Pharmacokinetics and Metabolism of Streptochlorin and Its Synthetic Derivative, 5-Hydroxy-2′-isobutyl Streptochlorin, in Mice

Yuanyuan Zhou, Yeong Jung Choi, Eunyeong Kim, Mun Hwan Oh, Hee Jae Shin,
Sang Kyum Kim, and Kiho Lee*

*College of Pharmacy, Korea University; Sejong 30019, Republic of Korea: b College of Pharmacy, Chungnam National University; Daejeon 34134, Republic of Korea; c Marine Natural Products Chemistry Laboratory, Korea Institute of Ocean Science and Technology (KIOST); Gyeonggi 15627, Republic of Korea: and d Biomedical Research Center, Korea University Guro Hospital; Seoul 08308, Republic of Korea.

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The purpose of this study was to investigate the pharmacokinetics and metabolism of streptochlorin and its derivative 5-hydroxy-2′-isobutyl streptochlorin (HIS) in mice. Plasma concentration of streptochlorin declined rapidly resulting in a high systemic plasma clearance (CL_s) (5.8±1.7 L/h/kg), a large volume of distribution (V_s) (1.4±0.9 L/kg) and a short half-life (t_1/2) (0.4±0.1 h) after a single intravenous administration (5 mg/kg). Oral bioavailability (F) was 10.3±3.4% after a single oral administration (10 mg/kg). HIS also showed a rapid plasma decline with a high CL_s (11.3±8.8 L/h/kg), a high V_s (0.8±1.0 L/kg) and a short t_1/2 (0.07±0.004 h) following intravenous administration. It was not detected in plasma after oral administration. Metabolic stability studies using mouse liver microsomes and S9 fractions predicted a high hepatic clearance for both compounds, consistent with the in vivo data. Metabolite identification studies revealed three metabolic pathways for streptochlorin: monoxygenation, glucuronidation of the indole moiety and oxidative opening of the 4-chlorooxazole ring. HIS was metabolized via monoxygenation of the isobutyl chain and glucuronidation of the indole ring. These results may aid in structural optimization to mitigate the metabolic liability of streptochlorin.

Key words streptochlorin; 5-hydroxy-2′-isobutyl streptochlorin; pharmacokinetics; metabolism

Terrestrial organisms have been a rich source of natural products for drug discovery and development for thousands of years. Since the mid-twentieth century, studies have shown that marine creatures, especially microbes, have provided many bioactive compounds with novel structures. These include compounds such as ziconotide which is an analgesic agent for severe and chronic pain treatment, trabectedin, an agent for severe and chronic pain treatment, trabectedin, an antagonist of the actinomycete Streptomyces sp. as a new antibiotic. 4) Over the past decades, it has demonstrated a variety of biological activities, including antiangiogenic efficacy in capillary tube formation and vascular endothelial growth factor (VEGF)-induced cell invasion in human umbilical vein endothelial cells, via the nuclear factor kappa B signaling pathway. 5) It has shown anticancer and antitumor efficacy in solid tumors and liver cholangiocarcinoma models in BALB/c nude mice. 6–8) Furthermore, it exhibited antifungal activity in passive cutaneous anaphylaxis reaction and 2,4-dinitrofluorobenzene-induced allergic dermatitis in female BALB/c mice via the tyrosine-protein kinase/proto-oncogene tyrosine-protein kinase and spleen tyrosine kinase signaling pathways. 9) And finally, anti-inflammatory effects in the murine lipopolysaccharide-induced acute lung injury model via Toll/interleukin-1 receptor domain-containing adaptor protein inducing interferon-β (TRIF)-dependent signaling pathways. 10)

Streptochlorin contains a privileged scaffold of 5-(3-indolyl)oxazole, which is incorporated in many natural products, including pimprinine, labradorin, martefragin A and almozole C. 11) Efforts have been made to synthesize derivatives to improve its efficacy. 12–14) Among them 5-hydroxy-2′-isobutyl-streptochlorin (HIS) has demonstrated effective anti-inflammatory activities. 15) It was shown to inhibit inflammatory responses in mouse models of lipopolysaccharide-induced acute lung injury and Escherichia coli-induced peritonitis, via TRIF-dependent signaling and inflammasome activation. 16) However, the drug disposition profiles following administration of streptochlorin and HIS in animals remains unknown. Metabolites formed from the compounds are also unexplored, which may provide clues about structure optimization for better pharmacokinetic properties and/or discovery of active metabolites.

In this report, the pharmacokinetics of streptochlorin and HIS were investigated after intravenous (i.v.) and oral (p.o.) administration in mice. Permeability was assessed in parallel artificial membrane permeability assay (PAMPA) to assess their passive membrane permeabilities. Metabolic stability was examined in mouse liver microsomes (MLMs) and liver S9 fractions fortified with relevant cofactors. Their metabolites were also identified and the corresponding metabolic pathways have been proposed.

MATERIALS AND METHODS

Chemicals Streptochlorin and HIS were provided by
Korea Institute of Ocean Science and Technology (Ansan, Republic of Korea) with a purity >99%. Streptochlorin was isolated from Streptomyces sp. as described previously.5) HIS was synthesized as described previously.15) Indole-3-carboxylic acid, atenolol, metoprolol, nadolol, propranolol, ranitidine, verapamil, chloramphenicol, hydrochlorothiazide, ketoprofen, glipizide, reduced nicotinamide adenine dinucleotide phosphate (NADPH), uridine 5'-diphosphogluconic acid (UDPGA), 3'-phosphoadenosine-5'-phosphosulfate (PAPS), dimethyl sulfoxide (DMSO), β-α-tocopheryl polyethylene glycol 1000 succinate, l-α-phosphatidyethanolamine, dodecane, ethanol and Kolliphor® EL were purchased from Sigma-Aldrich (U.S.A.). Pooled male CD-1 MLMs and pooled male CD-1 mouse liver S9 fractions were purchased from BD Biosciences (U.S.A.). Potassium phosphate buffer (0.1 M, pH 7.4) was purchased from Biosesang (Seongnam, Republic of Korea). MultiScreen® 96-well filter plates and transport receiver plates were purchased from Merck Millipore (U.S.A.). Indole-3-glyoxylamide (purity >98%) was purchased from Celldum Science (Republic of Korea). All chemicals were of the highest purity available.

**PAMPA** PAMPA assay was performed in 96-well microtiter and microfilter plate assembly as described previously15) with the following modifications. Sample analyses were conducted using an Agilent 6460 triple quadrupole LC-MS/MS system equipped with dual AJS ESI ion source and Agilent 1200 series HPLC system in multiple reaction monitoring (MRM) mode. Chromatographic separation was performed on Phenomenex Kinetex C18 column (2.1×50 mm, 2.6 μm) at 40°C using deionized water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). Reference samples were run with gradient elutions at a flow rate of 0.4 mL/min: elution parameters were as follows: beginning with 2% B and holding for 0.5 min, ramp to 35% B over 1.5 min, and then ramp to 98% B over 8 min. The injection volume was 5 μL. Retention times for atenolol, metoprolol, nadolol, propranolol, ranitidine, verapamil, chloramphenicol, hydrochlorothiazide, ketoprofen and glipizide were 3.94, 4.52, 4.20, 4.99, 3.99, 5.76, 5.49, 4.51, 7.29 and 6.90 min, respectively. Samples of streptochlorin or HIS were run with the following gradient elution program at a flow rate of 0.4 mL/min: beginning with 45% B and holding for 2 min, ramp to 95% B over 1 min, and then hold for 3 min. Retention times for streptochlorin, HIS and glipizide were 4.76, 4.78 and 4.09 min, respectively. Ion source parameters of the mass spectrometer were set as follows: capillary voltage 4000 V, drying gas 12 L/min, nebulizer gas 35 psi, sheath gas 10 L/min, sheath gas heater 350°C and a drying gas temperature of 325°C. Mass spectral data were acquired in negative auto MS/MS mode with full scan MS/MS modes (F 110 V, CE 30 eV). The mass scan rate was 8 spectra/s for both MS and MS/MS scan modes.

To determine the degradation half-life (t1/2), the analyte versus internal standard peak area ratios were calculated from acquired full scan mass data using Agilent Mass Hunter Quantitative Analysis Software (ver.B.5.0) and converted to percentage remaining, using the 0 min peak area values as 100%. t1/2 was calculated using the following equation: t1/2 = 0.693/k, where k is the first-order degradation rate constant, which was estimated by one-phase exponential decay non-linear regression analysis of the degradation time course using GraphPad Prism® 6 (GraphPad Software, La Jolla, CA, U.S.A.). Apparent intrinsic clearance (CLint) was calculated using the following equation16,17):

\[ CL_{\text{int}} = \frac{k}{f_{\text{u,mic}}} \times \frac{\text{mL incubation}}{\text{mg microsomes}} \times \frac{45 \text{ mg microsomes}}{\text{g liver}} \times \frac{87.5 \text{ g liver}}{\text{kg body wt}} \]

\[ CL_{\text{int}} = \frac{k}{f_{\text{u,s9}}} \times \frac{\text{mL incubation}}{\text{mg liver S9}} \times \frac{143 \text{ mg liver S9}}{\text{g liver}} \times \frac{87.5 \text{ g liver}}{\text{kg body wt}} \]

The scaling factor for mouse liver S9 fractions (143 mg/g liver) was obtained from the literature.18,19) Fractions unbound in microsomes (fucmic) and S9 (fus9) were measured in triplicate by equilibrium dialysis described previously20) fucmic values of streptochlorin and HIS were 0.33±0.04 and 0.09±0.02, respectively. fus9 values were 0.68±0.04 and 0.33±0.02 for streptochlorin and HIS, respectively. Hepatic plasma clearance (CLp) and hepatic blood clearance (CLb, H.) were calculated from CLint values using the ‘well-stirred’ model of the

In Vitro Metabolic Stability Study in MLMs and Mouse Liver S9 Fractions  Microsomal incubations were conducted in 0.1 M potassium phosphate buffer (pH 7.4) in eight-well cluster tubes. The final reaction mixture contained 1 mg/mL mouse liver microsomes, 1 mM NADPH and 1 μM streptochlorin or HIS in a final volume of 160 μL. The mixture was warmed at 37°C for 5 min and then initiated by addition of the cofactor. The reactions were terminated at 0, 1, 2, 5, 10, 15, 30 min by adding equal volume of ice-cold acetonitrile containing glipizide (1 μg/mL) as the internal standard. The tubes were vortexed, sonicated and centrifuged at 3000×g for 20 min. The supernatant was analyzed by Agilent 6530 Q-TOF LC-MS/MS system equipped with dual AJS ESI ion source and Agilent 1200 series HPLC system in a negative auto MS/MS scan mode.

For S9 stability experiments, 1 μM streptochlorin or HIS was incubated at 37°C for 5 min in 50 mM Tris buffer (pH 7.5) containing 1 mg/mL mouse liver S9 fractions, 8 mM MgCl2, 25 μM alamethicin and methanol (0.25 v/v%). The reaction was then initiated by adding cofactors NADPH, UDPGA and PAPS (final concentration: 1 mM) and terminated at 0, 1, 2, 5, 10, 15, 30 min by adding an equal volume of ice-cold acetonitrile containing glipizide (1 μg/mL) as the internal standard. The final incubation volume was 160 μL. The resulting mixtures were vortexed, sonicated and then centrifuged at 3000×g for 20 min. The supernatant was analyzed as described above for the microsomal incubations.

Chromatographic separation was performed on an Agilent Eclipse C18 column (2.1×100 mm, 2.6 μm) maintained at 40°C using a mobile phase consisting of deionized water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The samples were analyzed in a linear gradient elution at a flow rate of 0.6 mL/min: starting with 95% A for 2 min, ramping to 95% B in 2 min, then holding for 3 min. The injection volume was 5 μL. Retention times for streptochlorin, HIS and glipizide were 4.54, 4.58 and 4.41 min, respectively. Ion source parameters of the mass spectrometer were set as follows: capillary voltage 4000 V, drying gas 12 L/min, nebulizer gas 35 psi, sheath gas 10 L/min, sheath gas heater 350°C and a drying gas temperature of 325°C. Mass spectral data were acquired in negative auto MS/MS mode with full scan MS/MS modes (F 110 V, CE 30 eV). The mass scan rate was 8 spectra/s for both MS and MS/MS scan modes.

To determine the degradation half-life (t1/2), the analyte versus internal standard peak area ratios were calculated from acquired full scan mass data using Agilent Mass Hunter Quantitative Analysis Software (ver.B.5.0) and converted to percentage remaining, using the 0 min peak area values as 100%. t1/2 was calculated using the following equation: t1/2 = 0.693/k, where k is the first-order degradation rate constant, which was estimated by one-phase exponential decay non-linear regression analysis of the degradation time course using GraphPad Prism® 6 (GraphPad Software, La Jolla, CA, U.S.A.). Apparent intrinsic clearance (CLint) was calculated using the following equation16,17):

\[ CL_{\text{int}} = \frac{k}{f_{\text{u,mic}}} \times \frac{\text{mL incubation}}{\text{mg microsomes}} \times \frac{45 \text{ mg microsomes}}{\text{g liver}} \times \frac{87.5 \text{ g liver}}{\text{kg body wt}} \]

\[ CL_{\text{int}} = \frac{k}{f_{\text{u,s9}}} \times \frac{\text{mL incubation}}{\text{mg liver S9}} \times \frac{143 \text{ mg liver S9}}{\text{g liver}} \times \frac{87.5 \text{ g liver}}{\text{kg body wt}} \]
liver as follows:\textsuperscript{21} CL_H = \frac{Q_{st}}{f_u \cdot f_a \cdot CL_{int}}(Q_{st} + f_u \cdot CL_{int}/R_b) and CL_{H,B} = \frac{Q_{st} \cdot f_a \cdot CL_{int}}{(Q_{st} + f_u \cdot CL_{int})/R_b}. Mouse hepatic blood flow ($Q_{st}$) was 90 mL/min/kg.\textsuperscript{20} Fraction unbound in plasma ($f_a$) was determined by the same method used for f_a, mic and $f_{a,sp}$, $f_u$ values of streptochlorin and HIS were 0.06±0.01 and 0.03±0.01, respectively. Blood to plasma concentration ratio ($R_b$) was determined following incubation in fresh whole blood as described previously.\textsuperscript{22} $R_b$ values were 1.23±0.06 and 0.88±0.14 for streptochlorin and HIS, respectively. Fraction unbound in blood ($f_u$) was calculated as follows: $f_u = f_a/R_b$. Hepatic extraction ratio ($E_H$) was calculated as: $E_H = CL_H/CL_{int}$.

**Metabolite Identification** The MLMs and S9 incubations with cofactors were carried out as described above in ‘in vitro’ metabolic stability study’ section with 50 µM test compounds. The reactions were terminated after 1 h by the addition of ice-cold acetonitrile containing glipizide as the internal standard. The samples were kept at -20°C for 20 min. The supernatant was analyzed by Agilent 6530 Q-TOF LC-MS/MS system equipped with dual AJS ESI ion source and Agilent 1200 series HPLC system in a negative auto MS/MS scan mode.

For in vivo metabolite identification, mouse plasma samples were obtained and prepared for analysis as described below in ‘in vivo’ pharmacokinetic study’ following a single IV dose of streptochlorin or HIS at 5 mg/kg.

Chromatographic separation was performed on Agilent Eclipse C18 column (2.1×100 mm, 3.5 µm) maintained at 40°C using a mobile phase consisting of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The samples were analyzed by gradient elution at a flow rate of 0.6 mL/min: starting with 95% A for 2 min, ramping to 40% B over 2 min, ramping to 60% B over 3 min, ramping to 95% B over 2 min and maintaining for 3 min. The injection volume was 5 µL. Retention times for streptochlorin, HIS and glipizide were 6.33, 6.49 and 5.98 min, respectively. Ion source parameters of the mass spectrometers were set as follows: capillary voltage 4000 V, drying gas 12 L/min, nebulizer gas 30 psi, sheath gas 10 L/min, sheath gas heater 350°C and a drying gas temperature of 325°C. The samples were transferred to eight-well tube strips. Five microliter aliquots of plasma samples were vortexed, sonicated and centrifuged at 3000×$g$ for 20 min. Fifty microliters of the supernatant mixed with 100 µL of 50% acetonitrile was subjected to analysis using Agilent 6460 Triple Quadrupole LC-MS/MS system equipped with dual AJS ESI ion and an Agilent 1200 series HPLC system. Chromatographic separation was performed on a Phenomenex Kinetex C18 column (2.1×50 mm, 5 µm). The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The samples were run with a gradient elution program at a flow rate of 0.6 mL/min at 40°C (starting with 5% B for 2 min, ramping to 45% B over 0.5 min, ramping to 95% B over 1.5 min, holding for 2 min). The injection volume was 15 µL. Retention times for streptochlorin, HIS and glipizide were 3.99, 4.01 and 3.89 min, respectively. Ion source parameters of the mass spectrometers were set as follows: capillary voltage 3500 V, drying gas 12 L/min, nebulizer gas 30 psi, sheath gas 6 L/min, sheath gas heater 300°C and a drying gas temperature of 300°C. Mass spectrometer conditions were optimized for the detection of each analyte in negative MRM mode: m/z 217→153 (F 70 V, CE 4 eV), 289→253 (F 70 V, CE 5 eV) and 444→319 (F 130 V, CE 20 eV) for streptochlorin, HIS and glipizide, respectively. The linear calibration ranges for streptochlorin and HIS were 50–1000 and 50–1000 ng/mL ($r^2$=0.995). The precision and accuracy of the assay were evaluated using QC samples prepared in triplicate at 2, 200 and 3000 ng/mL. The precision was assessed by examining the relative standard deviation (RSD) of the QC samples. Comparison of average measured concentration versus the corresponding nominal concentration of the QC samples was used to assess accuracy. Both the accuracy (90–120%) and precision (RSD<20%) of the assay were within the acceptable range.

Pharmacokinetic parameters were calculated by noncompartmental analysis using PKSolver.\textsuperscript{24}

**Statistical Analysis** Results are expressed as mean± standard deviation (S.D.). Statistical analysis was performed by Student’s t-test using GraphPad Prism\textsuperscript{TM} 6 and p-values smaller than 0.05 were considered statistically significant.

**RESULTS**

**Permeability of Streptochlorin and HIS in PAMPA** Membrane permeability of streptochlorin and HIS was investigated using the PAMPA system. The apparent permeability coefficient ($P_{app}$) value of streptochlorin (86.0±9.9 nm/s) was higher than those of all five, high permeability reference compounds included in this study (Fig. 1). The $P_{app}$ value of HIS was 48.9±4.2 nm/s, which was significantly higher than those
of four of the high permeability reference compounds including the U.S. Food and Drug Administration (FDA)-recommended high permeability marker metoprolol (15.7±1.5 nm/s, \(p<0.001\))\(^{25}\) (Fig. 1). These results suggest that both streptochlorin and HIS are likely to readily permeate across biological membranes including the intestinal membrane via passive diffusion.

**Metabolic Stability of Streptochlorin and HIS in MLMs and S9 Fractions** The metabolic stability of streptochlorin and HIS was investigated *in vitro* in MLMs and S9 fractions fortified with various cofactors.

Streptochlorin concentration declined rapidly with \(t_{1/2}\) of 0.73 min when incubated with MLMs in the presence of NADPH (Table 1, Fig. 2); no significant change was observed in its concentration when cofactor was absent (data not shown), indicating that streptochlorin is likely metabolized by CYP or flavin-containing monoxygenase in the liver. \(CL_{H}\) and \(CL_{H,B}\) values of streptochlorin predicted from the \(CL_{int}\) value using the ‘well-stirred’ model were 95.2 and 77.4 mL/min/kg, corresponding to a high \(E_{H}\) of 0.86 (Table 1). When incubated in mouse liver S9 fractions in the presence of NADPH, UDPGA and PAPS, streptochlorin declined rapidly with \(t_{1/2}\) of 2.76 min (Table 1, Fig. 2); no significant change in its concentration was observed without cofactors (data not shown). \(CL_{H}\) and \(CL_{H,B}\) values converted from \(CL_{int}\) were 79.1 and 64.3 mL/min/kg. The corresponding \(E_{H}\) was 0.71 (Table 1).

HIS was also metabolized rapidly with \(t_{1/2}<2\) min in MLMs and S9 fractions fortified with cofactors (Table 1, Fig. 2). \(CL_{H}\) and \(CL_{H,B}\) values predicted from \(CL_{int}\) were 69.1 and 78.6 mL/min/kg, with the corresponding \(E_{H}\) value of 0.87 in MLMs. While in S9 fractions, \(CL_{H}\) and \(CL_{H,B}\) values were 77.3 and 87.8 mL/min/kg for S9 fractions, corresponding to a high \(E_{H}\) value of 0.98 (Table 1).

**In Vitro Metabolite Identification for Streptochlorin and HIS in MLMs and S9 Fractions** Metabolite formation from streptochlorin and HIS was investigated *in vitro* using MLMs and S9 fractions at a higher concentration (50 \(\mu\)M) of the test compounds in the presence of cofactors. Structure elucidation for the metabolites was performed using accurate mass data and MS/MS spectra obtained using a Q-TOF LC-MS/MS system. Representative chromatograms and mass spectral data for the putative metabolites are shown in Fig. 3, Tables 2 and 3.

A total of 10 putative metabolites of streptochlorin were identified *in vitro*; among them, five metabolites (M1–M5) were detected in the microsomal incubations and eight metabolites (M2 and M4–M10) in the S9 incubations (Fig. 3A). Streptochlorin eluting at 6.33 min had a \(m/z\) value of 217.0174 ([M–H]\(^−\)) (Fig. 3A); its MS/MS spectrum showed product ions at \(m/z\) 153.0465 formed by the loss of CO (28 Da) and HCl (36 Da), \(m/z\) 126.0292 formed by the loss of HCN (27 Da), and \(m/z\) 102.0360 formed by the loss of the methylene group from the indole ring (Fig. 3A, Table 2). M1 and M2 were eluted at 5.07 and 5.22 min, respectively (Fig. 3A). Their deprotonated molecular ions had \(m/z\) 233.0118 ([M–H]\(^−\)), 16 Da greater than that of streptochlorin, indicating addition of one oxygen atom.

![Fig. 1. Permeability of Streptochlorin and HIS in PAMPA](image)

Fig. 1. Permeability of Streptochlorin and HIS in PAMPA

Open (atenolol, ranitidine, hydrochlorothiazide and nadolol) and closed (metoprolol, propranolol, chloramphenicol, verapamil and ketoprofen) bars represent low and high permeability references, respectively. Nadolol was not detected in the receiver side. Data are mean±S.D. (\(n=3\)).

![Fig. 2. Metabolic Stability of Streptochlorin and HIS in Mouse Liver Microsomes and S9 Fractions](image)

Fig. 2. Metabolic Stability of Streptochlorin and HIS in Mouse Liver Microsomes and S9 Fractions

Streptochlorin and HIS were incubated at 37°C with either NADPH-fortified MLM or mouse liver S9 fractions fortified with NADPH, UDPGA and PAPS simultaneously. Time course of test compound depletion is expressed as % of initial concentration remaining during the incubations. Each point represents mean±S.D. (\(n=3\)).

<table>
<thead>
<tr>
<th>Compound</th>
<th>(t_{1/2}) (min)</th>
<th>(CL_{int}) (mL/min/kg)</th>
<th>(CL_{H}) (mL/min/kg)</th>
<th>(CL_{H,B}) (mL/min/kg)</th>
<th>(E_{H})</th>
<th>(t_{1/2}) (min)</th>
<th>(CL_{int}) (mL/min/kg)</th>
<th>(CL_{H}) (mL/min/kg)</th>
<th>(CL_{H,B}) (mL/min/kg)</th>
<th>(E_{H})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptochlorin</td>
<td>0.73</td>
<td>11327.1</td>
<td>95.2</td>
<td>77.4</td>
<td>0.86</td>
<td>2.76</td>
<td>4620.2</td>
<td>79.1</td>
<td>64.3</td>
<td>0.71</td>
</tr>
<tr>
<td>HIS</td>
<td>1.67</td>
<td>18154.9</td>
<td>69.1</td>
<td>78.6</td>
<td>0.87</td>
<td>0.25</td>
<td>105105</td>
<td>77.3</td>
<td>87.8</td>
<td>0.98</td>
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</table>

Table 1. Prediction of \(CL_{H}\), \(CL_{H,B}\) and \(E_{H}\) of Streptochlorin and HIS in Mice

Mouse liver S9 fractions fortified with NADPH, UDPGA and PAPS
Fig. 3. Representative Extracted Ion Chromatograms (EICs) and Fragmentations for Streptochlorin, HIS and Their Putative Metabolites from in Vitro Incubations in Mouse Liver Microsomes or S9 Fractions

(A) EICs and fragmentations of streptochlorin and its putative metabolites, (B) EICs and fragmentations of HIS and its putative metabolites.
ring (Table 2). M3 eluting at 5.15 min had suggesting that the monooxygenation had occurred on the indole ions at respectively (Fig. 3A); and all had deprotonated molecular retention times of M7, M8 and M9 were 4.30, 4.45 and 4.63 min, i.e., monooxygenation atom by the biotransformation reaction, i.e., monooxygenation (Table 2). Their MS/MS spectra showed three product ions of values 16 Da greater than those of streptochlorin, i.e., m/z 169.0398, 142.0258 and 118.0306, suggesting that monooxygenation had occurred on the indole ring (Table 3). M10 with a retention time of 4.85 min had monooxygenation had occurred on the indole ring (Table 2), suggesting that it was an M5 glucuronide; its isotope pattern indicated monooxygenation (loss of a glucuronic acid moiety), glucuronidation occurred during their biotransformation (Table 2). Their product ion spectra showed product ions at and 85.0299 were characteristic of the glucuronide moiety. and M5 were confirmed by matching retention times, accurate masses, and MS/MS fragmentation patterns using authentic compounds (data not shown). M6 eluting at 4.24 min had m/z ([M−H]−) value of 160.0404 (Fig. 3A, Table 2). Its product ion observed at could be formed from either and 85, 113, 193. M4 was proposed as 2-(1H-indol-3-yl)-2-oxoacetamide with a molecular formula of C15H15ClN2O2 (Figs. 3A, 6). The chemical structures of M4 and M5 were proposed as 1H-indol-3-carboxylic acid (Figs. 3A, 6). M4 eluted at 4.68 min with m/z 187.0508 ([M−H]−), 28 Da lower than that of M3 (Fig. 3A, Table 2). Its product ions (m/z 144.0381, 116.0496) were identical to those of M3 (Table 2), indicating that M4 also contained a structural moiety converted to indolyl-3-carboxaldehyde during collision-induced dissociation (CID). M4 was proposed as 2-(1H-indol-3-yl)-2-oxoacetamide with a molecular formula of C10H14N2O2 (Figs. 3A, 6). M5 eluting at 4.65 min had m/z ([M−H]−) value of 160.0404 (Fig. 3A, Table 2). Its product ion observed at m/z 116.0487 could be formed by neutral loss of CO2 suggesting the presence of a carboxylic acid (Table 2). M5 was proposed as 1H-indole-3-carboxylic acid (Figs. 3A, 6). The chemical structures of M4 and M5 were confirmed by matching retention times, accurate masses, and MS/MS fragmentation patterns using authentic compounds (data not shown). M6 eluting at 4.24 min had m/z ([M−H]−) value of 336.0725, 176Da greater than that of M5 (Fig. 3A, Table 2), suggesting that it was an M5 glucuronide; its product ions observed at m/z 193.0338, 175.0568, 113.0240 and 85.0299 were characteristic of the glucuronide moiety. The product ion at m/z 193.0338 could be formed from either an ether or acyl glucuronide by cleavage of the C–O bond linking glucuronic acid and aglycone, suggesting that M6 was acyl glucuronide of M5 (Table 2). In sum, a metabolic pathway of streptochlorin is proposed in Fig. 4A based on these results.

A total of 5 tentative metabolites of HIS were detected in vitro; among them, 2 metabolites (M1 and M2) were detected in the microsomal incubations and 4 metabolites (M2 and M3–M5) in the S9 incubations (Fig. 3B). HIS eluting at

### Table 2. Mass Spectral Data of Streptochlorin and Its Putative Metabolites

<table>
<thead>
<tr>
<th>Compound</th>
<th>r&lt;sub&gt;t&lt;/sub&gt; (min)</th>
<th>Molecular formula</th>
<th>Precursor ion (m/z, [M−H]−)</th>
<th>Product ion (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptochlorin</td>
<td>6.33</td>
<td>C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;15&lt;/sub&gt;ClN&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>217.0174</td>
<td>217.0172</td>
</tr>
<tr>
<td>M1 (monooxygenation)</td>
<td>5.07</td>
<td>C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;15&lt;/sub&gt;ClN&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>233.0118</td>
<td>233.0117</td>
</tr>
<tr>
<td>M2 (monooxygenation)</td>
<td>5.22</td>
<td>C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;15&lt;/sub&gt;ClN&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>233.0118</td>
<td>233.0121</td>
</tr>
<tr>
<td>M3 (dioxoazolidinone)</td>
<td>5.15</td>
<td>C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;15&lt;/sub&gt;ClN&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>215.0457</td>
<td>215.0447</td>
</tr>
<tr>
<td>M4 (oxoacetamide)</td>
<td>4.68</td>
<td>C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;15&lt;/sub&gt;ClN&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>187.0508</td>
<td>187.0501</td>
</tr>
<tr>
<td>M5 (carboxylic acid)</td>
<td>4.65</td>
<td>C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;15&lt;/sub&gt;ClN&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>160.0404</td>
<td>160.0391</td>
</tr>
<tr>
<td>M6 (glucuronidation of M5)</td>
<td>4.24</td>
<td>C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;15&lt;/sub&gt;ClN&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>336.0725</td>
<td>336.0715</td>
</tr>
<tr>
<td>M7 (monooxygenation + glucuronidation)</td>
<td>4.30</td>
<td>C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;15&lt;/sub&gt;ClN&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>409.0444</td>
<td>409.0415</td>
</tr>
<tr>
<td>M8 (monooxygenation + glucuronidation)</td>
<td>4.45</td>
<td>C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;15&lt;/sub&gt;ClN&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>409.0444</td>
<td>409.0438</td>
</tr>
<tr>
<td>M9 (monooxygenation + glucuronidation)</td>
<td>4.63</td>
<td>C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;15&lt;/sub&gt;ClN&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>409.0444</td>
<td>409.0434</td>
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<tr>
<td>M10 (glucuronidation)</td>
<td>4.85</td>
<td>C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;15&lt;/sub&gt;ClN&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>393.0494</td>
<td>393.0480</td>
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</table>

r<sub>t</sub>: retention time, Bold: most abundant product ion.

### Table 3. Mass Spectral Data of HIS and Its Putative Metabolites

<table>
<thead>
<tr>
<th>Compound</th>
<th>r&lt;sub&gt;t&lt;/sub&gt; (min)</th>
<th>Molecular formula</th>
<th>Precursor ion (m/z, [M−H]−)</th>
<th>Product ion (m/z)</th>
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</thead>
<tbody>
<tr>
<td>HIS</td>
<td>6.55</td>
<td>C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;15&lt;/sub&gt;ClN&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>289.0749</td>
<td>289.0755</td>
</tr>
<tr>
<td>M1 (monooxygenation)</td>
<td>5.11</td>
<td>C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;15&lt;/sub&gt;ClN&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>305.0693</td>
<td>305.0709</td>
</tr>
<tr>
<td>M2 (monooxygenation)</td>
<td>5.48</td>
<td>C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;15&lt;/sub&gt;ClN&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>305.0693</td>
<td>305.0698</td>
</tr>
<tr>
<td>M3 (monooxygenation + glucuronidation)</td>
<td>4.59</td>
<td>C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;15&lt;/sub&gt;ClN&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>481.0989</td>
<td>481.0995</td>
</tr>
<tr>
<td>M4 (monooxygenation + glucuronidation)</td>
<td>4.79</td>
<td>C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;15&lt;/sub&gt;ClN&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>481.0989</td>
<td>481.0977</td>
</tr>
<tr>
<td>M5 (glucuronidation)</td>
<td>5.16</td>
<td>C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;15&lt;/sub&gt;ClN&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>465.1048</td>
<td>465.1050</td>
</tr>
</tbody>
</table>

r<sub>t</sub>: retention time, Bold: most abundant product ion.
6.55 min had \( m/z \) 289.0758 ([M−H]) (Fig. 3B); its product ion spectrum showed product ions at \( m/z \) 253.0974 formed by the loss of HCl, \( m/z \) 168.0315 formed by the loss of 3-methylbutan-1-imine moiety, and \( m/z \) 140.0372 formed by the loss of CO from the product ion of \( m/z \) 168.0315 (Table 3). M1 and M2 eluted at 5.11 and 5.48 min, respectively, with \( m/z \) 305.0693 ([M−H]) 16 Da greater than that of HIS (Fig. 3B), and were assigned as monooxygenated metabolites of the parent compound. The product ion spectra of M1 and M2 showed fragment ions at \( m/z \) 169.0402, \( m/z \) 141.0427 and \( m/z \) 113.0235, suggesting that the monooxygenation had occurred on the isobutyl chain (Table 3). M5 with a retention time of 5.16 min had \( m/z \) 289.0966 formed by the loss of the glucuronic acid moiety, \( m/z \) 253.0966 formed by the loss of HCl, and \( m/z \) 113.0232, a characteristic product ion of glucuronic acid (Table 3). The retention times of M3 and M4 were 4.59 and 4.79 min, respectively (Fig. 3B); both metabolites had \( m/z \) 481.0989 ([M−H]) (Fig. 3B), which is 16 Da higher than that of M5, indicating they were formed by monooxygenation and mono-glucuronidation of the parent (Fig. 4B). The product ion spectra of M4 showed product ions at \( m/z \) 269.0905 formed by the simultaneous loss of glucuronic acid and HCl, \( m/z \) 163.0218 characteristic of glucuronide, and \( m/z \) 169.0362 indicative of monooxygenation on the isobutyl moiety (Table 3). The proposed metabolic pathway of HIS is shown in Fig. 4B.

**Pharmacokinetics of Streptochlorin and HIS in Mice**

The pharmacokinetics of streptochlorin and HIS were investigated following a single intravenous (5 mg/kg) or oral
After IV administration, the plasma concentration of streptochlorin decreased rapidly in a multi-exponential manner and fell below the quantitation limit (<15.6 ng/mL) after 2 h (Fig. 5A). Streptochlorin had a high systemic plasma clearance ($CL_p$) of 5.8±1.7 L/h/kg and a high blood clearance ($CL_b$) of 4.7±1.4 L/h/kg, close to the hepatic blood flow of the mouse (5.4 L/h/kg), and a short $t_{1/2}$ of 0.4±0.1 h (Table 4). It also had a high volume of distribution at steady state ($V_{ss}$) of 1.4±0.9 L/kg well exceeding the total body water, suggesting that it distributes well outside the vasculature (Table 4). Streptochlorin was absorbed rapidly with a peak time ($t_{max}$) of 10 min following the oral dose (Fig. 5A, Table 4). The $t_{1/2}$ was similar to IV administration (Table 4). The plasma levels were below the quantitation limit (<15.6 ng/mL) 1 h after dosing (Fig. 5A). The oral bioavailability ($F$) was 10.3±3.4% (Table 4).

The plasma concentration of HIS also declined rapidly after IV administration and fell below the quantitation limit (<15.6 ng/mL) after 30 min (Fig. 5B). HIS had a high $CL_p$ of 11.3±8.8 L/h/kg and $CL_b$ of 12.8±10.0 L/h/kg far exceeding the hepatic blood flow of the mouse, and a short $t_{1/2}$ of 0.071±0.004 h (Table 4). The $V_{ss}$ was 0.8±1.0 L/kg. The pharmacokinetic parameters could not be determined following an oral dose of HIS because its plasma levels were below the quantitation limit throughout the time course.

**In Vivo Metabolite Identification in Mice**

Metabolite identification was conducted using mouse plasma obtained for the pharmacokinetics studies following a single i.v. dose of streptochlorin or HIS at 5 mg/kg. Metabolites were identified using the same method described above in the *in vitro* metabolite identification study.

M3 and M10 were detected in the plasma following the streptochlorin administration (Fig. 5C). The $t_{max}$ of M3 was the same as the first plasma sampling time (i.e., 5 min) of the pharmacokinetics study and its plasma level declined rapidly...
thereafter (Fig. 5C), indicating that the metabolite was both formed and rapidly cleared in the body. M10 also had a shorter $t_{\text{max}}$ and $t_{1/2}$ indicating rapid formation and clearance of the metabolite, although its plasma peak areas were much higher than those of M3 throughout the time course (Fig. 5C).

M2, M4 and M5 were detected in plasma following the HIS administration (Fig. 5D). In a similar manner to streptochlorin, all the detected metabolites showed a short $t_{\text{max}}$ and $t_{1/2}$ indicating rapid formation and clearance (Fig. 5D). The plasma peak areas of M5 were much higher than those of other detected metabolites throughout the time course (Fig. 5D).

DISCUSSION

In this study, we report the metabolism and pharmacokinetics of streptochlorin and its derivative HIS, in mice. The results of these studies may give us a better understanding of metabolic fate and disposition properties of 5-(3-indolyl) oxazole-containing natural products.

Streptochlorin showed a high $CL_{\text{H}}$ and $CL_{\text{E}}$, close to the hepatic blood flow in mice. Streptochlorin was metabolized rapidly in in vitro metabolic stability studies resulting in a high predicted $CL_{\text{H}}$ and $CL_{\text{E}}$, consistent with the in vivo data. The metabolism of streptochlorin was slower in the liver S9 fractions compared to the microsomes. This may suggest that streptochlorin is metabolized mainly by CYP as liver S9 fractions generally show about 20–25% CYP activity compared to liver microsomes.

Streptochlorin had a low oral bioavailability (10.3 ± 3.4%). This may be attributed to extensive hepatic first-pass metabolism based on its high $CL_{\text{E}}$ and $E_{\text{H}}$ predicted from the in vitro metabolic stability studies. The high PAMPA permeability suggests that intestinal permeation was not likely rate limiting for its oral absorption unless efflux transporters were involved. Molecular properties known to be associated with oral drug absorption, such as molecular weight (MW) (218.6), $c\log P$ (2.60), polar surface area (PSA) (33.6 Å²), number of hydrogen bonds (donors=1, acceptors=3) and number of rotatable bonds (1), were all within ranges consistent with its high membrane permeability.

HIS also showed a high $CL_{\text{H}}$ and $CL_{\text{E}}$ in vivo, consistent with the in vitro metabolic stability data. HIS was shown to be metabolized faster in the S9 fractions compared to the microsomes, indicating that phase II biotransformation was the predominant metabolic pathway in vitro. HIS was not detected in the plasma after an oral dose given at 10 mg/kg. This is in agreement with the in vitro metabolic stability data predicting extensive hepatic first-pass metabolism, i.e., predicted $E_{\text{H}} = 1$. Its high PAMPA permeability and optimal molecular properties (MW: 290.1, $c\log P$: 3.67, PSA: 53.9 Å²), number of hydrogen bond donors: 2, number of hydrogen bond acceptors: 4, number of rotatable bonds: 3) again suggest that the intestinal permeation was not likely to be the main reason for poor oral absorption.

A total of 10 putative metabolites were identified following the in vitro stability studies of streptochlorin; mass spectral data indicated that they were formed by monoxygenation, glucuronidation, a combination of both, and the oxidative opening of the 4-chlorooxazole ring. Two that were formed by oxidation and glucuronidation respectively, were detected as circulating metabolites in vivo in mice.

Oxidative cleavage of the 4-chlorooxazole ring produced three unusual metabolites: M3 (indole-oxazolidine-2,4-dione), M4 (indole-3-glyoxylamide) and M5 (indole-3-carboxylic acid). Formation of M3 from streptochlorin appears to involve two steps, an oxidation at C2 position of the oxazole ring followed by an oxidative dechlorination (Fig. 6). Oxidation at the C2 position of oxazole has been reported previously. The platelet aggregation inhibitor ditaazole, is primarily metabolized to 2-oxazolone by sequential N-dealkylation followed by oxidative deamination. 2-Benzooxazolone metabolite has been found during metabolism of the muscle relaxant oxazolamine in rats. Further, the antibactericidal agent delamanid, provides another example of oxidative deamination on the 2-amino oxazole. It has also been reported that without any substituent at the C2 position, oxazole has been metabolized to 2-oxazolone by aldehyde oxidase. The electron-deficient $sp^2$ carbon atom is susceptible to attack by a nucleophile, like oxygen of water. Oxazole has a carbon–hydrogen bond adjacent to an aromatic nitrogen atom, which is characteristic of substrates of aldehyde oxidases. However, streptochlorin metabolized to 2-oxazolone was NADPH-dependent, thereby indicating involvement of CYP, not aldehyde oxidase. Oxidative dehalogenation is a common reaction of aromatic ring halogen substituents. Oxidative dehalogenation of the 4-halide group of oxazole ring has been reported before. For instance, the reaction of 2-methyl-4-bromo-5-phenyloxazole with NaOH yields 2-hydroxyphenylacetic acid via the intermediate 4-oxazolone. In fact, replacement of the 2-chloro group on oxazole with other moieties is an extremely facile process in the preparation of 2-amino-, 2-alkoxy- and 2-hydrazoneoxazoles. Therefore, the 4-chloride of streptochlorin oxazole was likely replaced by a hydroxyl group followed by tautomerization to the ketone metabolite M3. It is proposed that an oxidative cleavage of oxazolidinedione ring of M3 results in the formation of oxoacetamide metabolite M4 (Fig. 6). The proposed mechanism involves oxidation of the C5 atom of oxazolidinedione ring and a subsequent ring opening accompanying the loss of CO₂. Many drugs such as vinclozolin,
famoxadone, pentoxazone, oxadixyl, myclozolin, dichlozoline and chlozolinate have oxazolidinedione rings. A 2-hydroxyl-acetoamide metabolite of the fungicide vinclozolin was detected in rat liver microsomes; it could also be produced in aqueous buffers at basic pH, suggesting that it was the product of non-enzymatic hydrolysis of the ester group.\(^{38,39}\) A similar 2-hydroxyl-acetamide metabolite was found after administration of famoxadone in rats.\(^{40}\) 2-Oxoacetamide metabolites have also been found previously. An oxoacetamide metabolite of the herbicide pentoxazone was formed by opening the oxazolidinedione ring followed by decarboxylation, and was the principal metabolite produced by microbial strains in paddy field soil.\(^{41}\) The fact that this oxoacetamide metabolite can be produced abiotically, indicates a mechanism by hydrolysis of ester group. An oxazolidinedione-containing hypoglycemic agent underwent oxazolidinedione ring cleavage to form both keto-amide and 2-hydroxyl-acetamide entities in rat, dog, monkey and human liver microsomes.\(^{42}\) Likewise, the conversion of M3 to M4 may be concurrent with decarboxylation, although the exact mechanism was not determined here. M4 itself was found in several marine sources, such as marine sponges \(Rhopaloeides\) odorabile and \(Hyrriotos\) sp., marine sponge \(Spongiosorites\) sp.,\(^{43}\) marine sponge \(Cryptpsula\) pallasiana sp.\(^{45}\) It is also known to be an intermediate in the synthesis of marine natural products, such as arborescindines,\(^{46}\) and is an endogenous ligand for aryl hydrocarbon receptor for treatment of cancer or immune system diseases.\(^{47}\) The carboxylic acid metabolite M5 was proposed as the hydrolytic product of M4 (Fig. 6). A similar mechanism was found in isatin (1H-indoline-2,3-dione) metabolism in rats.\(^{30,48}\) Isatin contains a cyclic \(\alpha\)-ketoamide structure, which was formed from indole by oxidation at C2 and C3 positions. Cleavage of the C–C bond between two carbonyl carbons resulted in N-formylanthranilic acid having a carboxylic acid group and a formamide group. Then N-formylanthranilic acid produced anthranilic acid and formate, the latter being the source of CO\(_2\) detected in the expired air. Likewise, when M4 was hydrolyzed to M5, formamide or its degradation products CO and NH\(_3\) may be formed (Fig. 6). The identities of M4 and M5 were further verified by comparison with authentic standards using chromatograms and mass spectra.

A total of 5 putative metabolites were identified with the \textit{in vitro} metabolic stability studies of HIS; mass spectral data indicated that they were formed by monooxygenation on the isobutyl group and glucuronidation. In contrast to streptochlorin, metabolites formed by oxidative ring opening of the oxazole moiety were not observed. Incorporation of an isobutyl side chain at the C2 position appears to prevent the oxazole ring from being opened by blocking oxidation at that position, although the new side chain itself was extensively metabolized. The 5-hydroxy group as well as the nitrogen atom of the indole ring could be glucuronidated in HIS. This additional site for phase II metabolism appears to expedite its metabolic clearance, as HIS metabolism was faster than streptochlorin in liver S9 fractions, but not in liver microsomes.

**CONCLUSION**

In conclusion, both streptochlorin and HIS have high systemic clearance and poor oral bioavailability, a suboptimal pharmacokinetic profile for systemic therapy agents. These pharmacokinetic properties appear to be mainly due to extensive metabolism. Therefore, structural optimization to mitigate this metabolic stability issue may lead to discovery of drug
candidates with acceptable pharmacokinetic properties.

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Conflict of Interest The authors declare no conflict of interest.

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