Identifying client proteins of the HSP70 co-chaperone network

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Introduction

Hypertrophic cardiomyopathy (HCM) is the most common genetically inherited cardiovascular disease that affects one in 500 of the general population.[1] HCM, a known cause of heart failure and sudden cardiac death, is characterized by left ventricular (LV) hypertrophy (>13 mm LV end diastolic wall thickness).[2] ~900 pathogenic variants have been identified in genes encoding eight sarcomere proteins within cardiomyocytes.[3] Variants in MYBPC3, which encodes the sarcomere protein called myosin binding protein-C (MyBP-C), are present in 50% of familial HCM cases.[4] The majority of MYBPC3 variants cause premature termination codons, triggering nonsense-mediated mRNA decay or degradation of truncated protein through the ubiquitin-proteasome system (UPS).[5] In hearts from HCM patients, truncated protein is undetectable and levels of wild-type (WT) MyBP-C are reduced by 24% compared to controls, suggesting a heterozygous MYBPC3 genotype that results in haploinsufficiency of MyBP-C.[6] However, haploinsufficiency is not observed in patient-derived human inducible pluripotent stem cell-derived cardiomyocyte (hiPSC-CM) lines, indicating a compensatory mechanism that could potentially be utilized therapeutically to restore levels of MyBP-C in HCM patients with a MYBPC3 pathogenic truncating variant. [7] That compensatory mechanism may be associated with the 70-kDa heat shock protein (HSP70) co-chaperone network, which plays an important role in protein homeostasis by recognizing client proteins for UPS-mediated degradation. MyBP-C is a known client of HSP70.[8] Thus, understanding the full range of HSP70 clientele and their interacting proteins is critical to identifying all the effects of modifying protein-protein interactions (PPIs) within the HSP70 co-chaperone network in attempts to restore MyBP-C levels in HCM patients with a MYBPC3 pathogenic truncating variant.

Aim 1: Identify likely additional clients of HSP70 by applying a chemical probe *in* vitro.
The chemical probe JG-231 was previously developed to act as an allosteric inhibitor of the PPI between HSP70 and one of the co-chaperone bcl2-associated athanogene 3 (BAG) proteins.[9] This helps push HSP70 to its ADP-bound state, in which it has high substrate affinity for its client protein–in this case MyBP-C–thus favoring the degradation of MyBP-C toward UPS-mediated degradation (**Fig 1**).[10] Preliminary data show that JG-231 reduces levels of MyBP-C via this mechanism *in* vitro. I hypothesize that after applying JG-231 *in vitro* to hiPSC-CMs, other sarcomere proteins besides MyBP-C, a known client, will be significantly increased or decreased. Such proteins can be considered likely additional client sarcomere proteins of HSP70.

Aim 2: Assess the efficacy of JG-231 to reduce MyBP-C levels *in vivo*.
Additionally, JG-231 has never been used *in vivo* before to monitor its effect on MyBP-C levels. Therefore, JG-231 was also assessed for its efficacy *in vivo* so that it can be used to identify alternative HSP70 clients in future experiments using animal models.

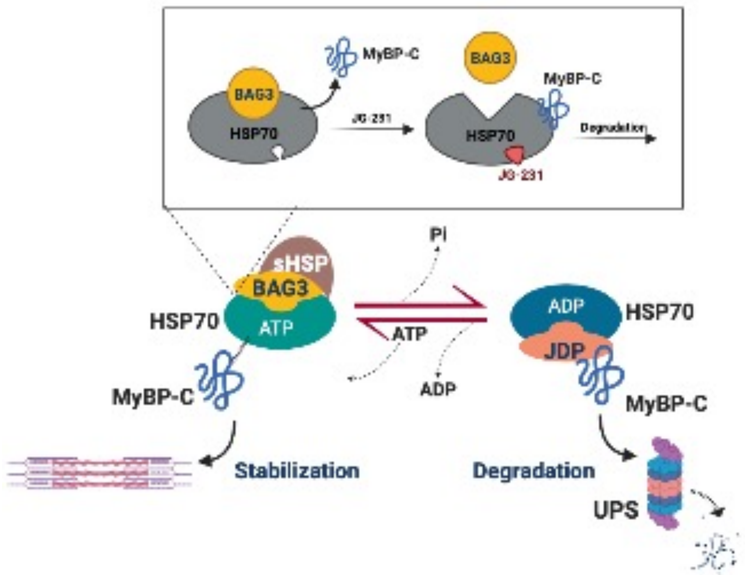

Fig 1 The HSP70 co-chaperone network and allosteric inhibition of the HSP70-BAG3 PPI by JG-231

Methods

Applying JG-231 *in vitro* to identify additional HSP70 clients

Varying concentrations of JG-231 were applied to WT hiPSC-CMs suspended in media. Dimethyl sulfoxide (DMSO) was also applied and served as a negative control. The treated cells incubated for 24 hours and then they were lysed and harvested. The lysate was measured using antibodies for cardiac troponin T (TNNT2), Filamin-C (FLNC), and myosin (**Fig 2**).[10] Relative protein abundance was measured using AlphaLISA immunoassays, which bind antibodies and beads to targeted protein analytes.[11] Those specific antibodies were quantified proteins of interest among several others since they yielded clearly-defined concentration-dependent curves when they were first measured on varying concentrations of untreated WT hiPSC-CMs and human heart tissue via AlphaLISA.

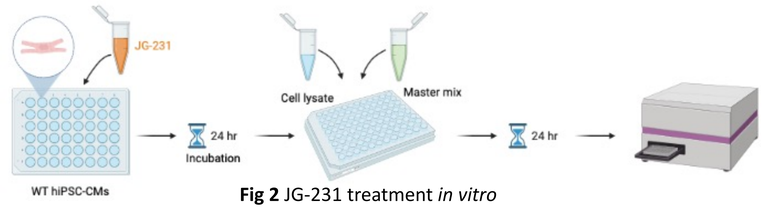

Fig 2 JG-231 treatment *in vitro*

Applying JG-231 *in vivo* to test for chemical efficacy

Five WT and seven heterozygous (for an MYBPC3 knock-in truncating variant) 12 week-old male and female mice were injected with JG-231 or a PBS/DMSO vehicle control. Preliminary data show that JG-231 derivatives are non-toxic in mice.[12] Mice were injected intraperitoneally (IP) three times a week for four weeks at a four mg/kg dosage. Hearts were harvested after four weeks of injections, homogenized, and tested for different protein levels at varying concentrations via AlphaLISA. It is important to note that ancestral mice from this line do not display haploinsufficiency.

Results

The results of JG-231 treatment *in vitro* showed that MyBP-C and TNNT2 had consistent concentration-dependent decreases relative to the DMSO vehicle control in abundance. Myosin and FLNC, however, increased until 1.25 or 2.5 uM [JG-231], respectively, and then decreased thereafter. The abundance of most proteins approaches 0% of DMSO past 5 uM of JG-231, suggesting that JG-231 may have toxic effects on the cells at high concentrations (**Fig 3**).

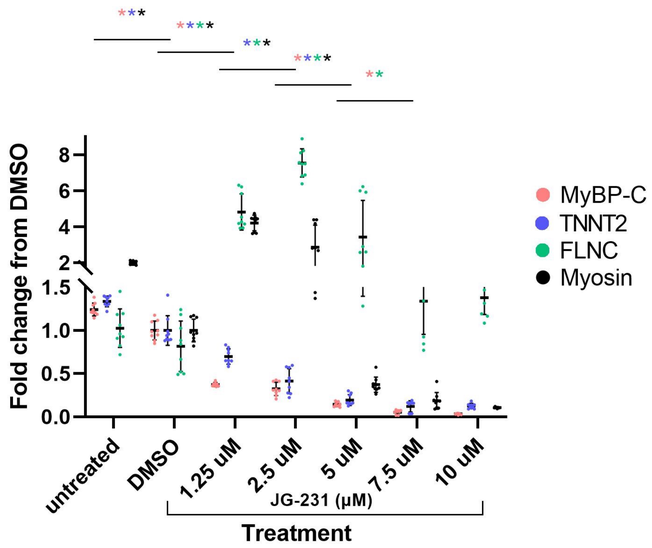

Fig 3 JG-231 treatment of WT hiPSC-CM; DMSO does not affect the cells and thus served as a negative control; * in the corresponding color of the protein signifies a significant difference in fold change from DMSO between two concentrations of JG-231 as determined by one-way ANOVA and post-hoc Tukey tests.

JG-231 did not significantly affect MyBP-C levels within the groups of WT or heterozygous truncating mice. Both genotypes also displayed similar responses to JG-231 (**Fig 4**).

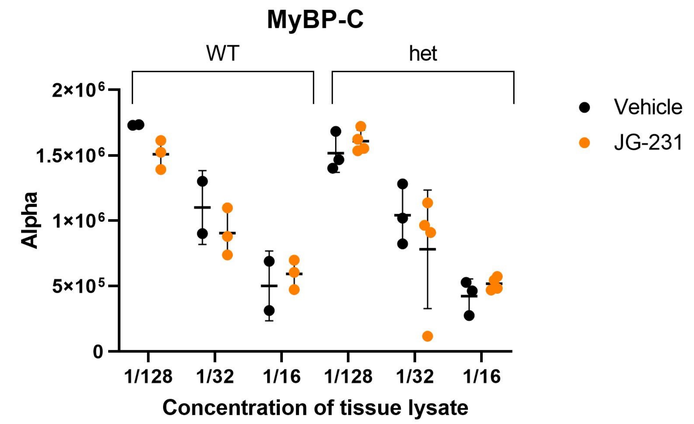

Fig 4 JG-231 applied *in vivo*; alpha represents relative protein abundance

Summary & Conclusions

Proteins that are significantly changed *in* vitro in response to JG-231 may be additional HSP70 clients that are degraded by HSP70. TNNT2 displayed a similar concentration-dependent decrease in response to JG-231 as MyBP-C, indicating that it may be an alternative client of HSP70. Although FLNC and myosin may also be alternative clients, it is unclear why they displayed an initial concentration-dependent increase. It is speculated that instead of facilitating FLNC and myosin's stabilization as seen in MyBP-C, BAG3 instead facilitates their degradation and thus they are stabilized by inhibiting the BAG3-HSP70 PPI, which is supported by the current literature.[13] The eventual decrease of all proteins may be due to toxicity effects of JG-231. TNNT2, FLNC, and myosin are all affected by JG-231 and thus are identified as candidates for further investigation; I am currently replicating the *in vitro* experiment in new cell lines and quantifying the cellular toxicity of JG-231. Other potential HSP70 client proteins may also be tested in future studies.

Applying JG-231 *in vivo* revealed that JG-231 has little to no effects on MyBP-C levels as it has been seen to have *in vitro*. In the context of a complex living organism, there may be compensatory mechanisms at play to restore levels of MyBP-C. Data is currently being analyzed to investigate if additional protein clients were affected *in vivo*. Studies in the near future will replicate the *in vivo* experiment using alternative dosages of JG-231.

This project helped strengthen our understanding of the HSP70 co-chaperone network, which informs future research for developing a therapy for restoring MyBP-C levels in patients with a MYBPC3 pathogenic truncating variant.

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