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Synthesis and Characterization of Novel Indole Derivatives Reveal Improved Therapeutic Agents for Treatment of Ischemia/Reperfusion (I/R) Injury

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To develop more potent therapeutic agents with therapeutic efficacy for ischemia/reperfusion (I/R) injury, we linked an antiinflammatory moiety (1,3-dioxane derivative) to the key pharmacophoric moiety of melatonin. We hypothesized that the resulting new indole derivatives might induce a synergistic protection against oxidative damage associated with I/R injury. Our results indicate that one of these indole derivatives (**7**) manifests potent antiinflammatory antioxidant effects and exerts a protective effect against skeletal muscle injury and associated lung injury following limb I/R in rats.

Introduction

Acute ischemia of the lower limb is frequently encountered in many disease states and surgical procedures, including acute atherosclerotic thrombosis, abdominal aortic aneurysm repair, traumatic arterial injuries, and thromboembolic events in the lower extremities.[1] Although reperfusion is unavoidable, there is evidence that reperfusion induces additional cellular injury. In addition to muscle necrosis and edema, reperfusion of the ischemic limb may lead to systemic complications such as respiratory distress syndrome and multiple organ dysfunction.[2] In relation to this, remote lung injury is associated with a high degree of morbidity and mortality. Currently, there are no completely effective treatments for remote organ injury.

The primary literature indicates that reactive oxygen species (ROS[a]) and inflammatory leukocytes play an important role in the pathogenesis of limb I/R injury. Along these lines, antioxidant therapy and/or inhibition of postischemic neutrophil infiltration may have therapeutic efficacy in various organs during I/R injury.[3] Melatonin represents an endogenous antioxidant that can directly scavenge a variety of reactive oxygen and nitrogen species, and it has been shown to have significant protective effects in various experimental models of reperfusion injury.[4] Conversely, 1,3-dioxane derivatives have been reported to possess antiinflammatory and anticancer properties and protective effects against reperfusion injury via antiproliferative and antiinflammatory activities in human neutrophils and tumor cells.[5] Recently, we have characterized a new class of 2,5-disubstituted 1,3-dioxanes, some of which possess antiinflammatory properties superior to those of aspirin.[6] In contrast to the classical, acidic NSAIDs (nonsteroidal antiinflammatory drugs), the new 1,3-dioxane analogues are basic. To develop more effective therapeutic agents to treat I/R injury, we have linked an antiinflammatory moiety (1,3-dioxane derivative) to the key pharmacophoric moiety of melatonin with the objective of developing a new indole derivative that would provide a synergistic protection against downstream oxidative damage associated with I/R injury. To examine the structure−activity relationships, we have synthesized several related indole derivatives with particular focus on the methoxy groups, the indole heterocycle, and the spatial disposition.

Chemistry

Indole derivatives **3**−**10** can be readily prepared via a Pictet−Spengler condensation. Starting from the mixed bisacetal **1** at 50 °C using oxalic acid and silica gel as the catalyst, the mixed bisacetal is converted into the diastereomeric aldehyde **2** in good yield (80%). On treatment of **2** with 5-methoxytryptamine in a Pictet−Spengler condensation, (1*R*)-1-substituted indole derivative **3** was obtained stereospecifically in good yield. With tryptamine replacing 5-methoxytryptamine in the Pictet−Spengler condensation, the cyclization product **4** was obtained in high yield. However, on treatment of **2** with L-tryptophan methyl ester in a Pictect−Spengler condensation, two diasteresomers **5** and **6** were obtained in yields of 55% and 30%, respectively. Subsequently, some difficulties were encountered during the deprotection of the *N*-phenylacetyl group of **3**−**6**. We observed racemization associated with the strong basic deprotection conditions. To avoid this pitfall, we attempted to remove the *N*-phenylacetyl protecting group of **3**−**6**, employing enzymatic hydrolysis. Since penicillin acylase from *Escherichia coli* can recognize a broad range of substrates, we examined this enzyme and found that it was very useful in the removal of *N*-phenylacetyl protecting groups of the dioxacycloalkanes. Indeed, the mild and neutral enzymatic approach provided a reliable avenue toward developing our desired compounds. Thus, upon treatment of an aqueous solution of **3**−**6** with the penicillin acylase at pH 6.0 and 37 °C, the *N*-phenylacetyl protecting group was removed almost quantitatively and produced the expected **7**−**10** without undesired side reactions.

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[a] Abbreviations: I/R, ischemia/reperfusion; ROS, reactive oxygen species; HOMO, highest occupied molecular orbital; TNF-α, tumor necrosis factor-α; MDA, malondialdehyde; MPO, myeloperoxidase.

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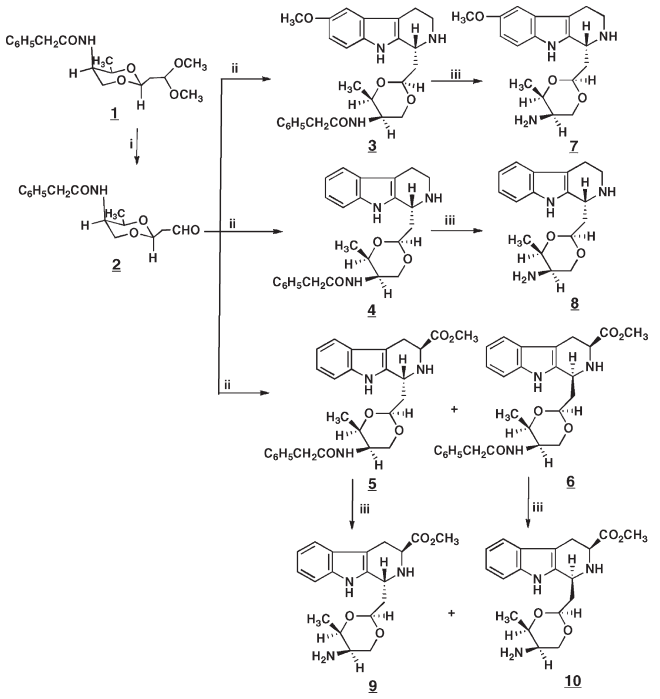
Scheme 1. Synthesis of Indole Derivatives **3**−**10**[a]



[a] Reagents and conditions: (i) oxalic acid, silica gel; (ii) 5-methoxytryptamine, tryptamine, L-tryptophane methyl ester; (iii) penicillin acylase, room temp, pH 6.0.

Moreover, HPLC analysis indicated that no racemization occurred during the enzymatic hydrolysis (Scheme 1).

Results and Discussion

With the indole derivatives in hand, we systematically undertook an investigation of the antiinflammatory activity of these compounds, beginning first with a xylene-induced ear edema model assay. All of the newly synthesized compounds manifested inhibition against xylene-induced ear inflammation in mice when compared with vehicle (Table 1, See Supporting Information), suggesting potent antiinflammatory activities for derivatives **3**−**10**. The most potent compounds, **7** and **9**, exhibited equivalent or even higher antiinflammatory activity than the standard reference drug indomethacin. Subsequently, **3**, **5**, **7**, **9** with substantial antiinflammatory activities were administrated in a series of lower concentrations to develop a detailed pharmacological activity profile. Oral administration of **3**, **5**, **7**, and **9** at doses of 5.0, 10.0, and 20.0 mg/kg yielded a dose-dependent antiinflammatory response in the xylene-induced mouse ear edema test (Table 2, see Supporting Information). We observed a significant enhancement in the antiinflammatory capacity of **7** and **9** with doses above 5.0 mg/kg.

Compared to the parent compounds **1** and **2**, the antiinflammatory activities of **3**−**10** were significantly enhanced. It is known that indolic compounds are efficient free radical scavengers and antioxidants.[7] At sites of inflammation, increased free radical activity is associated with activation of neutrophil NADPH oxidase and/or the uncoupling of a variety of redox systems.[8] Such conditions generally result in impairment of the antioxidant defense system. Thus, improving intracellular responses via enhanced antioxidant capabilities may bolster the maintenance of balance between ROS and antioxidant defense systems. Accordingly, we hypothesize that the improved antiinflammatory activities of the newly synthesized **3**−**10** may be linked to the antioxidant-enhancing contributions of the indole moieties. On the other hand, the indole structures influence the antioxidant efficiency and properties of biological systems at quite different extents and levels. For example, **7** displayed the most potent antiinflammatory activity (81.5% inhibition). We hypothesize that the indole nucleus of **7** functions to maintain the two key pharmacophoric moieties (5-methoxy group of melatonin and 1,3-dioxacycloalkane ring) in an optimal configuration, allowing them to adopt the required orientation for interactions within the receptor-binding pocket. Further, compared to the *N*-phenylacetyl substituted precursors **3**−**6**, we propose that the uninhibited status of the 5-amino moiety of the 1,3-dioxacycloalkanes of **7**−**10** results in a significant improvement of the antiinflammatory activities and further suggests that the amino group may be pivotal for maximal antiinflammatory capacity. As is well-known in all pharmaceutical processes, a minor structural alteration can lead to a marked influence of the antiinflammatory activity, which raises the possibility that the improved biological profiles observed for **7**−**10** might result from a synergistic effect. Nonetheless, the formation of a hydrogen bond with the 5′-amino moiety may be critically important during the receptor binding process. Thus, the potent antiinflammatory capacities of **7**−**10**, at least partially, may result from a facile protonation of the amine moieties, which in turn facilitates better access of the molecule to the cellular membrane. Of interest, although sharing common chemical structures, the antiinflammatory activity of (1*S*,3*S*)-**10** was significantly less (45.1% inhibition) than that of (1*R*,3*S*)-**9** (74.3% inhibition). A similar trend was observed in (1*R*,3*S*)-**5** (60.0% inhibition) and (1*S*,3*S*)-**6** (40.6% inhibition). These latter observations imply that the spatial disposition of the 1,3-dioxane ring also plays an important role in the biological activity of these indole derivatives.

Inflammation is a complex phenomenon involving multiple cellular and molecular interactions in a variety of mechanistic interactions. To further confirm their antiinflammatory activities, **3**−**10** were evaluated in the carrageenan-induced paw inflammation rat model. Inflammation induced by carrageenan is acute, nonimmune, and highly reproducible, and this model system represents another useful phlogistic test system in which to investigate the systemic nature of antiinflammatory agents.[8,9] After subcutaneous injection of carrageenan, edema, hyperalgesia, and erythema immediately develop in the test subjects. These signs of inflammation result from the concerted actions of several proinflammatory agents (including bradykinin, histamine, tachykinins, complement and reactive oxygen and nitrogen species). In response, neutrophils migrate to sites of inflammation and generate proinflammatory ROS.[8,9] The inflammatory response is readily quantified by an increase in paw size (edema). As shown in Table 3 (see Supporting Information), all of the newly synthesized indole derivatives effectively inhibited paw edema at 3 h following carrageenan administration. Of note, **7** and **9** exhibited antiinflammatory activity comparable to that of indomethacin. Accordingly, on the basis of results in the murine ear edema model and the paw edema carrageenan rat model, **7** and **9** exerted significant antiinflammatory activity during the acute phase of inflammation.

The antiinflammatory effect of the newly synthesized indole derivatives in vivo most likely occurs via the inhibition of certain key enzymes involved in inflammation and/or cell signaling pathways. 1,3-Dioxane derivatives have been reported recently to possess PKC (protein kinase C) inhibitory activity and exert antiinflammatory, anticancer, and reperfusion injury protective effects via their antiproliferative and

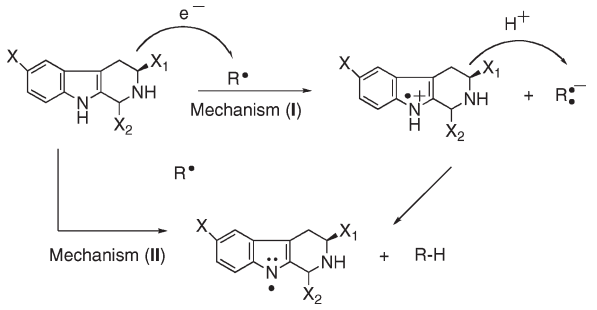


Figure 1. Possible mechanisms of action of indole derivatives.

antiinflammatory activities in human neutrophils and tumor cells.[5] Accordingly, we characterized the mechanism of actions of our new indole derivativesin a PKC inhibition assay. However, preliminary findings indicated that most of these newly synthesized compounds displayed weak PKC inhibitory activity (data not shown), suggesting that PKC might not be the target protein (at least in vitro). Further experiments are in progress to ascertain the protein target(s) of these indole derivatives, with a particular focus on cyclooxygenase and lipoxygenase and on phosphoinositide 3-kinase systems. Mechanistic insights for these new indole analogues may provide beneficial information for the future development of anti-ischemia agents.

During I/R injury, various ROS are produced and induce cellular and tissue damage. Hydrogen peroxide (H_2O_2) induces DNA strand breaks and base damage via a mechanism requiring transition metal ions. Metals (i.e., copper ion) present in biological systems can react with H_2O_2 via Fenton reactions to produce hydroxyl radicals, which result in DNA strand breakage. The hydroxyl radical can react rapidly with almost any cellular biomolecule.[8,9] Accordingly, we felt it was critically important to evaluate the free radical scavenging activity of the indole derivatives that manifested maximal antiinflammatory activity (**3**, **5**, **7**, **9**) against ROS, especially hydroxyl radicals.

Rat pheochromocytoma (PC12) cells originate from the adrenal medulla and synthesize and release catecholamines. These cells are very sensitive to oxidative stress. The PC12 cell model system has been well established for in vitro ischemia studies.[10] Cell survival, as determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction, was markedly decreased after PC12 cells were exposed to free radicals. We found that preincubation of PC12 cells with the indole derivatives **7**/**9** or the incubation of cells with these compounds following exposure to free radicals resulted in improved viability of PC12 cells which was diminished by free radical exposure, suggesting that derivatives **7**/**9** were efficient scavengers of active radical species. Preincubating cells with **7**/**9** induced a stronger effect, and this probably was due to a combination of antioxidant activity and membrane permeability. From Table 4 (see Supporting Information), we observed that **7** exhibited better radical scavenging activity than **9**. In particular, the scavenging effect of **7** is more pronounced for $^{\bullet}OH$ and H_2O_2, suggesting that **7** is acting as a scavenger of hydroxyl radicals generated through the Fenton reaction. With regard to the effectiveness of **7**/**9** against oxidative cellular death, we speculate that the antioxidant activities of these indole derivatives are directly correlated with the presence of the indole nucleus. The indole moiety of **7**/**9** most likely is the reactive center of interaction with oxidants because of its high resonance stability and very low activation energy barrier toward free radical reactions. The antioxidative capacities of **7**/**9** may well be mediated via scavenging of reactive oxygen radicals and forming a stable indole radical at the pyrrole ring. Interestingly, compared with indole derivatives **7**/**9**, although sharing the common indole moiety and having similar chemical structures, the respective precursors of **7**/**9**, **3**/**5**, displayed much weaker free radical scavenging capacities. We propose that the bioavailability of the respective compounds or the actual intracellular concentration might influence the extent of the free radical scavenging effect in vitro. We speculate that indole derivatives **7**/**9** possess enhanced hydrophilic and lipophilic characteristics compared to precursors **3**/**5**. It remains probable that indole derivatives **7**/**9** are more effective at penetrating the biological membrane, and this may explain their enhanced free radical scavenging capacity in vitro in comparison to their respective precursors.

In relation to antioxidant and antiinflammatory activities, **7** with the methoxy group appears more active than **9** which lacks this group. We hypothesized that the electronic properties of the indole ring could be manipulated by the presence of the 5-methoxy group in such a way to make **7** more likely to react with radical species. Along these lines, we further hypothesized that this radical scavenging effect might be ascribed to the electron releasing properties of the ethereal oxygen, making the doublet in the nitrogen atom more accessible for a possible electron transfer. Alternatively, the introduction of a 5-methoxy group might result in higher antioxidant activity by conferring higher stability to the aroxyl radical via electron delocalization.

In relation to the above, we have proposed two possible radical scavenging mechanisms for indole derivatives in Figure 1. Mechanism I involves electron transfer from the antioxidant to the active radical, which produces a cation radical and an anion. The electron transfer is followed by proton transfer from the cation radical to the anion. A second potential mechanism II involves direct transfer of hydrogen between the antioxidant and the active radical.

The antioxidant activity and/or radical scavenging effect of antioxidants is correlated with selected molecular and biochemical parameters, such as the highest occupied molecular orbital (HOMO) energy, the net charge, and the difference in heat of formation between antioxidant and its radical, the latter reflecting the O−H bond dissociation energy. Along these lines, the ionization energy of the HOMO can be employed as a measure of a compound's capacity to participate in oxidant scavenging.[11] Our preliminary findings suggest that the linking of 1,3-dioxane to the key pharmacophoric moiety of melatonin increases the antioxidant potential. For example, the HOMO energy of melatonin is −10.425 eV. In comparison, the HOMO energy of compound **7** is −9.463 eV. As a general rule, the higher the HOMO energy, the more active the compound is as an antioxidant.[11] Theoretically, **7** should possess higher radical trapping potential than melatonin. In addition, on the basis of the lowest energy conformations, the charge distributions, and the high positive charge of the hydrogen atoms bound to the nitrogen atoms, we predict that the indole cation radical of **7** possesses significant N−H acidity and therefore can easily release protons, which may facilitate free radical scavenging (Figure 2).

Limb I/R injury drives the generation and transport of numerous proinflammatory molecules (O_2^{-}, NO, H_2O_2, cytokines, and arachidonic acid metabolites) into the systemic circulation, which further promotes local and systemic inflammation.[1,2] Beyond the regional injury, limb I/R injury may also yield a systemic inflammatory response resulting in

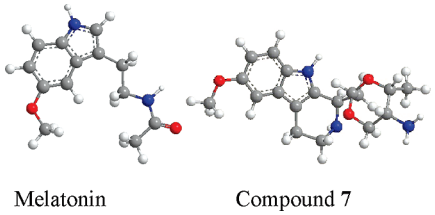


Figure 2. Predicted lowest energy conformations of melatonin and **7**.

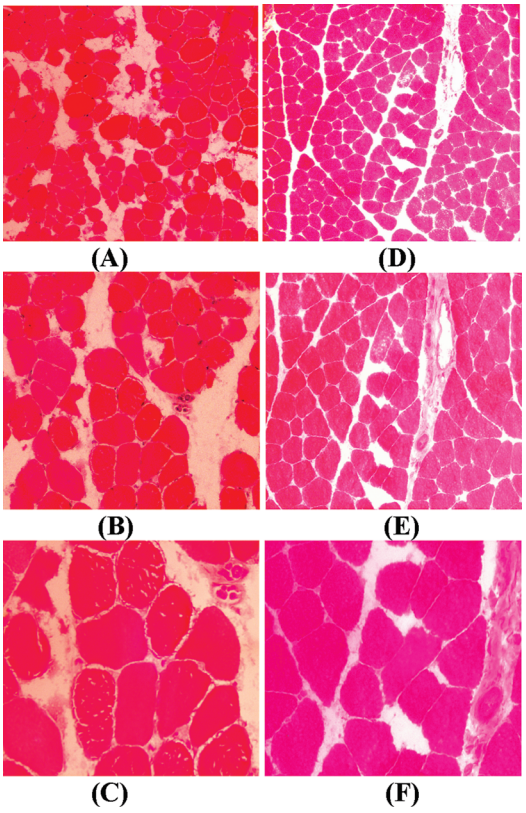


Figure 3. Hematoxylin-eosin-stained frozen cross-section of skeletal muscle. (A−C) Representative muscle tissue sections were taken from the rats exposed to limb I/R. (D−F) Representative muscle tissue sections were taken from I/R + **7** (20 mg/kg) treated rats. Tissue sections were stained with H&E (hematoxylin and eosin). Magnification: (A, D) ×100; (B, E) ×200; (C, F) ×400.

multiple organ failure. The lung appears to be a primary organ susceptible to systemic inflammatory responses, likely related to the high respiratory quotient. Remote lung injury, induced by hind limb I/R injury, is characterized by accumulation of inflammatory infiltrates, increased microvascular permeability, alveolar capillary endothelial cell injury, and pulmonary edema. Accordingly, we addressed the effects of regional and systemic effects of I/R injury by examining if **7** could exert protection against local and remote organ injury induced by hind limb I/R injury in the rats. To perform these studies, we employed a rubber band tourniquet model in the rat as the model of hind limb I/R injury. In this rodent model, the collateral blood flow around the femoral and iliac arteries can be effectively occluded to ensure complete limb ischemia. After 3 h of bilateral lower limb ischemia, tourniquets are released and reperfusion is performed for 4 h. This model system is associated with local and systemic proinflammatory responses. We found that after 3 h of ischemia followed by 4 h of reperfusion, muscle edema, neutrophil accumulation, and muscle cell necrosis were apparent by histological analysis in

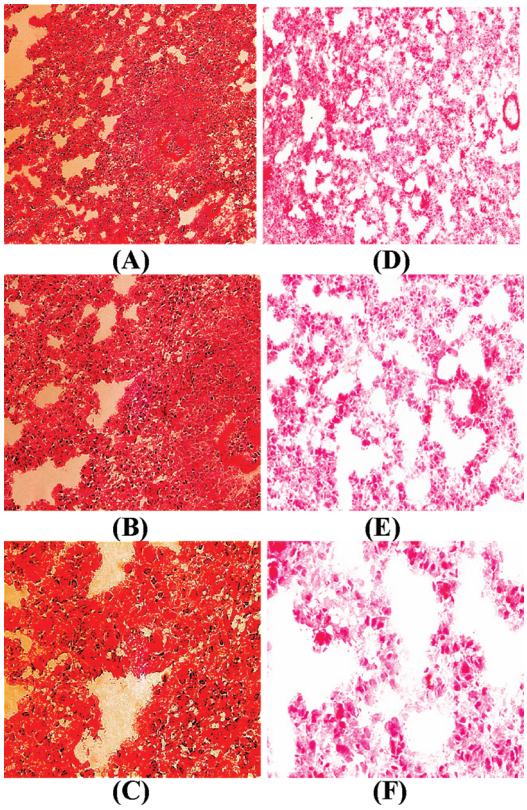


Figure 4. Hematoxylin-eosin-stained frozen cross-section of lung tissue. (A−C) Representative lung tissue sections were taken from the rats exposed to limb I/R injury. (D−F) Representative lung tissue sections were taken from I/R injury + **7** (20 mg/kg) treated rats. Tissue sections were stained with H&E (hematoxylin and eosin). Magnification: (A, D) ×100; (B, E) ×200; (C, F) ×400.

the I/R group rats (Figure 3A−C). As well, there was also significant remote organ injury in the lung, characterized by increased permeability, edema, and neutrophil sequestration (Figure 4A−C). Since free radical formation and inflammation play an important role in I/R injury, we investigated the putative beneficial effects of **7** in limb I/R injury by monitoring the change in serum tumor necrosis factor-α (TNF-α), malondialdehyde (MDA), and myeloperoxidase (MPO) in tissues.[12,13] Hind limb I/R promotes the inflammatory response by stimulating the production of inflammatory cytokines and chemokines.[1,2] TNF-α is an important mediator of local and remote organ injury following hind limb I/R. We found that the TNF-α levels were dramatically increased in the I/R injury group at the end of reperfusion compared to that of the sham group (Table 5, see Supporting Information). This indicated that the inflammatory damage induced by hind limb I/R injury appeared to be systemic. In the treatment group administered **7**, there was a significant fall in the serum levels of proinflammatory cytokines at the end of reperfusion period compared to the I/R injury (e.g., no treatment) group, suggesting that TNF-α increased in the systemic circulation following I/R injury, which could be effectively inhibited by intervention with **7**.

With regard to biomarkers of oxidative damage, MDA (malondialdehyde) represents an important intermediate of lipid peroxidation and therefore is generally taken as a representative surrogate for lipid peroxidation. We found that the MDA levels of plasma and lung were significantly increased in the I/R injury group compared to the sham group (Table 5, see Supporting Information). Conversely, MDA levels in plasma and lung were significantly lower than that of the I/R injury + **7**

group. Elevated MDA levels in plasma and lung suggested decreased levels of endogenous antioxidants and indicated that **7** significantly attenuated downstream lipid peroxidation and cellular injury. This beneficial effect might be partly related to the antioxidant and scavenging capacities of **7** for reactive $^{\bullet}OH$ and H_2O_2 species.

During hind limb ischemia/reperfusion, leukocytes may reenter the systemic circulation. These activated neutrophils may cause local and remote organ damage, especially in the lungs. After adhering to the pulmonary microvascular endothelium, neutrophils exert their toxic effects through the release of proteolytic enzymes and free radicals.[2] We found that lung MPO activity in the I/R injury group was significantly increased (Table 5, see Supporting Information). Treatment with **7** significantly attenuated this effect. The decrease in lung MPO activity and attenuation of lung histopathology (Figure 4D−F) indicated that **7** could protect the remote organ (lung) against limb I/R injury. This protective effect of **7** most likely resulted from an inhibition of neutrophil infiltration and lipid peroxidation. This proposed inhibition is also associated with tissue edema as evaluated by the *W*/*D* ratio (wet to dry tissue weight ratio) (Table 6, see Supporting Information), which revealed that **7** was effective at attenuating the lung damage associated with limb I/R injury.

Skeletal muscle ischemia is associated with an increase in vascular permeability, which is characterized by swollen and edematous muscle after acute limb injury. Our histological results (Figure 3A−C) revealed a significant separation between muscle fiber bundles, suggesting the presence of interstitial edema and increased microvascular permeability. Microvascular permeability is an indicator of skeletal muscle injury following hind limb I/R. As for other parameters measured, this muscle edema was significantly attenuated with **7** treatment (Figure 3D−F). As well, signs of pulmonary edema consisting of interstitial thickening and a high degree of neutrophil infiltration in the lung capillary vessels and interstitium (Figure 4A−C) was vastly improved following intervention with **7** (Figure 4D−F). The histological results further confirmed that **7** could reduce the tissue edema and protect local and remote organ against hind limb I/R injury. We speculate that the efficacy in protection for indole derivative **7** against local and remote organ injury results from the combined effects of inhibiting the release of free radicals and proinflammatory cytokines.

Conclusion

We have designed and synthesized several related indole derivatives with particular focus on the methoxy group, the indole heterocycle, and the spatial composition. The antiinflammatory activities of all newly synthesized indole derivatives **3**−**10** were examined in the xylene-induced ear edema murine model and the carrageenan-induced paw inflammation model in the rat. The potent radical scavenging activity of **7** and **9** was examined in rat pheochromocytoma (PC12) cell survival assays, this cell line being exquisitely sensitive to free radical damage. Considering its superior antiinflammatory and antioxidant capacity, **7** was further evaluated in a hind limb I/R injury animal model, which revealed its significant capacity to reduce lipid peroxidation and local (muscle) and remote organ (lung) injury induced by hind limb I/R injury in rats. These findings suggest that indole derivative **7** may become an important agent to attenuate the severity of I/R injury for future investigations, although the precise cellular mechanism of action for this compound remains to be determined.

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Supporting Information Available: Details of the synthetic procedures and characterization for **1**−**10** and details of biological tests. This material is available free of charge via the Internet at http://pubs.acs.org.

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