Pharm Res (2011) 28:2669–2679
DOI 10.1007/s11095-011-0476-8

EXPERT REVIEW

Novel Therapies Targeting Inner Mitochondrial Membrane—From Discovery to Clinical Development

Hazel H. Szeto • Peter W. Schiller

Received: 21 February 2011 / Accepted: 6 May 2011 / Published online: 3 June 2011

ABSTRACT Mitochondrial oxidative stress and dysfunction have been implicated in the aging process and in numerous chronic diseases. The need for therapies that can protect and/or improve mitochondrial function is obvious. However, the development of mitoprotective drugs has been hampered by a number of challenges, and there are at present no approved therapies for mitochondrial dysfunction. This article describes the original discovery, preclinical development, and clinical development of a novel class of small peptide molecules that selectively target the inner mitochondrial membrane and protect mitochondrial function. These compounds have the potential to be a paradigm-shifting approach to the treatment of mitochondrial dysfunction, which underlies many common diseases, including cardiorenal, neurologic, and metabolic disorders.

KEY WORDS ischemia-reperfusion injury · metabolic disorders · neurodegenerative diseases · oxidative stress · Szeto-Schiller peptides

ABBREVIATIONS

ATP	adenosine triphosphate
BBB	blood-brain barrier
CypD	cyclophilin D
Cyt c	cytochrome c
ETC	electron transport chain
IMM	inner mitochondrial membrane
iv	intravenous
MCAT	overexpression of catalase targeted to mitochondria
MPP^+	1-methyl-4-phenylpyridium
MPT	mitochondrial permeability transition
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
OMM	outer mitochondrial membrane
ROS	reactive oxygen species
sc	subcutaneous
SOD	superoxide dismutase
TPP^+	triphenylphosphonium ion

H. H. Szeto (✉)
Department of Pharmacology
Joan and Sanford I. Weill Medical College of Cornell University
1300 York Avenue
New York, New York 10021, USA
e-mail: hhszeto@med.cornell.edu

P. W. Schiller
Laboratory of Chemical Biology and Peptide Research
Clinical Research Institute of Montreal
Montreal, Quebec, Canada

INTRODUCTION

Mitochondria are intracellular organelles found in most eukaryotic cells that are responsible for the generation of adenosine triphosphate (ATP), the predominant source of cellular energy for the body. Mitochondrial disorders resulting from genetic mutations in mitochondrial DNA or nuclear DNA that encode mitochondrial proteins generally present with deficits involving multiple organ systems, especially neuropathies, myopathies, cardiomyopathies, and diabetes (1). In addition to their primary role as energy providers for cellular processes, mitochondria are also the major producers of intracellular reactive oxygen species (ROS) and are themselves highly susceptible to the damaging effects of free radicals. Mitochondrial oxidative damage leads to reduced ATP production and further increases in ROS production that can eventually lead to declining cellular function and cell death. Mitochondrial oxidative stress and dysfunction have been implicated in the aging process and in numerous chronic diseases, including

diabetes, ischemic heart disease, heart failure, acute and chronic kidney diseases, and neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease (2–5). Furthermore, many of the side effects of approved drugs are now recognized to be due to mitochondrial toxicity (6). The need for therapies that can protect and/or improve mitochondrial function is obvious. However, the development of mitoprotective drugs has been hampered by a number of challenges, and there are at present no approved therapies for mitochondrial diseases.

The two biggest impediments to the development of mitoprotective drugs are the lack of a specific molecular entity to target and the difficulty of delivering the therapeutic agent to the proper mitochondrial compartment.

Mitochondria are intracellular organelles composed of two membranes, an outer mitochondrial membrane (OMM) and an inner mitochondrial membrane (IMM). The OMM is similar in composition to the plasma membrane and is freely permeable to small lipophilic molecules. In addition, the OMM contains VDAC (voltage-dependent anion channel) and other channels that are permeable to small hydrophilic molecules and allows the transfer of metabolites in and out of mitochondria. The mitochondrial matrix enclosed by the IMM contains not only mitochondrial DNA but also the major enzymes of the tricarboxylic acid cycle, which provides reducing equivalents in the form of NADH and $FADH_2$ to the electron transport chain (ETC) on the IMM. The IMM is further compartmentalized into numerous cristae that greatly increase the surface area of the IMM. The four protein complexes of the ETC (complex I–IV) reside on these cristae in the IMM, and the proton gradient generated across the inner membrane as a result of electron transfer from complex I to complex IV drives the production of ATP by the F0F1 ATPase (complex V). The IMM is unique in that it has a very high concentration of proteins and is rich in cardiolipin, a very anionic phospholipid that has four fatty acid chains rather than two. This special composition makes the IMM highly impermeable to all molecules including small cations.

Modern drug discovery has focused on developing therapies that target a specific molecular entity in the disease process, such as receptors, enzymes, or transcription factors. The discovery process is then aided by high throughput assays and chemical libraries for rapid screening of likely drug candidates that would act on the target of interest. The development of mitoprotective therapies has been hampered by the lack of simple high-throughput assays that can assess activity of the ETC or mitochondrial ATP synthesis. In addition, mitochondrial ATP synthesis is a process that requires many mitochondrial components to function properly, and it is therefore difficult to target a single molecular entity for drug development. One exception might be the putative mitochondrial permeability transition (MPT) pore. The MPT pore is a high conductance channel that is thought to be formed by the VDAC on the OMM, the adenine nucleotide translocator on the IMM, and cyclophilin D (CypD) in the matrix (see reviews (7,8)). MPT can be induced by high mitochondrial Ca^{2+}, inorganic phosphate, and oxidative stress. Opening of the MPT pore causes a sudden increase in permeability of the IMM, resulting in swelling of the mitochondrial matrix, rupture of the outer membrane, mitochondrial uncoupling, and release of cytochrome (cyt) c into the cytosol. The loss of mitochondrial cyt c would inhibit electron transport (7), while cytosolic cyt c is known to trigger the caspase cascade and apoptosis (8). The pharmaceutical industry is actively developing CypD inhibitors because compounds that bind to CypD, such as cyclosporine A, can inhibit MPT.

The site of action is also a significant challenge for development of mitoprotective compounds. To be effective, CypD inhibitors need to be delivered into the mitochondrial matrix. Most molecules have difficulty penetrating the IMM, with the exception of highly lipophilic molecules. Compounds that get to mitochondria also tend to inhibit mitochondrial function, either by inhibiting the protein complexes of the ETC, uncoupling the proton gradient, or directly inhibiting the ATP synthase (see reviews (6,9)). More than 60 types of xenobiotics are known to inhibit complex I, including the fibrates and thiazolidinediones (10,11). Some of the non-steroidal anti-inflammatory drugs and other lipophilic weak acids can dissipate the proton gradient across the IMM and reduce ATP synthesis (12). Other well-known drugs, such as propranolol and local anesthetics, can directly inhibit the ATP synthase (9). All of these xenobiotics can produce off-target inhibition of cellular energy.

For this reason, recent drug development efforts have focused their attention on reducing mitochondrial oxidative stress. Oxygen normally serves as the ultimate electron acceptor in the ETC and is reduced to water in the process of oxidative phosphorylation. Excessive electrons in the ETC, however, can lead to electron leak and generate superoxide anion, especially at complex I and complex III (see review (13)). Superoxide from complex I is released into the matrix, while complex III can release superoxide into the matrix as well as the intermembrane space (14). The proteins and cardiolipin of the IMM are therefore most vulnerable to oxidative damage (15). Oxidative damage to the protein complexes of the ETC inhibits enzyme activity, and cardiolipin peroxidation results in loss of cardiolipin and dissociation of cyt c from the IMM (16). Cyt c is the mobile electron carrier responsible for shuttling electrons from complex III to complex IV. Being a cationic protein, cyt c binds with high affinity to the anionic cardiolipin on the IMM. Loss of cyt c from the IMM will reduce ATP

synthesis and further increase electron leak and ROS generation, setting up a feed-forward cycle of ROS-induced ROS production (17). Mitochondrial DNA, which is associated with the IMM, is also susceptible to oxidative damage because it is not protected by histones, and this can further inhibit activity of the electron transport chain and reduce mitochondrial ATP production (18).

Mitochondrial ROS are normally countered by a number of antioxidant enzymes in mitochondria, including Mn superoxide dismutase (SOD), which converts superoxide to hydrogen peroxide (H_2O_2), and glutathione peroxidase and catalase, which convert H_2O_2 to water (19). Oxidative stress takes place when there is either excessive production of ROS or diminished endogenous antioxidant capacity. It should be noted that the antioxidant enzyme systems become dysfunctional under oxidative stress, resulting in defense failure and further exacerbation of oxidative damage (20,21). In turn, this will result in further activation of the mitochondrial ROS generating sites and set in motion a self-sustaining and amplifying feed-forward cycle between ROS generation and mitochondrial dysfunction.

Mitochondrial ROS clearly contributes to age-related pathologies and lifespan. Deletion of MnSOD accelerated aging and decreased lifespan in mice (22,23), while overexpression of MnSOD increased lifespan in Drosophila melanogaster (24), although not in mice (25). Interestingly, overexpression of catalase targeted to mitochondria (MCAT) extended lifespan in mice and reduced cardiac pathology, cataracts, tumor burden, and insulin resistance (26–30). In contrast, overexpression of catalase in peroxisomes or nucleus had no protective effect (26). These studies suggest that control of mitochondrial H_2O_2 can reduce age-related pathologies and increase lifespan. Mitochondrial oxidative stress can be minimized either by scavenging excessive ROS or reducing mitochondrial ROS emission. But, most importantly, the therapy needs to be delivered to the mitochondrial compartment to be effective.

Conventional antioxidants, such as coenzyme Q (CoQ), vitamin E, and N-acetylcysteine, have not been particularly effective because of their limited distribution to mitochondria. This has led to several attempts in improving mitochondrial delivery of available antioxidants. The most common method for targeting compounds to mitochondria takes advantage of the potential gradient (150–180 mV) that is generated as a result of the proton gradient across the IMM. Conjugating lipophilic antioxidants to a cation can result in 100–1,000-fold accumulation in the mitochondrial matrix (31). Triphenylphosphonium ion (TPP^+) has successfully been used to deliver a number of natural antioxidants, like CoQ (MitoQ) and plastoquinone (SkQ1), into the mitochondrial matrix (31,32). The effectiveness of this approach, however, is limited to mitochondria with normal potential, while excessive matrix uptake of these lipophilic cations can depolarize mitochondria. Both MitoQ and SkQ1 are currently in early clinical trials, and their efficacy and safety remain to be determined.

THE CHANCE DISCOVERY OF A NOVEL CLASS OF MITOPROTECTIVE COMPOUNDS

The Szeto-Schiller (SS) peptides are the first compounds that selectively target and concentrate in the IMM where ATP and free radical production take place. The SS peptides were discovered by serendipity while Hazel H. Szeto and Peter W. Schiller were working on a family of dermorphin analogs that showed very high affinity and selectivity for the μ opioid receptor (see Table I) (33). SS-01 (H-Tyr-D-Arg-Phe-Lys-NH_2) and SS-02 (H-Dmt-D-Arg-Phe-Lys-NH_2; Dmt = 2′6′-dimethyltyrosine) (originally abbreviated as DALDA and [Dmt^1]DALDA, respectively, in published papers) are highly polar water-soluble tetrapeptides that carry 3+ net charge at physiologic pH. As with all opioid peptides, SS-01 and SS-02 both have Tyr (or Dmt) in the N-terminus, which is necessary for high affinity binding to opioid receptors. The substitution of Dmt for Tyr in SS-02 further increased binding affinity to the μ receptor (33). It was a big surprise when SS-02 demonstrated potent analgesic activity after subcutaneous (*sc*) administration to rodents (34), suggesting that it was capable of crossing the blood-brain barrier (BBB). It has generally been thought that only highly lipophilic compounds are capable of crossing the BBB.

Subsequent studies in cell cultures showed that SS-02 readily penetrated a variety of cell types without the need for specific transporters or receptors (35). Furthermore, SS-02 was able to translocate through a monolayer of polarized epithelial cells, consistent with its ability to cross the BBB. This was highly unexpected, given the molecular size of SS-02 (640), the highly polar peptide backbone, and the presence of 3+ net charge (the N-terminal amino group and the side chains of Arg and Lys). The key discovery was the observation by confocal laser scanning microscopy that an SS-02 analog containing the small fluorescent anthraniloyl label showed an intracellular distribution similar to that of Mitotracker TMRM, suggesting that the peptide was targeted to mitochondria (36). Mitochondrial uptake of SS-02 was confirmed using both [^{3}H]SS-02 and the fluorescent labeled analog in isolated mitochondria. Surprisingly, although SS-02 has a 3+ net charge at physiologic pH, its mitochondrial uptake did not depend on mitochondrial potential, suggesting that it was not distributed into the mitochondrial matrix. Potential-independent uptake is a significant advantage when dealing with diseased mitochondria that are likely to have reduced mitochondrial potential. In contrast, TPP^+-conjugated

Table I Opioid Receptor Binding Data of SS Peptides

Compound	K_i (nM)[a]		
	μ[b]	δ[b]	κ[c]
H-Tyr-D-Arg-Phe-Lys-NH_2 (SS-01)	1.69 ± 0.25	>10,000	4,230 ± 360
H-Dmt-D-Arg-Phe-Lys-NH_2 (SS-02)	0.143 ± 0.015	2,100 ± 310	22.3 ± 4.2
H-Phe-D-Arg-Phe-Lys-NH_2 (SS-20)	63.0 ± 0.7	>10,000	2,190 ± 240
H-D-Arg-Dmt-Lys-Phe-NH_2 (SS-31)	287 ± 9	>10,000	>10,000

[a] Values represent means of 3–6 determinations ± SE. [b] Displacement of [^{3}H]DAMGO (μ-selective) and [^{3}H]DSLET (δ-selective) from rat brain membrane binding sites. [c] Displacement of [^{3}H]U69,593 (κ-selective) from guinea pig brain membrane binding sites

compounds, such as MitoQ and SkQ1, are not taken up by depolarized mitochondria and will even be released from the mitochondrial matrix when mitochondrial potential drops (31). Mitochondria fractionation studies ultimately revealed that >85% of SS-02 was found in the fraction containing IMM (36), making it the first compound to selectively target the IMM where the ETC resides. It was estimated that SS-02 concentrates >1,000-fold in the IMM when compared to extracellular concentrations. Unlike MitoQ and SkQ1, SS-02 does not cause mitochondrial depolarization, even at high concentrations, because it is not distributed into the matrix.

Opioid Actions of SS-02

Before SS-02 was recognized as a mitochondria-targeted antioxidant, it was known to be a highly potent and effective opioid analgesic (34). Opioid receptors are seven transmembrane receptors found on the plasma membrane that are coupled to G proteins. There are three types of opioid receptors: μ, δ and κ. SS-02 binds with very high affinity to the μ opioid receptor but has much lower affinity for the δ and κ receptor, thus making it highly selective for the μ receptor (33,37) (see Table I). The binding affinity of SS-02 for the μ receptor (0.14 nM) is 7-fold greater than that of morphine (1 nM). SS-02 is an agonist at all three opioid receptors as shown by G protein binding assays (37). The selectivity and agonist activity of SS-02 was also confirmed using standard bioassays based on inhibition of electrically evoked contractions of the guinea pig ileum (GPI) and the mouse vas deferens (MVD) (33). The GPI assay is usually considered as representative for μ opioid receptor interactions, even though the ileum does also contain κ opioid receptors. In the MVD assay, activity is primarily mediated by δ opioid receptors, even though μ and κ receptors also exist in this tissue. The results showed that SS-02 is a very potent agonist at the μ receptor (33) (Table II). The EC50 for SS-02 in the GPI assay is 1.41 ± 0.29 nM, as compared to 29.3 ± 2.2 nM for morphine. *In vivo* analgesic testing in mice revealed that SS-02 is 36 times more potent than morphine when administered subcutaneously and as much as 833 times more potent than morphine when administered into the intrathecal space (34).

The very high affinity of SS-02 for the μ opioid receptor makes it undesirable as a mitoprotective compound. In addition to analgesia, the μ opioid receptor is associated with adverse effects, including constipation, respiratory depression, tolerance, and dependence. It was therefore important to develop other mitochondria-targeted peptide compounds that do not have high affinity for opioid receptors.

Development of Non-opioid Analogs of SS-02

Peptides are usually not cell-permeable because of the tendency of the peptide backbone to form hydrogen bonds with water. The endogenous opioid peptide, met-enkephalin (H-Tyr-Gly-Gly-Phe-Met-OH), is a pentapeptide that can only penetrate cells by receptor-mediated uptake or by other transporter systems (38). Although SS-02 is a little smaller, it carries 3+ net charge at physiological pH. Unexpectedly, SS-02 diffused rapidly into a variety of cell types without the need for receptors or transporters (35). Furthermore, the permeability coefficient of SS-02 across a monolayer of epithelial cells was >100-fold larger compared to four analogs of met-enkephalin, none of which contain charged residues (35,39). On the other hand, cell penetration is also negligible for oligopeptides consisting of less than six charged amino acid residues such as Lys or Arg (40), suggesting that basic residues alone cannot explain the cellular permeability of SS-02. The unique feature of SS-02 is its alternating aromatic-cationic structural motif, where aromatic residues (Dmt and Phe) alternate with basic residues (Arg and Lys). This motif allows for intramolecular cation-π interaction between the electron-rich π ring (Dmt or Phe) and the adjacent cation (Arg or Lys). The additional methyl groups on Dmt further increases electron density on the π ring. Cation-π energies are of the same order of magnitude as hydrogen bonding energies, and the π rings may shield the cation charge and enhance membrane penetration. Thus, a decision was made to retain this aromatic-cationic motif in the design of non-opioid analogs of SS-02.

It has long been appreciated from studies of opioid peptides that the hydroxyl group on the N-terminal Tyr is essential for opioid activity (41). The substitution of Dmt for Tyr further increases opioid binding affinity (33) (see

Table II Bioassays for Opioid Activity

Compound	GPI[a] EC50 (nM)[c]	MVD[b] EC50 (nM)[c]
Morphine	29.3 ± 2.2	155 ± 31
H-Dmt-D-Arg-Phe-Lys-NH_2 (SS-02)	1.41 ± 0.29	23.1 ± 2.0
H-Phe-D-Arg-Phe-Lys-NH_2 (SS-20)	687 ± 88	755 ± 115
H-D-Arg-Dmt-Lys-Phe-NH_2 (SS-31)	45,400 ± 4,100	42,700 ± 13,600

[a] Guinea pig ileum assay (representative of μ opioid receptor activity. [b] Mouse vas deferens assay (representative of δ opioid receptor activity. [c] Mean of 3–6 determinations ± SE

Table I). A relatively simple way to reduce opioid affinity would be to replace the Dmt of SS-02 with a Phe, and this analog (H-Phe-D-Arg-Phe-Lys-NH_2) was synthesized as SS-20. However, Dmt is the amino acid residue that can scavenge free radicals (42), and SS-20 would not have that capability. A second approach was to change the order of the amino acid residues in SS-02 so that the Dmt is not the N-terminal amino acid, while preserving the alternating aromatic-cationic motif. This resulted in SS-31 (H-D-Arg-Dmt-Lys-Phe-NH_2).

The binding affinity of SS-20 and SS-31 to μ, δ and κ opioid receptors was determined by competitive displacement of [^{3}H]DAMGO, [^{3}H]DSLET, and [^{3}H]U69,593, respectively, from guinea pig brain membranes (33). These three radioligands are considered to be selective for the μ, δ and κ opioid receptor, respectively, and are routinely used for assessment of binding affinity (37). The results are shown in Table I. Replacement of Dmt with Phe resulted in 450-fold decrease in affinity for the μ receptor and lack of appreciable binding to either δ or κ opioid receptors. Changing the Dmt from position 1 to position 2 resulted in 2,000-fold lower affinity of SS-31 for the μ receptor and no appreciable binding to δ or κ receptors.

The loss of opioid activity was confirmed using the GPI and MVD bioassays, and the results are summarized in Table II. SS-20 is more than 100-fold less potent than SS-02 in both the GPI and MVD assay, while SS-31 is at least 30,000-fold less potent than SS-02 in the GPI assay and 2,000-fold less in the MVD assay. Thus, both SS-20 and SS-31 are essentially devoid of opioid activity.

To further confirm that SS-31 lacks opioid activity, SS-31 was administered to mice intrathecally, and pain sensitivity (nociception) was measured by the classic tail-flick assay. This is an acute nociceptive assay in which radiant heat is directed to the tail of a mouse or rat, and the latency of the mouse to remove its tail from the heat source is recorded. In mice, it was found that SS-31, even at doses >1,000 times the ED50 of SS-02, did not elicit any antinociceptive response (H.H. Szeto, unpublished results).

Mitochondrial Uptake of SS-31 and SS-20

The conservation of the alternating aromatic-cationic motif appears to be sufficient for cell penetration and mitochondria targeting. [^{3}H]SS-31 was readily taken up by N_2A neuroblastoma cells, and steady-state concentrations were obtained in 30 min, suggesting that SS-31 is metabolically stable and diffuses freely across the cell membrane (43). The intracellular concentration of SS-31 was estimated to be ~6 times the extracellular concentration. Mitochondrial uptake of [^{3}H]SS-31 was demonstrated using isolated mouse liver and brain mitochondria. Uptake of [^{3}H]SS-31 by mitochondria was very rapid, with maximal levels (30–50%) reached before 2 min (43). The uptake of SS-31 was concentration-dependent with no evidence of saturation up to 10 μM. SS-31 was estimated to concentrate ~5,000-fold in mitochondria.

Cell penetration of SS-20 was demonstrated with confocal laser scanning microscopy in normal lung fibroblasts as well as cancer cells (44). SS-20 was found to co-localize with Mitotracker CMXRos, suggesting mitochondrial localization. Furthermore, structure-activity studies showed that the aromatic-cationic motif was sufficient for cell permeability and mitochondrial targeting and that other natural and unnatural aromatic and basic amino acids can be utilized to enhance the library of mitochondria-targeting peptides (44).

Mitochondrial Actions of SS Peptides

Unlike TPP^+ that only serves as a mitochondrial delivery vector for other compounds, the SS peptides have intrinsic activity in mitochondria. The Dmt-containing analogs have intrinsic antioxidant activity because Tyr is known to scavenge oxyradicals, forming relatively unreactive tyrosyl radicals that can be followed by radical-radical coupling to give dityrosine or react with superoxide to form tyrosine hydroperoxide (42). Dmt has the same or even greater scavenging ability because of its greater electron density on the aromatic ring. SS-02 and SS-31 were shown to dose-dependently scavenge hydrogen peroxide, hydroxyl radical, and peroxynitrite *in vitro* (36,45). SS-20, which does not contain a Tyr or Dmt, was unable to scavenge these ROS. Because these peptides are targeted directly to the IMM, SS-02 and SS-31 are positioned to scavenge H_2O_2 from both the matrix as well as the intermembrane space. Both SS-02 and SS-31 reduced spontaneous H_2O_2 emission from isolated mitochondria under basal conditions (36,45).

SS-02 and SS-31 can also inhibit lipid peroxidation (36,45) and are therefore ideally situated to prevent cardiolipin peroxidation from hydroxyl radicals.

The selective targeting and concentration of SS-02 and SS-31 in the IMM make them extremely potent in reducing mitochondrial oxidative stress in intact cells. Both SS-02 and SS-31 inhibited cell death caused by the lipid hydroperoxide (*tert*-butylhydroperoxide) in nanomolar concentrations (36,43). Treatment with SS-31 reduced intracellular ROS and mitochondrial depolarization and prevented caspase activation and apoptosis (43). An extracellular concentration of 1 nM can produce a concentration of >1 μM in the IMM, which is well within the concentration determined to be sufficient to protect mitochondria in isolated mitochondrial studies (36). SS-31 also inhibited the increase in mitochondrial superoxide and mitochondrial depolarization in hepatocytes treated with hypochlorous acid and prevented cell death (46). When endothelial cells were subjected to shear stress, 1 nM SS-31 prevented the increase in mitochondrial superoxide and upregulation of heme oxygenase-1 (47).

In addition to their antioxidant actions, the SS peptides can also promote mitochondrial respiration. 1-methyl-4-phenylpyridium (MPP^+), the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is known to inhibit mitochondrial complex I activity and inhibits mitochondrial O_2 consumption and ATP production (48). Remarkably, both SS-31 and SS-20 dose-dependently abolished the inhibition of O_2 consumption and ATP production caused by MPP^+ in isolated mouse liver mitochondria (49). The mitochondrial protection provided by the SS peptides was readily demonstrated in intact cells. Both SS-31 and SS-20 dose-dependently prevented MPTP-induced cell death in dopamine cells (49). Improved mitochondrial respiration should reduce ROS generation. Thus, even though SS-20 cannot scavenge free radicals, it can still act as a mitochondrial antioxidant by reducing ROS generation (50).

Other than their direct action on the mitochondrial ETC, the SS peptides can also inhibit MPT and mitochondrial swelling. SS-02 and SS-31 were both effective in preventing mitochondrial swelling, mitochondrial depolarization, and cyt c release in isolated mitochondria elicited by Ca^{2+} overload (36). Furthermore, SS-31 and SS-20 inhibited mitochondrial swelling induced by MPP^+ in isolated mitochondria and prevented MPTP-induced apoptosis in dopamine cells (49).

In summary, the SS peptides exert multiple protective functions on mitochondria. They promote mitochondrial respiration and ATP synthesis, reduce ROS generation, and inhibit mitochondrial swelling. In addition, Dmt-containing SS peptides (SS-02 and SS-31) can also directly scavenge mitochondrial ROS.

Preclinical Efficacy of SS Peptides

The hypothesis that mitochondrial ROS contributes to the aging process is supported by recent studies showing that lifespan is significantly increased in transgenic C57BL/6J mice that overexpress human catalase in cardiac and skeletal muscle mitochondria (MCAT mice) (26). Catalase is normally localized in the peroxisome, and lifespan was not increased in transgenic mice that overexpress catalase in peroxisome or nucleus (26). In addition to increased lifespan, MCAT mice have been shown to have less age-related pathology, including cataracts, hearing loss, inflammation, tumor burden, cardiac lesions, and insulin resistance (27–29,51). MCAT mice have also been reported to have improved exercise performance (52) and to be protected against insulin resistance induced by high fat diet (53), cardiomyopathy caused by zidovudine (54), and hypertensive cardiomyopathy (55).

The *in vivo* efficacy of the SS peptides has been evaluated in a number of disease animal models, and they have demonstrated remarkable efficacy as mitochondria protectants. In some studies, the protective actions of SS-31 were similar to the protection seen in MCAT mice (53,55), providing evidence that SS-31 acts by reducing mitochondrial oxidative stress.

Ischemia-Reperfusion Injury

Mitochondrial dysfunction and oxidative stress play a major role in ischemia-reperfusion injury. Oxidative phosphorylation is inhibited during ischemia and may deteriorate further upon reperfusion due to opening of the MPT pore resulting in mitochondrial depolarization, impaired ATP synthesis, and further increase in ROS production. SS-02, SS-31, and SS-20 have all been shown to reduce myocardial ischemia-reperfusion injury in *ex vivo* and *in vivo* studies. Specifically, cardiac stunning was attenuated by all the SS peptides in isolated guinea pig hearts when given prior to global ischemia (45,56). When SS-31 and SS-20 were administered after ischemia, only SS-31 was effective in reducing myocardial stunning, suggesting that while SS-20 can precondition the heart and reduce ischemic damage, only the scavenging analogs can counter the oxidative burst upon reperfusion and serve as post-conditioning agents (45,57). *In vivo* studies showed that SS-02 and SS-31 both reduced cardiac infarct size when administered prior to occlusion of the left anterior descending coronary artery in rats (58). Further *in vivo* studies showed that this myocardial protection also occurs when SS-31 is administered immediately prior to reperfusion and the return of coronary blood flow. Both peptides significantly improved myocardial ATP content and decreased lipid peroxidation in rats after reperfusion, suggesting most likely that SS-20 can

reduce mitochondrial ROS production by improving mitochondrial bioenergetics (45).

Renal ischemia-reperfusion injury is the most common cause of acute kidney injury. A recent study showed that treatment with SS-31 limited tubular cell apoptosis and necrosis after prolonged ischemia in rats and provided significant improvement in renal biomarkers 24 h later (59). Electron microscopy revealed that SS-31 prevented mitochondrial swelling during ischemia and protected the integrity of the mitochondrial membranes. The preservation of mitochondrial integrity significantly increased mitochondrial respiration, accelerated ATP recovery upon reperfusion, and minimized tubular cell death. The protection of tubular cells with SS-31 was associated with reduced oxidative stress and inflammatory response and accelerated tubular cell proliferation.

SS-31 has also been reported to protect reduced glutathione status in the brain, significantly attenuated infarct size, and reduced hemispheric swelling when administered following transient occlusion of the middle cerebral artery in mice (58). SS-31 also protected skeletal muscles and preserved normal cellular architecture after 3 h of hindlimb ischemia in mice caused by a tourniquet (60).

These preclinical studies support mitochondrial protection as an upstream target for pharmacological intervention in ischemia-reperfusion injury. The SS peptides hold tremendous promise for use in the setting of anticipated ischemic intervals, such as free tissue transfer, cell and solid organ transplantation, and many other medical and surgical procedures.

Neurodegenerative Diseases

Mitochondrial impairment and oxidative damage are intimately involved in the pathogenesis of neurodegenerative diseases. An additional impediment in the development of therapies for these diseases is their ability to penetrate the BBB. The SS peptides can readily cross the BBB (34), and they have demonstrated *in vivo* efficacy in animal models of Parkinson's disease and amyotrophic lateral sclerosis (ALS).

In a model of Parkinson's disease, SS-31 and SS-20 protected dopamine neurons from the cytotoxic effects of MPTP and prevented striatal dopamine depletion (49). Immunohistologic staining for tyrosine hydroxylase showed complete preservation of dopamine neurons in the substantia nigra. These findings are consistent with the mitochondrial and cell culture studies described above.

A point mutation in SOD1 (CuZn SOD) is known to be associated with familial ALS in humans (61). Mice with a G93A mutation in SOD1 develop a phenotype that closely mimics human ALS with tremors, gait impairment, and paralysis. Treatment of G93A SOD1 mice with SS-31 led to significant delay in the onset of resting tremors, improved rotarod performance, and prolonged survival (62). The improved outcome was associated with a significant reduction in motor neuron cell loss in the lumbar spinal cord, and several oxidative biomarkers were markedly decreased (62).

These studies indicate that the SS peptides can indeed penetrate the BBB and exert neuroprotective actions. A recent study showed that SS-31 can prevent amyloid β toxicity in neuroblastoma N_2A cells and significantly increased neurite outgrowth in primary neurons from AβPP transgenic mice, a mouse model of Alzheimer's disease (63). These findings support SS-31 as potential treatment for Alzheimer's disease.

Muscle Atrophy and Weakness

Mechanical ventilation is a life-saving measure that is often required for medical conditions such as acute lung injury, sepsis, heart failure, chronic obstructive lung disease, neuromuscular disorders, and drug overdose. Prolonged mechanical ventilation is associated with significant diaphragmatic weakness resulting from both myofiber atrophy and contractile dysfunction and is the most frequent cause of weaning difficulty (64,65). Recent studies revealed that mechanical ventilation-induced oxidative stress in the diaphragm leads to proteolysis via activation of calpain and caspase-3 (66). Administration of SS-31 as an intravenous (*iv*) infusion to rats during mechanical ventilation prevented increase in diaphragmatic mitochondrial ROS emission and protected against diaphragmatic protein oxidation, calpain and caspase-3 activation, myofiber atrophy, and contractile dysfunction (67). These findings indicate that mitochondria are a primary source of ROS production in the diaphragm during prolonged mechanical ventilation and that SS-31 may reduce weaning difficulty.

Extended periods of skeletal muscle inactivity also result in loss of muscle mass and strength. Mitochondrial oxidative stress plays a central role in proteolytic pathways in skeletal muscle (68), and SS-31 was recently shown to attenuate muscle atrophy in rats following hindlimb immobilization (69). These findings suggest that the SS peptides may be beneficial in preventing muscle atrophy resulting from chronic bed rest or spaceflight, cancer cachexia, and sarcopenia due to aging, or medications such as the antiretroviral drugs.

Metabolic Syndrome

Reduced mitochondrial ETC function in skeletal muscles is also associated with obesity, insulin resistance, and type 2 diabetes (70). High fat diet leads to mitochondrial dysfunction and elevated ROS production in skeletal muscle (53). Administration of SS-31 was able to inhibit the increase in

skeletal muscle mitochondrial H_2O_2 production following high fat diet in rats, and this prevented the development of insulin resistance (53). Similar findings were seen in MCAT mice, demonstrating that SS-31 can mimic the protective effect of mitochondrial catalase overexpression.

Heart Failure

Systemic hypertension induces left ventricular hypertrophy, fibrosis, and diastolic dysfunction. The renin-angiotensin-aldosterone system plays a central role in hypertensive cardiovascular diseases. Angiotensin binds to the angiotensin receptor-1 and stimulates ROS formation by NADPH oxidase, which has been shown to stimulate mitochondrial ROS and mitochondrial dysfunction (71). A recent study showed that angiotensin-induced cardiomyopathy was ameliorated by SS-31 treatment and was not observed in the MCAT mice (55). These findings support a role for mitochondrial oxidative stress in heart failure and provide a strong rationale for investigating the clinical application of SS-31 for treatment or prevention of hypertensive cardiovascular diseases.

Clinical Development of SS Peptides

Based on these very promising preclinical efficacy data, the SS peptides have entered into clinical development with a for-profit commercial sponsor using a modified form of SS-31. A brief summary of the progress made to date with this modified form of SS-31 is provided below.

Drug Metabolism and Pharmacokinetic Studies

One major drawback of small peptide molecules is their susceptibility to rapid enzymatic degradation. Met-enkephalin (H-Tyr-Gly-Gly-Phe-Met-OH) is highly susceptible to peptidases, and its elimination half-life is less than 2 min when administered systemically. The incorporation of D-Arg and amidation of the C-terminus greatly enhanced the plasma stability of SS-02 and provided an elimination half-life of 1.77 h in sheep (72). No degradation of SS-02 was found when incubated in sheep plasma for up to 4 h at 37°C (72).

Stability of SS-31 has been determined in mouse, rat, dog, monkey, and human plasma. There was little degradation of SS-31 when incubated at 37°C for 1 h in all species. Longer incubation showed fastest degradation in rat and mouse plasma ($t_{1/2}$ ~ 1.8 and 3.6 h, respectively), but SS-31 was much more stable in dog, monkey, and human plasma ($t_{1/2}$ ~ 7.7, 4.3 and 30.8 h, respectively).

Liver microsomal preparations from mouse, rat, dog, monkey, and human were used to determine the metabolic stability of SS-31, according to standard guidelines. Dextromethorphan was used as a positive control in these assays. With the exception of the dog, no appreciable loss of SS-31 was observed when incubated in any of the other microsomal preparations for up to 1 h at 37°C. Approximately 96.7% of the parent compound remained at the end of 60 min incubation in the human liver microsomal preparation. The results predict a very low *in vivo* clearance of 0.54 ml/min/kg for SS-31 in humans, and SS-31 would be classified as a compound of low clearance (<2.2 ml/min/kg) (73).

The *in vivo* pharmacokinetics of SS-31 have been determined in rats, dogs, and non-human primates. The plasma level curves after *iv* and *sc* injections in rats are shown in Fig. 1. There was no significant gender difference in rats, and the results shown in Fig. 1 are combined data from both genders. Following *iv* injection of 1 mg/kg, plasma concentration of SS-31 declined rapidly with an apparent terminal half-life of about 0.8 h. Plasma concentration at 8 h was 0.8 ng/ml, or ~1 nM, which is effective concentration seen in majority of cell culture studies (36,43,47,49,63,74). The calculated pharmacokinetic parameters for SS-31 from *iv* administration are summarized in Table III. Consistent with its very polar property, the apparent volume of distribution of SS-31 is very small, being only 40% of total body water. SS-31 is rapidly absorbed after *sc* administration, with peak plasma levels detected within 15 min. By comparing area under the curve

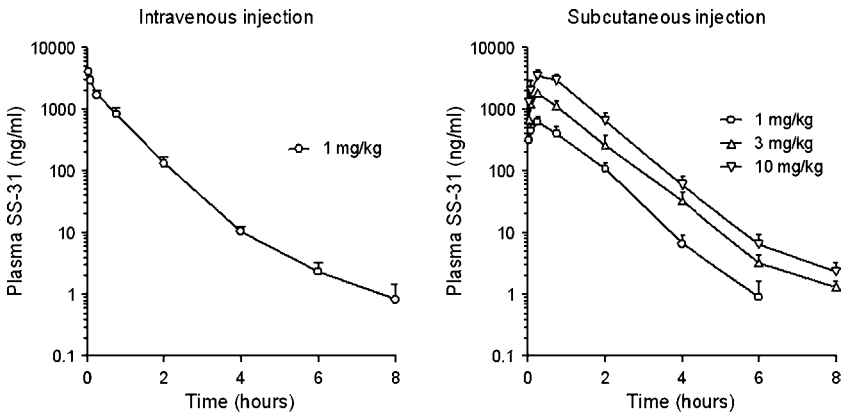

Fig. 1 Plasma levels of SS-31 in rats following intravenous injection (*left panel*) and subcutaneous injection (*right panel*). Data are plotted as mean ± SE; $n = 6$ in each group.

Table III Pharmacokinetic Parameters of SS-31 After *I.V.* Administration

	C_{max} (ng/ml)	$AUC_{0\text{-}t}$ (ng.h/ml)	MRT (h)	$T_{1/2}$ (h)	CL (L/h/kg)	V (L/kg)
Rat ($n=6$)	4,162 ± 584	2,123 ± 274	0.60 ± 0.04	0.80 ± 0.14	0.478 ± 0.065	0.288 ± 0.034
Dog ($n=3$)	2,374 ± 422	2,117 ± 240	0.76 ± 0.07	0.78 ± 0.14	0.476 ± 0.054	0.358 ± 0.026
Monkey ($n=2$)	3,291	3,279	1.06	1.05	0.304	0.323

C_{max}, maximal concentration; $AUC_{0\text{-}t}$, area under curve from time 0-t; MRT, mean residence time; $T_{1/2}$, elimination half-life; CL, clearance; V, apparent volume of distribution

(AUC) after *iv* (2,123 ng.h/ml) and *sc* (815 ng.h/ml) administration, the bioavailability of *sc* SS-31 is estimated to be 38%. Plasma levels and drug exposure (as determined by AUC) were dose-proportional from 1 to 10 mg/kg, doses that span the dose range used in preclinical efficacy studies (49,53,55,58,59,62,75).

Single dose *iv* dose studies in dogs and cynomolgus monkeys show that there is little species difference in the pharmacokinetics of SS-31 among rats, dogs and monkeys (see Table III). Bioavailability of SS-31 after *sc* administration was higher in the dog (72.7%) and monkey (81.4%) compared to rat (38%).

Investigational New Drug Application and Clinical Trials. An Investigational New Drug application has been opened with the U.S. FDA, and Phase 1 trials with the modified form of SS-31 are ongoing. In the completed first-in-human Phase 1clinical trial, the test compound was administered *iv* for 4 h to five cohorts of healthy volunteers. Preliminary data suggest that the compound exhibits the same linear pharmacokinetics as described for animals. There was no change in pupillary reaction from pre-dose to 1, 2, or 4 h post-dose as assessed by pupil size of reaction to light. Similarly, there was no change from pre-dose to 1, 2, or 4 h post-dose in the degree of subject sedation as assessed by the Ramsay-scale Sedation Assessment. As measured, mean changes in expiratory volume between baseline and 0.5 and 4 h post-dose were also not considered clinically significant in any volunteer. Furthermore, mean changes in histamine between baseline and 15 min, 30 min, 1 h, and 4 h were not considered clinically significant in any volunteer. These results suggest that SS-31 lacks any appreciable opioid activity. It is anticipated that a proof-of-concept Phase 2 clinical trial for ischemia-reperfusion injury will begin this year in patients with first-time acute ST-segment elevation myocardial infarctions.

The Role of Serendipity in Drug Discovery

Serendipity has played a significant role throughout the history of drug discovery. In fact, many of the most effective and most widely used therapeutic agents today arose through serendipity. Over the past three decades, the drug discovery paradigm has shifted more and more to a target-based approach aided by computer modeling, chemical libraries, and high throughout screening. Despite enormous investments by the pharmaceutical industry, the results of this modern paradigm have been very disappointing, and new drug development has been in a sharp decline over the past decade. Why has this new paradigm not worked well? One reason is that we simply do not understand enough about complex diseases to identify validated targets. Another reason is that the use of rational drug design and high throughput systems tend to remove any chance of serendipitous discoveries.

Serendipity played a dominant role in the initial discovery of the SS peptides. This platform would never have been discovered by rational design because we simply do not know enough about the targets for mitochondria protection or the structural requirements for mitochondria targeting. This chance discovery was followed by rational peptide design rather than a high throughput screen using peptide libraries. Likewise, rational design was involved in the design of analogs that have suitable chemical, pharmacokinetic, and pharmacodynamic properties. Preclinical studies to date suggest that these peptides may have therapeutic potential in numerous clinical disorders with unmet needs. The first candidate compound is in early clinical trial and other compounds are in preclinical development. In the meantime, studies are ongoing to further explore the mechanisms of action of this novel class of mitoprotective compounds. These compounds also provide valuable tools to explore mitochondrial biology and the role of mitochondrial dysfunction in aging and associated illnesses.

DISCLOSURES

The SS peptides described in this article are licensed for commercial research and development to Stealth Peptides Inc., a clinical stage biopharmaceutical company, in which the authors have financial interests.

REFERENCES

1. Taylor RW, Turnbull DM. Mitochondrial DNA mutations in human disease. Nat Rev Genet. 2005;6:389–402.

2. Wanagat J, Dai DF, Rabinovitch P. Mitochondrial oxidative stress and mammalian healthspan. Mech Ageing Dev. 2010;131:527–35.
3. Ren J, Pulakat L, Whaley-Connell A, Sowers JR. Mitochondrial biogenesis in the metabolic syndrome and cardiovascular disease. J Mol Med. 2010;88:993–1001.
4. Martin LJ. Mitochondrial and cell death mechanisms in neurodegenerative diseases. Pharmaceuticals (Basel). 2010;3:839–915.
5. Forbes JM, Coughlan MT, Cooper ME. Oxidative stress as a major culprit in kidney disease in diabetes. Diabetes. 2008;57:1446–54.
6. Scatena R, Bottoni P, Botta G, Martorana GE, Giardina B. The role of mitochondria in pharmacotoxicology: a reevaluation of an old, newly emerging topic. Am J Physiol Cell Physiol. 2007;293:C12–21.
7. Crompton M. The mitochondrial permeability transition pore and its role in cell death. Biochem J. 1999;341(Pt 2):233–49.
8. Green DR, Reed JC. Mitochondria and apoptosis. Science. 1998;281:1309–12.
9. Wallace KB, Starkov AA. Mitochondrial targets of drug toxicity. Annu Rev Pharmacol Toxicol. 2000;40:353–88.
10. Scatena R, Martorana GE, Bottoni P, Giardina B. Mitochondrial dysfunction by synthetic ligands of peroxisome proliferator activated receptors (PPARs). IUBMB Life. 2004;56:477–82.
11. Brunmair B, Lest A, Staniek K, Gras F, Scharf N, Roden M, *et al.* Fenofibrate impairs rat mitochondrial function by inhibition of respiratory complex I. J Pharmacol Exp Ther. 2004;311:109–14.
12. Moreno-Sanchez R, Bravo C, Vasquez C, Ayala G, Silveira LH, Martinez-Lavin M. Inhibition and uncoupling of oxidative phosphorylation by nonsteroidal anti-inflammatory drugs: study in mitochondria, submitochondrial particles, cells, and whole heart. Biochem Pharmacol. 1999;57:743–52.
13. Turrens JF. Mitochondrial formation of reactive oxygen species. J Physiol. 2003;552:335–44.
14. Muller FL, Liu Y, Van Remmen H. Complex III releases superoxide to both sides of the inner mitochondrial membrane. J Biol Chem. 2004;279:49064–73.
15. Lesnefsky EJ, Hoppel CL. Cardiolipin as an oxidative target in cardiac mitochondria in the aged rat. Biochim Biophys Acta. 2008;1777:1020–7.
16. Paradies G, Petrosillo G, Paradies V, Ruggiero FM. Oxidative stress, mitochondrial bioenergetics, and cardiolipin in aging. Free Radic Biol Med. 2010;48:1286–95.
17. Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial ROS-induced ROS release: an update and review. Biochim Biophys Acta. 2006;1757:509–17.
18. Hebert SL, Lanza IR, Nair KS. Mitochondrial DNA alterations and reduced mitochondrial function in aging. Mech Ageing Dev. 2010;131:451–62.
19. Andreyev AY, Kushnareva YE, Starkov AA. Mitochondrial metabolism of reactive oxygen species. Biochemistry (Mosc). 2005;70:200–14.
20. Kono Y, Fridovich I. Superoxide radical inhibits catalase. J Biol Chem. 1982;257:5751–4.
21. Yamakura F, Taka H, Fujimura T, Murayama K. Inactivation of human manganese-superoxide dismutase by peroxynitrite is caused by exclusive nitration of tyrosine 34 to 3-nitrotyrosine. J Biol Chem. 1998;273:14085–9.
22. Li Y, Huang TT, Carlson EJ, Melov S, Ursell PC, Olson JL, *et al.* Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. Nat Genet. 1995;11:376–81.
23. Lebovitz RM, Zhang H, Vogel H, Cartwright Jr J, Dionne L, Lu N, *et al.* Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. Proc Natl Acad Sci USA. 1996;93:9782–7.
24. Sun J, Folk D, Bradley TJ, Tower J. Induced overexpression of mitochondrial Mn-superoxide dismutase extends the life span of adult Drosophila melanogaster. Genetics. 2002;161:661–72.
25. Jang YC, Perez VI, Song W, Lustgarten MS, Salmon AB, Mele J, *et al.* Overexpression of Mn superoxide dismutase does not increase life span in mice. J Gerontol A Biol Sci Med Sci. 2009;64:1114–25.
26. Schriner SE, Linford NJ, Martin GM, Treuting P, Ogburn CE, Emond M, *et al.* Extension of murine life span by overexpression of catalase targeted to mitochondria. Science. 2005;308:1909–11.
27. Treuting PM, Linford NJ, Knoblaugh SE, Emond MJ, Morton JF, Martin GM, *et al.* Reduction of age-associated pathology in old mice by overexpression of catalase in mitochondria. J Gerontol A Biol Sci Med Sci. 2008;63:813–22.
28. Dai DF, Santana LF, Vermulst M, Tomazela DM, Emond MJ, MacCoss MJ, *et al.* Overexpression of catalase targeted to mitochondria attenuates murine cardiac aging. Circulation. 2009;119:2789–97.
29. Lee HY, Choi CS, Birkenfeld AL, Alves TC, Jornayvaz FR, Jurczak MJ, *et al.* Targeted expression of catalase to mitochondria prevents age-associated reductions in mitochondrial function and insulin resistance. Cell Metab. 2010;12:668–74.
30. Wolf N, Penn P, Pendergrass W, Van Remmen H, Bartke A, Rabinovitch P, *et al.* Age-related cataract progression in five mouse models for anti-oxidant protection or hormonal influence. Exp Eye Res. 2005;81:276–85.
31. Murphy MP, Smith RA. Targeting antioxidants to mitochondria by conjugation to lipophilic cations. Annu Rev Pharmacol Toxicol. 2007;47:629–56.
32. Antonenko YN, Avetisyan AV, Bakeeva LE, Chernyak BV, Chertkov VA, Domnina LV, *et al.* Mitochondria-targeted plastoquinone derivatives as tools to interrupt execution of the aging program. 1. Cationic plastoquinone derivatives: synthesis and *in vitro* studies. Biochemistry (Mosc). 2008;73:1273–87.
33. Schiller PW, Nguyen TM, Berezowska I, Dupuis S, Weltrowska G, Chung NN, *et al.* Synthesis and *in vitro* opioid activity profiles of DALDA analogues. Eur J Med Chem. 2000;35:895–901.
34. Zhao GM, Wu D, Soong Y, Shimoyama M, Berezowska I, Schiller PW, *et al.* Profound spinal tolerance after repeated exposure to a highly selective mu-opioid peptide agonist: role of delta-opioid receptors. J Pharmacol Exp Ther. 2002;302:188–96.
35. Zhao K, Luo G, Zhao GM, Schiller PW, Szeto HH. Transcellular transport of a highly polar 3+ net charge opioid tetrapeptide. J Pharmacol Exp Ther. 2003;304:425–32.
36. Zhao K, Zhao GM, Wu D, Soong Y, Birk AV, Schiller PW, *et al.* Cell-permeable peptide antioxidants targeted to inner mitochondrial membrane inhibit mitochondrial swelling, oxidative cell death, and reperfusion injury. J Biol Chem. 2004;279:34682–90.
37. Zhao GM, Qian X, Schiller PW, Szeto HH. Comparison of [Dmt1]DALDA and DAMGO in binding and G protein activation at mu, delta, and kappa opioid receptors. J Pharmacol Exp Ther. 2003;307:947–54.
38. Hauser M, Donhardt AM, Barnes D, Naider F, Becker JM. Enkephalins are transported by a novel eukaryotic peptide uptake system. J Biol Chem. 2000;275:3037–41.
39. Lang VB, Langguth P, Ottiger C, Wunderli-Allenspach H, Rognan D, Rothen-Rutishauser B, *et al.* Structure-permeation relations of met-enkephalin peptide analogues on absorption and secretion mechanisms in Caco-2 monolayers. J Pharm Sci. 1997;86:846–53.
40. Tunnemann G, Ter-Avetisyan G, Martin RM, Stockl M, Herrmann A, Cardoso MC. Live-cell analysis of cell penetration ability and toxicity of oligo-arginines. J Pept Sci. 2008;14:469–76.
41. Horn AS, Rodgers JR. Structural and conformational relationships between the enkephalins and the opiates. Nature. 1976;260:795–7.

42. Winterbourn CC, Parsons-Mair HN, Gebicki S, Gebicki JM, Davies MJ. Requirements for superoxide-dependent tyrosine hydroperoxide formation in peptides. Biochem J. 2004;381:241–8.
43. Zhao K, Luo G, Giannelli S, Szeto HH. Mitochondria-targeted peptide prevents mitochondrial depolarization and apoptosis induced by tert-butyl hydroperoxide in neuronal cell lines. Biochem Pharmacol. 2005;70:1796–806.
44. Horton KL, Stewart KM, Fonseca SB, Guo Q, Kelley SO. Mitochondria-penetrating peptides. Chem Biol. 2008;15:375–82.
45. Szeto HH. Mitochondria-targeted cytoprotective peptides for ischemia-reperfusion injury. Antioxid Redox Signal. 2008;10:601–19.
46. Whiteman M, Spencer JP, Szeto HH, Armstrong JS. Do mitochondriotropic antioxidants prevent chlorinative stress-induced mitochondrial and cellular injury? Antioxid Redox Signal. 2008;10:641–50.
47. Han Z, Varadharaj S, Giedt RJ, Zweier JL, Szeto HH, Alevriadou BR. Mitochondria-derived reactive oxygen species mediate heme oxygenase-1 expression in sheared endothelial cells. J Pharmacol Exp Ther. 2009;329:94–101.
48. Fonck C, Baudry M. Rapid reduction of ATP synthesis and lack of free radical formation by MPP+ in rat brain synaptosomes and mitochondria. Brain Res. 2003;975:214–21.
49. Yang L, Zhao K, Calingasan NY, Luo G, Szeto HH, Beal MF. Mitochondria targeted peptides protect against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxicity. Antioxid Redox Signal. 2009;11:2095–104.
50. Szeto HH. Development of mitochondria-targeted aromatic-cationic peptides for neurodegenerative diseases. Ann NY Acad Sci. 2008;1147:112–21.
51. Someya S, Xu J, Kondo K, Ding D, Salvi RJ, Yamasoba T, *et al.* Age-related hearing loss in C57BL/6J mice is mediated by Bak-dependent mitochondrial apoptosis. Proc Natl Acad Sci USA. 2009;106:19432–7.
52. Li D, Lai Y, Yue Y, Rabinovitch PS, Hakim C, Duan D. Ectopic catalase expression in mitochondria by adeno-associated virus enhances exercise performance in mice. PLoS ONE. 2009;4:e6673.
53. Anderson EJ, Lustig ME, Boyle KE, Woodlief TL, Kane DA, Lin CT, *et al.* Mitochondrial H2O2 emission and cellular redox state link excess fat intake to insulin resistance in both rodents and humans. J Clin Invest. 2009;119:573–81.
54. Kohler JJ, Cucoranu I, Fields E, Green E, He S, Hoying A, *et al.* Transgenic mitochondrial superoxide dismutase and mitochondrially targeted catalase prevent antiretroviral-induced oxidative stress and cardiomyopathy. Lab Invest. 2009;89:782–90.
55. Dai DF, Chen T, Szeto H, Nieves-Cintron M, Summer V, Santana LF, *et al.* Mitochondrial targeted antioxidant peptide ameliorates hypertensive cardiomyopathy. J Am Coll Cardiol. 2011; in press.
56. Wu D, Soong Y, Zhao GM, Szeto HH. A highly potent peptide analgesic that protects against ischemia-reperfusion-induced myocardial stunning. Am J Physiol Heart Circ Physiol. 2002;283:H783–91.
57. Song W, Shin J, Lee J, Kim H, Oh D, Edelberg JM, *et al.* A potent opiate agonist protects against myocardial stunning during myocardial ischemia and reperfusion in rats. Coron Artery Dis. 2005;16:407–10.
58. Cho J, Won K, Wu D, Soong Y, Liu S, Szeto HH, *et al.* Potent mitochondria-targeted peptides reduce myocardial infarction in rats. Coron Artery Dis. 2007;18:215–20.
59. Szeto HH, Liu S, Soong Y, Wu D, Ganger M, Darrah S, *et al.* Mitochondria-targeted peptide accelerates ATP recovery and reduces injury following ischemia-reperfusion injury. J Am Soc Nephrol. 2011; in press.
60. Bavinck NHP, Jimenez N, Millet BA, Szeto HH, Spector JA. SS-31: a mitochondria-specific therapy that provides significant protection against ischemia-reperfusion injury in muscle *in vitro* and *in vivo*. Washington, DC: Northeastern Society of Plastic Surgeons; 2010.
61. Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, *et al.* Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature. 1993;362:59–62.
62. Petri S, Kiaei M, Damiano M, Hiller A, Wille E, Manfredi G, *et al.* Cell-permeable peptide antioxidants as a novel therapeutic approach in a mouse model of amyotrophic lateral sclerosis. J Neurochem. 2006;98:1141–8.
63. Manczak M, Mao P, Calkins MJ, Cornea A, Reddy AP, Murphy MP, *et al.* Mitochondria-targeted antioxidants protect against amyloid-beta toxicity in Alzheimer's disease neurons. J Alzheimers Dis. 2010;20 Suppl 2:S609–31.
64. Levine S, Nguyen T, Taylor N, Friscia ME, Budak MT, Rothenberg P, *et al.* Rapid disuse atrophy of diaphragm fibers in mechanically ventilated humans. N Engl J Med. 2008;358:1327–35.
65. Powers SK, DeCramer M, Gayan-Ramirez G, Levine S. Pressure support ventilation attenuates ventilator-induced protein modifications in the diaphragm. Crit Care. 2008;12:191.
66. Whidden MA, Smuder AJ, Wu M, Hudson MB, Nelson WB, Powers SK. Oxidative stress is required for mechanical ventilation-induced protease activation in the diaphragm. J Appl Physiol. 2010;108:1376–82.
67. Powers SK, Hudson MB, Nelson WB, Talbert EE, Min K, Szeto HH, *et al.* Mitochondria-targeted antioxidants protect against mechanical ventilation-induced diaphragm weakness. Crit Care Med. 2011, doi:10.1097/CCM.0b013e3182190b62.
68. Powers SK, Kavazis AN, McClung JM. Oxidative stress and disuse muscle atrophy. J Appl Physiol. 2007;102:2389–97.
69. Min K, Smuder AJ, Kwon OS, Kavazis AN, Szeto HH, Powers SK. Mitochondrial-targeted antioxidants attenuate immobilization-induced skeletal muscle atrophy. Anaheim: Experimental Biology; 2010.
70. Ritov VB, Menshikova EV, Azuma K, Wood R, Toledo FG, Goodpaster BH, *et al.* Deficiency of electron transport chain in human skeletal muscle mitochondria in type 2 diabetes mellitus and obesity. Am J Physiol Endocrinol Metab. 2010;298:E49–58.
71. Doughan AK, Harrison DG, Dikalov SI. Molecular mechanisms of angiotensin II-mediated mitochondrial dysfunction: linking mitochondrial oxidative damage and vascular endothelial dysfunction. Circ Res. 2008;102:488–96.
72. Szeto HH, Lovelace JL, Fridland G, Soong Y, Fasolo J, Wu D, *et al.* *In vivo* pharmacokinetics of selective mu-opioid peptide agonists. J Pharmacol Exp Ther. 2001;298:57–61.
73. Lave T, Dupin S, Schmitt C, Valles B, Ubeaud G, Chou RC, *et al.* The use of human hepatocytes to select compounds based on their expected hepatic extraction ratios in humans. Pharm Res. 1997;14:152–5.
74. Thomas DA, Stauffer C, Zhao K, Yang H, Sharma VK, Szeto HH, *et al.* Mitochondrial targeting with antioxidant peptide SS-31 prevents mitochondrial depolarization, reduces islet cell apoptosis, increases islet cell yield, and improves posttransplantation function. J Am Soc Nephrol. 2007;18:213–22.
75. Cho S, Szeto HH, Kim E, Kim H, Tolhurst AT, Pinto JT. A novel cell-permeable antioxidant peptide, SS31, attenuates ischemic brain injury by down-regulating CD36. J Biol Chem. 2007;282:4634–42.