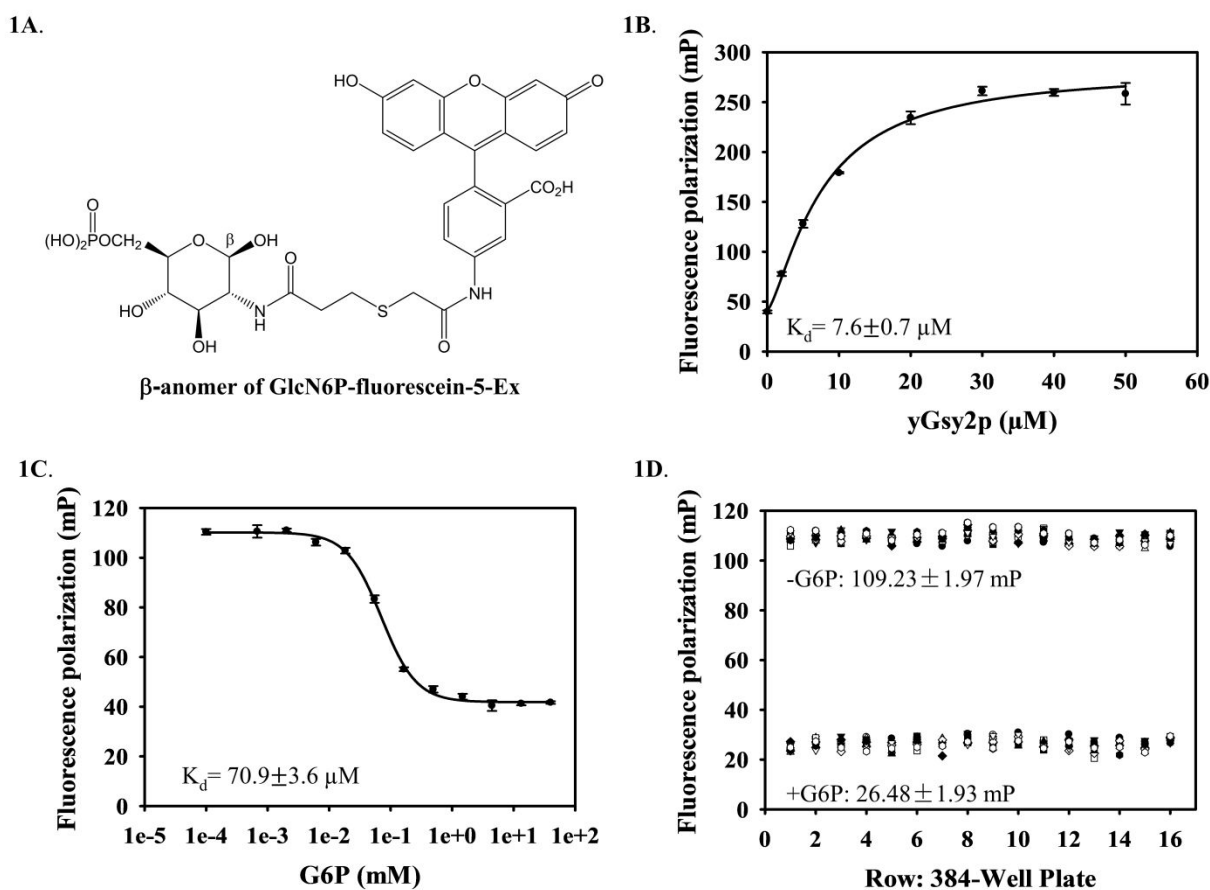
Figure 1. Development of fluorescence polarization assay for HTS. (A) 2-(6-Hydroxy-3-oxo-3H-xanthen-9-yl)-5-(2-((3-oxo-3-((((2R,3R,4R,5S,6R)-2,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)amino)propyl)thio)acetamido)benzoic acid (β-anomer of GlcN6P-fluorescein-5-Ex). (B) Saturation binding isotherm for GlcN6P-fluorescein-5-Ex

binding to yGsy2p. The binding affinity was determined by adding GlcN6P-fluorescein-5-Ex to

a final concentration of 20 nM in the presence of varying yGsy2p concentrations (0 to 50 µM).

K_d= 7.6 $\pm$ 0.7 µM. Averages of triplicate assays ± SEM are shown. (C) Displacement of

GlcN6P-fluorescein-5-Ex binding to yGsy2p by G6P. A mixture of yGsy2p and GlcN6P-

fluorescein-Ex was added to various G6P concentrations (0.68 µM to 40 mM). K_d= 70.9 $\pm$ 3.6

µM. Averages of triplicate assays ± SEM are shown. (D) Z′-factor determination: fluorescence

polarization values of the bound (-G6P), and the free (+2 mM G6P) GlcN6P-fluorescein-5-Ex

are shown, for the 16 rows with each row containing 12 columns of either the bound or free state.

The graphs depict a representative experiment from at least three independent experiments.

Hits identification and validation

We used the aforementioned FP displacement assay to screen the 50K ChemBridge Diversity

library at a final assay concentration of 10 µM against the yGsy2p enzyme. The HTS was

adapted to the 384-well plate format using three columns for the DMSO negative controls and

one column for unlabeled G6P as the positive control, resulting in a total of 157 screening plates

(**Supplemental Table 1 and 2**). We used a Z'-score threshold of -1, corresponding to a

separation of 3 standard deviations between μ_c (means of the control DMSO signal) and μ_s

(means of the library sample signal) as a signal cutoff (**Supplemental Table 2**). These standards

produced 117 hits and an overall 0.23% hit rate. Initial stock availability led to re-purchasing of

110 compounds (designated **H1-H110**) from ChemBridge and validation of their activity using

the standard ^{14}C-glucose incorporation assay at 100 µM concentration. This approach validated

16 hits with greater than 40% inhibition of yGsy2p activity (**Figure 2A**).

Since the yeast yGsy2p enzyme was used as a surrogate for the human hGYS1 enzyme, we next

examined if the 16 validated compounds were also active as inhibitors of hGYS1 activity. To

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accomplish this necessary confirmation, we redesigned the hGYS1 construct to create a

constitutively active enzyme through deletion of the C-terminal phosphorylation domain

(residues 635-737) and through the substitution of the two N-terminal phosphorylation sites

(Ser8, Ser11) with Asn residues (designated hGYS1Δ634S8,11N) (**Figure 2B**). Purification of

this truncated form of GS in significant quantity led to an enzyme that exhibited an activation

state of ~0.2, a ratio of GS activity in the absence over the presence of G6P as an index of the

phosphorylation state of the enzyme[37]. Unlike the heavily phosphorylated, full-length enzyme

produced in insect cells that had an activation state of <0.01[38], this truncated form of GS was

well suited for validation assays designed to look for inhibitors because the enzyme was neither

overly inhibited by phosphorylation nor rendered insensitive to the effects of G6P. At a test

concentration of 100 μM, only 1 hit, namely a substituted imidazole, (*rac*)-2-methoxy-4-(1-(2-

(1-methylpyrrolidin-2-yl)ethyl)-4-phenyl-1*H*-imidazol-5-yl)phenol (**H23**), demonstrated greater

than 20% inhibition using this truncated hGYS1 (**Figure 2C and 2D**).

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2A.

2B.

2D.

Screen triaging strategy

2C.
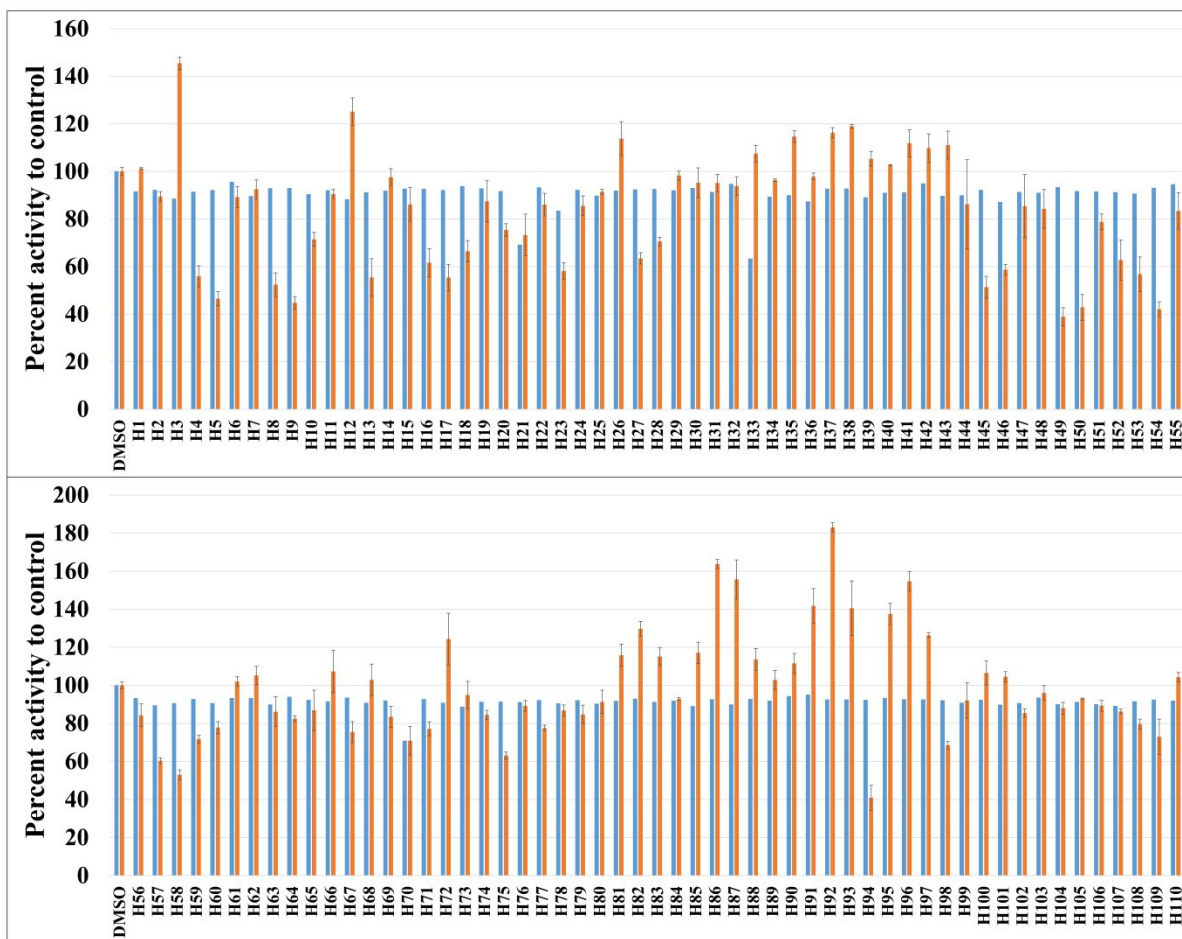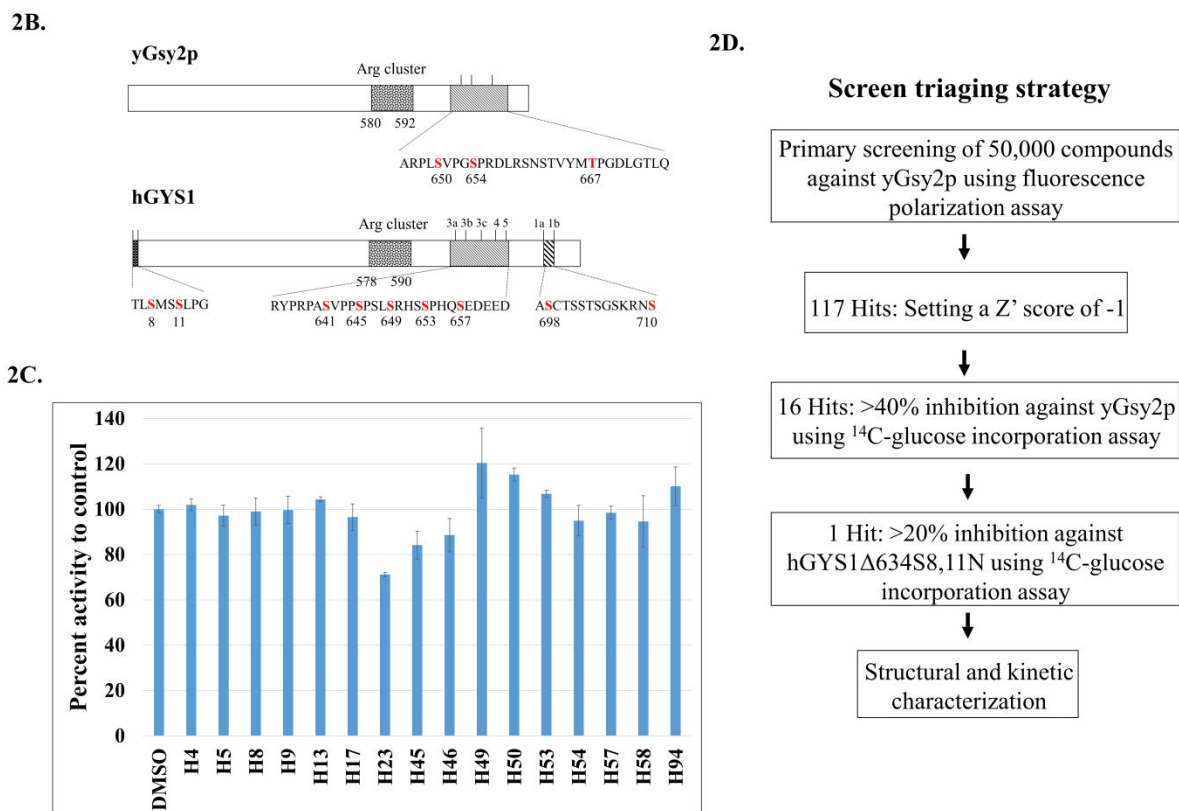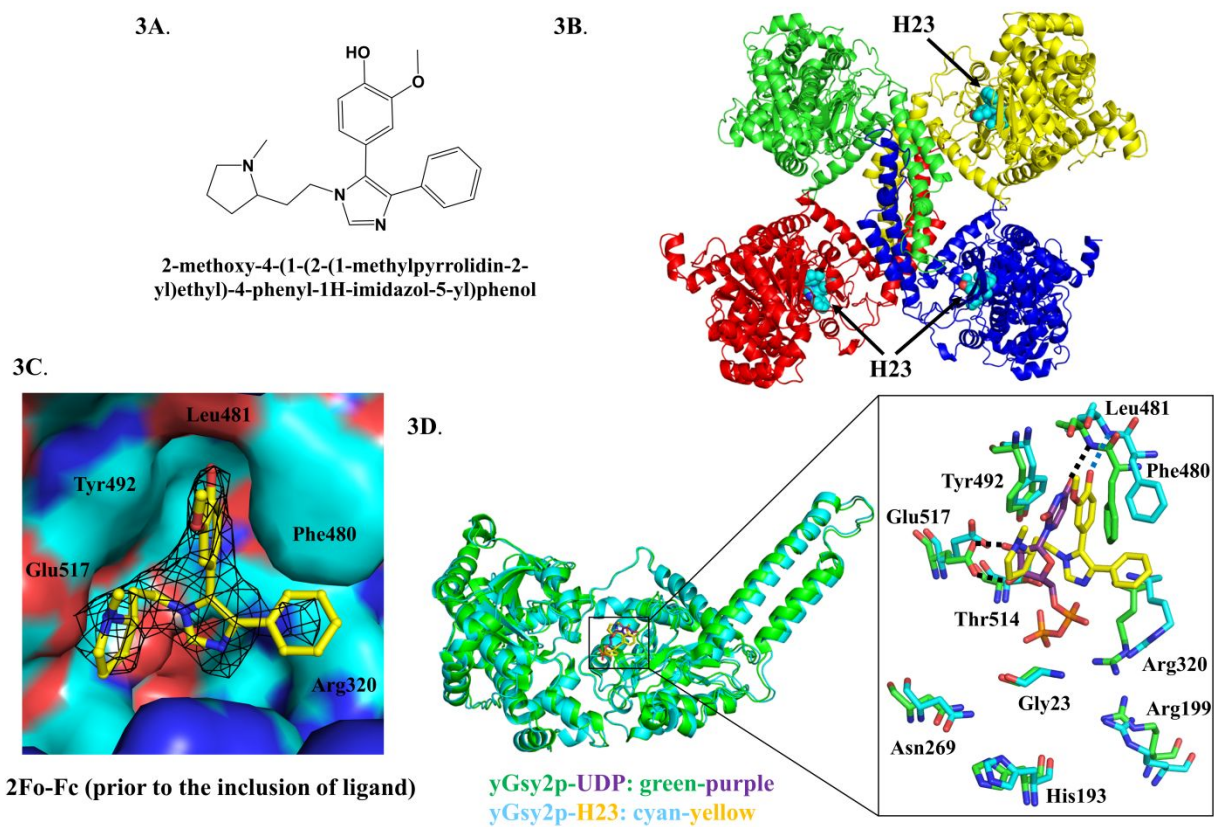

Figure 2. Hits identification and validation. (A) In the FP assay, setting a Z'-score threshold of

-1 gave 117 initial hits, out of which 110 (designated **H1-H110**) were re-purchased and tested.

The blue column represents the percentage of fluorescence polarization signal to control. The

hits were screened at 10 μM in single measurement. The orange column is the percentage of

yGsy2p activity to control measured through ^{14}C-glucose incorporation assay. The hits were

tested at 100 μM. Averages of triplicate assays ± SEM are shown. (B) Schematic representation

of yGsy2p and hGYS1 protein. Phosphorylation residues are shown in red. To generate

hGYS1Δ634S8,11N mutant, hGSY1 was truncated at position 634 with mutations of Ser8/11 to

Asn to eliminate potential phosphorylation sites. (C) Percentage of hGYS1Δ634S8,11N activity

to control measured through ^{14}C-glucose incorporation assay. The hits were tested at 100 μM.

Averages of triplicate assays ± SEM are shown. (D) Screening triaging strategy.

Crystal structure of the H23-yGsy2p complex

Although there are no mammalian GS crystal structures, the structures of two eukaryotic

enzymes, namely yGsy2p from *Saccharomyces cerevisiae*[10] and GS from *Caenorhabditis*

elegans[39] encouraged our undertaking crystallization experiments. As previously described, yeast

Gsy2p existed as tetramer in the crystal structure, and each subunit contained two Rossmann-fold

domains with the catalytic site in the interdomain cleft[10]. In the crystal packing environment in

crystals of the activated form of GS, one subunit appeared 13.3º more closed than the other three

"open" subunits[40]. We successfully determined the crystal structure of the **H23**-yGsy2p complex

to a resolution of 2.85 Å (**Table 1**), and we observed **H23** binding in three of the four subunits,

all of which corresponded to the "open" domain positions (**Figure 3B**). The structure showed

H23 bound within the active site of GS in a location that overlapped with the binding site for

UDP-glucose (UDPG)[40] (**Figure 3C and 3D**). Structural alignment of the R589/592A2·UDP

complex to the WT·**H23** complex using their C_α carbons generated an overall root mean square

deviation (rmsd) of 0.36 Å, indicating a high degree of similarity. The binding of **H23** to yGsy2p

was mediated by hydrogen-bond formation between the phenolic hydroxyl group of **H23** and the

nitrogen backbone of Leu481; hydrophobic interactions of the 2-methoxyphenol sandwiched

between Tyr492 and Phe480; van der Waals interactions of the benzene group with the side

chains of Phe480 and Arg320; and additional van der Waals interactions of the *N*-

methylpyrrolidine moiety with Tyr492, Thr514, and Glu517 (**Figure 3D**).

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Table 1. Structural data and refinement statistics

Data collection		Refinement	
Space group	*I222*	No. reflections	95326
Cell dimensions		R_{work}/R_{free}	0.20/0.26
a, b, c (Å)	192.3, 206.6, 205.0	r.m.s. deviations	
α, β, γ (°)	90.0, 90.0, 90.0	Bond lengths (Å)	0.010
Resolution (Å)	50-2.85	Bond angles (°)	1.44
R_{merge}	0.091 (0.766)	Ramachandran plot	
R_{meas}	0.100 (0.843)	Preferred/Allowed (%)	99.01
R_{pim}	0.040 (0.347)	Outliers (%)	0.99
CC1/2	0.999 (0.857)	B-factors	
$I/\sigma(I)$	20.3 (2.0)	Protein	Chain A, 80.1; B, 83.7; C, 91.9; D, 94.6
Completeness (%)	99.7 (100)		
Redundancy	6.3 (5.5)	Ligand (H23)	Chain A, 100.9; C, 128.3; D, 107.5

3A.

2-methoxy-4-(1-(2-(1-methylpyrrolidin-2-yl)ethyl)-4-phenyl-1H-imidazol-5-yl)phenol

3B.

3C.

2Fo-Fc (prior to the inclusion of ligand)

3D.

yGsy2p-UDP: green-purple
yGsy2p-H23: cyan-yellow

Figure 3. Crystal structure of the H23-yGsy2p complex (PDB: 6U77). (A) Chemical structure of **H23**. (B) Ribbon diagram representation of the crystal structure of **H23**-yGsy2p complex. **H23** is represented by space-filling models in cyan, and binds to three subunits of yGsy2p which are colored differently. (C) The electron density for **H23** prior to the inclusion of the ligand in refinement. The map shown is the original unbiased 2Fo-Fc map contoured at 1 standard deviation. (D) Stick representation of the superposed UDP (purple) and **H23** (yellow) structures and their interactions with the surrounding amino acids.

Kinetic characterization of H23

In a study of the inhibitory potential for **H23** against yeast and human GS using the standard ^{14}C-glucose incorporation assay, **H23** exhibited IC_{50} values of 280 μM and 263 μM in the absence and presence, respectively, of G6P for yGsy2p (**Figure 4A**). The similarity in IC_{50} values indicated **H23** was not in direct competition with G6P. Under subsaturating G6P concentrations, the IC_{50} values of **H23** against either hGYS1Δ634S8,11N or wild-type hGYS1 were 161 μM and 875 μM, respectively (**Figure 4B**). Even though the presence or absence of G6P did not impact **H23** potency, the activity state of the human GS enzyme had a five-fold effect on the binding of **H23**.

To understand **H23** mode of inhibition, we performed co-variation experiments by simultaneously varying the concentrations of UDPG at different fixed concentrations of **H23** and fitting the kinetic data against the competitive, non-competitive and uncompetitive inhibition equations. For yGsy2p, the inhibition data with **H23** was consistent with a competitive mode of inhibition with respect to varied UDPG, with K_i values of 370 μM in the presence of G6P, and 290 μM in the absence of G6P. UDP displayed a similar competitive mode of inhibition with a K_i value of 350 μM (**Figure 4C**).

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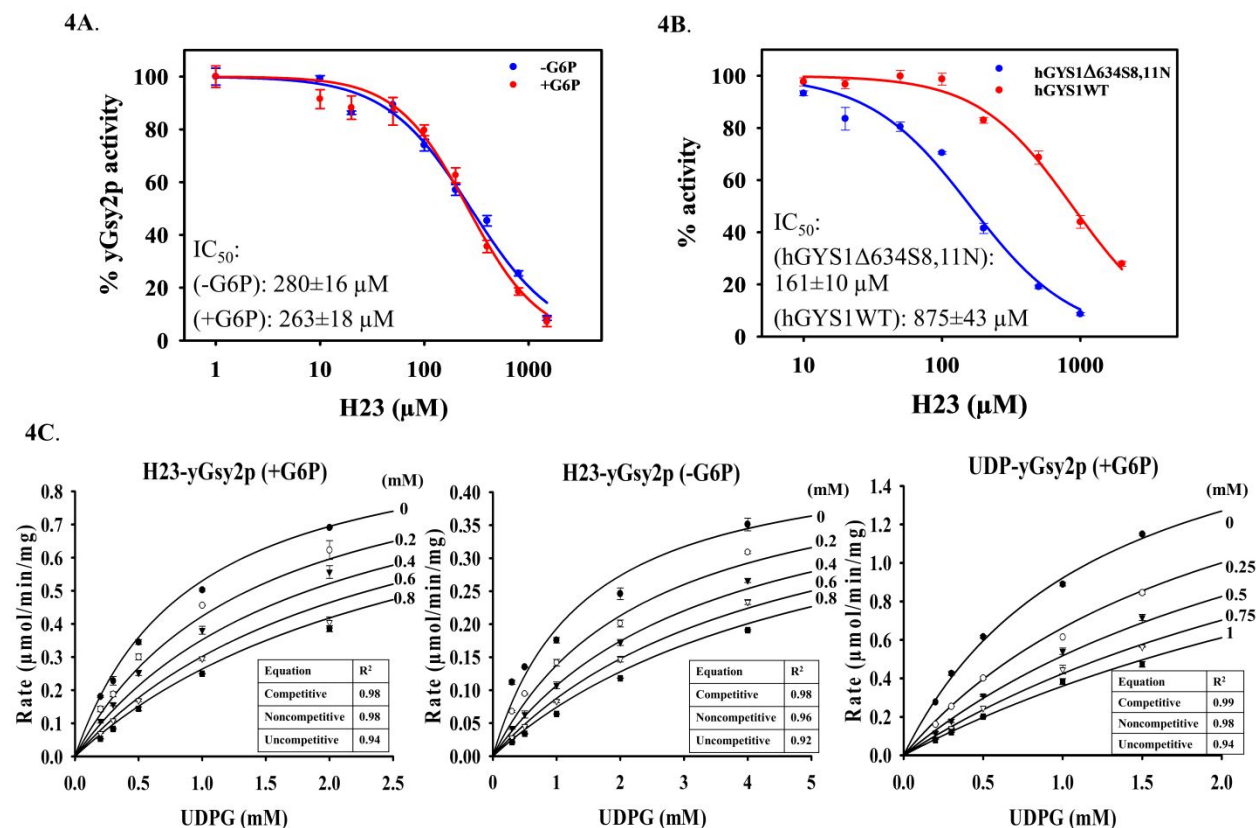
Figure 4. Kinetic characterization of H23. (A) Inhibition of **H23** to yGsy2p in the absence or

presence of G6P. (B) Inhibition of **H23** to hGYS1Δ634S8,11N and hGYS1 wild type. All IC_{50}

curves represent one of three experiments performed using triplicate measurements for each

condition, with mean ± SEM shown. (C) Michaelis-Menten curves fit to the competitive

inhibition equation for varied **H23** versus UDPG. The values of goodness of fit (R^2) are shown

for all equations evaluated. **H23** has a K_i of 370 ± 30 μM in the presence of G6P and a K_i of 290

± 20 μM in the absence of G6P. UDP is used as a positive control for competitive inhibition

against varied UDPG for yGsy2p, which displayed a K_i of 350 ± 10 μM. The reported K_i values

are the mean ± SEM from three independent experiments in duplicate.

Development of structure-activity relationships for analogs of H23 toward hGYS1

The overall protein sequence alignment demonstrates 55% sequence identity between yGsy2p and hGYS1. Because the amino acids in the binding site for **H23**, including Arg320, Phe480, Leu481, Tyr492, and Glu517 in yGsy2p, are completely conserved across yeast and human species (**Supplemental Figure 2**), the structural information derived from **H23**-yGsy2p crystal structure provided a useful guide for structure-activity studies focused on hGYS1. We examined a total of 491 analogs that shared at least 50% structural similarity with **H23**, and we tested their activities against yGsy2p, hGYS1Δ634S8,11N, and wild-type hGYS1 using the ^{14}C-glucose incorporation assay. Our initial kinetic studies showed that a five-membered, heteroaryl (HA) core with a phenyl group at the A_1 position, and a second, vicinal phenyl group were essential elements of **H23** (**Table 2**). Consequently, analog development modified the HA core, A_1 and substructures at R_1-R_7 positions shown in the structure in **Table 2**. In this study, all **H23** analogs possessed one of the following HA cores: imidazole (designated as HA_1), pyrrole (designated as HA_2) or pyrazole (designated as HA_3). Active compounds appeared in structures with any of these three cores, but the most potent compounds had a pyrazole (HA_3) scaffold. Commercial libraries were the source of HA_1 and HA_2 compounds in **Table 2**, and a three-step synthesis provided the HA_3 compounds as outlined in **Scheme 1**.

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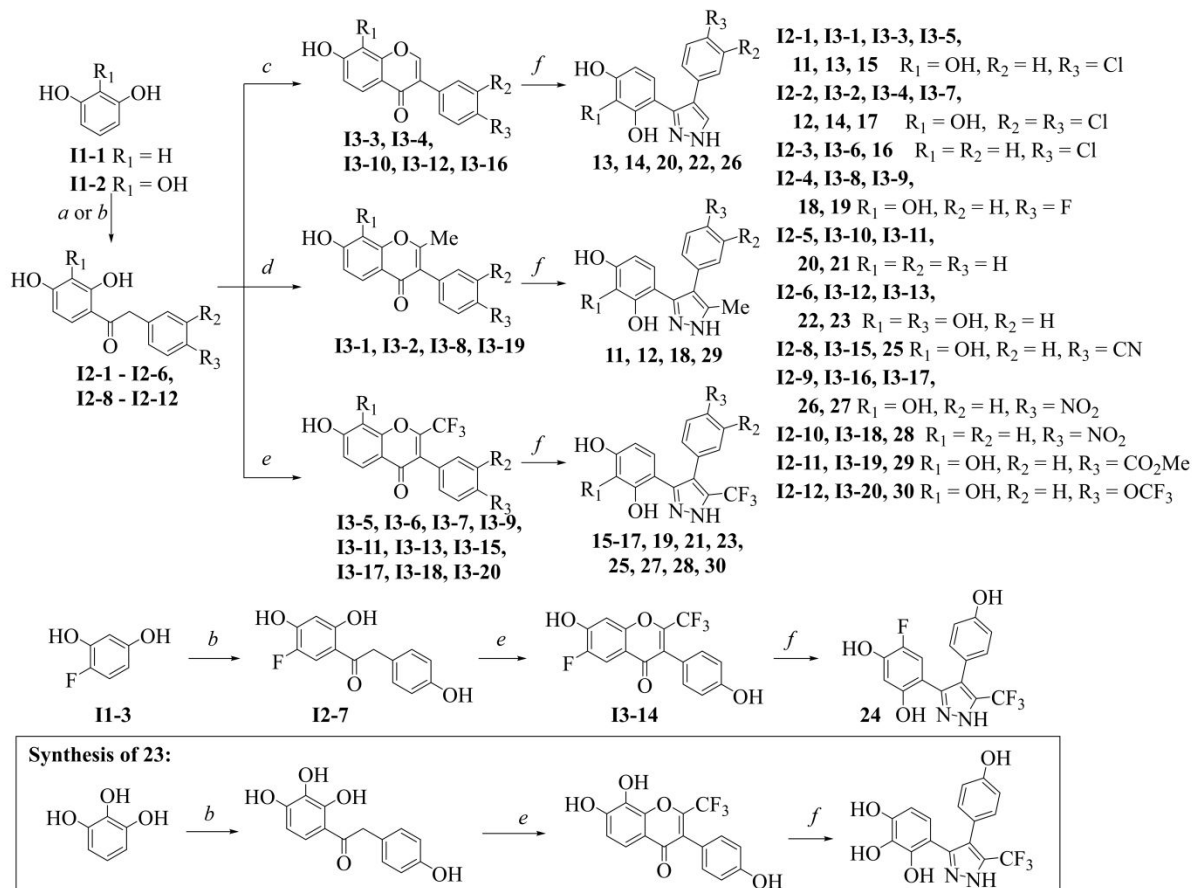
Scheme 1. Synthesis of pyrazoles 11-30. Reagents and conditions: (*a*) (i) arylacetonitrile,

BF$_3$·Et$_2$O, HCl (gas), rt, 6-8 h, (ii) H$^+$/H$_2$O, 0.5-2 h; (*b*) (i) arylacetic acid, BF$_3$·Et$_2$O, 80-90 ^{0}C, 2

h, (ii) H$_2$O; (*c*) (i) DMF, BF$_3$·Et$_2$O, POCl$_3$, 50-60 ^{0}C, 2 h, (ii) H$_2$O; (*d*) (i) Ac$_2$O, KOAc, reflux, 8

h, (ii) H$_2$SO$_4$, EtOH, reflux, 0.5 h; (*e*) (i) (CF$_3$CO)$_2$O, pyridine, rt, 48-120 h, (ii) H$_2$O; (*f*)

N$_2$H$_4$·H$_2$O, reflux, 0.5-6 h.

A step-by-step program of modifying positions in **H23** involved changes designed either to

augment or diminish interactions with substituents in **H23** and the adjoining residues in yGsy2p.

Briefly, starting from the imidazole core (HA$_1$), we determined that the R$_1$ hydroxyl group that

formed a hydrogen bond with the peptide nitrogen of Leu481 was essential to retain good

inhibitory activity (*i.e.*, compare **H23** *versus* **1** in **Table 2**). The addition of an extra methylene

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unit to the R_7 chain eliminated inhibitory potential (**4** *versus* **3**). In a similar fashion, the most potent of the pyrrole HA_2 compounds also had a hydroxyl group at R_1 position (**7** *versus* **9** or **10**). Interestingly, substitution of a carboxyl group at R_7 position improved the potency to hGYS1 by 2-fold (IC_{50} values for **H23** *versus* **7**), a finding that suggested different conformational binding modes for **H23** and **7** for hGYS1.

Because none of the analogs within the HA_1 or HA_2 series generated noteworthy improvements in potency, we turned to the pyrazole (HA_3) series. Within the HA_3 series, SAR studies demonstrated the importance of a hydrogen bond acceptor at the R_1 position as displayed in the following order of potency: -OH > $-NO_2$ > -CN > -F > -H > -Cl (i.e., **23** *versus* **27**, **25**, **19**, **21** and **15**). When a chlorine is present at the R_1 position, adding a second chlorine substituent at the R_2 position improved potency (**12** *versus* **11** or **14** *versus* **13** or **17** *versus* **15**). The most potent compounds in this series had a pyrrogallol group at the R_{4-6} positions, and the hydroxyl groups on this substituent conferred inhibition to GS even if the R_1 position was non-optimal. For instance, **11** was 16-fold more potent than **H23**. Strikingly, the *meta*-hydroxyl group (R_5) was critical to binding. Substitution of this hydroxyl groups with just hydrogen decreased potency significantly (**16** *versus* **15** or **28** *versus* **27**). SAR studies also indicated that substituents at the R_7 position had an effect on activity in the following order of $-CF_3$ > -H > $-CH_3$ (**15** or **13** *versus* **11**; **17** or **14** *versus* **12**). In summary, analog development initiated from the substituted imidazole **H23** led to a substituted pyrazole, namely 4-(4-(4-hydroxyphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-5-yl)pyrogallol (**23**) that had an *in vitro* IC_{50} value of 2.75 μM, an improvement in potency toward hGYS1 of more than 300-fold.

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Table 2. Structure-activity relationships for H23 analogs
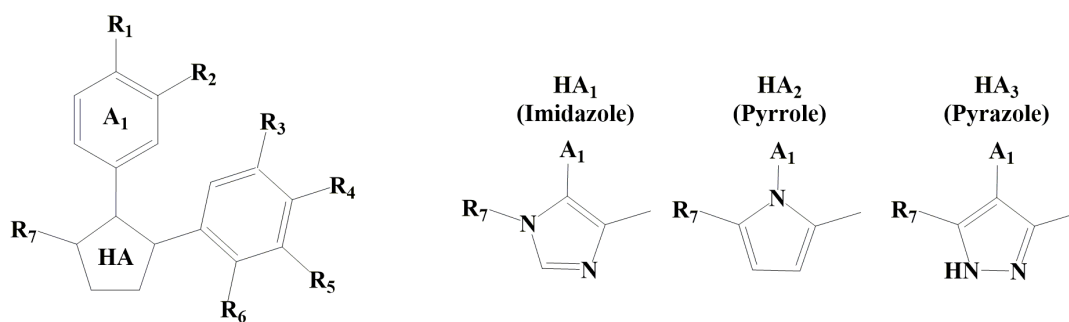
Compound	HA	A_1	R_1	R_2	R_3	R_4	R_5	R_6	R_7	%Activity @ 300μM compound yGsy2p	hGYS1Δ 634S8,11N	hGYS1	IC_{50} (μM) hGYS1
H23	HA_1	phenyl	OH	OCH_3	H	H	H	H	(pyrrolidine)	36.5±0.6	46.5±0.4	77.1±1.2	875±43
1	HA_1	pyridine	H	H	H	H	H	H	(pyrrolidine)	NI	93.3±1.5	NI	ND
2	HA_1	pyridine	H	H	H	H	H	H	(imidazole)	72.7±0.4	94.4±2.2	NI	ND
3	HA_1	phenyl	OH	H	H	H	H	H	(imidazole)	66.6±1.3	56.6±1.3	93.4±2.8	ND
4	HA_1	phenyl	OH	H	H	H	H	H	(imidazole)	94.8±2.9	NI	NI	ND
5	HA_2	phenyl	OH	H	H	H	H	H	CH_3	79.7±1.0	84.3±0.5	NI	ND
6	HA_2	phenyl	OH	H	H	H	H	H	(phenyl)	86.0±3.1	NI	NI	ND
7	HA_2	phenyl	OH	H	H	H	H	H	(COOH)	40.1±0.9	40.1±0.4	58.9±0.3	384±28
8	HA_2	phenyl	OH	H	H	OCH_3	H	H	(COOH)	34.5±0.4	50.6±3.0	84.0±0.1	ND
9	HA_2	phenyl	$CONH_2$	H	H	H	H	H	(COOH)	49.3±0.6	40.6±0.1	78.7±0.9	ND
10	HA_2	phenyl	F	H	H	H	H	H	(COOH)	84.7±0.1	72.1±4.1	87.5±0.4	ND
11	HA_3	phenyl	Cl	H	H	OH	OH	OH	CH_3	3.52±0.67	6.47±0.09	4.34±0.94	52.9±3.8

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Compound	HA	A$_1$	R$_1$	R$_2$	R$_3$	R$_4$	R$_5$	R$_6$	R$_7$	% Activity @ 20 µM compound			IC$_{50}$ (µM)
										yGsy2p	hGYS1Δ 634S8,11N	hGYS1	hGYS1
11	HA$_3$	(phenyl)	Cl	H	H	OH	OH	OH	CH$_3$	NI	NI	91.8±0.3	52.9±3.8
12	HA$_3$	(phenyl)	Cl	Cl	H	OH	OH	OH	CH$_3$	NI	81.7±2.7	84.7±2.5	ND
13	HA$_3$	(phenyl)	Cl	H	H	OH	OH	OH	H	NI	79.4±0.9	85.4±0.1	ND
14	HA$_3$	(phenyl)	Cl	Cl	H	OH	OH	OH	H	76.7±0.5	26.4±2.3	52.5±4.4	19.5±1.3
15	HA$_3$	(phenyl)	Cl	H	H	OH	OH	OH	CF_3	51.5±0.4	11.8±2.4	47.5±1.7	19.8±0.5
16	HA$_3$	(phenyl)	Cl	H	H	OH	H	OH	CF_3	NI	87.9±1.5	NI	ND
17	HA$_3$	(phenyl)	Cl	Cl	H	OH	OH	OH	CF_3	47.4±4.4	5.28±0.16	12.2±1.3	13.7±0.6
18	HA$_3$	(phenyl)	F	H	H	OH	OH	OH	CH$_3$	NI	89.0±1.5	94.0±2.4	ND
19	HA$_3$	(phenyl)	F	H	H	OH	OH	OH	CF_3	45.7±12.4	11.7±0.1	47.8±7.0	14.1±0.8
20	HA$_3$	(phenyl)	H	H	H	OH	OH	OH	H	NI	25.2±1.6	84.7±2.7	ND
21	HA$_3$	(phenyl)	H	H	H	OH	OH	OH	CF_3	51.8±1.8	5.68±0.16	47.2±1.2	16.4±0.6
22	HA$_3$	(phenyl)	OH	H	H	OH	OH	OH	H	6.21±0.59	23.0±1.9	22.6±0.4	9.82±1.06
23	HA$_3$	(phenyl)	OH	H	H	OH	OH	OH	CF_3	0.97±0.01	-1.01±0.37	-0.26±0.34	2.75±0.08
24	HA$_3$	(phenyl)	OH	H	F	OH	H	OH	CF_3	65.2±2.4	74.2±4.0	78.4±0.9	72.4±6.5
25	HA$_3$	(phenyl)	CN	H	H	OH	OH	OH	CF_3	51.2±1.5	8.14±0.65	42.0±1.5	10.1±0.8

Compound	HA	A$_1$	R$_1$	R$_2$	R$_3$	R$_4$	R$_5$	R$_6$	R$_7$	% Activity @ 20 μM compound			IC$_{50}$ (μM)
										yGsy2p	hGYS1Δ634S8,11N	hGYS1	hGYS1
26	HA$_3$	(phenyl)	O–N$^+$–O$^-$ (nitro)	H	H	OH	OH	OH	H	78.4±5.1	85.0±2.6	66.8±3.7	34.0±7.6
27	HA$_3$	(phenyl)	O–N$^+$–O$^-$ (nitro)	H	H	OH	OH	OH	CF$_3$	18.8±0.5	10.5±0.1	1.43±0.13	6.54±0.09
28	HA$_3$	(phenyl)	O–N$^+$–O$^-$ (nitro)	H	H	OH	H	OH	CF$_3$	82.3±7.5	92.7±1.6	89.2±6.4	ND
29	HA$_3$	(phenyl)	O–C–O (ester)	H	H	OH	OH	OH	CH$_3$	90.6±5.8	NI	80.1±3.6	ND
30	HA$_3$	(phenyl)	O–CF$_3$	H	H	OH	OH	OH	CF$_3$	91.6±0.6	40.3±2.3	81.4±2.1	ND

For HA$_3$ Scaffold, IC$_{50}$(S) were determined using a cut-off of 70% activity to hGYS1. Compound 24 is an exception since it has a unique F at R$_3$ position. NI: no inhibition, greater than 95% activity; ND: not determined. Values are the mean ± SEM from at least three independent experiments in duplicate.

Kinetic characterization of compound 23

In order to understand the molecular features of our most potent analog, compound **23**, we examined the kinetic characteristics of its interaction with GS (**Figure 5A**). Compound **23** has a competitive mode of inhibition for wild-type hGYS1, with a K$_i$ value of 1.31 ± 0.14 μM (**Figure 5B**). In light of the SAR and our kinetic results, we hypothesized that the binding of **23** to the active site of yGsy2p resembled the binding of **H23** in the active site. In this model, the hydroxyl group at R$_1$ position formed a hydrogen bond with the nitrogen backbone of Leu481. The three hydroxyl groups in the pyrogallol subunit formed hydrogen bonds with Thr514 or Glu517. Additional structural flexibility of hydrogen-bond formation depended on the relative position of **23** in the binding pocket. As our SAR study showed, the hydroxyl group at R$_5$ position was important to confer inhibition, and an additional hydrogen-bond formation between Arg320 and the pyrazole ring may also strengthen binding, particularly when an electron-withdrawing

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trifluoromethyl group was present, and diminish binding when electron-donating methyl group

was present in the same position in the pyrazole ring (**Figure 5C**). To validate whether

compound **23** bound within the active site as modelled, we generated mutation within the active

site where Y513 was mutated to L513. This mutation, Y513L, did not abolish GS activity.

Unlike other active site mutations that almost completely eliminated enzyme activity, the Y513L

mutant decreased GS catalytic activity by only 10-fold. Consistent with our modelled mode of

binding, we found that inhibition by **23** toward Y513L was compromised compared to wild type
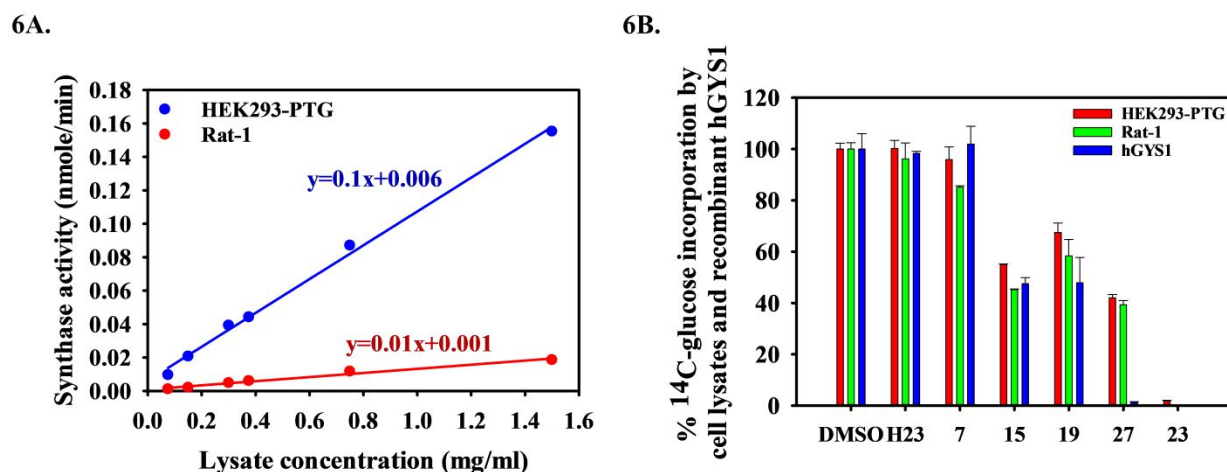
(**Figure 5D**).
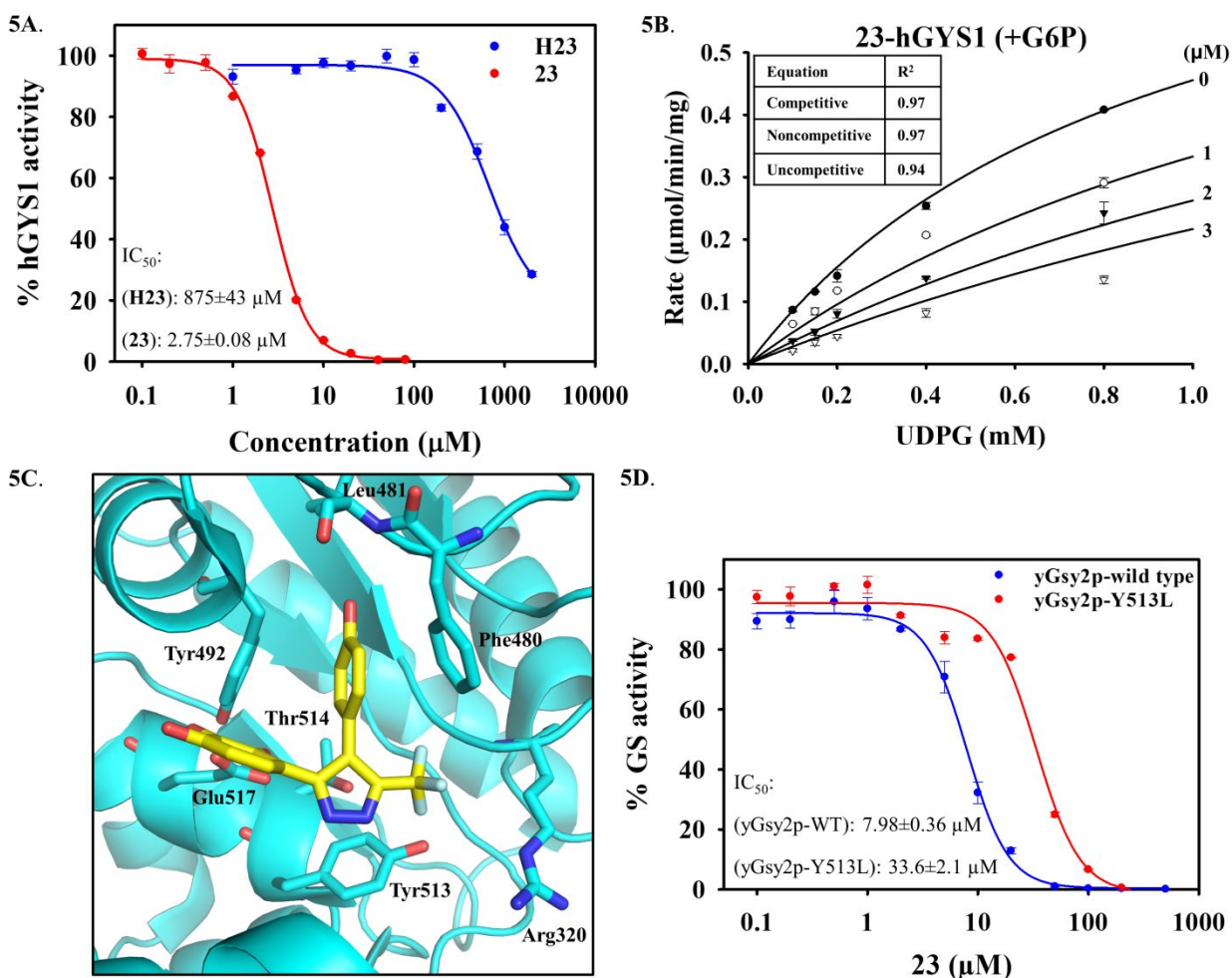
Figure 5. Kinetic characterization of 23. (A) Dose response curves for parent compound **H23**

and the most potent analog **23** against wild-type hGYS1. (B) Michaelis-Menten curve of

competitive inhibition for **23** versus UDPG. The values of goodness of fit (R^2) are shown for all

equations. **23** shows a K_i of 1.31 ± 0.14 µM under the tested condition. K_i value for **23** is the

mean $\pm$ SEM from three independent experiments performed using duplicate measurements for

each condition. (C) Hypothetical binding model of **23** to GS in the active site. The hydroxyl

group at R_1 position forms a hydrogen bond with the nitrogen backbone of Leu481. The three

hydroxyl groups flanking the benzene form hydrogen bonds with Tyr514 or Glu517. An

additional hydrogen bond is formed between Arg320 and the pyrazole ring, which is

strengthened when electron-withdrawing group (like CF_3) is present and diminished when

electron-donating group (like CH_3) is present. Manual docking of **23** in the active site was

performed based on SAR and mode of inhibition studies. (D) Validation of **23** binding in the

active site. Compound **23** showed decreased potency against the active-site mutant Y513L

compared with wild-type yGsy2p. All IC_{50} curves represent one of three experiments performed

using triplicate measurements for each condition, with mean $\pm$ SEM shown.

Inhibition of glycogen synthase activity in cell lysates

We next examined these substituted pyrazoles as inhibitors of GS activity in cell lysates.

Cultured cells normally do not accumulate large amounts of glycogen, largely due to the high

levels of phosphorylation and the resulting low activity state of GS[26]. Lysates from two cell lines

were prepared for GS activity measurement: the HEK293-PTG overexpressing PTG, a regulatory

subunit of protein phosphatase 1, that recruits the phosphatase to glycogen where it promotes the

dephosphorylation and activation of GS[41,42], and glycogen accumulation; and the Rat-1

fibroblasts which have low GS activity and glycogen as previously described[43,44]. The HEK293-

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PTG cells have a seven-fold increase in the GS activity ratio from 0.02 to 0.15. To determine GS

activity in lysates and optimize the conditions for measurement, we measured the incorporation

of ^{14}C-glucose into glycogen at 0.2 mM UDPG and 1 mM G6P initially as a function of different

lysate concentrations. We observed a linear increase in GS activity within the range of 0.075-1.5

mg/ml lysate. Under the conditions of the assay, the lysate from the HEK293-PTG cells had 10-

fold more activity than the Rat-1 cell lysate (**Figure 6A**). To limit substrate utilization to under

10%, we used 0.15 mg/mL HEK293-PTG lysate and 0.75 mg/mL Rat-1 lysate with imidazole

H23, pyrrole **7** and pyrazoles **15**, **19**, **27** and **23**. When tested at 100 μM, **H23** and **7** did not

significantly inhibit GS activity in lysates. However, the remaining four substituted pyrazoles,

namely **15**, **19**, **27** and **23**, reduced synthase activity in both HEK293-PTG and Rat-1 cell lysates

by >30%. Consistent with its greatest potency toward purified enzyme, pyrazole **23** exhibited

almost complete inhibition of synthase activity in lysates (**Figure 6B**). In summary, these

analogs targeted GS activity in the context of the glycogen particles present in cellular lysates

with potencies similar to those observed in purified enzyme preparations.

6A.

6B.

Figure 6. Inhibition of glycogen synthase activity in cell lysates. (A) Synthase activity in

HEK293-PTG and Rat-1 cell lysates in the presence of 0.2 mM UDPG and 1 mM G6P. The data

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was fit to linear regression line with equations showing synthase activity rate (nmole/min, y-value) under various lysate concentrations (mg/ml, x-value). The data represent averages of triplicate assays ± SEM. (B) Percent of ^{14}C-glucose incorporation to control (DMSO) by cell lysates and recombinant hGYS1 in the presence of **H23** and its analogs. For cell lysates and recombinant hGYS1, 100 μM and 20 μM compounds were used respectively. Averages of triplicate assays ± SEM are shown.

DISCUSSION

The suppression of glycogen accumulation emerged as an attractive therapeutic approach for GSDs whose etiology derived from excessive glycogen storage. Animal models supported this approach in which genetic or chemical depletion of glycogen alleviated disease symptoms in models of Lafora disease[27,28,29,30,45], Pompe disease[31], and Cori disease[32]. Interestingly, recent studies suggested that type 2 diabetes (T2D) might also be within the GSD spectrum, as glycogen accumulation in pancreatic β cells under hyperglycemia contributed to the pathology of β-cell dysfunction[16,17].

Only a few studies identified molecules that targeted glycogen accumulation, including the widely-used glucose lowering T2D drug metformin[46,47], mTORC1 inhibitor rapamycin that indirectly suppress GS activity through signaling regulation[31], GYS2 RNAi that mediates enzyme reduction[32], and the newly developed antibody-enzyme fusion that can degrade polyglucosan[45]. However, these molecules either targeted glycogen synthesis in an indirect manner, or present a challenge for the delivery of therapeutics to the central nervous system. In this study, we adapted a high-throughput fluorescence polarization assay to screen directly for small-molecule modulators of GS. A HTS of a commercial 50K chemical library identified 117 primary hits against yGsy2p, and through ^{14}C-glucose incorporation assay only 1 hit was

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validated as hGYS1 inhibitor (**Figure 2**). The low translation between these two assays might lie in the nature of these two systems, since fluorescence polarization assay is affinity-based whereas the radiochemical assay is activity-based. In addition, the kinetic aspects of the two enzyme systems differ as the impact of regulatory input on the enzyme results in different outcomes where regulation of yeast GS by G6P primarily impacts k_{cat}, whereas in mammalian systems the impact is primarily on K_m for substrate[48,49]. There is no evidence that the kinetic steps in catalysis differ between the two enzymes, but it is clear that the manner in which the rates for those individual steps are impacted by regulatory input does differ. A more sensitive assay directly targeting hGYS1 is likely to offer better screening outcome. Nonetheless, our FP assay provided a novel HTS assay as a starting point, and did indeed identify a competitive inhibitor of UDPG, namely (*rac*)-2-methoxy-4-(1-(2-(1-methylpyrrolidin-2-yl)ethyl)-4-phenyl-1*H*-imidazol-5-yl)phenol (**H23**), that bound within the active site of yeast yGsy2p. This outcome was supported by both an X-ray structure and by kinetic studies using **H23** and analogs developed in a subsequent SAR study. The site of interaction was surprising because the assay identified small-molecule inhibitors that displaced G6P binding. However, a close inspection of the known, eukaryotic GS structures demonstrated that the UDPG and G6P binding sites resided on opposing ends of the same alpha-helix, with Tyr492 stacked against the uracil ring of UDP and His500 forming a hydrogen bond with the phosphate moiety of G6P (**Supplemental Figure 3**). Considering this structural proximity and the cooperative nature of the structural transitions in GS, it was not surprising that binding at one site could transmit structural information to the other site and that under the subsaturating conditions of our HTS assay, binding of compounds within the active site could promote displacement of the fluorophore from the G6P site.

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Humans have two isoforms of GS, namely hGYS1 and hGYS2. The fact that hGYS1 is universally expressed in most tissues while hGYS2 is restrictively expressed in liver prompted us to identify inhibitors against hGYS1 in the hope of diminishing brain glycogen stores, aberrantly accumulated in some GSDs such as Lafora disease[26]. The high sequence homology between hGYS1 and hGYS2 enzymes could make it difficult to develop isoform-specific inhibitors. Nonetheless, inhibition of hGYS2 might aid in the therapeutic efficacy of GSDs with excessive glycogen accumulation in liver that would ultimately lead to liver damage. Recently, Pursell *et al.* showed that GYS2 inhibition with RNAi prevents liver injury in mouse models of Cori disease and had no adverse effects[32].

Mutations in GYS1[50] or GYS2[51] in rare GSDs lead to decreased glycogen in muscle and liver respectively. Disruption of the mouse GYS1 gene resulted in 90% perinatal lethality, likely due to cardiac developmental problems during embryogenesis, but the surviving mice were ostensibly normal and lived normal lifetimes[52]. Disruption of GYS2 in mice largely mimicked the phenotype of human GSD0 patients, namely tendencies to post-prandial hyperglycemia and to hypoglycemia upon fasting but compatible with a relatively normal life[51,53]. Given that small-molecule inhibition will not have the penetrance of genetic defects, inhibition of GS activity by small-molecules is unlikely to elicit extreme phenotypes, and small-molecule inhibition remains as a potentially valuable means of treating these devastating diseases.

CONCLUSIONS

In summary, this study described a strategy for developing a high-throughput FP assay for the screening of small-molecule inhibitors of GS, the binding mode of a leading imidazole **H23** validated through both X-ray crystallographic and kinetic data; and an SAR study leading to analogs, such as pyrazole **23**, with low micromolar potency. These outcomes suggest that

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targeting glycogen synthase with small-molecule inhibitors represents an attractive approach for developing new therapeutics for diseases in the GSD family. Unlike other enzyme-based strategies where complete inhibition is the ultimate objective, a partial reduction of GS activity may be sufficient to alleviate unwanted and damaging levels of glycogen deposition in the neural tissue of patients suffering from Lafora disease[27,28,30]. The challenges of developing any therapeutic, nevertheless, remain the same, and the gulf that stretches between our identification of a pyrazole inhibitor **23** with activity in the low micromolar range and a therapeutic candidate is wide and deep. Initial efforts will involve microsomal studies to evaluate anticipated pharmacokinetic concerns about unwanted redox reactions of pyrazole **23** to 4-(4*H*-pyrazol-4-ylidene)- or 2,3-dihydroxy-4-(3*H*-pyrazol-3-ylidene)cyclohexa-2,5-dien-1-ones. We will use a combination of synthesis (*i.e.*, SAR studies) and computational modeling to identify analogs that avoid this concern and retain desired physiochemical properties (*i.e.*, water solubility; bioavailability); we will evaluate any promising, new leading structures for potential toxicity issues (*e.g.*, hERG studies); and we will explore biotinylated analogs to confirm the specificity of these pyrazole analogs for the desired target. These initial, encouraging results bode well for the future development of small-molecule strategies to study and potentially treat GSDs.

EXPERIMENTAL SECTION

Materials

The 50K compound Diversity set library, the primary hits **H1-H110**, and analogs **1-4**, **7-9** were purchased from ChemBridge Corporation (San Diego, CA). Analogs **5**, **6** and **10** were purchased from Vitas-M Laboratory (Champaign, IL). The purity of these purchased compounds were > 95% based on the spectra (either LC/MS or NMR) provided by the vendors. All pyrazoles were characterized and validated by both LC/MS and NMR analyses.

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Synthesis and purification of GlcN6P-fluorescein-5-Ex

The tracer was synthesized by using a standard coupling reaction between an amine and N-hydroxysuccinimidyl (NHS) ester. The reaction included: 100 mM $NaHCO_3$ (pH 8.4), 130 mM glucosamine-6-phosphate (GlcN6P) pH 8.0, 41.6% DMSO and 14 mM fluorescein-Ex, succinimidyl ester. The reaction was incubated for 1 hour at 37°C followed by an overnight incubation at 25ºC with continuous stirring, the reaction was stopped by addition of Tris-HCl (pH 8.0) to a final concentration of 0.2 M. Purification of GlcN6P-fluorescein-5-Ex was carried out by HPLC on a semi-preparative Luna C18 column (250×10 mm, 5 μm) from Phenomenex. The eluents used were 25 mM NH_4OAc (pH 5.5) (E1) and 100% methanol (E2). Elution was performed by the following gradient: T_0= 5% (v/v) E2, T_{10}= 50% (v/v) E2, T_{30}= 50% (v/v) E2, T_{45}= 75% (v/v) E2, T_{55}= 5% (v/v) E2 at a flow rate of 4 mL/min. The fractions containing the UV-containing fractions were collected, dried using a SpeedVac, dissolved in water and stored at -20°C. The fractions containing GlcN6P-fluorescein-5-Ex were identified and confirmed by mass spectrometric analysis. The final tracer concentration was determined by UV spectroscopy (Abs_{492}~9.2×10^4 M^{-1} cm^{-1}).

Fluorescence polarization assay

All the fluorescence polarization (FP) experiments were performed on an EnVision multimode plate reader (Perkin Elmer) with the λ_{ex}= 485 nm and λ_{em}= 535 nm. The FP experiments were performed in 384-well, black, flat bottom microplates at 25ºC. After addition of all reagents, plates were spun down for a minute at 1000g in a centrifuge followed by measurement of the FP signals, where each well was flashed 10 times and the average values were used. All polarization values were expressed as milli-Polarization units (mP), calculated from equation 1:

$$mP = (1000) * \frac{S - G * P}{S + G * P} \qquad\qquad (1)$$

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Where: S= fluorescence intensity measured when the excitation and emission polarizers are parallel and P= fluorescence intensity measured when the excitation and emission are perpendicular and G= grating factor that corrects for instrument bias. All the nonlinear regression analyses were performed by fitting the experimental data to the defined equations using SigmaPlot version 13.0.

Determination of the GlcN6P-fluorescein-5-Ex/yGsy2p equilibrium dissociation constant

The concentration of the tracer was initially varied from 0-100 nM in order to determine the optimal assay concentration. Based on total fluorescence intensity and mP values, the optimal concentration was determined to lie between 10-40 nM in the final assay. The binding affinity for the tracer for yGsy2p was determined by adding the tracer to a final concentration of 20 nM to each well in the presence of varying yGsy2p concentrations (0 to 50 µM) in a final volume of 50 µL. The final assay buffer consisted of 15 mM Tris-HCl (pH 7.8) and 15 mM NaCl, the plate was incubated at 25ºC for 10 minutes before reading. The dissociation constant, $i.e.$ K_d was calculated by fitting the experimental data to equation 2:

$$f = y_0 + \frac{a * x}{b + x} \qquad (2)$$

Where f=mP, y_0=mP$_{min}$, a= mP$_{max}$-mP$_{min}$, b= K_d and x= Concentration of yGsy2p. Experiments were performed in triplicate.

Activation of yGsy2p or hGYS1 in the presence of G6P or GlcN6P

The activation of hGYS1 or yGsy2p in the presence of G6P or GlcN6P was determined using the radiochemical assay previously described[33] and the data was fit to equation 2. However, the parameters were defined as: f= % activation, y_0 = % activation$_{min}$, a=% activation$_{max}$ - % activation$_{min}$, x= Concentration of G6P or GlcN6P and b= AC$_{50}$. Experiments were performed in triplicate.

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Competitive displacement experiments

G6P was serially diluted in 15 mM Tris-HCl (pH 7.8) to provide a final concentration ranging

from 0.68 µM to 40 mM. The reaction mixture contained a final concentration of 15 mM Tris-

HCl (pH 7.8), 15 mM NaCl, 20 nM GlcN6P-fluorescein-5-Ex, 4.2 µM yGsy2p and varying

concentrations of G6P in a final volume of 50 µL. The plate was incubated for 10 minutes at

25ºC before reading. The K_d was determined by fitting the observed mP changes to equation 3:

$$f1 = \mathrm{mP}min + \frac{(\mathrm{mP}max - \mathrm{mP}min)}{\left(1 + \frac{x}{EC50}\right)^{\wedge}(-Hillslope)} \tag{3}$$

Where $f1$= mP, EC_{50}= K_d, x= Concentration of G6P. Experiments were performed in triplicate.

Determination of Z′-factor

To determine the quality of the FP-displacement assay for adaptation for HTS, the Z′-factor was

calculated using equation 4:

$$Z' = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|} \tag{4}$$

where σ_p and σ_n are the standard deviations of the signal for the positive and negative controls.

For the negative control 2 µL of H_2O was added and for the positive control 2 µL of G6P was

added to a final concentration of 2 mM in a 384-well plate. The protein sample was prepared in

25 mM Tris-HCl (pH 7.8), 25 mM NaCl, 7 µM yGsy2p, 40 nM tracer and dispensed by a Multi-

drop 384 liquid dispenser (Titertek) into the wells with either H_2O or G6P. The plate was spun

down in a centrifuge followed by measurement of the FP signals.

Expression and purification of yGsy2p and hGYS1

The His-tagged yGsy2p recombinant enzyme was expressed in BL21 (DE3) *Escherichia coli* and

purified using a two-step procedure including affinity chromatography on Ni^{2+}-nitrilotriacetic

acid-agarose and ion exchange purification on Q-sepharose column[10]. The hGYS1 in the pFL

vector[38] was modified by deleting the intein-chitin binding domain fusion at the C-terminus and replacing it with a simple non-cleavable 6 × His-tag at the C-terminus. Purification of the construct was achieved using Ni-NTA resin (Qiagen product #31314) following manufacturer's instructions. Separately, a construct of hGYS1 was generated in which the C-terminus was truncated at position 634 and the N-terminal phosphorylation sites at positions 8 and 11 were simultaneously mutated to Asn residues, that avoids solubility issues associated with mutation of these residues to Ala, in order to generate the hGYS1Δ634S8,11N construct. This construct was also fused to the same C-terminal 6 × His-tag for purification using Ni-NTA resin.

Determination of kinetic parameters

Enzyme activity of GS was determined using UDPG as a substrate through ^{14}C-glucose incorporation assay by monitoring the amount of radiolabeled glucose being incorporated into glycogen[33]. Unless otherwise noted, yGsy2p activity was measured in reaction solution containing 0.3 mM UDPG in the absence or presence of 0.04 mM G6P. The activity of mutant hGYS1Δ634S8,11N enzyme was measured using 0.2 mM UDPG and 0.4 mM G6P, while the activity of wild-type hGYS1 was measured using 0.2 mM UDPG and 1 mM G6P. All kinetic data analyses were performed using the program package SigmaPlot (version 13.0) by fitting the data to the appropriate kinetic equation. The IC$_{50}$ curves for **H23** and its analogs were fit to the four parameter logistic equation. Titration experiments for Michaelis–Menten curves were performed by covarying inhibitor and substrate concentrations. The reaction mixture contained 5 μg/ml yGsy2p, varied UDPG (0.2-8 mM in the absence or presence of 7.2 mM G6P) and **H23** concentrations (0-0.8 mM). All data were fit to competitive, non-competitive and uncompetitive inhibition models in SigmaPlot (Version 13.0). Appropriate model was selected through analysis of goodness-of-fit and the residuals of those fits. All experiments include the controls contained

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2% (v/v) DMSO. The values presented here are the averages ± the standard errors of the mean of

three independent experiments with duplicate measurements for each data point in each

experiment.

Crystallization and structure determination

Yeast Gsy2p crystals were obtained using hanging drop vapor diffusion method[10]. Briefly, the

protein solution was prepared at 3 mg/ml containing 25 mM G6P. The protein solution was

mixed with crystallization reservoir solution containing 0.1 M Bis-Tris, pH 5.9 and 13-15%

PEG300. The crystals were soaked with compounds (0.5 mM) on sitting drop plates to obtain

inhibitor-bound yGsy2p complex. The crystals were cryo-protected and frozen. Diffraction data

sets were collected using X-ray crystallography at the Advanced Photon Source at beamline 19-

ID, operated by the Structural Biology Center at Argonne National Laboratory. The data sets

were then indexed, integrated and scaled using the HKL3000 program package. The structures

were solved by molecular replacement using MOLREP, as implemented in the Collaborative

Computational Project Number 4 (CCP4) program suite. The G6P bound yGsy2p-R589/592A2

mutant structure (pdb code: 3NB0) was used as the model for molecular replacement. The

structures were initially refined with a single round of rigid body refinement for individual

domains, followed by iterative rounds of restrained refinement with the application of domain-

based TLS and NCS restraints using REFMAC5 as implemented in CCP4. COOT (version

0.7.2.1) was used to visually inspect and manually adjust the refined models.

Compound synthesis

Synthesis of pyrazoles **11-30** was performed in a 3-step procedures which include 1) synthesis of

polyhydroxydeoxybenzoins; 2) ring-closure reaction for the synthesis of isoflavones with or

without de-acetylation of phenolic groups; and 3) recyclization reaction of chromones under

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hydrazine actions (**Scheme 1**). Initial Hoesch reaction of substituted polyphenols and

arylacetonitriles in boron trifluoride etherate with passing of anhydrous HCl led to formation of

A-ring polyhydroxylated deoxybenzoins **I2-1 – I2-5** and **I2-9 – I2-12**[54]. Alternative condensation

of pyrrogallol or 4-flouroresorcinol with phenylacetic acids in boron trifluoride etherate at

heating was performed for the synthesis of 4'-hydroxy- or 4'-cyano deoxybenzoins **I2-6 – I2-8**[55].

Ring-closure reaction with Vilsmeier reagent or trifluoroacetic anhydride in pyridine after work-

up with water afford 2-unsubstituted or 2-trifloromethyl isoflavones. Synthesis of 2-methyl

isoflavones **I3-1**, **I3-2**, **I3-8**, and **I3-19** was performed by reaction of polyhydroxydeoxybenzoins

with acetic anhydride in presence of potassium acetate with the following deacylation in ethanol

without purification of intermediate acetates. Target pyrazoles **11-30** were synthesized by the

reaction of synthesized 2 (un)substituted isoflavones **I3-1 – I3-20** with hydrazine hydrate in

ethanol under reflux.

Analysis of GS activity from cell lysates

HEK293-PTG cells were generated by transfecting HEK293 cells[56] with the plasmid pCDH-

FLAG-PTG, harboring the mouse PTG coding region and the hygromycin antibiotic resistance

gene utilizing Lipofectamine following manufacturer specifications. Mixed clones were selected

for ~10 days in the presence of 0.2 mg/mL hygromycin, expanded and stored in liquid N_2.

Analyses of protein expression, GS activity ratio in the absence and presence of saturating

concentrations of UDPG (4.4 mM) and of G6P (7.2 mM) and glycogen levels indicated that the

protein was expressed, the GS activity ratio was increased 7-fold, from 0.02 in control cells to

0.15 in transfected cells, and that glycogen was increased by 90-fold. Quantitation of the

expression of PTG is difficult because the basal levels are very low, undetectable under our

conditions.

For lysate preparation the HEK293-PTG cells were cultured in 100 mm plate with 5.5 mM αMEM, 10% FBS, 6 μg/mL penicillin, 10 μg/mL streptomycin, and 0.2 mg/ml hygromycin. Rat-1 cells were cultured in 100 mm plate with 25 mM DMEM, 10% FBS, 6 μg/mL penicillin and 10 μg/mL streptomycin. Cells were grown for 3-4 days till confluency. Before harvest, cells were washed twice with 5 mL ice-cold GS buffer (50 mM Tris-HCl pH 7.8, 20 mM EDTA, 25 mM KF). Then 400 μL GS homogenization buffer (50 mM Tris-HCl pH 7.8, 10 mM EDTA, 2 mM EGTA, 100 mM NaF) with protease inhibitor (0.1 mM TLCK, 10 μg/ml leupeptin, 1 mM benzamidine, 0.5 mM PMSF, 1 mM Na_3VO_4) and β-mercaptoethanol (0.4%) plus Triton × 100 (0.2%) were added to each 100 mm plate. Plates were frozen on liquid N_2 and scraped. Then lysates were transferred to 2 ml Eppendorf tube, sonicated for 15 seconds twice on ice, and placed on nutator for 10 minutes at 4 ºC. Protein concentrations were measured using the Bradford reagent and indicated that the protein concentration was the same in both lysates. GS activity was initially measured with varying concentration of the lysates. For monitoring the effect of small-molecules on GS, HEK293-PTG cell lysates were diluted 10-fold while Rat-1 cell lysates were diluted 2-fold in homogenization buffer to achieve steady-state kinetics under the conditions we used in ^{14}C-glucose incorporation assay[33]. GS activity in cell lysates was measured in 50 mM Tris-HCl buffer at pH 7.8 with 6.7 mg/ml glycogen, and subsaturating concentrations of UDPG (0.2 mM) and G6P (1 mM) in the absence or presence of 100 μM **H23** and its analogs. All assays including the controls contained 2% (v/v) DMSO.

Generation of yGsy2p Y513L mutant

To characterize the binding pattern of compound **23**, active site mutation Y513L was made without necessarily compensating enzyme activity. Point mutation of Y513L was performed using QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) in the pET-28A

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harboring the yeast Gsy2p cDNA[49] using forward primer 5′-

CTACGAGCCTTGGGGTCTCACACCTGCAGAATGTAC-3′ and its complement primer, and

was confirmed by DNA sequencing. The mutant protein was purified exactly the same way as

was yGsy2p. However, the yield was significantly less compared to WT protein. Additionally,

the activity of Y513L mutant is around 10-fold lower than WT enzyme. Kinetics experiments

with Y513 mutant were performed under saturating G6P concentration (7.2 mM).

ASSOCIATED CONTENT

Supporting Information

Supplemental Figures S1-S3; Supplemental Tables S1-S2; Vendor Supplied Compound Purity;

LC/MS and NMR Confirmation of Author Provided Compound Purity (PDF)

Molecular Formula Strings (CSV)

Accession Code

The structure-factors and derived atomic coordinates for the complex between **H23** and yeast

Gsy2p have been deposited with the RCSB under the code 6U77. Authors will release the atomic

coordinates and experimental data upon article publication.

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Author Contributions

B.T. conducted most of the experiments and summarized all data. V.M.C. conducted the

fluorescence polarization assay for high-throughput screening. M.S.F., S.P.B., G.P.M., P.W., and

D.S.W. synthesized compounds. K.K.M. assisted with structural characterization. A.A.D.R

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designed the expression of the His-tagged hGYS1 proteins and generated the HEK293-PTG

cells. C.A.M. purified recombinant hGYS1 protein. B.T. and T.D.H. formed the concept,

designed experiments, analyzed data, and wrote the draft manuscript. B.T., D.S.W., A.A.D.R.,

P.J.R. and T.D.H. reviewed and revised the manuscript.

The authors declare no competing interests.

Conflict of Interest

Thomas D. Hurley holds significant financial equity in SAJE Pharma, LLC and Maze

Therapeutics. Peter J. Roach and Anna A. DePaoli-Roach hold equities with Maze Therapeutics.

However, none of the work described in this study is based on or supported by either company.

David S. Watt has partial ownership in a private venture, Epionc, Inc., to develop small-molecule

inhibitors for cancer treatment. In accord with University of Kentucky policies, David S. Watt

has disclosed this work to the University of Kentucky's Intellectual Property Committee and

complied with stipulations of the University's Conflict of Interest Oversight Committee.

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ABBREVIATIONS USED

GSD: glycogen storage disorder; GS: glycogen synthase; yGsy2: yeast glycogen synthase 2; hGYS1: human glycogen synthase 1; SAR: structure-activity relationship; GlcN6P: glucosamine-6-phosphate; G6P: glucose-6-phosphate; PTG: protein-targeting-to-glycogen; HTS: high-throughput screening; UDPG: Uridine diphosphate glucose; FP: fluorescence polarization.

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