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14. ABSTRACT Our goal is to discover drugs that stabilize the normal structure of superoxide dismutase 1 as a means to slow the progression of ALS caused by mutations in this protein. To accomplish this goal, we developed an assay that is based on the observation that the luciferase enzyme can be split into 2 halves. These 2 halves can be forced to reconstitute an active enzyme if they are brought together by some force. In our assay, this force is the normal interaction that occurs when 2 individual SOD1 proteins come together to form a normal active enzyme. Using recombinant DNA, we create fusion proteins of SOD1 and each half of the luciferase enzyme. In the past year, we have characterized and optimized an assay that measures the strength of interaction between SOD1 proteins by measuring luciferase activity. We have demonstrated that the assay reports the stability of normal dimeric interactions, and interactions between normal and disease-associated mutant SOD1. Information obtained during the screen helped us identify a candidate compound that may stabilize SOD1 dimer interactions. We have also used the assay to perform an initial screen of a drug library.

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1. Introduction

Mutations in the human copper-zinc superoxide-1 dismutase (**SOD1**) gene are established as a prevalent cause of the familial form of ALS (**fALS**). Dissociation of the mutant-harboring SOD1 dimer is believed to be the rate-limiting step in the subsequent processes that lead to neuropathology. In the present study, we proposed to develop and validate novel HTS assays having the capacity to detect small molecules that modulate multiple aspects of SOD1 protein-protein interactions (**PPI**). Our hypothesis is that drugs that kinetically stabilize the SOD1 dimer—either by increasing monomer subunit association or decreasing dimer dissociation— will arrest the downstream cascade of cellular events that cause mutant-SOD1 aggregation, and ultimately motor neuron toxicity. Our first Aim is to develop, characterize and optimize a high throughput screening (**HTS**) assay to discove
r drugs that stabilize SOD1 tertiary structure. To accomplish this goal, we developed a split-luciferase complementation assay (**SLCA**) using Gaussia luciferase (**gLuc**) fused to wild-type (WT) and/or mutant SOD1 (Fig. 1). In our implementation of the HTS assay, the two portions of gLuc are fused to either WT or mutant SOD1 and these fusion proteins are expressed separately in CHO cells. The cell lysates are mixed and incubated to allow any homodimers in the lysate to dissociate to monomers, which then re-associate over time, bringing the two gLuc fragments into proximity to reconstitute their activity. Studies in our first Aim were to better characterize this assay and optimize it for 384 wells. Our second Aim, was to apply a highly innovative, multiplexed drug discovery approach using selected chemotypes from diverse chemical libraries, as well as intelligently-selected, binary combinations of FDA-approved drugs, to potentially discover allosteric, combination modulators of SOD1 interactions. Based on the design of the assay, we envisioned that we could identify two types of molecules that might be useful; those that stablize dimeric structure and thos that block dimer formation. Successfully achieving both of our Aims over the course of the two-year program has the potential to discover pre-therapeutic lead candidates that allosterically modulate SOD1 protein interactions supra-additively and, potentially, combination-drug leads with a fast-track development path to clinical trials.

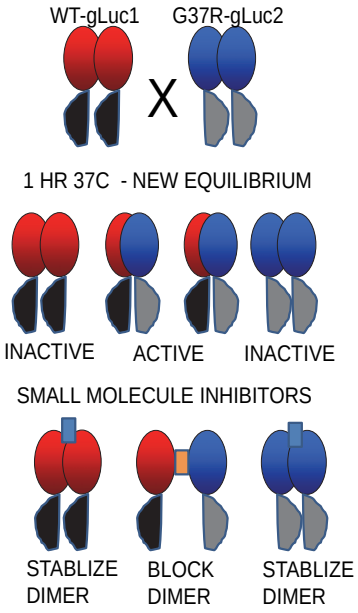


Figure 1: Possible SOD1 subunit exchange MOA. *The top portion of each chimera* represents an SOD1 isoform, the bottom part is a *gLuc* 'tail'. See text for details.

2. KEYWORDS AND ABBREVIATIONS

α-synuclein (aSyn or aS)
bovine serum albumin (BSA)
cysteine (cys)
dithiothreitol (DTT)
disuccinimidyl suberate (DSS)
familial amyotrophic lateral sclerosis (fALS)
Gaussia luciferase (Gluc)
glutahione (GSH)
high throughput screening (HTS)
Lankenau Chemical Genomics Center (LCGC)
mechanism of action (MOA)

natural products (NP)
orthogonal pooled screening (OPS)
protein interactions (PPI)
relative luminesence units (RLU)
shifted transversal (pooled) design (STD)
size-exclusion chromatography (SEC)
sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)
split-luciferase complementation assay (SLCA)
superoxide-1 dismutase (SOD1)
Wild-type SOD1 fused to N-terminal portion of gLuc (WT-LI)
Wild-type SOD1 fused to C-terminal portion of gLuc (WT-L2)
G37R fALS varaint of SOD1 fused to N-terminal portion of gLuc (G37R-LI)
A4V fALS varaint of SOD1 fused to N-terminal portion of gLuc (A4V-LI)
G37R fALS varaint of SOD1 fused to C-terminal portion of gLuc (G37R-L2)
A4V fALS varaint of SOD1 fused to C-terminal portion of gLuc (A4V-L2)

3. ACCOMPLISHMENTS

MAJOR GOALS OF THE PROJECT

SA1: Develop an innovative high-throughput approach for characterizing SOD1 protein-protein interactions (PPI); demonstrate the idea can be translated for a new way to discover drugs that kinetically stabilize the SOD1 heterodimer (Figure 1).

SA1: Statement of Work (SOW) Milestones

i. Determine that the SOD1 split-luciferase, complementation assay ("**SLCA"**) reaction that will be used in drug screening reports the formation of dimers (as depicted by **Fig. 1**), rather than non-specific aggregate formation.
    a. Use size-exclusion and gel electrophoresis chromatography to show that the molecules producing luciferase activity in the SLCA assay have sizes expected for dimers of the fusion proteins and not an aggregate.
    b. Competition experiments will be used for both assay characterization and determination of drug MOA. At equilibrium, excess native SOD1 should attenuate Gluc activity when added to the SLCA mixture undergoing dimer exchange.
ii. Optimize SOD1-SLCA using luciferase activity measurements. Determine IC_{50} values for confirmed hits and mode of action (**MOA**) for compounds detected by SLCA.
iii. Generate SLCA constructs for A4V-SOD1:Gluc1 and A4V-SOD1:Gluc2, validate the assay and apply this assay to our HTS screening effort.

SA2: A**pply an innovative, multiplexed drug discovery approach using selected chemotypes from diverse chemical libraries, *as well as intelligently-selected, binary combinations of FDA-approved drugs,* to *potentially discover allosteric, combination modulators of hSOD1 interactions***.

SA2: SOW Milestones

i. Apply shifted-transversal design to prepare an orthogonal-pooled library containing 310 FDA-approved drugs as ~45,000 binary pairs within ~1,000 combination wells. Add known single-actives together in positive-control wells to demonstrate data-deconvolution works.

ii. Screen combinatorial library in A4V mutant homodimer and WT-G37R heterodimer SOD1 subunit exchange SLCA and confirm activities of active pairs.
iii. Similarity-searching and testing of active leads. Characterize MOA for combination-actives.
iv. Demonstrate cellular activity for hits detected from combinatorial screening. Implement fallback strategy of applying pooled screening of common chemical libraries, if required.

Accomplishments towards Specific Aim 1

SA1 ia) Confirmation that luciferase activity generated in the SLCA assay is primarily generated by dimers formed by the SOD1-gLuc proteins (conducted at the University of Florida)

Lysates from CHO cells individually expressing SOD1 (WT or mutant G37R variant) fused to the split, complementary halves of Gluc (designated L1 or L2) were mixed and incubated for 1 hr at 37^0C, the reaction mix was subjected to size-exclusion chromatography (SEC). The elution profile of the SEC column was characterized by passing purified preparations of known proteins through the column to determine the fraction number in which these proteins eluted. We then fractionated the SLCA assays and aliquots of each were transferred to microtiter plates and measured for Gluc activity with coelenterazine. As shown by ***Figure 2***, peak of luciferase activity for mixtures of WT-L1 and WT-L2 eluted in fractions that we estimate correspond to proteins of approximately ~52 kD, which is a close approximation to what would be expected for a dimer of WT-SOD1-gLuc1 and WT-SOD1-gLuc2 (SOD1 dimer = ~32 kD; Gluc = ~19 kD; total expected for SOD1-Gluc = 51 kD). Heterodimers WT-L1 with mutant G37R-L2 eluted at a similar range. We also examined the migration of heterodimers between WT-L1 and an experimental variant of SOD1, (C111S), which we has been reported to be less sensitive to oxidative modification and oligomerization (1). Heterodimers of WT-L1 and C111S-L2 migrated to a size similar to WT-L1 and G37R-L2 (**Figure 2**). Although the apparent MW of these molecules was somewhat larger than expected, the migration of proteins in SEC is influenced by both the shape and size of the molecule and the C111S and G37R heterodimers with WT SOD1 may have a conformational difference from WT homodimers that influences the migration. Virtually no Gluc activity was observed in fractions with an apparent MW of 102 kD or above, which would be expected for a tetramer or higher order oligomers. Overall the data are consistent with the outcome we expected; namely, that the luciferase activity generated in the ex vivo SLCA assay is the result of SOD1-dimerization.

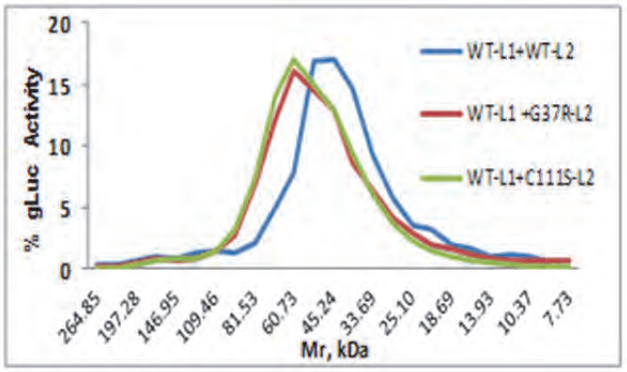


Fig. 2: Luciferase activity separated by size exclusion chromatography. Mixtures of cell lysates were incubated for 1 hr, and then subjected to Sephadex size exclusion chromatography. Each fraction collected was analyzed for *Gaussia* luciferase activity. The graph plots gLuc activity as a percentage of total activity with X-axis indicating the predicted Mr for each fraction collected. The peak luciferase activity migrates very close to the predicted size of dimeric isoforms, and not oligomers.

Determination that the majority of SOD1-gLuc fusion proteins in the cell lysates is dimeric before mixing in the SLCA assay (conducted at the University of Florida).

To further characterize whether the individual SOD1-gLuc fusion proteins were dimers before mixing in the SLCA assay, we incubated the cell lysates with a crosslinker (1 mM DSS - disuccinimidyl suberate) before preparation for denaturing SDS-PAGE. After probing with an anti-GLuc antibody, clear bands are apparent in the WT-L1 and WT-L2 +DSS lanes, consistent with the size expected for a dimer (arrow in **Figure 3**), and the dark monomer bands in those lanes are concomitantly fainter. The L1 gLuc fragment is larger than the L2 fragment, explaining the slightly differing apparent size of the monomer bands. For controls we made lysates of the Monomeric WT-L1 and L2 constructs (presumed monomer). For the Mon-L1, there is no obvious dimer band. For the Mon-L2, there is some dimer-sized band apparent, but multimers (or higher MW cross-linked species) are also apparent. We interpret the latter observation as evidence that Mon-L2 is not specifically in a stable dimer. Untransfected cell lysates (NTf = non-transfected) contained no antibody reactive bands as expected. The lower band that is apparent in the WT-L1 and Mon-L1 samples is unknown at this time. It appears to be a breakdown product we frequently see but not identified. Immunoblots of the cell lysates used in these reactions with antibodies to SOD1 identified a single band (not shown), indicating that the breakdown products may be generated by cleavage within the SOD1 portion of the fusion proteins. These breakdown products would be unlikely to be able to form a stable dimer and would not contribute to the observed luciferase signal. **These data demonstrate that SOD1-Gluc fusion proteins present in the individual lysates exist predominantly as dimers before they are mixed in the ex vivo *reaction whereas data in Figure 2 indicate that the source of luciferase activity in the ex vivo SLCA assays are also dimers of the SOD1-L1 and -L2 fusion proteins. Taken together, the data are consistent with the interpretation that light formation by Gluc complementation occurs by SOD1 subunit exchange, as we originally hypothesized (depicted in Fig 1).***

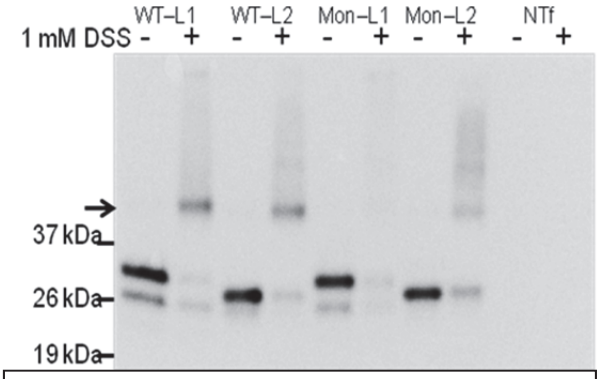


Fig. 3: Immunoblots of cell lysates incubated with and without the cross-linking agent DSS. See text for details.

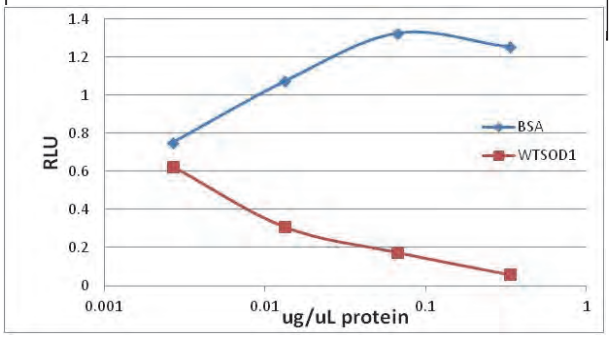
SA1 ib) Excess native WT SOD1 inhibits luciferase activity in the SLCA assay (conducted at the Lankenau Institute using cell lysates generated at the University of Florida).

If the observed luciferase activity in our SLCA assays depends on the dissociation of homodimers of each SOD1-gLuc protein after the individual lysates are mixed; and then, their re-association as a heterodimer, bringing the two halves of the gLuc reporter into complementary proximity, thereby reactivating light-generating enzymatic activity (as depicted by **Fig. 1**), then the addition of purified WT SOD1 to these SLCA reactions should act as a negative competitor. **Figure 4 demonstrates** that excess WT SOD1 (commercial source Sigma Inc) does inhibit luciferase activity in the SLCA assay as hypothesized (RLU are normalized to the control equilibration reaction, i.e., with no added protein). Concentration-dependent inhibition of the reaction by native WT-SOD1 was observed, with virtually complete inhibition occurring at 0.3 µg/µL of native WT-SOD1, or a concentration of 8 µM final. By contrast, we observed that the addition of BSA enhanced the apparent activity of the SLCA reaction. These data indicate that native, purified WTSOD1 added exogenously to the SOD1-SLCA abrogates the Gluc complementation reaction and provides a confirmation that SOD1 dimers can dissociate and freely exchange as first suggested by Dr. Borchelt in 1995 (2).

SA1 ii) Optimization and validation of SOD1-SLCA—specific effects of reducing agents and small molecule inhibitors (Conducted at the Lankenau Institute with cell lysates prepared at the University of Florida).

As noted above, we found that the addition of BSA enhances the signal, achieving a maximal effect at ~0.05% (***Figure 4***). We also found that DTT markedly enhanced the apparent dimerization of the WT-L1 + G37R-L2 heterodimer. The response to DTT was biphasic, with a sharp maximal stimulation observed at 100 µM (**Fig. 5**), which is 10-fold lower than typically is used for preventing the oxidation of free sulfhydral groups. We reasoned that DTT was inhibiting the coelenterazine + Gluc enzyme reaction, but that DDT-enhanced SOD1 dimerization supersedes this inhibitory effect, thus having a net stimulatory effect. We next compared the effect of a free cysteine, a milder reducing agent than DTT. ***Figure* 6** summarizes our findings on treatment of the reaction mix with thiol-reducing dithiothreitol (*DTT—green curves*), or cysteine (red curves). The fold-increases we measured for the SOD1-SLCA were normalized to untreated lysates (i.e., control = 1) and plotted on the left Y-axis in log-scale. The right axis shows the fold-effects of the two sulfhydryl reducing agents on native Gluc on at linear scale. At 100 µM, DTT caused a 46-fold enhanced L1/L2 response, whereas it reduced the native Gluc response by ~ 30%. At higher concentrations, DTT was inhibitory. Cysteine at 3 mM produced more profound enhancement of SOD1-L1/L2 luciferase activity, ~700 fold. Unlike DTT, cysteine also enhanced the Gluc response by 3-fold at the highest concentration tested. Luciferase activity produced in SLCA reactions containing G37R-L1 mixed with G37R-L2 was also enhanced by the addition of cysteine (~65 fold at 3 mM).

Fig 4: Mixed lysates containing WTSOD-L1 and G37R-L2 competing with purified WT SOD1 or BSA (90 min incubation). Readout is gLuc signal normalized to no protein control.



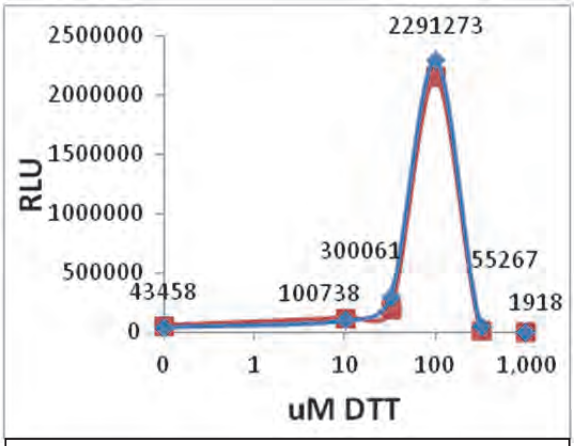


Figure 5: Low DDT concentrations markedly enhance SOD1 dimer formation. L1-WT and G37R-L2 were incubated with increasing concentrations of DTT for 3.5 hrs. The data shown are two replicates from a single experiment.

We compared the effects of DTT and cysteine in an SLCA developed with α-synuclein (aSyn) fusion proteins, which we intended to use in a counter screen. Our aSyn SLCA used a-Syn fused to gLuc1 and to gLuc2. These proteins are expressed separately and then mixed ex vivo in a fashion similar to what we describe above for the SOD1 SLCA assays. Unlike SOD1 having four cysteine groups, the aSyn protein does not contain cysteine. The addition of 100 µM DTT to the aSyn-SLCA inhibited the formation of luciferase activity (**Figure 7**). Cysteine caused only a very modest increase of 2-3 fold in light output from lysates containing aSyn-L1 and aSyn-L2 fusion proteins. **Thus, we concluded the increased luciferase activity in our SOD1 SLCA assays by sulfhydryl reducing agents is due to specific enhancement in the dimerization of SOD1 isoforms, rather than due to an artifact pertaining to the Gluc-coelenterazine reactions.**

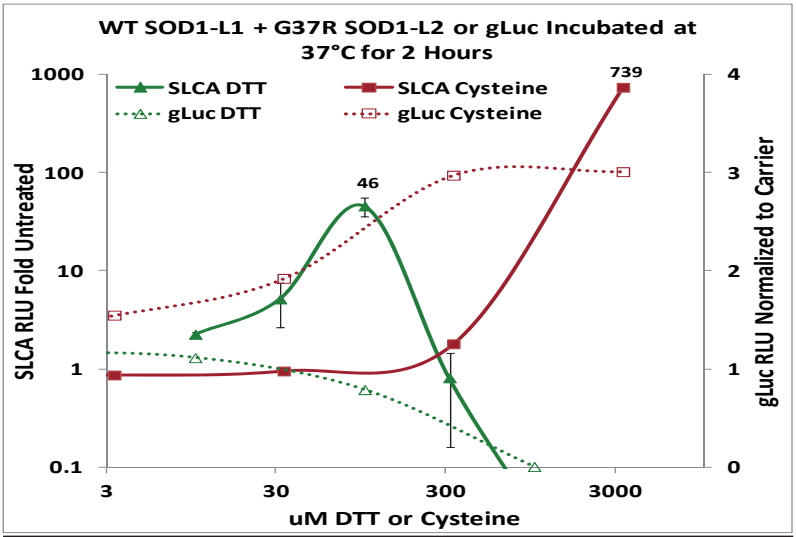


Fig. 6: Thiol reducing agents enhance WT-L1 + G37R-L2 Luciferase activity. Red plots are show the cysteine effect on SOD1-SLCA (solid-red plot, with effect magnitude shown on left Y-axis) or on Gluc (dotted red plot, fold-effect magnitude shown on right Y-axis). Green plots are show the DDT effect on SOD1-SLCA (solid-green plot, with effect magnitude shown on left Y-axis) or on Gluc (dotted green plot, with fold-effect magnitude shown on right Y-axis). Data are means of 2-3 replicates from one experiment, which repeatedly gave similar results.

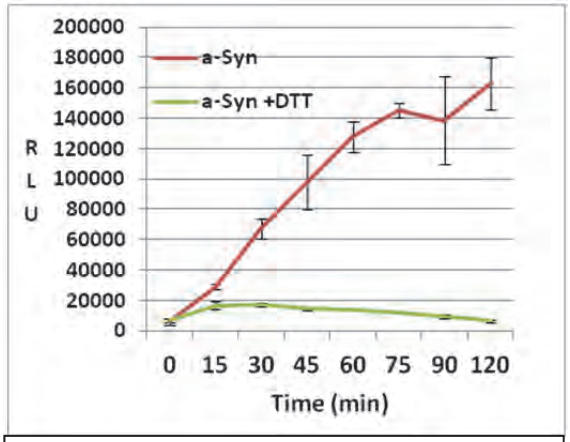


Figure 7: 100 μM abrogates a-synuclein (aS) dimer formation. Lysates from sells expressing L1-aS or aS-L2 with mixed and incubated with DDT for the indicated times. Data are averages ± SD of triplicates.

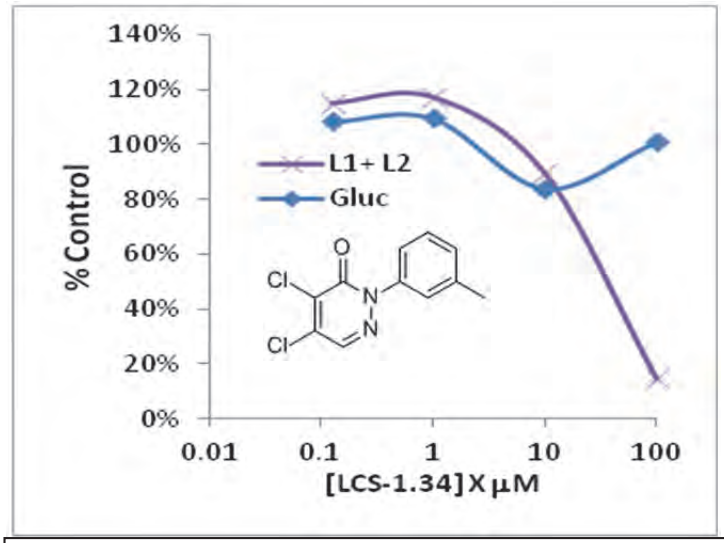
These findings offer several options in optimizing these SOD1 SLCA reactions. Luciferase output may be enhanced by the addition of BSA, DTT, or cysteine. Depending upon how we may want to run the screen (see below), the addition of these reagents could be used to enhance activity.

Candidate approach for identifying lead compounds (conducted at the Lankenau Institute with cell lysates prepared at the University of Florida)

Recently, it was reported that 4,5-dichloro-2-m-tolylpyridazin-3(2H)-one (**Fig. 8**) inhibited the enzymatic activity of SOD1 (3). This compound, called LCS-1.34, was found in a phenotypic HTS assay that slowed the growth of lung cancer cells in vitro. We searched LCGC's internal 250,000 small molecule collection and found the identical structure in our inventory (designated as LCGC-142623). Cell lysates each containing L1WT or G37RL2 were mixed and incubated for 1 hr at 370C with increasing concentrations of the compound and then measured for light output. We found that the compound reported by Somwar et al [Figure 8, right panel, reproduced from (3)] as having an IC50 for inhibiting SOD1 of ~3 uM inhibited the SLCA reaction with an apparent IC50 of ~30 uM. The compound did not significantly inhibit native Gluc at 100 μM, the highest concentration tested (Figure 8). It remains for us to establish that LCS-1.34's MOA for inhibiting SOD1 enzymatic activity is disruptive to the SOD1 dimer. If so, it might offer a useful tool molecule for further validating that the SOD1-SLCA can detect molecules known to modulate dimer formation.

SA1.iii) Develop A4V-SOD1 SLCA for HTS (conducted at the Lankenau Institute using cell lysates generated at the University of Florida; recombinant DNA work at the Univ of Florida).

Because the A4V mutation in SOD1 is far more common in North America, we recognized the need to develop an assay that specifically examines this mutant, in case there is mutant to mutant variation and because such patients will likely be the most frequent for any subsequent clinical trials.



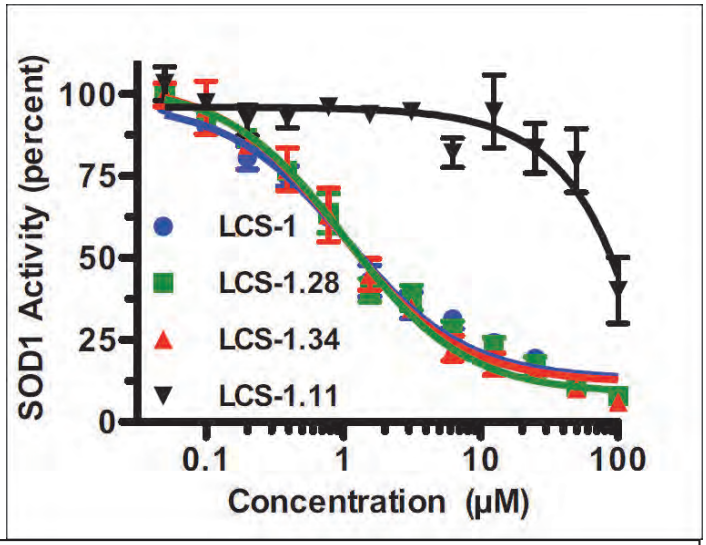


Figure 8: Effects of LCS-1.34—an inhibitor of SOD1 enzyme activity—on apparent hetero-dimerization of L1-WT/G37R-L2. The compound was published as a strong 'hit' in a phenotypic, cellular lung cancer screen, in which SOD1 inhibitors slow proliferation; the published SOD1 potency for LCS-1.34 is ~3 mM (right panel). We found (left panel) that its IC50 for inhibiting dimerization is ~30 mM. Lysates expressing native Gluc were relatively insensitive to the compound.

We have succeeded in expressing and testing the reagents required for development and optimization of an A4V-SOD1 assay in the SLCA format (**Figure 9**). Mixtures of A4V-L1 and -A4V-L2 yielded luminenence output ~8-fold above background, whereas /WT-L1 and -A4V-L2 mixtures yielded considerably greater output of light (60-fold). Interestingly, we found the luciferase activity produced by A4V-L1/L2 mixtures was less sensitive competition with purified WTSOD1 (not shown). We do not fully understand what this outcome means. It may mean that source of luciferease from mixtures of A4V-L1 and A4V-L2 are not native-like dimers, but some other type of association.

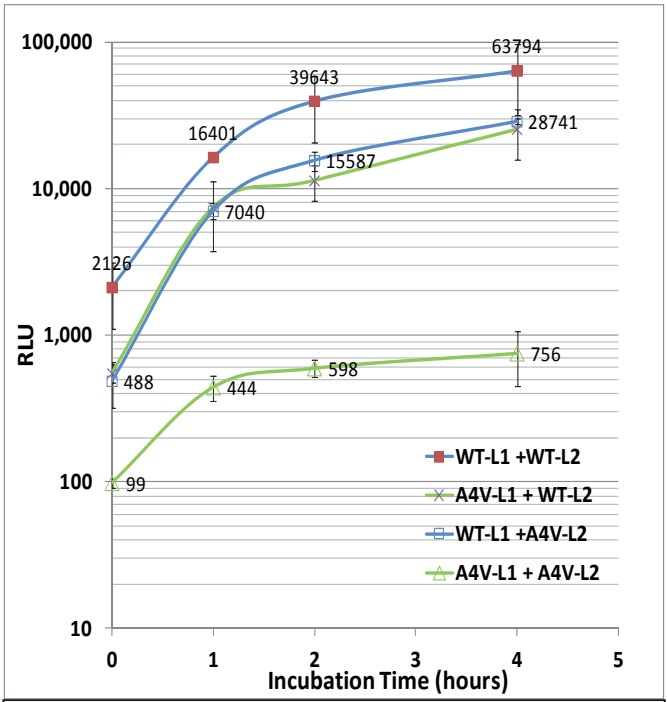


Figure 9: The A4V SOD1-Mutant forms homodimers, and heterodimers with WT-SOD1.

We next sought to test compounds reported by others to stablize A4V-SOD1 homodimers (4,5). Using computational (virtual) screening and *in vitro* aggregation assays, Ray et al (5) identified potential lead compounds that stabilized the A4V-SOD1 mutant, preventing its dissociation in response to chaotropes, and subsequent aggregation. One of the best such stabilizers was reported as: 4-bromo-2-{(E)-[(4- fluorophenyl) imino]methyl}phenol which we had in LCGC's chemical collection designated as LCGC-37845 (shown as RED in **Figure 10**). We tested this compound, along with analogs similar to other SOD1-stabilizing structures reported by Ray et al', in both the A4V (**Fig. 10 left**) and WT (**Fig 10 right**) SLCA homodimer formats. None of them significantly enhanced luciferase activity. We note,

however, that Wright et al (6) reported that these compounds did not bind at the dimer interface as originally described by Ray et al and thus whether these compounds are genuine stabilizers of A4V SOD1 homodimers is uncertain. Our data provide additional evidence that these compounds may not be useful.

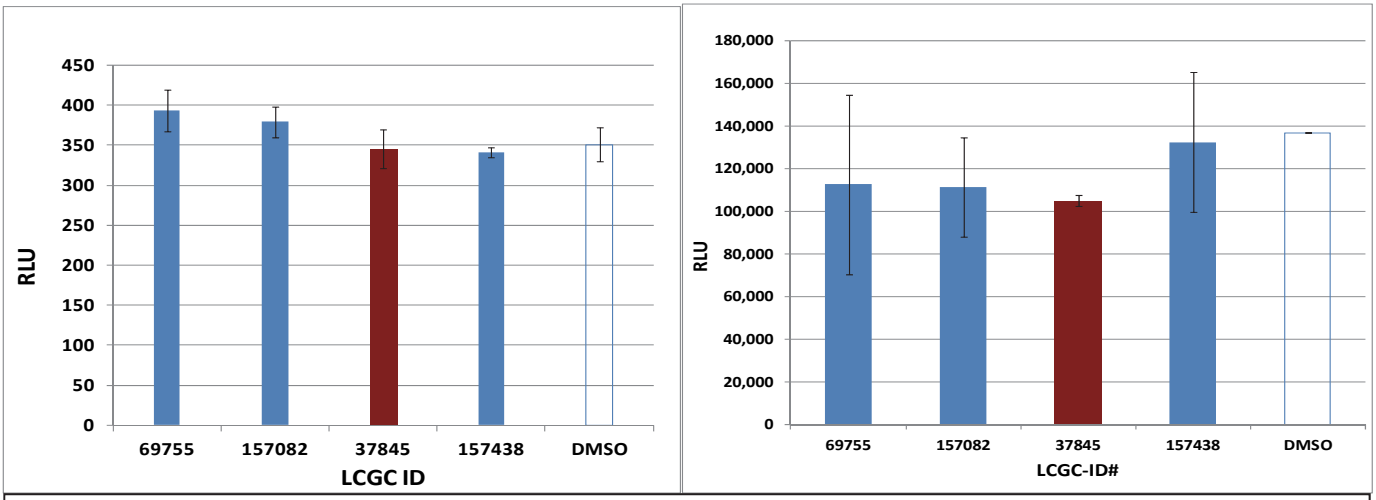
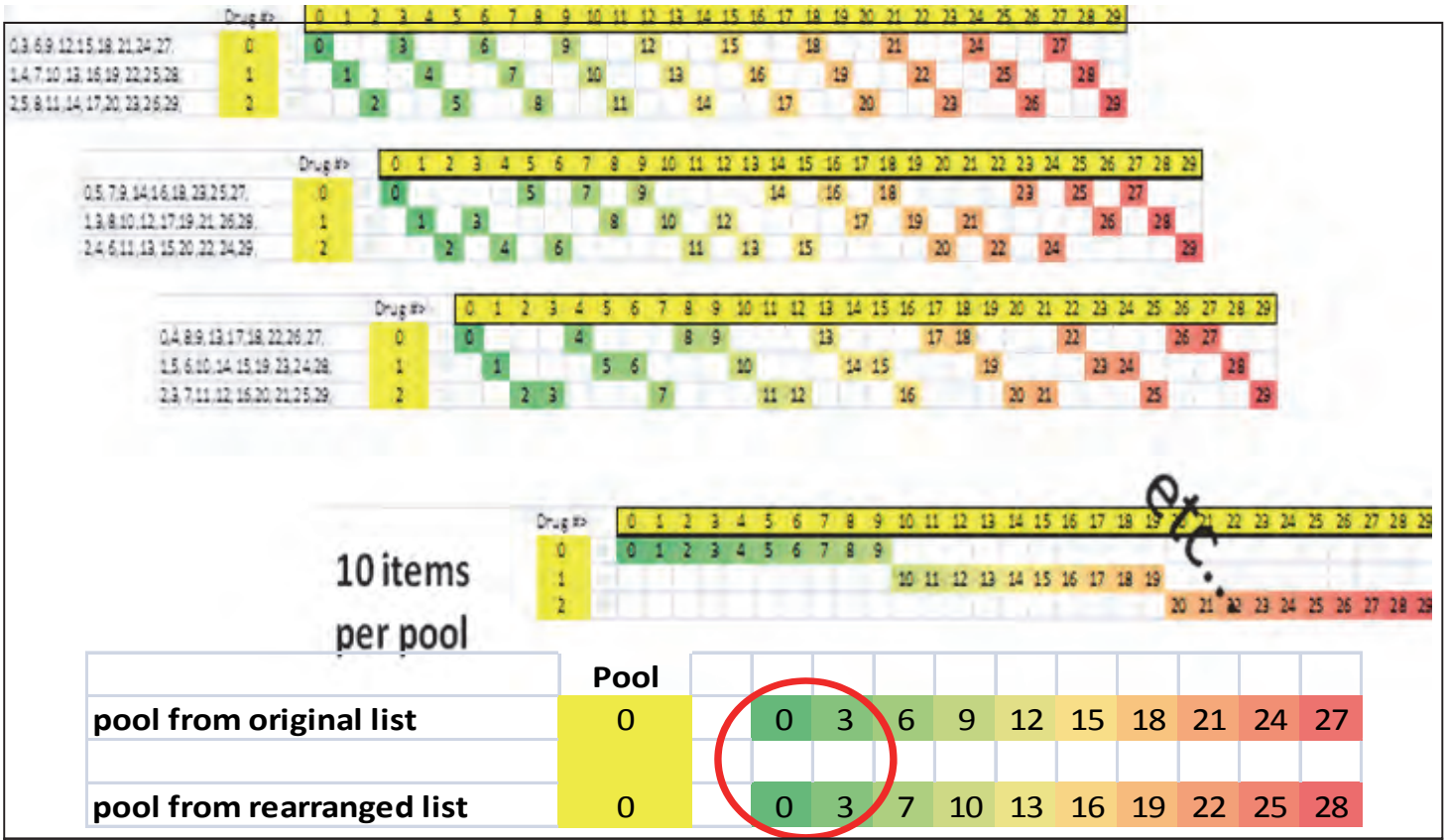


Figure 10: Effect for compounds reported to stabilize SOD1 in the L1/A4V-A4V/L2 (13a) and L1/WT-WT/L2 (13b) SLCA. Compounds purported to stabilize SOD1 had no effect in stabilizing A4V or WT homodimer isoforms. Data are averages ± SD from triplicate determinations in one experiment that was repeated.

SA2 i) Apply shifted-transversal design to prepare an orthogonal-pooled library containing 310 FDA-approved drugs as ~45,000 binary pairs within ~1,000 combination wells. Add known single-actives together in positive-control wells to demonstrate data-deconvolution works. (conducted at the Lankenau Institute using cell lysates generated at the University of Florida).

The highly innovative approach we proposed to use relies on the concept of orthogonal 10-pooling of '*binary-paired-drugs*', as depicted by ***Figure 11***. The objective of STD is to set the stage to pinpoint specific drug combinations with synergistic activity within arrays of combinatorial mixtures of FDA-approved drugs by their 'uniquely redundant' appearance in 'orthogonal (parallel) wells'. We derived an algorithm to direct the automated pipettor through the steps of creating 48,000 non-redundant pairings in 10-pools prepared from 310 stock-solutions as source positions. Once a given STD-matrix array is created, it can readily be screened in any number of assays. C*ompounds that stabilize the G37R mutant homodimer should delay the onset of disease in either of the two transgenic models, we therefore selected G37R homodimer (G37RL1 mixed with G37RL2) for the initial screen.*



	Pool											
pool from original list	0	0	3	6	9	12	15	18	21	24	27
pool from rearranged list	0	0	3	7	10	13	16	19	22	25	28

Figure 11: Shifted Transversal Design (STD). STD provides an efficient algorithm for creating pools that contain all possible pairings from amongst n compounds by the formula pairs = n*n/2; so 310 drugs yield ~ 48,000 pairs. The figure depicts the iterative process for the creation of pools containing 10 compounds each from a mini-library of n = 30 compounds (numbered 0 to 29). To form orthogonal pools, the process is repeated with a different ordering of the same compounds. Pair 0,3 is the only common pair shared between a pool from the original list and one from the rearranged list, shared by two pools each containing 45 pairs.

Figure 12, depicts the 384-well plate layout we have adopted for all HTS assays to discover the first SOD1 dimer stabilizers. **Figure 13** summarizes the QC/QA tracking data captured for every screened plate; shown here is a representative screened plate of STD 10-pooled compounds. For the HTS studies, the final assay volume in PBS buffer was 50 µL, containing 1.0 µL of each lysate, incubated for 3 hours, followed by measurement of Gluc complementation using a Biotek Synergy plate reader, equipped with dual reagent injectors, in the 'flash' mode. The average fold induction for cysteine on every plate was used to define the maximum response, which was normalized to 100,000 RLU. This corrects for varying response magnitude on different plates within and between screening runs. Each drug appears 111 times within in the 10-pools of a STD library from a given set of 310 drugs. Luciferases are sensitive to non-specific inhibition from organic molecules, including by DMSO (**Fig. 14**). The STD 10-pools generally were slightly inhibitory (~2,000 RLU per 100,000 normalized RLU), representing ~-2% to -5% inhibition, with most ~-2% (**Fig. 15**). In order to provide for orthogonal representation and de-convolution of >99% of all the possible pairs, the STD algorithm was applied thrice; each set of resulting wells is termed a 'matrix.' Each matrix comprises 1,147 pools, thus three matrices nets 3,441 pools, whose screening guarantees (to at least 99%) orthogonal de-convolution of every possible pairing of compounds from amongst the 310 drugs (47,895 possible pairs). *Every drug appears in 37 pools per matrix, thus 111 times overall in the three orthogonal matrices.*

Figure 12: 384-well Plate Layout for HTS. 'Mix' (n = 8) refers to 1 L each of G37RL1 + G37R L2 lysates diluted in PBS buffer to a final volume of 50 L. Similarly, the Blue section is Mix + 1 L of DMSO (n = 16). The dark and light red sections (n = 8, each) contain cysteine at 3.3 mM or 0.3 mM, serving as the internal standard for the assay. The grey wells are where 1 L aliquots of compound pools as 'dots' were added in advance as assay-ready plates.

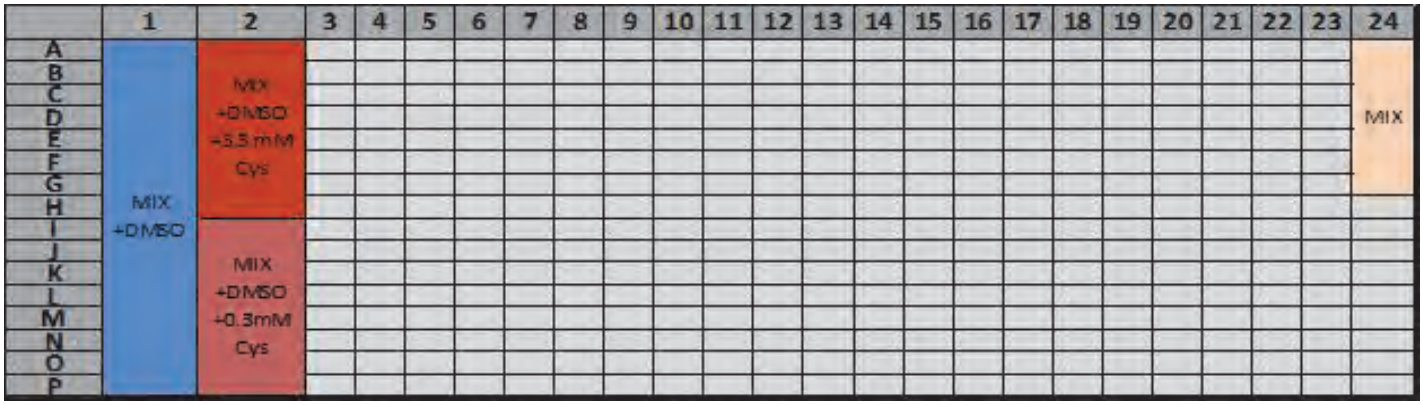


Figure 13: Representative QC/QA Metrics for HTS: Positive and Negative Controls. The Z'-Factor (right Y-axis) is calculated for each plate using both the values for the induction (left Y-Axis; log-scale) by 3.0 mM and 0.3 mM cysteine (Cys), respectively, and the DMSO-containing wells. The signal window, thus, is defined as ~17-fold (3.3 mM Cys) above a background of ~2400 RLU. By including 'no-DMSO' wells, we can monitor sensitivity of assay to slight inhibition elicted by the solvent.

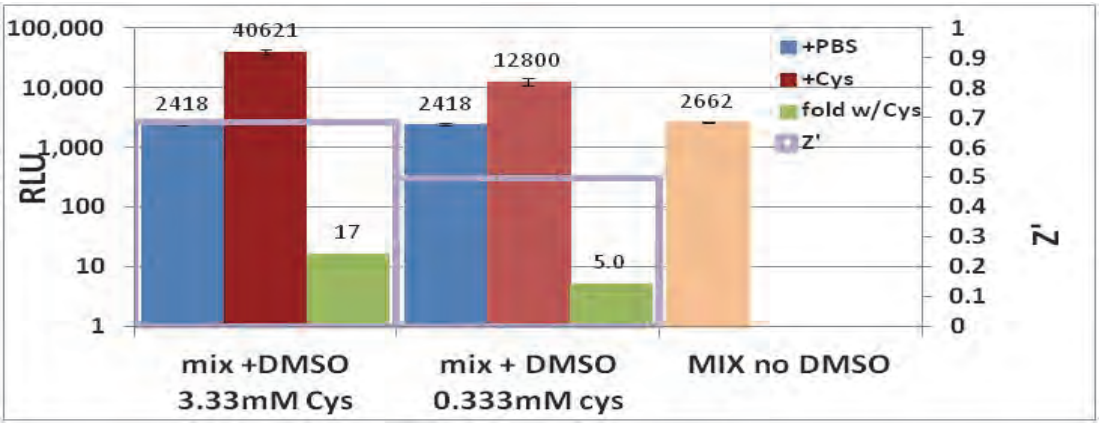
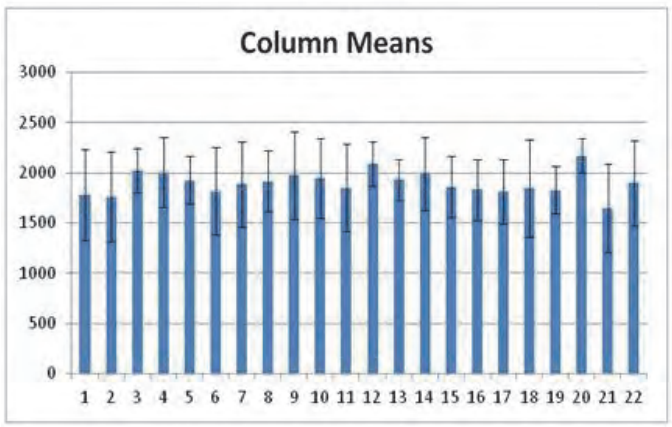


Figure 14: Representative QC/QA Metrics for HTS: Row and Column Means. The mean ± SD values for each of 16 Rows and each of 22 columns of a representative 384-well plate are shown. The first two columns the control and reference wells and are not shown. The last column includes only 8 wells containing compound (see Fig 19).



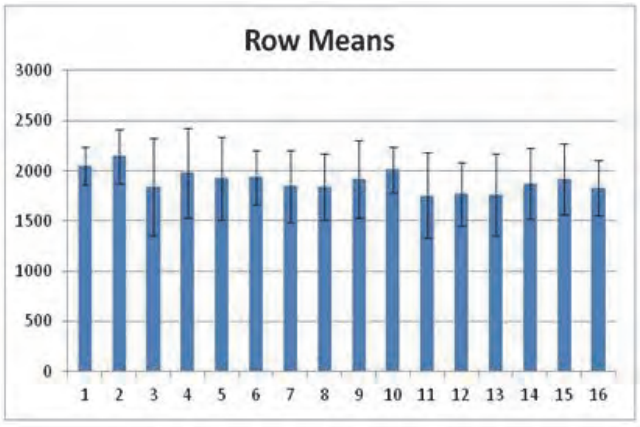
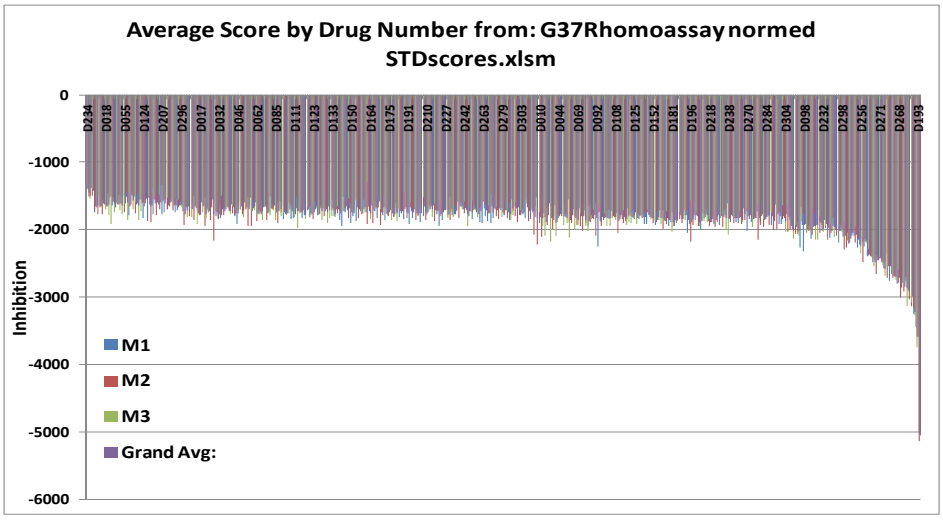


Figure 15: Plot of normalized average scores for individual drugs. The averaged and individual scores of all 210 drugs, each appearing in 111 wells in a total of three orthogonally-pooled matrixes are shown.



SA2 ii. Screen Combinatorial library in A4V mutant homodimer and WT-G37R heterodimer SOD1 subunit exchange SLCA and confirm activities of active pairs (conducted at the Lankenau Institute with cell lysates prepared at the University of Florida).

In a slight departure from what was originally proposed, we decided that our first screen would be in SLCA reactions from mixtures of G37R-L1 and G37R-L2. We chose this tact for 2 reasons. First, we had noted that the libraries we used to create the pools contained inhibitor of native gLuc activity, potentially creating a large number of false positive. Second, we noted that the luciferase activity of G37R-L1 and G37R-L2 reactions was far lower than that of WT-L1 mixed with G37R-L2 creating an opportunity to screen for compounds that increase luciferase activity by stabilizing the G37R homodimer. We were encouraged towards this screen by the observation that cysteine robustly enhance luciferase activity from G37R-L1/L2 SLCA reactions. We successfully applied STD to a subset of 310 FDA-approved drugs, generating all possible 47,895 pairs for testing in the G37R homodimer screen. Coverage of all possible pairs was achieved by applying the shifted-transversal design (**STD, Figure 11**) (7), a mathematical algorithm uniquely specifying members for 10-pools containing 45 pairs per well. Drug selections represented broad structural classes in a largely non-redundant manner from a set of ~1,000 FDA-approved drugs. Drugs were proportionally diluted according to their published maximum tolerated doses prescribe clinically. For example, potent drugs having poor therapeutic indices were diluted accordingly before pooling. In this way, we enhance the likelihood for discover pharmacologically relevant drug synergies. To consider a combination drug for repositioning, the components must have a reasonable therapeutic index, particularly for chronic administration to patients. This excludes many drug classes, such as cancer chemotherapeutics and narcotics.

We prepared the first STD-combination set with the aid of the automated pipetting of a Packard Multiprobe® workstation. This allowed validating fundamental operations with dye samples to ensure proper execution of source-to-destination transfers, and to examine the pipettor log-files. The procedure required several days of largely a 'hands-off' automated operation. An audit-log of pipettor movements from sources to destinations was saved. An Excel workbook was constructed with the aid of V-Basic and MS-Access macros to automatically de-convolute screening data generated from the STD library. The workbook has capability to determine both the single compounds and compound pairs coincident in wells that satisfy any specified score interval or simple threshold.

Score Source:	G37Rhomoassay normed STDscores.xlsm			1253.196	SD All CMPDS
Average Scores:				-1838.099	Avg. ALL CMPDS
drug	M1	M2	M3	Grand Avg:	# SD's
D234	-1443.21	-1386.49	-1367.210083	-1398.972	0.4
D074	-1457.45	-1507.64	-1535.087172	-1500.060	0.3
D221	-1373.91	-1490.14	-1393.727899	-1419.258	0.3
D001	-1744.67	-1696.07	-1504.485571	-1648.407	0.2
D006	-1665.25	-1773.04	-1487.720915	-1642.004	0.2
D013	-1646.48	-1673.94	-1626.379653	-1648.934	0.2
D015	-1554.29	-1775.14	-1486.411045	-1605.281	0.2
D018	-1619.36	-1631.47	-1697.825747	-1649.551	0.2
D022	-1459.53	-1656.4	-1778.128955	-1631.352	0.2
D025	-1528.57	-1492.53	-1908.984171	-1643.359	0.2
D027	-1523.98	-1580.27	-1745.522889	-1616.592	0.2
D043	-1662.29	-1598.05	-1608.417313	-1622.920	0.2
D053	-1483.56	-1620.51	-1718.83013	-1607.632	0.2
D054	-1616.15	-1526.67	-1770.384452	-1637.736	0.2

Figure 16: **No individual drug was active in G37R homodimer screen**. The best of 14 highest-scoring drugs deviated only 0.4 SD units from the average activity for all compounds in the entire screen, which is not a statistically significant effect.

Number	Drug	Pair Score:	pair	# SD's
D009	AMLODIPINE BESYLATE			
D018	LOPERAMIDE HYDROCHLORIDE	293	D059,D119	1.7
D026	MEPHENTERMINE SULFATE	210	D038,D234	1.6
D038	ERYTHROMYCIN ETHYLSUCCINATE	188	D119,D277	1.6
D059	AZELASTINE HYDROCHLORIDE	188	D222,D263	1.6
D062	AMIKACIN SULFATE	103	D009,D269	1.5
D070	RAMIPRIL	100	D074,D113	1.5
D073	ROPINIROLE	84	D153,D215	1.5
D074	KETOCONAZOLE	75	D038,D159	1.5
D088	PHENOXYBENZAMINE HYDROCHLORIDE	73	D104,D235	1.5
D098	ATROPINE SULFATE	65	D026,D070	1.5
D104	SULFADIMETHOXINE	63	D018,D119	1.5
D113	ESTROPIPATE	46	D207,D301	1.5
D119	ZOLPIDEM	46	D196,D288	1.5
D133	ESZOPICLONE	37	D167,D242	1.5
D153	MELATONIN	26	D098,D225	1.5
D157	MECAMYLAMINE HYDROCHLORIDE	24	D088,D234	1.5
D159	METHOXSALEN	12	D133,D157	1.5
D167	PROCAINAMIDE HYDROCHLORIDE	11	D073,D207	1.5
D196	ROSIGLITAZONE	8	D062,D234	1.5
D207	CHLORAMPHENICOL HEMISUCCINATE			
D215	ETHINYL ESTRADIOL			
D222	TRIHEXYPHENIDYL HYDROCHLORIDE			
D225	PRACTOLOL			
D234	FLURBIPROFEN			
D235	PROCHLORPERAZINE EDISYLATE			
D242	DYPHYLLINE			
D263	BEKANAMYCIN SULFATE			
D269	MIGLITOL			
D277	NORFLOXACIN			
D288	KETOTIFEN FUMARATE			
D301	OUABAIN			

Figure 17: Summary of highest paired-drug scores. As combination-pairs, their deviation from the average activity for the entire screen was more apparent at up to 1.7 S.D. units from the mean. However, their activity scores were very low and not statistically significant.

Further analysis of the scores on a per-drug basis reveals that the 14 best average scores obtained from all 111 pools where a drug appears reach only about 0.4 SD units over the overall average (**Figure 16**), well below any reasonable threshold for further consideration. Thus, in devonvoluting the pooled data, no individual compound yielded significant light enhancement. This is not surprising, given as we screened these compounds as singles (data not shown) and could detect no light enhancement to signify G37R homodimer stabilization. We did detect somewhat better enhancer-activity from pools containing specific combinations, rather than for single compounds alone. This is an encouraging finding at this early stage. However, the top scoring pairs at best reach about 1.7 standard deviations above the overall average, not quite meeting the accepted significance threshold of 2 SD's. The top 19 pairs are listed in **Figure 27**, with a key to the 32 comprised drugs. As a 'reality check', the three best scoring pools from each plate (30 pools) were selected and queried for coincident drug pairs amongst them. Five of these 19 best pairs were instantly discovered using this simple method, giving reassurance that the workbook algorithms are working (actual analysis is not shown).

Summary of Key Research Accomplishments (Milestones)

1. We demonstrated that the luciferase activity in our SLCA reaction is generated predominately from dimers, and not high MW multimers or aggregates.
2. The SOD1-SLCA was sensitive to competitive inhibition by native (purified) hWT-SOD1 protein.
    - The data support that the SLCA reaction proceeds by protein subunit exchanges between dimeric isoforms of SOD1 that appear specific and saturable, probably occurring at the dimer binding interface.
3. We succeeded in optimizing and formating the SOD1-SLCA in the 384-well microplate suitable for HTS.
4. We developed a A4V-SLCA and it is ready for HTS.

5. By applying a variant of orthogonal pooled screening (OPS) called the shifted transversal design (STD), we were able to successfully prepare and screen a binary-pair, combinatorial library with 310 FDA-approved drugs, representing all possible ~48,000 combination pairs—in triplicate—contained within ten 384-well microplates.
    - This represents ~12-fold compression, offering significantly enhanced efficiency for new leads discovery.
6. We screened the first run of STD-OPS using a G37R homodimer for the SLCA. The assay performed very well based on metrics commonly used by HTS scientists; however, no combination-hits were found.

OPPORTUNITIES FOR TRAINING AND PROFESSIONAL DEVELOPPEMENT

Nothing to report.

DESSEMINATION OF RESULTS TO COMMUNITIES OF INTEREST

Nothing to report (manuscript in preparation).

PLANS FOR NEXT REPORTING PERIOD

The 'combinatorial box' we prepared will be tested with the G37R-WTSOD1 heterodimer and the A4V-A4V homodimer, and possibly the A4V-WTSOD1 heterodimer, in the SLCA formats we have established and validated. We will prepare other STD combinatorial sets, and also include mixing of ~160 natural products with proven bioactivities and that are ostensibly safe for pharmaceutical applications. We intend to combine them with ~160 generic drugs. Given the greater chemical diversity inherent in natural products (NP), the drug-NP combinations will interrogate broader swaths of combinatorial and pharmacological space. We also will investigate a conditional-HTS paradigm, also called 'synthetic lethal', by running the STD set in the presence and absence of a low concentration of cysteine to determine if we can discover 'synergins' that enhance the effect of a sub-maximal concentration of cysteine. The mechanism of action for LCS-1.34 will be determined by measuring the enzymatic activity of SOD1 in the presence and absence of the compound, in addition to the steps we delineated in our original application including testing whether the LCS-1.34 compound can inhibit the aggregation of mutant SOD1.

4. IMPACT

IMPACT ON THE DEVELOPMENT OF PRINCIPAL DISCIPLINE(S) OF THE PROJECT

The SOD1-SLC assays we have developed appear to recapitulate physiologically-relevant properties of SOD1 functional biology that some have proposed play important roles in ALS neuropathogenesis, particularly heterodimer formation between WT and mutant SOD1 isoforms. We show for the first time that the interactions between mutant and WT SOD1 isoforms are specific and that WT and mutant SOD1 dimers readily exchange subunits. We also now have evidence that AV4 mutant of SOD1 can readily heterodimerize with WT monomers. *To our knowledge, this is the first demonstration of heterodimerization between the A4V mutant and WT SOD1 proteins*. The formation of heterodimeric WT mutant SOD1 is hypothesized to account for the apparently toxic synergy between mutant and WT SOD1 toxicity; in that co-expression of WT SOD1 generally accelerates disease caused by mutant SOD1. Our data suggest a potential mechanism by which a misfolded conformation in mutant SOD1 might be propagated to WT SOD1 through repeated exchanges of SOD1 subunits between dimers.

We have observed profound enhancement of luciferase activity by the reducing agents DTT and cysteine. *We believe that the basis for the profound increases in SOD1 apparent dimerization in response to reducing agents involves SH-reduction, potentially involving the cysteine at position 111 of SOD1; and that based on findings from others, the MOA could involve GSH removal from oxidized SOD1.* Experimental and computational results from other investigators demonstrated that Cys-111 glutathionylation induces structural rearrangements that modulate stability of both wild type and fALS mutant SOD1. McAlary et al (8) showed that glutathionylation potentiates benign SOD1 variants to toxic forms associated with amyotrophic lateral sclerosis. Redler et al (9) reported that glutathionylation results in profound destabilization of SOD1 dimers resulting in monomer accumulation. They showed the marked rightward shift in K_D value for SOD1-GSH was due to decreases in the SOD1 association rate, which was corrected by treatment with DTT to remove the glutathione moiety. The explanation for the observed destabilization caused by post-translational SOD1 glutathionylation is due to steric hindrance by the C111-GSH modification at cysteine-111, which is closely situated ventral to the dimer interface. Others have confirmed the finding that glutathionylation promotes monomer formation by slowing the formation rate of SOD1 dimers isolated from human erythrocytes (10). Monomer of mutant SOD1, particularly in the oxidized (GSH) form, may be important contributors to pathologic aggregate formation.

IMPACT ON OTHER DISCIPLINES

Nothing to Report.

IMPACT ON TECHNOLOGY TRANSFER

Nothing to Report.

IMPACT ON SOCIETY BEYOND SCIENCE AND TECHNOLOGY

Nothing to Report

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5. CHANGES/PROBLEMS

CHANGES IN APPROACH AND REASONS FOR CHANGE

In our original proposal we had planned to focus our HTS effort on SLCA reactions involving mixtures of WT-gLuc and G37R-gLuc (or A4V-gLuc). In this mix of WT and mutant SOD1, we were expecting hits to decrease luciferase activity by stabilizing the dimers of each fusion protein – inhibiting the exchange of subunits. The known presence of inhibitors of native gLuc in our compound library was a potential issue that we were aware of and planned to deal with by counter screen. As noted above, because of some advantages in regard to false hit rates, our first screen used mixtures of G37R-gLuc1 and G37R-gLuc2. We determined that this screen could be easier to run because there would be few false-hits to follow up. Unfortunately, our screen of the G37R-gLuc1 and G37R-gLuc2 SLCA reactions produced no hits. We are now in the process of preparing new batches of cell lysates from WT-gLuc1 and G37R-gLuc2 to run the HTS for this heterodimer pairing. We will also prepare cell lysates for an HTS with WT-gLuc1 and A4V-gLuc2. If time and funds permit, we will run an HTS for A4V-gLuc1 and A4V-gLuc2.

Actual or anticipated problems for delays and actions or plans to resolve them.

Nothing to report

Changes that had significant impact on expenditures

Nothing to report.

Changes in human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

6. PRODUCTS

Nothing to report (manuscript in preparation).

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

INDIVIDUALS THAT HAVE WORKED ON THE PROJECT

UNIVERSITY OF FLORIDA

Name: David R. Borchelt
Project Role: PI
Researcher Identifier
Nearest person month worked: 1 cal mo
Contribution to Project: Oversight of the project. Interpretation of research outcomes. Communication with subaward PI, Dr. Reichman.
Funding Support:

Name: Hilda Brown
Project Role: Research Manager
Researcher Identifier
Nearest person month worked: 10 cal mo
Contribution to Project: Ms. Brown was responsible for all of the benchwork performed at the University of Florida. She generated and validated the new A4V-gLuc constructs. She performed the SEC experiments and cross-linking experiments described in SA1ia. She generated the cell lysates to be shipped to Dr. Reichman at the Lankenau for optimization studies and the initial HTS screen.
Funding Support:

CHANGES IN ACTIVE SUPPORT

No change in effort commitment to the present project is planned.

NEW GRANTS FUNDED IN THE AWARD PERIOD

ALSA8210 (Golde, PI) 07/01/2015 – 06/30/2016 0.24 cal mo
ALS Association $80,000 total direct costs
"Exploring the positive effect of rAAV-IL-10 in an ALS mouse model"
This study seeks to determine the mechanisms by which over-expression of IL-10 delays the onset of MND in the G93A mouse model of ALS, while also seeking to augment the efficacy of this treatment.
Role: Investigator

ALSA8252 (Chakrabarty, PI) 07/01/2015 – 06/30/2016 0.3 cal mo
ALS Association $80,000 total direct costs
"Chemokine based immunobiotherapeutics in ALS "
This study will determine whether the beneficial effects of IL-10 in the G93A mouse model of ALS can be augmented by co-manipulation of chemokine pathways.
Role: Co-Investigator

1R01NS092788-01 (Borchelt, PI) 07/01/2015-06/30/2020 2.4 cal. mo.
NIH/NINDS $218,750 direct/yr
"Modeling the progression of SOD1-linked motor neuron disease"
The symptoms of amyotrophic lateral sclerosis (ALS) seem to spread along neuroanatomical pathways to engulf the motor nervous system, and the rate at which symptoms spread dictates how long patients live. In preliminary studies, we have provocative evidence that spinal cords of mice that model ALS caused by mutations in superoxide dismutase 1 (SOD1) contain entities that can mediate "transmission" of motor neuron disease to genetically vulnerable mice. Our planned studies will further develop these models and define the

general transmissibility of SOD1-linked motor neuron disease. We will establish models in which disease can be focally induced by injection of misfolded SOD1 (in tissue homogenates) into the sciatic nerve. We will use these models to investigate mechanisms of cell to cell spread of misfolded SOD1.

No Number (Ayers/Borchelt, MPI) 06/01/2015-05/31/2017 No salary
The Robert Packard Center for ALS Research at Johns Hopkins $60,000 total direct costs (no cost ext)
"Role of misfolded wild-type SOD1 in cases of sporadic ALS"

This study will investigate whether spinal tissues from sporadic ALS patients may contain entities that can "transmit" or induce SOD1 misfolding to mice that are genetically vulnerable (expressing low levels of a mutant SOD1 fused to yellow fluorescent protein).

1P50 AG047266-01A1 (Golde, PI) 08/01/2015-07/31/2020 1.2 cal mo
University of Florida – Mt Sinai Medical Center AD Research Center
ADRC-Project 3 (Borchelt, PI) $119,282 direct costs/yr

The goals of project 3 are to 1) Develop robust mouse models of mixed AD inclusion pathologies in which one or more elements of the pathology spreads from the periphery into the CNS or spreads between substructures; and 2) Develop recombinant antibody therapies that can target multiple misfolded proteins that spread into or within the CNS.

GRANTS THAT ENDED IN THE AWARD PERIOD

Packard Center for ALS (Borchelt-PI) 09/01/2012-02/28/2015
Johns Hopkins University "Propagation of toxic SOD1 conformations in ALS"

P01 NS049134-10 (Valentine - PI) NIH/NINDS 08/01/2010-04/30/2015
NIH/NINDS "Molecular Mechanisms of SOD1-linked ALS"

5Az04 Ed and Ethel Moore Alzheimer's Disease Research Program (Lewis, PI) 01/15/2015-12/31/2015
"Developing biotherapies for Alzheimer's Disease"

5AZ05 Ed and Ethel Moore Alzheimer's Disease Research Program (Dore, PI) 01/15/2015-12/31/2015
"Alternative to COX-2 inhibitors in AD: Therapeutic potential of PGE2 EP1 receptor selective antagonist."

5R21NS083006-02 (D. Borchelt, J. Lewis - MPI) NIH/NINDS 09/01/2013-12/31/2015
"Proteostasis in Neurodegenerative Disease"

PARTNER ORGANIZATIONS

Organization Name: **Lankenau Insitute for Medical Research**
Location of Organization: **Wynnewood, PA**
Partners contribution to the proejct: This project is a collaboration between Dr. David R. Borchelt (University of Florida) and Dr. Melvin Reichman (Lankenau Institute). Dr. Reichman's laboratory staff have participated in the project, generating some of the data relevant to Specific Aim 1 and all of the data relevant to Specific Aim 2.

8. SPECIAL REPORTING REQUIREMENTS:

A separate report of the activities of Dr. Reichman's laboraotry will be provided.

9. APPENDICES: None.

AD_____
http://mrmc.amedd.army.mil/index.cfm?pageid=researcher_resources.technical_reporting

AWARD NUMBER: W81XWH-15-1-0128

TITLE: Inhibitors of SOD1 interaction as an approach to slow the progressive spread of ALS symptoms

SUBAWARD PRINCIPAL INVESTIGATOR: Melvin Reichman

RECIPIENT: Lankenau Institute for Medical Research

REPORT DATE: 07/13/2016

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

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14. ABSTRACT **Purpose: 1)** Develop superoxide dismutase-1 (SOD1) split-luciferase complementation assays (SLCA) for high throughput screening (HTS). **2)** Apply SOD1-SLCA with novel multiplexed-HTS to discover combination-drug leads. **Scope**: Our ultimate objective is to discover drugs that kinetically stabilize mutant SOD1 isoforms implicated in ALS. **Results**: We successfully optimized SLCA in the 384-well format for HTS of mutant SOD1 dimer isoforms, including mutant G37R and A4V homodimers, as well as heterodimers of G37R and A4V with WT-SOD1. We applied a shifted transversal design to prepare ~48,000, binary-combinations from a set of 310 FDA-approved drugs, compressed into less than ten 384-well plates (>10-fold efficiency). We tested this first combination drug set in the G37R homodimer SLCA, but did not find an active combination-drug. **Significance**: While we have not yet found a drug development candidate, we showed that SOD1 heterodimers readily form and that the redox state of SOD1 impacts its kinetic behavior. We will expand the scope of HTS to new combinatorial libraries and additional mutant-SOD1 homodimer and heterodimer isoforms to achieve our ultimate objective.

15. SUBJECT TERMS
Amyotrophic Lateral Sclerosis (ALS); Superoxide Dismutase-1 (SOD1); Drug Discovery

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1. Introduction

Mutations in the human copper-zinc superoxide-1 dismutase (SOD1) gene are established as a prevalent cause of the familial form of ALS (fALS). Dissociation of the mutant-harboring SOD1 dimer is believed to be the rate-limiting step in the subsequent processes that lead to neuropathology. In the present study, we proposed to develop and validate novel HTS assays having the capacity to detect small molecules that modulate multiple aspects of SOD1 protein-protein interactions (PPI). Our hypothesis is drugs that kinetically stabilize the SOD1 dimer—either by increasing monomer subunit association or decreasing dimer dissociation—will arrest the downstream cascade of cellular events that cause mutant-SOD1 aggregation, and ultimately motor neuron toxicity. Our first Aim is to develop, characterize and optimize a high throughput screening (HTS) assay to discover drugs that stabilize SOD1 tertiary structure. To accomplish this goal, we developed a split-luciferase complementation assay (SLCA) using Gaussia luciferase (Gluc) fused to wild-type (WT) and/or mutant SOD1 (Fig. 1). In our implementation of the HTS assay, the two portions of Gluc are fused to either WT or mutant SOD1 and these fusion proteins are expressed separately in CHO cells. The cell lysates are mixed and incubated to allow any homodimers in the lysate to dissociate to monomers, which then re-associate over time, bringing the two Gluc fragments into proximity to reconstitute their activity. Studies in our first Aim were to better characterize this assay and optimize it for 384 wells. Our second Aim, was to apply a highly innovative, multiplexed drug discovery approach using chemotypes intelligently-selected from a set of FDA-approved drugs to potentially discover allosteric, combination modulators of SOD1 interactions. Based on the design of the assay, we envisioned that we could identify two types of molecules that might be useful; those that stablize dimeric structure and those that block dimer formation. Successfully achieving both of our Aims over the course of the two-year program has the potential to discover combination drugs that allosterically modulate SOD1 protein interactions supra-additively and potentially have a fast-track development path to clinical trials.

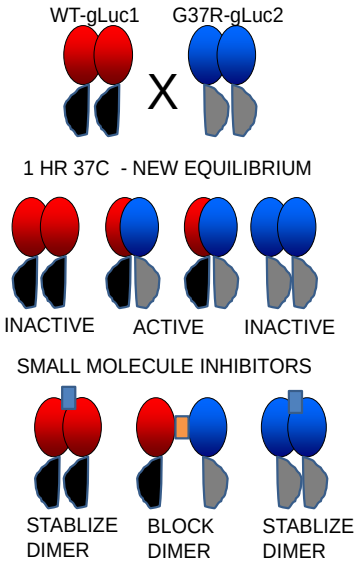


Fig. 1: Possible SOD1 subunit exchange MOA. *The top portion of each chimera* represents an SOD1 isoform, the bottom part is a *Gluc* 'tail'. See text for details.

2. KEYWORDS AND ABBREVIATIONS

α-synuclein (aSyn or aS)
bovine serum albumin (BSA)
cysteine (cys)
dithiothreitol (DTT)
disuccinimidyl suberate (DSS)
familial amyotrophic lateral sclerosis (fALS)
Gaussia luciferase (Gluc)
glutahione (GSH)
high throughput screening (HTS)
Lankenau Chemical Genomics Center (LCGC)
mechanism of action (MOA)
natural products (NP)
orthogonal pooled screening (OPS)
protein interactions (PPI)

relative luminesence units (RLU)
shifted transversal (pooled) design (STD)
size-exclusion chromatography (SEC)
sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)
split-luciferase complementation assay (SLCA)
superoxide-1 dismutase (SOD1)

3. ACCOMPLISHMENTS

MAJOR GOALS OF THE PROJECT

SA1: Develop an innovative high-throughput approach for characterizing SOD1 protein-protein interactions (PPI); demonstrate the idea can be translated for a new way to discover drugs that kinetically stabilize the SOD1 heterodimer (Figure 1).

SA1: Statement of Work (SOW) Milestones

i. Determine that the SOD1 split-luciferase, complementation assay ("**SLCA**") reaction that will be used in drug screening reports the formation of dimers (as depicted by **Fig. 1**), rather than non-specific aggregate formation.
   a. Use size-exclusion and gel electrophoresis chromatography to show that the molecules producing luciferase activity in the SLCA assay have sizes expected for dimers of the fusion proteins and not an aggregate.
   b. Competition experiments will be used for both assay characterization and determination of drug mode of action (MOA). At equilibrium, excess native SOD1 should attenuate Gluc activity when added to the SLCA mixture undergoing dimer exchange.

ii. Optimize SOD1-SLCA using luciferase activity measurements. Determine IC_{50} values for confirmed hits and MOA for compounds detected by SLCA.

iii. Generate SLCA constructs for A4V-SOD1:Gluc1 and A4V-SOD1:Gluc2, validate the assay and apply this assay to our HTS screening effort.

SA2: **Apply an innovative, multiplexed drug discovery approach using selected chemotypes from diverse chemical libraries, *as well as intelligently-selected, binary combinations of FDA-approved drugs,* to *potentially discover allosteric, combination modulators of hSOD1 interactions***.

SA2: SOW Milestones

i. Apply shifted-transversal design to prepare an orthogonal-pooled library containing 310 FDA-approved drugs as ~48,000 binary pairs within ~3,000 combination wells. Add known single-actives together in positive-control wells to demonstrate data-deconvolution works.

ii. Screen combinatorial library in A4V mutant homodimer and WT-G37R heterodimer SOD1 subunit exchange SLCA and confirm activities of active pairs.

iii. Similarity-searching and testing of active leads. Characterize MOA for combination-actives.

iv. Demonstrate cellular activity for hits detected from combinatorial screening. Implement fallback strategy of applying pooled screening of common chemical libraries, if required.

Accomplishments towards Specific Aim 1

SA1 ia) Confirmation that luciferase activity in the SLCA assay is primarily generated by dimers formed by the SOD1-Gluc proteins (conducted at the University of Florida)

Lysates from CHO cells individually expressing SOD1 (WT or mutant G37R variant) fused to the split, complementary halves of Gluc (designated L1 or L2) were mixed and incubated for 1 hr at 37°C, the reaction mix was subjected to size-exclusion chromatography (SEC). The elution profile of the SEC column was characterized by passing purified preparations of known proteins through the column to determine the fraction number in which these proteins eluted. We then fractionated the SLCA assays and aliquots of each were transferred to microtiter plates and measured for Gluc activity with coelenterazine. As shown by **Fig. 2**, the peak of luciferase activity for mixtures of WT-L1 and WT-L2 eluted in fractions that we estimate correspond to proteins of approximately ~52 kD, which is a close approximation to what would be expected for a dimer of WT-SOD1-Gluc1 and WT-SOD1-Gluc2 (SOD1 dimer = ~32 kD; Gluc = ~19 kD; total expected for SOD1-Gluc = 51 kD). Heterodimers WT-L1 with mutant G37R-L2 eluted at a similar range. We also examined the migration of heterodimers between WT-L1 and an experimental variant of SOD1, C111S, which we has been reported to be less sensitive to oxidative modification and oligomerization (1). Heterodimers of WT-L1 and C111S-L2 migrated to a size similar to WT-L1 and G37R-L2 (**Fig. 2**). Although the apparent MW of these molecules was somewhat larger than expected, the migration of proteins in SEC is influenced by both the shape and size of the molecule and the C111S and G37R heterodimers with WT SOD1 may have a conformational difference from WT homodimers that influences the migration. Virtually no Gluc activity was observed in fractions with an apparent MW of 102 kD or above, which would be expected for a tetramer or higher order oligomers. Overall the data are consistent with the outcome we expected; namely, that the luciferase activity generated in the *ex vivo* SLCA assay is the result of SOD1-dimerization.

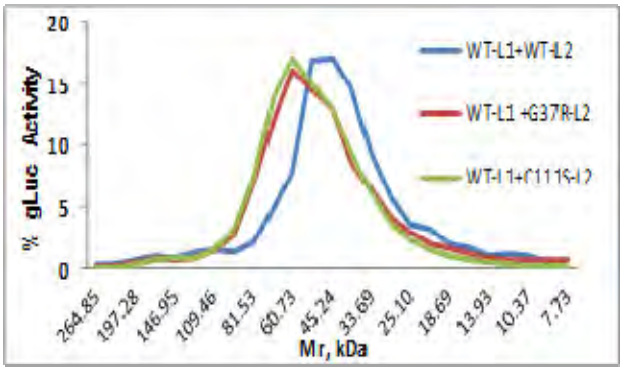


Fig. 2: Luciferase activity separated by size exclusion chromatography. Mixtures of cell lysates were incubated for 1 hr, and then subjected to Sephadex size exclusion chromatography. Each fraction collected was analyzed for *Gaussia* luciferase activity. The graph plots Gluc activity as a percentage of total activity with X-axis indicating the predicted Mr for each fraction collected. The peak luciferase activity migrates very close to the predicted size of dimeric isoforms, and not oligomers.

Determination that the majority of SOD1-Gluc fusion proteins in the cell lysates is dimeric before mixing in the SLCA assay (conducted at the University of Florida).

To further characterize whether the individual SOD1-Gluc fusion proteins were dimers before mixing in the SLCA assay, we incubated the cell lysates with a crosslinker (1 mM DSS - disuccinimidyl suberate) before preparation for denaturing SDS-PAGE. After probing with an anti-Gluc antibody, clear bands are apparent in the WT-L1 and WT-L2 +DSS lanes, consistent with the size expected for a dimer (arrow in **Fig. 3**), and the dark monomer bands in those lanes are concomitantly fainter. The L1 Gluc fragment

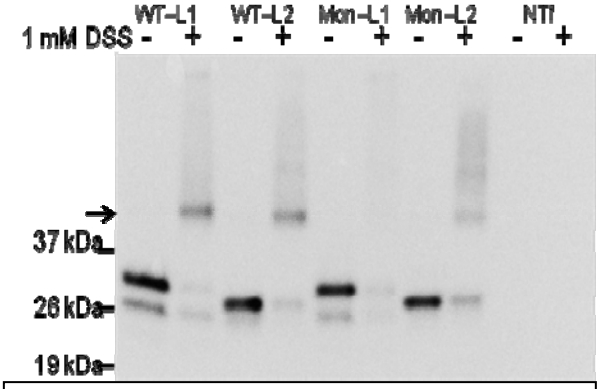


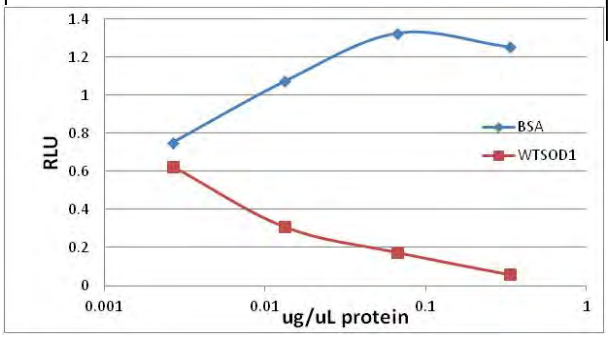
Fig. 3: Immunoblots of cell lysates incubated with and without the cross-linking agent DSS. See text for details.

is larger than the L2 fragment, explaining the slightly differing apparent size of the monomer bands. For controls we made lysates of the Monomeric WT-L1 and L2 constructs (presumed monomer). For the Mon-L1, there is no obvious dimer band. For the Mon-L2, there is some dimer-sized band, but multimers (or higher MW cross-linked species) are also apparent. We interpret the latter observation as evidence that Mon-L2 is not specifically in a stable dimer. Untransfected cell lysates (NTf = non-transfected) contained no antibody reactive bands as expected. The lower band that is apparent in the WT-L1 and Mon-L1 samples is unknown at this time. It appears to be a breakdown product we frequently see but is unidentified. Immunoblots of the cell lysates used in these reactions with antibodies to SOD1 identified a single band (not shown), indicating that the breakdown products may be generated by cleavage within the SOD1 portion of the fusion proteins. These breakdown products would unlikely be able to form a stable dimer and would not contribute to the observed luciferase signal. *These data demonstrate that SOD1-Gluc fusion proteins present in the individual lysates exist predominantly as dimers before they are mixed in the* ex vivo *reaction whereas data in Figure 2 indicate that the source of luciferase activity in the* ex vivo *SLCA assays are also dimers of the SOD1-L1 and -L2 fusion proteins. Taken together, the data are consistent with the interpretation that light formation by Gluc complementation occurs by SOD1 subunit exchange, as we originally hypothesized (depicted in Fig 1).*

SA1 ib) Excess native WT SOD1 inhibits luciferase activity in the SLCA assay (conducted at the Lankenau Institute using cell lysates generated at the University of Florida).

If the observed luciferase activity in our SLCA assays depends on the dissociation of homodimers of each SOD1-Gluc protein after the individual lysates are mixed; and then, their re-association as a heterodimer, bringing the two halves of the Gluc reporter into complementary proximity, thereby reactivating light-generating enzymatic activity (as depicted by **Fig. 1**), then the addition of purified WT SOD1 to these SLCA reactions should act as a negative competitor. **Fig. 4** demonstrates that excess WT SOD1 (commercial source Sigma Inc.) does inhibit luciferase activity in the SLCA assay as hypothesized (RLU are normalized to the control equilibration reaction, i.e., with no added protein). Concentration-dependent inhibition of the reaction by native WT-SOD1 was observed, with virtually complete inhibition occurring at 0.3 µg/µL of native WT-SOD1, or a concentration of 8 µM final. By contrast, we observed that the addition of BSA enhanced the apparent activity of the SLCA reaction. These data indicate that native, purified WTSOD1 added exogenously to the SOD1-SLCA abrogates the Gluc complementation reaction and provides a confirmation that SOD1 dimers can dissociate and freely exchange as first suggested by Dr. Borchelt in 1995 (2).

Fig. 4: Mixed lysates containing WTSOD-L1 and G37R-L2 competing with purified WT SOD1 or BSA (90 min incubation). Readout is Gluc signal normalized to no protein control.



SA1 ii) Optimization and validation of SOD1-SLCA—specific effects of reducing agents and small molecule inhibitors (Conducted at the Lankenau Institute with cell lysates prepared at the University of Florida).

As noted above, we found that the addition of BSA enhances the signal, achieving a maximal effect at ~0.05% (***Fig. 4***). We also found that DTT markedly enhanced the apparent dimerization of the WT-L1 + G37R-L2 heterodimer. The response to DTT was biphasic, with a sharp maximal stimulation

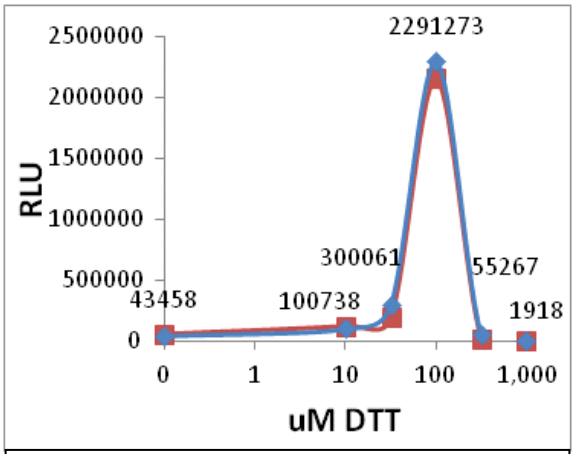


Fig. 5: Low DDT concentrations markedly enhance SOD1 dimer formation. L1-WT and G37R-L2 were incubated with increasing concentrations of DTT for 3.5 hrs. The data shown are two replicates from a single experiment.

observed at 100 µM (**Fig. 5**), which is 10-fold lower than typically is used for preventing the oxidation of free sulfhydral groups. We reasoned that DTT was inhibiting the coelenterazine + Gluc enzyme reaction, but that DDT-enhanced SOD1 dimerization supersedes this inhibitory effect, thus having a net stimulatory effect. We next compared the effect of free cysteine, a milder reducing agent than DTT. We next compared the effect of free cysteine, a milder reducing agent than DTT. The data in ***Fig.* 6** summarize our findings on treatment of the reaction mix with thiol-reducing dithiothreitol (*DTT—green curves*), or cysteine (red curves). The fold-increases we measured for the SOD1-SLCA were normalized to untreated lysates (i.e., control = 1) and plotted on the left Y-axis in log-scale. The right axis shows the fold-effects of the two sulfhydryl reducing agents on native Gluc on at linear scale. At 100 µM, DTT caused a 46-fold enhanced L1/L2 response, whereas it reduced the native Gluc response by ~ 30%. At higher concentrations, DTT inhibited the L1/L2 response. Cysteine at 3 mM produced marked enhancement of SOD1-L1/L2 luciferase activity at ~700 fold. Unlike DTT, cysteine also enhanced the Gluc response by 3-fold at the highest concentration tested. Luciferase activity produced in SLCA reactions containing G37R-L1 mixed with G37R-L2 was also enhanced by the addition of cysteine (~65 fold at 3 mM, data not shown).

We compared the effects of DTT and cysteine in an SLCA developed with α-synuclein (aSyn) fusion proteins, which we intended to use in a counter screen. Our aSyn SLCA used a-Synn to Gluc1

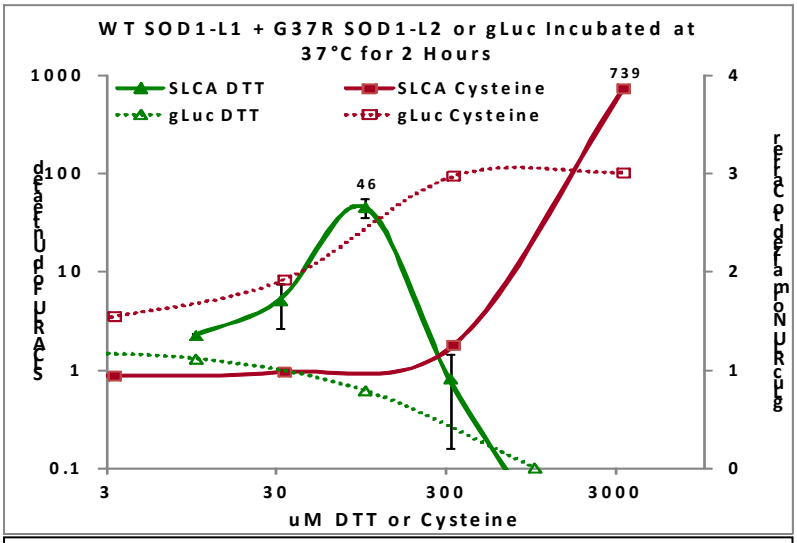


Fig. 6: Thiol reducing agents enhance WT-L1 + G37R-L2 Luciferase activity. Red plots are show the cysteine effect on SOD1-SLCA (solid-red plot, with effect magnitude shown on left Y-axis) or on Gluc (dotted-red plot, fold-effect magnitude shown on right Y-axis). Green plots are show the DDT effect on SOD1-SLCA (solid-green plot, with effect magnitude shown on left Y-axis) or on Gluc (dotted-green plot, with fold-effect magnitude shown on right Y-axis). Data are means of 2-3 replicates from one experiment, which repeatedly gave similar results.

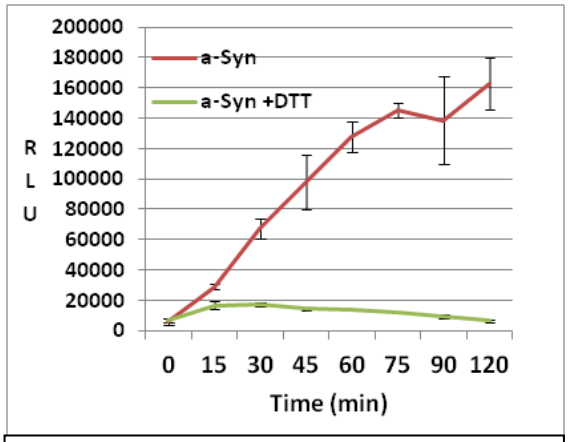


***Fig. 7: 100 µM abrogates a-synuclein (aS) dimer formation*.** Lysates from cells expressing L1-aS or aS-L2 with mixed and incubated with DDT for the indicated times. Data are averages ± SD of triplicates.

and to Gluc2. These proteins are expressed separately and then mixed *ex vivo* in a fashion similar to what we describe above for the SOD1 SLCA assays. Unlike SOD1 having four cysteine groups, the aSyn protein does not contain cysteine. The addition of 100 uM DTT to the aSyn-SLCA inhibited the formation of luciferase activity (**Fig. 7**). Cysteine caused only a very modest increase of 2-3 fold in light output from lysates containing aSyn-L1 or aSyn-L2 fusion proteins. *Thus, we concluded the increased luciferase activity in our SOD1 SLCA assays by sulfhydryl reducing agents is due to specific enhancement in the dimerization of SOD1 isoforms, rather than due to an artifact pertaining to the Gluc-coelenterazine reactions.* These findings offer several options in optimizing these SOD1 SLCA reactions. Luciferase output may be enhanced by the addition of BSA, DTT, or cysteine. Depending upon how we may want to run the screen (see below), the addition of these reagents could be used to enhance activity.

<u>Candidate approach for identifying lead compounds (conducted at the Lankenau Institute with cell lysates prepared at the University of Florida)</u>

Recently, it was reported that 4,5-dichloro-2-m-tolylpyridazin-3(2H)-one (**Fig. 8**) inhibited the enzymatic activity of SOD1 (10). This compound, called LCS-1.34, was found in a phenotypic HTS

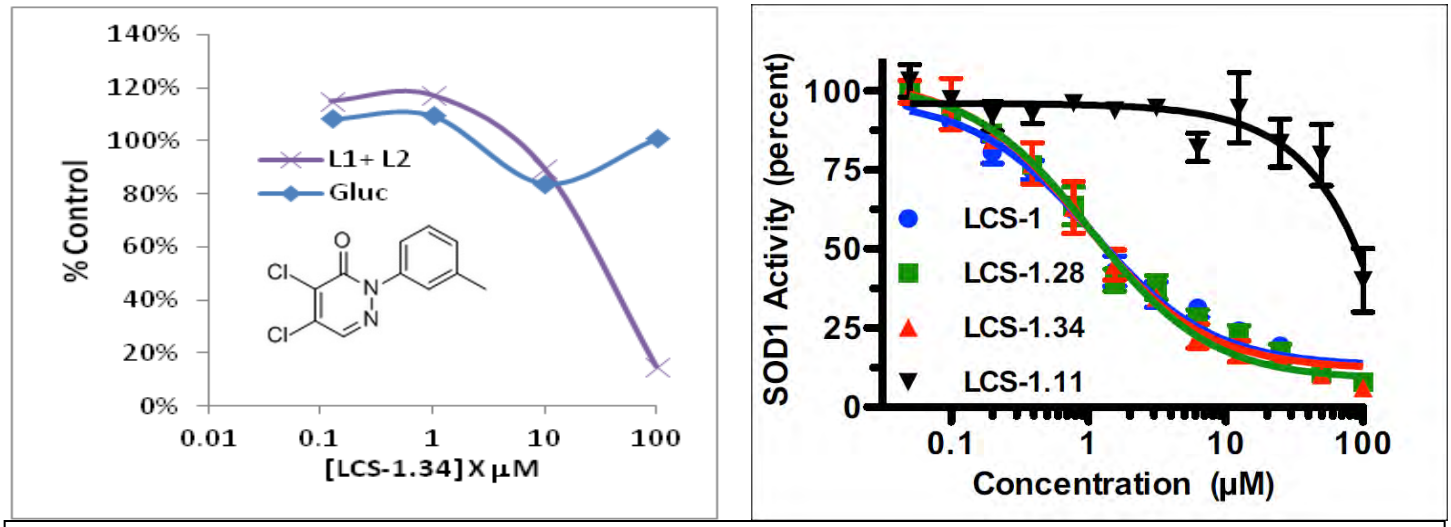


Fig. 8: Effects of LCS-1.34, an inhibitor of SOD1 enzyme activity, on apparent hetero-dimerization of L1-WT/G37R-L2. The compound was published as a strong 'hit' in a phenotypic, cellular lung cancer screen, in which SOD1 inhibitors slow proliferation; the published SOD1 potency for LCS-1.34 is ~3 µM (right panel). We found (left panel) that its IC_{50} for inhibiting SOD1 heterodimerization is ~30 mM. Lysates expressing native Gluc were relatively insensitive to the compound.

assay that slowed the growth of lung cancer cells in vitro. We searched LCGC's internal 250,000 small molecule collection and found the identical structure in our inventory (designated as LCGC-142623). Cell lysates each containing L1WT or G37RL2 were mixed and incubated for 1 hr at 37°C with increasing concentrations of the compound and then measured for light output. We found that the compound reported by Somwar *et al*. (Figure 8, right panel, reproduced from (10) as having an IC_{50} for inhibiting SOD1 of ~3 µM inhibited the SLCA reaction with an apparent IC_{50} of ~30 µM. The compound did not significantly inhibit native Gluc at 100 µM, the highest concentration tested (Figure 8). It remains for us to establish that LCS-1.34's MOA for inhibiting SOD1 enzymatic activity is disruptive to the SOD1 dimer. If so, it might offer a useful molecular tool for further validating that the SOD1-SLCA can detect molecules known to modulate dimer formation.

SA1.iii) Develop A4V-SOD1 SLCA for HTS (conducted at the Lankenau Institute using cell lysates generated at the University of Florida; recombinant DNA work at the Univ. of Florida)

Because the A4V mutation in SOD1 is far more common in North America, we recognized the need to develop an assay that specifically examines this mutant, in case there is mutant to mutant variation and because such patients will likely be the most frequent for any subsequent clinical trials. We succeeded in expressing and testing the reagents for development and optimization of an A4V-SOD1 assay in the SLCA format (**Fig. 9**). Mixtures of A4V-L1 and -A4V-L2 yielded luminenence output ~8-fold above background, whereas /WT-L1 and -A4V-L2 mixtures yielded considerably greater output of light (60-fold). Interestingly, we found the luciferase activity produced by A4V-L1/L2 mixtures was less sensitive competition with purified WTSOD1 (not shown). We do not fully understand what this outcome means. It may mean that source of luciferease from mixtures of A4V-L1 and A4V-L2 are not native-like dimers, but some other type of association.

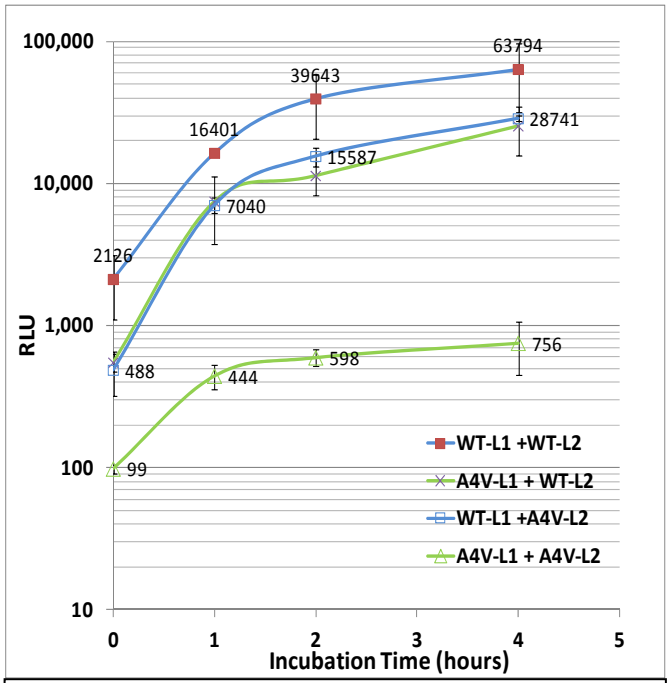


Fig. 9: The A4V SOD1-Mutant forms homodimers, and heterodimers with WT-SOD1.

We next sought to test compounds reported by others to stablize A4V-SOD1 homodimers (3,4). Using computational (virtual) screening and *in vitro* aggregation assays, Ray *et al*. (4) identified potential lead compounds that stabilized the A4V-SOD1 mutant, preventing its dissociation in response to chaotropes, and subsequent aggregation. One of the best such stabilizers was reported as: 4-bromo-2-{(E)-[(4- fluorophenyl) imino]methyl}phenol which we had in LCGC's chemical collection designated as LCGC-37845 (shown as red in **Fig. 10**). We tested this compound, along with analogs similar to other SOD1-stabilizing structures reported by Ray *et al*., in both the A4V (**Fig. 10a left**) and WT (**Fig. 10b right**) SLCA homodimer formats. None of the compounds significantly enhanced luciferase activity. We note, however, that Wright *et al*. (5)

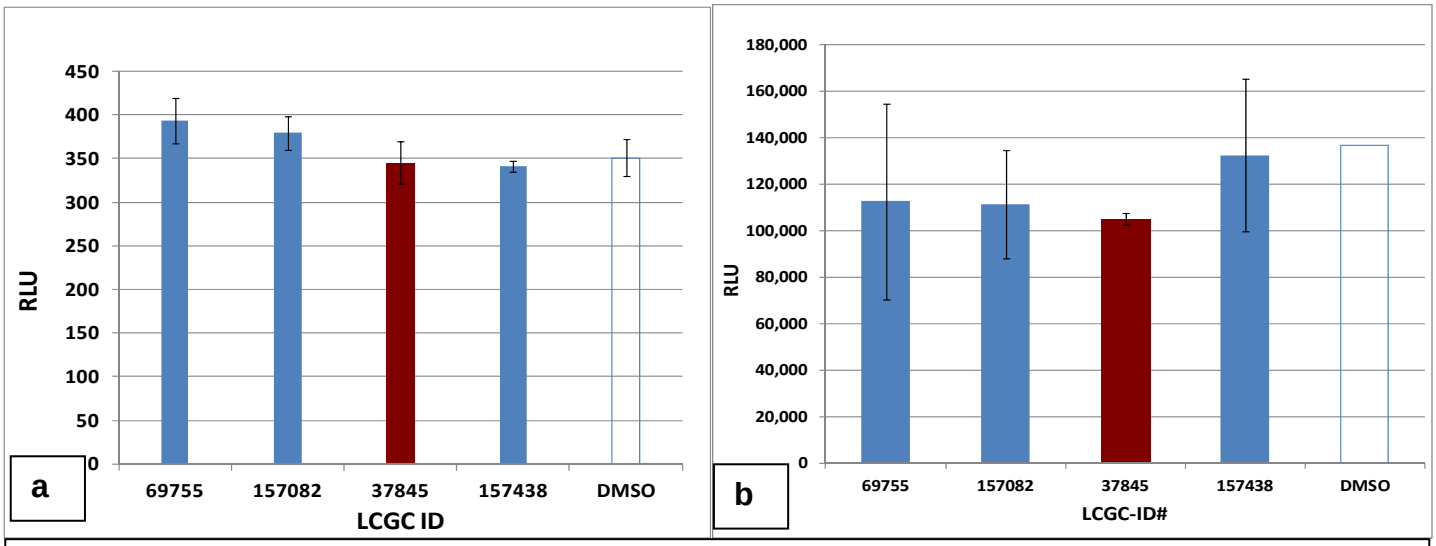


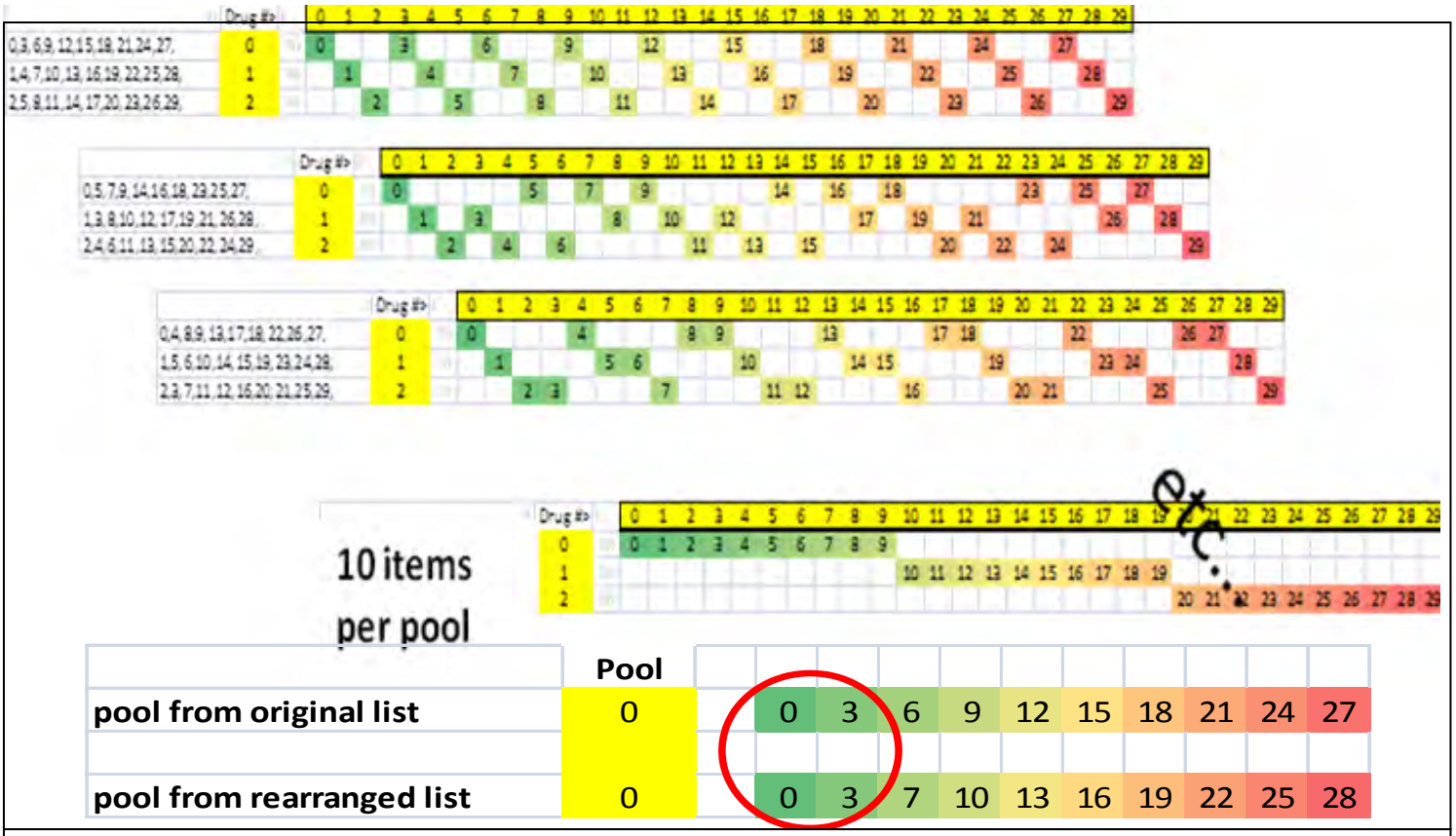
Fig. 10: Effect for compounds reported to stabilize SOD1 in the L1/A4V-A4V/L2 (13a) and L1/WT-WT/L2 (13b) SLCA. Compounds purported to stabilize SOD1 had no effect in stabilizing A4V or WT homodimer isoforms. Data are averages ± SD from triplicate determinations in one experiment that was repeated.

reported that these compounds did not bind at the dimer interface as originally described by Ray *et al*. and thus whether these compounds are genuine stabilizers of A4V SOD1 homodimers is uncertain. Our data provide additional evidence that these compounds may not be useful.

SA2 i) Apply shifted-transversal design to prepare an orthogonal-pooled library containing 310 FDA-approved drugs as ~45,000 binary pairs within ~1,000 combination wells. Add known single-actives together in positive-control wells to demonstrate data-deconvolution works (conducted at the Lankenau Institute using cell lysates generated at the University of Florida).

The highly innovative approach we proposed to use relies on the concept of orthogonal 10-pooling of '*binary-paired-drugs,*' as depicted by **Fig. 11**. The objective of STD is to set the stage to pinpoint specific drug combinations with synergistic activity within arrays of combinatorial mixtures of FDA-approved drugs by their 'uniquely redundant' appearance in 'orthogonal (parallel) wells'. We derived an algorithm to direct the automated pipettor through the steps of creating 48,000 non-redundant pairings in 10-pools prepared from 310 stock-solutions as source positions. Once a given STD-matrix array is created, it can readily be screened in any number of assays. *Compounds that stabilize the G37R mutant homodimer should delay the onset of disease in either of the two transgenic models available in Dr. Borchelt's laboratory; therefore, we selected G37R homodimer for the initial screen.*

Fig. 12 depicts the 384-well plate layout we have adopted for all HTS assays to discover the first SOD1 dimer stabilizers. **Fig. 13** summarizes the QC/QA tracking data captured for every screened plate; shown here is a representative screened plate of STD 10-pooled compounds. For the HTS studies, the final assay volume in PBS buffer was 50 µL, containing 1.0 µL of each lysate, incubated for 3 hours, followed by measurement of Gluc complementation using a Biotek Synergy plate reader, equipped with dual reagent injectors, read in the 'flash' mode. The average fold induction for cysteine on every plate was used to define the maximum response, which was normalized to 100,000 RLU. This corrects for varying response magnitude on different plates within and between screening runs. Each drug appears 111 times within in the 10-pools of a STD library from a given set of 310 drugs. Luciferases are sensitive to non-specific inhibition from organic molecules, including by DMSO (**Fig. 14**). The STD 10-pools generally were slightly inhibitory (~2,000 RLU per 100,000 normalized RLU), representing ~-2% to -5% inhibition, with most ~-2% (**Fig. 15**). In order to provide for orthogonal representation and de-convolution of >99% of all the possible pairs, the STD algorithm was applied thrice; each set of resulting wells is termed a 'matrix.' Each matrix comprises 1,147 pools, thus three matrices nets 3,441 pools, whose screening guarantees (to at least 99%) orthogonal de-convolution of every possible pairing of compounds from amongst the 310 drugs (47,895 possible pairs). *Every drug appears in 37 pools per matrix, thus 111 times overall in the three orthogonal matrices.*



	Pool											
pool from original list	0	0	3	6	9	12	15	18	21	24	27
pool from rearranged list	0	0	3	7	10	13	16	19	22	25	28

Fig. 11: Shifted Transversal Design (STD). STD provides an efficient algorithm for creating pools that contain all possible pairings from amongst n compounds by the formula pairs = n*n/2; so 310 drugs yield ~ 48,000 pairs. The figure depicts the iterative process for the creation of pools containing 10 compounds each from a mini-library of n = 30 compounds (numbered 0 to 29). To form orthogonal pools, the process is repeated with a different ordering of the same compounds. Pair 0/3 is the only common pair shared between a pool from the original list and one from the rearranged list, shared by two pools each containing 45 pairs.

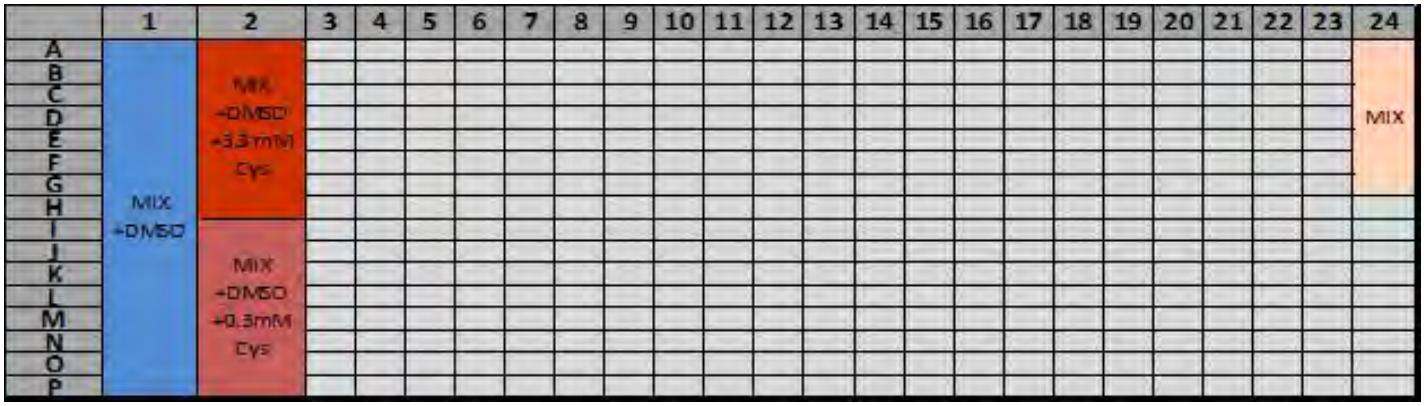


Fig. 12: 384-well Plate Layout for HTS. 'Mix' (n = 8) refers to 1 μL each of G37RL1 + G37R L2 lysates diluted in PBS buffer to a final volume of 50 μL. Similarly, the Blue section is Mix + 1 μL of DMSO (n = 16). The dark and light red sections (n = 8, each) contain cysteine at 3.3 mM or 0.3 mM, serving as the internal standard for the assay. The grey wells are where 1 μL aliquots of compound pools as 'dots' were added in advance as assay-ready plates.

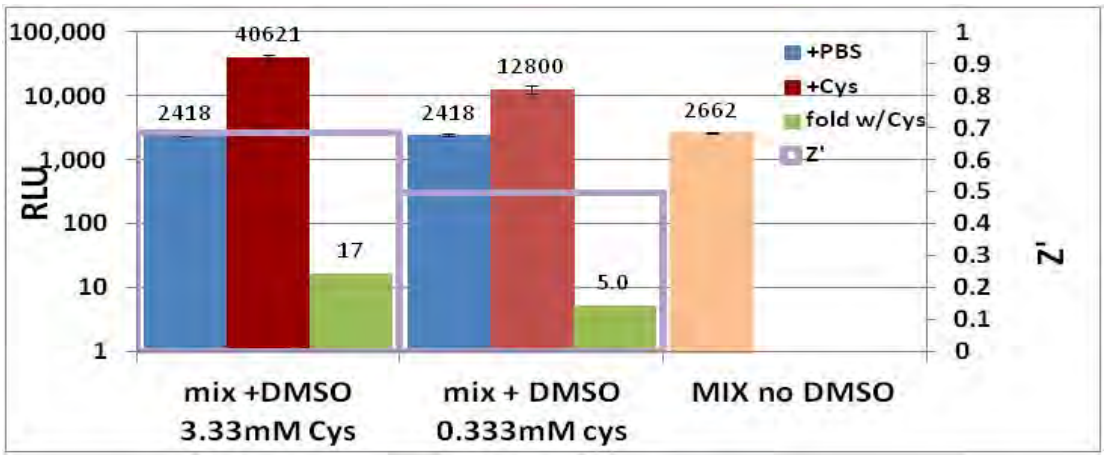


***Fig. 13: Representative QC/QA Metrics for HTS*:** Positive and Negative Controls. The Z'-Factor (right Y-axis) is calculated for each plate using both the values for the induction (left Y-Axis; log-scale) by 3.0 mM and 0.3 mM cysteine (Cys), respectively, and the DMSO-containing wells. The signal window, thus, is defined as ~17-fold (3.3 mM Cys) above a background of ~2400 RLU. By including 'no-DMSO' wells, we can monitor sensitivity of assay to slight inhibition elicted by the solvent.

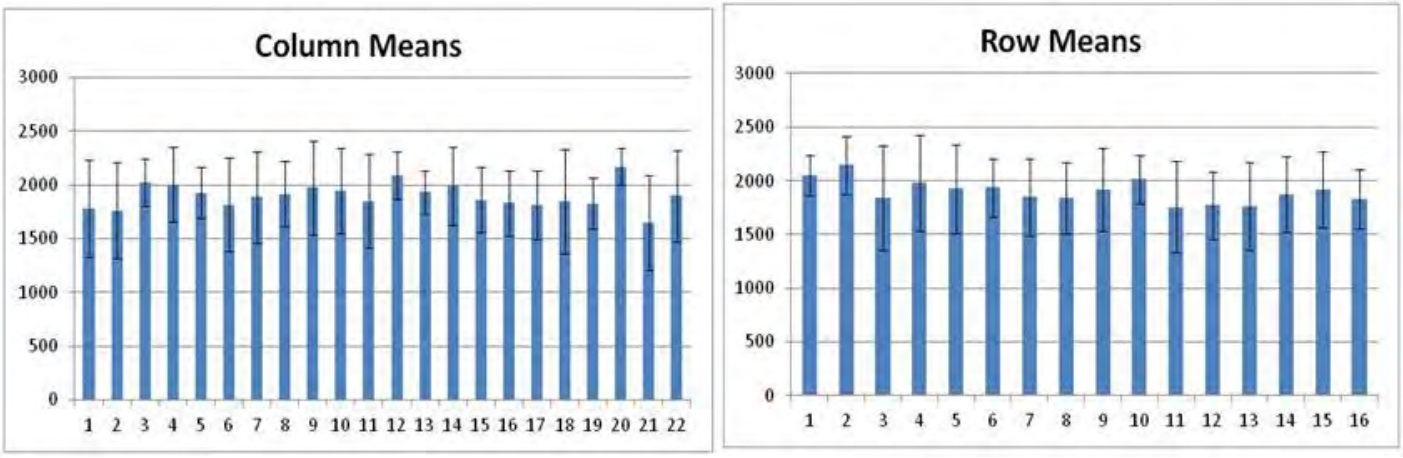


Fig. 14: Representative QC/QA Metrics for HTS: Row and Column Means. The mean ± SD values for each of 16 Rows and each of 22 columns of a representative 384-well plate are shown. The first two columns the control and reference wells and are not shown. The last column includes only 8 wells containing compound (see Fig 19).

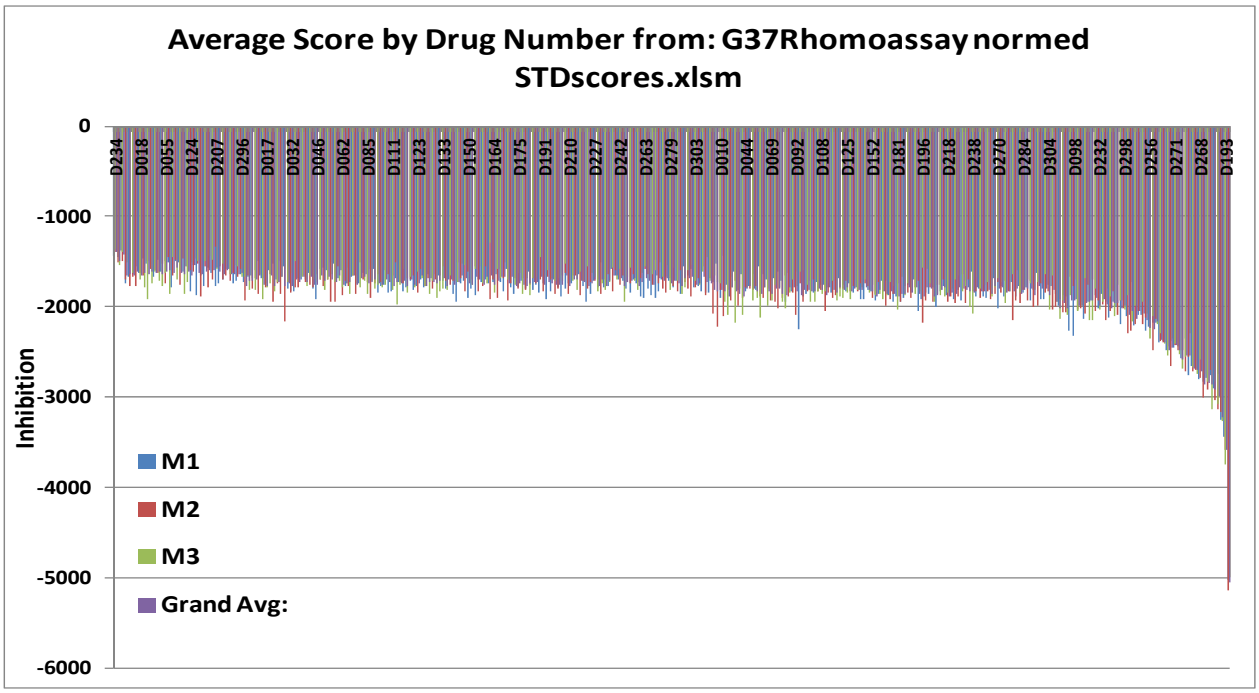


Fig*. 15: Plot of normalized average scores for individual drugs*. The averaged and individual scores of all 310 drugs, each appearing in 111 wells in a total of three orthogonally-pooled matrixes are shown.

SA2 ii. Screen Combinatorial library in A4V mutant homodimer and WT-G37R heterodimer SOD1 subunit exchange SLCA and confirm activities of active pairs (conducted at the Lankenau Institute with cell lysates prepared at the University of Florida).

In a slight departure from what was originally proposed, we decided that our first screen would be in SLCA reactions from homodimeric mixtures of G37R-L1 and G37R-L2. We chose this tact for 2 reasons. First, we had noted from pilot screening the frequency of non-specific Gluc inhibitors, potentially creating a large number of false positives. Second, we noted that the luciferase activity of G37R-L1 and G37R-L2 reactions was far lower than that of WT-L1 mixed with G37R-L2 creating an opportunity to screen for compounds that increase luciferase activity by stabilizing the G37R homodimer. We were encouraged towards this screen by the observation that cysteine robustly enhanced luciferase activity from G37R-L1/L2 SLCA reactions. We successfully applied STD to a subset of 310 FDA-approved drugs, generating all possible 47,895 pairs for testing in the G37R homodimer screen. Coverage of all possible pairs was achieved by applying the shifted-transversal design (**STD, Fig. 11**) (6), a mathematical algorithm uniquely specifying members for 10-pools containing 45 pairs per well. Drug selections represented broad structural classes in a largely non-redundant manner from a set of ~1,000 FDA-approved drugs. Drugs were proportionally diluted according to their published maximum tolerated doses prescribe clinically. For example, potent drugs having poor therapeutic indices were diluted accordingly before pooling. In this way, we enhance the likelihood for discovering pharmacologically relevant drug synergies. To consider a combination drug for repositioning, the components must have a reasonable therapeutic index, particularly for chronic administration to ALS patients. This excludes many drug classes, such as cancer chemotherapeutics and narcotics.

We prepared the first STD-combination set with the aid of the automated pipetting of a Packard Multiprobe® workstation. This allowed validating fundamental operations with dye samples to ensure proper execution of source-to-destination transfers, and to examine the pipetter log-files. The procedure required several days of largely a 'hands-off' automated operation. An audit-log of pipetter movements from sources to destinations was saved. An Excel workbook was constructed with the aid of V-Basic and MS-Access macros to automatically de-convolute screening data generated from the STD library. The workbook has capability to determine both the single compounds and compound pairs coincident in wells that satisfy any specified score interval or simple threshold.

Score Source:	G37Rhomoassay normed STDscores.xlsm			1253.196	SD All CMPDS
Average Scores:				-1838.099	Avg. ALL CMPDS
drug	M1	M2	M3	Grand Avg:	# SD's
D234	-1443.21	-1386.49	-1367.210083	-1398.972	0.4
D074	-1457.45	-1507.64	-1535.087172	-1500.060	0.3
D221	-1373.91	-1490.14	-1393.727899	-1419.258	0.3
D001	-1744.67	-1696.07	-1504.485571	-1648.407	0.2
D006	-1665.25	-1773.04	-1487.720915	-1642.004	0.2
D013	-1646.48	-1673.94	-1626.379653	-1648.934	0.2
D015	-1554.29	-1775.14	-1486.411045	-1605.281	0.2
D018	-1619.36	-1631.47	-1697.825747	-1649.551	0.2
D022	-1459.53	-1656.4	-1778.128955	-1631.352	0.2
D025	-1528.57	-1492.53	-1908.984171	-1643.359	0.2
D027	-1523.98	-1580.27	-1745.522889	-1616.592	0.2
D043	-1662.29	-1598.05	-1608.417313	-1622.920	0.2
D053	-1483.56	-1620.51	-1718.83013	-1607.632	0.2
D054	-1616.15	-1526.67	-1770.384452	-1637.736	0.2

***Fig. 16**: **No individual drug was active in G37R homodimer screen**.* The best of 14 highest-scoring drugs deviated only 0.4 SD units from the average activity for all compounds in the entire screen, which is not a statistically significant effect.

Further analysis of the scores on a per-drug basis reveals that the 14 best average scores obtained from all 111 pools where a drug appears reach only about 0.4 SD units over the overall average (**Fig. 16**), well below any reasonable threshold for further consideration. Thus, in devonvoluting the pooled data, no individual compound yielded significant light enhancement. This is not surprising, given as we screened these compounds as singles (data not shown) and could detect no light enhancement to signify G37R homodimer stabilization. We did detect somewhat better enhancer-activity from pools containing specific combinations, rather than for single compounds alone. This is an encouraging finding at this early stage. However, the top scoring pairs at best reach about 1.7 standard deviations above the overall average, not quite meeting the accepted significance threshold of 2 SD's. The top 19 pairs are listed in **Fig. 17**, with a key to the 32 comprised drugs. As a 'reality check', the three best scoring pools from each plate (30 pools) were selected and queried for coincident drug pairs amongst them. Five of these 19 best pairs were instantly discovered using this simple method, giving reassurance that the workbook algorithms are working (actual analysis is not shown).

Number	Drug
D009	AMLODIPINE BESYLATE
D018	LOPERAMIDE HYDROCHLORIDE
D026	MEPHENTERMINE SULFATE
D038	ERYTHROMYCIN ETHYLSUCCINATE
D059	AZELASTINE HYDROCHLORIDE
D062	AMIKACIN SULFATE
D070	RAMIPRIL
D073	ROPINIROLE
D074	KETOCONAZOLE
D088	PHENOXYBENZAMINE HYDROCHLORIDE
D098	ATROPINE SULFATE
D104	SULFADIMETHOXINE
D113	ESTROPIPATE
D119	ZOLPIDEM
D133	ESZOPICLONE
D153	MELATONIN
D157	MECAMYLAMINE HYDROCHLORIDE
D159	METHOXSALEN
D167	PROCAINAMIDE HYDROCHLORIDE
D196	ROSIGLITAZONE
D207	CHLORAMPHENICOL HEMISUCCINATE
D215	ETHINYL ESTRADIOL
D222	TRIHEXYPHENIDYL HYDROCHLORIDE
D225	PRACTOLOL
D234	FLURBIPROFEN
D235	PROCHLORPERAZINE EDISYLATE
D242	DYPHYLLINE
D263	BEKANAMYCIN SULFATE
D269	MIGLITOL
D277	NORFLOXACIN
D288	KETOTIFEN FUMARATE
D301	OUABAIN

Pair Score:	pair	# SD's
293	D059,D119	1.7
210	D038,D234	1.6
188	D119,D277	1.6
188	D222,D263	1.6
103	D009,D269	1.5
100	D074,D113	1.5
84	D153,D215	1.5
75	D038,D159	1.5
73	D104,D235	1.5
65	D026,D070	1.5
63	D018,D119	1.5
46	D207,D301	1.5
46	D196,D288	1.5
37	D167,D242	1.5
26	D098,D225	1.5
24	D088,D234	1.5
12	D133,D157	1.5
11	D073,D207	1.5
8	D062,D234	1.5

Fig. 17: Summary of highest paired-drug scores. As combination-pairs, their deviation from the average activity for the entire screen was more apparent at up to 1.7 S.D. units from the mean. However, their activity scores were very low and not statistically significant.

Summary of Key Research Accomplishments (Milestones)

1. We demonstrated that the luciferase activity in our SLCA reaction is generated predominately from dimers, and not high MW multimers or aggregates.
2. The SOD1-SLCA was sensitive to competitive inhibition by native (purified) hWT-SOD1 protein.
    - The data support that the SLCA reaction proceeds by protein subunit

exchanges between dimeric isoforms of SOD1 that appear specific and saturable, probably occurring at the dimer binding interface.

3. We succeeded in optimizing and formating the SOD1-SLCA in the 384-well microplate suitable for HTS.
4. We developed a A4V-SLCA and it is ready for HTS.
5. By applying a variant of orthogonal pooled screening (OPS) called the shifted transversal design (STD), we were able to successfully prepare and screen a binary-pair, combinatorial library with 310 FDA-approved drugs, representing all possible ~48,000 combination pairs—in triplicate—contained within ten 384-well microplates.
   - This represents ~12-fold compression, offering significantly enhanced efficiency for new leads discovery.
6. We screened the first run of STD-OPS using a G37R homodimer for the SLCA. The assay performed very well based on metrics commonly used by HTS scientists; however, no combination-hits were found.

OPPORTUNITIES FOR TRAINING AND PROFESSIONAL DEVELOPMENT

Nothing to report.

DISSEMINATION OF RESULTS TO COMMUNITIES OF INTEREST

Nothing to report (manuscript in preparation).

PLANS FOR NEXT REPORTING PERIOD

The 'combinatorial box' we prepared will be tested with the G37R-WTSOD1 heterodimer, as we had originally proposed, and the A4V-A4V homodimer, and possibly the A4V-WTSOD1 heterodimer, in the SLCA formats we have established and validated. We will prepare other STD combinatorial sets, and also include mixing of ~160 natural products with proven bioactivities and that are ostensibly safe for pharmaceutical applications. We intend to combine them with ~160 generic drugs. Given the greater chemical diversity inherent in natural products (NP), the drug-NP combinations will interrogate broader swaths of combinatorial and pharmacological space. We also will investigate a conditional-HTS paradigm, also called 'synthetic lethal', by running the STD set in the presence and absence of a low concentration of cysteine to determine if we can discover 'synergins' that enhance the effect of a sub-maximal concentration of cysteine. The mechanism of action for LCS-1.34 will be determined by measuring the enzymatic activity of SOD1 in the presence and absence of the compound, in addition to the steps we delineated in our original application including testing whether the LCS-1.34 compound can inhibit the aggregation of mutant SOD1.

4. IMPACT

IMPACT ON THE DEVELOPMENT OF PRINCIPAL DISCIPLINE(S) OF THE PROJECT

The SOD1-SLC assays we have developed appear to recapitulate physiologically-relevant properties of SOD1 functional biology that may play important roles in ALS neuropathogenesis, particularly heterodimer formation between WT and mutant SOD1 isoforms. We show for the first time that the interactions between mutant and WT SOD1 isoforms are specific and that WT and mutant SOD1 dimers readily exchange. We also now have evidence that AV4 mutant of SOD1 can readily heterodimerize with WT monomers. *To our knowledge, this is the first demonstration of*

heterodimerization between the A4V mutant and WT SOD1 proteins. The formation of heterodimeric WT mutant SOD1 is hypothesized to account for the apparently toxic synergy between mutant and WT SOD1 toxicity; in that co-expression of WT SOD1 generally accelerates disease caused by mutant SOD1. Our data suggest a potential mechanism by which a misfolded conformation in mutant SOD1 might be propagated to WT SOD1 through repeated exchanges of SOD1 subunits between dimers.

We have observed profound enhancement of luciferase activity by the reducing agents DTT and cysteine. *We believe that the basis for the profound increases in SOD1 apparent dimerization in response to reducing agents involves SH-reduction, potentially involving the cysteine at position 111 of SOD1; and that based on findings from others, the MOA could involve GSH removal from oxidized SOD1.* Experimental and computational results from other investigators demonstrated that Cys-111 glutathionylation induces structural rearrangements that modulate stability of both wild type and fALS mutant SOD1. McAlary *et al*. (7) showed that glutathionylation potentiates benign SOD1 variants to toxic forms associated with amyotrophic lateral sclerosis. Redler *et al*. (8) reported that glutathionylation results in profound destabilization of SOD1 dimers resulting in monomer accumulation. They showed the marked rightward shift in K_D value for SOD1-GSH was due to decreases in the SOD1 association rate, which was corrected by treatment with DTT to remove the glutathione moiety. The explanation for the observed destabilization caused by post-translational SOD1 glutathionylation is due to steric hindrance by the C111-GSH modification at cysteine-111, which is closely situated ventral to the dimer interface. Others have confirmed the finding that glutathionylation promotes monomer formation by slowing the formation rate of SOD1 dimers isolated from human erythrocytes (9). Monomers of mutant SOD1, particularly in the oxidized (GSH) form, may be important contributors to pathologic aggregate formation.

IMPACT ON OTHER DISCIPLINES

Nothing to Report.

IMPACT ON TECHNOLOGY TRANSFER

Nothing to Report.

IMPACT ON SOCIETY BEYOND SCIENCE AND TECHNOLOGY

Nothing to Report

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5. CHANGES/PROBLEMS

CHANGES IN APPROACH AND REASONS FOR CHANGE

In our original proposal we had planned to focus our HTS effort on SLCA reactions involving mixtures of WT-Gluc and G37R-Gluc (or A4V-Gluc). In this mix of WT and mutant SOD1, we were expecting hits to decrease luciferase activity by stabilizing the dimers of each fusion protein – inhibiting the exchange of subunits. The known presence of inhibitors of native Gluc in our compound library was a potential issue that we were aware of and planned to deal with by counter screen. As noted above, because of some advantages in regard to false hit rates, our first screen used mixtures of G37R-Gluc1 and G37R-Gluc2. We determined that this screen could provide cleaner data because there would be few false-hits to follow up. Unfortunately, our screen of the G37R-Gluc1 and G37R-Gluc2 SLCA reactions produced no hits. We are now in the process of preparing new batches of cell lysates from WT-Gluc1 and G37R-Gluc2 to run the HTS for this heterodimer pairing. We will also prepare cell lysates for an HTS with WT-Gluc1 and A4V-Gluc2. If time and funds permit, we will run an HTS for A4V-Gluc1 and A4V-Gluc2.

Actual or anticipated problems for delays and actions or plans to resolve them.

Nothing to report

Changes that had significant impact on expenditures

Nothing to report.

Changes in human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

6. PRODUCTS

Nothing to report (manuscript in preparation).

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

INDIVIDUALS THAT HAVE WORKED ON THE PROJECT

LANKENAU INSTITUTE FOR MEDICAL RESEARCH

Name:	**Melvin Reichman**
Project Role:	PI-subaward
Researcher Identifier	MelReichman
Nearest person month worked:	1 Calendar Month
Contribution to Project:	Co-PI. Oversight of the LIMR effort on project. Interpretation of research outcomes, in coordination with Project PI, Dr. Borchelt.

Funding Support:

Name:	**Preston Scott Donover**
Project Role:	Associate Scientist
Researcher Identifier	
Nearest person month worked:	2 Cal. Months
Contribution to Project:	Mr. Donover devised and executed the shifted transversal design method for multiplexing the FDA-approved drug set(s). He helped validate the SOD1-SLCAs and also characterized the activities of the literature compounds described in the report.

Name:	**Amanda Schabdach**
Project Role:	Laboratory Technician II
Researcher Identifier	
Nearest person month worked:	3 Cal. Months
Contribution to Project:	Ms. Schabdach performed the assay optimization and validation for HTS of the drug combinations in the G37R homodimer SOD1-SLCA. She also performed HPLC/MS characterization of literature reference compounds.

Funding Support:

CHANGES IN ACTIVE SUPPORT: No change in effort to the present project is planned.

NEW GRANTS FUNDED IN THE AWARD PERIOD

Title: Accelerating drug discovery for ALS
Supporting Agency: The Amyotrophic Lateral Sclerosis Association
ID: 16-IIP-279
Time Commitment: 1.2 CM
PI: Melvin Reichman, Ph.D.
Name and address of the Funding Agency's Grant Officer: Mark Yard (mark@alsa-national.org; Tel# 203-641-1158)
Performance Period: 8/1/15-7/31/18

Level of Funding: $80,000 (direct costs for current year); $240,000 3/yr Total Funding (direct costs for total grant period)
Project's Goals: We will apply a new HTS approach that uses one efficient bioassay to identify single-active and, potentially, combination-drug leads that kinetically stabilize the SOD1 mutant-harboring dimer. We were recently awarded a patent for this novel application of orthogonal-pooled screening.

GRANTS THAT ENDED IN THE AWARD PERIOD

None

PARTNER ORGANIZATIONS

Organization Name: **University of Florida**
Location of Organization: **Gainesville, FL**
Partners contribution to the proejct: This project is a collaboration between Dr. David R. Borchelt (University of Florida) and Dr. Melvin Reichman (Lankenau Institute). Dr. Reichman's laboratory staff have participated in the project, generating some of the data relevant to Specific Aim 1 and all of the data relevant to Specific Aim 2.

8. SPECIAL REPORTING REQUIREMENTS: None.

9. APPENDICES: None.