**Synthesis and Biological Evaluation of Andrographolide Derivatives as Potential Anti-Inflammatory Agents**

**ABSTRACT**

**Background** Persistent inflammation might induce lipid metabolism disorders, which affected a significant proportion of the population worldwide, such as serious infections, cardiovascular diseases and autoimmune diseases. 14-Alpha-Lipoic acylandrographolide (AL-1) was previously synthesized and shown good activity to treat inflammatory diseases in our lab. However, AL-1 has a poor solubility in water and hygroscopicity, which brings problems in drug delivery.

**Aim** To enhance the water solubility of AL-1 and its anti-inflammatory activity.

**Method** By modifying its structure, we designed and synthesized the andrographolide derivative hydrochloric salt AL-2, which contains two glycine groups compared with AL-1. Then, the anti-inflammatory activity of AL-2 was evaluated in vitro.

**Results and Conclusions** Both AL-1 and AL-2 could inhibit the release of NO in inflammatory cells. In addition, the water-solubility of AL-2 was improved as well as its cytotoxicity was reduced. Therefore, these results suggested that AL-2 may be a potential drug candidate in the treatment of inflammation.

**KEYWORDS** andrographolide derivative, inflammation, glycine, solubility, hygroscopicity

**INTRODUCTION**

Inflammation is increasingly being recognized as a risk factor and significant mechanistic contributor inseparably linked to the development of serious diseases, such as ulcerative colitis (UC), which is a chronic non-specific inflammatory bowel disease (IBD). Oxidative stress is an important player in the pathogenesis of inflammatory. The activity of reactive oxygen and nitrogen species (ROS/NS) has been found to correlate with pathological changes observed in the inflammatory disease. The method to reduce or prevent the cumulative damage of ROS/NS-induced injury has been researched for many years. The currently available medications are limited, such as amino salicylates, steroids and immunosuppressants. Although these drugs have curative effects in clinic, their side effects are troubled, such as drug dependency and cytotoxicity. Therefore, the efficient drugs and other therapies are urgently needed in clinic.

Andrographolide (Andro) is the major bioactive ingredient in *Andrographis paniculata*. Modern pharmacological research reveals that Andro has a broad range of beneficial pharmacological effects, including anti-inflammation, anti-cancer, antibacterial, antiviral, etc. Andro had a good effect on anti-inflammatory by inhibiting the NF-κB connected with DNA and reducing the expression of inflammation protein. Therefore, 14-Alpha-Lipoic acylandrographolide (AL-1) was designed and synthesized in our lab. Pharmacological research showed that AL-1 reduced reactive oxygen species (ROS) and nitric oxide (NO) generation induced by inflammation. AL-1 was a novel andrographolide derivative, with conjugating andrographolide and alpha lipoic acid (Scheme 1). AL-1 inhibited the inflammatory response via lowering the level of inflammatory cytokines and myeloperoxidase (MPO) activity, which suggested that it is promoted as one of the most promising drugs in treatment for inflammation. However, because of the instability and...
poor water-solubility of AL-1, the modification of AL-1 should be performed.

Water solubility is extremely important physical chemistry property of organic small molecule drugs. Good water solubility will contribute to the improvement of efficacy and pharmacokinetic properties. Normally, drugs of lower solubility will bring a series of problems, for example, low solubility can affect metabolism in the body and it is difficult for poorly water-soluble drugs to be made into oral or intravenous agents. To improve water solubility, structure modification is a straight and effective method, including salt formation, polar group introduction, liposolubility reduction. Salt formation is the most commonly employed method for modifying aqueous solubility. It is widely applied in three aspects when the drug have been into salts. First of all, salt can improve the drug into medicinal properties. It can change the solubility of the drug, improve drug compliance and improve the stability of the drug, and so on. Second, salt can optimize preparation and purification process. It can remove the impurities in drugs which cannot be into salt. Finally, salt, a property of good medicine, can obtain patent protection and extend the patent protection of the prototype drug. The solubility of drugs can affect the pharmacokinetic properties, chemical stability, and the choice of dosage form.

To improve its druggability, the andrographolide derivative hydrochloric salt AL-2 was designed and synthesized, with the two hydroxyl groups of AL-1 conjugated with two glycine groups. Finally, the protecting groups were cleaved by using dry hydrogen chloride gas in diethyl ether solvent and the hydrochloric salt AL-2 was obtained (Scheme 1). Through the determination of water-soluble and pharmacological experiments, microculture tetrazolium (MTT) assay was used to determine cell activity. The comparative results of AL-1 with AL-2 showed that AL-2’s solubility was improved in water and its cytotoxicity was reduced. Therefore, AL-2 may be a potential new drug candidate in the treatment of inflammation.

**MATERIALS AND METHODS**

**Chemistry**

In the experiment, raw material AL-1 was synthesized in our lab having 97% purity by high performance liquid chromatography (HPLC) analysis. Boc-Glycine was purchased from Qiude Biological Chemical Company (Shanghai, China). Other chemicals were purchased from the Tianjin Fuyu Chemical Factory (Tianjin, China). $^1$H NMR and $^{13}$C NMR spectra were recorded on a Bruker AV 300/400 spectrometer at 300/400 MHz. Mass spectra were recorded on an HP 1100 LC/MSD spectrometer (HP, Palo Alto, USA). High-resolution mass spectra were obtained on an SYNAPT G2 Mass Spectrometry (Weters, USA). The solubility was measured by HPLC (Agilent, USA). The melting point was measured by XT3A micro-melting point apparatus. Chromatographic purification was performed with silica gel (200–300 mesh) and seen under UV light at 254.

**14-Alpha-Lipoic acyl andrographolide (AL-1)**

Compound AL-1 was synthesized in our laboratory. Yellow solid, $^1$H NMR (400 MHz, CDCl3) δ 6.98 (t, J = 6.7 Hz, 1H), 5.91 (d, J = 5.8 Hz, 1H), 4.85 (s, 1H), 4.58 – 4.42 (m, 2H), 4.25 – 4.06 (m, 2H), 3.50 (ddd, J = 27.3, 14.8, 7.1 Hz, 2H), 3.29 (d, J = 6.8 Hz, 1H), 3.12 (dd, J = 34.9, 21.0, 15.3 Hz, 4H), 2.52 – 2.26 (m, 6H), 2.01 – 1.74 (m, 7H), 1.74 – 1.58 (m, 5H), 1.35 – 1.10 (m, 7H), 0.65 (s, 3H). $^{13}$C NMR (400 MHz, CDCl3) δ 174.42, 170.52, 151.92, 148.21, 125.32, 110.23, 81.76, 73.10, 69.12, 65.52, 57.66, 57.24, 56.59, 44.27, 41.66, 40.26, 39.92, 39.12, 38.43, 35.92, 35.26, 30.12,
29.57, 26.75, 26.02, 25.11, 24.16, 16.59. MS (ESI) m/z 539.3 [M + H]+.

14-Alpha-Lipoic acylandrographolide-13, 19-di-tert-butoxycarbonyl-glycine (AL-2a)

To compound AL-1 (500 mg, 0.9 mmol) in 30 mL anhydrous CH2Cl2 at 0°C was added N-T-butoxycarbonyl-glycine (Boc-Gly, 0.8 g, 4.5 mmol) and 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI, 0.9 g, 4.5 mmol), then the catalyst 4-Dimethylamino-pyridine (DMAP, 20 mg) was added to the solution. The reaction was allowed to continue for 5 h. Later, the mixture was washed with saturated sodium chloride solution and extracted with dichloromethane (3 × 100 mL). The combined organic layers were dried over anhydrous Na2SO4 and concentrated in vacuo. The residue was purified by column chromatography using ethyl acetate and petroleum (1:1) as eluent to afford AL-2a as a white solid (285 mg, 75% yield), m.p.: 153-155°C.

A high performance liquid chromatography method was established to detect the solubility of AL-1 and AL-2. The Microsorb-MV (5 μM, 4.6 mm × 250 mm) column was adopted, the mobile phase of AL-1 and AL-2 were 0.05% KH2PO4 (PH 3.5)—CH3OH (25: 75) and 0.35% K2HPO4 (PH 10.0)—CH3OH (20: 80), respectively, at the flow rate of 1.0 mL min−1, and the detection wavelength was 234 nm. First, the reference substance solution of AL-1 (0.05, 0.1, 0.6, 0.8, 1, 2 mg/L) was made of methanol, while the reference substance solution of AL-2 (0.03, 0.05, 0.1, 0.5, 3, 5 g/L) was made of purified water, we measured the peak area with HPLC and get the linear regression equation, which put the peak area as the ordinate and the concentration as the abscissa. Then, we can calculate the drug solubility in the saturated solution according to the standard curve.

PHARMACOLOGY

Cell culture

The mouse RAW 264.7 was grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 100 units/mL penicillin/streptomycin sulfate. The cells were incubated in a humidified 5% CO2 atmosphere at 37°C. LPS from E. coli was used as the stimuli for all the experiments at a final concentration of 1 μg/mL.

Microculture tetrazolium (MTT) assay for cell viability

The RAW 264.7 cells were plated at a density of 2 × 104 cells per well in 96-well plates per 100 μL of medium. For determination of cell viability, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylthiazolium bromide (MTT) assay was performed. Fifty microliters of MTT (Sigma-Aldrich) was added to each well, and the cells were cultured for another 4 hours at 37°C under a 5% CO2 atmosphere. The supernatant was discarded, and 150 μL of dimethyl sulfoxide was added to each well to dissolve the formazan formed. The optical density was measured using a plate reader at 490 nm. The optical density of the formazan formed by the untreated cells was defined as 100%.

ROS production assay

The intracellular formation of ROS was quantified by fluorescence with DCF-DA. The RAW 264.7 cells (2 × 104 cells/well) were plated in fluorescence microtiter 96-well
plates for 24h. The cells were then treated with different concentrations of each test compound and incubated for 24 h. RAW 264.7 cells were loaded with 20 μM DCF-DA in Hank’s buffered salt solution (HBSS) and incubated for 30 min in the dark. After washing out the excess probe, the fluorescence was measured at 480/20 nm excitation and 520/20 nm emission in a fluorescence multi-detection reader (Synergy HT Multidetection Microplate Reader; BioTek, VT).

**NO production assay**

The RAW 264.7 cells were plated at 2 × 10⁴ cells/well in 96 well plates, and then incubated with or without LPS (1 μg/mL) in the absence or presence of AL-1 or AL-2 for 24 h. NO levels in culture media were determined using the Griess reaction assay and presumed to reflect NO levels. Briefly, 100 µL of cell culture medium was mixed with 100 µL of Griess reagent (equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid, and 0.1% (w/v) naphthylethlenediamine dihydrochloride), incubated at room temperature for 10 min. The absorbance was measured at 540 nm using a microplate reader (PowerWaveXS). Fresh culture media were used as blanks in all experiments. NO levels in the samples were read off a standard sodium nitrite curve.

**RESULTS AND DISCUSSION**

**Chemistry**

The synthesis of AL-2 was described in Scheme 1. Compound AL-1 was synthesized by using the method reported by our group⁸. It was converted to the intermediate compound AL-2a through esterification with Boc-Glycine in the presence of EDCI and DMAP. Then, it was treated with anhydrous HCl to remove protection groups (Boc) and afford AL-2 as hydrochloride salt. Glycine was selected to add on the AL-1, because it was the simplest amino acid and a kind of non-essential amino acid for human.

**SOLUBILITY**

Due to the poor solubility, the standard solution of AL-1 was prepared in methanol. While AL-2’s standard solution was prepared in distilled water. The result was shown in Fig. 1. Typical equations of the standard curves of AL-1 and AL-2 were \( y = 35.983x - 1.3374 \) and \( y = 3.7949x - 40.843 \), respectively, with good correlation coefficient (\( R^2 = 0.9991 \) and 0.9997, respectively) during the appropriate concentration range. According to the standard curve, the water solubility of AL-1 was 0.0705 mg/L, the water solubility of AL-2 was 4.76 g/L. Therefore, the solubility in water of AL-2 was greatly increased without stronger hygroscopicity.

**PHARMACOLOGY**

**Effects on cell viability in LPS-induced RAW 264.7 cells**

The effects of AL-1 and AL-2 on the viability of RAW 264.7 cells were determined by a colorimetric MTT assay after 24 h treatments. The data were expressed as percent cell viability compared to control. AL-2 did not cause any cytotoxicity at 10 μM in RAW 264.7 cells. As shown in Fig. 2 both AL-1 and AL-2 had anti-inflammatory activity. Although the anti-inflammatory cell activity of AL-2 was a little weakness than AL-1’s, AL-2’s cytotoxicity was greatly reduced, especially on concentration of 10 μM, the reason might be that salt could reduce drug stimulation to reduce the toxicity.

**Effects on the production of ROS in LPS-induced RAW 264.7 cells**

In general, results showed that the generation of intracellular ROS decreased significantly in RAW 264.7 cells, which were, respectively, treated with AL-1 and AL-2 (Fig. 3). AL-1 and AL-2, respectively, compared with the model group. The elevated ROS levels were decreased by 22.82%, 42.85% and 44.12% after pretreatment with...
Fig. 2 Effects of AL-1 and AL-2 on cell viability in LPS-induced RAW 264.7 cells. Cells were treated with the indicated concentration of AL-1, AL-2 and LPS for 24 h. Cell viabilities were assessed using MTT assay. Each value represents means ± SD of six independent experiments. **P < 0.01 indicates differences from the unstimulated control group. *P < 0.05 and **P < 0.01 indicates differences from the LPS-treated group.

AL-1 at doses of 0.01, 0.1 and 1 μM, while the elevated ROS levels were decreased by 16.08%, 16.42% and 19.94% after pretreatment with AL-2 at doses of 0.01, 0.1, and 1 μM. These results demonstrated that AL-2 could significantly reduce the level of ROS.

Fig. 3 Effects of AL-1 and AL-2 on LPS-induced ROS production in RAW 264.7 cells. The level of intracellular ROS was measured with DCF-DA. The formation of ROS in the cells was evaluated by the arbitrary fluorescence unit and described as fold induction test via vehicle. Each value represents mean ± SD of six independent experiments. **P < 0.01 indicates differences from the unstimulated control group. *P < 0.05 and **P < 0.01 indicates differences from the LPS-treated group.

Fig. 4 Effect of AL-1 and AL-2 on the NO production in LPS-induced RAW 264.7 cells. The cells were treated with LPS only or with AL-1 and AL-2 ranging from 0.01 to 1 μM for 24 h. The culture media were collected, and the nitric oxide concentration was measured by the Griess reaction. Each value represents mean ± SD of triplicate experiments. **P < 0.01 indicates differences from the unstimulated control group, and **P < 0.01 indicates differences from the LPS-treated group.

Effects on the production of NO in LPS-induced RAW 264.7 cells

A growing number of studies have shown that intestinal homeostasis is controlled by the corresponding NO, but the excessive production of NO may be an important pathophysiologic basis of IBD. The NO levels were measured using Griess agent according to a published method16. As shown in Fig. 4 the production of NO
decreased significantly in RAW 264.7 cells, which were, respectively, treated with AL-1 and AL-2. The results showed that AL-1 and AL-2 were similar in reducing the production of NO. The reason might be that salt only changes the physical properties of AL-1.

CONCLUSION

In conclusion, a new andrographolide derivatives AL-2 was designed and synthesized. Both AL-1 and AL-2 had an active effect on inflammatory cells. Among these, two compounds, AL-2 had better solubility in water and lower cytotoxicity. AL-2 may be valuable for the treatment of inflammation. To explore the mechanism of action for this novel compound AL-2, we design the further experiments and the results will be reported in due course.

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