Modulators of proteostasis: therapeutic targets and diagnostic markers to halt and reverse atrial fibrillation

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Chapter 15

General discussion and future perspectives

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State of the art on atrial fibrillation

AF is the most common age-related cardiac arrhythmia accounting for one-third of the hospitalizations for arrhythmia disorders with an annual cost of 13 billion euro in the European Union and an alarming growing incidence due to the Western life-style [1, 2]. With the present trend, almost 30 million North Americans and Europeans will be affected with AF by 2050 [3]. AF is a progressive disease and is associated with substantial morbidity and mortality, with stroke, myocardial infarction, and heart failure being the most critical complications [2, 4-6]. AF is considered as an 'electrical' disease, and the current treatment is directed at the electrical refractoriness of the atria [7], however does not halt or cure the disease. Therefore, research should be aimed at unraveling of the root causes of AF which may lead to novel therapies directed at these root causes.

Emerging evidence indicates that the so called electropathology represents a root cause of AF. Electropathology consists of structural damage in the atrial myocardium which underlies electrical conduction abnormalities. The degree of electropathology is related to the stage of AF and becomes more severe over time [8-11]. Despite the efforts of changing electrical refractoriness of atrial cardiomyocytes with the current treatment, the therapies do not affect structural damage and therefore, damage accumulates in the cardiomyocytes resulting in further loss of sarcomeres (myolysis), fragmentation of the sarcoplasmic reticulum and change in mitochondria shape [12], which drive progression of the arrhythmia. One can imagine that excitation-to-contraction coupling becomes more and more difficult with the accumulation of structural damage and, as such, also impairs electrical conduction [13, 14]. The deterioration of the cardiomyocytes explains the progression of the disease, and why AF recurs in at least 50% of the patients within 12 months after treatment [15, 16]. Obviously, there is an urgent need for improvement of AF therapies, that aim to halt and reverse the structural damage in AF patients.

Treatment challenges in atrial fibrillation: identification of novel druggable targets

RhoA pathway

Previous research demonstrated that derailment of proteostasis – proteostasis being the homeostasis of proteins, regulating protein synthesis, folding, trafficking, disaggregation and degradation in cells – plays a key role in structural damage in AF [2, 17]. Molecular chaperones, like HSPs, are essential in maintaining proteostasis – by assisting the correct folding of new proteins, refolding of misfolded proteins, detaching aggregated proteins and removing toxic proteins to ensure correct function – which underlies a healthy cell [18]. Previous research showed exhaustion of HSPs in patients with persistent AF and lower levels of HSPs correlated with a higher degree of myolysis [11]. Several pathways are involved in the failing heat shock response, among which is the RhoA stress signaling pathway (Figure 1), which is activated during AF progression [19, 20]. Pathological RhoA activation has been found to suppress the cardio-protective heat shock response in HL-1 atrial cardiomyocytes by impairing the binding of heat shock factor 1 (HSF1) to the heat shock element in the promotor sequence of the *hsp* genes [21]. Pre- or post-treatment with the potent HSP-booster geranylgeranylacetone (GGA) could not overcome the HSPA1A-suppressive effect of RhoA activation. Genetic inhibition of RhoA, however, boosted the heat shock response. The mechanism behind the prohibition of RhoA for HSF1 to bind the HSE needs to be further investigated, but this pathway opens novel insights in potential druggable targets within the RhoA pathway which increase cardio-protective HSP levels in AF.

Table 1. **Key pathways underlying structural damage in AF**

Pathway	Target	Drug	Biomarker
RhoA	RhoA	CCG-1423 [52] Rhosin [53]	unknown
Heat shock response	HSPs	GGA, GGA*-59 [29, 30, 39]	HSPB1 [15, 16]
Mitochondria	mitochondrial Ca^{2+} uniporter	Ru360 [41]	Serum cfc-mtDNA [51]
	electron transport system	SS31 peptide [41]	
	fragmentation (fission) mitochondria	mdivi-1 [54]	

Heat shock response

Priming the heat shock response (Figure 1) in cardiomyocytes by pharmacological pretreatment with GGA or genetic overexpression of HSPB1 is an established intervention in experimental models for AF to maintain proper function after tachypacing and protect against structural damage [11, 19, 22-26]. As genetic manipulation in humans is not obvious, pharmacological halting of existing structural damage and contractile dysfunction to prevent AF progression or even reversing it, would be clinically highly relevant. Notwithstanding GGA's protective effects, the poor physicochemical properties of GGA, including its lipophilic nature and limited solubility, pose a serious disadvantage to its druggability in AF. The gut mucosal distribution pattern owing to GGA's hydrophobic character hinders its systemic bioavailability [27, 28], probably needing high dosages to treat AF. To overcome these disadvantages, various GGA-derivatives with improved physicochemical properties, compared to GGA, were synthesized and tested for their ability to induce HSPs in HL-1 cardiomyocytes [29]. The best HSP inducers were tested for their protection against tachypacing-induced calcium transient loss in HL-1 atrial cardiomyocytes. The cardio-protective actions of these HSP inducers were HSPB1 dependent, and also protected against tachypacing-induced heart wall dysfunction in *Drosophilas.* Subsequently, GGA and GGA-derivatives were tested in the newly developed AF-reversibility model [30]. In this model, GGA and three GGA-derivatives, of which GGA*-59 was superior, showed a significant restoration from tachypacing-induced calcium transient loss in HL-1 cardiomyocytes. GGA*-59 likely confers its protective and recovery effects on contractile dysfunction by enhancing HSF1 hyperphosphorylation with the subsequent induction of cardio-protective HSP expression. How GGA and GGA-derivatives prolong hyperphosphorylation needs to be further elucidated. There may be a role for RhoA herein, as post-translational prenylation with C15 (farnesyl) or C20 (geranylgeranyl) isoprenoids mediates translocation of RhoA to the plasma membrane resulting in activation of the downstream RhoA signaling pathway [31-33]. GGA and GGA-derivatives may compete with endogenous geranyl-groups, which could lead to inhibition of physiological RhoA activation, resulting in enhanced binding of HSF1 to the HSE in the promotor region of *hsp* genes. That GGA pre- and post-

treatment could not overrule RhoA activation by calpeptin and subsequent HSPA1 depression in the study of Meijering, et al. [21], might be attributed to the timing of GGA treatment or due to the extent of artificial RhoA activation by calpeptin, which may be greater than during physiological occurring RhoA activation. Yet, the effect of GGA on RhoA needs further investigation by genetic ablation, competition and enhanced binding experiments to elucidate its exact role.

The protective actions by GGA and GGA-derivatives seems critically dependent on boosting of HSPB1 expression, as their protective effect was abrogated by siRNA against HSPB1 [22, 29]. HSPB1 (co)-localize at the myofilaments, stabilizing the sarcomeric proteins, including alpha-actinin, actin and myosin [11, 34, 35]. Thereby, HSPB1 may shield the contractile proteins from AF-induced cleavage by cysteine proteases, such as calpain [26, 36, 37]. Given that GGA-derivatives enhance HSP expression and protect from tachypacing-induced contractile dysfunction, which was abrogated after suppression of HSPB1, we appoint HSPB1 to be one of the most important players in the cardio-protective effect of GGA-derivatives. In addition, GGA*-59 and HSPB1 itself are also able to accelerate recovery from tachypacing-induced structural changes [30]. HL-1 cardiomyocytes treated with GGA*-59 or recombinant HSPB1 (rcHSPB1) after tachypacing revealed increased levels of HSPB1 expression. GGA*-59 and rcHSPB1 accelerated recovery from tachypacing-induced calcium transient loss and restored mRNA and protein levels of (acetylated) α-tubulin and sarcomeric protein levels of cardiac troponin I and troponin T. Furthermore, GGA*-59 enhanced recovery of depolymerized (acetylated) α-tubulin fractions, which coincides with elevated HSPB1 binding. GGA*-59 treatment did not change tachypacing-induced calpain activity, but did normalize tachypacing-induced HDAC6 activity. These findings point to HSPB1 protecting the microtubule network, possibly by direct binding of HSPB1 to HDAC6 [38], suppressing HDAC6's activity and preventing deacetylation, depolymerization and subsequent degradation of α-tubulin by calpain [30]. Moreover, the HSP inducer GGA*-59 and recombinant HSPB1 accelerate recovery from tachypacing-induced structural damage and contractile dysfunction in HL-1 cardiomyocytes, indicating that HSP induction is an interesting target to potentially reverse AF-induced remodeling. One feature which makes GGA and GGA-derivatives interesting as

drugs, is that they boost the HSR in cardiomyocytes upon stress (when pre-treated with a mild, sub-lethal HS or after tachypacing) and not under non-stressed conditions [29], indicating that augmentation of the HSR by GGA and its derivatives is confined to stressed cells. In clinical perspective, this might indicate that side effects due to enhanced HSR are limited, if existent at all. Accordingly, it is of interest to further explore the mechanism of GGA*-59 in *in vivo* models, for example *Drosophilas* and atrial pacemaker stimulated dog models for AF, and investigate its pharmacokinetics, pharmacodynamics and safety for eventual future applications in the clinic.

Yet, despite its less favorable physicochemical therapeutic profile, GGA is already marketed in various Asian countries and considered a safe drug. Therefore, GGA itself may already be further explored in clinical AF. Since it was unknown whether GGA can induce HSPs in the human heart, a recent study obtained proof of concept for its HSP-inducing effect in the human heart [39]. Three days of oral GGA treatment, prior to coronary artery bypass grafting surgery of patients with coronary artery disease, associated with higher HSPB1 and HSPA1 expression levels in right and left atrial appendages and higher HSPB1 levels present at the myofilaments. These findings pave the way to a further study on the role of GGA and/or GGA-derivatives as a protective compound in experimental and clinical (post-operative) AF.

Mitochondria

Recent research findings indicate that mitochondrial dysfunction plays a role in AF (Figure 1) [40, 41]. This was demonstrated in tachypaced HL-1 atrial cardiomyocytes and atrial appendages of AF patients, by increased (transcription of) mitochondrial stress chaperones, including HSPD1, fragmentation of the mitochondrial network and aberrant ATP levels [41]. Inhibition of the mitochondrial Ca^{2+} uniporter with Ru360, prevented tachypacing-induced mitochondrial calcium transient loss in HL-1 cardiomyocytes and decreased heart wall contractions in *Drosophilas*, normalized cellular ATP levels and protected the mitochondrial network from tachypacing-induced fragmentation, but did not

normalize transcription levels of HSPD1 and HSPE1 in HL-1 cardiomyocytes. The rationale behind these results is that AF-induced calcium overload in cardiomyocytes is partially buffered by the mitochondria. However, extended periods of mitochondrial Ca^{2+}-buffering reduce mitochondrial Ca^{2+} uptake, which, in turn, negatively affects mitochondrial respiration [42]. These findings of altered ATP levels in AF patients suggest an initial compensatory mechanism to sustain the high heart rate of AF, which gets exhausted when AF persists for a longer period of time. Therefore, targeting mitochondria may represent a novel therapeutic strategy to counteract AF-induced mitochondrial dysfunction, as well as other cardiac diseases, including heart failure, myocardial infarction, ischemic heart disease, dilated cardiomyopathy, diabetic cardiomyopathy and hypertension-induced cardiomyopathy, which are also associated with mitochondrial dysfunction [43, 44]. In addition, to fuel future mitochondrial drug studies, measurement of the degree of mitochondrial damage in serum samples may aid in patient-tailored therapy.

Novel mechanism-based biomarkers to aid in AF patient-tailored therapy

The current clinical staging of AF is inaccurate. AF is diagnosed with a surface electrocardiogram when a patient already suffers from AF, presenting with palpitations or thromboembolic complications. Subsequent staging of AF is based on (symptomatic) duration of AF. Diagnosis and staging of AF is, logically, challenging in patients with asymptomatic or very short-lasting episodes of AF. Moreover, the current staging of AF is not related to the degree of electropathology: the amount of electrical ànd structural changes in the atria. Current therapy is aimed at alleviation of electrical refractoriness, but as cardiomyocytes contain and accumulate sustainable structural damage, the electrical conduction and excitation-to-contraction coupling is impaired and hamper functional recovery to sinus rhythm, especially in the more advanced stages of the disease where there is more damage. The current therapy is symptomatic, and therefore unable to cure the disease. AF diagnosis and staging using mechanism-based biomarkers may guide patient tailored therapy.

Heat shock proteins as biomarker in AF

One such marker may represent HSPs. Therefore, HSP levels in serum and atrial tissue samples of patients with and without AF were enrolled in the Halt & Reverse trial [45]. This trial investigates whether HSP levels in serum and atrial tissue discriminate between control and AF (stage) and/or predict clinical outcome after AF treatment. Serum HSPB1, HSPA1, HSPB7 and HSPD1 levels of controls and patients with symptomatic AF, measured at baseline prior to electrical cardioversion (ECV) or pulmonary vein isolation (PVI) to treat AF, did not correlate to the presence or stage of AF, compared to control, or to AF recurrence. However, HSPB1 levels were increased in follow-up serum samples received from PVI patients at 3, 6 and 12 months, who developed AF recurrence within one year post-PVI. These results suggest that HSPB1 levels may be useful in predicting recurrence of AF after ablative therapy. Whether serum HSPB1 levels can also be used to select patients for HSP-inducing therapy is not known yet, but a recent study reveals that AF patients with high HSP levels at baseline and who underwent treatment for 3 months with the HSP-inducing compound L-glutamine, show normalization of HSP and metabolite levels compared to patients with low HSP levels at baseline, indicating that serum HSPB1 levels may guide patient-tailored therapy [46].

The role of HSP levels in serum and atrial tissue was further expanded in AF and control patients undergoing elective cardiothoracic surgery for either coronary artery bypass grafting and/or mitral valve surgery and/or aortic valve surgery and/or first correction of a congenital heart defect (mostly atrial septal defects), combined or not with a Maze procedure (AF patients). Neither baseline nor follow-up serum HSP levels correlated to the presence of AF, compared to control, AF stage, post-operative AF (control patients) or AF recurrence in AF patients who underwent Maze treatment. Also tissue (RAA and LAA) levels of HSPB1, HSPA1, HSPB5 and pHSF1 were similar between control and AF groups. On the contrary, RAA HSPA5 levels were significantly lower in longstanding persistent AF and HSPD1 levels higher in persistent AF and in the total AF group compared to controls in sinus rhythm. Also, both HSPA1 and HSPA5 RAA levels were higher in control patients with post-operative AF, compared to patients without post-operative AF. Interestingly, in the group of AF patients who

received Maze treatment on top of the treatment for their underlying heart disease, HSPB1 RAA levels were significantly lower and HSPA5 LAA levels higher in patients with AF recurrence within 1 week after the surgery. These findings indicate that HSPA5 and HSPD1 atrial tissue levels are related to more persistent stages of AF, suggesting a role for endoplasmic reticulum and mitochondrial stress to drive AF progression. In addition, atrial tissue levels of HSPB1 are indicative for AF recurrence after AF-maze surgery and HSPA1 and HSPA5 are suggestive for the development of post-operative AF in control sinus rhythm patients. These observations indicate a role for myolysis, endoplasmic reticulum and mitochondrial stress to drive AF progression. Unfortunately, the atrial tissue levels did not correlate with the serum levels, indicating that in patients undergoing surgery, serum HSP levels cannot be used as a marker for AF.

The next step is to determine whether the HSP levels in serum and atrial tissue correlate with the degree of electrical conduction abnormalities in atrial appendages. It is conceivable that HSP levels correlate with the degree of electropathology. Therefore, further research is warranted to elucidate the correlation between HSP levels and electropathology (i.e. the actual AF stage) [47]. The exact role of HSP levels in the prediction of the stage of AF, duration of AF episodes and AF after treatment may be elucidated in future studies and also whether increased HSP levels are a result of AF or precedes AF (recurrence). Studies with longitudinal blood sampling (for HSP measurements) and continuous monitoring of electrical parameters may pin point possible associations.

Mitochondrial DNA as biomarker in AF

Cell-free circulating mitochondrial DNA (cfc-mtDNA) has been successfully used as a biomarker for conditions associated with mitochondrial dysfunction [48-50]. Cfc-mtDNA may represent an interesting biomarker to stage AF and predict AF recurrence. The level of cfc-mtDNA (COX3 and ND1) in serum was found to be associated with AF stage and with recurrence of AF, especially in male patients with paroxysmal AF undergoing ECV and PVI treatment, and not cardiac surgery [51]. In addition, increased levels of cfc-mtDNA in the medium of *in vitro* tachypaced HL-1 cardiomyocytes

were associated with enhanced mitochondrial damage and stress in these cardiomyocytes. AF may trigger the mtDNA release from atrial cardiomyocytes into the circulation upon mitochondrial damage, especially during the earlier stage of AF (paroxysmal AF), to become exhausted in the later stages, as observed in (longstanding) persistent AF patients. The applicability of cfc-mtDNA as a reliable biomarker for AF needs further exploration in future studies including larger patient populations.

Conclusion

In this thesis, a new part of the complex pathways underlying structural damage in AF has been unraveled. We identified RhoA, the heat shock response and mitochondrial dysfunction as key pathways driving AF. In addition, drugs with optimal physicochemical properties and directed at these key pathways halt and reverse AF, suggesting clinical applicability. Finally, modulators of these pathways, including HSPB1 and cfc-mtDNA may represent markers to identify the stage of AF and patients at risk to develop post-operative AF. Therefore, these findings may aid in the selection of mechanism-based patient tailored therapy.

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