Chapter 8

Summary, general discussion and future perspectives
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Atrial Fibrillation (AF) is the most common cardiac arrhythmia in the clinic. The prevalence of AF is related age, sex, race and geographic locations [1]. AF is rare in children and young adults, while it becomes more common in elderly people with age over 75 years old [2, 3]. In each age group, the prevalence of AF in female is lower than in male [3], and in the Netherlands, about 45,000 patients are diagnosed with AF each year [4]. AF is a persistent and progressive disease, that causes alterations of electrophysiology and structure of atrial cardiomyocytes. Electrical remodeling is characterized by abbreviation of the atrial effective refractory period (ERP), which has been observed in animal models of AF and patients with AF [5-7]. More importantly, in various models and patients with AF, the structural remodeling was found in atrial cardiomyocytes, including degradation of sarcomeres, aberrantly shaped mitochondria, accumulation of glycogen, fragmented sarcoplasmic reticulum, and disorganized fibers orientation in atrial tissue [8, 9]. Also, the structural impairment was found to affect the conduction of electric signals around the atria, and the orientation of the organized fibers in atria is related to the direction of conduction of electric signals [10-12]. It was found that the structural remodeling underlies the electrical remodeling, which is termed as electropathology [13].

It is known that the electrical remodeling is completely restored to normal within a few days after electrical cardioversion in both animal models and clinical AF [14, 15]. While, this therapy is only temporarily effective and AF still recurs in more than 87% of patients [16, 17]. As for the pharmacological cardioversion, the treatments are only moderately effective and limited by its pro-arrhythmic and non-cardiovascular side effects [18]. The limited effectiveness of the cardioversion therapies is due to sustainable changes of myocardium structure, which derail the electrical coupling and functional recovery to sinus rhythm after cardioversion therapies [13]. Importantly, when patients are diagnosed with AF, they often present with a certain degree of electropathology. Therefore, there is an urgent need for improvement of AF therapies, that aim at reversing the structural remodeling of AF patients. Hereto, preliminarily, we established a reversibility HL-1 cardiomyocyte model for AF, in this model, structural changes were induced by tachypacing and maintained during up to 24 hours recovery of cardiomyocytes. Next, tachypaced HL-1 cardiomyocytes were post-treated with drugs that target different modifiable endpoints with the aim to accelerate recovery from tachypacing. By using this reversibility model, we screened various drugs. In this thesis, we screened several HSP inducers, including GGA and GGA-derivatives, as well as PARP1 inhibitor ABT888 in chapter 5 and chapter 6, respectively. In addition, also various drugs, targeted at PARP1, HDAC3 and HDAC5, were screened to test whether they can protect against AF promotion, by adding the drug before induction of tachypacing in atrial cardiomyocytes, *Drosophila* and isolated rat atrial cardiomyocytes (see chapter 4, 6 and 7). The key modifiable targets that we targeted in the prevention and/or reversibility therapy, and the major findings of this thesis are summarized in figure 1.
Aging, hypertension, heart failure, obesity, diabetes and lifestyle are risk factors that contribute to AF progression. The key modifiable targets that are involved in structural, metabolic remodeling and epigenetic changes during AF progression, are pharmacologically modulated, to prevent and reverse AF progression in terms of contractile function normalization, in various experimental models for AF. Specifically, the microtubule network, NAD+, HDAC3 and HDAC5 are the main targets that play an important role in induction of structural, metabolic remodeling and epigenetic changes, as well as HSPs is also a crucial modifiable target for preventing and reversing structural and contractile remodeling induced by AF. P: Prevention Therapy; R: Reversibility/Rescue Therapy; P+R: both therapies are applied. Part of this figure is modified from the review paper [19].

**Figure 1.** Aging, hypertension, heart failure, obesity, diabetes and lifestyle are risk factors that contribute to AF progression. The key modifiable targets that are involved in structural, metabolic remodeling and epigenetic changes during AF progression, are pharmacologically modulated, to prevent and reverse AF progression in terms of contractile function normalization, in various experimental models for AF. Specifically, the microtubule network, NAD+, HDAC3 and HDAC5 are the main targets that play an important role in induction of structural, metabolic remodeling and epigenetic changes, as well as HSPs is also a crucial modifiable target for preventing and reversing structural and contractile remodeling induced by AF. P: Prevention Therapy; R: Reversibility/Rescue Therapy; P+R: both therapies are applied. Part of this figure is modified from the review paper [19].

**Therapeutic application of heat shock proteins in AF and other cardiac diseases**

**State of the art and new discoveries in AF**

Protein homeostasis (proteostasis) is in charge of regulating protein synthesis, trafficking, disaggregation and degradation across the cell. In particular, the molecular chaperones, heat shock proteins (HSPs) play a crucial role in proteostasis, they are involved in and guard the correct folding of proteins, refolding of misfolded proteins, and assist in the clearance of toxic proteins, to ensure that proteins and cellular processes function normally [20]. HSPs have been suggested as a promising therapeutic target for AF. In previous studies, it was shown that the enhancement of HSPB1 in cardiomyocytes, by heat shock, HSP inducer GGA and HSPB1 transfection, prevented the tachypacing-induced degradation of sarcomeres and contractile dysfunction of atrial cardiomyocytes [21, 22]. Not only HSPB1, the genetic transfection of other small HSPs, also prevented structural remodeling and contractile dysfunction in tachypaced cardiomyocyte and Drosophila models for AF [23, 24]. Moreover, GGA was also found to prevent atrial conduction abnormalities under ischemic and failing heart conditions in dog and rat models [25, 26]. Some extended protective effects GGA-derivatives
and HSPB1 delivered in intervening AF progression were found in the thesis. In chapter 5, we found that both the HSP inducer GGA59 and recombinant HSPB1 (rcHSPB1) transfection could reverse the tachypacing-induced contractile dysfunction and disruption of microtubule network, and suppress the HDAC6 activity in HL-1 cardiomyocytes. Particularly, rcHSPB1 experiments helped us to understand that HSPB1 itself was sufficient to restore the tachypacing-induced microtubule damage and contractile dysfunction of HL-1 cardiomyocytes. Although, the effect of structural reversal by HSPB1 alone is not as strong as GGA59. This discrepancy in reversal effects between GGA59 and HSPB1 maybe due to the observation that GGA59 not only restored the microtubules at the protein level, it also re-upregulated the transcription levels of all relevant α-tubulin encoding genes, and restored the protein expression of cTnI and cTnT. While, HSPB1 slightly upregulated the mRNA expression of one α-tubulin encoding gene, tuba1a, and didn’t restore the protein expression of cardiac troponins. This observation triggers further studies on the mechanisms of how GGA59 and HSPB1 work differently in restoring structural remodeling induced by tachypacing. GGA59 might partly have effect through its HSPB1 inducing effect and also via other pathways, including possible changes on geranylation of proteins.

**Role of HSPs in clinical AF**

In addition to the pre-clinical AF studies. HSP levels are also altered in atrial tissue and/or serum samples of patients with AF. A correlation between HSP levels and the occurrence and recurrence of AF was observed. For example, it was found that higher level of HSPA1A in tissue of patients with AF correlates with lower occurrence of postoperative AF [27]. Also, it was reported that the level of HSPB1 in atrial tissue of patients with paroxysmal AF is higher compared to patients without AF, but also patients with persistent AF [21], which indicated that the atrial tissue level of HSPB1 contributes to diagnosis of AF and identification of different stages of AF. Moreover, the maintenance of sinus rhythm of AF patients who underwent the mitral valve replacement, was correlated with high activity of HSF1 and high expression level of HSPB1 and HSPA1A in atrial tissue of patients, compared to patients who showed recurrence of AF after the surgery, suggesting that activation of the HSF1-HSPs pathway may be involved in the maintenance of restored sinus rhythm, or the low level of HSF1 and HSPs could predict the recurrence of AF after surgery [28]. Moreover, HSP60 level in atrial tissue and plasma of patients with AF was found to correlate with severity of tissue remodeling, implying that low HSP60 levels may predict the maintenance of restored sinus rhythm [28]. Taken together, specific HSPs have the potential to indicate the stage of AF, and the chance of occurrence and recurrence of AF in the clinic. It is of interest to discover the correlation between circulating HSPs and the progression of AF in patients, so that in combination of other markers, such as troponin, ANP, BNP and ST2 [29-31], the stage of AF in patients can be determined more accurately. In addition, the HSP inducers that have been tested in preclinical studies and on the market are listed in table 1.

**Promising effects of HSPs in other cardiac diseases**

Beyond the exploration of HSPs in AF, there are numerous preclinical studies describing a role of HSPs, in *in vivo* models for various cardiac diseases. For example, one study revealed that HSP-inducing compound GGA attenuated cardiac hypertrophy, fibrosis and cardiac dysfunction, via enhancing the expression level of HSPB1 and HSPB8 in mice with desmin-related cardiomyopathy [32]. Heat stress-
induced HSP expression and HSPA1A gene transfection protected against ischemic injury in the heart of rats [33, 34]. Recently, another study used acute injection of 3E10-Fv carried HSP72, a single-chain variable fragment (Fv) of monoclonal antibody (3E10) which facilitate a rapid transport of HSP72 into cells, into rabbits with ischemic myocardium injury, and these rabbits revealed a reduced amount of myocardial apoptosis, decreased infarct size and improved left ventricular function [35]. Not only HSPA1A, also some small HSPs, including HSPB1, HSPB5, HSPB7 and HSPB8, showed a cardioprotective effect in "in vivo" models for heart failure and myocardium ischemia [32, 36-39], and in "ex vivo" models for myocardium infarction [40].

Taken together, the finding suggests that enhancing specific HSP levels in the heart is a promising approach to prevent or even reverse cardiac dysfunction during the progression of AF, via targeting of cellular proteostasis and structural remodeling, thereby normalizing the contractile function of cardiomyocyte.

**Therapeutic application of targeting metabolic remodeling in AF**

The normal contractile function of a cardiomyocyte is not only dependent on the healthy function of its contractile units and cytoskeleton, but also significantly relies on the metabolic status of a cardiomyocyte, so that the energy demand will be met to support the normal excitation and contraction of a cardiomyocyte. In fact, alterations in energy metabolism has been found in a number of studies of "in vivo" models for AF [41-43] and patients with AF [44-46]. The irregular and rapid excitation and contraction of the atrium requires a much higher energy supply, due to metabolic abnormalities occurring in the cardiomyocytes along AF progression. If it is difficult to meet the elevated energy requirement in this scenario, and therefore metabolic remodeling contributes to the contractile dysfunction of cardiomyocytes [45, 47, 48]. The abnormal cellular metabolism and energy generation could be a therapeutic target for AF.

**The energy rescuer, nicotinamide adenine dinucleotide (NAD\(^+\)) in AF and other cardiac diseases**

It is known that NAD\(^+\) is a critical element of cell metabolism and energy production [49]. There are a few studies on the impact of NAD\(^+\) on electrophysiology of atrial cardiomyocytes in the heart of rats and rabbits [50-52]. It was revealed that the extracellular NAD\(^+\) significantly affects the action potential duration and contractile function of the myocardium of rats [50]. In our study (chapter 6), we discovered that AF-induced DNA damage causes NAD\(^+\) depletion, leading to contractile dysfunction of cardiomyocytes in multiple experimental models for AF. In line, similar findings were also observed in atrial appendages from patients with AF, implying that the DNA damage resulted in over-activation of a DNA repair enzyme PARP1, which in turn induced NAD\(^+\) depletion resulting in contractile dysfunction. Indeed, the inhibitor of the NAD\(^+\) consuming enzyme PARP1 prevented NAD\(^+\) depletion, oxidative stress and contractile dysfunction in HL-1 atrial cardiomyocytes, adult rat atrial cardiomyocytes and *Drosophila*, and it also prevented the derailment of electrophysiology and ion channel expressions in atrial cardiomyocytes, which suggests that AF progression could be prevented and reversed by inhibiting PARP1 or supplementation of NAD\(^+\) or its precursors. The two protective drugs, PARP1 inhibitor ABT888 and olaparib, which were investigated in the thesis, may represent novel therapeutic strategy to treat AF. Since they are both tested on phase II in clinical trials for treating solid tumors, ovarian cancer and breast cancer, they may enter a clinical trial in AF patients.
on a short term. Particularly, olaparib has been approved by FDA and EMA, therefore represent an interesting candidate to treat AF (table 1) [53-55].

In comparison with the limited studies of NAD$^+$ in AF, the protective effects of NAD$^+$ are more extensively identified in other cardiac diseases. For example, it was found that the cellular NAD$^+$ level was significantly depleted in both in vitro and in vivo models for cardiac hypertrophy, and the NAD$^+$ treatment blocked the hypertrophic response, through activation of anti-hypertrophy mechanisms mediated by a HDACIII member Sirt3 [56]. In addition, a recent study revealed that NAD$^+$ was decreased in heart tissue of mouse with LMNA cardiomyopathy, that showed ventricular dysfunction. Furthermore, supplementation of a NAD$^+$ precursor, nicotinamide riboside (NR), restored the ventricular dysfunction and increased the survival of mice with LMNA cardiomyopathy [57]. Similar protective effects of NAD$^+$ precursors were also observed in two recent studies with mouse models of heart failure [58, 59].

In chapter 6, we describe findings related to the beneficial effects of elevated NAD$^+$ level by direct NAD$^+$ supplementation and inhibition of PARP1. These findings are in line with protective effects of NAD$^+$ and its precursors in animal models for heart failure and cardiac hypertrophy, showed by other researchers [56, 58, 59]. It suggests that prevention and restoration of NAD$^+$ depletion is a highly promising pharmacological strategy for treating AF. In particular, the NAD$^+$ precursor NR, that was recently developed as a dietary ingredient (Niagen®), was also recently reported to have high tolerability and effectiveness in healthy middle-aged and older men and women [60]. Therefore, NR represents a potential candidate for testing in a large animal model for the prevention and recovery from AF progression, and also in the clinical AF.

**What is known about other modifiable targets regarding metabolic remodeling in AF?**

Apart from supplementation of NAD$^+$ as a promising therapeutic approach, to prevent or rescue cardiac dysfunction in AF and other cardiac diseases, metabolic remodeling can be suppressed by targeting other modulators involved in the energy metabolism. For instance, the alterations of (peroxisome proliferator-activated receptor) PPAR-α, PPAR-γ, (AMP-activated protein kinase) AMPK activity and fatty acid (FA) oxidation are associated with atrial contractile and structural remodeling in AF [48]. Therefore, the agonists of PPAR-α, PPAR-γ, and AMPK, as well as the antagonists of FA oxidation were also studied in various experimental models for AF. In a rabbit model for AF, the PPAR-α agonist fenofibrate prevented the loss of ATP, ADP and AMP, reduced the accumulation of lipid droplets and glycogen, also normalized the derailed metabolic modulators at both mRNA and protein level, such as mCPT-1, MCAD and SREBP1, in atrium tissue of the rabbits. It was further demonstrated that fenofibrate prevented tachypacing-induced metabolic remodeling through PPAR-α/sirtuin 1/PGC-1α in HL-1 cardiomyocytes [61]. Moreover, PPAR-γ agonists, especially pioglitazone could attenuate the atrial structural changes and AF progression in rabbits with heart failure and rabbits with diabetes [62, 63]. In addition, a few studies provide evidence that the AMPK activation is a compensatory response to heart diseases caused by metabolic stress, with the aim to generate more energy and also to reduce energy consumption in the heart [64-68]. In addition, the AMPK activators 5-aminoimidazole-4-carboxamide (AICAR) protected against tachypacing-induced impairment of calcium handling and contractile dysfunction in isolated dog atrial cardiomyocytes [68]. Metformin,
another AMPK activator, attenuated oxidative stress and sarcomere degradation in tachypaced HL-1 atrial cardiomyocytes [69]. Taken together, the promising outcomes of the compounds targeting conservation of the cardiomyocyte metabolism in experimental model systems for AF, indicate that the metabolic remodeling may represent a valuable target to prevent AF progression. Especially compounds that aid in the reversibility of structural remodeling and therefore attenuate AF progression is clinically highly relevant.

**Therapeutic application of targeting epigenetics in AF**

Accumulating evidence reveal that altered epigenetic regulation contributes to the pathogenesis of AF [70, 71]. Specifically, histone deacetylases (HDACs) deacetylate histones, structural and contractile proteins, and thereby contributes to structural and contractile remodeling and progression of AF [72].

**State of the art and new discoveries about HDACs in AF**

Several studies describe beneficial effects of HDAC inhibitors on prevention of AF-induced remodeling in cardiomyocytes, adipocytes and inflammatory cells. Seki et al. showed that a class I HDAC inhibitor CI-994 decreased the amount of fibrosis and inflammation in atrial tissue of dogs with sustained AF via prevention of the amount of intra-atrial adipocytes and immune cells infiltration [73]. Lugenbiel et al. found TSA, a pan-HDAC inhibitor for class I and class II HDACs, to reduce or upregulate the mRNA and protein expression levels of multiple potassium channel subunits, resulting in prolonged the action potential duration (APD) in tachypaced HL-1 cardiomyocytes [74]. Zhang et al. discovered that in both *in vitro* and *in vivo* models, inhibition of the catalytic tubulin deacetylase domain of HDAC6 via pharmacological and genetic approaches protected against the derailed calcium handling and contractile dysfunction in tachypaced HL-1 cardiomyocytes. Inhibition of HDAC6 activity preserved the integrity of microtubule network, and prevented calpain-induced degradation of depolymerized α-tubulin in tachypaced HL-1 cardiomyocytes and dogs [75].

In chapter 7, we found that class I HDAC3 and class Ila HDAC5 play a key role in AF progression. We modulated HDAC3 and HDAC5 levels by knock down and overexpression of their genes and applied HDAC3 inhibitor or HDAC5 nuclear boosters in tachypaced HL-1 cardiomyocytes and *Drosophila*. Suppression of HDAC3 and overexpression of HDAC5, genetically or pharmacologically, prevented tachypacing-induced contractile dysfunction in cardiomyocytes and *Drosophila*. It is intriguing to find out that tachypacing-induced phosphorylation and thereby translocation of HDAC5 from the nucleus to the cytosol, results in expression of MEF2 related fetal gene program, and expression of β-MHC and BNP that underlies AF promotion in cardiomyocytes. In line, increased HDAC3 expression levels and activity levels, increased HDAC5 phosphorylation as well as the MEF2-related fetal genes expression were further confirmed in the atrial tissue of patients with persistent AF. Together, the findings indicate that HDAC3 and HDAC5 represent interesting therapeutic targets and HDAC3 inhibitor and HDAC5 nuclear boosters are potential drug candidates for the future treatment of clinical AF.

**Status of clinical trials with HDAC inhibitors**

Similar to PARP1 inhibitors, the effect of HDAC inhibitors have been substantially studied in cancer. There are currently four novel HDAC inhibitors that have been approved for the clinical treatment of
cancer, and over five HDAC inhibitors are in phase III clinical trials as anti-cancer drugs [76]. In AF, multiple HDAC inhibitors have been studied in experimental model systems, including tachypaced HL-1 cardiomyocytes and Drosophila for AF [77]. Among them, the class I HDAC inhibitor CI-994 and class III HDAC inhibitor nicotinamide are in clinical trials for the treatment of different cancers. The class I and II HDAC inhibitor TSA has been reported to be only used in preclinical studies, due to its toxic effects. TSA is a hydroxamic acid HDAC inhibitor and in the same class of HDAC inhibitors as suberoylanilide hydroxamic acid (SAHA). SAHA has been approved by FDA for the treatment of cancer since 2006. The findings indicate that SAHA might be a superior drug to protect against AF progression in patients [76, 78, 79]. In addition, HDAC inhibitors that have been tested in preclinical and clinical studies are listed in table 1 and table 2 [73, 75, 79-85].

**HDACs and Heat Shock Proteins**

In chapter 1 till chapter 7, we investigated the roles of HSP, PARP1 and HDACs in AF progression. Also, the application of compounds that regulate HSP, PARP1 and HDACs in various experimental model systems for AF are discussed. In fact, HSP and HDACs modulate each other, which has been identified in some non-cardiac diseases. The impacts of HDACs on some HSPs have been studied extensively. Especially HDAC inhibitors have effect on the expression of specific HSPs and function.

**HDACs inhibition and role on HSPC, HSPA1A and HSPB1 function**

The most studied example of HDACs influencing HSP function is the effect of HDAC inhibition on HSP90 (HSPC) function, and its application in the treatment of cancer [86-88] and neurodegenerative diseases [89]. The hyper-acetylation of HSP90 by HDAC inhibitors causes loss of HSP90 chaperone function, and HSP90 dysfunction was suggested as the major mechanism driving the protective effects of HDAC inhibitors, such as anti-inflammation [90]. In addition, several studies confirmed that HDAC6 inhibition alters HSP90 function. The effect of HDAC6 inhibition on HSP90 is likely due to the interaction of HDAC6 and HSP90 within a complex they form with ATP, HSF1, HSPA1A, client proteins and co-chaperones of HSP90. Since HDAC6 is inhibited, the acetylated HSP90 and free HSF1 result in changes of many cellular processes, leading to protective effects. For instance, in a breast cancer cell model, HDAC6 inhibition decreased ATP-conjugation to HSP90 via HSP90 acetylation, once the HSP90 complex was disrupted, one HSP90 client protein, an oncoprotein HER-2, underwent degradation via the proteasome system, and thereby inhibited the proliferation of breast cancer cells [91].

Two studies have found that pan-HDAC inhibitors increased HSP70 (HSPA1A) protein expression level and thereby revealed beneficial effect in animal models for ischemic brain damage [92, 93]. Studies elucidated that class I and II HDAC inhibitors upregulated HSPA1A mRNA and protein expression levels via increasing the HSPA1A promoter activity in mouse and human embryonic stem cells, and neurons as well as Drosophila [94-97]. Apart from HDAC inhibitors enhanced HSPA1A expression via HSF1 activity, other pathways were also found to be involved. For instance, HDAC inhibition increased the acetylation level of transcription factor Sp1, leading to the increased binding between Sp1 and HSPA1A promoter, which gave rise to HSPA1A induction in neurons [95]. Also, the PI3-kinase/Akt signaling was found to be involved in the induction of HSPA1A by HDAC inhibition in neurons [95]. In addition to HSP90, HSPA1A can also be deacetylated by HDAC6. Inhibition of HDAC6 led to the hyperacetylation of HSPA1A, which also disrupted the chaperone function of HSP90, resulting in
degradation of its client proteins, including HER-2 and thereby initiating cell cycle arrest and cell apoptosis. It was due to this effect of HDAC6 inhibition, that HDAC6 inhibitors were tested as anticancer drugs in several clinical trials [98].

Various studies showed that one of the class III sirtuin family members, Sirt1 regulated the HSF1-HSE binding activity and HSPA1A expression in various cells types. Overexpression of Sirt1 enhanced the HSF1-HSE binding activity and also prolonged the longevity of 293T cells upon the heat stress. The observation also indicates that the deacetylation of HSF1 promotes the HSF1 binding affinity to HSE in the DNA [99]. In line with this study, Sirt1 inhibitors decreased HSF1 binding activity and reduced HSPB1 protein expression level, thereby attenuating the heat shock response (HSR) upon the heat stress [100]. Again, the findings indicate that deacetylation of HSF1 is critical to the HSF1-HSE binding activity, expression of specific HSPs and the HSR. Sirt1 might play an important role in cellular protein homeostasis by regulating the HSF1-HSE binding activity. The relation between HDAC inhibition and regulation of HSF1 and HSPs may also apply to the findings that suppression of HDAC6 activity and induction of HSPB1 by GGA59 in the HL-1 cardiomyocyte reversibility model for AF (chapter 5).
Table 1. Drugs that are at preclinical and clinical studies for AF (HSP inducers and PARP inhibitors).

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Experimental model</th>
<th>Consequence</th>
<th>Clinical status</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geranylgeranyacetone</td>
<td>HSP inducer (GGA)</td>
<td>Dog atrial myocyte</td>
<td>Calcium current↑</td>
<td>anti-ulcer drug on market in Japan</td>
<td>[21, 22, 25]</td>
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<td></td>
<td></td>
<td>HL-1 cardiomyocyte</td>
<td>APD↑</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Drosophila</td>
<td>Contractile function↑</td>
<td></td>
<td></td>
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<tr>
<td>GGA-derivative: GGA59</td>
<td>HSP inducer</td>
<td>HL-1 cardiomyocyte</td>
<td>Reverse CaT</td>
<td>Preclinical</td>
<td>Chapter 5</td>
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<td></td>
<td></td>
<td></td>
<td>Reverse (acetylated)α-tubulin (mRNA &amp; protein level)</td>
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<td></td>
<td></td>
<td></td>
<td>Reverse cTnI, cTnT (protein level)</td>
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<td></td>
<td></td>
<td></td>
<td>HDAc6 activity↓</td>
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<tr>
<td>Nicotinamide</td>
<td>PARP1 inhibitor</td>
<td>HL-1 cardiomyocyte</td>
<td>TP-induced PARylation↑</td>
<td>phase III, laryngeal cancer</td>
<td>Chapter 6 &amp; [79]</td>
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<td></td>
<td></td>
<td>Class III HDAC</td>
<td>NAD↑</td>
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<td></td>
<td></td>
<td>inhibitor</td>
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<tr>
<td>3-aminobenzamide (3-AB)</td>
<td>PARP inhibitor</td>
<td>HL-1 cardiomyocyte</td>
<td>TP-induced PARylation↑</td>
<td>Preclinical</td>
<td>Chapter 6</td>
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<tr>
<td></td>
<td></td>
<td>Drosophila</td>
<td>NAD↑</td>
<td></td>
<td></td>
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<tr>
<td>ART888 (Veliparib)</td>
<td>PARP1/2 inhibitor</td>
<td>HL-1 cardiomyocyte</td>
<td>CaT↑, APD, APD dispersions↓, excited area↑</td>
<td>Phase I, solid tumors, leukemia and BRCA-deficient tumors; Phase II, glioblastoma multiforme; Phase III, Ovarian cancer</td>
<td>Chapter 6 &amp; [53, 54]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TP-induced PARylation↓, oxidative protein, DNA damage↑</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Rat adult atrial cardiomycyte</td>
<td>CaT↑, TP-induced PARylation↓, NAD↑</td>
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<td></td>
<td></td>
<td>Drosophila</td>
<td>Contractile function↑</td>
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<tr>
<td>Olaparib</td>
<td>PARP1/2 inhibitor</td>
<td>HL-1 cardiomyocyte</td>
<td>APD↑, APD dispersions↓, excited area↑</td>
<td>Phase I, BRCA deficient</td>
<td>Chapter 6 &amp; breast cancer; [53, 54, 55]</td>
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<tr>
<td></td>
<td></td>
<td>Drosophila</td>
<td>Contractile function↑</td>
<td>Phase II, malignancy; FDA and EMA approved drug, ovarian cancer on 2014; FDA approved drug, germline BRCA mutant breast cancer on 2018</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Rat adult atrial cardiomycyte</td>
<td>CaT↑</td>
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Excited area=excited cell surface area
Table 2. Drugs that are at preclinical and clinical studies for AF (HDAC inhibitors).

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Experimental model</th>
<th>Consequence</th>
<th>Clinical status</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-acetylvaline (C-994)</td>
<td>Classi HDAC inhibitor</td>
<td>HopX mouse Dog</td>
<td>Atrial fibrosis, AF duration ↓ Fibrosis, AF duration ↓ Immune cells infiltration ↓ TNFα, IL-6 ↓ Leptin and adiponectin ↓</td>
<td>Phase I, solid tumors and advanced cancer; Phase II pancreatic cancer</td>
<td>Chapter 6 &amp; [73, 80, 81, 82, 83]</td>
</tr>
<tr>
<td>Tricostatin A (TSA)</td>
<td>Pan-HDAC inhibitor</td>
<td>HopX mouse Dog</td>
<td>Connexin 40 expression ↑ Atrial Fibrosis and arrhythmia ↓</td>
<td>Preclinical</td>
<td>[84]</td>
</tr>
<tr>
<td>MPT0604</td>
<td>Pan-HDAC inhibitor</td>
<td>Rabbit</td>
<td>Ryr and NCX expression ↑ ADP ↑ AF duration ↑</td>
<td>Preclinical</td>
<td>[85]</td>
</tr>
<tr>
<td>Tubacin</td>
<td>HDAC6 inhibitor</td>
<td>HL-1 cardiomyocyte Drosophila</td>
<td>CaT ↑ e-tubulin deacetylation ↓ e-tubulin degradation ↓ Depolymerized α-tubulin ↑ Polymerized α-tubulin ↑ Contractile function ↑</td>
<td>Preclinical</td>
<td>[75]</td>
</tr>
<tr>
<td>Tubastatin A</td>
<td>HDAC6 inhibitor</td>
<td>Dog</td>
<td>CaT, cell shortening ↑</td>
<td>Preclinical</td>
<td>[75]</td>
</tr>
</tbody>
</table>

HopX mouse: increased HDAC activity, ventricular hypertrophy associated with atrial fibrosis and AF inducibility; CaT: Calcium Transient

Future perspectives and challenges

Based on what we have found in chapter 5, we established a HL-1 cardiomyocyte reversibility model for AF. This reversibility model for AF can be utilized as a drug screening system to test a wide range of drugs for their roles on recovery from structural and contractile remodeling. We screened several HSP-inducing compounds GGA and GGA-derivatives and identified one compound, GGA59, with superior effect on protection and recovery from AF remodeling. In future research related to GGA59, we suggest the following experiments. Firstly, demonstrate whether the protective effect of GGA59 is solely dependent on HSPB1 expression. This can be tested by suppression of HSPB1 expression after tachypacing of HL-1 cardiomyocytes, which are post-treated with GGA59 for 24h. As we observed in chapter 5, 24 hours of posttreatment with GGA59 revealed a protective effect on contractile function in tachypaced HL-1 cardiomyocytes. Secondly, I suggest to examine whether GGA59 and HSPB1 cause specific modifications on the promoter region of α-tubulin genes, therefore upregulating the α-tubulin gene expression. We may measure the effect via transfection of HL-1 cardiomyocytes with the reporter construct containing the luciferase gene under the control of α-tubulin promoter, in combination with GGA59 or recombinant HSPB1 posttreatment after tachy pacing.

It is also interesting to investigate whether GGA59 alters geranylation of proteins, such as α-tubulin, RhoA and cardiac troponins, thereby affecting their biological activities. It is known that proteins prenylation affects the cellular localization of the proteins, the prenylated proteins prefer to locate at cellular membrane, and the unprenylated proteins stay in the cytosol [101]. By separating the cellular membrane from the cytosol in GGA59 post-treated tachypaced HL-1 cardiomyocytes, we may detect changes of (geranylated) proteins in cellular membranes and cytosolic fractions. These findings will elucidate whether proteins show altered geranylation by GGA59, which may contribute to the
observed protective effects. Also, we may directly detect the geranylated proteins with geranylation specific antibodies [102].

A number of clinical trials have been conducted with both PARP1 inhibitors and HDAC inhibitors for the treatment of cancer. These trials paved the way for future clinical trials with drugs that revealed protective effects as described in chapter 6 and chapter 7. As for the PARP1 inhibitor ABT888 and olaparib, we can consider to apply these two drugs in a large animal model for AF. We observed that ABT888 also accelerates the recovery from contractile dysfunction, and restored the NAD$^+$ levels in the tachypaced HL-1 cardiomyocytes. Therefore, future research may also focus on testing both ABT888 and olaparib in various reversibility models for AF, other than HL-1 cardiomyocytes, such as isolated adult atrial cardiomyocyte and Drosophila, or even a large animal model for AF, to verify whether the two PARP1 inhibitors can reverse AF progression. Likewise, it will be also intriguing to test HDAC3 inhibitor and HDAC5 nuclear boosters in the reversibility models for AF. Isolated adult atrial cardiomyocytes (as described in chapter 6), or the immortalized atrial myocyte (iAM) that is originally derived from rat atrial cardiomyocytes [103], are suitable cellular model systems to be utilized to perform reversibility experiments as described (chapter 5). In these model systems, several HDACs, PARP1 and HSP drugs can be tested for their effect on recovery from structural and contractile remodeling. Furthermore, as for the Drosophila, we can use two stages of Drosophila to establish the reversibility model. The first stage is the prepupae, as we normally use to perform experiments on. However, the technical difficulty is the treatment of drugs to prepupae after tachypacing, and the short time window (2-3 hours) for measuring the heart wall movements in prepupae. The second stage is the adult fly. We can induce AF in adult fly by tachypacing it with its head and posterior abdomen touched to the conductive electrode gel [104], followed by conducting the semi-intact heart dissection preparation, as in the heart tubes of Drosophila will be exposed to the outside [105]. In one of the steps of the preparation, we can incubate the semi-intact heart with drugs for a desired time, such as 15min, followed by measurement of parameters of cardiac contractile function [106]. However, we are not certain about how practical and effective this whole posttreatment experiment in adult fly will be, since it is uncertain that whether the exposed semi-intact heart will still respond to drugs after tachypacing and early part of the dissection preparation. Even though, it is interesting and worth to explore this model system in the near future.

In conclusion, in this thesis, we tested various drugs directed at HSP induction, PARP1 inhibition, NAD$^+$ conservation and HDAC activity. Several of these drugs reveal beneficial effect on the prevention of structural and contractile remodeling in experimental model systems for AF. To test whether drugs can aid in the recovery from remodeling, a unique reversibility model was developed by utilizing HL-1 cardiomyocytes. Also, in this model, several drugs accelerated recovery from tachypacing-induced remodeling. Since most of patients with AF reveal structural and contractile remodeling, the development of this model is clinically highly relevant.
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