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REVIEW

First-in-class cardiolipin-protective compound as a therapeutic agent to restore mitochondrial bioenergetics

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A decline in energy is common in aging, and the restoration of mitochondrial bioenergetics may offer a common approach for the treatment of numerous age-associated diseases. Cardiolipin is a unique phospholipid that is exclusively expressed on the inner mitochondrial membrane where it plays an important structural role in cristae formation and the organization of the respiratory complexes into supercomplexes for optimal oxidative phosphorylation. The interaction between cardiolipin and cytochrome *c* determines whether cytochrome *c* acts as an electron carrier or peroxidase. Cardiolipin peroxidation and depletion have been reported in a variety of pathological conditions associated with energy deficiency, and cardiolipin has been identified as a target for drug development. This review focuses on the discovery and development of the first cardiolipin-protective compound as a therapeutic agent. SS-31 is a member of the Szeto-Schiller (SS) peptides known to selectively target the inner mitochondrial membrane. SS-31 binds selectively to cardiolipin via electrostatic and hydrophobic interactions. By interacting with cardiolipin, SS-31 prevents cardiolipin from converting cytochrome *c* into a peroxidase while protecting its electron carrying function. As a result, SS-31 protects the structure of mitochondrial cristae and promotes oxidative phosphorylation. SS-31 represents a new class of compounds that can recharge the cellular powerhouse and restore bioenergetics. Extensive animal studies have shown that targeting such a fundamental mechanism can benefit highly complex diseases that share a common pathogenesis of bioenergetics failure. This review summarizes the mechanisms of action and therapeutic potential of SS-31 and provides an update of its clinical development programme.

LINKED ARTICLES
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Abbreviations
Cyt *c*, cytochrome *c*; ETC, electron transport chain; IMM, inner mitochondrial membrane; IR, ischaemia-reperfusion; LV, left ventricle; MPT, mitochondrial permeability transition; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; SS, Szeto-Schiller; TAC, transverse aortic constriction; TPP^+, triphenylphosphonium ion

Introduction

Defects in energy metabolism represent a common thread among many age-associated complex diseases. A decline in bioenergetics underlies the general frailty of old age and a broad spectrum of metabolic and degenerative diseases. A wealth of research converges on the mitochondrion as the central player in cellular aging (Bratic and Trifunovic, 2010; Lee and Wei, 2012; Bratic and Larsson, 2013). Mitochondria produce about 90% of cellular energy, but they are also the major source of intracellular reactive oxygen species (ROS) and play a central role in the initiation and execution of apoptosis. As energy output declines, the most energetic tissues are preferentially affected, resulting in degenerative changes in the CNS, heart, kidney and muscle. Age-related decline in mitochondrial bioenergetics has been observed in these tissues and is associated with a decline in function in both experimental animals and humans (Short *et al.*, 2005;

Judge and Leeuwenburgh, 2007; Figueiredo *et al.*, 2009). The universality of bioenergetic failure in age-associated complex diseases has led to the idea that restoration of mitochondrial bioenergetics may present a common approach to the treatment of disorders as diverse as heart failure, chronic kidney disease, skeletal muscle weakness and neurodegenerative diseases.

As mitochondrial oxidative stress has long been considered the primary aetiology behind aging and mitochondrial dysfunction, the majority of drug discovery efforts to date have centred on compounds that reduce mitochondrial ROS. Mitochondria-targeted antioxidants that are based on delivery of known redox agents to the mitochondrial matrix by conjugation to delocalized cations [such as triphenylphosphonium ion (TPP^+)] have been reviewed extensively (Skulachev *et al.*, 2009; Smith *et al.*, 2012). Although these compounds can reduce mitochondrial ROS, recent studies suggest they inhibit mitochondrial bioenergetics (Fink *et al.*, 2012; Reily *et al.*, 2013).

More recently, cardiolipin, the phospholipid that is uniquely expressed on the inner mitochondrial membrane (IMM), has been identified as a potential drug target because of its central role in the structural formation of cristae membranes and organization of the respiratory components of the electron transport chain (ETC), as well as serving as the platform for initiation of apoptosis (Schlame and Ren, 2009; Schug and Gottlieb, 2009; Sorice *et al.*, 2009; Paradies *et al.*, 2010). Changes in cardiolipin not only alter fluidity and folding of the IMM, but can profoundly alter the organization and function of respiratory complexes. Cardiolipin peroxidation and loss of cardiolipin has been associated with aging and several metabolic and degenerative diseases (Han *et al.*, 2005; Chicco and Sparagna, 2007; Shi, 2010; Claypool and Koehler, 2012). Recent lipidomic studies have identified cardiolipin as a target for drug development for traumatic brain injury, Parkinson's Disease, cancer, diabetes and the metabolic syndrome (Bayir *et al.*, 2007; Gross and Han, 2007; Han *et al.*, 2007; Kiebish *et al.*, 2008; Ji *et al.*, 2012; Tyurina *et al.*, 2013). This review will focus on the discovery and development of the first class of compounds that targets cardiolipin on the IMM and optimizes cristae architecture, improves mitochondrial bioenergetics and reduces ROS generation. The Szeto-Schiller (SS) peptides have been evaluated in numerous preclinical disease models involving bioenergetics failure, and the lead compound (Bendavia™) is currently in Phase II clinical trials for several clinical indications.

Mitochondrial bioenergetics declines with age

Aging is known to result in biochemical and functional alterations in many components of the mitochondrial ETC, resulting in reduced efficiency of electron transport, inhibition of oxidative phosphorylation (OXPHOS), and increased ROS generation (Lesnefsky and Hoppel, 2006). Catalytic activity of complexes I, III and IV all decline with age in liver, brain, heart and skeletal muscle (Lenaz *et al.*, 1997; Paradies *et al.*, 1997; Fannin *et al.*, 1999; Lesnefsky *et al.*, 2001a; Moghaddas *et al.*, 2003; Boveris and Navarro, 2008; Petrosillo *et al.*, 2008). These defects in respiratory complex activity are reflected in reduced state 3 respiration and coupled respiration in isolated mitochondria (Kim *et al.*, 1988a,b; Paradies and Ruggiero, 1990; Short *et al.*, 2005; Boveris and Navarro, 2008; Figueiredo *et al.*, 2009; O'Toole *et al.*, 2010). A significant decrease in coupled respiration and ATP synthesis was also found in skeletal muscles from aged mice and humans (Marcinek *et al.*, 2005; Conley *et al.*, 2013).

Loss of efficiency in electron transport is expected to increase electron leak and ROS generation in aging. There is substantial disagreement, however, about whether mitochondrial ROS production increases with age (Muscari *et al.*, 1990; Drew *et al.*, 2003; Moghaddas *et al.*, 2003; Suh *et al.*, 2003; Judge *et al.*, 2005; Sen *et al.*, 2007). These discrepancies may be caused by variation in endogenous antioxidant capacity in different tissues and cell types (Suh *et al.*, 2003; Judge *et al.*, 2005). Despite the lack of consistent evidence showing increased mitochondrial ROS production, there is good evidence that the mitochondrial glutathione redox status (GSH:GSSG) shifts progressively towards oxidation with age (de la Asuncion *et al.*, 1996; Rebrin *et al.*, 2003; Suh *et al.*, 2003) and there is a significant decrease in mitochondrial GSH levels in brain, skeletal muscle and liver of senescent animals (Rebrin and Sohal, 2004; Perluigi *et al.*, 2010), indicating mitochondrial oxidative stress.

Aging leads to alterations in lipid composition of the IMM

Lipids interact dynamically to form transient arrangements and influence the fluidity of lipid membranes. Lipids also interact with proteins and modulate the structural organization of proteins on the membrane, allowing for multimeric protein complexes and higher order supercomplexes (Bogdanov *et al.*, 2008). Since the ETC resides on the IMM, changes in lipid composition of the IMM can profoundly affect mitochondrial bioenergetics. The age-associated decline in OXPHOS may result from alterations in lipid composition of the IMM (Gomez and Hagen, 2012). The defect in OXPHOS involves all respiratory complexes, including a reduction in cytochrome *c* (cyt *c*). Interestingly, the decrease in complex IV activity in cardiac mitochondria could be reversed by the addition of exogenous phospholipid liposomes, suggesting that the defect in OXPHOS may occur secondary to a defect in the phospholipid environment of complex IV (Lesnefsky and Hoppel, 2003).

The IMM contains predominantly phosphatidylcholine, phosphatidylethanolamine and cardiolipin. While phosphatidylcholine and phosphatidylethanolamine are found on other membranes, cardiolipin is only expressed on the IMM (Osman *et al.*, 2011). Cardiolipin is synthesized from phosphatidylglycerol by cardiolipin synthase on the inner face of the IMM, followed by a transacylation reaction to achieve the desirable acyl side chains (Schlame *et al.*, 2000). The acyl chains can vary but are predominantly comprised of linoleic acid (18:2) in the heart. Unlike other phospholipids, cardiolipin is a lipid dimer with two phosphate head groups and four acyl side chains, thus giving it a conical structure (Figure 1). To a lesser extent, phosphatidylethanolamine also

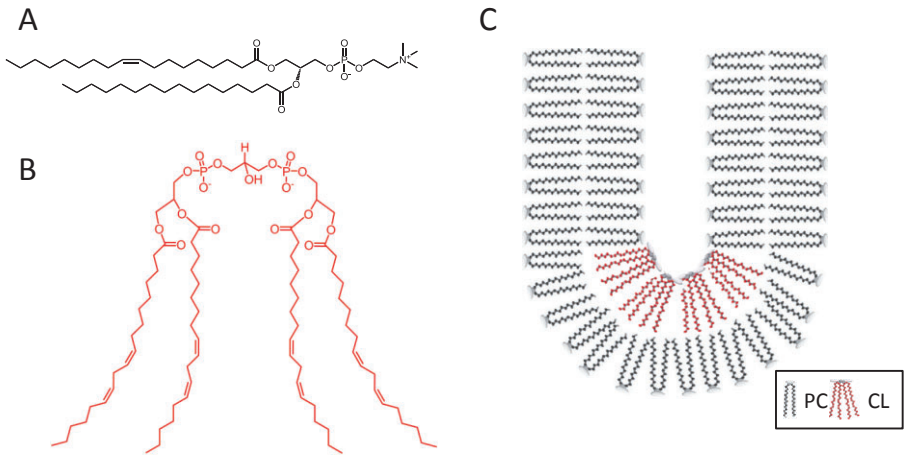

Figure 1

Cardiolipin promotes curvature in lipid membranes due to its unique conical structure. (A) Chemical structure of phosphatidylcholine (PC). (B) Chemical structure of cardiolipin (CL). (C) Cardiolipin exerts lateral pressure in a lipid bilayer to induce a negative curvature.

has a cone shape. As a result, these two phospholipids do not form bilayers. On a membrane with other lipids, they exert a lateral pressure that modulates membrane curvature (Frey and Mannella, 2000; Osman *et al.*, 2011) (Figure 1). Thus cardiolipin is particularly important for cristae formation, and deficiency in cardiolipin results in loss of cristae membranes (Acehan *et al.*, 2007) (Figure 2). Cardiolipin also plays an important role in maintaining inner membrane fluidity and osmotic stability, and decreases the energy required to create folds or cristae in the IMM (Shibata *et al.*, 1994; Nichols-Smith *et al.*, 2004).

Cardiolipin also helps to organize the respiratory complexes into supercomplexes to facilitate optimal electron transfer among the redox partners (Zhang *et al.*, 2002; Pfeiffer *et al.*, 2003; Mileykovskaya and Dowhan, 2009; Kiebish *et al.*, 2012; Bazan *et al.*, 2013) (Figure 2). Many of the respiratory complexes and carrier proteins require cardiolipin for optimal assembly and function (Fry and Green, 1981; Hoch, 1992; Mileykovskaya *et al.*, 2005; Zhang *et al.*, 2005; Chicco and Sparagna, 2007; Wittig and Schagger, 2009; Schwall *et al.*, 2012). Disruption of supercomplex formation can enhance ROS generation from complex I (Maranzana *et al.*, 2013). Cardiolipin also plays a role in anchoring cyt *c* to the IMM and facilitates electron transfer from complex III to complex IV (Rytomaa and Kinnunen, 1994, 1995).

A decline in cardiolipin content with age has been reported in mitochondria from brain, liver and heart (Vorbeck *et al.*, 1982; Paradies *et al.*, 1997; Hagen *et al.*, 1998; Sen *et al.*, 2007). This loss of cardiolipin may be due to changes in cardiolipin synthase activity, alterations in cardiolipin remodelling or cardiolipin peroxidation. The loss of cardiolipin can explain the decreased mitochondrial membrane fluidity reported in aged rats (Lee *et al.*, 1999). It is also consistent with the findings that age-related decline in complex IV activity can be restored by exogenously added cardiolipin or cyt *c* (Paradies *et al.*, 1997; O'Toole *et al.*, 2010; Petrosillo *et al.*, 2013).

Cardiolipin peroxidation, mitochondrial permeability transition and cell death

In addition to its important role in maintaining cell viability, cardioloipin can also play an important role in cell death, especially when it is oxidized. Cardiolipin is particularly vulnerable to oxidative damage because of its high content of unsaturated fatty acids and its location near the site of ROS production (Paradies *et al.*, 2011). Cardiolipin can be oxidized by mitochondrial H_2O_2, but this oxidation is greatly enhanced in the presence of cyt *c* (Kagan *et al.*, 2005; Basova *et al.*, 2007; Wiswedel *et al.*, 2010). Interaction of cyt *c* with cardiolipin promotes cyt *c* unfolding and dramatically enhances the protein's peroxidase activity. Native cyt *c* has a compact tertiary structure with its haem iron coordinated to Met80 and His18. Because of its hexacoordinated iron, native cyt *c* has very low peroxidase activity. Studies with cyt *c* and cardiolipin liposomes have reported substantial unfolding of cyt *c* that can disrupt the Met80 ligation and exposes the haem iron to H_2O_2 (Hanske *et al.*, 2012; Muenzner *et al.*, 2013). Other investigators have proposed that one or two acyl chains of cardiolipin can penetrate deep into the hydrophobic core of cyt *c* and loosen the Met80-Fe axial bond (Kalanxhi and Wallace, 2007; Sinibaldi *et al.*, 2008; Sinibaldi *et al.*, 2010).

The oxidation of cardiolipin produces kinks in the acyl chain that disturbs cardiolipin microdomains on the IMM and causes the loss of curvature (Figure 3). Cardiolipin peroxidation also disrupts supercomplexes and causes cyt *c* to be detached from the IMM. All of this results in inhibition of mitochondrial respiration and sets the stage for apoptosis (Gonzalvez and Gottlieb, 2007; Schug and Gottlieb, 2009). Oxidized cardiolipin synergizes with Ca^{2+} to induce opening of the mitochondrial permeability transition (MPT) pore (Petrosillo *et al.*, 2007). The identity of the MPT pore remains

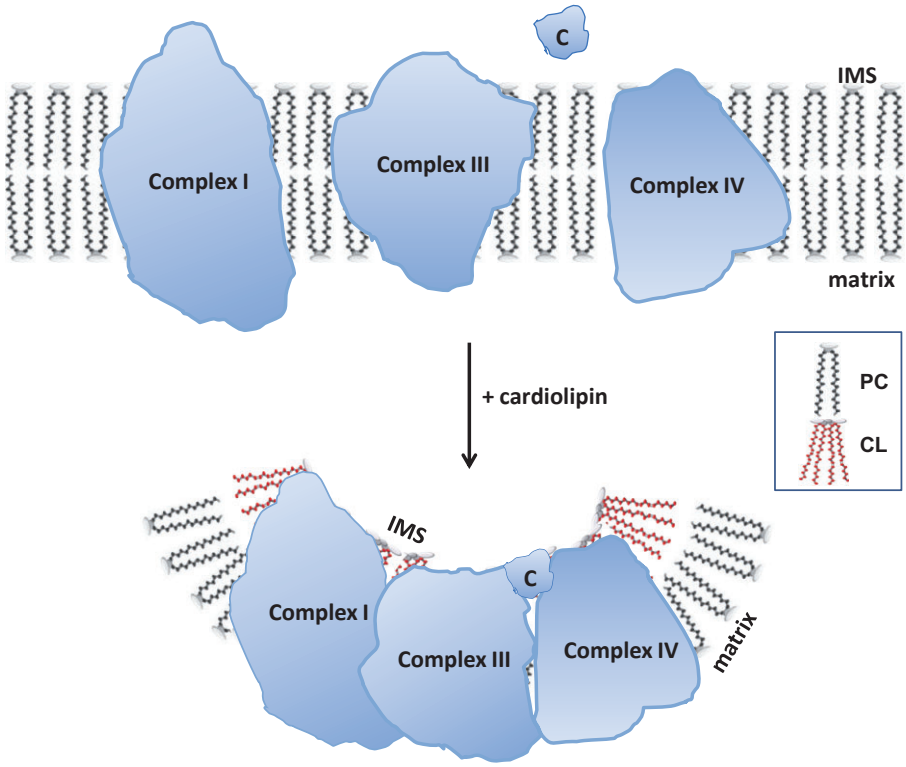

Figure 2

Cardiolipin is important for cristae structure and supercomplex formation on the inner mitochondrial membrane (IMM). The protein complexes of the electron transport chain reside on cristae membranes and cardiolipin (CL) provides curvatures on the IMM to increase surface area for the respiratory complexes. Cardiolipin also helps to organize the respiratory complexes into supercomplexes to facilitate electron transfer among the redox partners. Lastly, cardiolipin anchors the highly cationic cyt *c* (C) via electrostatic interaction to bring it in close proximity to Complex III and Complex IV for efficient electron transfer. IMS: intermembrane space.

elusive, but it is believed to be a non-specific pore which allows free passage of any molecules <1.5 kDa (Halestrap, 2009). The voltage-dependent anion channel, the adenine nucleotide translocator and the F_0,F_1-ATP synthase have all been postulated to be part of the MPT pore (Bernardi, 2013). Mitochondrial permeability transition disrupts the permeability barrier of the IMM and leads to collapse of the mitochondrial potential and uncoupling of oxidative phosphorylation (Halestrap, 2009). In addition, MPT causes the release of cyt *c* and other proapoptotic proteins into the cytosol where they trigger the caspase cascade and cell death by apoptosis (Shidoji *et al.*, 1999; Jiang and Wang, 2004; Ott *et al.*, 2007). Severe depletion of ATP will result in cell death by necrosis.

Cardiolipin as a target for drug development

Cardiolipin peroxidation and depletion have been reported in a variety of pathological conditions associated with energy deficiency, including skeletal muscle weakness, heart failure, neurodegenerative diseases, diabetes and ischaemia-reperfusion (IR) injury. Compounds that can inhibit cardiolipin peroxidation and preserve cardiolipin may potentially be beneficial for these diseases.

Attempts at designing molecules to inhibit cardiolipin peroxidation have been very limited. The mitochondria-targeted electron scavenger XJB-5–131 (4-amino-TEMPO conjugated to hemigramacidin S) was reported to inhibit cardiolipin peroxidation, reduce apoptotic neuronal cell death and improve behaviour in a rat traumatic brain injury model (Bayir *et al.*, 2007). This compound may inhibit cardiolipin peroxidation by reducing mitochondrial H_2O_2, but there is no evidence that it directly inhibits cyt *c* peroxidase activity. A mitochondria-targeted inhibitor of cyt *c* peroxidase was shown to inhibit cardiolipin peroxidation *in vitro* and protect against radiation injury (Atkinson *et al.*, 2011). The imidazole-substituted fatty acid was designed to replace Met80 as the sixth coordinate on the haem iron, and TPP^+ was used to deliver it to mitochondria. It is unclear whether locking the haem iron with imidazole affects the electron carrying capacity of cyt *c*, and TPP^+ has been shown to inhibit mitochondrial bioenergetics (Fink *et al.*, 2012; Reily *et al.*, 2013). The imidazole approach may also have off-target effects due to potential interaction with other haem proteins. A third approach used a poorly peroxidizable TPP^+ conjugated octadecanoic acid to remodel the endogenous pool of cardiolipin to reduce cardiolipin peroxidation (Tyurina *et al.*, 2012). This approach has only been investigated in cultured cells, and it is not known what effect this modified cardiolipin may have on mitochondrial respiration. It is important

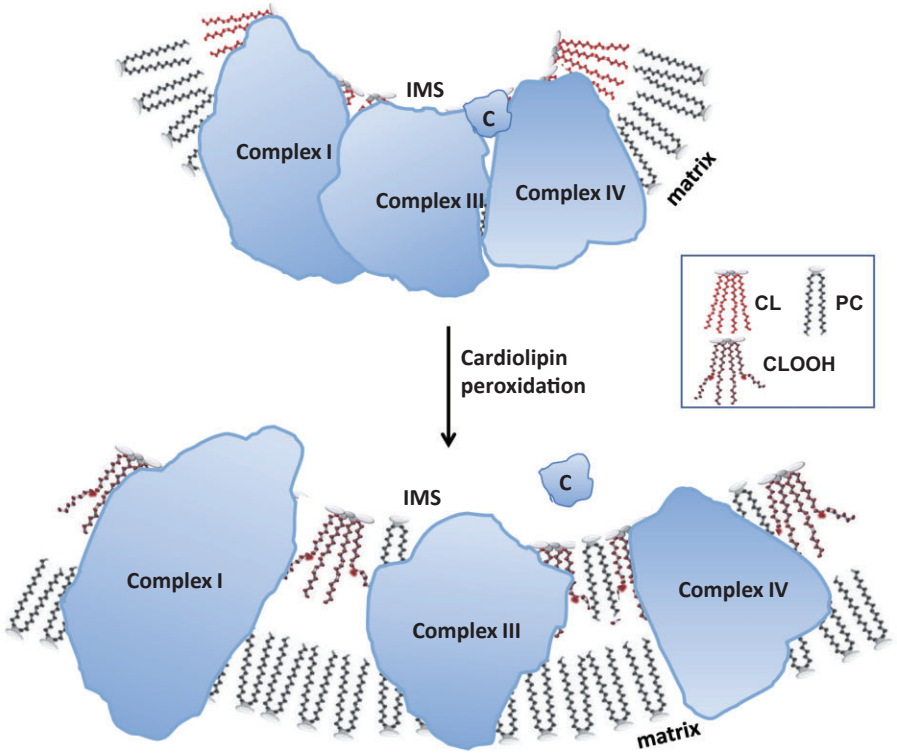

Figure 3

Cardiolipin peroxidation destabilizes cardiolipin microdomains on the inner mitochondrial membrane (IMM) and disrupts supercomplexes. Cardiolipin (CL) is particularly vulnerable to oxidative damage because of its high content of unsaturated fatty acids. Peroxidation of the acyl chains alters the structure of cardiolipin (CLOOH) and prevents cardiolipin from aggregating into microdomains or rafts on the IMM. The breakdown of cardiolipin rafts abolishes cristae curvatures and disrupts the organization of respiratory complexes into higher order supercomplexes. Peroxidation of cardiolipin also reduces its affinity for cyt *c* (C) and sets the stage for cyt *c* release into the cytosol and apoptosis. IMS: intermembrane space.

that any attempt at inhibiting cyt *c* peroxidase activity does not destroy the vital function of cyt *c* as an electron carrier.

Discovery of a new class of small molecules that target cardiolipin

The SS peptides represent a unique class of mitochondria-targeted compounds and their chance discovery was described in a recent review (Szeto and Schiller, 2011). These are synthetic tetrapeptides having an alternating aromatic-cationic motif, among which SS-31 (D-Arg-2′6′-dimethylTyr-Lys-Phe-NH_2) is the most extensively studied (Figure 4A). Despite being very water soluble, the SS peptides are remarkably cell permeable, and they are readily taken up and act on all cell types, including endothelial cells, renal and intestinal epithelial cells, myotubes, cardiomyocytes, macrophages and neurons (Zhao *et al.*, 2003; 2005; 2004; Cho *et al.*, 2007b; Han *et al.*, 2009; Zhu *et al.*, 2010; 2011; Andersson *et al.*, 2011; Calkins *et al.*, 2011; Li *et al.*, 2011; Dai *et al.*, 2011a; Gilliam *et al.*, 2012; Kloner *et al.*, 2012; Birk *et al.*, 2013b). The mechanism of cellular uptake for these polar peptides is consistent with diffusion as their uptake is linear and independent of receptor- or transporter-mediated processes (Zhao *et al.*, 2003). Confocal microscopy with fluorescent-labelled analogues suggested that these compounds are localized to mitochondria (Zhao *et al.*, 2004). In addition to fluorescent labels, the intracellular distribution of these peptides can be visualized using biotinylated analogues (Birk *et al.*, 2013a). Using isolated mitochondria, uptake of the SS peptides was found to be rapid and independent of mitochondrial potential, and fractionation studies revealed that the peptides are concentrated on the IMM rather than in the mitochondrial matrix (Zhao *et al.*, 2004; 2005).

It was recently discovered that SS-31 selectively binds to cardiolipin via both electrostatic and hydrophobic interactions (Birk *et al.*, 2013b). Aladan (Ald), a polarity-sensitive fluorescent amino acid whose emission maximum (λmax) undergoes a blueshift in a hydrophobic environment (Cohen *et al.*, 2002), was used to probe whether the highly water-soluble SS-31 can interact with lipids. Ald was incorporated into the peptide sequence of SS-31 by substituting Ald for Phe (Figure 4B). Only anionic phospholipids (phosphatidylserine and cardiolipin) caused a blueshift in the λmax of [ald]SS-31, whereas the zwitterionic phospholipids (phosphatidylcholine and phosphatidylethanolamine) had no effect (Figure 4C). As phosphatidylserine is negligible in the IMM, cardiolipin is the primary target for SS-31 in the IMM. These results led us to propose that electrostatic interaction between the two basic amino acids on SS-31 and the phosphate head groups of cardiolipin aligns the aromatic residues within the hydrophobic acyl chain region. The interaction

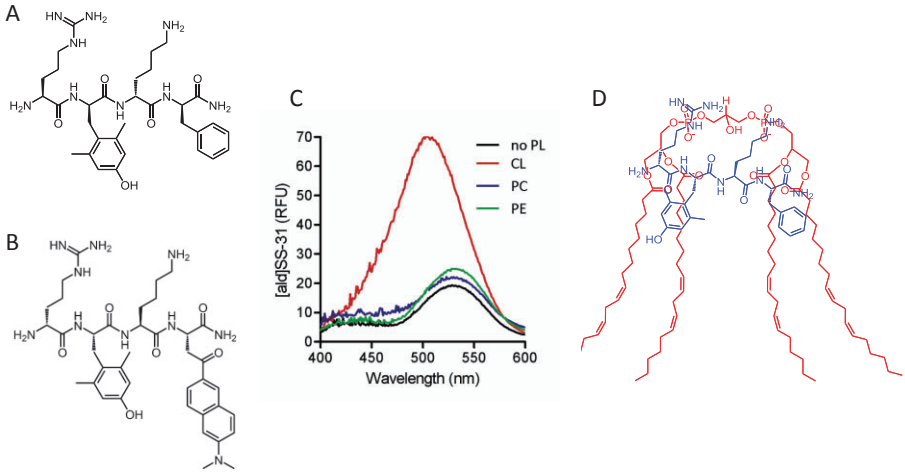

Figure 4

SS-31 selectively binds to cardiolipin. (A) Chemical structure of SS-31 (D-Arg-dimethylTyr-Lys-Phe-NH_2). (B) Chemical structure of [ald]SS-31. The Phe^4 in SS-31 is replaced by aladan (ald), a polarity-sensitive fluorescent amino acid. (C) Fluorescence emission spectra of [ald]SS-31 in the absence of phospholipids (no PL), or in the presence of cardiolipin (CL), phosphatidylcholine (PC) or phosphatidylethanolamine (PE). Addition of cardiolipin caused a shift of the emission maximum (λmax) from 530 to 500 nm. (D) Proposed model showing the interaction of SS-31 with cardiolipin. Electrostatic interaction between the two cationic moieties of SS-31 (Arg and Lys) and the phosphate head groups of cardiolipin aligns the aromatic residues (dimethyl-Tyr and Phe) within the hydrophobic acyl chain region of cardiolipin.

between SS-31 and cardiolipin occurs at 1:1 molar ratio (Figure 4D). The insertion of the aromatic groups into the lipid environment has now been confirmed by nuclear magnetic resonance (Birk *et al.*, 2013a).

Interaction of SS-31 with cardiolipin changes cyt *c* activity

Cyt *c* plays major roles in mitochondrial respiration and in apoptosis, and this delicate balance between life and death is regulated by its interaction with cardiolipin. Electrostatic interaction between cyt *c* and cardiolipin brings cyt *c* in close proximity to the respiratory complexes for optimal electron transfer. However, hydrophobic interaction between cyt *c* and cardiolipin can result in unfolding of the tertiary structure of cyt *c* and converts this electron carrier into a peroxidase (Figure 5). Thus cyt *c* is Janus-faced with two contrasting functions, one promoting life and one promoting death. We have found that the interaction of SS-31 with cardiolipin favours the electron carrier over the peroxidase in cyt *c*.

SS-31 potently inhibited cardiolipin-induced cyt *c* peroxidase activity *in vitro* as well as in isolated mitochondria, indicating that SS-31 can inhibit peroxidase activity of endogenous cyt *c* in mitochondria (Birk *et al.*, 2013b). Interaction of SS-31 with cardiolipin had no effect on the binding of cyt *c* to cardiolipin. When cyt *c* was added to the [ald]SS-31/cardiolipin complex, the fluorescent signal was dramatically quenched, indicating that the peptide/cardiolipin complex is localized within angstroms of the haem which is a large resonance acceptor (Birk *et al.*, 2013b). Structural studies with circular dichroism revealed that SS-31 effectively prevented the unfolding of cyt *c* by cardiolipin and protected the Met80-haem ligation, thus preventing any peroxidase activity (Birk *et al.*, 2013b).

Importantly, SS-31 appears to have no inhibitory effect on the electron carrier function of cyt *c*. By disrupting the Met80-Fe ligand, cardiolipin lowers the redox potential of cyt *c* and inhibits its reduction by glutathione or ascorbate (Basova *et al.*, 2007). SS-31 itself has no effect on cyt *c* reduction by glutathione or ascorbate, but it was able to prevent the inhibition caused by cardiolipin at a 1:1 peptide : cardiolipin ratio (Birk *et al.*, 2013a). Furthermore, addition of SS-31 to isolated mitochondria significantly increased state 3 respiration initiated by substrates for complex I or II, or when cyt *c* was directly reduced by N,N,N′,N′-tetramethyl-p-phenylenediamine/ascorbate, suggesting that protecting cardiolipin can improve the activity of each component of the ETC (Birk *et al.*, 2013a). Importantly, SS-31 had no effect on state 4 respiration indicating that SS-31 does not promote electron flux by uncoupling mitochondria. SS-31 promotes the efficiency of coupled respiration by increasing P/O ratio and this was confirmed by increase in the rate of ATP synthesis (Birk *et al.*, 2013a). Thus, SS-31 targets cardiolipin and interacts with cyt *c* to protect the Met80-haem bond and inhibits peroxidase activity while improving π-π* interaction to promote electron transfer and ATP synthesis. By modulating the interaction between cardiolipin and cyt *c*, SS-31 favours cyt *c* as an electron carrier and minimizes its role as a peroxidase (Figure 5).

SS-31 protects mitochondrial cristae, promotes ATP synthesis and inhibits mitochondrial permeability transition

During ischaemia, the rapid drop in ATP leads to elevation in cytosolic calcium followed by increase in mitochondrial calcium. Calcium induces mitochondrial ROS and cardiolipin peroxidation (Lesnefsky *et al.*, 2001b; Petrosillo *et al.*, 2004;

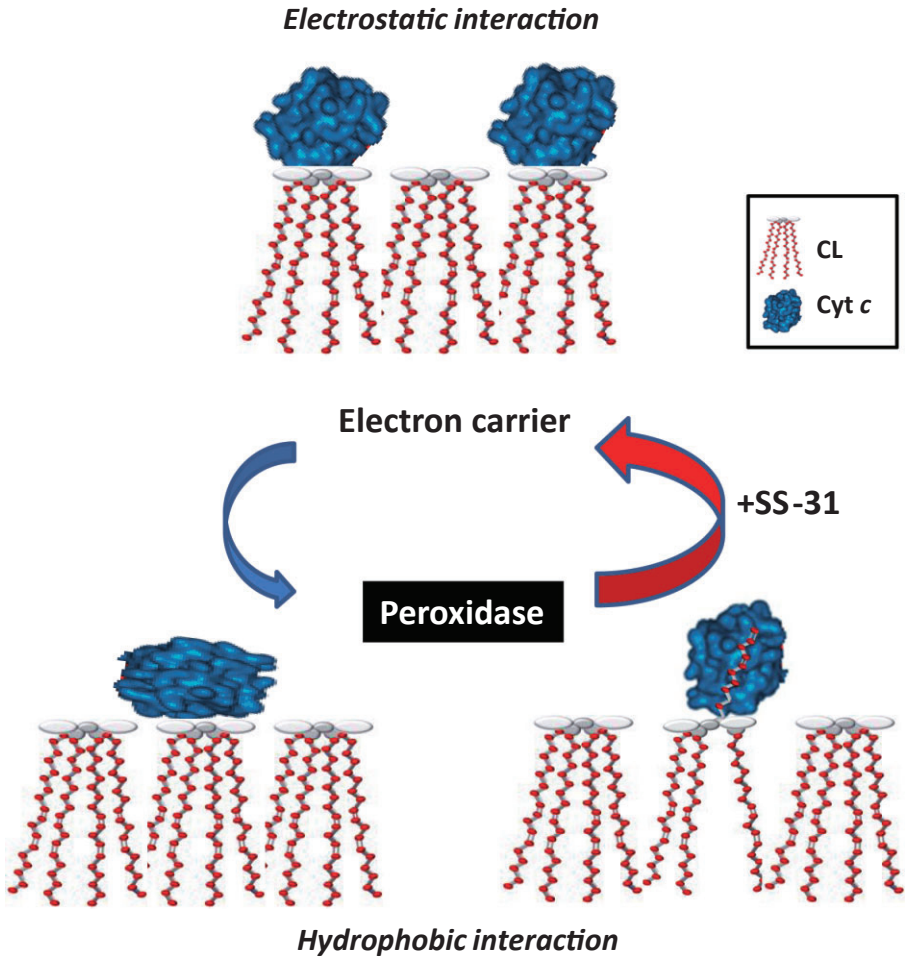

Figure 5

Interaction of SS-31 with cardiolipin favours the electron carrier over the peroxidase in cyt *c*. Electrostatic interaction between cyt *c* and cardiolipin brings cyt *c* in close proximity to the respiratory complexes for optimal electron transfer. However, hydrophobic interaction between cyt *c* and cardiolipin results in unfolding of the tertiary structure of cyt *c* and converts this electron carrier into a peroxidase by disrupting the Met80-haem ligation. The SS-31/cardiolipin complex localizes to within angstroms of the haem group to protect the Met80-haem coordination and inhibits peroxidase activity while improving π–π^* interaction to promote electron transfer and ATP synthesis.

Chen *et al.*, 2008; Paradies *et al.*, 2009). We found that calcium can directly stimulate cyt *c* peroxidase activity *in vitro*, and SS-31 can completely prevent this peroxidase activity with EC_{50} less than 1 μM (Birk *et al.*, 2013b). By targeting mitochondrial cardiolipin and inhibiting cardiolipin peroxidation, SS-31 protects mitochondrial cristae and facilitates ATP recovery in ischaemic tissues (Birk *et al.*, 2013b).

Interruption of renal blood flow for 45 min in the rat resulted in dramatic swelling of tubular cell mitochondria and loss of cristae membranes (Figure 6A). Some mitochondria remained swollen in early reperfusion, while others showed disrupted outer mitochondrial membranes and spilling of matrix materials into the cytoplasm, consistent with MPT (Figure 6C). In contrast, cristae membranes remained intact in the SS-31 treated kidneys at the end of ischaemia (Figure 6B) and after 5 min reperfusion (Figure 6D). Mitochondrial permeability transition led to sustained inhibition of mitochondrial respiration; with ATP levels down by more than 30% even 1 h after return of blood flow (Szeto *et al.*, 2011). Mitochondria treated with SS-31 showed improved mitochondrial respiration and complete recovery of ATP after 1 h. By accelerating ATP recovery after ischaemia, SS-31 protected epithelial cells from apoptotic and necrotic cell death. These studies demonstrate that by binding to cardiolipin and inhibiting cardiolipin peroxidation, SS-31 protected the structure of the mitochondrial cristae during ischaemia, inhibited MPT pore opening and accelerated ATP recovery immediate upon reperfusion.

Therapeutic potential of SS-31 in age-associated diseases

Numerous animal studies have demonstrated the remarkable efficacy of SS-31 in diverse diseases associated with bioenergetic failure (see Table 1). The effective dose(s) of SS-31 in the different disease models are summarized in Table 1. Minimal

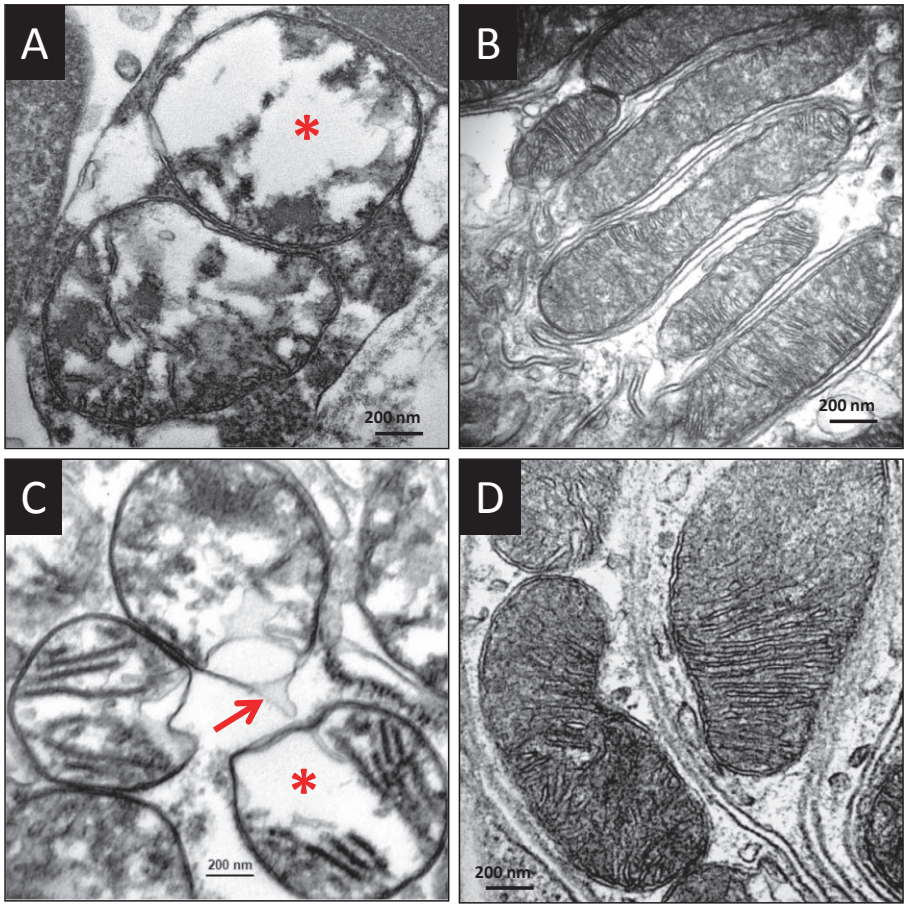

Figure 6

SS-31 protects mitochondrial cristae during ischaemia and prevents mitochondrial permeability transition. Rats were treated with saline or SS-31 (2 mg kg^{-1}) before occlusion of renal blood flow for 45 min. Kidney sections were obtained either at the end of ischaemia or after 5 min reperfusion and examined by transmission electron microscopy. (A) Representative electron microscopic image of proximal tubular cells from saline-treated animal at the end of ischaemia shows swollen mitochondria with loss of cristae and matrix material (*). (B) Mitochondria from SS-31 treated kidneys looked normal and were elongated with finely stacked cristae membranes even after 45 min ischaemia. (C) Mitochondria from saline-treated animals remained swollen (*) after 5 min reperfusion and some of them showed breaks in the outer mitochondrial membrane and loss of matrix contents into the cytosol (arrow), consistent with mitochondrial permeability transition. (D) Mitochondria from SS-31 treated sections were normal with many cristae stacks. All images are taken at 80 000 × magnification.

effective dose in rodents is 0.1–0.5 mg kg^{-1} given subcutaneously (s.c.) or in the peritoneum (i.p.), and dose-dependent responses were observed between 0.1 and 5 mg kg^{-1} (Yang *et al.*, 2009; Szeto *et al.*, 2011). Plasma levels of SS-31 are dose-proportional over these dose ranges (Szeto and Schiller, 2011). In general, doses are lower for larger animal models (dogs, sheep and pigs). It is beyond the scope of this review to present all published reports, and some of these studies have been reviewed previously (Szeto, 2008a,b; Szeto and Schiller, 2011). This review will concentrate on the most recent findings and those preclinical studies that have provided the rationale for clinical trials with SS-31. In addition to studies described in detail below, SS-31 has been reported to be beneficial for chronic kidney disease, metabolic syndrome, neurodegenerative diseases and drug-induced mitochondrial toxicity (Petri *et al.*, 2006; Mizuguchi *et al.*, 2008; Anderson *et al.*, 2009; Yang *et al.*, 2009; Duan *et al.*, 2013; Huang *et al.*, 2013; Toyama *et al.*, 2013). It should be pointed out that SS-31 is also referred to as MTP-131 or Bendavia in the literature. The nomenclature used in the original paper will be used in this review.

Efficacy of SS-31 in IR injury

In addition to acute renal ischaemia, SS-31 is effective against myocardial ischaemia and cerebral ischaemia. Treatment with SS-31 prior to coronary artery ligation significantly reduced infarct size, decreased severity of arrhythmias and reduced lipid peroxidation in rats (Cho *et al.*, 2007a). SS-31 also reduced infarct size in a mouse model of cerebral ischaemia and attenuated glutathione depletion even when administered at the onset of reperfusion (Cho *et al.*, 2007b).

A recent paper reported the results of a collaborative study from three independent laboratories on the effects of Bendavia in acute cardiac IR injury (Kloner *et al.*, 2012). Bendavia

Table 1

Summary of preclinical studies with SS-31

Ischaemia-reperfusion models	Species	Dose	Route	Time of administration	I/R duration	Major findings	Reference
Isolated perfused hearts	Guinea pig	1 nM	perfusate	Starting 20 min before ischaemia and throughout reperfusion	30′ I/60′ R	preserved contractile force preserved ATP content ↓ infarct area ↓ lipid peroxidation	Szeto (2008b)
Isolated perfused hearts	Guinea pig	1 nM	perfusate	Starting before ischaemia and throughout reperfusion	20′ I/2h R	↓ infarct area	Kloner *et al.* (2012)
Isolated perfused hearts	Guinea pig	1 nM	perfusate	Starting at onset of reperfusion	20′ I/2h R	↓ infarct area	Kloner *et al.* (2012)
Isolated perfused hearts	Diabetic rats	1 nM	perfusate	Starting at onset of reperfusion	20′ I/2h R	↓ infarct area	Sloan *et al.* (2012)
Left anterior descending coronary artery ligation	Rat	3 mg kg^{-1}	i.p.	30 min before ligation 5 min before reperfusion	60′ I/60′R	↓ infarct area, ↓ arrhythmias ↓ lipid peroxidation	Cho *et al.* (2007a)
Left anterior descending coronary artery ligation	Sheep	0.05 mg kg^{-1} h^{-1}	i.v.	Starting 30 min before reperfusion	60′ I/3h R	↓ infarct area	Kloner *et al.* (2012)
Left anterior descending coronary artery ligation	Rabbit	0.05–0.1 mg kg^{-1} h^{-1}	i.v.	Starting either 10 min before or at reperfusion	30′ I/3h R	↓ no-reflow	Kloner *et al.* (2012)
Middle cerebral artery occlusion	Mice	2 or 5 mg kg^{-1}	s.c.	immediately after reperfusion, or at 6, 24 or 48 h after reperfusion	30′ I/72h R	↓ infarct volume ↓ glutathione depletion	Cho *et al.* (2007a)
Renal artery occlusion	Rats	0.5, 2 or 5 mg kg^{-1}	s.c.	30 min before ischaemia 5 min before reperfusion, 2 h after reperfusion	45′ I/24h R	protected mitochondrial structure after ischaemia ↑ ATP synthesis, ↑ GSH ↓ lipid peroxidation, ↓ tubular apoptosis and necrosis, improved kidney function	Szeto *et al.* (2011)
Renal artery occlusion	Rats	2 mg kg^{-1}	s.c.	30 min before ischaemia 5 min before reperfusion	30′ I/24h R	Protected renal mitochondria and ATP synthesis ↓ tubular apoptosis ↓ renal dysfunction	Birk *et al.* (2013b)
Islet transplantation	Donor mice Islets Recipient mice	3 mg kg^{-1} 1 nM 3 mg kg^{-1}	s.c.	24 h before islet harvest All isolation reagents *bid* x 10 days		↑ islet yield ↓ islet apoptosis, ↑ post-transplantation islet graft function	Thomas *et al.* (2007)
Renal artery stenosis	Pigs	0.05 mg kg^{-1} h^{-1}	i.v.	Continuous administration for 4 h during renal angioplasty	4 weeks	↑ renal blood flow and GFR ↓ microvascular rarefaction, ↓oxidative stress, tubular injury and fibrosis	Eirin *et al.* (2012)

Table 1

Continued

Heart Failure models	Species	Dose	Route	Time of administration	Duration of study	Major findings	Reference
Ang II hypertensive cardiomyopathy	Mice	3 mg kg^{-1} day^{-1}	osmotic pump	Concurrent with angiotensin exposure	4 weeks	Prevented left ventricular hypertrophy Mitigated diastolic dysfunction Prevented cardiac fibrosis	Dai *et al.* (2011b)
$G\alpha_q$ overexpression	Mice	3 mg kg^{-1} day^{-1}	osmotic pump	age 12–16 weeks	4 weeks	Ameliorated diastolic dysfunction and cardiac hypertrophy ↑ systolic function and myocardial performance	Dai *et al.* (2011b)
Thoracic aortic constriction	Mice	3 mg kg^{-1} day^{-1}	osmotic pump	At time of aortic constriction	4 weeks	Protected cardiac mitochondria Ameliorated cardiac enlargement and systolic dysfunction Prevented cardiac fibrosis	Dai *et al.* (2013)
Chronic ischaemia heart failure	Dogs	0.05 mg kg^{-1} h^{-1}	i.v.	Ejection fraction ~30%	2 h	↑ ejection fraction, stroke volume, cardiac output	Sabbah *et al.* (2012)
Chronic ischaemia heart failure	Dogs	0.5 mg kg^{-1}	s.c.	Daily injection when ejection fraction ~30%	3 months	↑ myocardial ATP synthesis ↑ systolic function	Sabbah *et al.* (2013)
Kidney Failure models	**Species**	**Dose**	**Route**	**Time of administration**	**Duration of study**	**Major findings**	**Reference**
Obstructive renal fibrosis	Rats	1 or 3 mg kg^{-1}	i.p.	Daily starting 1 day before obstruction	14 days	↓ interstitial fibrosis ↓ tubular apoptosis ↓ macrophage infiltration ↑ tubular proliferation	Mizuguchi *et al.* (2008)

Table 1

Continued

Skeletal muscle models	Species	Dose	Route	Time of administration	Duration of study	Major findings	Reference
Mechanical ventilation diaphragm weakness	Rats	3 mg kg^{-1}, followed by 0.15 mg kg^{-1} every 3 h	s.c.	Onset of mechanical ventilation	12 h	Prevented ↑ mitochondrial H_2O_2 and lipid and protein oxidation Prevented diaphragmatic contractile dysfunction and atrophy	Powers *et al.* (2011)
Immobilization-induced hindlimb muscle atrophy	Mice	1.5 mg kg^{-1}	s.c.	Daily starting at onset of casting	14 days	Prevented ↓ in mitochondrial respiration and ↑ mitochondrial H_2O_2 Mitigated hindlimb muscle atrophy	Min *et al.* (2011)
Immobilization-induced hindlimb muscle atrophy	Rats	3 mg kg^{-1}	s.c.	Daily starting at onset of casting	7 days	Prevented ↓ in mitochondrial respiration and ↑ mitochondrial H_2O_2 Prevented hindlimb muscle atrophy	Talbert *et al.* (2013)
Age-related muscle weakness	Aged mice	3 mg kg^{-1}	s.c.	Daily starting at age 27 months	1 h or 8 days	One dose rapidly reversed muscle energy deficits and improved fatigue resistance eight daily doses improved whole body endurance	Siegel *et al.* (2013)
Burn-induced muscle loss	Mice	5 mg kg^{-1}	s.c.	Post-burn days 1,3 and 7	7 days	Prevented muscle apoptosis ↑ glucose tolerance ↓ oxidized proteins	Lee *et al.* (2011)

Table 1

Continued

Metabolic studies	Species	Dose	Route	Time of administration	Duration of study	Major findings	Reference
Insulin resistance induced by high fat diet	Rats	5 mg kg^{-1}	i.p.	Daily concurrent with onset of high fat diet	6 weeks	↓ mitochondrial H_2O_2 Prevented insulin resistance	Anderson *et al.* (2009)
Burn-induced insulin resistance	Rats	4 mg kg^{-1}	i.p.	Immediately after burn injury and at 12 and 24 h	24 h	Ameliorated burn-induced insulin resistance	Carter *et al.* (2011)
Burn-induced hypermetabolism	Rats	2 mg kg^{-1} followed by 4 mg kg^{-1} day^{-1}	i.v. osmotic pump	immediately after burn injury	7 days	↓ O_2 consumption and CO_2 production ↓ UCP1 expression in brown adipose tissue	Yo *et al.* (2013)
Burn-induced skeletal muscle bioenergetics	Rats	3 mg kg^{-1}	i.p.	30 min pre-burn and immediately post-burn, or immediately post-burn only	7 days	↑ ATP synthesis rate at 6 h ↑ mitochondrial redox status restore mitochondrial coupling	Righi *et al.* (2013)
Diabetic retinopathy	Diabetic rats	3 mg kg^{-1}	s.c.	Daily starting 2 weeks after induction of diabetes	4 months	Protected retinal ganglion cells and capillary basement membranes protected blood-retinal barrier	Huang *et al.* (2013)
Neurodegenerative models	**Species**	**Dose**	**Route**	**Time of administration**	**Duration of study**	**Major findings**	**Reference**
Amyotrophic lateral sclerosis (SOD1 mutant)	Mice	5 mg kg^{-1}	i.p.	Starting at 30 days of age	till death	↑ survival and motor performance	Petri *et al.* (2006)
Parkinson's Disease (MPTP model)	Mice	0.1–10 mg kg^{-1}	i.p.	30 min before first MPTP dose, and 1 and 12 h after 3rd MPTP dose	7 days	Dose-dependent protection of dopaminergic neurons and dopamine and metabolite levels	Yang *et al.* (2009)
Drug-induced toxicity models	**Species**	**Dose**	**Route**	**Time of administration**	**Duration of study**	**Major findings**	**Reference**
Contrast dye (diatrizoate) nephropathy	Rats	3 mg kg^{-1}	i.p.	24 h and 0.5 h before, and 24 h after contrast dye	48 h	↓ renal dysfunction ↓ tubular injury ↓ renal oxidative stress	Duan *et al.* (2013)
Oxaliplatin neuropathic pain	Rats	10 mg kg^{-1}	s.c.	4 days after oxaliplatin administration	2 h	Reverse cold hypersensitivity but not mechanical allodynia	Toyama *et al.* (2013)
Oxaliplatin neuropathic pain	Rats	5 mg $kg^{-1}day^{-1}$	osmotic pump	Continuous treatment concomitant with 3 weekly oxaliplatin injections	4 weeks	Ameliorated cold hypersensitivity and mechanical allodynia	Toyama *et al.* (2013)

administered after the onset of ischaemia demonstrated cardioprotective effects in several IR models. Bendavia reduced infarct size in rabbits and sheep after coronary artery ligation, attenuated the extent of no-reflow in rabbits and reduced infarct size in isolated perfused guinea pig hearts. In cultured myocytes, Bendavia blunted bursts of intracellular ROS after hypoxia challenge, maintained mitochondrial potential and reduced cell death during reoxygenation. Importantly, the investigators reported that Bendavia is rapidly taken up by the myocardium even after ischaemia, consistent with our early report that mitochondrial uptake of SS-31 is potential-independent (Zhao *et al.*, 2004). The encouraging results of this study provided the rationale for testing Bendavia in a clinical trial for acute myocardial infarction with percutaneous coronary intervention (see below **Clinical development of Bendavia**).

Mortality from myocardial ischaemia is increased in diabetics, and animal models also show increased IR injury in diabetic hearts. A recent study found that cardiac mitochondria from diabetic hearts displayed significantly greater sensitivity to MPT pore opening (Sloan *et al.*, 2012). Diabetic animals treated with MTP-131 showed improved resistance to MPT pore opening. Diabetic hearts also showed increased infarct size after ischaemia *ex vivo*, and treatment with MTP-131 prior to reperfusion reduced infarct size in both non-diabetic and diabetic hearts (Sloan *et al.*, 2012).

Bendavia has even been shown to reduce reperfusion injury after chronic ischaemia. Atherosclerotic renal artery stenosis results in chronic renal ischaemia and hypertension. Percutaneous transluminal angioplasty and stenting corrects blood pressure but does not improve renal function, and this is believed to result from reperfusion injury in a chronically ischaemic organ. In a porcine model of renal artery stenosis, a short infusion of Bendavia at the time of angioplasty resulted in significant improvement in renal blood flow and glomerular filtration rate 4 weeks later (Eirin *et al.*, 2012). Mitochondrial biogenesis was restored, and microvascular rarefaction, apoptosis, oxidative stress, tubular injury and fibrosis all decreased. The potential of Bendavia in improving kidney outcomes in percutaneous renal angioplasty is currently being evaluated in a clinical trial (see below **Clinical development of Bendavia**).

Efficacy of SS-31 in organ transplantation

IR injury is also a challenge in transplantation. Islet cell transplantation is currently limited by islet cell loss during the isolation procedure and after transplantation. The use of SS-31 in the isolation procedure helped preserve mitochondrial potential, reduced islet cell apoptosis and increased islet cell yield (Thomas *et al.*, 2007). The investigators suggested that SS-31 may present a novel approach to optimize islet isolation, reduce the need for multiple pancreata to achieve insulin independence and increase the pool of eligible organ donors.

IR injury is also a major cause of delayed graft function and chronic graft dysfunction following renal transplantation. Unlike tubular epithelial cells that can regenerate after ischaemic injury, the endothelial cell lacks comparable regenerative potential, and peritubular capillary dropout has been observed following acute renal ischaemia (Basile *et al.*, 2001; Zhu *et al.*, 2004). The loss of peritubular capillaries results in persistent tissue hypoxia and chronic inflammation, resulting in release of profibrotic cytokines such as TGFβ and renal fibrosis. In a model of renal IR injury in the rat, administration of SS-31 at the time of ischaemia significantly reduced endothelial damage and ameliorated microvascular dropout, chronic inflammation and fibrosis (S. Liu and H. Szeto, unpublished). These results suggest that SS-31 may be beneficial in minimizing the risk of progression to chronic kidney disease following acute kidney injury. SS-31 may also improve graft survival in renal transplantation. SS-31 has also been shown to prevent obstructive renal fibrosis (Mizuguchi *et al.*, 2008).

Efficacy of SS-31 in heart failure

Heart failure is a major cause of mortality in the developed world and is most commonly the result of either ischaemic heart disease or chronic hypertension. The failing heart has been described as an energy-starved engine (Neubauer, 2007). The heart consumes 20–30 times its own weight in ATP every day, and 90% of the ATP is derived from mitochondrial oxidative phosphorylation. Heart failure is a mismatch between supply and demand of ATP. This mismatch may result from decreased oxygen and substrate delivery to the myocardium or from increased workload to the myocardium following hypertension. Current treatments for heart failure all rely on 'energy sparing' by decreasing workload – beta blockers for slowing heart rate, and ACE inhibitors and aldosterone antagonists to decrease pressure overload. However, there is no convincing evidence that they are effective for heart failure with preserved ejection fraction.

There is ample evidence that mitochondrial bioenergetics is compromised in heart failure, and considerable amount of experimental and clinical data suggest that targeting cardiac metabolism may be beneficial in treating heart failure (Rosca and Hoppel, 2010; Fillmore and Lopaschuk, 2013). There is significant decrease in myocardial ATP and phosphocreatine content in human heart failure (Beer *et al.*, 2002). During the progression of heart failure, there is a shift in substrate preference from fatty acids to glucose, with up-regulation of enzymes involved in glycolytic pathways (Ardehali *et al.*, 2012). The shift towards glucose metabolism improves myocardial contractile efficiency by increasing the ratio of ATP production to oxygen consumption. This has led to strategies that decrease fatty acid metabolism and/or increase glucose oxidation, and some of them are in early clinical trials (Jaswal *et al.*, 2011; Ardehali *et al.*, 2012; Fillmore and Lopaschuk, 2013).

Cardiac mitochondria in failing hearts have structural abnormalities and loss of cristae (Sabbah *et al.*, 1992; Karamanlidis *et al.*, 2011). There is decrease in activity of the individual respiratory complexes and in formation of supercomplexes, resulting in OXPHOS inhibition (Rosca *et al.*, 2008; Lenaz and Genova, 2012; Fillmore and Lopaschuk, 2013). The loss of supercomplexes may be related to the loss of cardiolipin in both human and experimental heart failure with alterations in cardiolipin biosynthesis and remodelling

(Sparagna *et al.*, 2007; Saini-Chohan *et al.*, 2009). These findings have led to the suggestion that dietary fatty acid intake may promote cardiolipin biosynthesis and improve mitochondrial bioenergetics and cardiac function. SS-31 represents a novel strategy to enhance mitochondrial bioenergetics in the failing heart by protecting cardiolipin to facilitate electron transfer. SS-31 has been evaluated in several experimental heart failure models.

Hypertensive heart failure model

The first reported study was a model of hypertensive cardiomyopathy in mice induced by angiotensin (Dai *et al.*, 2011a). Continuous administration of angiotensin for 4 weeks significantly increased both systolic and diastolic pressure. After 4 weeks, echocardiography showed twofold increase in left ventricular (LV) mass and 35% decline in diastolic function, with no change in fractional shortening. Simultaneous administration of SS-31 ameliorated the cardiac hypertrophy and diastolic dysfunction without reducing the pressor response to angiotensin. SS-31 also attenuated cardiac fibrosis from tissue remodelling. The non-mitochondrial targeted antioxidant, N-acetylcysteine, had no protective effect in this model, but induction of mitochondrial catalase 2 weeks before angiotensin infusion prevented cardiomyopathy (Dai *et al.*, 2011a,b). The results from this study support the use of SS-31 in hypertensive diastolic heart failure. There is currently no approved drug therapy for heart failure with preserved ejection fraction.

Pressure-overload heart failure model

SS-31 was subsequently evaluated in the classic transverse aortic constriction (TAC) model whereby the sudden increase in end-systolic pressure results in cardiac hypertrophy with progression to overt heart failure. In mice, TAC caused more than twofold increase in LV mass and significant dilation of the LV within 4 weeks (Dai *et al.*, 2013). There was also a significant decline in systolic function after TAC with 50% reduction in fractional shortening. Continuous delivery of SS-31 by osmotic mini pump over the 4 weeks completely ameliorated the cardiac hypertrophy and systolic failure. Myocardial fibrosis was significantly increased in this model, and this was also completely prevented by SS-31 treatment. SS-31 also abolished the metabolic switch from fatty acid oxidation to glucose metabolism. Thus the mechanism of action of SS-31 in the failing heart is entirely different from that of metabolic modulators which tend to further inhibit fatty acid metabolism.

Ultrastructural studies showed 30% reduction in mitochondrial cristae and a significant increase in mitochondrial oxidative damage that were both mitigated by SS-31. The major involvement of mitochondrial dysfunction in pressure-overload heart failure was further confirmed by examination of the cardiac proteome. Of the 538 proteins significantly changed after TAC, 30% were mitochondrial proteins, 25% were involved in metabolism and less than 10% represented regulatory proteins of the cytoskeleton. Majority of mitochondrial proteins declined in abundance after TAC, and 84% of them were attenuated by SS-31. The major pathways affected in heart failure induced by TAC were mitochondrial dysfunction/oxidative phosphorylation and citrate cycle, and these were abolished by SS-31 treatment. These results with the TAC model suggest that SS-31 may also be beneficial in heart failure with reduced ejection fraction, and clearly support a mitochondrial mechanism of action.

Myocardial ischaemia heart failure model

SS-31 was recently evaluated in the canine myocardial infarct model induced by repeated intracoronary embolizations with microspheres. This model manifests many of the sequelae of human heart failure including marked depression of LV systolic and diastolic function, LV dilatation and hypertrophy, reduced cardiac output and elevation of systemic vascular resistance. Sustained depression of cardiac function is seen in this model after embolization is discontinued, and this model has been used for evaluation of a number of experimental therapeutic agents.

In the first study, dogs with advanced heart failure received either Bendavia (0.05 mg kg^{-1} h^{-1}) or saline for 2 h. Bendavia significantly increased ejection fraction, stroke volume, cardiac output and LV contractility index (dP/dt) without increasing heart rate or decreasing systemic pressure or systemic vascular resistance (Sabbah *et al.*, 2012). These findings suggest that the improvement of LV function is likely to be the result of improved cardiac energetics, and Bendavia may be helpful in the treatment of patients with acute heart failure syndromes.

In the second study, dogs with advanced heart failure were randomized to 3 months therapy with subcutaneous injection of Bendavia (0.5 mg kg^{-1}) or saline once daily for 3 months. Bendavia treatment significantly improved ejection fraction and reduced LV end-diastolic pressure compared with the saline group (Sabbah *et al.*, 2013). Bendavia significantly improved mitochondrial state 3 respiration, increased mitochondrial potential, increased ATP synthesis and decreased MPT pore opening. Besides increasing coupling efficiency (P/O ratio) (Birk *et al.*, 2013a), Bendavia also protects the microvasculature after acute and chronic ischaemic injury and increases oxygen delivery to tissues (Eirin *et al.*, 2012; Kloner *et al.*, 2012). Thus Bendavia can increase ATP production with the same amount of oxygen available, and in addition, increase oxygen delivery to the failing heart. These studies using different animal models confirm that Bendavia improves myocardial function in advanced heart failure by promoting mitochondrial bioenergetics and suggest that Bendavia may be beneficial for both systolic and diastolic failure.

Efficacy of SS-31 in skeletal muscle aging

Aging is associated with loss of skeletal muscle mass and contractile dysfunction. There is evidence of impaired skeletal muscle mitochondrial bioenergetic function with age in humans and mice (Marcinek *et al.*, 2005; Amara *et al.*, 2007; Figueiredo *et al.*, 2009). Mitochondrial coupling efficiency and maximal ATP production in skeletal muscle of 30-month-old mice was significantly lower compared to 7-month-old mice (Marcinek *et al.*, 2005). This was associated with higher resting ADP and decreased energy charge (ATP/ADP). Studies

with isolated mitochondria revealed increased number of mitochondria but significantly reduced state 3 mitochondrial respiration and increased oxidative damage (Figueiredo *et al.*, 2009). This age-associated decline in skeletal muscle function and exercise intolerance results in significant health care costs, and there is no pharmacological treatment to rapidly reverse these mitochondrial deficits.

Siegel *et al.* (2013) recently reported that a single treatment with SS-31 restored *in vivo* mitochondrial energetics to 'young' levels in aged mice after only 1 h, while there was no effect in young mice. Age-related declines in resting and maximal mitochondrial ATP production, coupling of oxidative phosphorylation and cell energy state (phosphocreatine/ATP) were all rapidly reversed after SS-31 treatment. These effects of SS-31 in aged muscle were associated with a more reduced glutathione redox status and lower mitochondrial H_2O_2 emission. Skeletal muscle of aged mice were more fatigue-resistant after a single SS-31 treatment, and 8 days of SS-31 treatment led to increased whole-body endurance as measured by treadmill running. This rapid improvement in mitochondrial energetic cannot be due to repairing or replacing damaged mitochondria. Rather, it suggests that SS-31 can rapidly improve mitochondrial respiration by increasing the efficiency of the ETC, and this may be related to the ability of SS-31 to improve cardiolipin function and promote fluidity and the formation of supercomplexes on the IMM.

Efficacy of SS-31 in disuse muscle atrophy

Skeletal muscle weakness and atrophy commonly occur during prolonged periods of inactivity due to limb immobilization or prolonged bed rest, and is especially a problem in the aging population. Similarly, prolonged mechanical ventilation is associated with diaphragmatic weakness resulting from myofibre atrophy and contractile dysfunction that result in difficulty weaning off the machine. Muscle atrophy occurs as a result of reduction in protein synthesis and increased proteolysis, with proteolysis playing a more prominent role (Shanely *et al.*, 2002; 2004). In terms of the proteolytic systems, calpain, caspase-3, ubiquitin-proteosome and autophagy systems are all involved in protein breakdown during disuse muscle atrophy (Powers *et al.*, 2012). ROS are important activators of key proteases in skeletal muscle, and recent evidence suggests that mitochondria are a dominant source of ROS production during inactivity-induced muscle atrophy (Powers *et al.*, 2011). Disuse atrophy has been associated with loss of mitochondria, mitochondrial swelling, increased mitochondrial ROS production and impaired mitochondrial respiratory function (Muller *et al.*, 2007; Powers *et al.*, 2012). Mechanisms that have been proposed for increased mitochondrial ROS production in disuse atrophy include increased mitochondrial uptake of Ca^{2+}, and increased fatty acid hydroperoxides (Bhattacharya *et al.*, 2009; 2011). Free fatty acids can also decrease mitochondrial respiration and increase H_2O_2 production (Bhattacharya *et al.*, 2011). The role of cardiolipin peroxidation in muscle atrophy has not been investigated, but swollen mitochondria lacking cristae membranes were observed in the rat soleus muscle after hindlimb suspension (Powers *et al.*, 2012). Cardiolipin peroxidation could account for induction of the intrinsic apoptotic pathway. Recent studies have shown that mitochondrial oxidative stress is a requisite step towards the activation of muscle proteolysis. By promoting mitochondria respiration and reducing ROS generation, SS-31 has been shown to be effective in preventing disuse atrophy in animal models.

Mechanical ventilation induced diaphragmatic atrophy

Diaphragmatic atrophy and contractile dysfunction can be seen after 12 h mechanical ventilation in rats. Mechanical ventilation significantly increased mitochondrial state 4 respiration, mitochondrial H_2O_2 release and oxidative damage to proteins and lipids that were completely blocked by SS-31 treatment during mechanical ventilation (Powers *et al.*, 2011). As a result, diaphragmatic contractile dysfunction and fibre atrophy were also prevented by SS-31 treatment. Mitochondrial oxidative stress appears to be a required upstream signal for the activation of all key proteases, as SS-31 prevented activation of calpain and caspase-3, as well as 20S proteasome activity in the diaphragm. These results have significant clinical implications and suggest that SS-31 may have therapeutic potential in protecting the diaphragm and reduce weaning problems in patients after prolonged mechanical ventilation (Levine *et al.*, 2011).

Immobilization induced skeletal muscle atrophy

Similar protection was observed with SS-31 in hindlimb muscles following immobilization in mice and rats (Min *et al.*, 2011; Talbert *et al.*, 2013). Fourteen days of immobilization by cast in mice resulted in significant muscle atrophy, along with muscle oxidative damage, and activation of calpain and caspase-3 (Min *et al.*, 2011). Permeabilized muscle fibres from the soleus muscle revealed significant decrease in state 3 respiration and significantly increased mitochondrial H_2O_2 production in both soleus and plantaris muscles. Once-daily injection of SS-31 prevented mitochondrial respiratory inhibition and H_2O_2 production, and consequently mitigated protease activation and abolished atrophy of both soleus and plantaris muscles. Importantly, administration of SS-31 to normal mice had no effect on mitochondrial respiration or ROS production.

When rats were subjected to casting for 7 days, significant atrophy was observed in both the soleus and plantaris muscle (Talbert *et al.*, 2013). Casting caused significant reduction in state 3 mitochondrial respiration and increased mitochondrial H_2O_2 production. Immobilization was associated with activation of calpain and caspase-3 activity, as well as an increase in the proteasome system, with up-regulation of muscle-specific E3 ligases. Autophagy signalling was also increased, suggesting that all four proteolytic systems are involved in skeletal muscle atrophy. Daily SS-31 treatment prevented the decrease in state 3 respiration and increase in H_2O_2 production, and abolished the activation of all four proteolytic systems. SS-31 also prevented the down-regulation of anabolic signalling molecules during immobilization, such as the Akt/mTOR pathway. Consequently, SS-31

treatment prevented immobilization-induced atrophy. Thus mitochondrial oxidative stress appears to play a requisite role in inhibiting protein synthesis and activating proteolytic systems to result in skeletal muscle atrophy.

Previous research has shown that PGC-1α, a co-activator of the PPARγ, plays a major role in mitochondrial biogenesis and oxidative metabolism (Lin *et al.*, 2005). PGC-1α expression in skeletal muscle is down-regulated in muscle atrophy from denervation and fasting, and overexpression of PGC-1α is sufficient to attenuate the muscle atrophy (Sandri *et al.*, 2006). However, SS-31 did not prevent the down-regulation of PGC-1α during immobilization, suggesting that the protective effect of SS-31 is not mediated by PGC-1α-induced mitochondrial biogenesis.

Skeletal muscle wasting induced by burn trauma

Severe burn injury causes a major systemic catabolic response that is associated with delayed rehabilitation and results in increased morbidity and mortality. This catabolic response is associated with mitochondrial dysfunction, with decreased OXPHOS and increased mitochondrial ROS, in skeletal muscle (Padfield *et al.*, 2005; Khan *et al.*, 2008). Treatment of mice with a single dose of SS-31 immediately after burn injury increased ATP synthesis rate fivefold, reduced oxidative and endoplasmic reticulum stress, and prevented apoptosis in skeletal muscle (Lee *et al.*, 2011; Righi *et al.*, 2013). SS-31 also reduced insulin resistance in burn injury and prevented hypermetabolism by decreasing uncoupling protein 1 in brown fat (Carter *et al.*, 2011; Yo *et al.*, 2013).

The results from these experimental animal models are very encouraging and suggest that SS-31 may not only be beneficial for mechanical ventilation and burn injury, but also to prevent muscle deconditioning from prolonged bed rest, especially in the aged population.

Clinical development of Bendavia

Animal studies carried out by a large number of independent investigators support the use of SS-31 for a number of major diseases with large unmet needs, including acute coronary syndrome, acute kidney injury, stroke, heart failure, sarcopenia, cachexia, neurodegenerative diseases and many others. Given all of the very promising preclinical efficacy data obtained to date, and its excellent pharmacokinetic profile, SS-31 entered into clinical development with a for-profit commercial sponsor (Stealth Peptides Inc., Newton, MA, USA) in 2010 using a clinical formulation named Bendavia.

Pre-Investigational New Drug pharmacokinetic studies

Despite being a peptide molecule, SS-31 has excellent 'drug-like' properties. It is a water-soluble compound and very stable in solution. The incorporation of D-Arg and amidation of the C-terminus greatly enhanced the stability of SS-31 against peptidase degradation. The *in vitro* and *in vivo* pharmacokinetics of SS-31 in many animal species have already been summarized in a previous review (Szeto and Schiller, 2011) and will only be described briefly here.

SS-31 is rapidly absorbed after s.c. administration, with peak plasma levels detected within 15 min. Consistent with its very polar property; the apparent volume of distribution of SS-31 is very small, being only 40% of total body water. Distribution of ^{125}I-SS-31 to kidney, heart, liver, skeletal muscle and lung occurred within 30 min after s.c administration, with highest concentrations being found in the kidney (Siegel *et al.*, 2013; Birk *et al.*, 2013b). Being a highly water-soluble compound, there was little distribution to adipose tissue (Birk *et al.*, 2013b). Brain concentrations are low compared to plasma, but its excellent efficacy in protecting striatal dopamine neurons in a Parkinson's disease model, indicates that SS-31 can readily cross the blood-brain barrier (Yang *et al.*, 2009). In the isolated perfused guinea pig heart, approximately 25% of the dose in the perfusate was extracted per minute (Kloner *et al.*, 2012). SS-31 is entirely excreted by the kidneys in non-clinical and clinical studies, accounting for 100% of the peptide (parent and metabolites) in urine. The elimination half-life is ~2 h in rat, dog and monkey. This is sufficient for once-daily dosing to achieve pharmacological efficacy in these species. Most importantly, plasma levels and drug exposure (area under the plasma level curve) are dose-proportional within the dose range used in preclinical efficacy studies (Szeto and Schiller, 2011).

Phase I studies

Several phase I studies have assessed the safety, tolerability and pharmacokinetics of Bendavia in healthy male and female subjects with intravenous and oral dosing. Bendavia was reported to be well tolerated as an intravenous infusion over a wide dose range (0.01 mg kg^{-1} h^{-1} to 0.25 mg kg^{-1} h^{-1} over 4 h), achieving effective plasma levels even with the lowest dose. The oral formulation of Bendavia provides plasma concentrations shown to be cardioprotective in many models of acute and chronic cardiac diseases (see Table 1). Oral Bendavia appeared to be safe and was well tolerated with no serious adverse effects across a broad dose range with highly predictable pharmacokinetics.

Phase II studies

Based on the extensive data demonstrating the effectiveness of Bendavia in preclinical models of myocardial IR injury (Cho *et al.*, 2007a; Kloner *et al.*, 2012), the first multinational phase II clinical study with Bendavia is focused on IR and microvascular injuries for patients experiencing acute ST-segment elevation myocardial infarction (STEMI). The rationale and design of the EMBRACE-STEMI study has been published (Chakrabarti *et al.*, 2013). This is a randomized, double-blind, placebo-controlled trial enrolling patients with a first-time anterior STEMI undergoing primary percutaneous coronary intervention. Patients are randomized to receive Bendavia at 0.05 mg kg^{-1} h^{-1} or placebo as an intravenous infusion. This dose is based on pharmacokinetic/pharmacodynamic relationship in several animal models examining the ability of Bendavia to affect IR injury (see Table 1), and human pharmacokinetic data from Phase I studies (Chakrabarti *et al.*, 2013). The elimination half-life (~4 h) is similar in humans and preclinical models and does not suggest retention by tissues. The clearance of Bendavia from isolated perfused guinea pig hearts was also found to have a

similar half-time (Kloner *et al.*, 2012). The primary end point is infarct size measured by creatine kinase release and cardiac magnetic resonance imaging with gadolinium enhancement. Patient enrolment into the EMBRACE-STEMI trial began in June 2012 and the plan is to enrol 300 patients across 40 sites within the United States and Europe. Bendavia has the potential to fill an unmet need for patients having large heart attacks or experiencing poor revascularization due to microvascular no-reflow.

A second phase II study is for treatment of acute kidney injury and renal microvascular dysfunction in hypertension. This study was initiated based on the remarkable effectiveness of Bendavia in improving renal microvascular blood flow and glomerular filtration rate after angioplasty in pigs with atherosclerotic renal artery stenosis (Eirin *et al.*, 2012). Previous research has shown that angioplasty alone fails to reverse structural and functional deterioration in stenotic kidneys (Eirin *et al.*, 2011). This clinical study is also supported by other animal studies showing the effectiveness of Bendavia in preventing acute ischaemia kidney injury (Szeto *et al.*, 2011; Birk *et al.*, 2013b). The phase II clinical study is intended to assess Bendavia's improvement of renal function in patients with hypertension and severe unilateral renal artery stenosis after treatment with angioplasty. This is a randomized, placebo-controlled, single-centre study, and patients will receive either Bendavia (0.05 mg kg^{-1} h^{-1}) or saline for a maximum duration of 4 h. The primary outcome measure will be glomerular filtration rate at 8 weeks after angioplasty. Secondary outcome measures will include renal volume, regional renal blood flow, renal oxygenation and a number of inflammatory and oxidative biomarkers.

With chronic oral dosing, a third phase II clinical study is planned for the use of Bendavia in treatment of congestive heart failure. Bendavia offers a completely innovative approach to treating heart failure by improving mitochondrial bioenergetics for the energy-starved heart, and extensive preclinical data support such a clinical study (Dai *et al.*, 2011a; 2013; Sabbah *et al.*, 2012; 2013).

Concluding remarks

SS-31 provides an entirely novel approach to the treatment of diseases that at first glance appear to be totally unrelated. Common to all of them, however, is the loss of cellular energy that accounts for their failure to function properly. SS-31 is the first compound that targets a specific phospholipid on the IMM, cardiolipin, to alter membrane properties and modify the activity of the ETC protein complexes to improve mitochondrial bioenergetics. In aging or diseased tissues, SS-31 serves to recharge the powerhouse of the cells. Research has shown that targeting such a fundamental mechanism can benefit highly complex diseases with multiple morbidities, such as the cardiorenal syndrome, which share a common pathogenesis of energy failure. Importantly, SS-31 has no effect on normal mitochondria which accounts for its excellent safety profile. The ongoing clinical studies with Bendavia will allow this idea to be validated and hopefully bring relief to patients suffering from these chronic diseases.

Current drug discovery efforts tend to focus on protein targets and signalling pathways for an individual disease condition, and target identification is driven by genomics and proteomics. The emerging field of lipidomics is bringing attention to lipids as drug targets. Changes in lipid composition of a membrane cannot only alter fluidity and folding of a membrane, but can profoundly change the organization and function of numerous proteins on the membrane. The complexity of the lipidome equals or exceeds that of the proteome and provides enormous opportunities for drug development.

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Conflict of interest

The SS peptides described in this article have been licensed for commercial research and development to Stealth Peptides Inc, a clinical stage biopharmaceutical company, in which H.H.S and the Cornell Research Foundation have financial interests. H.H.S. is the scientific founder of Stealth Peptides International. The Research Program in Mitochondrial Therapeutics was established with a gift from Stealth Peptides International.

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