Graphical Abstract

Pharmacological protection of mitochondrial function mitigates acute limb ischemia/reperfusion injury

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ABSTRACT

We describe several novel curcumin analogues that possess both anti-inflammatory antioxidant properties and thrombolytic activities. The therapeutic efficacy of these curcumin analogues was verified in a mouse ear edema model, a rat arterial thrombosis assay, a free radical scavenging assay performed in PC12 cells, and in both *in vitro* and *in vivo* ischemia/reperfusion models. Our findings suggest that their protective effects partially reside in maintenance of optimal mitochondrial function.

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Acute limb ischemia, which associates with significant morbidity and mortality, has been linked to thrombosis, embolism, and trauma. The sudden decrease in blood flow to an extremity can result in acute limb ischemia and reperfusion which releases toxic free radicals and the potential threat of limb viability.¹ Early diagnosis and timely intervention is critical to prevent irreversible tissue damage and necrosis. Acute limb ischemia encompasses three progressive stages: 1) growth of the arterial thrombus leading to occlusion of collateral vessels; 2) edema resulting in elevated compartmental pressure which further restricts blood flow; and 3) narrowing and obstruction of the micro-vasculature.² Progression to stage 3 is indicative of sufficient occlusion that results in irreversible tissue edema. Prompt reperfusion of the ischemic limb is necessary to restore baseline organ function. Pharmacotherapeutics for acute limb ischemia usually entails intra-arterial application of thrombolytics (e.g., streptokinase, urokinase, recombinant tissue plasminogen activator, and reteplase). Reperfusion is mandatory in restoring blood flow, yet it is a physiological process that leads to cellular injury due to enhanced free radical production. Antioxidant supplementation is indicated for limb ischemia that can result in enhanced mitochondrial production of reactive oxygen species (ROS).^{3,4} Indeed, several studies have highlighted the capacity of antioxidants to reduce oxidative stress and mitigate claudication in patients with critical limb ischemia.³⁻⁵

Curcumin, isolated from turmeric, has numerous therapeutic properties, including antioxidant^{6,7}, anti-inflammatory⁸ and anti-cancer^{9,10}. Although curcumin represents a potential lead compound for drug design, a significant limitation of its direct use is its poor water and plasma solubility, which significantly impacts bioavailability.^{11,12} The pentapeptide Ala-Arg-Pro-Ala-Lys (ARPAK) derived from fibrinogen degraded by plasmin and corresponding to amino acids 43-47 of the human fibrinogen B chain, increases coronary and femoral artery blood flow¹³. Of particular interest is the fibrinogen-derived peptide fragment PAK, which is relatively small, easily synthesized and exhibits thrombolytic activities in several models^{14,15}. These observations have led us to ask whether curcumin and the fibrinogen-derived short peptide fragment could be combined into a single hybrid molecule that would have advantageous synergies. We hypothesized that the hybrid drugs would exhibit improved bioavailability with concomitant antioxidant and thrombolytic activities. The rationale of this approach involves linking two molecules with individual intrinsic activity into a single agent, thus packaging dual-activity into a single hybrid drug. In general, the hybrid drugs may offer various advantages including dosage compliance, minimized toxicity, novel combinatorial drug design, and more inexpensive preclinical evaluation. The current report summarizes the synthesis and biological evaluation of such hybrid drugs.

The preparation of new curcumin analogues followed the synthetic route outlined in **Scheme 1**. We performed the synthesis of the curcumin analogues **5a,b** via a two-step strategy as described.^{16,17} As a first step, pentane-2,4-dione and vanillin/3,4-di-methoxybenzaldehyde were subjected to a Pabon reaction to give the monoaryl intermediates **2a,b**, which were then subjected to a second Pabon reaction with a suitable aldehyde **4** to provide **5a,b**, respectively. **5a,b** was then subjected to deprotection and coupling reactions to provide compounds **7a,b**. Following deprotection reactions, **7a,b** was converted to **8a,b** and **9a,b**, respectively. Compounds **8a,b** were subjected to coupling reactions with Boc-Pro-Ala-Lys (Boc) in the presence of DCC/HOBt and subsequently deprotected to generate the target compounds, **11a,b**. The synthetic scheme is shown in **Fig. 1**.

Scheme 1: Synthesis of the curcumin analogues **5a,b**, **9a,b** and **11a,b**. In **5a,9a,11a**, R=H; in **5b,9b,11b**, R=CH₃.

Following our previously published protocol,¹⁸⁻²⁰ the newly synthesized curcumin analogues **11a,b** along with **9a,b** and **5a,b** were examined in the thread-induced venous thrombosis animal model. Here, the weights of thrombi are determined as outcome measures to assess their thrombolytic effects. The cotton threads serve as a thrombogenic surface onto which the thrombus forms, reaching a maximum mass after 2-3 hours. The prolonged, non-occlusive character of thrombogenesis in this model facilitates monitoring of thrombus progression as opposed to the initiation of thrombus formation, thus facilitating conditions that more closely resemble human pathophysiology with blood flow ongoing throughout the experiment. The structure of the cotton-threaded thrombus shows a composition of fibrin associated with tightly aggregated and distorted erythrocytes, comparable to human deep-vein thrombosis structure. Aspirin, glycoprotein (GP) IIb/IIIa inhibitors, and clopidogrel display an inhibitory effect on platelet activation and aggregation². Plasminogen gathers in the fibrin matrix. Fibrin-bound plasminogen will be converted by thrombolytic drugs to plasmin, the rate-limiting step in thrombolysis. Current recommendations state that all patients with critical limb ischemia must receive antiplatelet therapy². Aspirin's antithrombotic effect is mediated by inhibition of blood platelet aggregation. Aspirin blocks cyclooxygenase by acetylating the enzyme's active site. Inhibition of cyclooxygenase blocks production of thromboxane A₂. Thromboxane A₂ is an important prothrombotic agent, which causes activation and aggregation of platelets (an early

step in thrombosis). Accordingly, aspirin was chosen as the positive control in this assay.

The cumulative data is summarized in **Table 1**. We found that the thrombolytic activities of compounds **11a,b** (**11a**: 22.5 ± 3.3; **11b**: 25.8 ± 4.7mg; vs. NS: 42.1 ± 3.2mg, p<0.01) were significantly higher than **5a,b** (**5a**: 39.7 ± 4.2; **5b**: 40.1 ± 3.7 mg; vs. NS: 42.1 ± 3.2mg) at equivalent amounts (5 µmol/kg). Compounds **11a,b** significantly reduced thrombus mass in a dose-dependent fashion, but the mechanism of their thrombolytic activity remains to be defined.

Table 1. Effect of compounds **5a,b**, **9a,b** and **11a,b** on thrombus weight ($\bar{X}$ mean ±SD)

Compound	Wet thrombus ($\bar{X}$ ± SD mg)
NS	42.1 ± 3.2
Aspirin	28.5 ± 3.0 ^{a,b}
5a	39.7 ± 4.2
5b	40.1 ± 3.7
PAK	32.8 ± 3.1 ^a
9a	34.5 ± 3.6
9b	38.3 ± 4.4
11a	22.5 ± 3.3 ^{a,b}
11b	25.8 ± 4.7 ^{a,b}

N=8; Dose = 5 µmol/kg. ^a: Compared to normal saline (NS), p<0.05; ^b: In comparison to normal saline (NS), p<0.01.

Compounds **5a,b**, **9a,b**, and **11a,b**, were further evaluated for anti-inflammatory characteristics in a xylene-induced ear edema model,²¹⁻²⁶ which is widely used for screening anti-inflammatory potential. Aspirin, the classic and widely used NSAID, is commonly used as the positive control in the anti-inflammatory drug screening assays. Each test compound was initially tested at 1 µmol/kg. The results for test compounds **9a,b** and **11a,b** revealed variable extents of inhibition against xylene-induced inflammation (**Table 2**), but suggesting potent anti-inflammatory activities in all instances. Compounds **11a,b** showed the most significant activity.

Table 2. Anti-inflammatory activities of curcumin analogues **5a,b**, **9a,b**, **11a,b** against xylene-induced ear edema in mice

Compd.	Edema Weight ($\bar{X}$ ± SD mg)	Inhibition (%)
NS	4.89 ± 0.37	
Aspirin	2.43 ± 0.43	50.30
5a	3.75 ± 0.32	23.31
5b	3.66 ± 0.41	25.15
9a	2.88 ± 0.40 ^a	41.10
9b	2.97 ± 0.37 ^a	39.26
11a	1.86 ± 0.27 ^{a,b}	62.00
11b	2.07 ± 0.51 ^{a,b}	57.67

N=10; NS=Saline; Dose of tested compounds = 1 µmol/kg;

^a: Compared with NS, p<0.01; ^b: Compared with **5a,b**, p<0.01.

Overproduction of free radicals leads to the damage of biological molecules during ischemia/reperfusion (I/R). In light of the antioxidant potential of curcumin, we hypothesized that curcumin analogues **11a,b** would also display free radical scavenging activity against ROS. To test this hypothesis, the rat pheochromocytoma (PC-12) cell model^{18,19} was employed for *in vitro* ischemia studies, a cell line very sensitive to oxidative stress and commonly used to evaluate antioxidant compounds. Reduction of the dye MTT (3-(4,5-di-methyl-thiazol-2-yl)-2,5-diphenyltetrazolium-bromide) is utilized as a cell survival

biomarker for PC-12 cells exposed to free radicals. In this assay, A Fenton-reaction is employed to generate $\bullet\text{OH}$ radicals in situ. Compounds **5a,b** showed moderate $\bullet\text{OH}$ radical scavenging activity, while compounds **11a,b** exhibited good hydroxyl radical scavenging activity ($\text{EC}_{50}/\bullet\text{OH}$: **11a**: $25.6 \pm 4.5 \mu\text{M}$; **11b**: $31.4 \pm 4.7 \mu\text{M}$) (Fig. 1). Curcumin is also capable of binding ferrous and ferric ions via the hydroxyl and methoxyl groups on the phenolic ring, as well as the keto-enol moiety.^{6,7} Indeed, the hydroxyl radical scavenging activity of curcumin and its derivatives may be secondary to its iron chelating property.³²

Fig. 1. Free radical scavenging assay in PC12 cells. (Error bars indicate standard error of the mean from at least three independent experiments).

In situ $\bullet\text{NO}$ radical was generated from the nitric oxide donor and potent vasodilator S-Nitroso-N-Acetyl-D,L-Penicillamine (SNAP). Compounds **5a,b** showed weak $\bullet\text{NO}$ radical scavenging activity, whereas the curcumin analogues **11a,b** showed moderate scavenging activity towards $\bullet\text{NO}$ ($\text{EC}_{50}/\bullet\text{NO}$: **11a**: $42.1 \pm 2.7 \mu\text{M}$; **11b**: $61.3 \pm 5.3 \mu\text{M}$). Over-production of $\bullet\text{NO}$ has been implicated in various inflammatory-related pathologies where its cytotoxicity is facilitated by the formation of ONOO^- radical through interaction of $\bullet\text{NO}$ with $\text{O}_2^{\bullet-}$; therefore, removing $\bullet\text{NO}$ should be therapeutically beneficial. Curcumin has been reported to inhibit $\bullet\text{NO}$ production indirectly via inhibition of inducible nitric oxide synthase (iNOS) following the suppression of nuclear factor-kappaB (NF- κB) activation.^{6,7} Our findings suggest that the direct interaction of **11a,b** with $\bullet\text{NO}$ may partially contribute to anti-inflammatory activity despite displaying only moderate $\bullet\text{NO}$ scavenging activity.

We further generated $\text{O}_2^{\bullet-}$ radicals in situ using the enzymatic reaction of hypoxanthine and xanthine oxidase. Compounds **5a,b** showed low capacity for $\text{O}_2^{\bullet-}$ scavenging, whereas the curcumin analogues **11a,b** showed moderate scavenging activity for $\text{O}_2^{\bullet-}$ ($\text{EC}_{50}/\text{O}_2^{\bullet-}$: **11a**: $53.8 \pm 5.3 \mu\text{M}$; **11b**: $65.7 \pm 5.2 \mu\text{M}$). The $\text{O}_2^{\bullet-}$ radical has been reported to react preferably at the keto-enol moiety of curcumins.^{6,7} Our findings further highlight the relative importance of the keto-enol moiety. The curcumin-analogues **11a,b** were effective as $\bullet\text{OH}$ scavengers, but only moderately effective in scavenging $\bullet\text{NO}$ and $\text{O}_2^{\bullet-}$ radicals. Since these curcumin analogues are also potent hydrogen donors, electron donation mechanisms associated with bond cleavage may be another contributing factor to the protective effect of these agents as opposed to only a direct interaction with a radical like $\text{O}_2^{\bullet-}$.

Oxidative stress and mitochondrial apoptosis have been implicated in cell death observed during I/R. Previous studies suggest that ROS overproduction occurs during I/R, and the administration of antioxidants may exert protection against I/R-induced cell death. We addressed the putative protective effects of compounds **11a,b** in a human umbilical vein endothelial cell (HUVECs) system using a simulated I/R (s-I/R) induction approach.^{27,28} The cells were loaded with MitoTracker to assess mitochondrial morphological changes. We found that

mitochondria underwent distinct morphological changes when the cells were subjected to s-I/R. The mitochondrial morphology was classified into three categories: tubular (normal), intermediate (tubular with swollen regions), and fragmented (small and globular) (Fig. 2A). The method for quantification involved determining the percentage of cells with abnormal mitochondrial morphologies as a surrogate measure of the proportion of cells with fragmented mitochondria. When cells with intermediate or fragmented mitochondria were expressed as a percentage of the total cells counted (100 cells were counted per experiment and the data was averaged over four independent experiments per treatment), the non-treated cells contained predominantly long and evenly distributed tubular mitochondria throughout the cell.

Fig. 2. Detection of oxidative stress in the mitochondria during simulated I/R (s-I/R). (A) Typical confocal fluorescence images of HUVEC cells counterstained 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 (**11a**) during s-I/R (**11a** + I/R). (B) Cells with tubular, elongated mitochondria (%) subjected to s-I/R with/without drug treatment (#: 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 **11a,b** significantly attenuated the increase in MitoProbe fluorescence intensity during s-I/R. 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, $p < 0.01$).

During 2h of simulated reperfusion, the number of cells with intermediate and fragmented mitochondria increased significantly. The simulated ischemia was found to significantly increase the percentage of cells with intermediate or fragmented mitochondria when compared to the control cells, whereas reperfusion resulted in the majority of cells displaying fragmented mitochondria. The percentage of control cells exhibiting long tubular mitochondria decreased from $85.3 \pm 6.1\%$ (sham control) to $15.8 \pm 4.3\%$ (s-I/R alone), and the mitochondria with the intermediate as well as the fragmented morphology were predominantly aggregated in the perinuclear region. Interestingly, the addition of compound **11a,b** to the perfusate of control cells undergoing ischemia resulted in significant protection from s-I/R induced cell death, and attenuated the fragmented mitochondrial appearance. The cells treated with **11a,b** + s-I/R exhibited an increase in the percentage of cells with tubular mitochondria ($56.4 \pm 6.2\%$ (**11a** + s-I/R); $50.3 \pm 5.7\%$ (**11b** + s-I/R) vs. $15.8 \pm 4.3\%$ (s-I/R alone) (Fig. 2B).

To determine whether or not the mitochondrial protection provided by compounds **11a,b** was associated with mitigation of mitochondrial ROS formation, the mitochondrial ROS levels were measured following s-I/R using confocal imaging microscopy coupled with MitoProbe.²⁸⁻³⁰ By measuring the changes in fluorescence intensity of MitoProbe at different time points, the total ROS generation in the mitochondria was estimated. An ~2.3 fold increase was observed for mitochondrial ROS levels during simulated ischemia. Further, mitochondrial ROS rapidly increased within the first 10 minutes following reperfusion and was followed by mitochondrial swelling. The increase in mitochondrial ROS preceded mitochondrial swelling in some areas of the mitochondria, indicating that opening of the mitochondrial permeability transition pore (MPT) was occurring after mitochondrial ROS formation. Within 120 minutes of reperfusion, an ~6.4 fold elevation of ROS in the mitochondria was associated with simulated reperfusion; however, significant inhibition of mitochondrial ROS was achieved via administration of compounds **11a,b** as shown by the changes in fluorescence intensity of MitoProbe (Fig. 2C).

Enhanced mitochondrial ROS production, membrane potential depolarization, and mitochondrial swelling have been shown to trigger the opening of the MPT and translocation of the mitochondrial apoptotic protein cytochrome c from membrane to cytosol; therefore, we examined cytochrome c release in the presence/absence of compounds **11a,b** as a function of simulated I/R. Confocal images revealed that cytochrome c was localized to the mitochondrial area in the sham-control cells. After the cells were subjected to reperfusion, cytochrome c translocated to the cytosolic and nuclear regions, with simultaneous mitochondrial swelling (Fig. 3A). Pretreatment with compounds **11a,b** significantly inhibit cytochrome c release and mitochondrial swelling during s-I/R from $78.5 \pm 4.5\%$ (s-I/R alone) to $23.5 \pm 5.1\%$ (**11a** + s-I/R), or $33.7 \pm 5.3\%$ (**11b** + s-I/R) (Fig. 3B), which was also associated with a low production of mitochondrial ROS. Our data indicate that mitochondrial ROS

overproduction during s-I/R contributes to mitochondrial fragmentation and cytochrome c release that is prevented by compounds **11a,b**.

Tourniquet application is broadly employed in orthopedic, vascular, and reconstructive surgery to provide a bloodless surgical field, but associates with enhanced production of free radicals within the ischemic tissue prior to, and during, reperfusion. This can be exacerbated through enhanced production of inflammatory adhesion molecules and cytokines. We induced complete limb I/R injury in a rat model by occluding collateral blood flow around the femoral and iliac arteries using a rubber band tourniquet (RBT). The rat model underwent 3h of unilateral lower limb ischemia, and then 4h of reperfusion post tourniquet release.¹⁸ Compounds **11a,b** were then further examined to ascertain their capacity for protection against local and remote organ injury using this tourniquet-induced limb I/R model.

Fig. 3. 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 3 h 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 (**11a**) during s-I/R (**11a** + 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 **11a,b**. 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, $p < 0.05$).

compared with s-I/R alone, $p < 0.05$). Arrow indicates the redistribution of cytochrome c in fluorescence image.

In our model, routine histological evaluation (hematoxylin-eosin staining) of frozen cross-sections of skeletal muscle derived from sham group rats revealed myofibers of uniform size and shape that were tightly arranged and in essentially direct contact with separation by a thin level of endomysium (image not shown). Conversely, 3h of ischemia followed by reperfusion in our tourniquet model led to extensive interstitial edema and damage characterized by swelling and inflammatory cell infiltration (**Fig. 4**). Muscle tissue damage post reperfusion was accompanied by an increase in the lipid peroxidation byproduct malondialdehyde (MDA) (**Table 4**), suggesting a correlation between enhanced lipid peroxidation and oxygen free radicals. Intervention with **11a** prevented interstitial edema and left the cross-sectional area of the microvessel essentially unchanged (**Fig. 5**). The histological results indicated that treatment with **11a** resulted in only mild edema and subtle inflammatory cell infiltration in the skeletal muscle, with no muscle fibers from this group showing obvious histological changes. We further performed modified trichrome staining in order to quantify intramuscular collagen deposition, as increased deposition of connective tissue has negative consequences on contractile muscle function via decrease of myofiber occupancy. As shown in **Fig. 5B**, there was approximately a 2-fold increase (17~20% vs. ~9%) in percent area occupied by collagen in saline + I/R groups, relative to **11a** + I/R treated group. Overall, **11a** treatment improved muscle morphology and reduced fibrosis.

We also noted an irregular distribution of oxidative metabolism enzymes that was seen in the NADH-stained skeletal muscle tissues from the I/R rat group treated with tourniquet blood flow restriction. Typically, lightly stained fibers were categorized as Type II (fast-twitch glycolytic, mitochondrial-poor), while fibers with intense staining were categorized as Type I (slow-twitch oxidative, mitochondrial-rich). Type II fibers, possessing decreased antioxidant enzyme activities in comparison to Type I fibers, are predicted to be more susceptible to I/R induced damage. However, our results indicated that mitochondria-rich Type I fibers suffered greater damage compared to Type II fibers.

11a intervention significantly mitigated most of the preceding damage, although occasional “ragged red fibers” were still observed (lower row in **Fig. 4B**) in addition to occasional vacuolated fibers seen in H/E sections (lower row in **Fig. 4A**). The majority of muscle fibers were intact, with well-defined edges, consistent texture, and homogeneous morphology. Additionally, NADH reactions were weak in most fibers (lower row in **Fig. 4C**), reflecting substantial tissue protection associated with **11a** intervention following I/R treatment. Along these lines, we found that mitochondrial-rich Type I fibers could withstand I/R intervention with essentially no damage, suggesting that compound **11a** intervention protects oxidative muscles to some extent. These muscles, rich in mitochondria, would otherwise be expected to suffer extensive freed radical production and damage associated with I/R.

These observations strongly support the notion that curcumin analogue **11a** indeed is present in mitochondria. To assess the extent of tissue protection affiliated with a reduction in the production of ROS, mitochondrial ROS production was determined in skeletal muscle tissue using MitoProbe for skeletal muscle tissue staining. The fluorescence intensity of MitoProbe was significantly higher in muscle tissues derived from the saline +I/R rat cohort subjected to tourniquet-induced I/R, suggesting that reperfusion of ischemic skeletal muscle is associated with increased production of mitochondrial ROS. Conversely, the fluorescence intensity in muscle tissues pretreated with **11a** was

significantly attenuated compared with saline +I/R group (**Fig. 5A**). Our data suggests that increased mitochondrial ROS production results in substantial skeletal muscle mitochondrial dysfunction and necrosis. Representative transmission electron microscope (TEM) sections after I/R injury in our tourniquet-induced I/R group are shown in **Fig. 5B**. The mitochondria in the I/R + Saline group revealed considerable swelling with significant cristae loss, whereas the I/R + **11a** cohort revealed a considerable reduction in mitochondrial swelling with maintenance of normal cristae (**Fig. 5B**).

Local inflammation, associated with release of cytokines and other mediators, are important in the evolution of systemic complications following lower limb I/R. Accordingly, we examined pro-inflammatory cytokines in our model. The plasma levels of pro-inflammatory TNF- α and IL-6 revealed a significant increase in the I/R rat cohort subjected to tourniquet-induced I/R as compared to the sham cohort; these cytokine elevations were significantly attenuated in the **11a,b**-treated cohort, suggesting the ability of compound **11a,b** to reduce systemic progression of the inflammatory process (**Table 3**).

Fig. 4. Representative photomicrographs of histological sections of skeletal muscle tissues from saline + I/R and I/R + **11a** treatment groups. (A): H&E staining; (B) modified tri-chrome staining; (C) NADH staining.

Finally, the beneficial effects of **11a,b** was also highlighted through studies on tissue MDA, GSH levels and SOD, and CAT activity in the limb tourniquet-induced I/R injury rat model. The SOD and CAT activities, and GSH levels, were significantly reduced in the I/R cohort compared to the sham cohort, an effect reversed by intervention with **11a,b** treatment. Moreover, **11a,b** reversed the elevation of MDA induced by limb I/R using the tourniquet induction procedure (Table 4).

Table 3. Effect of curcumin analogues on the serum TNF- α and IL-6 levels in a limb I/R animal model

	TNF- α (pg/ml)	IL-6 (pg/ml)
Sham	18.7 $\pm$ 4.5	118.7 $\pm$ 25.4
I/R + Saline	185.1 $\pm$ 23.5	580.3 $\pm$ 95.2
11a + Sham	22.5 $\pm$ 6.1	120.3 $\pm$ 28.9
11b + Sham	23.2 $\pm$ 7.0	125.7 $\pm$ 31.0
I/R+ 5a	155.4 $\pm$ 27.3	450.8 $\pm$ 110.3
I/R+ 5b	160.7 $\pm$ 25.1	472.5 $\pm$ 102.5
I/R+ 11a	58.7 $\pm$ 12.5**	185.3 $\pm$ 89.2*
I/R+ 11b	62.0 $\pm$ 18.3**	202.4 $\pm$ 80.3*

N=8; *, p<0.05; **, p<0.01 compared with I/R + saline group.

Fig. 5. (A) Fluorescent staining images of skeletal muscle tissues taken from saline + I/R cohort (upper row) and from **11a**+ I/R rat cohort (lower row) (red: MitoProbe fluorescence; blue: Hoechst 33342 fluorescence) subjected to tourniquet-induced I/R; (B) interactive 3D surface plots of the merged images shown in (A).

To further explore the utility of our new curcumin analogues, we examined their ionization potentials. E_{HOMO} represents an energy measurement that is directly related to the ionization potential, and it characterizes the susceptibility of the molecule to attack by electrophiles. Conversely, E_{LUMO} gauges electron affinity of a compound and it estimates the susceptibility of the molecule toward attack by nucleophiles. E_{HOMO} and E_{LUMO} are not antioxidant estimates, but they can be connected 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 how it is related to the stability of radical forms of molecules. 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 compare the precursor **5a** and **11a**. Compound **11a** possessed the higher E_{HOMO} combined with the lower ΔE , and was a much more effective free radical scavenger than curcumin derivative **5a** (Fig. 6). The *in silico* results correlate well with our experimental evaluations.

Fig. 6. HOMO/LUMO plots for curcumin analogues **5a** and **11a**, and ΔE determines the chemical reactivity.

The degradation of curcumin in buffer is a free radical-driven incorporation of O_2 . The major product of the autooxidation of curcumin is a bicyclopentadione, which is formed by oxygenation and double cyclization of the heptadienedione chain. It has been suggested that the degradation of curcumin in buffer occurs through a mechanism similar to that of lipid peroxidation.³¹ As a first step of degradation, hydrogen is dissociated from the phenolic group to form a phenolic radical, which then transfers to the conjugated heptadienedione chain and leads to formation of bicyclopentadione derivatives of curcumin. Both the phenolic group and the conjugated heptadienedione chain play important roles in the degradation of curcumin. Curcumin analogues **11a,b** possess the higher E_{HOMO} with the lower ΔE and are much more effective free radical scavenger than the precursors **5a,b**. We therefore propose that the enhanced antioxidant activity of the curcumin analogues **11a,b** may increase their chemical stability and biological activity. As a result, these curcumin analogues can efficiently suppress the formation of the curcumin phenolic radical and mitigate degradation. Together, these results suggest the protective effects of curcumin analogues **11a,b** most likely associate with redox-dependent mechanism.

In conclusion, we have presented a new class of agents that can reverse the mitochondrial oxidative stress and associated apoptosis induced by I/R injury^{33,34}, employing both *in vitro* and *in vivo* models. Our results suggest that I/R injury induces mitochondrial remodeling, with a concomitant loss of selected

functions, and that selected curcumin analogue **11a,b** can facilitate recovery of mitochondrial reticular networks and cell rescue. Our studies lay the platform for a more comprehensive

characterization of the preclinical utility of these curcumin analogues in the treatment of I/R.

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Table 4. Effect of curcumin-PAK conjugates on the muscle antioxidant enzymes in a limb I/R animal model

	GSH ($\mu\text{mol/g}$)	SOD (U/mg protein)	CAT (U/mg protein)	MDA (nmol/g)
Sham	2.45 $\pm$ 0.21	1.12 $\pm$ 0.13	72.56 $\pm$ 4.89	21.35 $\pm$ 3.12
11a + Sham	2.32 $\pm$ 0.15	1.08 $\pm$ 0.17	70.15 $\pm$ 2.35	22.78 $\pm$ 4.21
11b + Sham	2.21 $\pm$ 0.43	1.00 $\pm$ 0.35	65.32 $\pm$ 4.59	23.12 $\pm$ 3.35
I/R + Saline	0.82 $\pm$ 0.19 ^b	0.41 $\pm$ 0.09 ^a	25.64 $\pm$ 5.29 ^a	45.78 $\pm$ 4.33 ^a
I/R+ 5a	1.41 $\pm$ 0.15	0.64 $\pm$ 0.06	44.65 $\pm$ 6.32	32.47 $\pm$ 3.19
I/R + 5b	1.55 $\pm$ 0.20	0.58 $\pm$ 0.08	42.44 $\pm$ 4.35	30.12 $\pm$ 2.48
I/R+ 11a	2.27 $\pm$ 0.24 ^c	1.07 $\pm$ 0.15 ^c	65.13 $\pm$ 5.39 ^d	20.31 $\pm$ 3.27 ^d
I/R+ 11b	1.75 $\pm$ 0.17 ^d	0.85 $\pm$ 0.12 ^c	51.91 $\pm$ 6.14 ^c	29.35 $\pm$ 4.10 ^c

N= 8; ^a: p<0.01; ^b: p<0.001 compared with the Sham-operated group;

^c: p<0.05; ^d: p<0.01; ^{****}: p<0.001 compared with the I/R + Saline group.