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**Introduction: SMA Foundation Mouse Drug Testing Pipeline**

The mission of the SMA Foundation is to accelerate the development of treatments for SMA. Testing potential drug compounds in SMA animal models is an important part of our work. Since 2003 the Foundation has spent over $27M on SMA mouse models through making them available to the research community, generating new models, supporting the SMA model repository at the Jackson Laboratory, and establishing and maintaining *in vivo* drug efficacy testing in a standardized platform. These activities are collaborative efforts that span several groups including universities, other non-profit research groups, contract research organizations, government agencies and biotech and pharmaceutical companies.

A key feature of the Foundation Research Program has been testing drugs in a standardized platform in a model of severe SMA (the Delta7 mouse). The standardization of the testing platform allows evaluation of drugs at a single laboratory using the same validated methods in each study, which in turn enables direct comparison of results from different drugs across studies. To date, we have tested 160 experimental compounds and approved drugs at PsychoGenics (PGI) (Tarrytown, NY). The initial goal was to screen compounds to identify already approved drugs that had beneficial effects in the Delta7 mice and could be tested in patients in the relatively near term. More recently the focus of drug screening has shifted to support development of new compounds specifically designed for SMA. We have concurrently expanded our testing program beyond the severe mouse model by actively pioneering the use of additional mouse models that exemplify milder forms of SMA.

This document provides details about the Foundation's in vivo testing program with PGI. It reviews the classes of compounds tested by the Foundation, presents a broad summary of the testing results and also discusses lessons learned from work with both severe and mild SMA animal models with next steps outlined.

**The Testing Platform**

The first goal in the collaboration between PGI and the Foundation was to examine different SMA mouse models and to select one for development of a standardized test battery with endpoints that have relevance to clinical disease features. The Delta7 mouse model, a widely-published representation of severe SMA developed by researchers at The Ohio State University, was chosen (Le et al. 2005). The Delta7 animal has an average lifespan of 14 days and displays severe muscle weakness, making it a model analogous to the most severe forms of SMA. Due to the animals' short lifespan, drug studies can be done relatively rapidly. The homozygous knockout animal in this model line completely lacks the mouse Smn gene but carries two copies of the human SMN2 gene, leading to a high expression level of truncated SMN transcripts, in which exon 7 is deleted (giving rise to the Delta7 name).

Generally, Delta7 mice are dosed starting at 3 days after birth (to allow for time to genotype the animals) and tested on various metrics until time of death. The key endpoints employed in the Foundation's Delta7 pipeline are survival, body weight and motor function. Staff trained to work with neonatal mice evaluate motor function through performance of a number of assays, including hind limb strength, rising from a laying to a standing position (righting), and turning around from a downward-facing to upward-facing position on an incline (geotaxis). These endpoints were validated for evaluator reliability and phenotype reproducibility across several groups of mice in both genders. These data have been used to devise protocols and study designs that are robust and sensitive enough to allow observation of a 20% improvement in these endpoints due to drug treatment. More details on the platform can be found in El-Khodor et al. 2008.

Significant resources have been devoted to the Delta7 drug testing effort over time, with nearly $20M spent to execute ~300 experiments involving 160 and counting compounds and supplements since 2005. The testing strongly resembles an iterative pharmaceutical company drug screening process in which the results of each test trigger go or no-go decisions for doing the next test. At the beginning, test doses are researched and tested in wild-type neonatal mice to

identify the maximum tolerated dose (MTD). After the MTD is identified, multiple doses are tested in Delta7 mice for efficacy in extending survival, increasing body weight and improving motor function, and changes in SMN transcript and protein where relevant. Compounds with no efficacy are not pursued, while those that produce efficacy signals are retested in a larger repeat studies. If the efficacy is repeated, further studies to explore the mechanism or effect of drug in tissues may also be done, and are usually tailored to the drug compound and its mechanism if known.

**Testing Drugs in a Standardized SMA Platform**

Thus far 31 different companies have submitted compounds for testing in the Delta7 pipeline and, together with the FDA-approved drugs and supplements, they span several mechanistic classes theorized to upregulate SMN and/or affect SMA-like symptoms. Many compounds were FDA-approved drugs and supplements internally selected by Foundation advisors, staff or academic collaborators, based on various therapeutic rationales for efficacy. Recently, several more new chemical entities and proprietary tool compounds have entered the pipeline. These entities were put forward by drug company collaborators who provide compounds and information relevant for their drugs and proposed mechanisms of action as well as supporting analysis of tissues generated in Delta7 drug studies (**Table 1**).

**Table 1: Drugs Tested by Mechanism in SMA Foundation Delta7 Pipeline**

| | SMN Upregulation | Neuroprotection | Neurotransmission | Energetics | Neurotrophins/ Growth Factors | Muscle Stimulation | Other |
|---|---|---|---|---|---|---|---|
| Drug # | 71 | 42 | 15 | 10 | 8 | 7 | 7 |
| Public | 17 | 11 | 15 | 10 | 2 | 3 | 7 |
| Private | 54 | 31 | 0 | 0 | 6 | 4 | 0 |
| Rationale | Restore protein loss that causes SMA | Prevent or reduce motor neuron death | Increase activity between neurons and muscles | Increase general cellular health and function | Provide support to motor neurons and muscle cells | Increase muscle strength | |
| Examples | Alt. splicing<br>Gene therapy<br>HDAC inhibitors | Riluzole<br>PPAR agonists | Lithium<br>Channel blockers<br>Agonists/antagonists | Supplements<br>Diets | Parathyroid hormone | Salbutamol<br>AICAR | Anti inflammatories |

Several compounds identified in early *in vitro* screens or publications as showing the ability to increase SMN protein levels were tested initially, including valproate, hydroxyurea and phenylbutyrate. While these demonstrated some benefits in the Delta7 mice, none of these aforementioned drugs produced significant improvements in all endpoints nor improved any one endpoint by greater than 20% compared to vehicle controls. This pattern was repeated with other available compounds, with the majority of them not improving the established Delta7 endpoints and a few improving one or two endpoints to a modest degree. Some combination therapies were attempted but in general they were poorly tolerated in the Delta7 mice or had no additive benefits, with the exception of L-carnitine and valproate, which had only modest additive benefits. Generally compounds that improved survival also increased body weight. Nutritional supplementation treatment with oils tended to increase body weight in the animals but had no other significant benefits on motor function or survival.

Ultimately, only a few compounds and classes were 'hits' that significantly impacted all key endpoints in the Delta7 mouse: extending survival, increasing body weight and improving motor function. Some results are still pending, but results to date show that these compounds tended to fall in the following categories: SMN alternative splicing compounds, pan-histone deacetylase (HDAC) inhibitors like trichostatin A and valproate; prednisolone also positively affected all Delta7 mouse endpoints (**Table 2,**). The few SMN upregulating compounds that were efficacious for all metrics also had the most robust effects on the key metrics of weight increase, survival extension and motor function improvement seen in the pipeline, with benefits greater than those seen with prednisolone. In particular, several proprietary compounds that act by upregulating SMN alternative splicing robustly extended survival and improved motor function far beyond that of valproate and trichostatin A, and thus this mechanism overall produced the greatest number of compounds that had significant beneficial effects.

Several compounds can be considered partial hits because they had modest impact on motor function in the Delta7 mice. These hits included troglitazone and other related peroxisome proliferator-activated receptor (PPAR) agonist compounds. These compounds were examined further to determine if there was any particular PPAR subtype that was responsible for the efficacy; however it appeared that the results were due to activity with multiple subtypes. Many other HDAC inhibitors were tested beyond valproate and trichostatin A, and not all of them were robustly effective in all metrics. For this class of compounds it is again unclear if there is any specific HDAC isotype that is responsible for activity in the Delta7 model. The available compounds that benefited motor function but not survival are intriguing. Such compounds came from a wide array of mechanistic classes and include compounds like salbutamol that had been reported to increase SMN in SMA patient cells. It is clear these compounds may also have other ways of affecting neuromuscular performance, and further work will be needed to truly understand their actions and their potential role as SMA drugs. Compounds like these could prove to be of symptomatic benefit for SMA patients but would require further testing in SMA mouse models and SMA patients to confirm this hypothesis.

**Table 2. Summary of SMA Foundation Delta7 Drug Testing Pipeline Results (some results are pending)**

| Metric Benefits | SMN Upregulation | Neuroprotection | Neurotransmission | Energetics | Neurotrophins/ Growth Factors | Muscle Stimulation | Other |
|---|---|---|---|---|---|---|---|
| All metrics, Survival >20% | 6 others | | | | | | -prednisolone |
| Survival, Weight (>15%) | -Valproate/L-carnitine<br>-trichostatin A*<br>-3 others | | -Mazindol | | | | |
| Motor Function (at least one metric) | -Valproate/L-carnitine<br>-Valproate*<br>-Hydroxyurea<br>-Phenylbutyrate*<br>-N-demethyl-trichostatin A<br>-Synthetic trichostatin A*<br>-Phenylbutylnitrone<br>-8 others | -Delta-2-Troglitazone<br>-Troglitazone<br>-10 others | -Rivastigmine*<br>-Mazindol | -Resveratrol<br>-Latrepirdine<br>-Nicotinimide | -3 others | -Terbutaline<br>-Salbutamol* | |
| No benefit | -Bortezomib<br>-Vorinostat<br>-Guanidine<br>-SAHA<br>-25 others including several HDAC inhibitors | -Erlotinib<br>-Lithium*<br>-Pioglitazone<br>-Fasudil<br>-HA1077<br>-Riluzole<br>-4-Phenyl Butyric Acid<br>-23 others | -Valproate/L-carnitine+4-Aminopyridine<br>-Clozapine<br>-Donepezil<br>-Pyridostigmine<br>-Diaminopyridine<br>-3,4-Diaminopyridine<br>-4-Aminopyridine<br>-Desipramine<br>-Rolipram<br>-Memantine<br>-MPEP | -Acetyl-L-Carnitine<br>-L-carnitine<br>-Bezafibrate<br>-Corn Oil<br>-Creatine<br>-Enfamil<br>-MCT Oil | -Parathyroid hormone<br>-Losartan<br>-3 others | -AICAR<br>-3 other | -Azathioprine<br>-Carvedilol<br>-Ibuprofen<br>-Imipramine<br>-Taltirelin<br>-Y27632 |

*Indicates multiple compound sources and formulations were tested.

The identities of proprietary compounds including several SMN alternative splicing compounds are not disclosed due to confidentiality obligations.

Overall, screening broadly with available compounds produced few strong hits in the Delta7 SMA mouse testing pipeline with proprietary new compounds designed to increase SMN through the alternative splicing mechanism, and the Foundation shifted its testing strategy to focus further testing on the proprietary new compounds. While robust hits were found infrequently in standardized Delta7 drug screening outside of the SMN alternative splicing compounds, the interpretation of this body of results may also require consideration of the model itself. Are the Delta7 mice too severe to be treated effectively by drugs whose mechanisms are not focused on increasing SMN? How do results in a severe neonatal disease model translate across the population of SMA patients? While the translatability of animal model results are always open to debate, it seems reasonable that a mouse model with less severe phenotypes may better

inform on drug efficacy for patients with milder disease. This rationale was the driving principle behind the Foundation's efforts to generate milder SMA model mice.

**Developing Models with Milder SMA Phenotypes**

In 2006 the Foundation entered into an agreement with Regeneron Pharmaceuticals to develop a series of mice that would better model milder forms of SMA for testing drugs. There is a general relationship between SMN2 copy numbers and severity of disease in SMA such that patients with milder disease tend to have more copies of SMN2. Regeneron produced a series of mouse lines that had an SMN2-like gene that was a hybrid of the mouse and human SMN gene (mouse-like for exons 1-6 and human-like for exons 7-8) and a different numbers of SMN2 genes (0,1,2 or 3) (**Table 3**). The resulting allelic series of mice were named A, B, C, D and homozygous mice (e.g. C/C) or crossed mice were produced (e.g. C/D). Surprisingly there was a stark difference in the survival of the mice and their phenotypes. Animals with fewer copies of SMN than the C/C mice (2) died before birth. C/C mice exhibit milder impairment of motor function than the Delta7 mice; however, like other SMA mouse models with smaller reductions of SMN, the C/C mice develop progressive necrosis of the tail, hindfeet and ears. The D/D mice show no obvious motor impairments, nor do they develop necrosis.

While the goal was to develop milder SMA models that could be used for motor activity and strength efficacy testing of drugs, the necrosis present in the C/C mice and the lack of obvious motor deficits in the D-allele mice complicates the use of the mice. Interpretation of motor and behavioral activity in C/C mice is confounded by their foot necrosis, but they are being effectively utilized to detect changes in SMN protein expression in drug studies. Other research with the allelic series mouse lines is ongoing, and is focused on identifying other morphological and physiological deficits in the C/C mouse CNS and muscle, characterizing crosses between allelic series mice as well as other SMA models. These lines are available at the Jackson Laboratories. (http://jaxmice.jax.org/list/ra1733.html)

**Table 3. Allelic Series SMA Mice**

| Line | Hybrid SMN1 Copies | SMN2 Copies | Phenotype | Jax Stock # BL6 | Jax Stock # FVB |
|---|---|---|---|---|---|
| A/A | 0 | 0 | Embryonic lethal | 7963 | 7955 |
| A/B | 1 | 0 | Embryonic lethal | | |
| A/C | 1 | 1 | Embryonic lethal | | |
| A/D | 1 | 3 | Viable | | |
| B/B | 2 | 0 | Embryonic lethal | 8453 | 8713 |
| B/C | 2 | 1 | Embryonic lethal | | |
| B/D | 2 | 3 | Viable | | |
| C/C | 2 | 2 | Viable, necrosis | 8714 | 8604 |
| C/D | 2 | 4 | Viable | | |
| D/D | 2 | 6 | Viable | 9378 | 9391 |

**Lessons Learned and Next Steps**

Taken as a whole, the results from the Delta7 testing platform have triggered a shift in the Foundation's screening strategy. We have found that proprietary compounds designed to upregulate SMN are the most efficacious class of compounds and are represented in the tables above by the handful of compounds with the most robust efficacy in all metrics. As a result, the current focus of the Foundation's Delta7 pipeline is to screen SMN-upregulating compounds to optimize drug development lead compounds and help select potential clinical development candidates. Available compounds were largely not efficacious in the Delta7 pipeline, and if motor benefits were seen they were modest. The new criteria for testing external compounds include having a sound therapeutic rationale, pre-existing pharmacokinetic data (with evidence of entry into the central nervous system if appropriate for the compound's mechanism), and the ability to support drug exposure and/or pharmacodynamic target engagement analyses.

Even when used in a standardized manner, the Delta7 model line is a challenging line for efficacy studies. Tissues from Delta7 mice can be used in studies to determine drug exposure and whether if drugs increase SMN levels in vivo,

however the small size of the neonatal tissues inherently limits the number of analyses possible. There were a number of compounds tolerated in MTD studies in wildtype mice that were not tolerated in the Delta7 mice, and this phenomenon is unpredictable. While a few compound extended the lifespan of the mice, the type of survival extension was not always the same for every drug. Some compounds extended the average survival of the majority of the mice, whereas other compounds appeared to produce much longer life extension in a smaller subpopulation of mice. The basis for this difference is unknown.

Given that the Delta7 model appears to be most amenable to SMN upregulating compounds, there is still a need to determine if milder phenotype mice will be responsive to a wider array of SMA therapeutic mechanisms. In the C/C model line, dosing regimens and lists of tolerated vehicles are in place while natural history data on their behavioral phenotypes and SMN levels are being further developed. In the meantime, the C/C mice are being used for assessing pharmacodynamic changes in SMN protein levels across a number of tissues. In conjunction with several academic collaborators and the Jackson Laboratory, the C/C mice are also being evaluated for evidence of quantitative muscle, motor neuron and neuromuscular junction abnormalities that could potentially be developed as endpoints for efficacy studies. Research on genetic crosses with the C/C and other mouse lines is underway, as it may be possible to generate a model that is slightly more severe than the C/C mouse or a model with motor phenotypes that lack necrosis like the recent lines described by the DiDonato or Chandler groups that have modifications to the mouse Smn gene (Gladman et al. 2010). To complement the C/C mouse analysis, these other mouse lines may also be imported for characterization when they become available.

In summary, the SMA Foundation is continuing in its efforts to accelerate development of therapeutics for SMA with standardized focused drug testing in both severe and mild phenotype SMA mouse models, as well as further exploration for mild mouse models suitable for motor efficacy testing. More detailed descriptions of the Delta7 pipeline drug screening results as well as the development of the SMA allelic series of mice are currently being prepared for peer-reviewed publications. Please contact the SMA Foundation at researchtools@smafoundation.org for any questions on this material.

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