Pharmacological modulation of connexin-formed channels in cardiac pathophysiology

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Coordinated electrical activity in the heart is supported by gap junction channels located at the intercalated discs of cardiomyocytes. Impaired gap junctional communication between neighbouring cardiomyocytes contributes to the development of re-entry arrhythmias after myocardial ischaemia. Current antiarrhythmic therapy is hampered by a lack of efficiency and side effects, creating the need for a new generation of drugs. In this review, we focus on compounds that increase gap junctional communication, thereby increasing the conduction velocity and decreasing the risk of arrhythmias. Some of these compounds also inhibit connexin 43 (Cx43) hemichannels, thereby limiting adenosine triphosphate loss and volume overload following ischaemia/reperfusion, thus potentially increasing the survival of cardiomyocytes. The compounds discussed in this review are: (i) antiarrythmic peptide (AAP), AAP10, ZP123; (ii) GAP-134; (iii) RXP-E; and (vi) the Cx mimetic peptides Gap 26 and Gap 27. None of these compounds have effects on Na+, Ca2+ and K+ channels, and therefore have no proarrhythmic activity associated with currently available antiarrhythmic drugs. GAP-134, RXP-E, Gap 26 and Gap 27 are pharmacological agents with a favorable clinical safety profile, as already confirmed in phase I clinical trials for GAP-134. These agents show an excellent promise for treatment of arrhythmias in patients with ischaemic cardiomyopathy.

Abbreviations
AAP, antiarrhythmic peptide; ADP, adenosine diphosphate; ATP, adenosine triphosphate; [Ca2+]i, intracellular Ca2+ concentration; CTP, cytoplasmic transduction peptide; Cx, connexin; ECG, electrocardiogram; ER, endoplasmic reticulum; ERP, effective refractory period; GPCR, G-protein coupled receptor; IV, intravenous; LAD, left anterior descending; LQT, long QT; MI, myocardial infarction; MW, molecular weight; PKA, protein kinase A; PKC, protein kinase C; PP1, protein phosphatase 1; SA, sinoatrial

Introduction

The normal heartbeat is the result of a coordinated contraction of individual cardiomyocytes that is synchronized through the electrical signal generated within the sinoatrial node and rapidly propagates via the specialized conduction system to the ventricles. From there, the depolarizing current moves through the cardiomyocytes that are characterized by a high degree of cell-to-cell coupling via gap junctions, allowing fast electrical conduction. Ventricular arrhythmias that disrupt the coordinated contraction result in cardiac arrest and sudden death, which is one of the major causes of death in ischaemic heart diseases (Zipes and Wellens, 1998). In the setting of acute myocardial infarction (MI), reperfusion is crucial for the prevention of irreversible cellular injury and the preservation of ventricular function. However, reperfusion itself may cause damage to...
cardiomyocytes (`reperfusion injury`) and result in ventricular arrhythmias (Cascio et al., 2001; Ovize et al., 2010). With the advent of improved therapy for re-establishing coronary flow and stenting to retain patent vessels, acute mortality at the time of MI has decreased (Pfeffer et al., 2003; Fox et al., 2010). On the other hand, the number of patients with heart failure due to post-MI remodeling with cardiac hypertrophy and dilation is increasing, and in this group arrhythmias are also an important cause of death. Several mechanisms lead to arrhythmias in ischaemic heart diseases. Cellular injury in acute ischaemia, as well as late-stage remodeling, can lead to abnormal triggered activity based on abnormal intracellular Ca\(^{2+}\) handling (Pogwizd et al., 2001). In all stages, the presence of non-conducting or poorly conducting areas in the myocardium, or of areas with an abnormal refractory period, can lead to re-entrant arrhythmias. Thus, reduction of gap junctional communication may result in the formation of a region of electrical conduction block, inducing the development of re-entrant arrhythmias (Duffy, 2008).

In the 1990s, a rational approach in the choice of ion channel blockers as an antiarrhythmic therapy was advocated based on the presence of re-entry or other mechanisms [Sicilian gambit – (Bigger et al., 1991)]. Later on, however, many drawbacks of these antiarrhythmic drugs have been recognized, such as the lack of efficacy and unacceptable side effects (Darbar et al., 2006). In a number of studies, class I and III antiarrhythmic drugs increased the mortality in patients with pre-existing cardiac diseases, such as a previous MI or heart failure (Weiss et al., 2000; Darbar et al., 2006). This increased mortality was the consequence of conduction slowing (class I) and the induction of torsades de pointes arrhythmias [polymorphic ventricular tachycardias associated with Q wave T wave (QT) interval prolongation] as observed with class III drugs (Naccarelli et al., 2000; Passman and Kadish, 2001). The proarrhythmic effect of some of the class I and III drugs makes their prophylactic use for ventricular arrhythmias a very controversial issue, especially in the context of ischaemia/reperfusion and MI.

More recently, because of the drawbacks of conventional antiarrhythmic drugs, implantable defibrillators have taken a very prominent place in arrhythmia management (Priori et al., 2003). However, these are not without problems: they have a high impact on the quality of life and high costs (Schron et al., 2002). Therefore, the discovery of novel antiarrhythmic drugs remains highly desirable. There is a need to look for novel targets, different from the classical ion channels. In the setting of ischaemic heart diseases, drugs that have preferential effects on cells in the border zone separating the ischaemic from the non-ischaemic area could offer specific advantages (Beardslee et al., 2000).

**Gap junctional communication and cardiac connexins**

As mentioned above, coordinated electrical activity of the heart is maintained by coupling of cardiomyocytes via gap junction channels prominently located at the intercalated disc, located at cardiomyocyte endings. These channels are composed of Cx proteins that form low-resistance conduits between cells. Cxs assemble into hexameric structures, called connexons or hemichannels. When two hemichannels of apposing cells interact, they open and form a functional gap junctional channel. Mammalian Cx proteins are encoded by 21 genes in the human genome (Willecke et al., 2002). The encoded proteins are designated CxMW, where MW represents the molecular weight of the protein in kDa. Cx43 is the predominant isoform in functional myocardium with a half-life of only 1.3 h (Figure 1). Cx40 is present in the conduction system and atrium, and Cx45 plays a role during development and is also found in adult hearts in the conduction system and at the border between myocytes and fibroblasts (Camelliti et al., 2006). Cx31.9 is present at the atrioventricular nodal region and can form functional hemichannels (Bukauskas et al., 2006). Furthermore, Cx43, Cx40 and Cx37 are present in endothelial cells (Brisset et al., 2009). Cx43 is also present at the subcellular level in endothelial cell and cardiomyocyte mitochondria where it contributes to the potassium flux into the mitochondrial matrix (Boengler et al., 2005; Miro-Casas et al., 2009; Rottlaender et al., 2010). The degree of intercellular communication depends on the open

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**Figure 1**

Cx43 in cardiomyocytes. Cx43 is a transmembrane protein with four transmembrane regions. After oligomerization in the endoplasmic reticulum (ER) and trans-Golgi network, hemichannels or connexons are formed. Docking of two hemichannels results in the formation of a gap junction channel. Cardiomyocytes are well-coupled cells via gap junctions located at the distal ends, called intercalated discs.
probability of the channels, which is (next to other modulating factors) influenced by the phosphorylation status. Phosphorylation controls cell-to-cell communication at several steps: (i) at the level of gene expression; (ii) assembly into gap junctions; (iii) channel gating; (iv) internalization; and (v) protein degradation. Phosphorylation occurs mainly on serine (S) residues (Lampe and Lau, 2000), although threonine (T) and tyrosine (Y) phosphorylation has also been observed (Crow et al., 1990; Swenson et al., 1990). Cx43 contains multiple phosphorylation forms, such as P0 (non-phosphorylated), P1, and P2 (phosphorylated forms; Musil and Goodenough, 1991). Phosphorylation at S364 and S365 leads to the P1 form, and phosphorylation at S325, S328 and S330 results in the P2 form (Solan and Lampe, 2007).

The closure of gap junctions during ischaemia, according to the hypothesis of ‘healing over’ of ischaemic cardiomyocytes, was first proposed by McCallister et al. (McCallister et al., 1979). This was supported by two lines of evidence: (i) the drastic changes in electrical coupling during ischaemia, which were explained by the closure of gap junctions (De Mello, 1987); and (ii) prominent cytosolic rearrangements during ischaemia, resulting in a redistribution of gap junctions (Kleber et al., 1987).

Indeed, in ischaemic heart disease, arrhythmias are often associated with reduced intercellular communication (both electrical and chemical) (Beardslee et al., 2000) due to malfunction of gap junctions, changes in Cx expression, alterations in the phosphorylation state and heterogeneous distribution that leads to ‘lateralisation’. This is caused by a redistribution of Cx43 from the intercalated discs to the lateral sides of the cardiomyocyte under ischaemic conditions (Kleber and Rudy, 2004).

One hour after the onset of ischaemia, there is a progressive increase in [Ca^{2+}], a decrease in intracellular pH, an elevation of protein phosphatase 1 activity, a reduced protein kinase A activity and a drop in intracellular adenosine triphosphate (ATP) levels (Jeyaraman et al., 2003; Turner et al., 2004; Lampe et al., 2006; Matsumura et al., 2006). All these events result in dephosphorylation of Cx43 at S325, S328, S330 and S365 (Beardslee et al., 2000; Lampe et al., 2006; Solan et al., 2007; Solan and Lampe, 2009). Dephosphorylation of the latter residue is necessary for phosphorylation at S368 by protein kinase C (PKC) (Ek-Vitorin et al., 2006). It is conceivable that S365 dephosphorylation alters the gating properties and the configuration of the protein, rendering gap junctions less sensitive to acidosis and increased [Ca^{2+}], (Solan et al., 2007; Solan and Lampe, 2009). Despite the redistribution of Cx43 during ischaemia causing a net lateralization effect of Cx43, the S368 phosphorylated form of Cx43 remains predominantly at the intercalated discs (Lampe et al., 2006). During cardiac ischaemia, gap junction uncoupling occurs after 15 min, and this is associated with (i) dephosphorylation of Cx43 (see above), (ii) the beginning of impulse slowing, (iii) increased anisotropy (Issa et al., 2009), (iv) unidirectional block, (v) re-entry arrhythmias and (vi) ventricular fibrillation (Smith et al., 1995; Lerner et al., 2000). An important consequence of cellular uncoupling is an increased dispersion of action potential duration and refractory period, which is pronounced in the border zone separating the ischaemic from the non-ischaemic area (Beardslee et al., 2000). During ischaemia, intracellular resistance may triple in value, and longitudinal conduction velocity can slow by 2.5-fold within 20 min (Kleber et al., 1987). These effects are probably the consequence of cardiomyocyte uncoupling secondary to the [Ca^{2+}] accumulation and acidification. The differences in Cx distribution, phosphorylation and function between the ischaemic and non-ischaemic zones are of major importance for arrhythmogenesis (Joziwak and Dhein, 2008).

The observed difference between the time course of electrical uncoupling and metabolic uncoupling during the early stages of ischaemia is significant. Electrical uncoupling of cardiomyocytes, determined by the ‘four-electrode method’, which measures tissue conductance in ventricular walls of the heart in vivo and ex vivo, occurred 10–20 min after the development of ischaemia-induced Ca^{2+}-overflow and rigor (Smith et al., 1995; Beardslee et al., 2000; Dhein, 2006). However, metabolic communication, measured by fluorescent dye transfer, persisted up to 30–60 min after no-flow ischaemia (Ruiu-Meana et al., 2001; Miura et al., 2004; 2007; Naitoh et al., 2006; 2009). The mechanisms responsible for these differences are unclear. During the later phases of ischaemia (between 1 and 3 h), the total amount of Cx43 is decreased as a consequence of increased proteinolysis, in combination with reduced transcription levels. This results in a lowered gap junctional communication (both electrical and metabolic coupling) and the onset of arrhythmias (Hatanaka et al., 2004; Miura et al., 2010). Although, previous studies have shown that gap junctional coupling in the myocardium is greatly reduced, it is never abolished after the onset of ischaemia.

Reduced expression and/or altered distribution of Cx43 have, furthermore, been described in patients suffering from other cardiac diseases, which were associated with an increased risk of arrhythmias, such as congestive heart failure, dilated cardiomyopathy, cardiac hypertrophy and Chagas’ disease (Saffitz et al., 1999; Severs et al., 2008).

In large clinical trials in heart failure patients, mostly after MI, β-blockers, such as metoprolol, reduced mortality and this was at least partly related to a reduction of sudden cardiac death (Navarro-Lopez et al., 1993). Apart from its antiarrhythmic effect, metoprolol increased Cx43 protein levels, but had no effect on Cx43 mRNA content nor on the phosphorylation and activation of protein kinases (Salameh et al., 2009; 2010). This might indicate that metoprolol stabilizes Cx43 gap junction plaques at the intercalated discs or prevents Cx43 degradation (Salameh et al., 2009; 2010). These results suggest that modulators of gap junctional communication might be an interesting therapeutic target.

**Antiarrhythmic peptides**

In the early 1980s, Aonuma et al. identified a natural antiarrhythmic peptide, AAP (Table 1) present in the bovine atrium (Aonuma et al., 1980; 1982) (Figure 2A). AAP (5–10 μM) restored the rhythmic contractions of isolated atria that had become irregular due to hypokalaemia and high levels of acetylcholine. Furthermore, this natural AAP also suppressed CaCl2-induced (Kohama et al., 1987), aconitine-induced and ouabain-induced ventricular arrhythmias in mice (Aonuma et al., 1983). Although the mode of action at that time was
## Table 1
Overview of antiarrhythmic peptides and Cx mimetic peptide, with selected references

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAP</td>
<td>H-Gly-Pro-4Hyp-Gly-Ala-Gly</td>
<td>Restores rhythmic movement in isolated atria beating in an arrhythmic manner</td>
<td>(Kohama et al., 1987)</td>
</tr>
<tr>
<td>AAP10</td>
<td>H-Gly-Ala-Gly-4Hyp-Pro-Tyr-CONH₂</td>
<td>Reduces the increased dispersion of action potential duration during regional ischaemia in isolated rabbit hearts</td>
<td>(Dhein et al., 1994)</td>
</tr>
<tr>
<td>HP-5</td>
<td>N-3-(4-hydroxyphenyl)-propionyl-Pro-Hyp-Gly-Ala-Gly-OH</td>
<td>Reduces the increased dispersion of action potential duration during regional ischaemia in isolated rabbit hearts</td>
<td>(Kjolbye et al., 2002)</td>
</tr>
<tr>
<td>ZP123</td>
<td>HjN-Gly-D-Ala-Gly-D-4Hyp-D-Pro-D-Tyr-Ac</td>
<td>Increases gap junctional communication and attenuates acidosis-induced uncoupling</td>
<td>(Kjolbye et al., 2003)</td>
</tr>
<tr>
<td>GAP-134</td>
<td>(2S,4R)-1-(2-aminoacetyl)-4-benzamidopyrrolidine-2-carboxylic acid hydrochloride</td>
<td>Reduces atrial defibrillation upon oral administration</td>
<td>(Butera et al., 2009)</td>
</tr>
<tr>
<td>RXP-E</td>
<td>Ser-Asp-Leu-Arg-Ser-Pro-Gln-Leu-His-Asn-Glu-Glu-Glu-Ser-Ala-Val-Pro-Phe-Tyr-Ser-His-Ser-His-Met-Val-Arg-Arg-Lys-Pro-Arg-Asn-Pro-Arg</td>
<td>Prevents acidification-induced uncoupling</td>
<td>(Shibayama et al., 2006)</td>
</tr>
<tr>
<td>CyRP-71</td>
<td>Cyclo-Arg-Arg-Pro-Pro-Tyr-Arg-Gln</td>
<td>More stable variant of RXP-E that prevents acidification-induced uncoupling</td>
<td>(Verma et al., 2009)</td>
</tr>
<tr>
<td>ZP2S19</td>
<td>Arg-Arg-Lys-(4hydroxy-l-benzoyl)</td>
<td>Binds to the carboxyterminal domain of Cx43 and stabilizes the open state</td>
<td>(Verma et al., 2010)</td>
</tr>
<tr>
<td>Gap 26</td>
<td>Val-Cys-Tyr-Glu-Lys-Ser-Phe-Pro-Iso-Ser-His-Val-Arg</td>
<td>Decreases infarct size and area at risk and closes Cx43 hemichannels</td>
<td>(Hawat et al., 2010)</td>
</tr>
<tr>
<td>Gap 27</td>
<td>Ser-Arg-Pro-Thr-Glu-Lys-Thr-Ile-Phe-Ile-Ile</td>
<td>Decreases infarct size and area at risk</td>
<td>(Przyklenk et al., 2005)</td>
</tr>
</tbody>
</table>

Overview of antiarrhythmic peptides and their effect on gap junctional communication.
Figure 2

(A) Chemical structure of AAP. (B) Chemical structure of AAP10. (C) cAAP10RG at the active site of the putative receptor after a docking simulation. The complementary interacting functional groups of both the fragments are marked black: distance c. 5 Å (Grover et al., 2001). (D) Chemical structure of ZP123. (E) Structure of GAP-134, $R^1 = \text{OH-group}$ and $R^2 = \text{NH}_2$-group. Figure adapted from (Piatnitski Chekler et al., 2009). AAP, antiarrythmic peptide.
unknown, later experiments indicated that they acted on gap junctions.

**AAP10**

Based on the naturally occurring AAP, several derivatives were synthesized, such as AAP10 (Table 1) (Dhein et al., 1994) and HP-5 (Table 1) (Kohama et al., 1987; Kjolbye et al., 2000; 2002). Both peptides reduced the increased dispersion of action potential duration during regional ischaemia in isolated rabbit hearts without an effect on: (i) heart rate; (ii) action potential duration and shape; (iii) the effective refractory period (ERP); (iv) contractility; and (v) mean coronary blood flow (Dhein et al., 1994; Kjolbye et al., 2002; Xing et al., 2003). These data suggest that AAP10 improves gap junctional conductance. Of these two peptides, AAP10 was chosen as the lead compound (Figure 2B) (Grover et al., 2001). A combination of two-dimensional nuclear magnetic resonance spectroscopy and mutational analysis identified the biologically active conformation, which was a semicyclic horseshoe-like structure. This bended structure, together with the electron density in the tyrosine-benzenne ring, and the presence of proline and hydroxyproline, were essential for normal functionality of the peptide (Grover et al., 2001; Butera et al., 2009). In addition, the alanine and hydroxyproline residues were involved in the binding of AAP10 to its interaction site [a G-protein coupled receptor (GPCR), see below]. Both AAP and AAP10 contained this semicyclic structure that might rotate in the receptor pouch (Dhein, 2002). It was shown that cyclic peptides derived from AAP10 were biologically active only when the ring structure was similar in size as the one from AAP10. cAAP10RG, a cyclic AAP10 peptide derivative constructed using a single CCF3 moiety as a bridge, had similar biological activities as AAP10 (Figure 2C) (Grover et al., 1998; 2001).

AAP10, used in the nanomolar concentration range (10–50 nM), increased gap junctional conduction in guinea pig cardiomyocytes (Dhein et al., 2001), in human atrial cardiomyocytes (Hagen et al., 2009), in neonatal rat cardiomyocytes, in pairs of adult guinea pig ventricular cardiomyocytes, and in HEK293 cells stably transfected with Cx43 or Cx45, but not with Cx40 (Weng et al., 2002). The lack of effect of AAP10 on Cx40 suggested that AAP10 probably exerts only minor effects on the cardiac conduction system, which is very valuable if the desired action is to be focused on the beating myocardium (Hagen et al., 2009). Furthermore, the effect of AAP10 has also been examined in a rabbit model of healed MI (Ren et al., 2006). Here, ventricular tachycardia was only induced in 2 out of 10 AAP10-treated rabbits, compared with 8 out of 10 vehicle-treated rabbits 3 months post-MI, suggesting that disturbed gap junctional communication also played a role in the pathogenesis of ventricular tachycardia in the non-acute phase of infarction. Moreover, AAP10 prevented the augmentation of transmural dispersion of repolarization and suppressed *torsade de pointes* arrhythmias in a canine long QT (LQT) model (Quan et al., 2009). In this model, non-phosphorylated Cx43 was significantly increased at sites of intercellular junctions. The properties of the gap junctions and the location of Cx43 at the intercalated discs were comparable between the control and the LQT2 group, indicating that the phosphorylation state of Cx43 is important for augmented transmural dispersion of repolarization in LQT2 models (Quan et al., 2009). The reduction of transmural dispersion of repolarization by AAP10 might be caused by enhanced electrotonic interactions between different cell types. Furthermore, treatment of human cardiomyocytes with AAP10 before or after CO2-induced acidosis prevented gap junctional uncoupling not only by inducing a steady increase in macroscopic conductance, but also by increasing metabolic coupling, assessed by lucifer yellow dye transfer (Hagen et al., 2009).

**ZP123**

AAP10 was chemically modified (rotation-inversion) to produce the antiarrhythmic drug ZP123 (rotigaptide; Table 1) (Figure 2D). In ZP123, the active amino acids were replaced by d-amino acids and the sequence was inverted so that the active groups were in the same position as in AAP10. ZP123 had an increased stability (1700 times higher) and a decreased clearance (140 times lower) in rat and human plasma compared with AAP10. The improved stability and decreased clearance are the result of the lowered degradation of d-amino acid peptides by peptidase activity in the circulation (Kjolbye et al., 2003). ZP123 increased Cx43 protein levels in a concentration-dependent manner in cultured neonatal ventricular cardiomyocytes after 24 h (Stahlhut et al., 2006). This effect was partly due to an increased Cx43 synthesis, and partly a consequence of a decreased degradation and phosphorylation. However, this peptide had no effect on cells expressing Cx26, Cx32 or Cx40, pointing to its specificity for Cx43 (Clarke et al., 2009; Dhein et al., 2009). Furthermore, ZP123 promoted electrical coupling, attenuated acidosis-induced uncoupling in cardiomyocytes and prevented the induction of epicardial re-entry ventricular tachycardia during left anterior descending (LAD) artery occlusion by inhibiting unidirectional conduction block (Elhoff et al., 2003; Xing et al., 2003; Haugan et al., 2005b; Stahlhut et al., 2006). Evidence from primary human osteoblasts (Jorgensen et al., 2005) and isolated hearts from mice and dogs (Kjolbye et al., 2003; Xing et al., 2003) suggested that the effect of ZP123 on cell-to-cell coupling was most pronounced during conditions with acute metabolic stress. ZP123 prevented increased dispersion of the action potential duration during ischaemia and acidosis in rabbit and guinea pig hearts (Dhein et al., 2003; Elhoff et al., 2003). Furthermore, this peptide also prevented: (i) ischaemia-induced slowing of the conduction velocity in isolated guinea pig hearts (Kjolbye et al., 2005), (ii) induction of re-entrant ventricular tachycardia 1–4 h after LAD ligation in the open-chest dog model (Xing et al., 2003), and (iii) reperfusion-induced arrhythmias due to the re-opening of the occluded artery (Hennan et al., 2006).

If conduction was parallel to the longitudinal fibre direction in explanted heart tissue samples of human patients with end-stage heart failure, ZP123 decreased the ERP, facilitated conduction and decreased the percentage of sites with conduction slowing. In some hearts, slowing of conduction
occurred also when propagation was perpendicular to the fibre direction (Wiegerinck et al., 2009).

Ventricular fibrillation associated with regional myocardial ischaemia requires higher defibrillation shock energy to successfully defibrillate. The energy required to effectively defibrillate is called the defibrillation threshold (Qin et al., 2002). The role of gap junctional communication on this defibrillation threshold is still uncertain: in isolated rabbit hearts, uncoupling of gap junctions using heptanol or 16-doxyl-stearic acid was associated with a reduced threshold (Qi et al., 2001). However, in porcine hearts, regional gap junctional uncoupling was associated with an increased defibrillation threshold (Sims et al., 2003). The latter result was subsequently confirmed by Dorian et al. (2005) in isolated rabbit hearts showing an increased chance of successful defibrillation (Dorian et al., 2005). This indicates the importance of cellular uncoupling in the electrical disorganization during induction of ventricular fibrillation.

ZP123 was able to revert established conduction-slowing 30 min after treatment was started. Although the onset of the action of ZP123 was very fast, the termination appeared to be much slower. During 30 min wash out of the drug, there was only a minor, non-significant reduction in conduction velocity compared with the control group (Haugan et al., 2005a). These effects were different from the effects observed after AAP10 wash out: in isolated pairs of cardiomyocytes the effect of AAP10 disappeared after 10 min wash out, while the effect on conduction velocity in the papillary muscle disappeared with 30 min wash out (Muller et al., 1999b). These differences might be explained by the increased stability of ZP123 compared with AAP10.

**ZP123 toxicity profile**

A preclinical toxicological profile of ZP123 was evaluated after single-dose injection and repeated dose injections in mice, rats and dogs. ZP123 was well-tolerated at 10 mg·kg⁻¹ body weight in dogs and 100 mg·kg⁻¹ body weight in rats even when given for 14 days. Additional single-dose intravenous (IV) bolus injections in rats and mice did not result in toxicity for doses up to 300 mg·kg⁻¹ body weight, indicating a large safety margin (Kjolbye et al., 2007). ZP123 was also non-genotoxic in a bacterial reverse mutation assay, a chromosome aberration assay and a mouse IV bolus micronucleus assay. Based on these data, the Food and Drug Administration approved the initiation of phase I clinical trials in healthy volunteers in September 2004, with a starting dose of 30 μg·day⁻¹, followed by continuous IV infusion or 2 mg IV bolus injection.

**Clinical trials**

Two phase I, single-dose or double-blinded, randomized, placebo-controlled studies using ZP123 were completed in March 2005. The results showed a low body clearance (133 mL·min⁻¹·kg⁻¹) and a half-life of 2.7 h after a single IV bolus injection and continuous IV infusion in healthy volunteers (Udata et al., 2006a). Between 61 and 84% of the injected ZP123 was excreted non-metabolized in the urine and no metabolites were detected in the plasma. However, because the peptide was excreted through the kidneys, there was a possibility for decreased clearance and an increased half-life in patients suffering from renal impairment. Clinical studies investigating the impact of renal impairment on ZP123 excretion are now underway. It is already established that patients can take ZP123 together with digoxin, a drug used to treat congestive heart failure and to slow the heart rate in patients with atrial fibrillation, without drug–drug interactions (Udata et al., 2006b). Despite the protecting effects of ZP123 during ischaemia and reperfusion, this peptide requires IV administration and is thus restricted to in-hospital use (Kjolbye et al., 2007).

Phase II studies investigating both the safety and tolerability of ZP123 in patients suffering from unstable angina or MI with or without ST-segment elevation, were initiated in June 2005 (Kjolbye et al., 2007). Although ZP123 has been developed to prevent life-threatening re-entry ventricular tachycardia or ventricular fibrillation, the first safety and tolerability studies were conducted on myocardial ischaemic patients with non-lethal ventricular arrhythmias (pre-ventricular beats and non-sustained ventricular tachycardia) for ethical reasons. Despite promising results, the further development of ZP123 as an antiarrhythmic agent was terminated after phase II clinical trials (NCT00137332), because of prioritization of clinical trials for GAP-134 (Zhang and Xiang, 2009). It is worth mentioning here that promoting gap junctional uncoupling with peptides like ZP123 and related compounds has an inherent danger to potentially stimulate hemichannel opening (Clarke et al., 2009). As a result, cellular ATP loss and volume overload via Cx43 hemichannels may be stimulated, thereby promoting cell death, especially in ischaemia, where the hemichannel fraction residing in the plasma membrane is already increased. Further stimulation of the hemichannel ATP leakage pathway (with ZP123 or AAP10) must thus, be avoided at any expense (see below).

**Mechanism of action of AAPs**

Both the naturally occurring AAP, the synthetic AAP10 and ZP123 activated PKCa and induced Cx43 phosphorylation at S368, leading to the opening of gap junction channels (Dhein et al., 2001; Weng et al., 2002; Axelsen et al., 2006; Jozwiak and Dhein, 2008). Axelsen et al. identified 13 different Cx43 serine phosphorylation sites, of which three were not described earlier (Axelsen et al., 2006). Within the first 7 min of ischaemia, S306 was fully dephosphorylated, whereas S330 became phosphorylated. Between 15–30 min, the time interval when gap junctional uncoupling occurred during ischaemia, S297 and S368 were dephosphorylated, resulting in gap junction uncoupling and the development of asystole (Beardslee et al., 2000). In contrast, treatment with ZP123 increased S297 and S368 phosphorylation at 30 min of ischaemia in heart tissue, resulting in a longer time interval before the development of ischaemia-induced asystole (Axelsen et al., 2006) (Figure 3).

AAP10 also reduced ischaemia-induced internalization of Cx43 in the ischaemic center and the border zone, while there was almost no effect in the non-ischaemic region.
Furthermore, AAP10 reduced the incidence of sustained type Ib ventricular fibrillation (Grover et al., 2001), through binding to a GPCR (200 kDa protein) with a $K_d$ of 0.88 nM and an $IC_{50}$ of 50 nM, as determined in isolated plasma membranes of rabbit hearts (Dhein et al., 1994). Binding studies revealed biphasic displacement, unmasking a high ($K_{d-high}$ ~19 nM) and a low ($K_{d-low}$ ~23 μM) affinity binding site (Weng et al., 2002). These results were verified by Jorgensen et al. (2005) in an in vivo experiment to determine the effect of ZP123 on bone strength and density 4 weeks after ovariectomy in rats. Here, ZP123 was either injected twice a day subcutaneously (300 nmol·kg$^{-1}$·day$^{-1}$) or administered via continuous intraperitoneal infusion (158 nmol·kg$^{-1}$·day$^{-1}$). During metabolic stress (oxygen and glucose deprivation plus low extracellular Ca$^{2+}$), a high affinity binding site with a $K_d$ of 0.1 nM and a receptor density of 15 fmol·mg$^{-1}$ protein using iodinated AAP10 ($[^{125}I]$-di-I-AAP10) was identified on osteoblastic cells. However, during physiological conditions, no specific binding sites for $[^{125}I]$-di-l-AAP10 could be demonstrated. Immobilizing AAP10 on an affinity chromatography column revealed the interaction with a 200 kDa membrane protein, which was further confirmed using cross-linking studies (Jorgensen et al., 2005). However, sequence analysis to further characterize this GPCR failed due to the low amount of protein. GPCR binding activated PKC$\alpha$ (Weng et al., 2002), resulting in Cx43 phosphorylation, correct incorporation of Cx43 into the membrane and improvement of gap junctional conductance (Grover et al., 2001). The activation of PKC$\alpha$, an isoform also present in cardiomyocytes, cannot be excluded. However, activation of this isoform induces a reduction rather than an increase in gap junctional coupling (Doble et al., 2000).

Interestingly, AAP10 acted only on Cx43 present at the intercalated discs, but not at the lateral sides. This suggests that AAP10 somehow interferes with the mechanisms responsible for the directed incorporation and localization of Cx43 in the plasma membrane. Furthermore, AAP10 maintained Cx43 at the polar membrane in the ischaemic zone, which might be due to reduced internalization from the cell pole or enhanced incorporation at that site (Jozwiak and Dhein, 2008).

**Effect of AAP on infarct size**

The effect of increasing gap junctional communication on infarct size was investigated in two in vivo studies. In the first study, MI was induced in 156 male rats by LAD artery ligation (Haugan et al., 2006). The rats were treated with ZP123 at three different concentrations for 3 weeks starting at the onset of ischaemia. ZP123 treatment was associated with a reduction in infarct size of 10–33% compared with 

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**Figure 3**

Schematic representation of the action of the antiarrhythmic peptides AAP10 and ZP123. Cx proteins form hexameric hemichannels that are transported to the plasma membrane. The dotted arrow represents the lateral movement of hemichannels in the plasma membrane on their way to being incorporated into a gap junctional plaque. AAP10 or ZP123 bind on their G-protein coupled receptor ultimately leading to the activation of PKC$\alpha$, with subsequent phosphorylation of Cx43 on S297 (Cx43$^{S297P}$), S330 (Cx43$^{S330P}$) and S368 (Cx43$^{S368P}$). The latter phosphorylation results in the opening of cardiac gap junctions and an increase in the conduction velocity, while Cx43$^{S297P}$ and Cx43$^{S330P}$ induce the internalization of gap junctions. AAP, antiarrythmic peptide; PKC, protein kinase C.
vehicle-treated rats, a decrease in the volume fraction of fibrotic tissue and an increase in the volume fraction of muscle tissue. There was, however, no effect on cardiac volumes, on the thickness of the left ventricular free wall or on the interventricular septum post-MI (Haugan et al., 2006).

Hennan et al. (2006) performed a second study using a dog model of ischaemia/reperfusion (60 min ischaemia followed by 4 h reperfusion). Treatment with ZP123 was started 10 min before reperfusion. The infarct size, expressed as percentage of the area at risk or the percentage of the left ventricle, was significantly lower after treatment with the highest dose of ZP123 (1 µg·kg⁻¹ bolus + 10 µg·kg⁻¹·h⁻¹ IV infusion) (Hennan et al., 2006). ZP123 treatment was associated with an increase in the presence of gap junctions at the area at risk, suggesting that ZP123 treatment might have prevented the ischaemia-induced downregulation/internalization of gap junctions. These results are in contradiction with some previous studies, in which non-selective gap junction blockers, such as heptanol might decrease the infarct size (Garcia-Dorado et al., 1997). However, other studies, using heptanol were unable to show an effect on the infarct size (Gyselbergh et al., 2001; Li et al., 2002). These variable effects observed in different studies with heptanol might be related to the concentration that was applied [loss of selectivity at concentrations >1 mM (Christ et al., 1999)] and differences in timing since the effect of heptanol is rapidly reversed. Furthermore, in a study by Przyklenk et al. who used the aspecific gap junction blocker heptanol (0.5 mM) and the Cx mimetic peptide Gap 27 (Table 1), a Cx channel blocker that corresponds to a sequence on the second extracellular loop of Cx43 (Evans et al., 2001; Leybaert et al., 2003; 6 µM, applied to the intact heart via catheterization of a proximal side port), the infarct size was also decreased (Evans et al., 2001; Leybaert et al., 2003; Przyklenk et al., 2005). The protective effect of Gap 27 on the infarct size (Przyklenk et al., 2005) is comparable in magnitude to the effects reported by (Hawat et al., 2010) with Gap 26 (Table 1), Gap 26 is a peptide that corresponds to a sequence on the first extracellular loop of Cx43 and that has been demonstrated to interact with the extra-cellular loops (Liu et al., 2006). The sometimes variable outcome of work with gap junction inhibitors may be related to actions of these substances on hemichannels, as well as on gap junction channels (Garcia-Dorado et al., 1997; 2002; Rodriguez-Sinovas et al., 2006; Miura et al., 2010). Gap 26 and Gap 27 peptides, for example, inhibit more rapidly hemichannel function in comparison to their uncoupling effects on gap junctions (Decrock et al., 2009a).

Furthermore, other, non-Cx-related mechanisms of cardioprotection, such as altered [Ca²⁺] dynamics, ATP preservation and PKCa activation, might also be responsible for the decreased infarct size after treatment with ZP123 (Kjolbye et al., 2007). Clearly, additional studies are required to better understand the cardioprotective pathway induced by ZP123, Gap 26 and Gap 27.

**Second generation antiarrhythmic peptides**

Based on the knowledge of the structure of AAP10 and ZP123, GAP-134 (Figure 2E and Table 1) was developed (Butera et al., 2009). GAP-134 is a small dipeptide analogue of ZP123 (MW: 291.3 Da), which is biologically active upon oral administration at an average plasma concentration of 250 nM, and reduces atrial fibrillation in a dog model (Rossman et al., 2009). The efficacy and potency of GAP-134 was similar to ZP123 (Butera et al., 2009). This compound had no effect on heart rate, arterial blood pressure or other electrocardiogram (ECG) parameters. The GAP-134 enhancing effect on conduction velocity and gap junctional coupling might lead to the use of GAP-134 (50 mg·kg⁻¹·body weight) as a preventive treatment for post-operative atrial fibrillation. Furthermore, GAP-134 is an effective antiarrhythmic compound in the setting of ischaemia/reperfusion-induced arrhythmogenesis in barbiturate-anesthetized, open-chest beagles. Hereby, GAP-134 had a robust cardioprotective effect that limited infarct size (Hennan et al., 2009). The effect of oral administration of GAP-134 (steady-state plasma concentration >100 nM) on conduction abnormalities and atrial fibrillation vulnerability was studied by Laurent et al. (2009) in a model of pacing-induced atrial myopathy (Laurent et al., 2009). Simultaneous pacing of the right atrium and ventricle induced severe left atrial dilation and increased atrial fibrillation vulnerability. GAP-134 decreased the atrial ERP in paced dogs only. These results were comparable with the results obtained after IV administration of ZP123 in a dog model of mitral regurgitation. GAP-134 enhanced gap junctional communication probably by an indirect route, as there was no change in Cx43 and Cx40 mRNA levels, nor in the spatial distribution of Cx43 in the atria after 14 days of oral GAP-134 administration (Laurent et al., 2009). Interestingly, GAP-134 had no effect on the electrophysiological properties of the healthy tissue. Phase I clinical trials testing GAP-134 on healthy volunteers were finished in February 2009 and demonstrated no serious side effects, and showed normal laboratory test results, and ECGs (NCT00783341).

**Hemichannels in cardiac ischaemia**

Hemichannels, the biogenetic precursors of gap junctions, are free hexameric plasma membrane channels not engaged in gap junctions. These channels open under several physiological and pathological circumstances, such as ischemic and metabolic inhibition (John et al., 2003). There is extensive evidence that hemichannels are involved in the extracellular release of ATP, which via its degradation products, adenosine diphosphate, and mainly adenosine, enhance coronary blood flow (Burnstock, 2006; Eltzschig et al., 2006; Evans et al., 2006; Kang et al., 2008). Uncontrolled release of ATP during cardiac ischaemia results in cellular ATP depletion and predispose the cardiomyocytes to cell death. Gap 26 interacts with one of the extracellular loops of Cx43 (Liu et al., 2006) and blocks Ca²⁺-triggered ATP release mediated by Cx43 hemichannels (Braet et al., 2003a,b; Leybaert et al., 2003; De Vuyst et al., 2009). Gap 26 (0.25 mg·mL⁻¹) added during 1 h of oxygen-glucose deprivation protected isolated rat cardiomyocytes against reperfusion-induced cell death (Shintani-Ishida et al., 2007). These results were confirmed in a recent study in which Gap 26, given before or after ischaemia, protected cardiomyocytes against LAD artery occlusion and reperfusion both in vitro (0.5 µM) and in vivo (1 µg·kg⁻¹ body
potential with the Cx43 carboxyterminal domain that binds RXP-E. These cyclic structures were more stable in the cytoplasm, and formed in this way an excellent platform for the next generation of compounds with maximal activity and minimal size to preserve gap junctional communication during arrhythmias (Verma et al., 2009). Recently, Verma et al. (2010) described the first RXP-derived peptidomimetic molecule with preserved activity as a gap junction opener: ZP2519 (Table 1) with a molecular weight of 619 Da (Verma et al., 2010). This molecule prevented acidification-induced uncoupling in cardiac gap junctions and in N2A cells stably transfected with Cx43 (Verma et al., 2010). ZP2519 had no influence on the gap junctional communication of Cx40 expressing cells.

Potential Limitations for the use of Cx channel modulators

Connexins, especially Cx43, are ubiquitous proteins that are expressed in multiple organs and tissues throughout the body (Laird, 2006). Thus, it is conceivable that therapeutic interventions (acute or chronic) directed at Cx channels will result in side effects. First of all, gap junctions play an important (but not always fully understood) role in processes like organogenesis, cell differentiation (Elias et al., 2008), cancer (Kandouz and Batist, 2010), wound healing (Trosko, 2007) and in cardiac tissues, healing over (McCallister et al., 1979). In addition to this, modulating gap junctional coupling may also interfere with the function of hemichannels, from which almost nothing is currently known concerning their role and function in vivo. For example, promoting gap junctional coupling may result in a stimulation of hemichannel function, with consequent loss of essential metabolites like ATP (Kang et al., 2008) and uncontrolled entry of Ca\(^{2+}\) into the cells (Kondo et al., 2000). Even the widely studied subject of the contribution of gap junctions in the spreading of cell death is still a controversial matter (Andrade-Rozental et al., 2000; Decrock et al., 2009b). Chronic administration of AAPs are expected to interfere with (counteract) the healing over effect, in which apoptotic cells are isolated by the closure of gap junctions, to prevent the spreading of apoptosis to neighbouring cells via an unknown pro-apoptotic molecule (Lin et al., 1998). The promotion of gap junctional coupling may, on the other hand, also bring in a ‘Good Samaritan Effect’ (Szybalska and Szybalski, 1962) which involves the spreading of cell death to the surrounding cells at risk in in vivo circumstances. Furthermore, Gap 26 doubled the viability of isolated cardiomyocytes exposed to 40 min ischaemia and 180 min reperfusion. The effect in isolated cardiomyocytes was attributed, based on whole-cell patch-clamp experiments, to the inhibition of hemichannel opening by Gap 26. Similar results were obtained with Gap 27 (6 \(\mu\)M-5 min infusion) in rabbit hearts (Przyklenk et al., 2005). Strikingly, it was reported that very low concentrations of Gap 26 (0.5 \(\mu\)M \textit{in vitro} or 1 \(\mu\)g/kg \textit{in vivo}) and Gap 27 (6 \(\mu\)M \textit{in vivo}) were effective in inhibiting hemichannel currents (Przyklenk et al., 2005; Hawat and Baroudi, 2009; Hawat et al., 2010), while the peptide concentrations used hitherto were rather in the 100 \(\mu\)M range to prevent hemichannel responses (De Vuyst et al., 2009). It is worth noting that AAP peptides discussed in the previous paragraphs have also been tested for their effects on hemichannel-related ATP release or dye uptake. The antiarrhythmic peptide AAP10 (50 nM) induced a threefold increase in the peak ATP release after 80 min ischaemia and a second smaller peak after 180 min ischaemia in cardiomyocytes (Clarke et al., 2009). In contrast, GAP-134 dose-dependently reduced dye uptake in C6 cells stably transfected with Cx43 (Rossman et al., 2009). As a result, GAP-134 may display beneficial effects both at the level of gap junctions (promoting cell-to-cell coupling) as well as at the level of hemichannels (limiting cellular ATP release and the increase in cell volume) (Butera et al., 2009).

Future perspectives: RXP-peptides as antiarrhythmic agents

RXP-E (Table 1) is a peptide that binds to the carboxyterminal domain of Cx43 with a \(K_d\) \(~\text{3.9}\ \mu\text{M}\). Nuclear magnetic resonance data showed that RXP-E induced a shift in the resonance peaks of amino acids D376 to D379 and amino acids N343 to K346 of Cx43 (Shibayama et al., 2006). These two amino acid stretches are involved in the pH-dependent dimerization of the carboxyterminal domain (Sorgen et al., 2004). RXP-E partially prevented acidification-induced uncoupling by increasing the stability of the open state without altering the unitary conductance (Shibayama et al., 2006). In a next step, the RXP-E peptide was fused to a cytoplasmic transduction peptide (CTP) (Kim et al., 2006) to facilitate cellular uptake of the peptide (Lewandowski et al., 2008). CTP-RXP-E did not modify conduction velocity in the neonatal rat ventricular cardiomyocytes during control conditions. However, when the cardiomyocytes were exposed to acidic conditions CTP-RXP-E preserved propagation of the action potential (Lewandowski et al., 2008). Furthermore, the core active structures (a linear octapeptide RRNYRRNY) were studied to identify the smallest Cx43 carboxyterminal domain binding molecule that was still active to regulate gap junctional communication (Verma et al., 2009). CyRPs were the first identified cyclic molecules that were able to bind Cx43 to stabilize the open state of the gap junction channels present at the plasma membrane. CyRP-71 (Table 1) showed the strongest homol-
activity. Furthermore, they have no effects on arterial blood pressure, heart rate or classical parameters of the ECG. Thus, these peptides might be ideally positioned to fulfill the need for antiarrhythmic reagents, that is, they are efficient and safe for patients to prevent fatal ventricular arrhythmias without any risk for proarrhythmic effects, hypotension, cardiac depression or organ toxicity.

Interestingly, AAPs preferentially act on poorly coupled cells (Müller et al., 1997a; Peters et al., 1997). It is possible that: (i) AAPs activate signal transduction pathways, which are only accessible during stress conditions; (ii) AAPs interfere with the stress response itself; or (iii) the receptor with which AAPs interact is only expressed during metabolic stress. Alternatively, if most gap junctions are in the open state at near maximal conductance during normal conditions, AAPs are expected to have limited effects because of the existing ‘luxury conduction’ state. By contrast, during ischaemia, there is an impaired gap junctional communication, making the effect of the AAPs more pronounced. Further research is required to identify which of these options determines the specificity of AAPs.

As is clear from this review, there are large differences between different animal models regarding the contribution of impaired gap junctional coupling to arrhythmogenesis. AAPs, however, offer the potential to be used as tools to dissect the role of disturbed gap junctional communication in cardiac arrhythmogenesis in different models. If the arrhythmia is based on poor gap junctional coupling, increasing gap junctional communication is expected to increase the conduction velocity and have antiarrhythmic effects. However, if gap junctions only play a minor role and the arrhythmia is based on other pathophysiological factors (e.g. ion channels and structural remodeling of the heart due to the presence of fibrosis), then AAPs will have only little effect on the ‘kiss of life’. Brain Res Brain Res Rev 32: 308–315.

Acknowledgements

Our research is supported by the Fund for Scientific Research Flanders, Belgium (FWO, grant nos. G.0354.07, G.0140.08 and 3G.0134.09 to L.L.), the Interuniversity Attraction Poles Program (Belgian Science Policy, project P6/31 to L.L.) and the German Research Foundation (Schu 843/7-2 to R.S.).

Conflict of interest

I hereby would like to confirm that there is no conflict of interest for any of the authors that have contributed to this review paper.

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