Anti-inflammatory, analgesic and antioxidant activities of novel kyotorphin-nitroxide hybrid molecules

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Mitochondrial oxidative damage contributes to a wide range of pathologies, including ischemia/reperfusion (I/R) injury, cardiovascular disorders and neurodegenerative diseases. Accordingly, protecting mitochondria from oxidative damage should possess therapeutic relevance. In the present study, we have designed and synthesized a series of novel kyotorphin-nitroxide hybrid molecules, and examined their free radical scavenging activities, in addition to their anti-inflammatory and analgesic activities. We have further characterized these compounds in a simulated I/R cellular model. Our findings suggest that the protective effects of kyotorphin-nitroxides partially reside in maintaining optimal mitochondrial function.

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Ischemia/reperfusion (I/R) injury represents tissue damage associated with the return of blood supply following an ischemic period. I/R is a general mechanism of damage relevant to many organs. The events of I/R trigger a systemic inflammatory response that can lead to cellular damage and even organ failure. It is widely felt that reactive oxygen species (ROS) and polymorphonuclear leukocytes play important roles in mediating I/R injury.¹⁻³ Numerous treatment strategies have been proposed to reduce I/R injury, such as ischemic pre-conditioning, post-conditioning, controlled reperfusion, and injection/infusion with various therapeutic agents.⁴⁻⁶ The ultimate therapeutic objective of these treatments is to reduce oxidative stress, inflammation, vascular injury, while simultaneously providing an energy supply to the reperfused organ. Among several cellular events postulated to contribute to reperfusion injury, bursts of ROS occurring during early reperfusion have been shown to contribute to the evolution of a leukocyte-mediated inflammatory reaction that exacerbates reperfusion-induced tissue injury. Accordingly, antioxidant supplementation and anti-inflammatory agents may represent a rationale therapeutic approach to alleviate I/R injury. However, conventional anti-inflammatory antioxidants have limited efficacy due to the difficulty of delivering them to mitochondria in a targeted manner. To address this limitation in our ability to effectively treat I/R, we seek to develop organelle-

targeting anti-inflammatory antioxidants to alleviate mitochondrial oxidative damage.

We and others have provided preliminary studies which indicate that nitroxides can attenuate oxidative damage in various I/R experimental models.⁷⁻¹⁷ The protective effects of nitroxides are attributed to their antioxidant capacities. In addition to directly scavenging free radicals, nitroxides also attenuate the formation of other reactive oxygen species (ROS) and reactive nitrogen species (RNS). Unlike antioxidants that act in a sacrificial mode, nitroxides can provide a unique type of catalytic protection. Nitroxides undergo one-electron redox reactions to yield the corresponding hydroxylamines and oxo-ammonium cations via electron transfer reactions. The hydroxylamine and oxo-ammonium cations can *com*-proportionate, yielding two nitroxide molecules. The non-radical species might also *com*-proportionate and yield the more stable radical form, thus regenerating themselves. Consequently, nitroxide radical, oxo-ammonium cation, and hydroxylamine can be present simultaneously in the tissue. These three forms can serve as self-restoring antioxidants through continuous exchange, and in this manner confer catalytic protective activity against oxidative damage. These unique features of nitroxides highlight a very novel role for this unique class of agents in protecting against I/R injury. Moreover, nitroxides readily penetrate the brain while permeating the cell membrane, which is unique in comparison to

many other biological antioxidants (e.g., SOD, glutathione peroxidase, etc). Thus, this class of agents is unique with respect to therapeutic potential for diseases and injuries related to oxidative stress.

Kytorphin (KTP; L-Tyr-L-Arg) has been isolated from bovine brain and shown to demonstrate analgesic activity in animal models, but only following central delivery. The therapeutic potential of KTP is primarily hampered in relation to low intestinal absorption. Accordingly, increasing peptide-cell membrane affinity represents a promising strategy to overcome this limitation. Here, our design principle is based on our hypothesis that covalent combination of kytorphin with nitronyl nitroxide (NN) will improve intestinal absorption of KTP. These constructs result from both a chemical rationale (NN renders KTP more lipophilic) and a physiological rationale (combining analgesic and free radical scavenging capacity). The complexity of tissue injury induced by I/R requires a multi-targeted treatment strategy, which we have adapted in the current studies. In our present study, conjugation of nitronyl nitroxide with KTP through a diamine linker proved to be a simple modification that converted kytorphin into an effective analgesic agent. Although there is no report about the application of kytorphin alone for I/R injury, it is known that anesthetics have a protective role against ischemia reperfusion injury.⁵ These protective effects have been attributed to pre- and post-conditioning effects in relation to apoptosis. It has been proposed that anesthetics modulate the interaction of PMN with the endothelial cell, and this may contribute their protective effects.⁵ We found that compound **9i,j** displayed potent analgesic, anti-inflammatory and free radical scavenging activities. Therefore these compounds were further characterized in a simulated I/R cellular model. Our findings suggest that their protective effects are at least partially reflected in the maintenance of mitochondrial function. Here, we report the design, synthesis and preliminary preclinical efficacy evaluation of novel kytorphin-nitroxide hybrid molecules.

Fig. 1. Chemical structures of newly synthesized kytorphin-nitroxide hybrid molecules **9a-j**.

The parental nitronyl nitroxide derivative (NN-7) was synthesized according to Ullman's procedure with minor modification.¹⁴⁻¹⁶ Synthesis was initiated with dinitro compound **1**, followed by the reduction with zinc in an ammonium chloride

(Zn/NH₄Cl) buffered solution to yield the intermediate bis(hydroxylamine) compound **3**, which was subsequently subjected to condensation with 4-hydroxybenzylaldehyde to generate the tetramethylimidazoline derivative **4** with moderate yield (52%). Oxidation by PdO₂ yielded compound **NN-5**. Treatment of **5** with BrCH₂COOC₂H₅ followed by hydrolysis in the presence of NaOH (2 mol/L) provided **NN-7** in good yield (90%). Synthesis of kytorphin-nitroxide hybrid molecules (**9a-j**) was straightforward, involving a simple coupling followed by deprotection (Scheme S1, SI). We employed hydrazinium monoformate as hydrogen donor, and magnesium as the catalyst, to facilitate the removal of some commonly used protecting groups during peptide synthesis. ESR spectra of these kytorphin-nitroxide hybrid molecules all exhibited nitronyl nitroxide free radical characteristics with spectra showing a five-line pattern at a 1/2/3/2/1 ratio, and there was no difference among the compounds tested.

The newly synthesized kytorphin-nitroxide hybrids (**9a-j**) were subjected to the tail flick assay to evaluate their analgesic activities. The tail flick is considered a spinal reflex, which is a commonly used acute pain (nociception) model.¹⁸ The analgesic capacities were expressed by the pain threshold variation (PTV), defined as the difference of pain threshold after drug administration minus basic pain threshold/basic pain threshold. All the test compounds except aspirin were prescreened for analgesic activity at a dose of 0.1 μ mol/kg. For each animal, PTVs were estimated at 30, 60, 90, 120, 150 and 180 min following vehicle or drug administration.

Our previous *in vitro* pharmacological studies of the peptide conjugates revealed that bioactivity was dependent on the linker type and the position of conjugation.¹⁷⁻²² With respect to the anchoring site, and based on our previous studies, two different conjugation positions were considered. Initially, KTP (L-Tyr-L-Arg) was conjugated with nitronyl nitroxide (NN) at the N-terminus to form **9a** or **9b**, respectively. Using the tail flick *in vivo* pain model, we found that the PTV profile of compound **9b** at a dose of 0.1 μ mol/kg was comparable to that of KTP, whereas compound **9a** exhibited moderate analgesic activity. Next, we further tested the importance of the individual amino acids for biological activities using single amino acid analogs. Initially, we attempted to replace Tyr by several Tyr analogues (3-methyltyrosine, 3-hydroxymethyltyrosine, 2-methoxytyrosine, 2-ethyltyrosine) to give compounds **9c-f**. Compared to **9a**, placement of a methyl group *ortho* to the phenol hydroxyl of Tyr (compound **9c**) significantly decreased PTV at all test time intervals. Compared to **9a**, *meta*-substitution of the tyrosine aromatic ring with an OMe group (**9e**) led to a significant diminution of PTV. Since it was uncertain whether the decreased activity with OMe resulted from the electronic effect of oxygen or the increased steric effect due to a larger group, the ethyl-substituted analogue **9f** was evaluated. The PTV profile of the ethyl derivative was similar to the OMe analogue, suggesting that electronic effects of the oxygen atom at the *meta*-position were non-disruptive. Theoretically, the presence of a hydroxymethyl *ortho* to the phenol hydroxyl in compound **9d** has the potential to disrupt hydrogen bonding or augment hydrogen bonding to active site groups. However, the observed effect of this group was minimal, suggesting that potential H-bonding between the hydroxymethyl and the phenol oxygen or active site groups did not play a major role. Taken together, the replacement at the phenolic functions of aromatic amino acids was considered detrimental to analgesic activity. We further evaluated replacement of arginine with Lys and Glu. Lys substitution (**9g**) had minimal effects. Compared to **9a**, Glu substitution (**9h**) resulted in a significant effect on PTV, as predicted.

Additionally, conjugation of nitronyl nitroxide with KTP through a diamine linker proved to be a simple modification that converted kyotorphin into an effective analgesic molecule. Among all test compounds, compounds **9i,j** were shown to possess significant and longer acting analgesic activities as revealed by their PTV profiles. At the dose of 0.1 $\mu\text{mol/kg}$, a statistically significant increase of PTV was found for **9j**, and it simultaneously induced a long-lasting inhibition in the tail flick assay (Table 1).

Unlike KTP, a dose- and time-dependent inhibition was detected in the tail flick test for our new nitroxide hybrids, and we observed different pharmacokinetic parameters for each compound. For example, the maximal pain threshold for aspirin was observed after 60 min, whereas for kyotorphin-nitroxide hybrids (i.e. **9a,g,i,j**) the value was 120 min post drug administration. The general duration of the analgesic action of these compounds was approximately 180 min. No analgesic effects were observed after oral administration of KTP, NN-7 or saline. To investigate dose-dependency effects, the compounds with significant analgesic activities (**9i,j**) were further examined in the same assay. Mice were administrated 0.05, 0.10 and 0.15 $\mu\text{mol/kg}$ of compound **9i,j** orally (Table 2). We observed a clear correlation between dose and analgesic activity. For example, even at the lowest dose of 0.05 $\mu\text{mol/kg}$, the PTV value of compound **9j** was still observed.

Table 3. Anti-inflammatory activities of **9a-j** against xylene-induced ear edema in mice

Compd.	Edema Weight (X $\pm$ SD mg)	Inhibition (%)
NS	5.09 $\pm$ 0.34	
Aspirin	2.55 $\pm$ 0.42	49.90
9a	3.01 $\pm$ 0.41 ^b	40.86
9b	3.65 $\pm$ 0.39	28.29
9c	3.59 $\pm$ 0.40	29.47
9d	3.50 $\pm$ 0.43 ^a	31.23
9e	3.60 $\pm$ 0.28	29.27
9f	3.55 $\pm$ 0.35	30.26
9g	3.21 $\pm$ 0.32 ^a	36.94
9h	3.45 $\pm$ 0.27 ^a	32.22
9i	2.51 $\pm$ 0.41 ^b	50.68
9j	1.35 $\pm$ 0.59 ^c	73.47
NN-7	3.58 $\pm$ 0.39 ^a	29.66
KTP	3.77 $\pm$ 0.47 ^a	25.93

N=10; NS=Saline; Dose of tested compounds = 0.1 $\mu\text{mol/kg}$;

^a: Compared with NS, $p < 0.01$; ^b: Compared with compound **KTP**, $p < 0.01$;

^c: Compared with NS, **KTP** and Aspirin, $p < 0.01$.

Table 4: Anti-inflammatory activities of **9i,j** at different doses

	Dose ($\mu\text{mol/kg}$)	Edema Weight (X $\pm$ SD mg)	Inhibition (%)
NS		5.09 $\pm$ 0.34	
9i	0.15	2.13 $\pm$ 0.24 **	58.15
	0.1	2.51 $\pm$ 0.41 **	50.68
	0.05	4.01 $\pm$ 0.45	21.21
9j	0.15	1.09 $\pm$ 0.25 **	78.58
	0.1	1.35 $\pm$ 0.59 **	73.47
	0.05	3.19 $\pm$ 0.26	37.33

N=10; **: Compared with groups at the dose of 0.05 $\mu\text{mol/kg}$, $p < 0.01$.

All kyotorphin-nitroxide hybrids were further evaluated for anti-inflammatory capacity in a xylene-induced ear edema model. Xylene-induced ear edema is widely used for rapid screening of compounds with anti-inflammatory capacity.²³⁻²⁸ Each kyotorphin-nitroxide hybrid molecule was initially tested at 0.1 $\mu\text{mol/kg}$, and most agents revealed inhibition against xylene-induced inflammation (Table 3), indicating substantial anti-inflammatory activities. Among all tested compounds, **9i,j** were

most effective in the ear edema paradigm. Because of the potent analgesic activity and anti-inflammatory capacities of **9i, j**, they were further examined at lower doses in the same assays to establish more detailed pharmacodynamic profiles. As shown in **Table 4**, we found a dose-dependent anti-inflammatory response for these compounds.

These newly synthesized kyotorphin-nitroxide hybrid molecules (**9a-j**) were subsequently analyzed for functional scavenging capacity against different active free radicals. The scavenging activities were evaluated against $\text{NO}\bullet$, H_2O_2 , $\bullet\text{OH}$ using cell survival assays in PC12 cells as described, with modification.^{16,17,20-23} The EC_{50} values are summarized in **Fig. 2**. Compounds **9a-j** exhibited varying degrees of scavenging capacity on different radicals. The scavenging capacity (EC_{50}) among all of the newly synthesized kyotorphin-nitroxide hybrid molecules (**9a-j**) ranged from 8.1 ± 2.1 to 77.8 ± 4.7 μM for $\text{NO}\bullet$, from 31.3 ± 3.2 to 95.8 ± 2.7 for H_2O_2 , and from 22.7 ± 3.4 to 78.6 ± 1.8 μM for $\bullet\text{OH}$. In particular, compound **9i** exhibited good scavenging capacity of active radical species ($\text{EC}_{50}/\bullet\text{NO}$: 19.5 ± 1.4 μM ; $\text{EC}_{50}/\text{H}_2\text{O}_2$: 32.5 ± 4.5 μM ; $\text{EC}_{50}/\bullet\text{OH}$: 33.9 ± 5.2 μM). Among all the test compounds, compound **9j** was the most potent compound in inhibiting $\text{NO}\bullet$ free radical activity with $\text{EC}_{50}/\bullet\text{NO}$: 8.1 ± 2.1 μM , and the scavenging capacity of $\bullet\text{OH}$ was also quite effective with $\text{EC}_{50}/\bullet\text{OH}$: 22.7 ± 3.4 μM . Compared to the parental compound NN-7, the $\bullet\text{NO}$ scavenging activity of **9i,j** was significantly enhanced. However, there is no statistically significant difference between kyotorphin-nitroxide hybrid molecules (**9a-j**).

Fig. 2. Free radical scavenging activities in PC12 cell survival assays

Since ROS overproduction occurs during I/R, and administration of chemical antioxidants may exert protection against I/R-induced cell death, we sought to examine the putative protective effects of compounds **9i,j** in a simulated I/R (sI/R) cellular model.²⁹ Human umbilical vein endothelial cells (HUVECs) were subjected to sI/R in a flow through chamber. Cells were loaded with MitoTracker to assess the mitochondrial morphological changes. We observed that the mitochondria undergo distinct morphological changes when cells were subjected to sI/R. The mitochondrial morphology was classified into three categories: tubular (normal), intermediate (tubular with swollen regions) and fragmented (small and globular) (**Fig. 3A**). For quantification, the percentage of cells with abnormal mitochondrial morphologies was determined and taken as a measure of the proportion of cells with fragmented mitochondria. HUVEC cells with intermediate or fragmented mitochondria were expressed as a percentage of the total cells counted (100 cells counted per experiment; data averaged for n=4 independent experiments/treatment; scorer blinded to treatment). Non-treated HUVEC cells contained mostly long tubular mitochondria, distributed evenly throughout the whole cell. During 2h of reperfusion, the number of cells with intermediate and fragmented mitochondria increased significantly. We found that simulated ischemia significantly increased the percentage of cells with intermediate or fragmented mitochondria compared to

control, whereas reperfusion primarily induced fragmented mitochondria. During simulated I/R, the percentage of control cells exhibiting long tubular mitochondria had fallen from $80.4 \pm 4.7\%$ (sham) to $11.3 \pm 2.1\%$ (s-I/R alone). Additionally, mitochondria with the intermediate and fragmented morphologies showed predominantly peri-nuclear mitochondrial aggregation in the control s-I/R condition. Interestingly, addition of compound **9i,j** to the perfusate throughout the control and ischemia periods resulted in significant protection from s-I/R induced cell death, as well as significant attenuation of the fragmented mitochondrial appearance. The cells treated with **9i,j** + s-I/R exhibited an increase in percentage of cells with tubular mitochondria to $41.5 \pm 3.7\%$ (**9i** + s-I/R)/ $55.7 \pm 4.5\%$ (**9j** + s-I/R) vs. $11.3 \pm 2.1\%$ (s-I/R alone) (**Fig. 3B**).

To determine whether the mitochondrial protection provided by compounds **9i,j** is due to anti-ROS formation, we measured mitochondrial ROS levels using confocal imaging microscopy coupled with MitoProbe for real-time monitoring of mitochondrial ROS production.^{30,31} By measuring the fluorescence intensity of MitoProbe change at different time points, the total ROS generation in the mitochondria was determined. We observed a ~1.9 fold rise of mitochondrial ROS levels during simulated ischemia, and found that mitochondrial ROS increased rapidly after cells were subjected to reperfusion (within the first 10 min) and accompanied by mitochondrial swelling and cell shrinkage. We observed that in some areas of the mitochondria, mitochondrial ROS increase preceded mitochondrial swelling, indicating that opening of the mitochondrial permeability transition pore (MPT) appeared after mitochondrial ROS formation. Within 120 min of reperfusion, a ~5.2 fold elevation of ROS in the mitochondria was induced by simulated reperfusion (**Fig. 3C**). However, administration of compounds **9i,j** significantly reduced mitochondrial ROS production. The inhibition of mitochondrial ROS by **9i,j** was quantitatively documented by the fluorescence intensity changes of MitoProbe.

Mitochondrial ROS over-production, membrane potential depolarization and mitochondrial swelling have been shown to trigger the opening of the MPT and to transpose the mitochondrial apoptosis protein (cytochrome c) from the mitochondrial membrane to the cytosol. Accordingly, we examined whether compound **9i,j** would prevent s-I/R-induced cytochrome c release. Confocal microscopy demonstrated that in the sham-control cell, cytochrome c was localized to the mitochondria. After cells were subjected to reperfusion, we found that cytochrome c was released to the cytosolic and the nuclear area. We also observed that the release of cytochrome c coincided with shrinkage of the cell and mitochondrial swelling (**Fig. 4A**). However, the s-I/R induced cytochrome c release and cell shrinkage was prevented by pretreatment with compound **9i,j**. As shown in **Fig. 4B**, pre-treatment of cells with compound **9i,j** inhibited cytochrome c release during s-I/R, reducing cytochrome c released cell number from $75.3\% \pm 5.2\%$ (s-I/R alone) to $47.8\% \pm 4.3\%$ (**9i** + s-I/R) or $27.8 \pm 4.5\%$ (**9j** + s-I/R). During this process, we also observed that mitochondrial ROS were reduced to baseline levels. These findings suggested that mitochondrial ROS overproduction during s-I/R contributed to mitochondrial fragmentation and cytochrome c release.

Fig. 3. Detection of oxidative stress in the mitochondria during simulated I/R (s-I/R). **(A)** Typical confocal fluorescence images of HUVEC cells counter-stained with MitoProbe (red fluorescence), MitoTracker (green fluorescence) and Hoechst 33342 (blue fluorescence): Fluorescence images of HUVEC cells from sham-control group (no s-I/R), cells were subjected to simulated ischemia alone (s-I) or s-I/R injury alone without drug treatment (s-I/R), and with the drug pretreatment (**9i,j**) during s-I/R (**9i,j** + I/R). **(B)** Cells with tubular, elongated mitochondria (%) subjected to s-I/R with/without treatment of NN-7 or **9i,j**. (#: Compared with sham at baseline, $p < 0.05$; *: compared with s-I/R alone, $p < 0.05$ (s-I/R alone); **(C)** By measuring the fluorescence intensity of MitoProbe changes at different time points, the total ROS generation in the mitochondria was determined. Increases in MitoProbe fluorescence relative to baseline values were evident during s-I/R. Pretreatment with **9i,j** significantly attenuated the increase in MitoProbe fluorescence intensity during s-I/R. The inhibition of mitochondrial ROS by **9i,j** was quantitatively measured by the fluorescence intensity changes of MitoProbe. Error bars indicate standard error of the mean from at least three independent experiments (#compared with sham at baseline, $p < 0.01$; ** compared with s-I/R alone, $p < 0.01$).

Fig. 4. Release of cytochrome c during simulated I/R. (A) HUVEC cells were stained with MitoProbe (green fluorescence). Cells were fixed at baseline, following simulated ischemia or after 3h of reperfusion. Cells were then immunostained for cytochrome c (red fluorescence); Fluorescence images of HUVEC cells from the sham group (no s-I/R), cells were subjected to simulated ischemia alone (s-I) or simulated I/R injury alone without drug (s-I/R), and with drug pretreatment (**9i,j**) during s-I/R (**9i,j** +I/R). (B) Cells showing redistribution of cytochrome c were quantified and expressed as a percent of the total cell population. A significant increase in the percentage of cells with cytochrome c release was observed in cells subjected to s-I/R. Cytochrome c release was significantly inhibited in cells pre-treated with **9i,j**. Error bars indicate standard error of the mean from at least three independent experiments (#: Compared with sham at baseline, $p < 0.05$; *: Compared with s-I/R alone, $p < 0.05$). Arrow indicates the redistribution of cytochrome c in fluorescence image.

Compounds **9i,j** appear to act as potent ROS scavengers that effectively counteract the rapidly formed mitochondrial ROS production associated with s/I/R. We also found that as long as compounds **9i,j** were administered before cells were subjected to reperfusion, they could reduce the damage to the mitochondria and prevent the subsequent s/I/R-induced apoptosis. These findings suggest that inhibition of mitochondrial ROS overproduction by compounds **9i,j** is mechanistically important in averting mitochondrial dysfunction and cell damage during I/R. The nitronyl nitroxide moiety, coupled with the diamine linker of the proper length, likely enable compounds **9i,j** to pass through

the plasma membrane and enter the mitochondrial compartment where they can effectively exert their protective effects.

To further explore the utility of our new nitroxide hybrids, we examined the ionization potentials of our optimized compounds **9i,j**. E_{HOMO} represents an energy measurement that is directly related to the ionization potential, and it characterizes the susceptibility of the molecule toward electrophilic attack. Conversely, E_{LUMO} gauges electron affinity of a compound and it estimates the susceptibility of the molecule to nucleophilic attack. E_{HOMO} and E_{LUMO} are not antioxidant estimates, but they can be related to the free radical scavenging activity of molecules. The energy difference between E_{LUMO} and E_{HOMO} orbital energies (ΔE) determines the overall chemical reactivity. ΔE estimates the ease of transition from the ground to the excited state, and it reflects the stability of molecular radicals. Higher E_{HOMO} values indicate better electron-donating properties. Conversely, lower E_{LUMO} values highlight the electron accepting properties of a molecule. The computational data obtained using these two parameters can be cross-correlated with the radical scavenging activity of compounds, and we applied these molecular orbital measurements to compounds **9i** and **9j**. Compound **9j** possessed the highest E_{HOMO} with the lowest ΔE and was the most effective free radical scavenger (Fig. 5). The *in silico* results correlate well with our experimental evaluations. Since the diamine linker can be readily protonated at physiological pH, the combination of nitronyl nitroxide and KTP through a diamine linker likely facilitates movement across the mitochondria inner/outer membranes via electrostatic forces. Potentially, synergetic effects may be gained from combining nitronyl nitroxide and diamine linker.

Fig. 5. HOMO/ LUMO plots for compounds KTP peptide, NN-7, **9i,j** and ΔE measurements.

Although mitochondria generate ROS, they are also cellular targets of ROS and mitochondrial dysfunction plays an important role in I/R injury. Oxidative insult to the mitochondria led to perturbation of its metabolic and redox status, altered membrane permeability and cytochrome c leakage, which triggered a cascade of apoptotic cellular events. Here, we have shown that mitochondrial function declines using *in vitro* simulated I/R cellular models, and have further demonstrated that oxidative stress exacerbates the associated cell damage. Our findings indicate that I/R injury stimulates mitochondrial remodeling,

trafficking, and loss of function, all of which can be mitigated by our novel nitroxide hybrids, compounds **9i,j**. Our data, indicating recovery of mitochondrial networks and inhibition of cytochrome c release, lay the groundwork for a more comprehensive characterization of nitroxide hybrids in the treatment of I/R.

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Table 1. In vivo analgesic activities of kyotorphin-nitroxide hybrids **9a-j** tested using tail flick method in mice

Agents	Pain threshold variation (X ± SD%)					
	30min	60min	90min	120min	150min	180min
NS	6.70 ± 4.98	9.76 ± 8.76	10.33 ± 9.04	9.57 ± 5.21	10.04 ± 7.94	10.75 ± 8.35
Aspirin	33.21 ± 5.32	66.32 ± 5.50	53.20 ± 6.21	25.21 ± 8.02	15.84 ± 4.65	20.56 ± 3.38
9a	12.53 ± 3.25 ^b	18.66 ± 4.54	31.45 ± 5.34	40.01 ± 4.95 ^c	34.14 ± 6.92 ^c	14.45 ± 4.16
9b	10.10 ± 2.13	14.31 ± 2.23	26.97 ± 3.21	13.18 ± 6.23	11.91 ± 6.24	10.05 ± 3.08
9c	10.24 ± 5.67	18.97 ± 6.14	22.10 ± 5.11	31.21 ± 4.52	13.25 ± 6.18	12.38 ± 4.94
9d	12.12 ± 4.33	19.13 ± 5.17	22.37 ± 6.42	25.78 ± 6.32	33.56 ± 5.17	20.14 ± 3.79
9e	11.37 ± 2.34	16.33 ± 4.77	22.47 ± 6.33	27.14 ± 5.78	24.12 ± 4.39	11.24 ± 3.77
9f	10.78 ± 5.33	17.31 ± 5.14	21.89 ± 4.67	22.78 ± 4.33	22.34 ± 6.12	15.04 ± 3.67
9g	12.56 ± 3.52	19.35 ± 4.13	29.31 ± 5.32	38.33 ± 5.04 ^c	33.73 ± 5.02 ^c	13.58 ± 4.34
9h	11.10 ± 2.39	13.44 ± 4.17	26.41 ± 4.32	13.58 ± 3.02	15.11 ± 2.13	14.22 ± 4.07
9i	15.28 ± 3.66	25.75 ± 4.28 ^b	35.74 ± 6.54 ^b	48.17 ± 5.77 ^d	37.11 ± 4.65 ^d	23.15 ± 3.78 ^b
9j	16.30 ± 3.32	29.36 ± 2.37 ^b	44.76 ± 6.45 ^b	60.32 ± 8.25 ^d	51.33 ± 9.22 ^d	22.01 ± 4.65 ^b
NN-7	9.10 ± 2.13	12.31 ± 2.83	20.97 ± 3.29	23.18 ± 6.23	21.11 ± 6.24	13.05 ± 3.08
KTP	16.08 ± 3.65	22.78 ± 3.41	25.35 ± 6.15	12.32 ± 2.98	12.11 ± 3.43	10.55 ± 3.24

N=10; NS=Saline; Dose of nitroxide-kyotorphin hybrids = 0.1 µmol/kg; ^a: Compared with NS, **NN-7** and **KTP**, p<0.05;

^b: Compared with NS, **NN-7**, **KTP**, p<0.01. ^c: Compared with Aspirin, p<0.05; ^d: Compared with Aspirin, p<0.01.

Dose of Aspirin = 165 µmol/kg; Dose of **NN-7** = 1 µmol/kg; Dose of **KTP** = 1 µmol/kg.

Table 2. Analgesic activities of **9i,j** at different doses using tail flick method in mice

	Dose (µmol/kg)	Pain threshold variation (X ± SD%)					
		30min	60min	90min	120min	150min	180min
NS		6.70 ± 4.98	9.76 ± 8.76	10.33 ± 9.04	9.57 ± 5.21	10.04 ± 7.94	10.75 ± 8.35
9i	0.15	17.89 ± 4.12	28.65 ± 4.11	32.33 ± 5.67	50.61 ± 4.59	39.13 ± 3.79	27.67 ± 5.34
	0.1	15.28 ± 3.66	25.75 ± 4.28 ^b	35.74 ± 6.54 ^b	48.17 ± 5.77 ^b	37.11 ± 4.65 ^b	23.15 ± 3.78 ^b
	0.05	7.48 ± 2.21	10.35 ± 3.16	13.57 ± 4.35	15.78 ± 4.65	11.91 ± 4.25	9.14 ± 3.15
9j	0.15	20.17 ± 4.29	33.47 ± 4.23	50.35 ± 5.17	68.42 ± 6.48	52.45 ± 7.18	30.34 ± 6.23
	0.1	16.30 ± 3.32	29.36 ± 2.37 ^b	44.76 ± 6.45 ^b	60.32 ± 8.25 ^c	51.33 ± 9.22 ^c	22.01 ± 4.65
	0.05	8.16 ± 2.13	12.31 ± 4.35	18.37 ± 4.72	25.36 ± 2.89 ^a	19.35 ± 2.45	16.28 ± 3.34

N=10; ^a: compared with NS, p<0.01; ^b: Compared with the same compound at the dose of 0.05 µmol/kg, p<0.05; ^c: Compared with the same compound at the dose of 0.05 µmol/kg, p<0.01.

References and notes

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