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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-inflammatory[8] and anticancer.[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 flow.[13] 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

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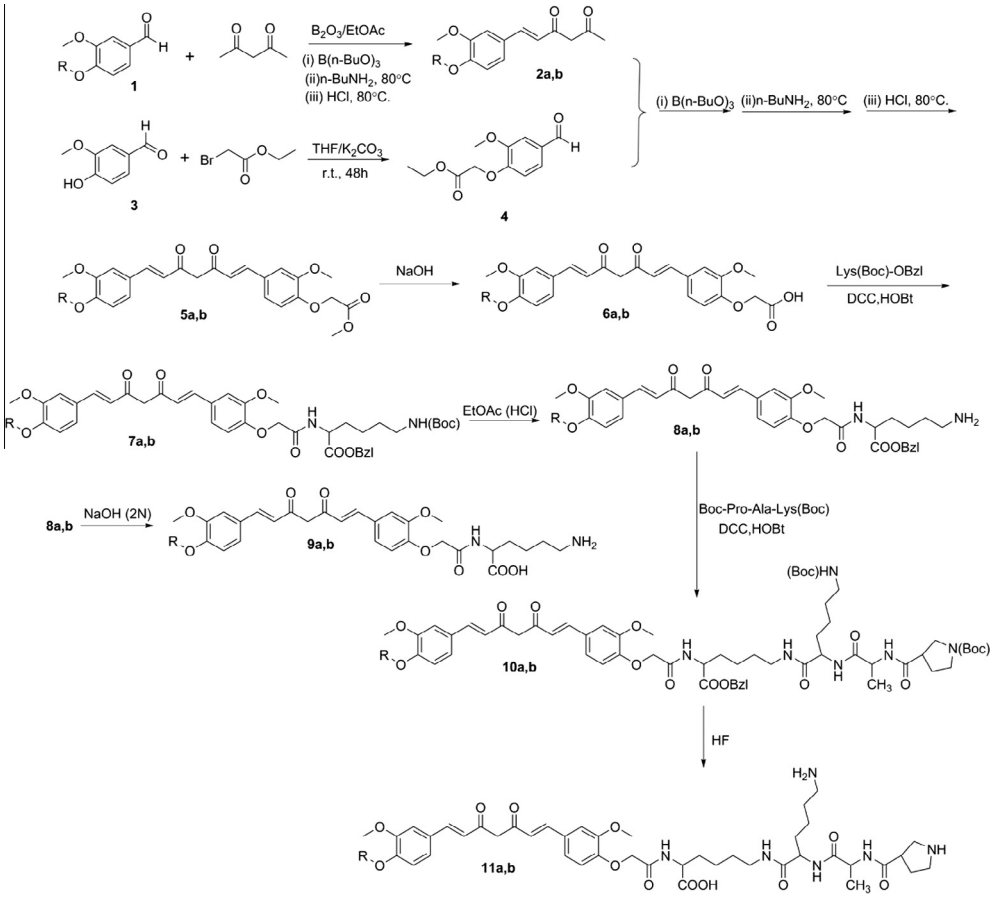
design, and more inexpensive preclinical evaluation. The current Letter summarizes the synthesis and biological evaluation of such hybrid drugs.

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 Figure 1.

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 h. 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 aggregation.[2] 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 therapy.[2] 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 ± 4.7 mg; vs NS: 42.1 ± 3.2 mg, p <0.01) were significantly higher than **5a**,**b** (**5a**: 39.7 ± 4.2; **5b**: 40.1 ± 3.7 mg; vs NS: 42.1 ± 3.2 mg) 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.

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



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 = CH_3.