Published in final edited form as:

Biochem J. ; 474(20): 3403–3420. doi:10.1042/BCJ20170469.

A novel image-based high throughput screening assay discovers therapeutic candidates for adult polyglucosan body disease

Leonardo J. Solmesky*,

Cell Screening Facility for Personalized Medicine, Department of Cell Research and Immunology, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel

Netaly Khazanov*,

Department of Chemistry, Bar Ilan University, Ramat Gan, Israel

Hanoch Senderowitz,

Department of Chemistry, Bar Ilan University, Ramat Gan, Israel

Peixiang Wang,

Program in Genetics and Genome Biology, The Hospital for Sick Children, University of Toronto, Toronto, Canada

Berge A. Minassian,

Program in Genetics and Genome Biology, The Hospital for Sick Children, University of Toronto, Toronto, Canada, and Department of Pediatrics, University of Texas Southwestern

Igor M. Ferreira,

Structural Genomics Consortium, Nuffield Department of Clinical Medicine, University of Oxford, Oxford, UK

Wyatt W. Yue,

Structural Genomics Consortium, Nuffield Department of Clinical Medicine, University of Oxford, Oxford, UK

Alexander Lossos,

Department of Neurology, Hadassah Medical Center, Hebrew University of Jerusalem Israel, Jerusalem, Israel

Miguel Weil, and

Cell Screening Facility for Personalized Medicine, Department of Cell Research and Immunology, The Sagol School of Neurosciences, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel

Corresponding authors: Or Kakhlon ork@hadassah.org.il, Miguel Weil miguelw@tauex.tau.ac.il.

*Contributed equally to this study.

Declarations of interest

The authors have no conflict of interest to declare.

Author contribution statement

LJS and OK designed, conducted and analyzed experiments and wrote the manuscript, NK and PW designed, conducted and analyzed experiments, MW, BAM and HS supervised experiments, wrote and critically reviewed the manuscript, BAM and AL critically reviewed the manuscript, IMF and WY conducted experiments and contributed important reagents.

Or Kakhlon

Department of Neurology, Hadassah-Hebrew University Medical Center, Jerusalem, Israel

Abstract

Glycogen storage disorders (GSDs) are caused by excessive accumulation of glycogen. Some GSDs (Adult Polyglucosan Body Disease (APBD), Tarui and Lafora diseases) are caused by intracellular accumulation of insoluble inclusions, called polyglucosan bodies (PB), which are chiefly composed of malconstructed glycogen. We developed an APBD patient skin fibroblast cell-based assay for PB identification, where the bodies are identified as amylase-resistant periodic acid-Schiff's (PAS) stained structures, and quantified. We screened the DIVERSet-CL 10,084 compound library using this assay in high throughput format and discovered 11 dose-dependent and 8 non dose-dependent PB-reducing hits. ~70% of the hits appear to act through reducing glycogen synthase (GS) activity which can elongate glycogen chains and presumably promote PB generation. Some of these GS inhibiting hits were also computationally predicted to be similar to drugs interacting with the GS activator protein phosphatase 1 (PP1). Our work paves the way to discovering medications for the treatment of PB-involving GSD, which are extremely severe or fatal disorders.

Keywords

APBD; Glycogen Storage Disorders; Image-based High Throughput Screening; Polyglucosans; Glycogen Synthase

Introduction

Glycogen storage diseases (GSDs) are caused by intracellular accumulation of insoluble inclusion bodies, chiefly composed of normal, or malconstructed glycogen. In GSD type IV, loss of function mutations in the glycogen branching enzyme (GBE, EC 2.4.1.18) reduces the number of glycogen branching points, generating amylopectin-like malconstructed glycogen, with longer outer chains and poorer solubility than normal glycogen. This malconstructed glycogen, or polyglucosan (PG), is deposited as the main constituent of inclusion bodies called PG bodies (PB)(1, 2) in most organs including liver, muscle, heart and the central and peripheral nervous systems. While accumulating in multiple tissues, PB lead to cell death only in neurons, where they can clog the narrow axons(3), or dendrites(4). This PB-mediated neuronal death was also confirmed in tissue culture(5). The main forms of GSD type IV are the Andersen Disease (OMIM 232500)(6), a fatal liver disorder with progressive cirrhosis of the organ leading to its failure, and the neurodegenerative disorder Adult Polyglucosan Body Disease (APBD, OMIM 263570)(7). In addition to GSD type IV, PB are also observed in Lafora Disease (LD, OMIM 254780)(8) and the type VII GSD (phosphofructokinase deficiency) Tarui Disease (OMIM 232800)(9).

The type IV GSD APBD is a rare genetic disorder in which PB accumulate as pathogenic inclusion bodies in the cytosol of brain cells(10). APBD is characterized by adult-onset progressive neurogenic bladder, gait difficulties (*i.e.*, spasticity and weakness) associated with upper and lower motor neuron involvement, sensory loss, predominantly in the distal

lower extremities, and sometimes mild cognitive difficulties(10, 11). Currently, no drug is available for treating APBD, or for any of the other PB-involving GSDs. On the other hand, it is important to discover new molecular entities that can be further developed into possible therapies for PB-involving GSDs, potentially pills, which are highly accessible.

High content screening (HCS), or image-based high throughput screening, is a method which allows therapeutic molecules discovery based on detection or quantification of phenotypic changes induced in cells. Such detection/quantification of phenotypic changes is based on the analysis of cell images employing image analysis software following exposure to tested molecules and specific staining(12, 13). HCS allows screening large numbers of molecules, while simultaneously obtaining multi-parametric information about their effects on cells. This information permits not only detecting candidates which achieve the desired effect, but also discarding *a priori* toxic compounds by pre-selecting for nontoxic molecules. Thus HCS can minimize drug attritions during pre-clinical and clinical phases(14).

In this work we opted for finding small molecules which can reduce PB levels and thus ameliorate APBD and other PB-related GSDs (Lafora, Andersen and Tarui diseases). Capitalizing on the fact that PB is an APBD hallmark and on the ability to visualize PB with periodic acid schiff (PAS)(15), we designed a novel high throughput screening assay for detecting PB and their reduction by small molecules. Using this assay we discovered hits which significantly and reproducibly lowered PB levels in APBD patient fibroblasts, ~70% of which we demonstrated to act, at least in part, through a reduction of GS activity.

Experimental/Materials and methods

High content (image-based) screening assay

All the steps of the assay were performed automatically using a Freedom Evo 150 liquid handling robot (Tecan). APBD patient-derived fibroblasts were seeded at a density of 800 cells/well in DMEM (Gibco) supplemented with 1mM sodium pyruvate (Gibco), 1% antibiotics (Biological Industries, Israel) and 5% fetal bovine serum (Hyclone) into 384 well microclear plates (Greiner). The plates were transferred by the robotic arm (RoMa) into an automated incubator (Liconic), integrated with the robotic system, where they were incubated at 37°C with 5% CO₂. Following 24h incubation, the medium was replaced by the robot using medium supplemented with 10µM of each screened compound (0.1% DMSO final concentration, all prepared by the robot). The screened compounds were part of the Diverset CL collection purchased from ChemBridge (10,084 out of 50,000 molecules). We then cultured these cells for another 24 h in the automated incubator, and, using the robot, washed them with PBS (Biological Industries, Israel), fixed them with 4% paraformaldehyde (PFA), washed again with PBS, neutralized the previously added PFA by ammonium chloride (Sigma Aldrich), washed with PBS, permeabilized with 0.1% Triton X-100 (Sigma Aldrich), and washed again with PBS. Following this, we used the robot to treat the fixed cells with 0.5 g/dl diastase (or amylase, Sigma Aldrich) for 5 minutes at 37°C in the automated incubator, washed with PBS, stained with PAS (Merck), Hoechst 33342 (Sigma Aldrich) and Cell Mask Deep Red (Molecular Probes) and washed with PBS. Images of stained cells were then acquired by the In Cell Analyzer 2000 (GE Healthcare) and analyzed

using the In Cell Investigator (GE Healthcare) applying an algorithm that quantified the PAS signal within each Cell Mask stained cell.

Image analysis and hit determination

Images were segmented based on the Cell Mask signal which was linked with the segmentation of the nuclei, employing an empirically established sensitivity and kernel size for allowing the best segmentation. Once these nucleus-cytoplasm complex objects were selected, the software calculated the number of these objects (cell number) as well as the intensity of the PAS signal within each of them. All the assay parameters (including the acquisition exposure times, objective, and the analysis parameters) were kept constant throughout the whole screening campaign for allowing comparison of signals obtained from different batches of assay within the campaign.

Dose-response curves

The assay was performed as mentioned above, but with compound concentrations of 1, 5, 10, and 50 μM , instead of a constant concentration of 10 μM . Dose response curves were fitted by nonlinear regression according to the Prism's GraphPad Statistical Package.

In vitro GS activity assay

The reaction was carried out in 96-well plates containing 50 μl of reaction volume (50 mM Tris, pH8, 20 mM EDTA, 25 mM KF, 2 mg/ml glycogen, 10 mM Glucose-6-Phosphate, 1.65 mM UDP[14C]glucose and 100 mM UDP-glucose). To test the inhibitory effect of the hits, we included each one in the reaction at a final concentration of 50 μM , or in different concentrations, as indicated, in the dose response curves. The reaction was initiated with the addition of 20 nM recombinant human GS1, followed by incubation at 37°C for 30 min. The reaction was then stopped with the addition of 100 μl cold 100% ethanol, and the total suspension was transferred into a 96-well filter plate. Vacuum was applied to remove the ethanol. Five washing steps with 150 μl of cold 66% ethanol then fully removed any non-incorporated UDP[14C]glucose, while the labelled glycogen stayed on the well filters. The plate was dried at 37°C incubator for 30 min. 50 μl of scintillation fluid were added to each well and the plate was read for 14C signal on Microplate scintillation and luminescence counter Top Count (Perkin Elmer).

Ligand Profiling

In order to identify potential bio-targets (required due to the phenotypic nature of the screen), active compounds were screened against the PharmaDB pharmacophore database using the Ligand Profile protocol, implemented in Discovery Studio (DS) version 4.1. PharmaDB is a database of pharmacophores created from the sc-PDB protein data bank (<http://bioinfo-pharma.u-strasbg.fr/scPDB/>). It consists of over 132,000 pharmacophore models, generated from 283 binding sites from 3678 unique proteins and 5608 unique ligands. Many protein targets are represented by multiple pharmacophores with shape constraints generated from excluded volumes.

Clustering of hits and known drugs

Hierarchical clustering was performed based on dendritic fingerprints using Canvas v. 2.7. The Kelley criterion was used to obtain an optimal number of clusters (16). The total number of clusters that was obtained in our analysis was five. However, four of these clusters included only 1–2 known drugs and only one cluster (the largest one, Supplementary Fig. 3) was informative and included all the hits (36) together with 21 of the 27 drugs found to interact with putative hit binding proteins which also bind carbohydrate derivatives (Supplementary Fig. 2).

Similarity analysis

Hit similarity to drugs was calculated using the Find Similar Molecules by Fingerprint protocol as implemented in DS. For a hit to be considered similar to a drug, the similarity score between them should have exceeded a threshold value of 0.5 calculated by either the MDL public key or the FCFC_4 fingerprint.

Results

A novel High Throughput Assay for detecting PB

Since in APBD neurons undergo degeneration due to PB accumulation, we decided to screen a small molecule library in order to find compounds capable of decreasing PB cellular content. For that purpose, we developed an assay based on PAS staining of PB in fibroblasts derived from an APBD patient's skin. We first conducted preliminary work to demonstrate that, unlike skin fibroblasts derived from healthy individuals, skin fibroblasts from APBD patients show detectable PB. We capitalized on the observation that PGs are resistant, up to certain ceiling concentration and exposure time, to the glycogen digesting enzyme amylase(1, 17). Therefore, to operationally define PG, we administered, post paraformaldehyde fixation, amylase to unaffected and APBD fibroblasts in order to digest soluble glycogen, sparing the relatively amylase-resistant PG, which consequently could be detected. As our results (Figure 1A) show, amylase resistant staining, and therefore PG and PB, were uniquely observed in APBD and not in control fibroblasts. Rapamycin, an established inhibitor of GS(5), not used as a therapeutic drug due to its toxicity in animal models (unpublished results), was used to demonstrate our ability to reduce PB pharmacologically. Furthermore, to anticipate the possible range of PB reduction by tested compounds, we have co-cultured patient and healthy control fibroblasts in different ratios (Figure 1B). Tested inhibitors can theoretically inhibit PB intensity only down to the value observed for control only cells, which do not contain polyglucosans. As can be seen in Figure 1B, increasing the ratio of control cells decreases the percentage of polyglucosan positive cells. Therefore, while the presence of polyglucosan is an all or none phenomenon in patient vs control fibroblasts, it can be quantified continuously in patient cells treated with polyglucosan reducing hit compounds. While the control cells are not the same patient cell line on which the HTS was applied, excluding their usage as a positive control, these control cells do demonstrate our ability to stain polyglucosans and in that sense validate our assay.

For both these preliminary observations and for the HCS, we optimized both amylase's cell exposure time and concentration to achieve the maximal extent of glycogen degradation

which spares PG. The chosen conditions following this optimization step were 0.5 g diastase/dl and 5 minutes digestion at 37°C. In addition, we optimized several other aspects of the assay establishing also the exposure time to compounds (1 day), the medium composition (5% FCS in DMEM), the employed fibroblasts passage (#11), the fixation technique (4% PFA for 10 minutes followed by ammonium chloride neutralization), the permeabilization (5 minutes by 0.1% Triton X-100), additional staining (Hoechst 33342 for nuclei and CellMask Deep Red for cytoplasm reference), plate type (384 well microplates) and cell density (800 fibroblasts/well).

Establishing criteria for the identification of PB-reducing compounds

Once optimized, we used the assay to screen 10,084 compounds from the Diverset CL collection (ChemBrige) by exposing APBD patient derived fibroblasts to these molecules at a 10 μ M concentration. Images were analyzed by performing segmentation of cells based on the Cell Mask Deep Red staining and using the Hoescht 33342 staining to determine if two cells were segmented together as a unique entity (in case they were too adjacent to allow segregation by the software). This feature allows reducing possible under segmentation. A representative image of the obtained staining is depicted in Figure 1C. Figure 1D shows the compiled data points of the screening, illustrating the approach employed for selecting hits. Briefly, once the cell area was established based on the Cell Mask Deep Red signal, the PAS signal in each of the cells was determined (corresponding to cell associated PB mean intensity). Cell area data averaged at the well level were normalized according to the overall well population in the entire screening campaign using the z-score method. Based on previous image training, we considered for further analysis only those wells treated with compounds whose induced normalized cell area population mean was above -1.5 z-score and showed at least 300 surviving cells (*i.e.* stained nuclei linked to Cell Mask Deep Red stained object) per well. The discarded data were presumably associated with cells affected by toxic compounds. In the remaining data we normalized the measured population mean PB intensity. Based on a series of images used for training, we considered as positive compounds only those where following treatment the normalized PB mean intensity was at least under -1.2 z-score of the scale. Following this approach, we selected 85 potential hits out of the 10,084 screened compounds (0.84%). Figure 1C shows that these selected molecules caused a decrease in PB mean intensity without any evidence of toxicity (no cell area or cell count reduction).

Discovery of drug candidates for treating PB-involving GSD

Following this primary screening, we decided to establish dose response curves for the 85 hits for further validation. For this purpose, we performed the same assay, except in compound concentrations of 0, 1, 5, 10, and 50 μ M. Figure 2 (A & C) shows the molecular formulae and dose response curves of 11 compounds selected as best dose-responders from the 85 hits. The dynamic range of the dose responses were relatively narrow, spanning a concentration range of 1–50 μ M. As Fig. 2B shows for two representative hits, above that range the hits become toxic.

While dose responsiveness is an accepted hit validation criterion(18), in our analysis we also included 8 compounds (Figure 3 A & C) which were effective in reproducibly lowering PB

levels, though by an apparently non dose-responsive manner in the concentration range used. Possibly applying these compounds at concentrations lower than the lowest concentration used in our assays (1 μ M) might have demonstrated dose-response for them as well. These compounds were also toxic above 50 μ M (Figure 3B).

The effect of PB-reducing hits on GS activity

Once PB-reducing hits were discovered, we decided to explore a possible mechanism by which these hits might lower PB levels. While instigated by malconstructed glycogen, PB are a more complex entity involving regulatory and scaffolding proteins(1). Therefore control of PB size may be mediated by several enzymes, able to generate PG (*e.g.*, GS and protein phosphatase-1 (PP1)), shape them (*e.g.* GBE), or degrade them (*e.g.*, glycogen debranching enzyme (GDE) and GP). However, the most straight forward PG-controlling enzyme with well-studied regulatory mechanism is GS. Therefore, we performed an *in vitro* assay to test hits effect on GS activity. As can be seen in Fig. 4A, we found that at a concentration of 50 μ M, 13 out of the 19 hits significantly reduce GS activity. This may suggest that the inhibitory effect we observed in PB reduction for most hits in cells is due to direct inhibition of the GS enzyme alone, or in combination with other modes of action, such as PP1 inhibition. In addition, all GS reducing compounds (#1, 2, 3, 4, 5, 9, 10, 11, 13, 14, 15, 16, 17) were dose responsive, even though the goodness of fit of the regression model was variable (Fig. 4B). Complete inhibition of GS was not observed for any hit (Fig. 4B), or even for the natural inhibitor of GS – UDP. We attribute this partial inhibition mainly to the presence of the GS activator glucose-6-phosphate, which can override GS inhibitors (19), in the assay buffer. In addition, the high concentrations of UDP-Glucose (100 mM) and its lower K_m compared to the K_i for UDP (20), probably also prevented complete inhibition by competitive inhibitors such as UDP (21).

Computational prediction of possible modes of action of the hits

Complementary to the above-described biochemical analysis of GS, to gain some preliminary insight into possible modes of action by which the identified hits have lowered PB levels, we resorted to computational analysis. We first confirmed that out of the 19 dose-dependent and non dose-dependent hits discovered (represented by 36 molecular entities where certain hits were represented by multiple protonation and tautomeric states, Supplementary Fig. 1) only two (compounds 6 and 30) were predicted to be promiscuous binders (based on a list of 28 fragments known to characterize promiscuous binders)(22). We then profiled the compounds against an extensive set of pharmacophore models derived from unique protein binding sites and ligands as detailed in Materials and Methods. This profiling procedure predicted protein sites which might interact with the hits. Using the UniHI software, we generated an interactome map of these proteins (Fig. 5A). From this interactome map, we have identified proteins which are drug targets. We then used gene ontology tools to filter out from the drug targets the proteins predicted to bind carbohydrate derivatives (Fig. 5B) under the assumption that these proteins will be more relevant to PG metabolism. UniHI has identified 27 drugs which interact with proteins which also bind carbohydrate derivatives (Supplementary Fig. 2). These 27 drugs were clustered together with the identified 19 (dose-dependent and non dose-dependent) hits. The largest cluster (Supplementary Fig. 3) included 21 drugs and all hits (including their possible protonation

(pH 7) and tautomeric states). About half of these drugs (9 out of 21, Drug Bank (<https://www.drugbank.ca/>) identifiers DB04012, DB01712, DB08520, DB02888, DB01723, DB00877, DB00337, DB00864, DB03621) interact with the GS activator protein phosphatase 1 (PP1), six directly (DB04012, DB08520, DB02888, DB00877, DB00864, DB03621) and three (DB01712, DB01723, DB00337) via the PP1 complexed FKBP1A protein. Moreover, four of the hits were found to be similar to at least five out of the nine PP1 binders. Interestingly, three of these four hits were above the median of GS inhibition potency (Table 1). This analysis thus suggests inhibition of PP1-mediated GS activation as a possible PB-lowering mechanism, which is alternative to direct inhibition of GS and common to some of the hits discovered. Indeed when two selected PP1 interacting drugs clustered with the hits, Pimecrolimus (DB00337) and Tacrolimus (DB00864), were added to APBD patient fibroblasts, they have significantly reduced PB staining (Fig. 6). However, as can be appreciated by the images, they have also significantly reduced cell number, suggesting a toxic potential. Such potential toxicity might put off possible clinical use for APBD, as is the case for other FDA approved GS inhibitors such as rapamycin (Fig. 1A). On the other hand, de novo discovered drugs and drug candidates, such as those described here, may, if shown to be safe, adequately replace potentially toxic FDA approved drugs.

Discussion

This work describes the early discovery of candidate molecules which could be developed into future leads for treating GSDs. With the exception of Pompe Disease (PD), which can be partially ameliorated using α -glucosidase enzyme replacement therapy, no effective therapies exist for GSDs. In particular, no molecules are available for reducing the pathogenic PB accumulation in the PB involving GSDs, GSD type IV (APBD and Andersen disease), GSD type VII (Tarui disease), and Lafora Disease (LD). This precluded the use of a positive control in our experiments. Instead, we performed the screening based on PB accumulation. We developed an empirical set of training images, established selected cut-offs and obtained population-based selection. This hit selection approach was also employed by others for assays lacking positive controls(23). However, while our assay inherently lacks a positive control, it has a clear advantage reflected in the cell type selected for it: It is well known that molecules discovered by animal models are not always well translated to human therapy(24). Therefore, our approach is novel in terms of being based on a human cell model, thus increasing the chances of translation to therapy.

To confirm that the measured output is the result of a true reduction in PB content and not of cell shrinkage (possibly caused by cell toxicity), our PB-detecting PAS assay was optimized by incorporating a third staining for cytoplasmic reference. This cytoplasmic reference allowed us to associate the measured PB mean intensity to a given cell and determine whether that cell area was significantly affected by the tested compound. This approach allowed us to confirm that the selected output reflects a true effect of the compound on the PB content, filtering out toxic compounds. Although PAS assay is broadly used to detect polysaccharides in histochemistry(25), this is, to our knowledge, its first use in a screening assay.

The distribution of hits among the plates (data not shown) was sufficiently homogeneous to suggest that the results cannot be attributed to an artifact resulting from an uneven staining of the plates. Only 11 dose-responding hits and 8 non dose-responding hits, with a stable effect, were selected for analysis. Typical dose-responding hit rates from experimental HTS can range between 0.01% and 0.14%(26), in high accordance with the hits rate observed for our 11 dose-responding candidates, which constitute 0.11% of the total screened molecules.

As APBD is a genetic condition, the GBE mutations implicated in it should produce a phenotype not only in neurons but also in cells not directly involved in the pathogenicity such as fibroblasts, which are easier to obtain and culture than neurons. Our preliminary results prior to the assay development (Fig. 1A) showed that APBD fibroblasts have a higher PB content than fibroblasts derived from unaffected subjects. Of note that, as opposed to APBD fibroblasts (both patient and mouse-derived), fibroblast cell lines from Lafora Disease (LD) patients or mice do not form PB *per se*. A likely explanation for this difference is that the replicating cell lines in LD, derived from adolescent patients as opposed to middle-aged to old ones in APBD, simply do not have the timespan required for full-fledged PB to materialize.

The screening assay we employed capitalized on phenotypic selection of hits based on a single readout (cell associated PG mean intensity), which can be caused by multiple factors, as suggested by the complexity of interactions among candidate hit interacting proteins (Fig. 5). Thus the observed compound effect in the analyzed images does not necessarily reveal whether the drug candidate is acting as a GS inhibitor, GBE stabilizer or by somehow promoting PG degradation. Nevertheless, our data (Fig. 4) suggest that a possible mode of action of ~70% of the hits (13 out of 19) is, at least in part, inhibition of GS activity, which stands to reason given that GS is the only enzyme capable of forming the α 1–4 interglucosidic bonds that constitute PG. Furthermore, our computational analysis, according to which half of the drugs clustered with all the hits interact with the GS activator PP1, supports interference of GS activation as a mechanism by which small molecules can lower PB levels. The GS inhibition mode of action has already been demonstrated for rapamycin, a drug used for treating tuberous sclerosis, which, while being a well-established autophagy inducer, actually suppressed PB levels not by autophagic clearance, but by inhibiting GS activity(5). Rapamycin reduced PG in APBD modeling neurons(5) and glycogen in mice modeling the GSD Pompe Disease(27). However its toxic effects in APBD mice (our unpublished data) disqualified it for further development. Since PB is the pathogenic factor in PB-involving GSDs, our findings that most PB reducing compounds are also mild GS inhibitors call for a GSD therapeutic strategy based on mild inhibition of GS activity. This strategy is also expected to lead to lower cytotoxicity. Of note that rapamycin, in addition to its pleiotropic effects, is a robust, rather than mild, inhibitor of GS activity (which it reduced by 70%(5)). The fact that some of our hits (*e.g.*, #4, Table 1) are GS inhibitors and at the same time similar to PP1 interacting drugs might lead, by future medicinal chemistry, to multi-targeting drugs: Single molecules which would only partially interfere with GS and PP1 thus still allowing them to sustain prioritized cell functions in which they are involved. Consequently, possible pleiotropic side effects are expected to be attenuated.

In one of the PB-associated GSDs, LD, it has been shown that a mere 30% reduction of GS activity, through knockout of the PP1 subunit (Ptg) that targets the phosphatase to GS, rescues the disease's animal model (28). Our drug candidates which achieve this threshold of GS activity reduction (Fig. 4) thus constitute a promising therapeutic strategy. On the other hand, PB reducing hits which did not inhibit GS (and even upregulated its activity, Fig. 4) point to the multifaceted regulation of PB levels, amenable not only to manipulation of their synthesis (by GS), but also of their degradation and shaping. Thus our data suggest that the hits discovered are indeed germane to net polyglucosan formation, either directly or indirectly. Work is underway to develop these hits further towards potential therapies for this severe group of disorders.

Finding an inhibitory effect of most hits on GS activity (Fig. 4) and the similarity found between hits and drugs interacting with the GS activator PP1 (Table 1) are especially important in view of the innate non-specificity of some screening assays, revealed e.g., by the so-called pan-assay interference compounds (PAINS)(29). Providing evidence in support of an expected and relevant mode of action, as GS inhibition is for lowering PB levels, should thus be exercised in all HTSs in order to prevent the "discovery" of false positive hits which may waste time and resources in futile research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the Mexican Friends of Tel Aviv University for their contribution in the establishment of the Cell Screening Facility for Personalized Medicine (CSFPM). We also thank Dr. Hamutal Engel from the Blavatnik Center for Drug Discovery, Tel Aviv University for her help in computational chemistry.

Funding information

This work was made possible thanks to grants from the Association Francaise contre les Myopathies (AFM) and the APBD Research Foundation (both to OK). LJS was supported by funds from the CSFPM, Tel Aviv University. BAM was supported by The National Institute of Neurological Disorders and Stroke of the National Institutes of Health (NIH) under award number P01 NS097197, the Ontario Brain Institute, the University of Toronto Michael Bahen Chair in Epilepsy Research and the University of Texas Southwestern Jimmy Elizabeth Wescott Chair in Pediatric Neurology.

Abbreviations list

APBD	Adult polyglucosan body disease
GSDs	Glycogen Storage Diseases
HCS	high content screening
PG	polyglucosans
PB	polyglucosan body
GBE	Glycogen branching enzyme
GS	Glycogen synthase

PAS	Periodic acid/Schiff base
FBS	Fetal bovine serum

References

1. Liu Y, Zeng L, Ma K, Baba O, Zheng P, Liu Y, et al. Laforin-malin complex degrades polyglucosan bodies in concert with glycogen debranching enzyme and brain isoform glycogen phosphorylase. *Mol Neurobiol.* 2014; 49(2):645–57. [PubMed: 24068615]
2. Wang Y, Ma K, Wang P, Baba O, Zhang H, Parent JM, et al. Laforin prevents stress-induced polyglucosan body formation and Lafora disease progression in neurons. *Mol Neurobiol.* 2013; 48(1):49–61. [PubMed: 23546741]
3. Lossos A, Klein CJ, McEvoy KM, Keegan BM. A 63-year-old woman with urinary incontinence and progressive gait disorder. *Neurology.* 2009; 72(18):1607–13. [PubMed: 19414729]
4. Girard JM, Stone SS, Lohi H, Blaszykowski C, Teixeira C, Turnbull J, et al. Phosphorylation prevents polyglucosan transport in Lafora disease. *Neurology.* 2012; 79(1):100–2. [PubMed: 22622857]
5. Kakhlon O, Glickstein H, Feinstein N, Liu Y, Baba O, Terashima T, et al. Polyglucosan neurotoxicity caused by glycogen branching enzyme deficiency can be reversed by inhibition of glycogen synthase. *J Neurochem.* 2013; 127(1):101–13. [PubMed: 23607684]
6. Bao Y, Kishnani P, Wu JY, Chen YT. Hepatic and neuromuscular forms of glycogen storage disease type IV caused by mutations in the same glycogen-branching enzyme gene. *J Clin Invest.* 1996; 97(4):941–8. [PubMed: 8613547]
7. Robitaille Y, Carpenter S, Karpati G, DiMauro SD. A distinct form of adult polyglucosan body disease with massive involvement of central and peripheral neuronal processes and astrocytes: a report of four cases and a review of the occurrence of polyglucosan bodies in other conditions such as Lafora's disease and normal ageing. *Brain.* 1980; 103(2):315–36. [PubMed: 6249438]
8. Roach PJ. Glycogen phosphorylation and Lafora disease. *Mol Aspects Med.* 2015; 46:78–84. [PubMed: 26278984]
9. Danon MJ, Servidei S, DiMauro S, Vora S. Late-onset muscle phosphofructokinase deficiency. *Neurology.* 1988; 38(6):956–60. [PubMed: 2966901]
10. Hellmann MA, Kakhlon O, Landau EH, Sadeh M, Giladi N, Schlesinger I, et al. Frequent misdiagnosis of adult polyglucosan body disease. *J Neurol.* 2015; 262(10):2346–51. [PubMed: 26194201]
11. Mochel F, Schiffmann R, Steenweg ME, Akman HO, Wallace M, Sedel F, et al. Adult polyglucosan body disease: Natural History and Key Magnetic Resonance Imaging Findings. *Ann Neurol.* 2012; 72(3):433–41. [PubMed: 23034915]
12. Solmesky LJ, Shuman M, Goldsmith M, Weil M, Peer D. Assessing cellular toxicities in fibroblasts upon exposure to lipid-based nanoparticles: a high content analysis approach. *Nanotechnology.* 2011; 22(49):494016. [PubMed: 22101838]
13. Dvash R, Khatchatourians A, Solmesky LJ, Wibroe PP, Weil M, Moghimi SM, et al. Structural profiling and biological performance of phospholipid-hyaluronan functionalized single-walled carbon nanotubes. *J Control Release.* 2013; 170(2):295–305. [PubMed: 23764531]
14. Solmesky, LJ., Adalist, J., Weil, M. Image-based high-content analysis, stem cells and nanomedicines: a novel strategy for drug discovery. In: Peer, D., editor. *Handbook of harnessing biomaterials in nanomedicine: preparation, toxicity and applications.* first. Singapore: Pan Stanford Publishing Pte; 2011.
15. Tabatabaei Shafiei M, Carvajal Gonczi CM, Rahman MS, East A, Francois J, Darlington PJ. Detecting glycogen in peripheral blood mononuclear cells with periodic acid schiff staining. *J Vis Exp.* 2014; (94)
16. Kelley LA, Gardner SP, Sutcliffe MJ. An automated approach for clustering an ensemble of NMR-derived protein structures into conformationally related subfamilies. *Protein Eng.* 1996; 9(11): 1063–5. [PubMed: 8961360]

17. Nikaido T, Austin J, Stukenbrok H. Studies in myoclonus epilepsy. 3. The effects of amyolytic enzymes on the ultrastructure of Lafora bodies. *J Histochem Cytochem.* 1971; 19(6):382–5. [PubMed: 4104134]
18. Inglese J, Auld DS, Jadhav A, Johnson RL, Simeonov A, Yasgar A, et al. Quantitative high-throughput screening: a titration-based approach that efficiently identifies biological activities in large chemical libraries. *Proc Natl Acad Sci U S A.* 2006; 103(31):11473–8. [PubMed: 16864780]
19. Bouskila M, Hunter RW, Ibrahim AF, Delattre L, Pegg M, van Diepen JA, et al. Allosteric regulation of glycogen synthase controls glycogen synthesis in muscle. *Cell Metab.* 2010; 12(5): 456–66. [PubMed: 21035757]
20. Gold AM. Kinetic mechanism of rabbit muscle glycogen synthase I. *Biochemistry.* 1980; 19(16): 3766–72. [PubMed: 6773553]
21. Sølling H. Studies on the allosteric properties of glycogen synthase I. *Eur J Biochem.* 1979; 94(1): 231–42. [PubMed: 108102]
22. Gilad Y, Nadassy K, Senderowitz H. A reliable computational workflow for the selection of optimal screening libraries. *J Cheminform.* 2015; 7:61. [PubMed: 26692904]
23. Bray, MA., Carpenter, A. Advanced Assay Development Guidelines for Image-Based High Content Screening and Analysis. In: Sittampalam, G.Coussens, N.Nelson, H.Arkin, M.Auld, D., Austin, C., editors. Assay Guidance Manual. Bethesda, MD: 2004.
24. Martic-Kehl MI, Schibli R, Schubiger PA. Can animal data predict human outcome? Problems and pitfalls of translational animal research. *Eur J Nucl Med Mol Imaging.* 2012; 39(9):1492–6. [PubMed: 22790876]
25. Hotchkiss RD. A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations. *Arch Biochem.* 1948; 16(1):131–41. [PubMed: 18921848]
26. Zhu T, Cao S, Su PC, Patel R, Shah D, Chokshi HB, et al. Hit identification and optimization in virtual screening: practical recommendations based on a critical literature analysis. *J Med Chem.* 2013; 56(17):6560–72. [PubMed: 23688234]
27. Ashe KM, Taylor KM, Chu Q, Meyers E, Ellis A, Jingozyan V, et al. Inhibition of glycogen biosynthesis via mTORC1 suppression as an adjunct therapy for Pompe disease. *Mol Genet Metab.* 2010; 100(4):309–15. [PubMed: 20554235]
28. Pederson BA, Turnbull J, Epp JR, Weaver SA, Zhao X, Pencea N, et al. Inhibiting glycogen synthesis prevents Lafora disease in a mouse model. *Ann Neurol.* 2013; 74(2):297–300. [PubMed: 23913475]
29. Baell J, Walters MA. Chemistry: Chemical con artists foil drug discovery. *Nature.* 2014; 513(7519):481–3. [PubMed: 25254460]

Summary statement – written for a broad audience, highlighting the key finding of your study

We have developed a unique assay for quantifying the levels of polyglucosans - toxic inclusion bodies containing malconstructed glycogen. This assay was implemented to discover small molecules able to reduce polyglucosans and thus potentially ameliorate glycogen storage disorders.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1. High Throughput Screening for compounds able to reduce PB

(A) Skin fibroblasts from an APBD (left) and control (middle) subjects and APBD subject treated overnight with 1 μ M rapamycin (right) were formaldehyde-fixed, amylase digested, stained with Hoechst (blue) and PAS (red), and then imaged by the InCell 2000 High Content Analysis system.

(B) Skin fibroblasts from healthy control and APBD patient were co-cultured in respectively 0:1, 1:3, 3:1 and 1:0 ratios. The cells were then stained with PAS and imaged as in (A). Representative images for the different patient cell co-culturing percentages and their quantification based on n=3 experiments are shown. (C) A representative image of cells showing the different stains employed (from left to right, as indicated in the figure, nuclear, PAS, Cell Mask and a merged image).

(D) APBD patient derived fibroblasts were cultured for 24h in DMEM (with 5%FBS) and then 10,084 unique compounds from the DiverSet CL library (ChemBridge) were added to each different well at a concentration of 10 μ M. Following an additional 24h incubation, the cells were fixed, the fixative was neutralized, and the cells were then permeabilized, treated with diastase and stained with Hoechst 33342, Cell Mask Deep Red and PAS. Images were then acquired and analyzed. Each well mean of cell area (based on Cell Mask Deep Red

staining) was normalized by z-scores according to the population mean of all the compounds. Compounds showing a toxic effect were discarded by keeping only those showing a z-score higher than -1.5 for their normalized cell area mean and more than 300 cells per well. Once this selection was made, the hits were selected based on the normalized PG mean intensity. Those compounds inducing a well-based PG mean intensity below -1.2 z-score were selected as hits. Thus, from 10,084 compounds screened, 85 were selected as hits. These selected compounds were subjected to the same assay once again employing concentrations of 0, 1, 5, 10 and 50 μ M. These dose response experiments narrowed down the 85 selected hits to 11 in which a concentration dependent decrease of cell associated PG mean intensity has been demonstrated.

Figure 2. Dose response of PB reducing hits

(A) Box plots depicting for each of the 11 concentration dependent hits discovered the effect of concentration on diastase resistant, cell associated, mean PAS intensity, normalized by z-score. Arrowheads, Median PAS intensity. The yellow boxes delineate upper and lower quartiles from the median, and upper and lower “whiskers” respectively show maximal and minimal mean PAS intensity values. Red dots, denote outliers which are values at least 1.5× (interquartile range (75th percentile – 25th percentile)) above or below the box.

(B) PAS staining images taken as in Fig. 1, demonstrating hit toxicity to cells in concentrations above 50 μM. Exemplary images for hits 5 and 7 are shown.

(C) Dose-response (DR) curves of the 11 concentration dependent hits discovered. Shown are DR curves of diastase resistant cell associated mean PAS intensities normalized by z-score. The zero concentration is excluded in all curves, as the concentrations are drawn in a log scale. Shown at the *bottom* are the EC₅₀ and R² data for the 11 hits.

C **Figure 3. Non dose-responding PB reducing hits**

(A) and (C) Data for hits which lowered mean normalized PAS intensity in a non-dose responding manner are presented as in Fig. 2 (A) and (C) respectively. (B) Demonstration of toxicity above 50 μM , as in Fig. 2(B), for representative hit number 14.

Figure 4. Hit effect on GS activity

(A) An in vitro GS enzyme activity assay (see Materials and Methods) was performed in order to test the effect of the 19 hits discovered on GS activity. Each hit compound was used at a final concentration of 50 μM. UDP serves as a positive control for reduction of GS activity. All hits, except for #10, have significantly changed GS activity with #6, 7, 8, 12, 18 and 19 increasing and the rest decreasing it ($n = 3$ experiments, $p < 0.05$, one-way ANOVA with Dunnett post-hoc test). See Table 1 for compounds names and molecular structures. (B) Dose response curves of the effect of the compounds on GS activity. Compound concentrations used are 3.125, 6.25, 12.5, 25, 50, 100, 200, 400, and 800 μM. Shown are also goodness of fit (R-square) and estimated EC50 values.

Figure 5. Protein targets of the hits

(A) An interactome of the predicted protein targets of the 36 discovered hits (including tautomers and protonation states). *Small yellow circles*, hit protein targets; *large yellow circles*, hit interacting proteins which serve as hubs for protein interaction; *red circles*, protein targets of the hits known to interact with drugs; *blue lines*, protein-protein interactions; *red arrows*, regulatory interactions. See <http://www.unihi.org/> for names and descriptions of gene symbols shown

(B) An interactome of the subsection of hit-protein targets known to bind drugs and predicted to bind carbohydrate derivatives (red circles in **(A)**).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 6. Effect on PB of known drugs clustered with the hits
Skin fibroblasts from an APBD subject were treated overnight with 1 μ M DMSO, Pimecrolimus, or Tacrolimus as indicated, formaldehyde-fixed, amylase digested, stained with Hoechst (blue) and PAS (red), and then imaged.

Table 1

Hit efficacy of GS inhibition compared to hit similarity to PP1 interacting drugs

Hit number and Name	Structure	Rank of Similarity to PP1 Interacting Drugs	Rank of Efficacy of GS Inhibition	% activity remaining
	UDP (positive control for GS inhibition)	Irrelevant. Not a hit.	1	47.6
#4: N-(2-methoxyethyl)-6-methyl-N-[(3-methyl-2-thienyl)methyl]-2-oxo-1,2-dihydropyridine-4-carboxamide	3	2	67.7
#3: 3-[2-(3-cyclopropyl-1,4,6,7-tetrahydro-5H-pyrazolo[4,3-c]pyridin-5-yl)-2-oxoethyl]-4-(3-methylbutyl)-2-piperazinone	Below cutoff	3	69.0
#14: 4-tert-butyl-2-(4H-1,2,4-triazol-4-yl)phenol	Below cutoff	4	72.3
#17: N-[3-(2,7-diazaspiro[4.5]dec-2-ylcarbonyl)-2-methylphenyl]-2-furamide	4	5	73.3

Hit number and Name	Structure	Rank of Similarity to PP1 Interacting Drugs	Rank of Efficacy of GS Inhibition	% activity remaining
#13: 2-(2-amino-4,6-dimethylpyrimidin-5-yl)-N-[(3S*,4R*)-4-ethoxytetrahydrofuran-3-yl]acetamide	Below cutoff	6	73.5
#1: 3-fluoro-4-{6-[(pyridin-3-yl)methyl]amino}-1H-pyrrolo[2,3-b]pyridin-4-yl]phenol	Below cutoff	7	77.3
#5: 3-{2-[2-[(3S*,4S*)-3-hydroxy-4-(2-naphthyl)piperidin-1-yl]-2-oxoethyl]-1,3-oxazolidin-2-one	Below cutoff	8	82.1
#16: 2-(4-methoxy-2-methylphenyl)-5-methylpyrimidin-4-amine	Below cutoff	9	86.5
#9: (1S*,6R*,9<1-methyl-3-(2-thienyl)-1H-pyrazol-5-yl]carbonyl]-3,9-diazabicyclo[4.2.1]nonan-4-one>	Below cutoff	10	87.1

Hit number and Name	Structure	Rank of Similarity to PP1 Interacting Drugs	Rank of Efficacy of GS Inhibition	% activity remaining
#11: N-[(5-ethylpyridin-2-yl)methyl]-N-methyl-2,1,3-benzoxadiazole-5-carboxamide 1-oxide	Below cutoff	11	90.5
#2: 6-[4-(2,6-difluoro-4-methoxybenzyl)piperazin-1-yl]pyrazine-2-carboxamide	Below cutoff	12	91.4
#15: N-[2-(4,5,6,7-tetrahydro-2H-indazol-3-yl)ethyl]-3-azaspiro[5.5]undecan-9-amine	Below cutoff	13	93.6
#10: N-[(1,5-dimethyl-1H-indazol-3-yl)methyl]-2-(3-pyrrolidinyl)benzamide	Below cutoff	14	96.2
#12: 7-(3-chlorophenyl)-4-(3-furyl)-2,3,4,5-tetrahydro-1,4-benzoxazepin-9-ol	Below cutoff	15	103.4

Hit number and Name	Structure	Rank of Similarity to PP1 Interacting Drugs	Rank of Efficacy of GS Inhibition	% activity remaining
#18: N-(2,4-dimethylphenyl)-3-[4-phenyl-5-(tetrahydrofuran-3-yl)-1H-imidazol-1-yl]propanamide	Below cutoff	16	105.0
#7: 4-isopropyl-2-methyl-6-{4-[(3-methylpyridin-2-yl)methyl]piperazin-1-yl}pyrimidine	Below cutoff	17	109.9
#19: (1S*,6R*)-9-<4-(methoxymethyl)-5-methylisoxazol-3-yl>carbonyl-3-methyl-3,9-diazabicyclo[4.2.1]nonan-4-one	2	18	114.2
#8: 5-methyl-1-[4-(4H-1,2,4-triazol-4-yl)phenyl]pyrrolidin-2-one	Below cutoff	19	115.4
#6: 4-[4-(3-fluorophenyl)-1H-pyrazol-5-yl]-1-[(1-isopropyl-1H-imidazol-2-yl)methyl]piperidine	Below cutoff	20	118.3