Online Supplement to "Treatments targeting inotropy" by Maack et al.

Additional aspects to β-AR agonists

The treatment of patients with HF with β-blockers improves symptoms, LV function and survival and has therefore become a cornerstone of HF therapy.[1] β-Blockers upregulate and resensitize β-ARs to endogenous and/or exogenous catecholamines. However, while this holds true for metoprolol (and presumably also bisoprolol), carvedilol fails to upregulate β-AR density[2] but nevertheless restores β-AR efficacy.[3] In patients with HF chronically treated with metoprolol, the potency and efficacy of the dobutamine-induced increase in cardiac output is maintained or even improved despite competitive antagonism of metoprolol with dobutamine at β_1-ARs.[4, 5] This is explained most likely by the concomitant resensitization and upregulation of β_1-ARs which appears to offset competitive antagonism of dobutamine-induced hemodynamic responses via β_1-ARs. In contrast, dobutamine-induced increases in cardiac output are blunted during treatment with carvedilol,[4] which may be explained by *i)* lack of upregulation of β_1-ARs by carvedilol and *ii)* irreversible binding of carvedilol to β-ARs which extends β-AR blockade beyond the elimination of carvedilol from the plasma.[6]

β_3-ARs are expressed in human cardiac myocytes and are resistant to homologous desensitization, but mediate effects that are antipathetic to β_1-ARs[7] and even reinforce the effects of β_1-ARs blockers;[8] they also exert antioxidant effects[9] and activate cGMP production, with potential beneficial effects on myocardial remodelling and LV function which are being tested clinically (ISRCTN 65055502).

Additional aspects to Levosimendan

A common alternative explanation why levosimendan does not prolong relaxation (other than through PDE3-inhibition) is that levosimendan´s binding to troponin C is Ca^{2+}-dependent.[10-15] It has been proposed that when cytosolic Ca^{2+} decreases during diastole, the Ca^{2+}-sensitizing effect by levosimendan (which would prolong relaxation) may disappear due to dissociation of levosimendan from troponin C.[10-15] These assumptions rely on High Performance Liquid Affinity Chromatography (HPLAC) experiments, in which, however, the retention time of levosimendan at the troponin C-HPLAC column was prolonged from ~5.5 to 9 minutes when Ca^{2+} was elevated from 100 µM to 30 mM.[10] In fact, levosimendan binds to troponin C covalently at millimolar Ca^{2+} and levosimendan concentrations, and it took several hours to resolve this covalent binding.[15]

Based on these results, it is currently unclear – if not rather unlikely – whether levosimendan can bind and unbind troponin C on a beat-to-beat basis in a millisecond and micromolar range, as would be required to explain the lack of relaxation prolongation by this mechanism. However, in a recent study using a fluorescence-based *in vitro* assay, the rates of Ca^{2+} dissociation-induced structural changes in cardiac troponin C were not slowed by levosimendan, suggesting that even in the absence of PDE3-inhibitory effects, relaxation may not be negatively affected by the mode of interaction of levosimendan with troponin C.[16] Further research is needed to fully understand the mechanisms of interaction between levosimendan and troponin C and how this affects cross-bridge kinetics.

Besides its effects on EC coupling, levosimendan also activates glibenclamide-sensitive *sarcolemmal* ATP-dependent K^+-currents (I_{KATP}), which may add to its vasodilating activity. However, the EC_{50} in ventricular cardiac myocytes is 4.7 µM,[17] i.e. I_{KATP} was activated at >100-fold higher concentrations than PDE3 inhibition. Furthermore, I_{KATP} does not play a role in regulating heart rate and force under normal, non-ischemic conditions.[18] Recent work suggested much more potent I_{KATP}-dependent effects in cremaster arterioles,[19] suggesting that this mechanism does play a role for vasodilation, at least in some vascular beds. However, PDE inhibitors also exert profound cAMP-dependent vasorelaxation (therefore the term “inodilator”). Levosimendan also activates *mitochondrial* K_{ATP}-channels,[20, 21] which play an important role in myocardial protection during ischemia/reperfusion in the context of pharmacological or ischemic “pre-“ and/or “post-conditioning”.[22, 23] Taken together, the cardiovascular profile of levosimendan is determined by a balanced effect on myofilament Ca^{2+} sensitivity (heart), PDE3-inhibition and sarcolemmal and mitochondrial I_{KATP} activation (heart and blood vessels, respectively). It is historically interesting to note that the PDE-inhibitory effect of levosimendan has been and still is largely ignored. This is unfortunate, since the dual (or even triple) mechanism of action on the heart (**Figure 5**) may be actually quite favourable in patients with HF.

Small-molecules targeting motor proteins

In patients with hereditary cardiomyopathies, disease-causing mutations frequently affect genes encoding sarcomeric proteins.[24, 25] In patients with HCM, these mutations typically increase myofilament Ca^{2+} sensitivity and force generation, while mutations occurring in DCM rather decrease Ca^{2+} sensitivity and force generation.[24, 25] The time-force integral as an important consequence of these mutations determines the type of remodeling (concentric versus eccentric

hypertrophy and dilation) through differential downstream activation of the mitogen-activated protein (MAP) kinase ERK1/2.[26] Accordingly, reducing myosin ATPase and force generation with a novel small-molecule inhibitor, MYK-461, reduced LV hypertrophy, fibrosis and maladaptive genomic remodeling in an animal model of HCM.[27] Meanwhile, MYK-461 obtained orphan drug status for obstructive HCM from the FDA in the United States. A phase 2 open-label pilot study (PIONEER-HCM) is currently evaluating the efficacy, pharmacokinetics, pharmacodynamics, safety, and tolerability of MYK-461 in subjects with symptomatic HCM and left ventricular outflow tract obstruction (https://clinicaltrials.gov/ct2/show/NCT02842242). Conversely, in an animal models of DCM with *reduced* myofilament Ca^{2+} affinity, treatment with the Ca^{2+} sensitizer pimobendan improved LV function and remodeling.[28] Therefore, therapies that increase myosin motor activity and/or Ca^{2+}-affinity may be of interest in the treatment of patients with DCM.

Another frequent effect of sarcomeric gene mutations is an accompanying reduction in thermal stability of myofilaments and tolerance against mechanical and oxidative stress. The resulting increase in the abundance of misfolded myosin may play an important role in the pathophysiology of cardiac diseases leading to HF.[29, 30] Here, the restoration of normal myosin homeostasis by pharmacological chaperones might provide an efficient means to interfere with the progression of HF. Re-evaluation of the molecular mechanism underlying the actions of the Ca^{2+} sensitizer EMD-57033 revealed its direct interaction with the myosin motor domain leading to activation of motor activity, increased thermal stability, and refolding of heat-inactivated β-cardiac myosin. Moreover, addition of EMD-57033 to heat-stressed cardiomyocytes suppresses the expression of the stress marker atrial natriuretic peptide.[31] The observed Ca^{2+} sensitization is predominantly prompted by EMD-57033-induced changes in myosin cross-bridge kinetics,[32, 33] which interfere with normal troponin-tropomyosin regulation.[34, 35] EMD-57033 shares the myosin-mediated modulation of Ca^{2+}-sensitivity with OM, and it binds to the same region of the myosin motor domain near the base of the lever arm and the N-terminal SH3-like subdomain.[31, 36] OM, however, does not share the pharmacological chaperone and modest PDE3-inhibitor co-activities displayed by EMD-57033.

The availability of a 2.6 Å structure of the human β-cardiac myosin motor domain (PDB code 4DB1) and detailed information of the binding sites and mode of action of small allosteric modulators of myosin activity expedite the use of rational drug-design approaches to generate myosin modulators with increased specificity for β-cardiac myosin and free of undesired co-activities, and have an optimized mode of action.[37] The rapid growth of computer processing power and the availability of suitable software application for *in silico* docking analysis and

pharmacophore modeling greatly facilitate the screening of large numbers of compounds from different compound classes. The highest scoring compounds are synthesized and their effectiveness is validated in assays that preserve the proper allostery of the cardiac actomyosin system. Here, the correct combination of human cardiac myosin, actin, tropomyosin, and troponin isoforms is required to study the consequences brought about by allosteric perturbations such as disease causing mutations, the binding of small molecules at an allosteric site, or combinations of both.

Together, the results obtained with EMD-57033 and OM indicate that development of new therapeutic small-molecule effectors can be targeted towards allosteric activators that augment systolic function. However, as discussed above, the consequences for diastolic function need to be critically considered.[31, 35, 36, 38] This strategy can be further developed in personalized medicine, thereby designing the exact compound needed to treat genetic and non-genetic forms of cardiac disease that involve allosteric modulation of important contractile proteins.

SERCA2a Gene Therapy

A common finding in all forms of human HF and preclinical HF models is the reduced kinetics of Ca^{2+} uptake from the cytoplasm into the SR (**Figure 1**).[39, 40] This reflects reduced gene and protein expression of SERCA2a, and reduced enzymatic activity due to abnormal post-translational modifications.[41-43] The direct sequelae of reduced SERCA2a activity are delayed clearance of cytoplasmic Ca^{2+} and abnormally high end-diastolic Ca^{2+} levels, leading to impaired myocardial relaxation.[40] Reduced SERCA2a activity also reduces SR Ca^{2+} load available for the next contraction, thereby limiting inotropic potential. The elevated diastolic Ca^{2+} also activates numerous maladaptive pathways and influences organelle function sensitive to Ca^{2+}, e.g. nuclear gene expression.

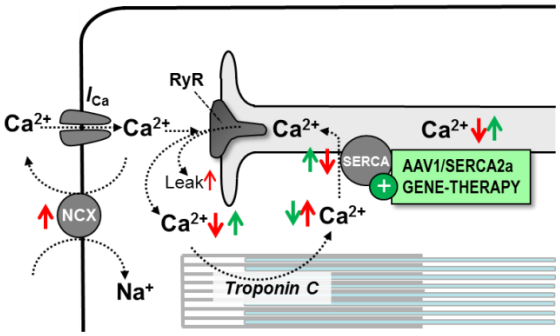

Figure S1: Concept of AAV1/SERCA2a gene therapy in patients with HF. By increasing SERCA2a mRNA and protein levels, increased SR Ca^{2+} uptake should increase systolic Ca^{2+} release from the SR and decrease diastolic Ca^{2+} levels in the cytosol, thereby improving systolic and diastolic function.

RyR, ryanodine receptor; NCX, Na^+/Ca^{2+} exchanger.

Small molecule approaches to stimulate SERCA2a activity in HF have hitherto been unsuccessful. In contrast, over the last 15 years, gene transfer of SERCA2a has evolved as a potentially effective technology to improve the contraction and relaxation of the failing heart in numerous preclinical HF models and isolated failing human cardiomyocytes (**Figure S1**).[44-47] In contrast to traditional positive inotropes discussed in the main manuscript, these beneficial effects upon contraction (inotropy) and relaxation occur in an energetically favourable manner, and also appear to reverse-remodel the pro-arrhythmic substrate of the failing heart via multiple mechanisms.[48, 49] There is also evidence of genetic reverse remodeling with partial or complete correction of the abnormal transcriptome[50] and miRome[51] observed in the failing heart, and microarchitectural reverse remodeling of the structural changes observed at the nanoscale.[52] The cumulative effect of all these changes is improvement in function and survival in a number of small and large animal preclinical models.

Based on these preclinical foundations, SERCA2a gene therapy was developed in a clinical trial programme pioneered by Roger Hajjar and colleagues. Clinical gene therapy utilizes the adeno-associated virus serotype 1 (AAV1) expressing the human SERCA2a gene under the control of the CMV promoter. AAV vectors are an attractive option for clinical cardiac gene therapy as they are non-pathogenic, deliver long lasting expression kinetics following a single infusion/injection and AAVs have relatively high tropism for the myocardium, allowing effective therapeutic gene expression which was previously a challenge with other viral and non-viral vectors.[53] A disadvantage of AAV gene delivery is broad immunization in target subjects, requiring comprehensive screening of HF patients for being AAV seronegative.

The CUPID trial program includes the first clinical gene therapy trials for HF, with the 3 year follow-up of the first 39 patients enrolled in the first phase 2a study, the CUPID 1 trial.[54, 55] The CUPID 1 trial has confirmed safety of this approach, at least in these small numbers of patients at 3 years, and some signals of potential efficacy. This lead to the larger phase 2b CUPID 2 trial, in which 243 patients with advanced HF from across Europe and the USA were treated with an intracoronary infusion of AAV1/SERCA2a or placebo.[56] Despite the promising results of the previous preclinical and clinical studies, however, AAV1/SERCA2a did not improve the primary endpoint of time to recurrent events, defined as hospital admission because of HF or ambulatory treatment for worsening HF.[56]

The underlying reasons for the neutral outcome of this study, despite solid preclinical evidence for efficacy are presently unclear. Most discussions focused on whether gene delivery had been

adequate in CUPID 2.[56, 57] The absolute levels of transgene detected in failing human hearts from the CUPID programme were low compared to the preclinical models, potentially reflecting both a low percentage of cardiomyocytes transduced, and low levels of SERCA2a in those with transgene. However this may be biased by the fact that heart samples were obtained from patients who deteriorated and proceeded to LVAD implantation or cardiac transplantation, who could be considered as 'non-responders'.

There are many factors which may have caused ineffective gene delivery, including the relatively low dose compared to other clinical AAV gene therapy trials and mode of infusion. When producing recombinant AAVs, not only full viruses, but also empty capsids or virus-like particles are generated. These resemble virus protein shells without the vector genome and were thought to interfere with virus transduction by competing for cell-surface receptors and contribute to antigen load, which may elicit a stronger immune response *in vivo*.[58] Therefore, in CUPID 2 the amount of empty viruses and the total particle load (but not of active virus) was reduced compared to CUPID 1.[55, 56] More recently, however, it was discovered that these empty virus capsids may adsorb neutralizing antibodies and thereby increase transfection efficiency.[59] Therefore, it remains open whether the lower total particle dose and in particular, the reduced number of empty virus capsids in CUPID 2 may have impaired virus transduction compared to CUPID 1.[56] The SERCA-LVAD trial (NCT00534703) is designed to prospectively measure SERCA2a gene delivery to the myocardium in all patients recruited, including those with neutralizing antibodies (excluded from the CUPID trials), and the results are awaited. An additional factor is that even if the gene is delivered effectively, post-transcriptional and post-translational factors may limit the production of SERCA2a protein and increased enzymatic activity which is the desired cellular effect of SERCA2a gene therapy.

It should be borne in mind though that the positive results of CUPID 1 in the high virus dose group were obtained in nine patients with virus application versus 14 patients randomized to placebo,[55] which may not exclude the play of chance in this small study population. What has been surprisingly less discussed is whether gene-therapy upregulating SERCA2a mRNA in patients with HF who are treated with guideline-recommended drugs is indeed a promising approach *per se*. Lowes et al.[60] observed that in patients with systolic HF who responded to the initiation of β-blocker treatment with an increase in LVEF by more than 5 absolute percent (26/32 patients), SERCA2a mRNA was upregulated 10-fold more than β_1-AR mRNA, suggesting that at least to some extent, β-blockers may improve LVEF in the long run by restoring defective Ca^{2+} handling by SERCA2a re-upregulation in failing hearts. In CUPID 2, 97% of patients receiving

AAV1/SERCA2a were treated with β-blockers, but only 6/9 patients (67%) in CUPID 1. However, these patients had disease progression with a reduced LVEF and high BNP or NT-proBNP despite β-blockers, in contrast to the responders reported by Lowes et al.,[60] in whom increased SERCA2a could be a surrogate of recovery. Therefore, it remains to be determined whether SERCA2a expression is still reduced in HF patients who have refractory HF which progresses despite β-blockers.

Despite its neutral outcome, CUPID 2 will hopefully stimulate further research into gene therapies.[57] The kinetics of AAV gene expression (2-4 weeks to achieve peak expression) are such that this will not be an option for acute HF, but this research field provides potential insights in how to achieve the optimal characteristics for a novel HF therapeutic.

Istaroxime

Istaroxime was designed in search of a digitalis-like drug with a safer profile. With a similar inhibitory effect on Na^+/K^+-ATPase and thus, intracellular Ca^{2+}, istaroxime also activates SERCA activity and thereby Ca^{2+} uptake into the SR, whereas digoxin increases SR Ca^{2+} leak.[61] Consequently, istaroxime has positive inotropic and lusitropic effects (accelerating relaxation) without increasing arrhythmias or altering heart rate or conduction in experimental HF models and human failing muscle strips.[62, 63] In the phase II randomized and controlled HORIZON-HF trial on 120 patients hospitalized for HF (EF≤35%), 6 hrs of istaroxime i.v. treatment increased systolic blood pressure and cardiac index and improved diastolic function and stiffness, associated with a reduction in pulmonary capillary wedge pressure (PCWP) and heart rate.[64, 65] In an ongoing trial, the effect of 24 hrs infusion of 2 different doses of istaroxime on cardiac hemodynamics and safety parameters will be assessed in 120 patients with acute decompensated heart failure, with an estimated completion in October 2018 (NCT02617446).

EF-hand Ca^{2+} binding motifs

About 50% of patients with HF have preserved ejection fraction (HFpEF), but impaired diastolic function. The mechanisms of diastolic dysfunction in patients with HFpEF are incompletely resolved, but may comprise alterations of the extracellular matrix (e.g., fibrosis), sarcomeres (post-translational modifications) and deranged Ca^{2+} and Na^+ handling.[66, 67] To improve cytosolic Ca^{2+} handling during diastole, a novel EF-hand Ca^{2+} binding motif was designed that facilitates fast Ca^{2+} transient decay while maintaining Ca^{2+} transient peak amplitude.[68] Mechanistically, these EF-hand motifs have increased Mg^{2+} affinity and decreased Ca^{2+} affinity relative to native

EF-hand motifs, thus delaying Ca^{2+} buffering until diastole. This corrects inherent physiological defects that have limited the use of native Ca^{2+} binding motifs to form optimized physiological delayed buffers to accomplish rapid cardiac relaxation. It was recently observed that by changing Ca^{2+} and Mg^{2+} buffering capacities of the EF-hand motif archetype protein, parvalbumin, not only accelerated cytosolic Ca^{2+} decay, but even increased the amplitude of cytosolic Ca^{2+} transients.[69] Therefore, this type of intervention could become useful to optimize Ca^{2+} handling not only in HFpEF, but also HF with reduced ejection fraction (HFrEF). Presently, however, no clinical trials with this approach are ongoing.

Targeting mitochondria – an alternative inotropic intervention?

Since mitochondrial ROS production is thought to be a key factor for the development and progression of HF, various strategies were developed to reduce oxidative stress in mitochondria.[70] One of these strategies is a mitochondrial-targeted tetrapeptide, SS-31, which binds to cardiolipin, an essential component of the inner mitochondrial membrane that is responsible for cristae formation and optimal assembly of the complexes of the respiratory chain to so-called "super-complexes".[71] SS-31 protects cardiolipin from damage by oxidative stress and thereby maintains proper function of the respiratory chain, avoiding aberrant slippage of electrons to O_2 to produce ROS.[71] In fact, SS-31 prevented the development of systolic and diastolic HF in various rodent HF models.[72-74] Recently, a study using SS-31 (syn.: MTP-131 or Elamipretide, formerly known as Bendavia) in a dog model of systolic HF revealed that this peptide not only improved systolic function in the long term (i.e., after 3 months of treatment), but also in the short-term. A 48-hour treatment with SS-31 increased LVEF and stroke volume and decreased end-systolic volume, with no changes of heart rate or systemic vascular resistance.[75] In fact, the increase in cardiac output by SS-31 (+25%) was comparable to the effect of omecamtiv mecarbil in a similar dog model of HF (+22% and +29%, respectively; **Table 3**).[36, 76] Since the failing heart is considered an "engine out of fuel",[77] and cardiac contractility – at least during stress situations – thought to be limited by ATP shortage,[78, 79] it may be plausible that improving mitochondrial function could improve contractility even acutely. Further research is needed to support these observations. SS-31 is currently tested in clinical phase II studies in patients with stable systolic HF ((NCT02788747) and decompensated HFrEF with hospitalization and fluid congestion (NCT02914665), respectively. In addition, improving energetic substrate utilization may have beneficial effects, as first clinical and experimental data have shown.[80, 81]

Along similar lines, drugs that target substrate metabolism of mitochondria have also shown positive effects in patients with HF. The putative mechanism of action of these drugs is inhibition of fatty acid utilization, facilitating a shift towards glucose oxidation which requires less oxygen per ATP yield. Trimetazidine, which inhibits β-oxidation of fatty acids, increased LVEF after short- (15 days) and long-term treatment in patients with ischemic cardiomyopathy[82] and improved phosphocreatine/ATP ratios after 90 days of treatment.[83] A meta-analysis of trials involving a total of 884 patients revealed that trimetazidine reduced hospitalization and NT-proBNP levels, reverse-remodeled the LV and improved symptoms, hospitalization, but not survival of patients with HF.[84] Perhexilline, which putatively inhibits uptake of fatty acids into mitochondria, improved LVEF, maximal oxygen consumption (VO_2max), resting and peak stress myocardial function, symptoms and energetics of cardiac and skeletal muscles in HF patients.[85, 86] These effects, however, appear to be independent of any switch of substrate utilization and therefore, the exact mechanism of this drug is still elusive.[85, 87] Although larger phase III clinical trials with these compounds are currently not available and not underway, these data nevertheless support the concept that improving mitochondrial energetics may be an alternative strategy to improve cardiac function with rather positive effects also on long-term outcome.

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