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Synthesis and Anti-inflammatory Evaluation of Novel Benzimidazole and Imidazopyridine Derivatives

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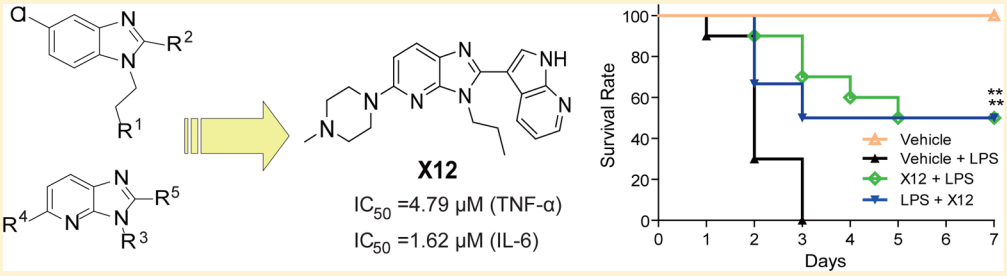
Supporting Information



ABSTRACT: Sepsis, an acute inflammatory disease, remains the most common cause of death in intensive care units. A series of benzimidazole and imidazopyridine derivatives were synthesized and screened for anti-inflammatory activities, and the imidazopyridine series showed excellent inhibition of the expression of inflammatory cytokines in LPS-stimulated macrophages. Compounds **X10**, **X12**, **X13**, **X14**, and **X15** inhibited TNF-α and IL-6 release in a dose-dependent manner, and **X12** showed no cytotoxicity in hepatic cells. Furthermore, **X12** exhibited a significant protection against LPS-induced septic death in mouse models. Together, these data present a series of new imidazopyridines with potential therapeutic effects in acute inflammatory diseases.

KEYWORDS: *benzimidazoles, imidazopyridines, anti-inflammation, macrophages, sepsis*

Sepsis, an acute inflammatory disease, is currently the 10th leading cause of death overall and accounts for major healthcare expenditures in the developing world.[1] It is widely believed that the incidence of sepsis and sepsis-related mortality will continue to rise. Research efforts in the field of sepsis have largely focused on the innate immune system and have conceptually viewed sepsis as a syndrome of hyperinflammation.[2,3] Under this paradigm, overzealous activation of the host inflammatory response, ostensibly intended for pathogen eradication, becomes dysregulated and consequently causes autoinjury to the host, leading to multiple organ dysfunction and death.[4] In this inflammatory storm, cytokines such as tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), and IL-1β play important roles.[5,6]

The pro-inflammatory cytokines, including TNF-α and IL-6, whose expression can be induced by endotoxin (lipopolysaccharide, LPS), share many cellular and molecular features in common and are deeply involved in sepsis and other inflammation-mediated diseases, such as rheumatoid arthritis, diabetic complications, cancer, atherosclerosis, and inflammatory bowel disease, by amplifying inflammatory signals in multiple pathways.[7,8] Therefore, therapeutically relevant candidates that target pro-inflammatory cytokines or inhibiting their expressions have attracted much attention in drug development for inflammatory disease. For example, improved survival following neutralization of these pro-inflammatory mediators by antibodies or specific inhibitors has been recently reported in animal models and clinical studies of sepsis.[9,10] Small molecule inhibitors of cytokine expression are emerging with a few having advanced into clinical trials.[11] Our group has previously designed and evaluated a lot of synthetic compounds as anti-inflammatory agents, some of which are undergoing preclinical assessment as antisepsis candidates.[12,13]

Recently, a series of benzimidazole derivatives were evaluated for anti-inflammatory activity in carrageenan-induced rat paw edema.[14] In addition, studies also showed that imidazopyridines possessed anti-inflammatory activity.[15,16] More recently, Ashwell et al.[17] reported the discovery and optimization of a series of imidazopyridines as selective and potent inhibitors of protein kinase B (PKB or AKT), which functions as a key signaling node in cell proliferation, survival, and inflammatory

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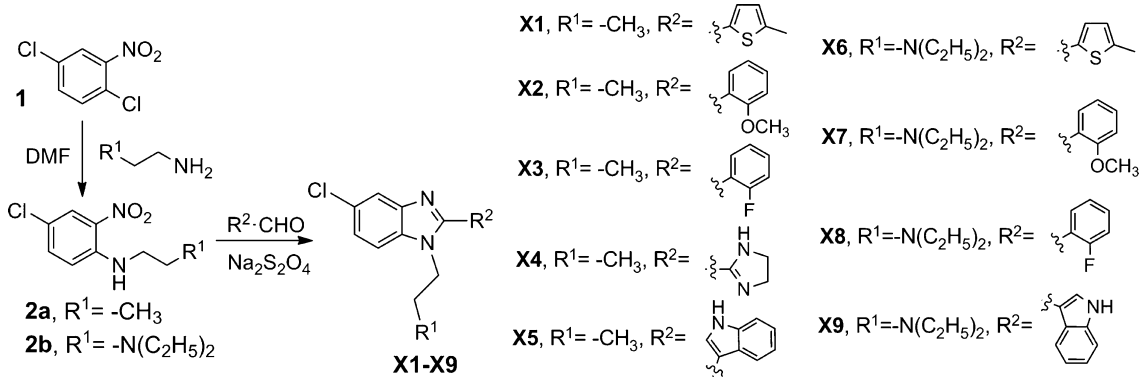
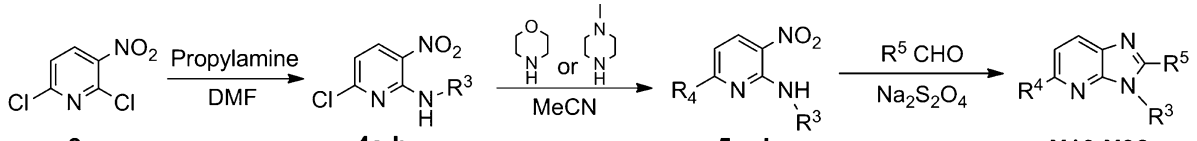
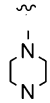
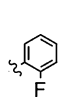
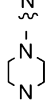
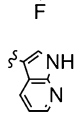
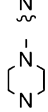
Scheme 1. General Synthesis and Chemical Structures of Benzimidazole Derivatives (X1−X9)



Scheme 2. General Synthesis and Chemical Structures of Imidazopyridine Derivatives (X10−X32)



Comp.	R^3	R^4	R^5	Comp.	R^3	R^4	R^5	Comp.	R^3	R^4	R^5
X10	*n*-pr			X18	*n*-pr			X26	H		
X11	*n*-pr			X19	*n*-pr			X27	H		
X12	*n*-pr			X20	*n*-pr			X28	H		
X13	*n*-pr			X21	*n*-pr			X29	H		
X14	*n*-pr			X22	*n*-pr			X30	H		
X15	*n*-pr			X23	H			X31	H		
X16	*n*-pr			X24	H			X32	H		
X17	*n*-pr			X25	H						

stress response. Thus, previous evidence prompted us to study the cytokine-inhibitory and antisepsis activities of new benzimidazole and imidazopyridine derivatives.

As shown in Schemes 1 and 2, two novel series of benzimidazoles (**X1−X9**) and imidazopyridines (**X10−X32**) have been synthesized. Briefly, substituted *ortho*-nitroanilines (**2a** and **2b**) were prepared by direct displacement of 1,4-dichloro-2-nitrobenzene (**1**) by amines at the reflux in dimethylformamide (DMF). Subsequently, a cyclization of *ortho*-nitroanilines by various aldehydes in sodium hyposulfite medium at reflux provided target benzimidazoles (**X1−X9**) with the yields ranging 30−70% (Scheme 1). In parallel, the synthesis of imidazopyridines derivatives (**X10−X32**) proceeded in the similar route (Scheme 2). Generally, 2-amino-6-chloro-3-nitropyridine (**4a**) and 2-amino-6-chloro-3-nitro-*N*-propylpyridin (**4b**), which were derived from nucleophilic substitution reactions of 2,6-dichloro-3-nitropyridine (**3**), were treated with morpholine or 1-methylpiperazine to afford 6-substituted derivatives (**5a−d**). Finally, the intermediates **5a−d** were cyclized with the various aldehydes in sodium hyposulfite medium to afford the target imidazopyridines (**X10−X32**). All of the new compounds were characterized by ^{1}H NMR and ESI-MS, and details on their synthetic and structural information are located in the Supporting Information. Before

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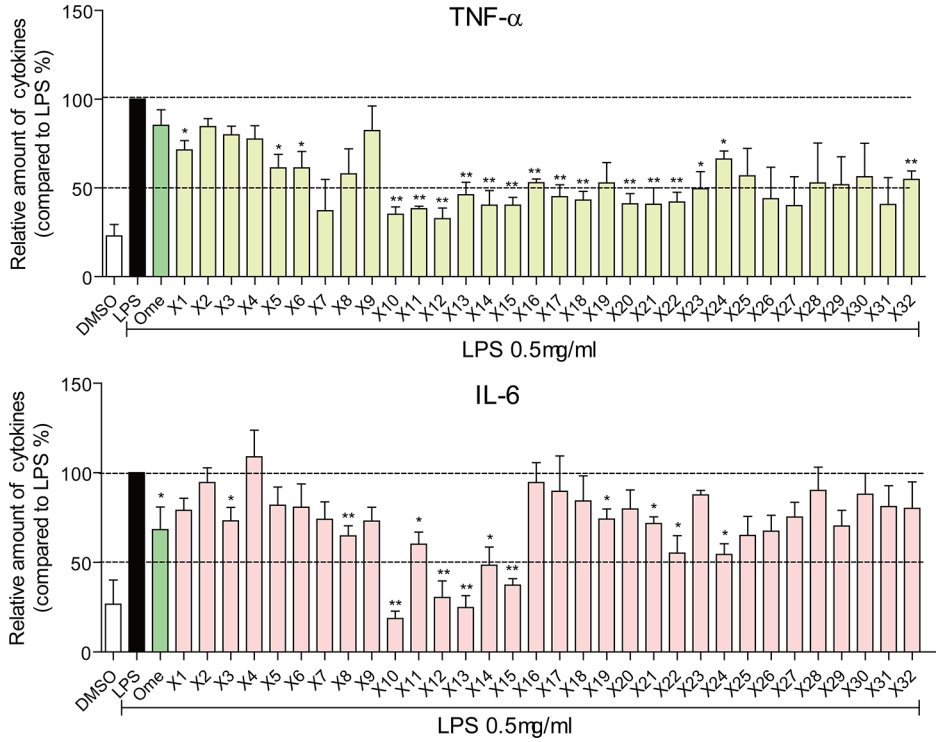

Figure 1. Benzimidazole and imidazopyridine derivatives inhibited LPS-induced TNF-α and IL-6 secretion in RAW 264.7 macrophages. Macrophages were plated at a density of 4.0×10^5/plate for overnight in 37 °C and 5% CO_2. Cells were pretreated with Ome or compounds (10 μM) for 2 h and then treated with LPS (0.5 μg/mL) for 22 h. TNF-α and IL-6 levels in the culture media were measured by ELISA and were normalized by the total protein. The results were expressed as the percentage of LPS control. Statistical significance relative to LPS group is indicated: *, $p < 0.05$; and **, $p < 0.01$.

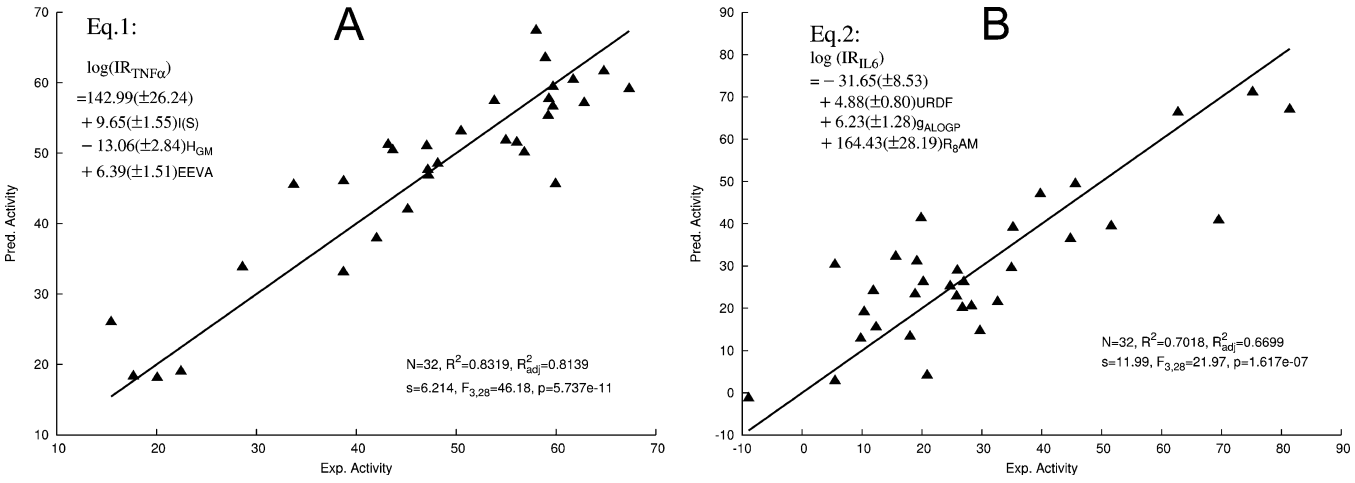

Figure 2. Plots of predicted activity against the corresponding experimental activity on TNF-α (A) and IL-6 (B) inhibition. *N*, the number of compounds taken into account in the regression; R^2, the multiple correlation coefficient; R_{adj}^2, adjusted multiple correlation coefficient; *s*, residual standard error; and the *F* value is related to the *F*-statistic analysis (Fischer test). The numbers in parentheses mean the standard deviation of the coefficients.

they were used in biological experiments, compounds were purified by silica gel column chromatography, and HPLC was used to determine their purity (≥95.0%).

Although inflammatory cytokines can be produced by various cells, macrophages are the prototypical cell source.[18] The 32 synthetic derivatives were evaluated for their ability to decrease the expression of TNF-α and IL-6 in mouse RAW 264.7 macrophages stimulated by LPS. Macrophages were pretreated with 10 μM compounds for 2 h and then incubated with 0.5 μg/mL LPS for 22 h. The amount of TNF-α and IL-6 in media was detected by enzyme-linked immunosorbent assays (ELISA) and normalized by protein concentration of cells harvested in homologous culture plates.

Figure 1 gives the cytokine-inhibitory activity of 32 derivatives with the benzimidazole-containing omeprazole (Ome) as an anti-inflammatory comparison.[19,20] The majority of these compounds exhibited higher inhibition than omeprazole against LPS-induced TNF-α and IL-6 expression. Compounds **X7**, **X10**−**X15**, **X17**, **X18**, **X20**−**X22**, **X26**, **X27**, and **X31** showed inhibition of TNF-α production in a range of

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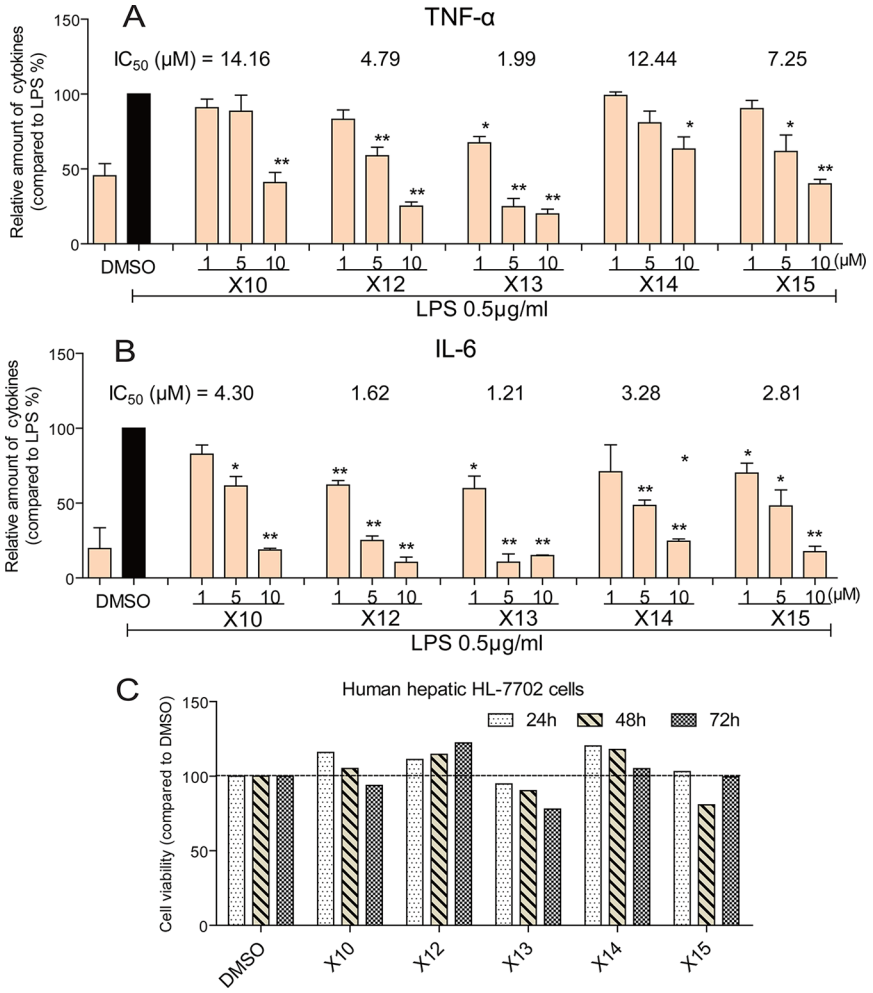

Figure 3. Cytokine-inhibitory activity and cytotoxicity of five active compounds. (A and B) Macrophages were plated at a density of 1.2×10^6/plate overnight at 37 °C and 5% CO_2. Cells were pretreated with specific compounds at indicated concentrations for 2 h, followed by LPS (0.5 μg/mL) treatment for 22 h. TNF-α (A) and IL-6 (B) levels in the culture medium were measured by ELISA and were normalized to the total protein amount. Statistical significance relative to the LPS group is indicated: *, $p < 0.05$; and **, $p < 0.01$. (C) Five imidazopyridines at 20 μM were tested for their cytotoxicity in human normal liver HL-7702 Cells. The cell viability was determined by MTT method after a treatment period of 24, 48, and 72 h.

50−65%. With regard to IL-6, compounds **X10** and **X12**−**X15** exhibited more than 50% inhibitory effects against IL-6 expression as compared to LPS control. Among the tested compounds, **X10** is the strongest inhibitor on LPS-induced TNF-α and IL-6 release with the inhibitory rates of 64.8 and 81.4%, respectively, as compared to the LPS control. According the bioscreening, it is observed that imidazopyridine derivatives showed stronger anti-inflammatory activity than benzimidazoles. Within the imidazopyridine series, compounds with *n*-propyl group on the R_3 and 1-methylpiperazine on the R_4 were the most active (**X10**−**X15**). Also, it should be noted that various heterocycles at the R_5 of the imidazopyridine have an important impact on activity.

To further demonstrate the SAR of these compounds and to evaluate the effects of various substituents on the activity, a quantitative SAR (QSAR) was calculated. The concrete description for QSAR study is shown in the Supporting Information, and the descriptors studied here are listed in Table S1 in the Supporting Information. The scatter plot of predicted versus experimental values is illustrated in Figure 2. Using three different variables, the statistically significant models, eq 1 and eq 2, were obtained for anti-TNF-α and anti-IL-6 activities of compounds, with relatively high regression coefficients (R^2) of 0.83 and 0.70, respectively. As discussed in the Supporting Information, the QSAR results indicate that the electronegativity and molecular polarizability may play an important role in the anti-inflammatory activity of benzimidazole and imidazopyridine derivatives.

Among tested compounds, the most promising compounds **X10**, **X12**, **X13**, **X14**, and **X15** were selected for further assessment of their dose-dependent inhibitory effects against LPS-induced TNF-α and IL-6 release, and their IC_{50} was determined. RAW264.7 macrophages were pretreated with compounds at a series of concentrations (1.0, 5.0, and 10 μM) for 2 h and were subsequently incubated with LPS (0.5 μg/mL) for 22 h. The release of TNF-α and IL-6 was determined by ELISA. The results are shown in Figure 3A,B, indicating a dose-dependent inhibition of LPS-induced TNF-α and IL-6 release by these derivatives. Accordingly, their IC_{50} values were determined, and all of them were under 10 μM except that of **X10** and **X14** against TNF-α release. **X13** exhibited the lowest IC_{50} values against the expression of both TNF-α (1.99 μM) and IL-6 (1.21 μM), respectively. The fact that the inhibition of TNF-α and IL-6 release by these compounds in a

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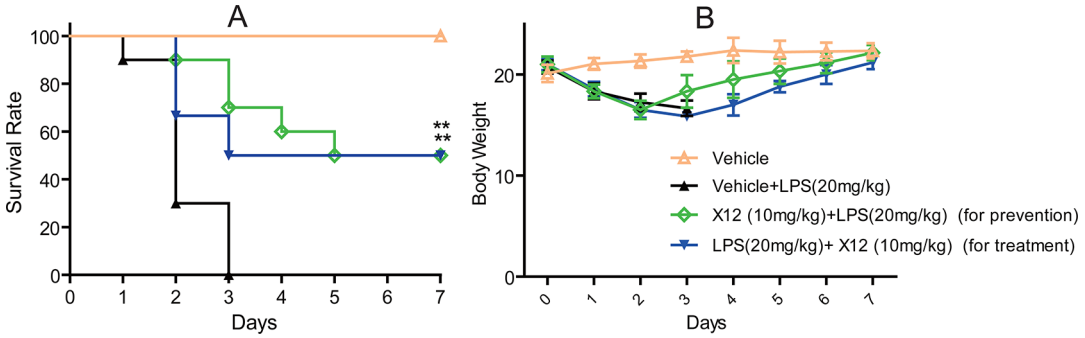

Figure 4. X12 improves survival of mice subjected to a lethal dose of LPS. Mice were treated with 15 mg/kg **X12** (iv) either 15 min before or 15 min after the injection of 20 mg/kg of LPS (iv). Survival (A) and body weight (B) were recorded for 7 days after the LPS injection at the interval of 1 day. Results from the summary of two different experiments are shown. n = 12 animals in each group (**p < 0.01 vs LPS group).

dose-dependent manner further suggests their potential as anti-inflammatory agents.

Before the in vivo evaluation, five active imidazopyridines **X10**, **X12**, **X13**, **X14**, and **X15** were tested for their cytotoxicity and safety in human normal hepatic cell line HL-7702. Cell viability was determined by MTT method after treatment with compounds for 24, 48, and 72 h. As shown in Figure 3C, **X13** shows a low toxicity, while the other four compounds at 20 μM, particularly **X12** and **X14**, are nontoxic in hepatic cells, indicating that they are relatively safe. Thus, combining with the anti-inflammatory activity and cytotoxicity of these compounds, we chose the representative compound **X12** for the next in vivo evaluation.

As a major endotoxin, LPS from Gram-negative bacteria has been implicated as a major cause of sepsis. A number of different approaches have been investigated to treat and/or prevent the septic shock associated with infections caused by Gram-negative bacteria, including blockage of one or more of the cytokines induced by LPS signaling.[21] Our data have demonstrated the inhibitory effects of these derivatives on LPS-induced cytokine release. For the potential clinical application, we further determine whether **X12** are able to attenuate endotoxin shock through inhibiting LPS-induced inflammatory response. **X12** was used in the form of **X12**-HCl for intravenous (iv) administration in acute inflammatory models. Mice were injected with LPS at the dosage of 20 mg/kg intravenously in the presence or absence of **X12** injection, and their survival rates were monitored for 7 days, respectively. As shown in Figure 4A, all animals treated with LPS alone died within 3 days as a result of the septic shock. In animals receiving **X12** at 15 mg/kg either 15 min prior to LPS injection (for preventive effect) or 15 min after LPS injection (for therapeutic effect), the survival rates were significantly increased as compared to that of the control group (50% survivals in both the prevention group and the treatment group, p < 0.01 in both groups v.s. LPS group). Meanwhile, the body weight of mice in the prevention group decreased during 0−2 days but regained slowly 2−7 days after LPS injection (Figure 4B), while mice in the treatment group lost their body weight till the third day after LPS injection. Thus, our data provide evidence for the anti-inflammatory effects of these imidazopyridine derivatives and **X12**'s potent prevention and treatment effects in sepsis. Although the anti-inflammatory mechanism and underlying molecular targets are still unknown, the beneficial effects of the imidazopyridines on LPS-induced inflammation will become an objective in the continuing research.

In conclusion, we have synthesized and evaluated two series of benzimidazole and imidazopyridine derivatives for their anti-inflammatory activities in LPS-stimulated macrophages. It was concluded that the imidazopyridine series showed better activity than the benzimidazole series. Combined with the QSAR analysis, an electron-donating substitution and low molecular polarizability in imidazopyridine skeleton are favorable to anti-inflammatory activities of imidazopyridine derivatives. Compound **X12** was selected for in vivo testing and was found to markedly decrease LPS-induced lethality in septic mice models. Thus, imidazopyridine derivatives may be promising for drug discovery as anti-inflammatory agents. Further studies should include testing of these new anti-inflammatory compounds in more animal models and examination of the underlying molecular mechanisms and direct targets at the transcriptional or post-transcriptional level.

■ ASSOCIATED CONTENT

Supporting Information

Full names, structures, and characterization of all compounds, and details regarding biological assays and quantitative structure−activity relationship (QSAR) study. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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