Diabetic Complications Consortium

Application Title: Coupling Pharmacogenetics and RNA-Seq to Identify Hsp70-dependent Gene Networks

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1. Project Accomplishments:

To date, approaches toward treating diabetic peripheral neuropathy **(DPN)** have focused on inhibiting pathogenic targets/pathways that are considered central effectors for mediating the pathophysiological progression of the disease (1). However, there has been limited translational success of this approach due, at least in part, to differences in the temporal and/or biochemical uniformity by which these targets/pathways contribute to the progression of DPN between individuals. In contrast, we have developed an *innovative* therapeutic strategy to improve myelinated/unmyelinated fiber function in DPN that does not rely on inhibiting a specific pathogenic mechanism of disease development, but is based on pharmacologic modulation of cytoprotective molecular chaperones such as heat shock proteins 90 and 70 **(Hsp90, Hsp70).**

Over the last 5 years, we have validated the potential utility of a novel class of compounds developed at the University of Kansas called novologues (2-5). Novologues are proprietary, non-toxic, orally bioavailable, small molecule inhibitors of Hsp90 that have shown exceptional promise in reversing multiple clinical indices of insensate DPN in wild type **(WT)** C57Bl/6 mice, Swiss Webster mice and BKS-db/db mice. This improvement temporally correlates with an increase in mitochondrial bioenergetics of adult sensory neurons. Although novologues bind to Hsp90, downstream induction of Hsp70 is required for drug efficacy since our first lead novologue, KU-32, did not improve mitochondrial bioenergetics nor reverse insensate DPN in diabetic Hsp70 knockout **(KO)** mice. Based on these published data, the goal of the DiaComp project was to further explore how Hsp70 may improve DPN by determining if novologue therapy altered select genes or gene networks within sensory neurons in an Hsp70-dependent manner following. The main project accomplishments to date are:

1) In a direct dose-comparison study to KU-32, we validated the efficacy of a new novologue, KU-596, in reversing insensate DPN **(Fig. 1)**, nerve conduction velocity deficits **(Fig. 2)** and improving sensory neuron mitochondrial bioenergetics **(Fig. 3)**. KU-596 maintains structural attributes of KU-32 that we previously identified as essential for neuroprotection, but replaces a component of the molecule that simplified its de novo synthesis and provided new IP and patent life. Under a licensing agreement we negotiated with Reata Pharmaceuticals, we are working with the company on developing an IND application to submit to the FDA for using KU-596 (now designated RTA901) in treating human insensate DPN.
2) C57Bl/6 mice were rendered diabetic and allowed to develop symptoms of insensate DPN for 12 weeks **(Fig 4)**. The mice were then treated with vehicle or 20 mg/kg KU-596 once a week for 4 weeks, which was sufficient to induce a 50% recovery in the measures of the insensate DPN.
3) mRNA was isolated from the dorsal root ganglia and used for RNA Seq analysis. Single read 100 RNA Seq analysis was performed on mRNA from 3-4 animals per group and the reads were mapped to the mouse genome using Top Hat. Transcript assembly was performed with CuffLinks and differential gene expression was ascertained using CuffDiff (6,7). Pathway analysis was used to identify genes enriched for various biologic processes and indicated that genes associated with inflammation were significantly upregulated in diabetic ganglia and these were broadly decreased by KU-596 treatment **(Fig.5).** An example is shown for the increase in genes linked with TNF signaling, the bulk of which were upregulated by diabetes **(Fig. 6)** and whose expression were significantly attenuated following KU-596 therapy **(Fig. 7)**. A representative sample of these genes is being validated by qPCR. Results in **Table 1** show the clear inverse relationship between the upregulation of genes associated with several cytokine networks and their reversal with KU-596 therapy.

Specific Aim:

Identify the Hsp70-dependent drug response phenotype to KU-596 therapy using RNA-Seq Analysis

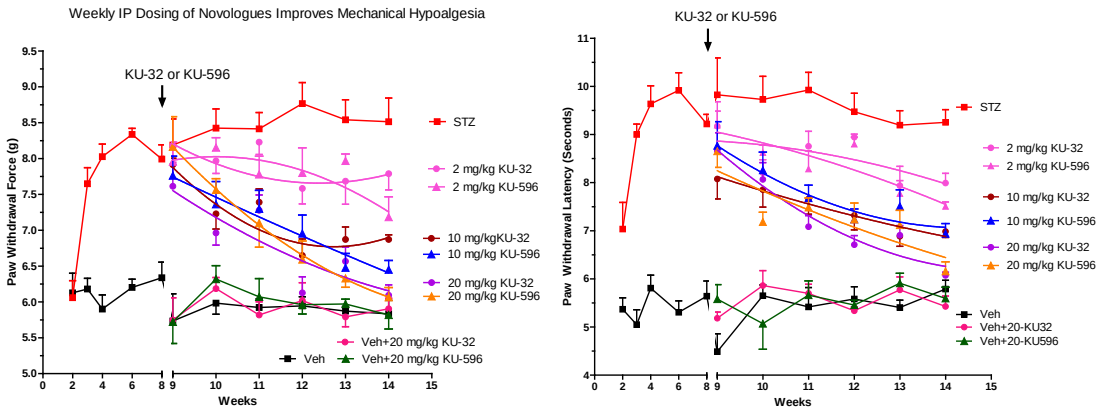


Fig 1- **KU-32 or KU-596 dose-dependently improve mechanical (upper) or thermal (lower) hypoalgesia with once a week dosing in diabetic mice.** Swiss Webster mice were rendered diabetic with streptozotocin and after 8 weeks of diabetes, treated once per week with the indicated dose of the novologues. Drugs were given via ip administration. Responses to mechanical and thermal stimuli were measured weekly after drug administration. Statistical differences are not indicated for clarity but 10 and 20 mg/kg novologue groups were significantly different from STZ from 10 weeks onward. (n=5-7 per group).

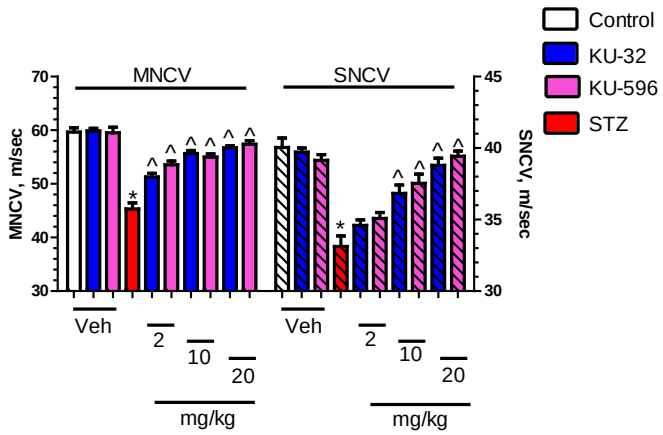


Fig 2- **KU-32 or KU-596 dose-dependently improves motor and sensory nerve conduction velocity (NCV) with once per week dosing in diabetic mice.** Swiss Webster mice were rendered diabetic with streptozotocin and after 8 weeks of diabetes, treated once per week with the indicated dose of the novologues. Drugs were given via ip administration. NCV was measured after 14 weeks of diabetes. *, $p < 0.05$ vs control; ^ , $p < 0.05$ vs STZ. Data are mean ± SEM, (n=5-7 per group)

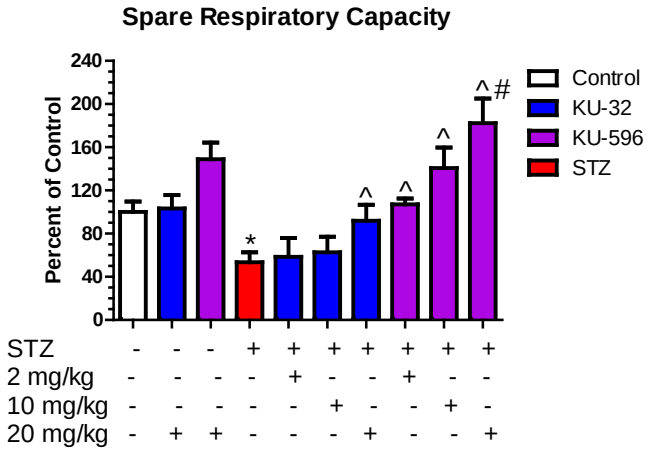
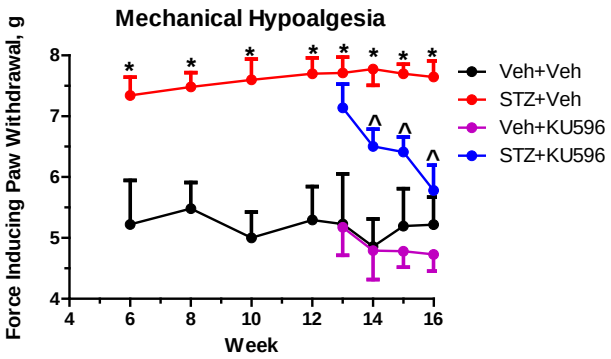


Fig 3- **KU-32 or KU-596 improves sensory neuron mitochondrial spare respiratory capacity (SRC).** Adult sensory neurons were isolated from each group of mice at week 14 and mitochondrial respiratory capacity was measured in the intact cells using an XF96 analyzer from Seahorse Bioscience. SRC is a measure of the mitochondrial reserve capacity for ATP production in response to cell stress. Diabetes significantly impairs SRC indicating that the neurons are functioning near their bioenergetic limits. Animals treated with KU-596 showed significantly improved SRC at all doses. This indicates that the drug was increasing the bioenergetic reserve capacity of the sensory neurons and enhancing their ability to match ATP production with demands. Data are mean ± SEM from 6 wells of neurons obtained from 3-4 mice per group. *, $p < 0.05$ vs control; ^ , $p < 0.05$ vs STZ.



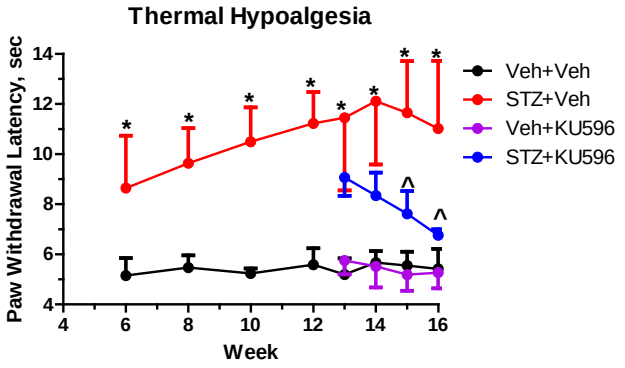


Fig 4- **Time course for development of sensory hypoalgesia and its partial reversal by 4 weeks of therapy with KU-596.** C57Bl/6 mice were rendered diabetic with streptozotocin and after 12 weeks of diabetes, treated once per week with 20 mg/kg of KU-596 given via oral gavage. Responses to mechanical and thermal stimuli were measured weekly after drug administration. Animals were sacrificed at 16 weeks and dorsal root ganglia used for isolating mRNA for RNA Seq analysis. *, $p < 0.05$ vs control; ^ , $p < 0.05$ vs STZ (n=6-8 per group).

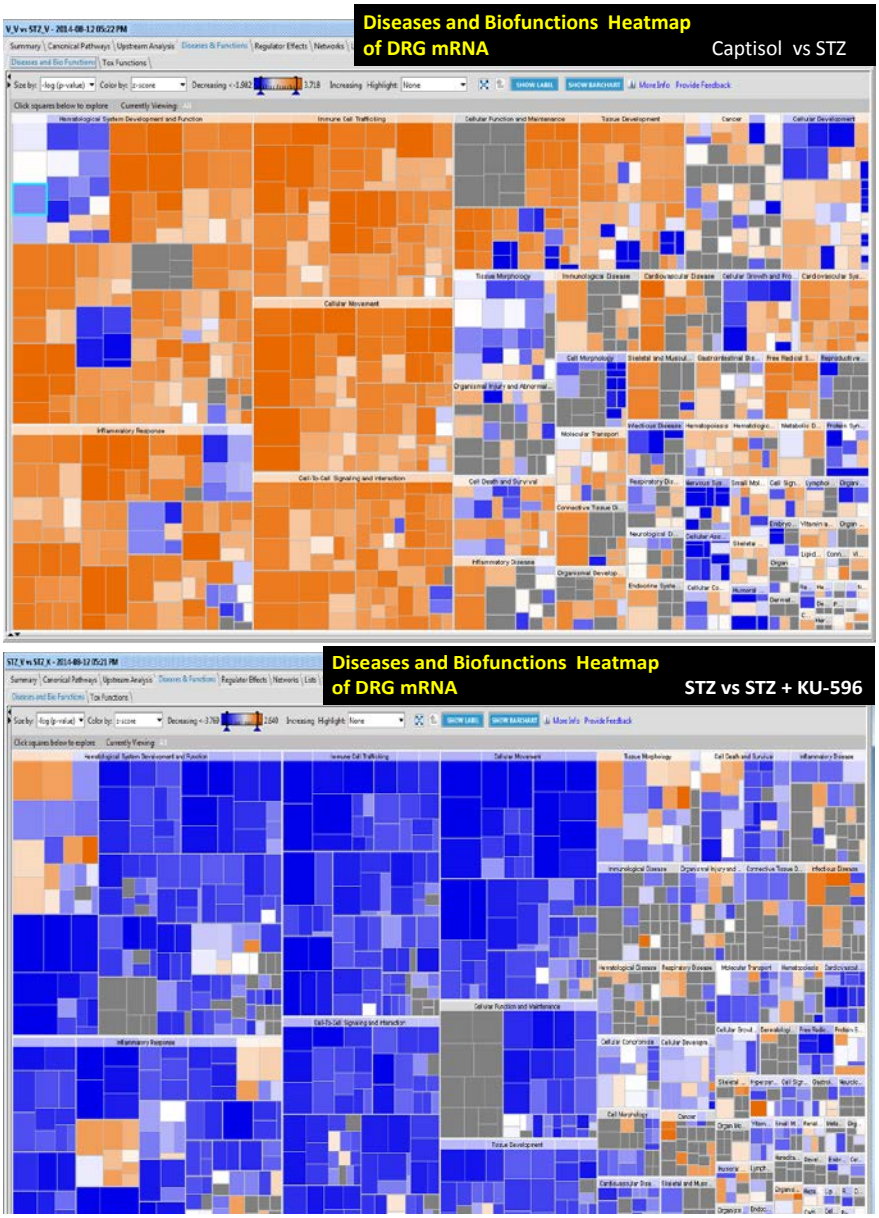


Fig. 5 **Diabetes Increased the Expression of Numerous Genes Associated with Inflammation (more orange) and the Expression of a Majority of these Genes was Decreased with KU-596 (blue).** Results are presented as a heatmap output from pathway analysis

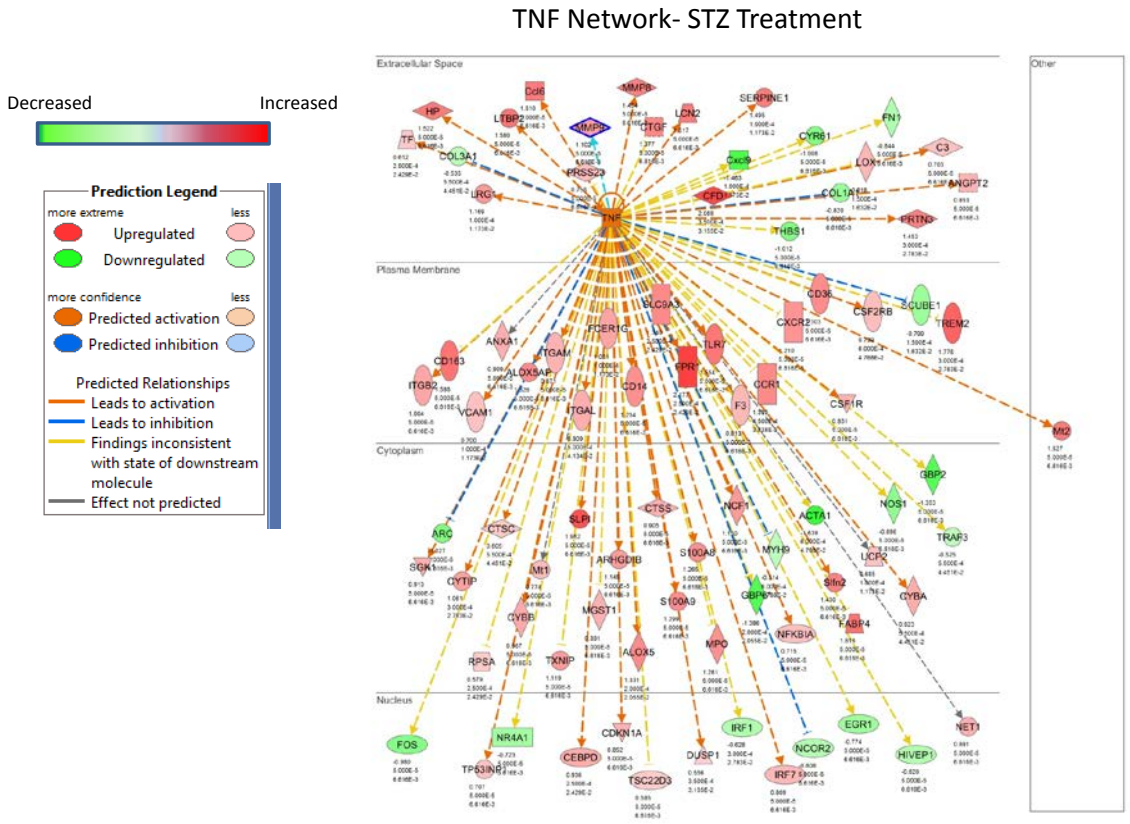


Fig 6- **Diabetes increased the expression of genes enriched in the TNF signaling pathway.** Pathway analysis identified that numerous genes associated with TNF signaling were significantly enriched and their mRNA expression increased by diabetes.

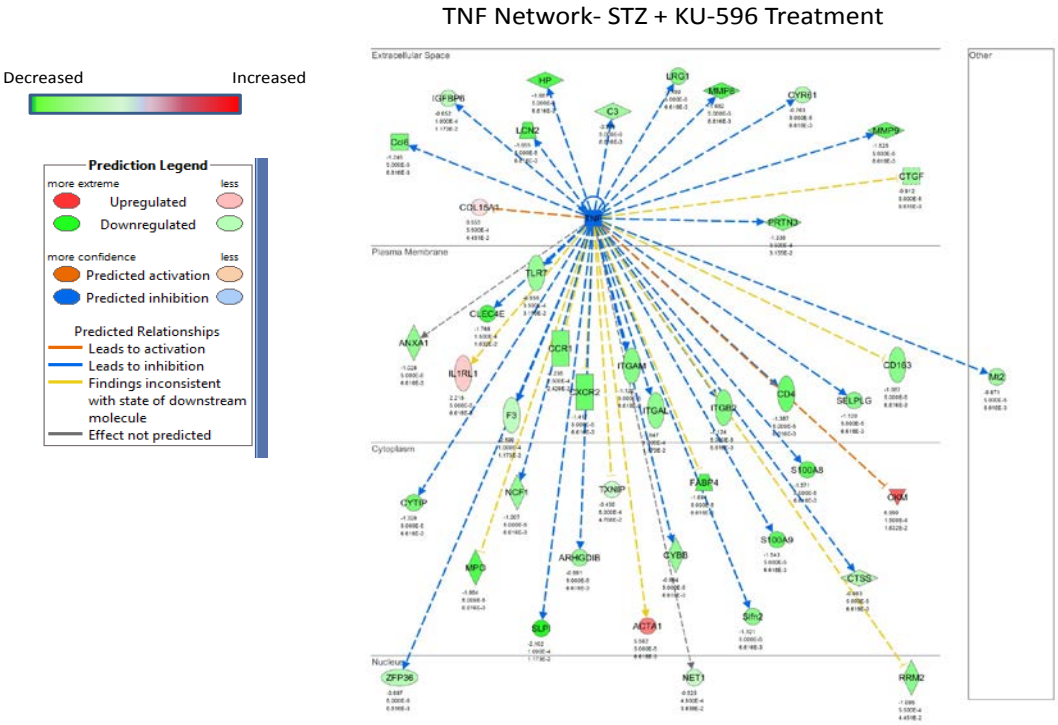


Fig 7. **KU-596 therapy decreased the expression of genes enriched in the TNF signaling pathway.** Pathway analysis identified that numerous genes associated with TNF signaling were significantly enriched and their mRNA expression decreased by KU-596.

Table 1- Effect of Diabetes on Increasing the Expression of Genes Associated with Several Cytokine Networks and that were Reversed by KU-596 Therapy

STZ Treatment

Upstream Regulator	Molecule Type	Predicted Activation State	Activation z-score	p-value of overlap
IFNG	cytokine	**Activated**	**2.165**	**6.19E-34**
TNF	cytokine	**Activated**	**2.212**	**7.00E-29**
IL6	cytokine	**Activated**	**2.628**	**8.86E-28**
CEBPE	transcription regulator	**Activated**	3.700	**1.44E-21**
SRF	transcription regulator	**Inhibited**	**-3.762**	**5.98E-13**

STZ + KU-596 Treatment

Upstream Regulator	Molecule Type	Predicted Activation State	Activation z-score	p-value of overlap
SRF	transcription regulator	**Activated**	**2.861**	**2.16E-22**
CEBPE	transcription regulator	**Inhibited**	**-3.563**	**1.96E-19**
IL6	cytokine	**Inhibited**	**-3.388**	**6.05E-18**
TNF	cytokine	**Inhibited**	**-3.222**	**1.48E-15**
IFNG	cytokine	**Inhibited**	**-3.483**	**5.61E-15**

Ongoing Work

1) Hsp70 KO mice are currently at 6 weeks of diabetes and will undergo a similar treatment with KU-596. This portion of the study was delayed as our Hsp70 KO colony that was housed in the University vivarium contracted murine hepatitis virus in Sept 2013. It took until Feb 2014 to get the virus cleared and another 4 months to re-establish the breeding colony to begin generating a sufficient number of animals for the study. The original vendor did not have any live stocks and recovery from cryo-preservation yielded only a single male, which was not overly helpful. Importantly, sufficient funds exist to support the completion of the animal work by the end of 2014 and the RNA Seq analysis in early 2015.
2) We are designing experiments that will more directly address whether attenuating aspects of TNF signaling following novologue therapy may improve sensory neuron bioenergetics.

Conclusions

At this point, our data clearly supports that KU-596 is an effective third generation novologue that has strong potential for development as a human therapeutic against insensate DPN. An unexpected finding of the work was the enrichment and increased expression of genes associated with inflammatory processes in mRNA isolated from the sensory ganglia. Moreover, the robust attenuation in the expression of these genes by KU-596 therapy points toward a mechanism of action that is associated with blunting numerous inflammatory genes. Since we show that KU-596 can also improve mitochondrial bioenergetics in sensory neurons isolated from the diabetic mice, this raises the possibility that a component of this response may be due to attenuating inflammatory processes in the ganglia. However, since novologues can increase mitochondrial function in purified sensory neurons that are subjected to hyperglycemic stress in vitro (8), the drug may have both direct and indirect effects on improving bioenergetics.

The DiaComp pilot funding has clearly provided a new direction in helping define the mechanism by which novologues have neuroprotective efficacy in DPN. Once accepted for publication, all raw data files from the RNA Seq analysis will be submitted to DiaComp.

2. Publications:

None so far. Anticipate one in 2015.

3. References

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