Synthesis, Bioactivity Evaluation, and Toxicity Assessment of Novel Salicylanilide Ester Derivatives as Cercaricides against *Schistosoma japonicum* and Molluscicides against *Oncomelania hupensis*

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A series of novel salicylanilide ester derivatives were synthesized, characterized, and evaluated for cercaricidal potential against *Schistosoma japonicum* and molluscicidal potential against *Oncomelania hupensis*. Four derivatives exhibited remarkable cercaricidal activity superior to that of niclosamide. Among them, the most active compound, 4-chloro-2-((2-methoxy-4-nitrophenyl)carbamoyl)phenyl 4-methoxybenzoate (compound 4c), showed a marked minimum effective cercaricidal concentration as low as 0.43 μ M and significant molluscicidal activity, with a 50% lethal concentration (LC_{50}) of 0.206 g/m². Particularly, compound 4c displayed 88-fold decreased fish toxicity on *Danio rerio* and 44-fold reduced cytotoxicity on human kidney HEK293 cells in comparison with the toxicity of niclosamide. The results indicated that 4c could serve as a promising drug candidate, with environmental safety properties, against *Schistosoma japonicum* at transmission stages. The preliminary molecular mechanism of target compounds in *Schistosoma japonicum* cercariae was also investigated. Salicylanilide ester derivatives exhibited an inhibitory effect on nitric oxide synthase (NOS) but no effect on lactate dehydrogenase (LDH) and acetylcholinesterase (AChE), and a strong and significant correlation between NOS inhibitory efficacy and cercaricidal activity was observed. In addition, 4c could downregulate the expression of NOS in a dose-dependent manner. These results suggested that NOS was probably one of the drug targets of salicylanilide esters.

Schistosomiasis japonica, caused by infection with *Schistosoma japonicum*, is recognized as a considerable economic and public health concern in Asia, including China, Indonesia, and the Philippines (1–3). According to estimates, over 58 million people are infected (4). Schistosome infection occurs through contact with water contaminated by cercariae, the free-living stage of the parasite shed from intermediate host *Oncomelania* snails (5). Although praziquantel is available for treatment, there is the fact that it cannot prevent individuals from reinfection. Repeated and intense exposure to cercariae could lead to an ineffective response of early-stage chronic cases in routine control programs, which would increase the risk of infections progressing to an advanced stage. A successful control program of the disease should be constructed as an integrated control scheme including cercaricidal methods, snail control programs, and chemotherapy.

Niclosamide has a long track record as a successful molluscicide against *Oncomelania* snails (6, 7). Moreover, it is effective for cercaria control both under laboratory conditions and in field experiments (8–10). However, niclosamide has relatively poor solubility in water and is a well-known environmentally hazardous chemical. It has been proved highly toxic to 18 species of fish (11). It exhibited strong to moderate cytotoxicity on several human cell lines (12, 13). In the last 2 decades, most of the modifications of niclosamide have focused on improving its solubility by altering formulations (e.g., as wettable powder, suspension concentrate, and polymeric controlled-release formulations) (14–17) or by linking it with surfactants (e.g., polyethylene glycols [PEGs]) (18–21). To the best of our knowledge, little effort has been made to reduce its toxicity through structure modification. The noticeable toxic effect on nontarget organisms restricts the broad use of

niclosamide as a cercaricide or molluscicide in an expanse of water.

In our previous studies, we occasionally found that introducing an ester substituent at the hydroxyl group of niclosamide and replacing the chlorine atom of nitroaniline with methoxyl resulted in a decrease in cytotoxicity. Based on these findings, in order to ascertain whether esterification of the hydroxyl group with different kinds of moieties was beneficial for reducing toxicity, the structural modification and optimization of niclosamide were performed. Twelve salicylanilide ester derivatives were synthesized. Their efficacy was determined in terms of (i) cercaricidal activity, (ii) molluscicidal activity, (iii) cytotoxicity on HEK293 cells, and (iv) acute lethal fish toxicity on *Danio rerio*.

However, the biological mechanism of niclosamide and its derivatives in cercariae still remains to be investigated. Some studies have proposed that niclosamide induces rapid death of cercariae through interacting with miscellaneous enzymes (8, 9). To ascertain the effect of salicylanilide esters on enzyme activity, a preliminary study was performed. Three important enzymes of *Schisto-*

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Soma japonicum cercariae were employed, including nitric oxide synthase (NOS), lactate dehydrogenase (LDH), and acetylcholinesterase (AChE).

In this work, we report the synthesis, cercaricidal and molluscicidal activity evaluation, and cytotoxicity and fish toxicity assessment of novel salicylanilide ester derivatives as potential drug candidates against *Schistosoma japonicum* at transmission stages. Preliminary structure-activity relationships (SARs) of the target compounds are discussed. In addition, an initial enzyme-inhibitory activity assay and reverse transcription-PCR (RT-PCR) were carried out, which hopefully provide a starting point for understanding the molecular mechanism of salicylanilide esters.

MATERIALS AND METHODS

Chemistry. Reagents and solvents were purchased from Sigma-Aldrich and were used without further purification. Melting points were measured with a B-540 Büchi apparatus and were uncorrected. ^1H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were recorded on a Bruker AM-400 spectrometer (400 MHz). Chemical shifts are given in ppm (δ) relative to tetramethylsilane (TMS) as an internal standard, and signals are indicated according to the following abbreviations: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; m, multiplet, etc. High-resolution mass spectra (HRMS) were recorded on a Thermo Q Exactive Orbitrap liquid chromatography-tandem mass spectrometry (LC-MS/MS) instrument. Thin-layer chromatography (TLC) was carried out using plate silica gel F254 (Merck). All yields are not optimized and generally represent the result of a single experiment.

Preparation of 5-chloro-2-hydroxy-N-(2-methoxy-4-nitrophenyl)benzamide (compound 3). 5-Chlorosalicylic acid (1.72 g, 10 mmol) and 2-methoxy-4-nitrophenylamine (1.68 g, 10 mmol) were dissolved in 30 ml of toluene. Then PCl_3 was added dropwise. The resulting mixture was refluxed for 14 h. The progress of the reaction was monitored by TLC. After the reaction, the precipitated solid was collected by filtration, washed with hot water, and purified by recrystallization from ethanol to afford a white solid, compound 3 (2.5 g). Yield 77.6%, melting point (mp): 206.6 to 207.3°C. LC-MS: $m/z = 323.71$ [$\text{M} + 1$] $^+$. ^1H NMR (400 MHz, CDCl_3) δ : 11.56 (s, 1H, OH), 8.71 (s, 1H, NH), 8.54 (d, 1H, $J = 7.2$ Hz, Ph-H [Ph is the abbreviation for phenyl]), 7.92 (d, 1H, $J = 7.2$ Hz, Ph-H), 7.78 (s, 1H, Ph-H), 7.43 (s, 1H, Ph-H), 7.37 (d, 1H, $J = 8.0$ Hz, Ph-H), 6.95 (d, 1H, $J = 8.4$ Hz, Ph-H), 4.15 (s, 3H, OCH_3).

General procedures for the synthesis of salicylanilide ester derivatives (compounds 4a to 4l). A corresponding acyl chloride (2 mmol) was slowly added dropwise to a cooled (0 to 5°C) mixture of compound 3 (2 mmol) and triethylamine (0.5 ml) in anhydrous CH_2Cl_2 (15 ml). After the mixture was stirred at room temperature for an additional 1 h, the reaction was quenched with water (10 ml). The resulting mixture was extracted with CH_2Cl_2 (three samples of 20 ml each). The combined organic phase was washed with brine, dried over anhydrous Na_2SO_4 , and concentrated under reduced pressure. The residues obtained were purified by recrystallization from ethanol to yield target compounds 4a to 4l.

4-Chloro-2-((2-methoxy-4-nitrophenyl)carbamoyl)phenyl benzoate (compound 4a). Yellow solid, yield 59%, mp: 161.3 to 162.4°C. HRMS: $m/z = 425.0521$ [$\text{M} - \text{H}$] $^-$, calculated: 425.0540, $\text{C}_{21}\text{H}_{14}\text{ClN}_2\text{O}_6^-$. ^1H NMR (400 MHz, CDCl_3) δ : 9.20 (s, 1H, NH), 8.65 (d, 1H, $J = 9.2$ Hz, Ph-H), 8.12 to 8.05 (m, 3H, Ph-H), 7.91 (dd, 1H, $J = 9.2$, 2.4 Hz, Ph-H), 7.72 (d, 1H, $J = 2.4$ Hz, Ph-H), 7.58 (dd, 1H, $J = 8.8$, 2.4 Hz, Ph-H), 7.38 to 7.20 (m, 4H, Ph-H), 3.58 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, CDCl_3) δ : 164.78, 162.24, 147.79, 147.09, 145.94, 143.66, 134.57, 132.91, 132.46, 131.16, 130.82, 129.67, 129.56, 125.64, 124.15, 118.28, 117.84, 105.25, 56.29.

4-Chloro-2-((2-methoxy-4-nitrophenyl)carbamoyl)phenyl 4-methylbenzoate (compound 4b). Yellow-brown solid, yield 91%, mp: 177.3 to 178.5°C. HRMS: $m/z = 439.0675$ [$\text{M} - \text{H}$] $^-$, calculated: 439.0697, $\text{C}_{22}\text{H}_{16}\text{ClN}_2\text{O}_6^-$. ^1H NMR (400 MHz, CDCl_3) δ : 9.10 (s, 1H, NH), 8.67

(d, 1H, $J = 9.2$ Hz, Ph-H), 8.10 to 8.05 (m, 3H, Ph-H), 7.89 (dd, 1H, $J = 9.2$, 2.4 Hz, Ph-H), 7.62 (d, 1H, $J = 2.2$ Hz, Ph-H), 7.55 (dd, 1H, $J = 8.8$, 2.4 Hz, Ph-H), 7.34 to 7.23 (m, 3H, Ph-H), 3.55 (s, 3H, OCH_3), 2.47 (s, 3H, CH_3). ^{13}C NMR (100 MHz, CDCl_3) δ : 164.86, 162.24, 147.79, 147.09, 145.94, 143.66, 133.57, 133.10, 132.46, 131.16, 130.82, 129.79, 129.06, 125.74, 125.15, 119.25, 117.84, 105.25, 56.01, 22.09.

4-Chloro-2-((2-methoxy-4-nitrophenyl)carbamoyl)phenyl 4-methoxybenzoate (compound 4c). Yellow-brown solid, yield 92%, mp: 162.1 to 163.3°C. HRMS: $m/z = 455.0625$ [$\text{M} - \text{H}$] $^-$, calculated: 455.0646, $\text{C}_{22}\text{H}_{16}\text{ClN}_2\text{O}_7^-$. ^1H NMR (400 MHz, CDCl_3) δ : 9.12 (s, 1H, NH), 8.70 (d, 1H, $J = 9.2$ Hz, Ph-H), 8.14 (dd, 2H, $J = 11.2$, 2.4 Hz, Ph-H), 8.06 (d, 1H, $J = 2.4$ Hz, Ph-H), 7.89 (dd, 1H, $J = 9.2$, 2.4 Hz, Ph-H), 7.62 (d, 1H, $J = 2.4$ Hz, Ph-H), 7.52 (dd, 1H, $J = 8.8$, 2.4 Hz, Ph-H), 7.22 (t, 1H, $J = 9.2$ Hz, Ph-H), 6.99 to 6.97 (m, 2H, Ph-H), 3.90 (s, 3H, OCH_3), 3.57 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, CDCl_3) δ : 164.91, 164.47, 162.30, 147.81, 147.19, 143.66, 133.61, 133.08, 133.01, 132.37, 131.14, 129.04, 128.15, 125.17, 120.64, 119.25, 117.84, 114.36, 105.27, 56.10, 55.90.

4-Chloro-2-((2-methoxy-4-nitrophenyl)carbamoyl)phenyl 4-cyanobenzoate (compound 4d). Yellow solid, yield 52%, mp: 230.4 to 231.5°C. HRMS: $m/z = 450.0469$ [$\text{M} - \text{H}$] $^-$, calculated: 450.0493, $\text{C}_{22}\text{H}_{13}\text{ClN}_3\text{O}_6^-$. ^1H NMR (CDCl_3) δ : 8.66 (s, 1H, NH), 8.61 (d, 1H, $J = 8.8$ Hz, Ph-H), 8.30 (d, 2H, $J = 8.4$ Hz, Ph-H), 7.92 to 7.88 (m, 2H, Ph-H), 7.83 (d, 2H, $J = 8.4$ Hz, Ph-H), 7.71 (s, 1H, Ph-H), 7.61 (dd, 1H, $J = 8.4$, 2.0 Hz, Ph-H), 7.28 (d, 1H, $J = 8.8$ Hz, Ph-H), 3.82 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, CDCl_3) δ : 163.54, 162.39, 147.72, 146.76, 143.92, 133.28, 133.07, 132.99, 132.96, 132.73, 132.50, 131.17, 131.06, 129.97, 129.79, 125.12, 119.14, 117.96, 117.94, 117.69, 105.50, 56.46.

4-Chloro-2-((2-methoxy-4-nitrophenyl)carbamoyl)phenyl 4-nitrobenzoate (compound 4e). Yellow-brown solid, yield 46%, mp: 209.5 to 210.1°C. HRMS: $m/z = 470.0367$ [$\text{M} - \text{H}$] $^-$, calculated: 470.0391, $\text{C}_{21}\text{H}_{13}\text{ClN}_3\text{O}_8^-$. ^1H NMR (CDCl_3) δ : 8.68 (s, 1H, NH), 8.60 (d, 1H, $J = 9.2$ Hz, Ph-H), 8.29 (dd, 2H, $J = 9.0$, 2.0 Hz, Ph-H), 7.92 to 7.81 (m, 4H, Ph-H), 7.71 (d, 1H, $J = 2.0$ Hz, Ph-H), 7.61 (dd, 1H, $J = 8.8$, 2.4 Hz, Ph-H), 7.26 (d, 1H, $J = 9.2$ Hz, Ph-H), 3.83 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, CDCl_3) δ : 163.54, 162.39, 147.72, 146.76, 143.92, 133.28, 133.07, 132.99, 132.96, 132.73, 132.50, 131.17, 131.06, 129.97, 129.79, 125.12, 119.14, 117.96, 117.94, 117.69, 105.50, 56.46.

4-Chloro-2-((2-methoxy-4-nitrophenyl)carbamoyl)phenyl 4-fluorobenzoate (compound 4f). Yellow solid, yield 81%, mp: 193.8 to 195.4°C. HRMS: $m/z = 443.0424$ [$\text{M} - \text{H}$] $^-$, calculated: 443.0446, $\text{C}_{21}\text{H}_{13}\text{ClFN}_2\text{O}_6^-$. ^1H NMR (400 MHz, CDCl_3) δ : 9.15 (s, 1H, NH), 8.61 (d, 1H, $J = 9.2$ Hz, Ph-H), 8.24 (dd, 2H, $J = 8.0$, 5.6 Hz, Ph-H), 7.97 (d, 1H, $J = 2.4$ Hz, Ph-H), 7.89 (dd, 1H, $J = 8.8$, 2.4 Hz, Ph-H), 7.61 (d, 1H, $J = 2.4$ Hz, Ph-H), 7.59 to 7.57 (m, 1H, Ph-H), 7.32 (d, 1H, $J = 8.8$ Hz, Ph-H), 7.26 (t, 2H, $J = 8.8$ Hz, Ph-H), 3.72 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, CDCl_3) δ : 170.14, 168.53, 167.14, 152.87, 151.65, 148.49, 138.25, 138.18, 138.09, 137.70, 137.03, 135.36, 134.31, 130.03, 129.59, 124.13, 122.28, 121.22, 121.00, 110.24, 61.07.

4-Chloro-2-((2-methoxy-4-nitrophenyl)carbamoyl)phenyl 4-chlorobenzoate (compound 4g). Brown solid, yield 64%, mp: 167.2 to 168.6°C. HRMS: $m/z = 459.0128$ [$\text{M} - \text{H}$] $^-$, calculated: 459.0151, $\text{C}_{21}\text{H}_{13}\text{Cl}_2\text{N}_2\text{O}_6^-$. ^1H NMR (400 MHz, CDCl_3) δ : 8.88 (s, 1H, NH), 8.61 (d, 1H, $J = 8.8$ Hz, Ph-H), 8.14 to 8.12 (m, 2H, Ph-H), 7.97 (d, 1H, $J = 2.4$ Hz, Ph-H), 7.90 (dd, 1H, $J = 8.8$, 2.4 Hz, Ph-H), 7.66 (d, 1H, $J = 2.4$ Hz, Ph-H), 7.58 (dd, 1H, $J = 8.4$, 2.4 Hz, Ph-H), 7.52 to 7.26 (m, 2H, Ph-H), 7.26 to 7.24 (m, 1H, Ph-H), 3.69 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, CDCl_3) δ : 164.09, 162.30, 147.72, 146.82, 143.75, 141.41, 133.41, 133.08, 132.70, 132.02, 130.70, 129.46, 129.43, 127.00, 125.10, 119.20, 117.90, 105.38, 56.25.

4-Chloro-2-((2-methoxy-4-nitrophenyl)carbamoyl)phenyl 4-bromobenzoate (compound 4h). Brown solid, yield 75%, mp: 162.3 to 163.6°C. HRMS: $m/z = 504.9601$ [$\text{M} - \text{H}$] $^-$, calculated: 504.9625, $\text{C}_{21}\text{H}_{13}\text{BrClN}_2\text{O}_6^-$. ^1H NMR (400 MHz, CDCl_3) δ : 8.87 (s, 1H, NH), 8.64 (d, 1H, $J = 8.8$ Hz, Ph-H), 8.06 to 8.03 (m, 2H, Ph-H), 7.97 (d, 1H, $J = 2.4$ Hz, Ph-H), 7.90 (dd, 1H, $J = 9.2$, 2.4 Hz, Ph-H), 7.68 to 7.65 (m, 3H,

Ph-H), 7.58 (dd, 1H, $J = 8.8, 2.4$ Hz, Ph-H), 7.25 (d, 1H, $J = 9.2$ Hz, Ph-H), 3.69 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, CDCl_3) δ : 165.20, 162.30, 147.72, 146.80, 143.77, 133.41, 133.08, 132.77, 132.46, 132.08, 130.69, 130.07, 129.45, 127.45, 125.10, 119.19, 117.91, 105.40, 56.25.

4-Chloro-2-((2-methoxy-4-nitrophenyl)carbamoyl)phenyl 3,4-difluorobenzoate (compound 4i). Yellow solid, yield 84%, mp: 166.3 to 167.9°C. HRMS: $m/z = 461.0327$ [$\text{M} - \text{H}$] $^-$, calculated: 461.0352, $\text{C}_{21}\text{H}_{12}\text{ClF}_2\text{N}_2\text{O}_6^-$. ^1H NMR (400 MHz, CDCl_3) δ : 8.81 (s, 1H, NH), 8.62 (d, 1H, $J = 8.8$ Hz, Ph-H), 8.14 to 8.12 (m, 1H, Ph-H), 7.94 to 7.89 (m, 2H, Ph-H), 7.69 (d, $J = 2.4$ Hz, 1H, Ph-H), 7.57 (dd, 1H, $J = 8.8, 2.4$ Hz, Ph-H), 7.27 to 7.25 (m, 1H, Ph-H), 7.02 to 6.94 (m, 2H, Ph-H), 3.77 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, CDCl_3) δ : 165.30, 162.37, 147.86, 146.47, 143.81, 135.06, 133.45, 133.03, 132.80, 130.52, 129.63, 125.11, 119.27, 117.88, 112.65, 112.43, 112.40, 105.89, 105.64, 105.41, 56.32.

4-Chloro-2-((2-methoxy-4-nitrophenyl)carbamoyl)phenyl thiophene-2-carboxylate (compound 4j). Yellow solid, yield 74%, mp: 180.6 to 181.8°C. HRMS: $m/z = 431.0081$ [$\text{M} - \text{H}$] $^-$, calculated: 431.0105, $\text{C}_{19}\text{H}_{12}\text{ClN}_2\text{O}_6^-$. ^1H NMR (400 MHz, CDCl_3) δ : 8.99 (s, 1H, NH), 8.69 (d, 1H, $J = 8.8$ Hz, Ph-H), 8.03 to 8.00 (m, 2H, Ph-H), 7.92 (dd, 1H, $J = 9.2, 2.4$ Hz, Ph-H), 7.74 (d, 1H, $J = 4.0$ Hz, Ph-H), 7.67 (d, 1H, $J = 2.8$ Hz, Ph-H), 7.57 (dd, 1H, $J = 10.0, 2.4$ Hz, thiophene-H), 7.28 (d, 1H, $J = 6.8$ Hz, thiophene-H), 7.26 to 7.20 (m, 1H, thiophene-H), 3.70 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, CDCl_3) δ : 162.26, 160.23, 147.85, 146.53, 143.73, 136.02, 135.18, 133.54, 133.05, 132.68, 131.71, 130.98, 129.28, 128.64, 125.04, 119.27, 117.88, 105.33, 56.19.

4-Chloro-2-((2-methoxy-4-nitrophenyl)carbamoyl)phenyl cyclopropanecarboxylate (compound 4k). Yellow solid, yield 35%, mp: 171.2 to 172.3°C. HRMS: $m/z = 389.0523$ [$\text{M} - \text{H}$] $^-$, calculated: 389.0540, $\text{C}_{18}\text{H}_{14}\text{ClN}_2\text{O}_6^-$. ^1H NMR (400 MHz, CDCl_3) δ : 9.15 (s, 1H, NH), 8.74 (d, 1H, $J = 8.8$ Hz, Ph-H), 7.98 to 7.94 (m, 2H, Ph-H), 7.79 (d, 1H, $J = 2.4$ Hz, Ph-H), 7.49 (dd, 1H, $J = 8.4, 2.4$ Hz, Ph-H), 7.16 (d, 1H, $J = 8.8$ Hz, Ph-H), 4.02 (s, 3H, OCH_3), 1.93 to 1.89 (m, 1H, CH), 1.16 to 1.13 (m, 2H, CH_2), 1.07 to 1.02 (m, 2H, CH_2).

4-Chloro-2-((2-methoxy-4-nitrophenyl)carbamoyl)phenyl but-3-enoate (compound 4l). Yellow solid, yield 44%, mp: 182.0 to 183.3°C. HRMS: $m/z = 389.0521$ [$\text{M} - \text{H}$] $^-$, calculated: 389.0540, $\text{C}_{18}\text{H}_{14}\text{ClN}_2\text{O}_6^-$. ^1H NMR (400 MHz, CDCl_3) δ : 10.66 (s, 1H, NH), 8.82 (d, 1H, $J = 9.2$ Hz, Ph-H), 8.25 (d, 1H, $J = 2.8$ Hz, Ph-H), 7.97 (dd, 1H, $J = 9.2, 2.4$ Hz, Ph-H), 7.77 (d, 1H, $J = 2.4$ Hz, Ph-H), 7.45 (dd, 1H, $J = 8.8, 2.8$ Hz, Ph-H), 6.99 (m, 1H, $J = 8.8$ Hz, Ph-H), 6.21 to 6.12 (m, 1H, =CH), 5.52 to 5.43 (m, 2H, CH_2 =), 4.81 (d, 2H, $J = 5.6$ Hz, CH_2), 4.01 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, CDCl_3) δ : 164.50, 162.54, 155.20, 148.18, 143.36, 134.58, 133.57, 132.60, 132.09, 127.53, 123.22, 119.86, 119.46, 118.04, 114.66, 105.43, 71.22, 59.48.

Cercaricidal activity assay. *Oncomelania hupensis* snails were collected from the western part of Dongting Lake, Hunan Province, China, and were transported to National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention. Snails were maintained in containers which were paved with wet filter paper. *Schistosoma japonicum* cercariae were collected from the infected *Oncomelania hupensis* snails. The obtained salicylanilide ester derivatives (compounds 4a to 4l) were diluted by dechlorinated water after being dissolved in dimethyl sulfoxide (DMSO). *Schistosoma japonicum* cercariae were plated in Microtiter 96-well plates (each well with 50 cercariae) and then exposed to serial concentrations of the target compounds. Assays were performed in triplicate. Niclosamide was used as a positive control, and DMSO was used as a negative control. Minimum effective concentrations against cercariae with 100% effectiveness were determined after a 30-min incubation at 25°C.

Molluscicidal activity assay. Healthy *Oncomelania hupensis* snails were collected from the western part of the Dongting Lake, Hunan Province, China. The snails were fed on boiled or oven-dried lettuce leaves and were acclimatized to laboratory conditions for 3 weeks. The assay was conducted using two methods, described below.

(i) Immersion method. The immersion method was carried out according to WHO procedures (22). Compounds 4c, 4f, and 4i were dissolved first in a small amount of dimethylformamide (DMF) and then added to dechlorinated water to obtain a 0.1% solution. The bioassay involved immersion of adult snails in a mixed aqueous solution of the investigated compounds at final concentrations ranging from 2.5 to 10 mg/liter at $25 \pm 1^\circ\text{C}$ under normal diurnal lighting. Ten snails were used for each concentration. The bioassays were performed in triplicate. After 24 h, the aquariums were decanted, and the snails were rinsed three times with dechlorinated water and offered lettuce leaves as food. The test snails were then left in water for another 48 h as a recovery period and examined to assess mortality. Niclosamide was used as a positive control, and DMF was used as a negative control. Snails were considered dead by evidence of discoloration, absence of muscle contraction, hemorrhage, and deterioration of the body tissues.

(ii) Spraying method. Six hundred grams of lake soil was put into each 20- by 25-cm stainless steel tray. Dechlorinated water was then added to make 40% humidity. One hundred living adult *Oncomelania hupensis* snails were then introduced into each tray. Concentrations of compound 4c between 0.0625 and 2 g/m² were sprayed into the trays. After 24 h, snails were removed from each tray, and numbers of dead snails were counted according to the same procedure used in the immersion method. Results were subjected to probit analysis to produce regression lines and to determine the 10%, 50%, and 90% lethal concentrations (LC_{10} , LC_{50} , and LC_{90} , respectively) (23).

Cytotoxicity assay on HEK293 cells. Cytotoxicity was assessed by MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay. A total of 6×10^4 human kidney cells (HEK293) were seeded in Microtiter 96-well plates for 24 h and subsequently treated with serial concentrations of compounds 4c, 4f, 4i, and 4l for 48 h. Then, 10 μl of MTT reagent (R&D Systems, Minneapolis, MN) was added. After incubation for 2 to 4 h at 37°C, 100 μl of detergent reagent was added to each well. Plates were left covered in the dark at 37°C for at least 2 h and then measured on an automated microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT) at 550 nm. Niclosamide was used as a positive control, and DMSO was used as a negative control. The concentration of drug for inhibition of 50% of cells (IC_{50}) was calculated using software for dose-effect analysis with microcomputers.

Acute lethal fish toxicity test. *Danio rerio* fish were used in the present study as test animals. In the laboratory, fish were reared in glass aquariums, each with a 60-liter capacity, continuously aerated through stone diffusers connected to a mechanical air compressor. Fish density was in the range of 12 fish per aquarium. Fish used in bioassay tests had an average weight of 0.3 ± 0.1 g and an average length of 20 ± 1 mm. Equal numbers of males and females were used throughout. Batches of new fish were maintained for at least 1 week in dechlorinated water to allow them to become acclimatized before any toxicity test was performed. Water temperature was maintained at $23 \pm 1^\circ\text{C}$ throughout. Fish were maintained in a natural illumination system under a normal prevailing photoperiod of 18 h of light and 6 h of dark. Toxicity tests were performed using apparently healthy fish of equal size, length, and age. Fish were starved from 1 day before tests and throughout the exposure time. Serial concentrations of compounds 4c and 4f were used to assess toxicity. The test solution of each concentration was prepared in a separate aquarium and replicated at least twice. The temperature of solutions was adjusted and maintained at $23^\circ\text{C} \pm 1^\circ\text{C}$. The pH value was adjusted and maintained at 7.0 ± 0.2 . Ten fish were introduced into each aquarium and confined for an exposure interval of 96 h. The vitality of the fish was monitored in real time. Niclosamide was used as a positive control. Negative-control fish were maintained in a similar aquarium that contained only fresh water and were observed throughout to make sure that experimental conditions did not pose any additional toxicity threats to the test fish. Dead fish were removed as soon as their death was confirmed. At the end of the exposure interval, final mortality records were made for both treated and control aquariums. Results were subjected to probit analysis to produce regres-

FIG 1 Routes of target compound synthesis (4a to 4l). Reagents and conditions are as follows: PCl_3 , toluene, reflux temperature (arrow a); Et_3N , CH_2Cl_2 , room temperature (arrow b).

sion lines and to determine the 10%, 50%, and 90% lethal concentrations (LC_{10} , LC_{50} , and LC_{90} , respectively) (23).

Enzyme activity assay. Soft tissues of *Schistosoma japonicum* cercariae were homogenized in phosphate-buffered saline (PBS; pH 7.4) and centrifuged at $12,000 \times g$ at 4°C . The supernatant was used for assays. The experimental procedures for NOS, LDH, and AChE activity assays were performed according to the technical bulletins of a nitric oxide synthase assay kit (KA1634; Abnova), lactate dehydrogenase activity assay kit (MAK066; Sigma-Aldrich), and acetylcholinesterase activity assay kit (MAK119; Sigma-Aldrich), respectively.

RT-PCR assay. *Schistosoma japonicum* cercariae were collected from the infected *Oncomelania hupensis* snails using the same method as described above. After the addition of compound 4c (at 1, 10, and $100 \mu\text{M}$), cercariae were incubated in RPMI 1640 medium at 37°C in 5% CO_2 for 4 h. Actin served as a control. Total RNA was extracted from *Schistosoma japonicum* cercariae using TRIzol (Invitrogen) according to the manufacturer's instructions. The quantity and quality of the RNA samples were assessed by a NanoDrop ND-2000 spectrophotometer and denaturing agarose gel electrophoresis. Random-primed reverse transcription (RT) was performed using $1.5 \mu\text{g}$ of total RNA according to the Super Script II kit protocol (Promega). Primer sequences were as follows: for NOS, 5'-ATATGGAACCAACAAAAGTGATTG-3' (forward) and 5'-GCGATTAAGCACATTCTGTTTTTA-3' (reverse) (amplification fragment of 276 bp); for actin, 5'-ATATGGCCGACGAGGAAGTCC-3' (forward) and 5'-GATTAGAAGCATTTCAGGTGAACAA-3' (reverse). PCR conditions were as follows: 94°C for 2 min, 94°C for 30 s, and 57°C for 45 s, with 33 cycles of 72°C for 50 s, followed by 72°C for 7 min. The PCR product was detected by agarose gel electrophoresis.

The crystallographic data have been assigned a deposition number at the Cambridge Crystallographic Data Center (CCDC accession number 910214).

RESULTS

Chemistry. The synthetic routes for the preparation of salicylanilide ester derivatives are summarized in Fig. 1. The target compounds 4a to 4l were synthesized in two steps, with overall yields of 27.2 to 71.4%. Amidation of 5-chlorosalicylic acid (compound 1) with 2-methoxy-4-nitrophenylamine (compound 2) in the presence of PCl_3 afforded the amide intermediate (compound 3). Esterification of compound 3 with appropriate acyl chlorides yielded the corresponding target compounds 4a to 4l. The structures of all target compounds were characterized by HRMS, ^1H NMR, and ^{13}C NMR. All

compounds were analyzed by HPLC, and the purity of each was confirmed to be over 98.5%. The single crystal structure of compound 4j was determined by high-resolution X-ray diffraction (Fig. 2).

Cercaricidal activity. The determination of cercaricidal activity of salicylanilide ester derivatives was carried out against *Schistosoma japonicum*. Niclosamide was used as a positive control. The results are summarized in Table 1. Four salicylanilide ester derivatives (compounds 4c, 4f, 4i, and 4l) exhibited significant cercaricidal activity. All of them showed improved potency in comparison with that of niclosamide. Compounds 4c, 4f, 4i, and 4l caused 100% mortality of *Schistosoma japonicum* cercariae with minimum effective concentrations (MECs) as low as 0.43, 0.45, 0.48, and $0.54 \mu\text{M}$, respectively. It is notable that 100% mortality was evident within only 30 min after exposure to the derivatives.

Molluscicidal activity. Based on the data obtained for cercaricidal activity, representative compounds (4c, 4f, and 4i) were se-

FIG 2 X-ray crystal structure of compound 4j (final R indices [R_e] = 0.0549).

TABLE 1 Cercaricidal activity of salicylanilide ester derivatives (4a to 4l) against *Schistosoma japonicum* after a 30-min exposure

Compound	Structure	MEC (μM) ^a
4a		>100
4b		>100
4c		0.43
4d		>100
4e		>100
4f		0.45
4g		>100
4h		>100
4i		0.48

TABLE 1 (Continued)

Compound	Structure	MEC (μM) ^a
4j		>100
4k		>100
4l		0.54
Niclosamide		0.63

^a MEC, minimum effective concentration against cercariae with 100% effectiveness.

lected for further evaluation of their molluscicidal activity against *Oncomelania hupensis* under laboratory conditions. Both immersion and spraying methods were employed. The results are summarized in [Tables 2](#) and [3](#).

A preliminary test was performed using the immersion method ([Table 2](#)). Compound 4c exhibited remarkable molluscicidal activity, causing 100% mortality at a concentration of 2.5 mg/liter, which was as effective as niclosamide. Compound 4i also showed satisfactory activity, with 76.67%, 96.67%, and 100% mortality at concentrations of 2.5, 5, and 10 mg/liter, respectively. Unexpectedly, a dramatic loss of potency was observed with compound 4f, with only 20% of snails killed at an exposure concentration of 10 mg/liter. The spraying method was carried out to determine the sublethal and lethal concentrations of the most active compound, 4c ([Table 3](#)). In the more accurate molluscicidal assay, compound 4c still presented significant molluscicidal activity, with LC_{10} , LC_{50} , and LC_{90} values of 0.055, 0.206, and 0.773 g/m², respectively. Particularly, the molar LC_{50} (MLC_{50}) value of compound 4c was 0.45 mmol/m², which was slightly superior to that of niclosamide (0.51 mmol/m²).

Toxicity assessment. (i) Cytotoxicity on HEK293 cells. In order to assess the toxicity of salicylanilide ester derivatives, selected target compounds (4c, 4f, 4i, and 4l) were tested for their cytotoxicity on HEK293 human kidney cells. The results are summarized in [Table 4](#). Generally, all of the tested compounds showed lower cytotoxicities than the value for niclosamide. Particularly, compounds 4c and 4f displayed 44- and 27-fold reduced cytotoxicities compared with the value for niclosamide (4c, IC_{50} of 80 μM ; 4f, IC_{50} of 50 μM).

TABLE 2 Molluscicidal activity of compounds 4c, 4f, and 4i against *Oncomelania hupensis* as determined by the immersion method after a 24-h exposure

Compound and concn (mg/liter)	No. of dead snails (<i>n</i> = 30) ^a	Mortality (%)
4c		
2.5	30	100
5	30	100
10	30	100
4f		
2.5	0	0
5	4	13.33
10	6	20
4i		
2.5	23	76.67
5	29	96.67
10	30	100
Niclosamide		
2.5	28	93.33
5	29	96.67
10	30	100
DMF	5	16.67
Dechlorinated water	3	10

^a *n*, total number of snails tested at each concentration.

(ii) **Acute lethal fish toxicity on *Danio rerio*.** *Danio rerio* is a commonly used model organism in ecotoxicological studies. An acute lethal toxicity test on *Danio rerio* is very useful as an early warning test for monitoring environmentally hazardous chemicals in water. Compounds 4c and 4f were selected for further fish toxicity assessment (Table 5). After a 96-h exposure, the results indicated that the sublethal and lethal concentrations of compound 4c on *Danio rerio* were very low (LC₁₀ of 19.53 mg/liter, LC₅₀ of 30.57 mg/liter, and LC₉₀ of 47.85 mg/liter). Remarkably, compound 4c demonstrated no toxicity to *Danio rerio* at the median lethal concentration of niclosamide (LC₅₀ of 0.25 mg/liter). No significant fish toxicity was observed with compound 4c at a

TABLE 3 Molluscicidal activity of compound 4c against *Oncomelania hupensis* as determined by the spraying method after a 24-h exposure

Compound and concn (g/m ²)	No. of dead snails (<i>n</i> = 100) ^a	Mortality (%)
4c ^b		
0.0625	18	18
0.125	22	22
0.25	58	58
0.5	84	84
1	91	91
2	100	100
Niclosamide ^c		
1	100	100
2	100	100

^a *n*, total number of snails tested at each concentration.^b For compound 4c, the LC₁₀, LC₅₀, and LC₉₀ values were 0.055, 0.206, and 0.773 g/m², respectively. The molar LC₅₀ (LC₅₀/molecular weight) was 0.45 mmol/m².^c For niclosamide, the LC₅₀ and LC₉₀ values were 0.168 and 0.551 g/m², respectively (42). The molar LC₅₀ (LC₅₀/molecular weight) was 0.51 mmol/m².**TABLE 4** Cytotoxicity and enzyme-inhibitory activity of salicylanilide ester derivatives (compounds 4a to 4l)

Compound	IC ₅₀ (μM)			
	Cytotoxicity on HEK293 cells	Enzyme-inhibitory activity		
		NOS	LDH	AChE
4a		>100	>100	>100
4b		>100	>100	>100
4c	80	11.5	>100	>100
4d		>100	>100	>100
4e		>100	>100	>100
4f	50	8.9	>100	>100
4g		>100	>100	>100
4h		>100	>100	>100
4i	2.5	11.4	>100	>100
4j		>100	>100	>100
4k		>100	>100	>100
4l	2.2	10.7	>100	>100
Niclosamide	1.8	10	26	5.2

concentration as high as 8 mg/liter. In contrast, only 0.3 mg/liter niclosamide caused 100% mortality. It is noteworthy that the MLC₅₀ of the most active compound, 4c, was 66.92 μM, making it 88-fold less toxic than niclosamide and indicating the considerably low toxicity of 4c on a single-molecule basis. Unfortunately, compound 4f did not exhibit the desired results. It produced 100% mortality at a concentration of 0.1 mg/liter.

Enzyme-inhibitory activity and expression of NOS detected by RT-PCR. Numerous reports have indicated that NOS, LDH, and AChE play important roles in biological and physiological processes of the schistosome; agents like compound 4c and niclosamide, which are highly toxic to cercariae, may cause an inhibitory effect on some important enzymes. Thus, salicylanilide ester derivatives were evaluated for their enzyme-inhibitory effects on NOS, LDH, and AChE in the soft tissues of *Schistosoma japonicum*

TABLE 5 Acute lethal toxicity of compounds 4c and 4f on *Danio rerio* after a 96-h exposure

Compound and concn (mg/liter)	No. of dead fish at the indicated time of exposure (<i>n</i> = 20) ^a				Mortality at 96 h (%)
	24 h	48 h	72 h	96 h	
4c ^b					
1	0	0	0	0	0
2	0	0	0	0	0
4	0	0	0	0	0
8	0	0	0	0	0
16	0	0	0	1	5
32	10	10	10	10	50
64	20	20	20	20	100
4f					
0.1	20	20	20	20	100
Niclosamide ^c					
0.3	20	20	20	20	100

^a *n*, total number of fish tested at each concentration.^b For compound 4c, the LC₁₀, LC₅₀, and LC₉₀ values were 19.53, 30.57, and 47.85 mg/liter, respectively. The molar LC₅₀ (LC₅₀/molecular weight) was 66.92 μM.^c For niclosamide, the LC₅₀ was 0.25 mg/liter after a 48-h exposure (43). The molar LC₅₀ (LC₅₀/molecular weight) was 0.76 μM.

FIG 3 The expression of NOS in *Schistosoma japonicum* cercariae detected by RT-PCR. Cercariae were treated with three different concentrations of compound 4c (1, 10, and 100 μ M). Sj, *Schistosoma japonicum*.

cercariae. According to the assay results (Table 4), niclosamide showed moderate to potent inhibitory activity on NOS, LDH, and AChE (NOS, IC_{50} of 10 μ M; LDH, IC_{50} of 26 μ M; AChE, IC_{50} of 5.2 μ M). On the other hand, although no LDH- and AChE-inhibitory activities were observed, some of the salicylanilide ester derivatives (compounds 4c, 4f, 4i, and 4l) exhibited significant NOS-inhibitory activity (4c, IC_{50} of 11.5 μ M; 4f, IC_{50} of 8.9 μ M; 4i, IC_{50} of 11.4 μ M; 4l, IC_{50} = 10.7 μ M) that was as potent as that of niclosamide. Interestingly, a very good correlation between the NOS-inhibitory efficacy and cercaricidal activity was observed, suggesting the potential of NOS to serve as one of the drug targets of salicylanilide esters.

To further confirm the assumption, we treated *Schistosoma japonicum* cercariae with compound 4c for 4 h at three different concentrations and monitored the expression of the NOS gene by RT-PCR. As shown in Fig. 3, compound 4c downregulated the expression of NOS in a dose-dependent manner. In particular, when cercariae were treated with 100 μ M 4c, the expression of NOS was significantly decreased.

DISCUSSION

Based on our previous findings, the structural modification of niclosamide focused on the esterification of the hydroxyl group. Introducing different ester groups resulted in remarkably different influences on cercaricidal activity. SAR studies revealed that, in general, appropriate ester moieties could improve or maintain potency. Compounds possessing 4-fluoro (4f), 3,4-difluoro (4i), and 4-methoxy (4c) phenyl esters showed potent cercaricidal activity. No cercaricidal activity was observed in the cases of compounds 4d, 4e, 4g, and 4h, which had CN, NO₂, Cl, and Br substituents in the *para*-position of the phenyl ring, respectively. On the other hand, introducing an allyl ester group (compound 4l) had a positive effect on potency. However, with the addition of a thiophene ring (4j) and cyclopropyl (4k), a sudden drop in potency was observed. Although a more comprehensive study is required to identify the optimal substituents at the phenyl group, current data suggest that introducing an appropriate substituted phenyl ring as an ester group of the scaffold is beneficial for cercaricidal activity.

We also evaluated the molluscicidal potential of three active compounds against *Oncomelania hupensis*. Compounds 4c and 4i exhibited considerable molluscicidal activity in immersion experiments, with activity equivalent to that of niclosamide. In addition, compound 4c presented potent molluscicidal activity in the more accurate spraying experiments, with LC_{10} , LC_{50} , and LC_{90} values of 0.055, 0.206, and 0.773 g/m², respectively. MLC_{50} is used in this study to compare the relative potency of the tested com-

pounds on a molecular basis. It is noteworthy that the MLC_{50} value of compound 4c was 0.45 mmol/m², which was superior to that of niclosamide (0.51 mmol/m²). The present results indicated that introducing a 4-methoxyphenyl or 3,4-difluorophenyl ester group on a salicylanilide scaffold could improve or maintain molluscicidal activity.

Importantly, when the cytotoxicities of four active compounds (4c, 4f, 4i, and 4l) were compared with the cytotoxicity of niclosamide, 4c and 4f showed much lower cytotoxicities (44- and 27-fold, respectively) on HEK293 cells. The cercaricidal activity of compound 4c was 186-fold more effective than its ability to kill human kidney cells. A similar pattern was also observed in the fish toxicity assay. After a 96-h exposure, compound 4c exhibited a very low 50% lethal concentration (LC_{50} of 30.57 mg/liter). The MLC_{50} value of 4c was 66.92 μ M, which makes it 88-fold less toxic than niclosamide. According to the Global Harmonization System (24), compound 4c is classified as a category 3 toxin to *Danio rerio* (10 mg/liter $\leq LC_{50} \leq$ 100 mg/liter), making it substantially less toxic than niclosamide (category 1, LC_{50} of $\leq$ 1 mg/liter). Unfortunately, compound 4f did not exhibit the desired assessment results: 100% mortality of *Danio rerio* was observed at a concentration of 0.1 mg/liter. It seemed that introducing a fluorine atom into the phenyl ester group caused high fish toxicity. The results demonstrated the environmental safety of the new synthetic compound 4c as a cercaricide and molluscicide, while it showed negligible toxicity to human kidney cells and *Danio rerio*. In the aspect of toxicity, compound 4c has achieved a noticeable improvement over niclosamide.

In order to understand the molecular mechanism of niclosamide and salicylanilide ester derivatives in cercariae, we investigated their enzyme-inhibitory activities. Three important enzymes, NOS, LDH, and AChE, were employed. Nitric oxide (NO) is a simple molecule that displays very important functions in many physiological and pathophysiological processes (25–27). It is produced by a variety of cells in vertebrates and invertebrates from the substrate L-arginine by NOS (28). Evidence of NO synthesis or NOS-like activity has been found in different locations of worms, including *Trichinella britovi*, *Hymenolepis diminuta*, and *Schistosoma japonicum* (29, 30). LDH, which plays an important role in the last step of glycolysis, is the representative enzyme in the biological anaerobic glycolysis pathway (31–34). It is well known that parasitic stages of the schistosome depend largely on anaerobic energy metabolism. Glycolytic enzymes are essential for the survival of the parasite and appear to be potential targets for chemotherapeutic attacks. Acetylcholine (ACh) has long been considered a neurotransmitter in the nervous systems of parasitic worms (35). The principal physiological role of AChE is believed to be termination of transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter (ACh) (36). ACh and AChE have been demonstrated to be involved in the motor activity of the schistosome (37–41). Their presence has been established at several developmental stages of the parasite, including cercaria, schistosomula, and adult worm.

According to the results, the molecular mechanisms of salicylanilide ester derivatives and niclosamide were a little different. Salicylanilide esters showed potent inhibitory activity on NOS but no effect on LDH and AChE. Niclosamide exhibited moderate to potent activity on all three enzymes. It seems as though introducing an ester group and replacing the chlorine atom of nitroaniline with methoxyl could selectively inhibit NOS but not LDH and

ACHe. Interestingly, a strong and significant correlation between NOS-inhibitory efficacy and cercaricidal activity was also observed. Further RT-PCR results indicated that the NOS gene was responsive to 4c treatment. The expression of NOS was significantly downregulated by compound 4c in a dose-dependent manner. The present work revealed that compound 4c could down-regulate NOS expression and inhibit its enzyme activity, which suggested that NOS was probably one of the drug targets of salicylanilide esters. By targeting NOS, they could influence the synthesis of NO, leading to the disorder and loss of various physiological functions, and eventually result in the death of cercariae.

Conclusions. In summary, a series of 12 salicylanilide ester derivatives were designed and prepared. A few derivatives showed a significant cercaricidal effect against *Schistosoma japonicum* and molluscicidal activity against *Oncomelania hupensis*. As we expected, the results of the toxicity assessment demonstrated that some of the active derivatives exhibited remarkably reduced toxicity compared with that of niclosamide. Moreover, SARs revealed that cercaricidal activity of the derivatives was well correlated with their NOS-inhibitory activities, and RT-PCR results indicated that compound 4c downregulated the expression of NOS in a dose-dependent manner; together, these results suggested that NOS probably was one of the drug targets. It is noteworthy that compound 4c showed not only potent cercaricidal and molluscicidal activity but also negligible toxicity to human kidney cells and *Danio rerio*, which indicates that it could be developed as a promising drug candidate against *Schistosoma japonicum* at the transmission stages. Field trials and further studies on the molecular mechanism of compound 4c are under way.

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