Diabetic Complications Consortium

Application Title: *In vivo* validation of novel compounds for treating diabetic kidney disease

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1. Project Accomplishments:

Diabetic Kidney Disease (DKD) is the major cause of kidney failure in the US and one of the fastest growing epidemics worldwide. DKD-related healthcare expenditures are extraordinarily high, exceeding $25 billion in 2011 for the Medicare population alone. There are no FDA approved treatments for DKD, and its prevalence continues to rise despite increased use of glucose- and blood pressure-lowering medications. Loss of specialized kidney cells, called podocytes, underlies the development and progression of DKD. The main challenge for discovering effective therapies has been the lack of understanding of the mechanisms that cause podocyte loss, as well as the lack of screening systems that capture the complex pathophysiology of DKD. Our group has shown that podocytes of DKD patients accumulate lipids in the form of lipid droplets (LDs), which in turn render them susceptible to injury and cell death under diabetic conditions. Furthermore, we demonstrated that decreasing LD accumulation in podocytes prevents renal disease in a mouse model of DKD. Conversely, we showed that increasing LD accumulation in podocytes accelerates development and progression of DKD. We developed a robust phenotypic assay utilizing immortalized human podocytes and utilized it to identify compounds that reduce LD accumulation. We screened > 45 million unique molecules and identified a novel class of compounds that block LD accumulation in podocytes, thereby protecting them from lipotoxicity and cell death. These hits were validated by resynthesis and re-testing in a clinically relevant assay utilizing sera from patients with DKD.

Our overall goal in this proposal was to test compounds *in vivo* to validate our therapeutic hypothesis. The initial compounds we had generated, however, turned out to have problematic physiochemical properties (precipitated in the osmotic pumps after a 1-2 days of implantation, thereby blocking delivery to the animals). To overcome this issue, we performed additional medicinal chemistry and generated a new compound, 2726-7, with similar bioactivity to the parent hit but with substantially improved physiochemical properties (~3 orders of magnitude better aqueous solubility). To further ensure that we do not run into the same problem with the pumps, we dissolved the compound in a biocompatible buffer that had been specifically formulated for preclinical testing of early-stage compounds. Our preliminary data suggests that the compound had a significant therapeutic effect *in vivo*. Our investigations into the mechanism of action (MoA) of our compound revealed that it prevents LD accumulation in podocytes by inducing lipophagy, thereby rescuing them from lipotoxicity and preserving their function.

Overall, despite the initial setback caused by the poor physiochemical properties of the first hit compounds, we have generated improved compounds and succeeded in demonstrating the validity of our therapeutic strategy *in vivo*. Importantly, we have identified a lead chemical series suitable

for further preclinical development, for which we will pursue follow-on funding, armed with the promising data that was generated in the DiaComp project.

2. Specific Aims:

Aim 1. Perform dose escalation studies to identify tolerated doses for two lead compounds.

Results: The compounds we had initially set to test in this aim turned out to have problematic physiochemical properties that precluded their use in *in vivo*. To overcome this issue, we synthesized several analogs based on the parent scaffold of 3369.278, with two main targets: 1) replacing the thiourea moiety which could become problematic during later stages of drug development, and 2) improving aqueous solubility. We tested these analogs in our phenotypic assay and found that one compound with substantially reduced cLogP, compound 2726-7 **(Fig 1)**, significantly attenuates LD accumulation and recovers cell numbers to unstressed control levels **(Fig. 2, 3A, 3B)**. Furthermore, compound 2726-7 significantly increased autophagosome steady-state numbers similar to the parent compound 3369.278, as assessed by levels of LC3+ puncta **(Fig. 3C)**.

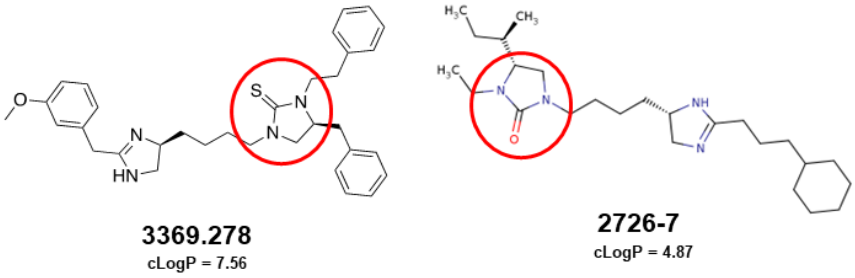

Figure 1. Chemical derivatization of 3369.278. Compound 2726-7 features substantially improved aqueous solubility (~ 3 orders of magnitude lower cLogP) and a regular urea moiety in place of the problematic thiourea.

Lipophagy is a process by which LDs are catabolized through autophagic pathways leading to lysosomal degradation of stored lipids into free fatty acids (FFAs). It can be induced as a coping response to lipotoxicity, and begins with the sequestration of LDs into autophagosomal membranes. We hypothesized that our compounds are inducing not only autophagy, but also lipophagy, to enable podocytes to overcome LD accumulation and lipotoxic stress. To test this hypothesis, we co-treated

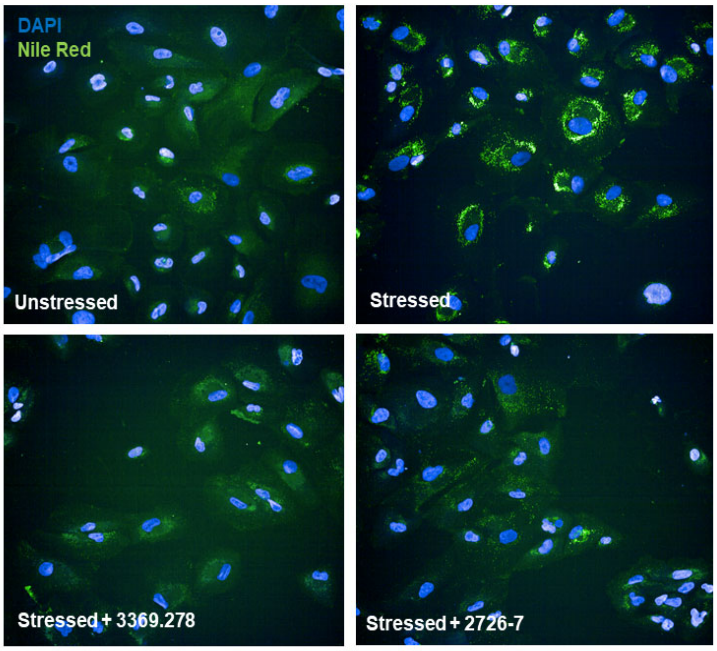

Figure 2. 2726-7 prevents LD accumulation in stressed podocytes. Single-field OPERA confocal image of differentiated podocytes under stressed or unstressed conditions, treated with the indicated compounds or vehicle control. Cells were stained with DAPI (nuclei, blue) and Nile Red (lipid droplets, green).

podocytes with Lalistat, an inhibitor of lysosomal acid lipase (LAL), thereby blocking a critical step in the lipohagy process. Our data show that Lalistat blunts the effect of 2726-7 in stressed podocytes, demonstrating that 2726-7 attenuates LD accumulation by inducing lipophagy **(Fig. 3D)**.

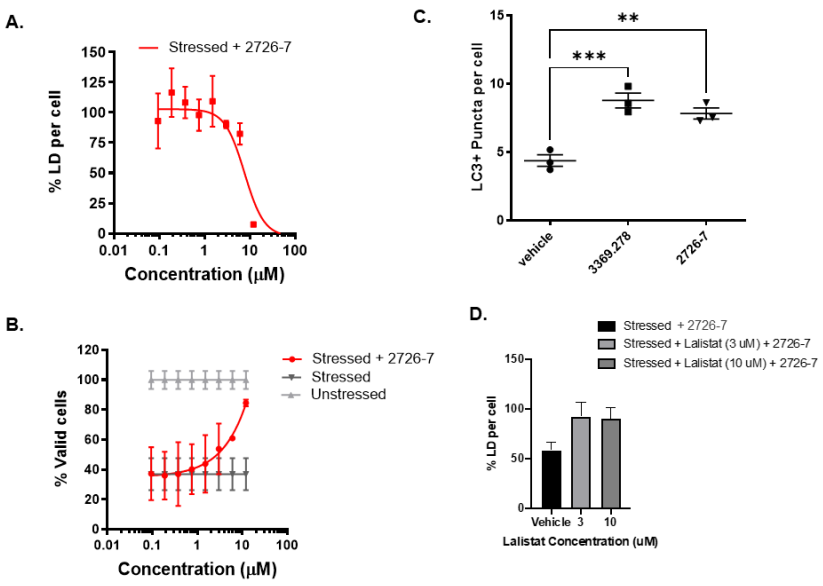

Figure 3. Characterization of Compound 2726-7. A) % LDs per cell and B) %Valid cells returned to unstressed levels with 2726-7 treatment. D) High content analysis revealed that 2726-7 increases mean LC3+ puncta per cell, similar to what is observed for the parent compound 3369.278. E) % LD per cell in stressed podocytes treated with Lalistat (0, 3, or 10 μM) and 2726-7 (5 μM). Data represent mean ± SEM, n=3 technical replicates.

For the dose escalation aim, our goal was initially to test the compound at several doses, with the maximum dose targeting a plasma concentration equal to the EC_{50} (EC_{50} is the concentration at which the compound shows half maximal activity in the *in vitro* primary screening assay), and two lower doses targeting plasma concentrations of EC_{50}/10 and EC_{50}/100. Unfortunately, COVID-related delays, supply chain disruptions, and CRO shutdowns severely hampered our scaleup synthesis to generate sufficient 2726-7 for performing the proposed experiments at all the proposed doses. Therefore, we proceeded to perform the *in vivo* efficacy testing with only the middle and the lowest doses.

Aim 2: Test the lead compounds in a mouse model of DKD.

Results: Finally, we tested 2726-7 in vivo in a mouse model of DKD, to assess its efficacy in reversing or delaying features of diabetic kidney disease. Our pharmacokinetic studies on this compound showed that, like many early-stage compounds, it has a relatively very short plasma half-life (<1 hr). This precludes bolus delivery modes, because the compound is rapidly eliminated in vivo and must be frequently

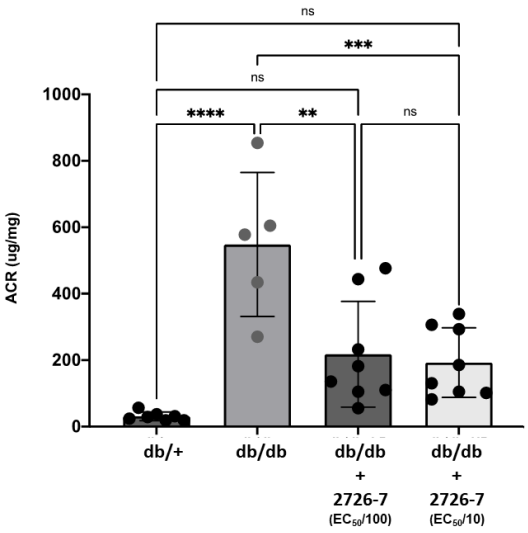

Figure 4. Compound 2726-7 decreases albuminuria in db/db mice. Albumin/creatinine ratio (ACR) of db/+ or db/db mice treated with two doses of compound 2726-7 compared to vehicle-treated controls historically produced in our lab. Both doses of compound 2726-7 result in a significant decrease in ACR. N>5; Data represent means ± SD; **p<0.01, ***p<0.001,****p<0.0001.

replenished. Therefore, we opted to deliver 2726-7 delivered via an ALZET osmotic pump (#2004), implanted subcutaneously according to the manufacturer's instructions. To avoid solubility issues from long-term incubation inside the pump in vivo, we dissolved the compound in a biocompatible buffer that had been specifically formulated for preclinical testing of early-stage compounds. The concentration of compound within the pump was set to 60 mg/mL or 6 mg/mL, targeting a steady-state plasma concentration of EC50/10 or EC50/100, respectively (EC50 is the concentration at which the compound shows half maximal activity in the primary assay). Type 2 diabetic 14-week-old db/db mice were treated with either compound or vehicle for four weeks. Our results show that 4-week treatment with either dose of compound 2726-7 significantly decreased the albumin/creatinine ratio (ACR) of db/db mice compared to db/db mice **(Fig. 4)**. Of note, our vehicle-treated db/db group showed high morbidity and all but one mouse either died or needed to be euthanized from severe weight loss. We postulate that the drug delivery vehicle - composed of Polyethylene glycol (PEG) 300 (25 % w/w), glycofurol (25 % w/w), cremophor ELP (25 % w/w), 200 proof ethanol (15 % w/w), and propylene glycol (10 % w/w) - was toxic to the db/db mice. This is further supported by the observation that none of the db/+ mice showed any signs of toxicity from the vehicle. Due to this unfortunate outcome with the db/db vehicle control group, we used historical data from age-matched db/db mice to gain preliminary insight into the efficacy of our compound. We found that db/db mice that received our compound had significantly lower ACR compared to age-matched mice. Interestingly, none of the mice that received 2726-7 showed any signs of toxicity, suggesting that treatment with this compound prevented vehicle-related toxicity observed in the vehicle-only db/db group, either directly or indirectly by treating lipid dysmetabolism in these mice. We believe that 2726-7 protects from the progression of diabetic kidney disease at least partially by protecting podocyte function, and that this attenuation likely contributed to rescuing the mice from possible vehicle-related toxicity.

An additional study is underway with vehicle-treated and saline-treated mice to further investigate the potential toxicity of the vehicle. If confirmed, the compound will be reformulated with a new vehicle, whose biocompatibility will be first verified in db/db mice (we previously did this in normal mice but have now learned that db/db can exhibit significantly lower tolerance to otherwise safe formulations due to their condition). We chose the current vehicle because it has been shown to be a good formulation for testing early-stage compounds with no published data to suggest that it has any adverse effects on mice. Nevertheless, if the vehicle proves toxic to db/db mice in the follow-up study, this will only underscore the therapeutic potential of our compound, as all db/db mice treated with 2726-7 survived to the endpoint and showed improvement in ACR with no signs of toxicity (normal weight gain, normal appearance, normal stools, etc).

3. Publications:

A manuscript will be submitted for publication in early 2023.