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Scopine as a Novel Brain-Targeting Moiety Enhances the Brain Uptake of Chlorambucil

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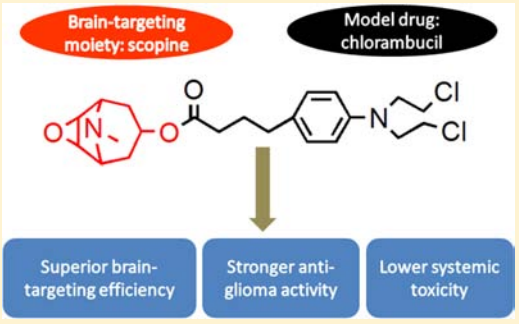
Supporting Information

ABSTRACT: The blood brain barrier (BBB) represents the biggest challenge for therapeutic drugs to enter the brain. In our study, we selected chlorambucil (CHL), an alkylating agent, as the model therapeutic agent, and used scopine as a novel brain-targeting moiety. Here, we synthesized Chlorambucil-Scopine (CHLS) prodrug and evaluated its brain-targeting efficacy. The tissue distribution study after i.v. injection revealed that the $AUC_{0\text{-}t}$ and C_{max} of CHLS in the brain were 14.25- and 12.20-fold of CHL, respectively. Specifically, CHLS accumulated in bEnd.3 and C6 cells in an energy-dependent manner. In C6 cells, superior anti-glioma activity with a significantly decreased IC_{50} of 65.42 nM/mL was observed for CHLS compared to CHL (IC_{50} > 400 nM/mL). The safety evaluation, including acute toxicity, pathology, and hematology study, showed minimal toxicity toward nontargeting tissues, and also reached a lower systemic toxicity at 5 mg/kg (i.v.). Our results suggested that scopine is a potential brain-targeting moiety for enhancing the brain uptake efficiency of CHL.



INTRODUCTION

The major obstacle for drug delivery to the brain is the blood brain barrier (BBB), which prevents nearly all large molecules and 98% of small molecular drugs from entering the central nervous system (CNS).[1] Although nanoparticle-based delivery systems, intrathecal administration strategy, nasal administration strategy, and BBB reversible open strategy were reported to challenge BBB and achieve successful brain accumulation, their targeting efficiency has remained relatively low and safety issues were observed when they were administered systemically.[2−4] Prodrug approaches, however, with relatively high brain targeting efficiency and low systemic toxicity have appeared as a promising strategy.

Multiple prodrug strategies have been developed to modify brain-limited drugs to operate their therapeutic effect in the CNS, such as (1) lipidization of the therapeutic molecule to enhance passive diffusion;[5] (2) chemical delivery systems (CDS) using a lipophilic carrier to facilitate penetration of the BBB and achieve retention in the brain by being oxidized to the hydrophilic form in the brain;[6−8] (3) carrier−drug conjugates whose transporters are located within the brain capillary endothelium, e.g., LAT 1, GLUT 1, SVCT 2;[9−11] and (4) ligand−drug conjugates which may undergo receptor mediated endocytosis, e.g., insulin, transferrin.[12,13]

In our previous works, we found that *N,N*-dimethyl amino as a modification group significantly enhanced the brain-uptake efficiency of dexibuprofen, naproxen, 5-fluorouracil, and dopamine.[14−17] *N,N*-Dimethyl amino is a linear chain tertiary amine, and whether a cyclic tertiary amine will have a similar brain targeting effect remains to be addressed. Therefore, we tested four cyclic tertiary amines (piperidinol, tropine, quinuclidinol, and scopine) as modification moieties for brain-targeting ability, and all the structures have an alcoholic hydroxyl group that could be easily conjugated to the carboxyl site of the model drug chlorambucil (CHL). CHL is an alkylating agent which has been extensively used clinically for cancer chemotherapy.[8] However, in previous research, CHL was proven to be greatly challenged by BBB so that it could not accomplish its antitumor activity in the CNS.[18] Thus, we selected chlorambucil as the model drug. We tested four cyclic tertiary amines conjugated CHL prodrugs and our results suggested that they all significantly enhanced the brain uptake of CHL. Among them, scopine showed the highest CHL concentration in the brain (data not shown). Thus, scopine was selected for the following study.

Scopine (Figure 1) is one of the metabolites of scopolamine which is a muscarinic antagonist used in the clinic to prevent motion sickness.[19] No pharmacological activity of scopine was reported and our study established for the first time the role of scopine as a brain-targeting moiety. Scopine has a simple and well-defined structure as well as a low degree of toxicity compared with macromolecular based targeting moieties.

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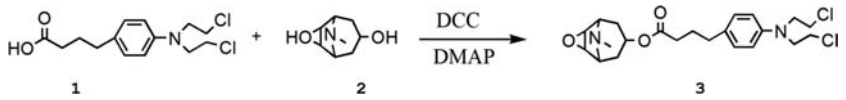

Figure 1. Schematic representation of the synthesis of CHLS. (1) CHL; (2) scopine; (3) CHLS.

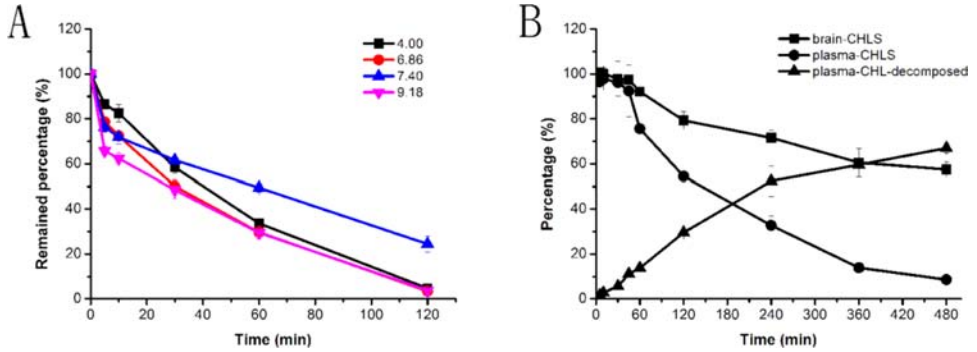

Figure 2. Stability of CHLS in (A) 0.1 M phosphate buffers at pH 4.00, 6.86, 7.40, and 9.18; (B) plasma and brain homogenates. Data represent mean ± SD (n = 3).

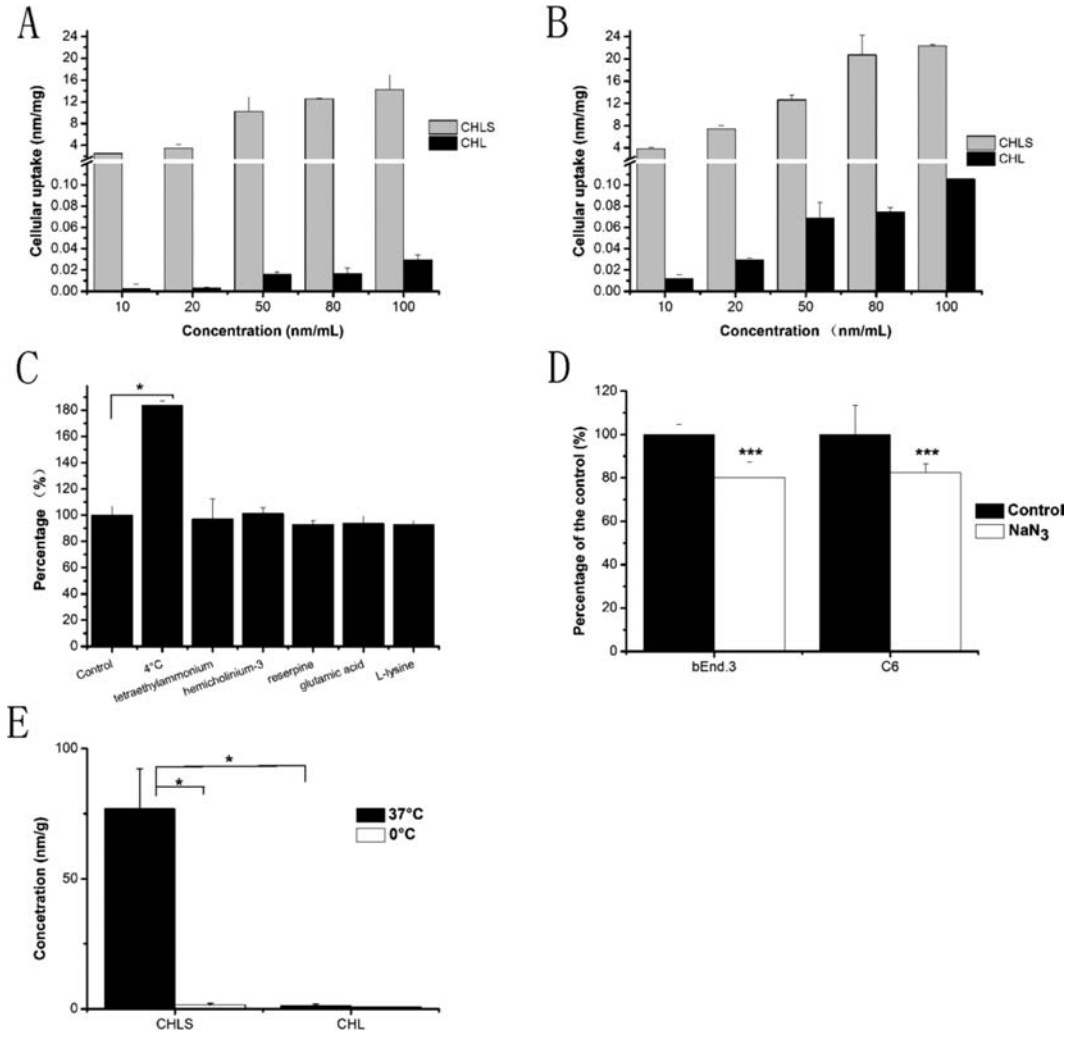

Figure 3. Cellular uptake of CHL and CHLS in bEnd.3 cells (A) and C6 cells (B). Cellular uptake of CHLS in bEnd.3 cells under different inhibitors (C). * indicates statistically different at $p < 0.05$. The inhibition effect of NaN_3 on cellular uptake of CHLS in bEnd.3 cells and C6 cells (D). *** indicates statistically different at $p < 0.001$ versus control group. In situ rat brain perfusion (E). Data represent mean ± SD (n = 5), * indicates statistically different at $p < 0.05$.

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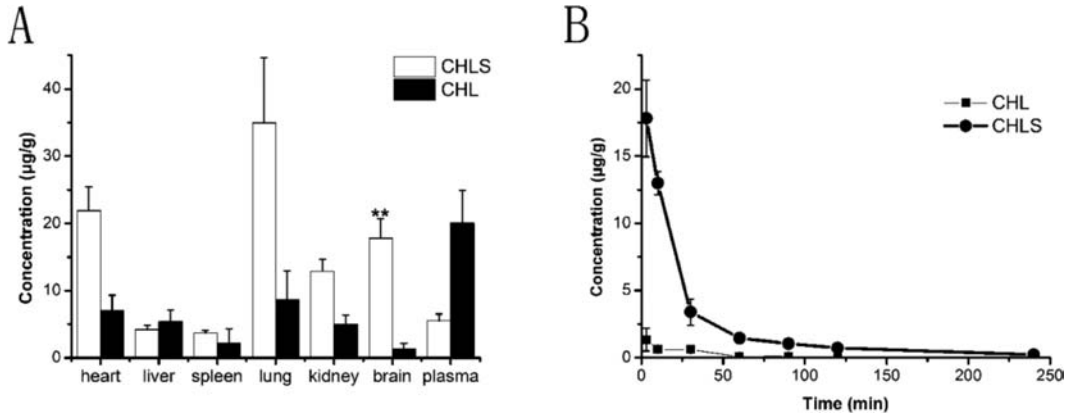

Figure 4. Tissue distribution of CHL and CHLS 5 min after administration (A) and the mean concentration−time curves of CHL and CHLS in the brain (B).[28] Data represent mean ± SD (n = 6), ** indicates significant differences at $p < 0.01$ versus CHL group.

In this study, we investigated the tissue distribution of the Chlorambucil−Scopine conjugate (CHLS, Figure 1), studied the cellular uptake and in situ rat brain perfusion efficiency, evaluated its anti-glioma activity on C6 cells, and conducted its safety evaluation as a novel drug.

RESULTS

Synthesis of Chlorambucil−Scopine (CHLS). CHLS was synthesized as outlined in Figure 1 and it was obtained as a colorless oil (yield 82.3%). ^{1}H NMR, ^{13}C NMR, and ESI-MS confirmed the assigned structure (Supporting Information Figure S1, S2, S3). The detailed results are shown below.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.05 (d, 2H, PhH, J = 8.4), 6.62 (d, 2H, PhH, J = 8.8), 4.99 (t, 1H, COOCH, J = 5.2), 3.71 (m, 4H, $(CH_2Cl)_2$), 3.64−3.59 (m, 6H, $N(CH_2)_2$, $O(CH)_2$), 3.17 (m, 2H, $N(CH)_2$), 2.56−2.51 (m, 5H, $OCOCH_2$, NCH_3), 2.26 (t, 2H, $PhCH_2$, J = 7.2), 2.14 (m, 2H, $CH_2\underline{CH_2}CH_2$), 1.88−1.56 (m, 4H, $OCH(\underline{CH_2})_2$); ^{13}C NMR (100 MHz, $CDCl_3$): 171.97, 144.17, 129.94, 129.40, 111.89, 65.46, 58.13, 56.02, 53.28, 42.21, 40.33, 33.88, 33.73, 30.89, 26.41; ESI-MS: m/z $[M + H]^+$: 441.1.

In Vitro Stability of CHLS. As shown in Figure 2, the half-lives of CHLS in 0.1 M phosphate buffers at different pH values were less than 1 h, and both the acidic and alkaline conditions accelerated the rate of hydrolysis. No CHL decomposition was observed in the aqueous media; as a result, the site of hydrolysis might not be the ester bond but the nitrogen mustard side. Nitrogen mustard compounds tend to degrade to mono and dihydroxy derivatives in aqueous media and thus CHLS might undergo hydrolysis via the same route.[20] However, in the plasma, CHLS was shown to hydrolyze via the cleavage of the ester bond and release CHL, and about 75.49% of total CHL remained after 8 h. In the brain homogenate, 57.46% of CHLS remained after 8 h, and only a small fraction of decomposed CHL was detected, possibly due to the lack of specific esterases in the brain. All these results indicated that CHLS was not that stable in aqueous media, but CHLS showed good stability in plasma and brain homogenate in vitro, which was probably due to the protein binding effect to decelerate the nitrogen mustard side hydrolysis.[21]

Cellular Uptake. The bEnd.3 cells are murine brain endothelial cells exhibiting endothelial properties and widely used as a model for mimicking brain capillary endothelial cells.[22−24] The C6 cells are a widely used brain glioma cell line initially derived from rat glial tumors by N-nitrosomethylurea.[25] As shown in Figure 3A,B, the cellular uptake of CHLS was concentration-dependent in both bEnd.3 cells and C6 cells. CHLS achieved a significantly higher level of accumulation in both cell lines than CHL at all concentrations investigated, and an extraordinary 1020 times improvement in bEnd.3 cells at 20 nM/mL was observed for CHLS. The significant differences in cellular uptake efficiency between CHLS and CHL illustrated that the conjugation of scopine extremely changed the cellular uptake activity of CHL.

Preliminary Uptake Mechanism Study. To study the uptake mechanism of CHLS, a series of inhibitors were preincubated with bEnd.3 cells and then their uptake rate was investigated. Tetraethylammonium (inhibitor of OCTs), hemicholinium-3 (inhibitor of the choline transport system), reserpine (inhibitor of vesicular monoamine transporter), and glutamic acid and L-lysine (inhibitors of amino acid transporters) displayed no inhibition effect on cellular uptake of CHLS in bEnd.3 cells. The effect of temperature was also investigated; however, CHLS showed greater cellular uptake efficiency at 4 °C than that of 37 °C, which might be due to the increased rate of hydrolysis of nitrogen mustards in the aqueous media at a higher temperature during the incubation time resulting in a lower concentration of CHLS at 37 °C compared to 4 °C (Figure 3C).

However, compared with controls, sodium azide significantly inhibited the cellular uptake of CHLS in bEnd.3 and C6 cells which showed 80.12% and 82.29% of cellular uptake, respectively, indicating an energy-dependent uptake mechanism of CHLS (Figure 3D).

In Situ Rat Brain Perfusion. As shown in Figure 3E, scopine significantly enhanced the brain uptake of CHLS, and the concentrations of CHLS and CHL in the right brain hemisphere at 37 °C were 76.93 ± 15.39 and 1.52 ± 0.65 nM/g, respectively. However, when the perfusion was performed at 0 °C, the brain uptake of CHLS significantly decreased to 1.21 ± 0.79 nM/g, but no significant differences were observed between 37 and 0 °C for CHL. The results revealed that the brain uptake of CHLS was an energy-dependent process, and CHL mostly likely achieved BBB penetration by passive diffusion.

The in situ rat perfusion technique which represents the BBB in normal physiological state is for studying brain uptake and its mechanism. The peripheral pharmacokinetics of a drug, such as binding to plasma proteins, absorption, metabolism, and clearance, which are different in rats and mice, are simplified.[9,26]

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In addition, Y. Sawada's group reported that the brain capillary permeabilities are similar in mice and rats.[27] As a result, the effect of temperature on the mechanism of CHLS uptake in rats can also apply to mice. The in situ brain perfusion model is well-established in rats, which needs to have the right common carotid artery catheterized to perfuse. The common carotid artery of adult rats is easy to dissect and catheterize, but for mice, their common carotid artery is too thin to catheterize. For these reasons, although we chose Kunming mice for other study in this paper, in this section, we selected Wistar rats.

Pharmacokinetics and Tissue Distribution. After i.v. injection, the ester bond of CHLS would gradually hydrolyze and release CHL. Thus, CHLS distribution was calculated as the sum of CHLS and CHL decomposed from CHLS. As shown in Figure 4A, the concentration of CHLS was less than that of CHL in plasma, but more in almost all organs. However, the relative uptake efficiency (Re) of brain was about 14.25, which was the highest among all organs. Moreover, the concentration efficiency (Ce) of brain was the highest as well. As Table 1 illustrated, CHLS accumulated more in the brain and displayed a longer elimination half-life than CHL. Also, the drug targeting index (TDI) was 6.20 for CHLS. All these results suggested that CHLS has a very good brain-targeting ability.

Table 1. Pharmacokinetic Parameters of CHLS and CHL in the Brain after Intravenous Administration (n = 6)

parameters	CHL	CHLS
$AUC_{0\text{-}t}$ (nM/mL·h)	2.03 ± 0.47	28.89 ± 1.60[a]
C_{max} (nM/mL)	4.82 ± 2.21	58.79 ± 9.42[a]
$T_{1/2}$ (h)	0.36 ± 0.08	0.78 ± 0.16[b]
Re	-	14.25
Ce	-	12.20
TDI	-	6.20

[a] $p < 0.001$. [b] $p < 0.01$ versus CHL group.

Alkylating Activity. The nitrogen mustards bind to cellular DNA to perform anticancer activity.[29] The DNA cross-linking alkylating activity of CHLS and CHL was thus compared as previously described.[30] CHL metabolizes extensively mainly via β-oxidation of its butyric acid side-chain in biological matrix and these metabolites are also bifunctional nitrogen mustards.[31,32] As a result, we tested not only the drugs alone, but also the total bifunctional nitrogen mustards after incubation with plasma and brain homogenate.

As shown in Figure 5A, the alkylating activity of CHLS was 71.23% compared with CHL. However, after incubation with plasma or brain homogenate, the alkylating activity of CHLS increased to 90.13% and 90.46% compared with CHL, respectively. Although the value of CHLS was still a little bit less than CHL, no significant differences between them were observed.

In Vitro Cytotoxicity against C6 Glioma Cells. Figure 5B shows the cytotoxicity of CHLS, CHL, and scopine against C6 glioma cells in vitro. The IC_{50} value of CHLS was 65.42 nM/mL, while CHL displayed an IC_{50} value higher than 400 nM/mL. Scopine displayed no cytotoxicity against C6 glioma cells under investigated concentrations.

Cell Cycle Assay. The histograms of the DNA content distribution in C6 cells with CHL and CHLS are shown in Figure 5C. In contrast to CHL, CHLS arrest more cell cycle progression in the G_2/M phase and fever cell population in the G_0/G_1 phase, showing its stronger mitotic activity. Furthermore, cell accumulation in the sub-G_1 phase was 1.24 ± 0.24%, 1.58 ± 0.47%, and 7.74 ± 1.03% for the control, CHL, and CHLS, respectively, indicating that CHLS could strongly induce apoptosis of C6 cells.[29,32,33]

Cell Apoptosis Assay. Cells stained with Annexin-V/PI were separated into four groups: viable (Annexin-V^-/PI^-), early apoptotic (Annexin-V^+/PI^-), late apoptotic or secondary necrotic (Annexin-V^+/PI^+), and necrotic (Annexin-V^-/PI^+) cells. As shown in Figure 5D, C6 cells treated with CHLS were most likely to be in the early apoptotic stage and the fraction of apoptotic cells was significantly higher than that of CHL group (99.6 ± 0.17% for CHLS versus 32.6 ± 6.4% for CHL), indicating CHLS's extraordinary pro-apoptotic effect.

Acute Toxicity Evaluation. After a single injection and 14 days of observation, the mortality of mice was calculated by SPSS 19 software to obtain LD_{50}. The LD_{50} of CHL was about 16.00 mg/kg, and its 95% confident interval was 14.59−17.55 mg/kg. The estimated LD_{50} for CHLS was around 22.05 mg/kg, and its 95% confident interval was 19.64−24.61 mg/kg (the value of CHLS has been converted to the equivalence of CHL). The results revealed that CHLS significantly decreased the acute toxicity of CHL.

Blood Cell Hemolysis. Most of the anticancer drugs induced blood cell hemolysis resulting in anemia in patients; thus, reducing the hemolysis of CHL was of great importance.[34] As shown in Figure 6A, CHLS significantly decreased the hemolysis of red blood cells from 70.30% to 25.95%, indicating its better blood compatibility than CHL.

Toxicity toward Nontargeting Tissues. The serum biological parameters 24 h after i.v. administration are listed in Table 2. No significant differences were observed among three groups for all parameters except the BUN. The level of BUN for CHLS and CHL was higher than the control ($p < 0.05$), but no significant differences in BUN were observed between CHL and CHLS, indicating some nephrotoxicity for CHL and CHLS, but the conjugation of scopine did not increase the nephrotoxicity of CHL. However, on Day 3, the BUN values for CHLS and CHL were back to normal (8.18 ± 0.92 and 7.93 ± 2.34 mM/L, repectively), showing that this nephrotoxicity of CHLS and CHL was reversible.

In the previous study, CHLS/CHL treatment showed the greatest level of bone marrow suppression 3 days after a single injection, and as a result, we measured hematological parameters on Day 3. As shown in Figure 6B, the WBC (white blood cells) counts were (9.48 ± 1.53), (3.23 ± 0.34), and (7.25 ± 1.10) × 10^9/L for the control, CHL, and CHLS, respectively. CHL induced a significant decrease in the WBC count compared with the control ($p < 0.05$). As for CHLS, although it was still lower than the control, the value was within the normal values of mice ((3.9−11.9) × 10^9/L).[35]

The histopathology evaluation was conducted after administration on Day 3, 7, and 14 and the hematoxylin and eosin (H&E) staining showed no necrosis, hyperemia, or inflammation for all organs tested in the control, CHL, and CHLS groups, especially lung, kidney, and heart in CHLS group which also had an improved accumulation of CHLS (Supporting Information Figure S4−6).

DISCUSSION

In our previous work, we found that linear chain tertiary amine *N*,*N*-dimethyl amino as a modification group significantly enhanced the brain-uptake efficiency of dexibuprofen, naproxen, 5-fluorouracil, and dopamine.[14−17] We then tested cyclic

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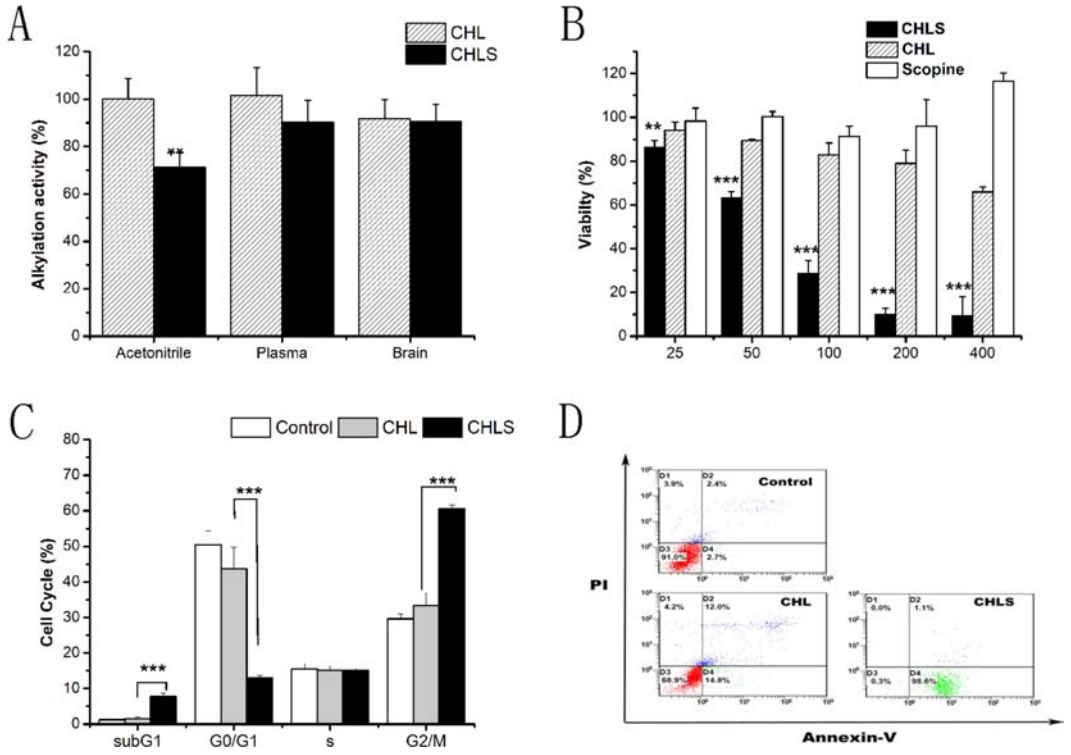

Figure 5. Anti-glioma activity of CHLS: (A) the alkylating avtivity of CHLS and CHL in acetonitrile and extraction solution after incubation with plasma and brain homogenate. Data represent mean ± SD (n = 3). ** indicates significantly different at $p < 0.01$ versus CHL group. (B) Cytotoxicity of CHL, CHLS, and scopine against C6 glioma cells. Data represent mean ± SD (n = 5). ** and *** indicate statistically different at $p < 0.01$ and $p < 0.001$ versus CHL group. (C) Flow cytometry analysis of the cell cycle for C6 cells in the presence of CHLS and CHL. Data represent mean ± SD (n = 5). *** represents significantly different at $p < 0.001$. (D) Representative quadrant plot obtained by FACS analysis in vitro showing the pro-apoptotic effect of CHL and CHLS.

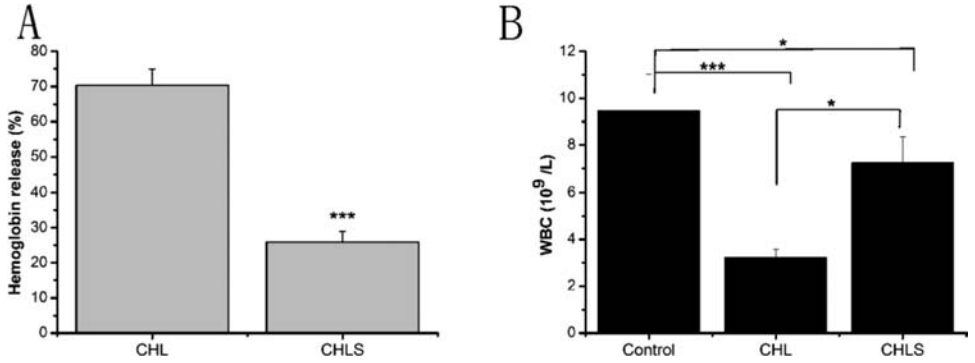

Figure 6. Effect of CHLS on red blood cells (A) and bone marrow suppression (B). * and *** represent significantly different at $p < 0.05$ and $p < 0.001$, respectively.

Table 2. Serum Biological Parameters 24 h after Intravenous Administration (n = 7)

parameters	control	CHL	CHLS
ALT (U/L)	39.8 ± 6.4	34.8 ± 11.7	37.0 ± 18.4
AST (U/L)	143.5 ± 34.5	133.3 ± 18.8	129.0 ± 27.2
BUN (mM/L)	7.75 ± 0.52	10.18 ± 1.05[a]	10.40 ± 0.79[b,c]
CREA (μM/L)	6.2 ± 1.0	5.3 ± 1.0	6.7 ± 0.6
UA (μM/L)	83.0 ± 22.2	74.8 ± 16.0	62.7 ± 20.8
CK (U/L)	1094.7 ± 426.4	1199.0 ± 234.4	1106.3 ± 180.6
LDH (U/L)	172.5 ± 26.1	180.5 ± 23.3	147.3 ± 26.6

[a] $p < 0.05$. [b] $p < 0.001$ versus the control. [c] $p > 0.05$ versus CHL group.

tertiary amines to find out whether they have a similar brain-targeting effect. As we expected, the cyclic tertiary amines, especially scopine, were shown to significantly enhance the brain uptake of CHL.

In the cellular uptake study, conjugation of scopine significantly improved the cellular uptake of CHL in both bEnd.3 cells and C6 cells, and the uptake was proven to be energy-dependent, indicating an active mode of transport. However, none of the common transporters under investigation, including organic cationic transporters, vesicular monoamine transporters, and amino acid transporters, were responsible for the active transport of CHLS in bEnd.3 cells.

In the in situ rat brain perfusion study, scopine significantly improved the brain uptake of CHL, which was also found to be energy-dependent. The result was consistent with the cellular

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uptake study, indicating that the brain uptake of CHLS might involve an active mode of transport.

The tissue distribution of CHLS showed that scopine not only improved the C_{max} and AUC_{0-t} of the brain, but also prolonged the elimination half-life of CHL, which would likely result in a sustained therapeutic effect. Based on these results, we hypothesized that the increased accumulation of CHLS in the brain may be orchestrated in a hierarchical manner. First, some specific transporters expressed within the brain capillary endothelium may actively transport CHLS into the brain. Second, the esterfication of CHL may increase its lipophilicity making it easier to penetrate cell membranes. Additionally, with the presence of an amino group, CHLS is positively charged under physiological conditions allowing it to interact with the negatively charged BBB.[36] The relative lower pH in the brain environment (intracellular pH ~ 7.0) than in the blood circulation (pH ~ 7.4) may induce ionization of tertiary amines of CHLS, which may also help CHLS to be retained in the brain.[37,38] Currently, studies are underway to help elucidate the specific mechanism behind CHLS transport through the BBB.

The carboxyl side in chlorambucil was reported to be pharmacokinetically active but not dynamic.[34] As a result, we hypothesized that conjugating scopine to CHL would not interfere with its pharmacological activity, and this was confirmed by the alkylating activity study. Though CHLS slowly hydrolyzed to its parent drug CHL in the brain, the prodrug could still exert its anti-glioma activity. In addition, the anti-glioma activity against C6 cells of CHLS was significantly improved compared to that of CHL. The cell cycle and cell apoptosis study revealed that CHLS could induce apoptosis of C6 cells, especially early apoptosis.

As shown in the biodistribution study, CHLS accumulated in lung, kidney, and heart besides brain. However, the histological examination and biochemical assessment showed that CHLS did not increase the toxicity to these off-target organs. Moreover, CHLS significantly reduced the acute toxicity and the hemolytic side-effect of CHL without inducing bone marrow suppression.

The BBB represents the major biological barrier for the brain targeted delivery of CHL, and only a small fraction of CHL can reach the brain. Conjugation of scopine to CHL was proven to greatly improve the brain uptake efficiency of CHL. Specifically, the fold of increase of AUC_{0-t} and C_{max} for brain (Re = 14.50, Ce = 12.20) were the highest among all tissues for CHLS. In addition, at the cellular level, scopine led to higher accumulation of CHL in bEnd.3 and C6 cells in an energy-dependent manner. Regarding increased accumulation of CHL in other organs, the fold of increase was less, which did not result in toxicity, while at the same time it provided the opportunity for CHL to perform its anti-glioma in the brain. Moreover, the term "brain-targeting" has been used to describe strategies that can increase the brain uptake efficiency of drugs. However, it remains a huge challenge to develop strategies that specifically deliver drugs to the brain without increasing nonspecific distribution.[39−41] Considering its greatly improved brain-targeting efficiency, we defined scopine as a brain-targeting moiety.

CONCLUSION

In summary, scopine as a brain-targeting moiety successfully improved the brain uptake efficiency of chlorambucil after intravenous injection in mice. The brain uptake of CHLS was proven to be an energy-dependent process resulting in significantly higher levels of accumulation in bEnd.3 and C6 cells compared to CHL. CHLS also displayed better anti-glioma activity against C6 cells with reduced systemic toxicity. Therefore, scopine represents a potential brain-targeting moiety which can be used to enhance the specific brain uptake of chlorambucil and other related therapeutic agent.

EXPERIMENTAL PROCEDURES

Materials. Chlorambucil (CHL, purity >98%) was purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). Scopine was obtained from Best-Reagent (Chengdu, China) with purity of 97%. Solutol HS 15 (polyoxyethylene esters of 12-hydroxystearic acid) was generously offered by BSFA (Shanghai, China). 3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and propidium iodide (PI) were obtained from Sigma-Aldrich (St Louis, MO, USA). Annexin V-fluorescein isothiocyanate (V-FITC) apoptosis kit was from Nanjing KeyGen Biotechnology (Nanjing, China). Pierce BCA protein assay reagent kit was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Plastic cell culture dishes and plates were purchased from Wuxi NEST Biotechnology Co. (Wuxi, China). The other chemicals were obtained from commercial sources. ^{1}H NMR and ^{13}C NMR analysis were performed on an AMX-400 Bruker Spectrometer. Chemical shifts were given in ppm (δ). Mass spectroscopy was performed by Agilent 1200 series RRLC system (USA).

The bEnd.3 cells were purchased from the American Type Culture Collection (Rockville, MD, USA) and C6 glioma cells were obtained from Nanjing KeyGen Biotechnology (Nanjing, China). Cells were cultured in DMEM with high glucose (Hyclone, USA) supplemented with 10% FBS (Hyclone, USA), 100 IU/mL penicillin, and 100 μg/mL streptomycin. Cells were cultured at 37 °C in a 5% CO_2 humidified environment incubator (Thermo Scientific, USA).

Kunming mice and Wistar rats were purchased from West China Experimental Animal Center of Sichuan University (Sichuan, China). All animal procedures for this study were approved by the Animal Ethical Experimentation Committee of Sichuan University according to the requirements of the National Act on the use of experimental animals (China).

Synthesis of Chlorambucil−Scopine (CHLS). Chlorambucil (303 mg, 1.0 mM), scopine (186 mg, 1.2 mM), and DMAP (61 mg, 0.6 mM) were dissolved in 50 mL anhydrous dichlormethane and added dropwise 10 mL anhydrous dichlormethane containing DCC (247 mg, 1.2 mM). The reaction mixture was then stirred at room temperature for about 4 h and then the solvent was evaporated. The residue was dissolved in ice-cold ethyl acetate and filtered to remove the reacted solid DCU. The filtrate was evaporated and applied to column packed with 100 g of silica gel with EA/PE (5:1, v/v) to yield the product.

Sample Preparation and LC-MS/MS Analysis. Samples for quantification were precipitated with at least 3 times (v/v) of acidified acetonitrile (0.1% formic acid, v/v), vortexed for 3 min, and centrifuged at 12 000 rpm for 10 min. Then, the supernatants were collected and filtered through a 0.22 μm hydrophobic membrane for subsequent LC-MS/MS injection. As for distribution and in situ rat brain perfusion, tissue samples were homogenized with twice normal saline (0.9%, m/v) and subsequent steps were identical to those describe above. All CHLS samples were under simultaneous determination of CHL and CHLS, and in cellular uptake and biodistribution section, decomposed CHL and CHLS were calculated as total CHLS.

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The detailed procedure and LC-MS/MS conditions were used as described before.[28]

In Vitro Stability of CHLS. The in vitro stability of CHLS was investigated in 0.1 M phosphate buffers (pH 4.00, 6.86, 7.40, and 9.18), freshly prepared rat plasma, and brain homogenates (33.3%), respectively. Briefly, 10 μL stock solution (4.86 μm/mL) was added into 5.0 mL preheated medium and mixed by vortex for 30 s. The mixture was then kept at 37 ± 0.5 °C. 20 μL samples were taken out at predetermined time intervals for LC-MS/MS analysis.

Cellular Uptake. The bEnd.3 cells or C6 cells were seeded into 6-well plates at a density of 2×10^5 and cultured for 24 h. Then the incubation medium was removed and replaced by CHLS or CHL in FBS-free culture medium at different concentrations for 0.5 h. After that, cells were washed with ice-cold PBS, trypsinized, harvested and centrifuged at 2000 rpm for 3 min. The pellet were then suspended in 200 μL ultrapure water and lysed by freeze−thawing cycle for 3 times to release the intracellular drugs. Twenty μL cell lysate sample was taken to determine the total cell protein content using BCA assay reagent kit (Pierce, USA). 150 μL cell lysate was added 450 μL acidified acetonitrile for protein precipitant for LC-MS/MS analysis. The uptake results were expressed as the amount of CHLS or CHL (nmol) against a unit weight (1 mg) of cellular protein.

Preliminary Uptake Mechanism Study. To study the uptake mechanism of CHLS, inhibitors including 100 nM/mL tetraethylammonium, 100 nM/mL hemicholinium-3, 100 nM/mL reserpine, 100 nM/mL glutamic acid, 100 nM/mL L-lysine, and 1 mg/mL sodium azide (NaN_3) were investigated. Briefly, bEnd.3 cells or C6 cells were preincubated with inhibitors for 15 min and then incubated with CHLS or CHL (10 nM/mL) for another 30 min at 37 °C. For the effect of temperature, cells were incubated with CHLS or CHL at 4 °C. After incubation, cells were treated similarly as described in the Cellular Uptake section.

In Situ Rat Brain Perfusion. Male Wistar rats (250 ± 20 g) were anesthetized with chloral hydrate (400 mg/kg, i.p.) and their right common carotids were perfused 30 s with perfusion medium containing: (1) CHL at 0 °C; (2) CHL at 37 °C; (3) CHLS at 0 °C; and (4) CHLS at 37 °C (n = 5). The detailed in situ rat brain perfusion method was according to Takasato et al.[9] After perfusion and washing with drug-free perfusion medium, the right brain hemisphere was collected, rinsed with ice-cold saline solution, wiped dry, weighed, and stored at −20 °C until analysis.

Pharmacokinetics and Tissue Distribution. 96 male Kunming mice (20 ± 2 g) were randomly divided into two groups. One received a single intravenous injection of CHL solution at 5 mg/kg and the other received CHLS injection at 7.26 mg/kg (equivalent to 5 mg/kg CHL) via tail vein. The solvent used for injection was saline solution (0.9%, w/v) containing 10% (w/v) Solutol HS 15 as the solubilizer. Blood and organ samples were collected 3, 10, 15, 30, 60, 90, 120, and 240 min after administration. Blood samples were collected in heparinized Eppendorf (EP) tubes and immediately centrifuged at 5000 rpm at 4 °C for 5 min, and then 100 μL supernatant was collected and stored at −20 °C until analysis. Tissue samples were rinsed with ice-cold saline solution, wiped dry, weighed, and stored at −20 °C.

Pharmacokinetic analysis was performed with Drug and Statistics Software (DAS 3.0; Mathematical Pharmacology Professional Committee of China, China), including the area under the concentration−time curve during the period of observation ($AUC_{0\text{-}t}$), the maximal concentration (C_{max}), and elimination half-life ($T_{1/2}$). The concentrations of CHLS were defined as total CHLS, including CHLS and decomposed CHL, and converted to the equivalence of CHL. To quantitatively calculate the brain targeting of CHLS, some parameters are defined as follows:

$$Re_{brain} = (AUC_{0-t,CHLS})_{brain}/(AUC_{0-t,CHL})_{brain}$$

$$Ce_{brain} = (C_{max,CHLS})_{brain}/(C_{max,CHL})_{brain}$$

$$TDI_{brain} = [(AUC_{0-t,CHLS})_{brain}/\sum_{non-targeting\ tissues}(AUC_{0-t,CHLS})]$$
$$/[(AUC_{0-t,CHL})_{brain}/\sum_{non-targeting\ tissues}(AUC_{0-t,CHL})]$$

Alkylating Activity. The alkylating activity of drugs and their metabolites was determined by spectrophotometric quantitation of their reaction-product after incubation with 4-(*p*-nitrobenzyl)-pyridine.[32,42]

100 μL fresh rat plasma or brain homogenates in EP tube was spiked with 5 μL acetonitrile containing 10 mM CHLS or CHL, mixed by vortex for 30 s, and incubated for 30 min at 37 °C. Then 900 μL acetonitrile/ethanol (1:1, v/v) was added and mixed by vortex for 3 min. The tube was centrifuged at 12 000 rpm for 10 min, and the supernatant which contained the drug itself and its active metabolites was collected. Then 0.2 mL of the supernatant was added to 1 mL acetate buffer (0.2 M, pH 5.6). 0.5 mL 4-(*p*-nitrobenzyl)-pyridine in acetonitrile (5%, w/v) was added and the mixture was incubated for 2 h at 37 °C. Finally, 3 mL 3-amino-1-propanol in tertiary butyl alcohol (25%, v/v) was added and the absorbance of the reaction product was determined by UV spectrometry (UV-220, Shimadz, Japan) at 560 nm.

As for the quantitation of CHLS and CHL alone, drugs were dissolved in acetonitrile to yield a final concentration of 1 mM. Subsequent steps were identical to that of the supernatant.

In Vitro Cytotoxicity against C6 Glioma Cells. C6 cells were seeded in a 96-well plate at a density of 1×10^4 per well and cultured at 37 °C in the presence of 5% CO_2 for 24 h. CHL and CHLS in FBS-free medium were added into each well at serial concentrations for another 24 h. The cytotoxicity of the two drugs against C6 cells was evaluated by the MTT assay.[43]

Cell Cycle Assay. Exponentially growing C6 cells (3×10^5 per well) were seeded in 6-well plates and incubated for 24 h. After that, CHLS or CHL (10 nM/mL) were spiked into each well followed by incubation for another 48 h. Cells were exposed to drug-free medium serving as the negative control. Subsequently, the adherent cells were digested and merged with the floating fraction. The harvested cells were fixed in 75% ethanol at 4 °C for 30 min, washed with ice-cold PBS, and permeablized with 0.1 mL Triton X-100 (0.1% v/v) for 15 min. After that, cells were stained with propidium iodide (PI, 20 μg/mL) and RNaseA (20 μg/mL) for 30 min in the dark at room temperature. DNA content in 2×10^4 cells/sample was measured by flow cytometry (Cytomics FC500; Beckman Coulter, Brea, CA, USA) and analyzed with MultiCycle software (Phoenix Flow Systems, San Diego, CA, USA).

Cell Apoptosis Assay. C6 cells were seeded in 6-well plates and treated similarly as described in Cell Cycle Assay to get the harvested cells. Harvested cells were incubated with AnnexinV-FITC and PI for another 15 min at room

temperature in the dark according to the instructions and immediately analyzed by flow cytometry.[33]

Acute Toxicity Evaluation. For acute toxicity study, Karber's method was used to determine the median lethal dose (LD_{50}).[44] 50 male Kunming mice (20 ± 2 g) were randomly divided into 5 groups. Each group received a single injection of CHLS or CHL via tail vein at different concentrations (n = 10). The class interval of CHLS was 0.81, while for CHL, it was 0.85. Then the mice were observed for mortality in the following 14 days. The LD_{50} and 95% confidence interval were calculated by SPSS software (v 19.0).[16,32,45]

Blood Cell Hemolysis. The whole blood of rat was stirred by a glass rod to remove the hemaleucin; then, 10-fold normal saline was added and centrifuged at 1500 rpm for 15 min. The supernatant was discarded and washed another three times with normal saline. The harvested erythrocytes were suspended in normal saline to a final concentration of 2% (v/v). CHLS and CHL were dissolved in normal saline containing 10% (w/v) Solutol HS 15. Then 0.5 mL drug solution (3.30 μM/mL) and 2 mL normal saline were added into 2.5 mL erythrocyte suspension. The mixture was incubated at 37 °C for 3 h. After centrifuging for 5 min at 1000 rpm, the supernatant was collected and the absorbance was measured at 413 nm by UV spectrometry. The positive control was pure water.

Toxicity toward Nontargeting Tissues. For hematology evaluation, 21 male Kunming mice (20 ± 2 g) were randomly divided into 3 groups and received a single intravenous administration of normal saline, CHL (5 mg/kg) or CHLS (7.26 mg/kg, equivalent to 5 mg/kg CHL) via tail vein (Day 0). On Day 1 and Day 3, 0.3 mL blood was collected in nontreated tubes and centrifuged at 5000 rpm for 10 min to obtain the serum samples for the measurement of serum alanine transaminase (ALT), aspartate transaminase (AST), urea nitrogen (BUN), creatinine (CREA), uric acid (UA), creatine kinase (CK), and lactate dehydrogenase (LDH) levels on Hitachi 7020 automatic biochemical analyzer (Hitachi Ltd. Japan). On Day 3, another 0.5 mL blood was collected into EDTA—2K (ethylenediaminetetraacetic acid dipotassium salt dehydrate)—treated tube for whole blood count on automatic hemoanalyzer.

For histopathology evaluation, 45 male Kunming mice (20 ± 2 g) were randomly divided into 3 groups and received a single intravenous administration of normal saline, CHL (5 mg/kg), or CHLS (7.26 mg/kg, equivalent to 5 mg/kg CHL) via tail vein (Day 0). On Day 3, 7, and 14, 5 mice in each group were sacrificed and the heart, liver, spleen, lung, kidney, and brain were quickly excised, rinsed with ice-cold PBS, and fixed in buffered formalin for hematoxylin and eosin staining for histological examination.

■ ASSOCIATED CONTENT

Supporting Information

Additional figures including and structure confirmation and histopathology examination. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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