Aconitine linoleate, a natural lipo-diterpenoid alkaloid, stimulates anti-proliferative activity reversing doxorubicin resistance in MCF-7/ADR breast cancer cells as a selective topoisomerase IIα inhibitor

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Abstract
Aconitine linoleate (1) is a lipo-diterpenoid alkaloid, isolated from Aconitum sinchiangense W. T. Wang. The study aimed at investigating the anti-proliferative efficacy and the underlying mechanisms of 1 against MCF-7 and MCF-7/ADR cells, as well as obvious the safety evaluation in vivo. The cytotoxic activities of 1 were measured in vitro. Also, we investigated the latent mechanism of 1 by cell cycle analysis in MCF-7/ADR cells and topo I and topo IIα inhibition assay. Molecular docking is done by Discovery Studio 3.5 and Autodock vina 1.1.2. Finally, the acute toxicity of 1 was detected on mice. 1 exhibited significant antitumor activity against both MCF-7 and MCF-7/ADR cells, with IC_{50} values of 7.58 and 7.02 μM, which is 2.38 times and 5.05 times more active, respectively than etoposide in both cell lines, and being 9.63 times more active than Adriamycin in MCF-7/ADR cell lines. The molecular docking and the topo inhibition test found that it is a selective inhibitor of topoisomerase IIα. Moreover, activation of the damage response pathway of the DNA leads to cell cycle arrest at the G0/G1 phase. Furthermore, the in vivo acute toxicity of 1 in mice displayed lower toxicity than aconitine, with LD_{50} of 2.2 × 10^5 nmol/kg and only slight pathological changes in liver and lung tissue, 489 times safer than aconitine. In conclusion, compared with aconitine, 1 has more significant anti-proliferative activity against MCF-7 and MCF-7/ADR cells and greatly reduces in vivo toxicity, which suggests this kind of lipo-alkaloids is powerful and promising antitumor compounds for breast cancer.

Keywords Aconitine linoleate · Cytotoxic activity · Topoisomerase IIα inhibitor · Acute toxicity · MCF-7 and MCF-7/ADR breast cancer cells · Molecular modeling

Introduction

According to the statistical data from the World Health Organization (WHO) in 2020, breast cancer has taken the top one place both in diagnose rate and mortality rate for female (Sung et al. 2021). However, its treatment has been hindered by multidrug resistance (MDR), leading to the urge for a new antitumor agent targeted specific target, especially for reversing the resistant problems. Among the various anticancer targets, DNA topoisomerases (topos) are well-known ones (Li and Liu 2001; Pommier 2006; McClendon and Osheroff 2007; Nitiss 2009a, b). Topos inhibitors are generally classified into topo I inhibitors as camptothecin (CPT) (Schoeffler and Berger 2008), and topo II inhibitors as amsacrine, etoposide and doxorubicin (Khadka and Cho 2013). However, the clinic use of current anticancer topoisomerase inhibitors has been limited by some major negative consequences. Firstly, most clinically active topo inhibitors are topo poisons, such as etoposide and doxorubicin, which usually need monitoring for severe genetic toxicity (Jun et al. 2015). Secondly, the severe dose-limiting toxicities and drug resistance of topo I inhibitors like CPT have emerged. Thirdly, the secondary malignancies led by the inhibition of
topo IIβ in the clinic use of the unselective topo II inhibitors have been reported (Felix 1998; Mistry et al. 2005; Azarova et al. 2007). For these reasons, many researchers have targeted topo IIα, rather than IIβ, on developing topo catalytic inhibitors, which induces cancer cell death through the elimination of the essential enzymatic activity, rather than topo poisons (Albert et al. 2013; Liang et al. 2019).

As a widely used traditional Chinese medicine, Aconitum possesses anti-inflammatory, analgesic, and anticancer activities (Kim et al. 2002; Liang et al. 2016b). Single Aconitum and its prescriptions have been documented for the treatment of cancer since the last century in China (Mingxin Tang and Sun 1986, He Shu et al. 2009, Chen 2015, Qiuping et al. 2016, Duan et al. 2018). However, the precise mechanisms of the Aconitum eliciting anticancer effects have not yet been fully clarified due to the diverse structures of compounds in Aconitum. Diterpenoid alkaloids, the main active components from Aconitum, have been reported as potential cytotoxic agents for decades (Li et al. 2005; Hazawa et al. 2009a, b; Wada et al. 2011, 2012, 2015a, b; Wada 2012; Liang et al. 2016a, 2017, 2018, Wada. 2019) (Fig. 1). But, the significant toxicities of diterpenoid alkaloids, such as the neurotoxicity and cardiotoxicity of aconitine (4), limited their further clinical application. Lipo-diterpenoid alkaloids, a kind of diterpenoid alkaloids bearing long ester groups (such as 1–3 in Fig. 1), are common components obtained from Aconitum, which have been reported with extensive pharmacological activities and low toxicity (Kitagawa et al. 1982; Isao et al. 1984; Hanuman and Katz 1994; Yamashita et al. 2018; Yamashita et al. 2020a, b). In 2017, our group first reported the obvious cytotoxic activities of lipo-diterpenoids 1 and 3, against HL-60, A-549, SMCC-7721, MCF-7, and SW480 cell lines, comparable to cisplatin (Liang et al. 2016a, 2017). Later, the good cytotoxic activities of one lipo-alkaloid against several tumor cell lines (A549, MDA-MB-231, MCF-7, KB, KB-VIN) also have been reported by the Koji Wada group (Koji Wada et al. 2019, Yamashita H, 2020a). Then, the highly selective index (SI>10) for compound 1 between MCF-7 and mouse fibroblast cells has surprised us, arousing a deeper pharmacological mechanism research in the current study.

In this work, aconitine linoleate (1) was investigated for its anti-proliferative activity in vitro against MCF-7 and Adriamycin-resistant subline MCF-7/ADR cell lines. In order to explore its mechanistic pathways, the lead compound 1 was performed extra investigations such as cell cycle analysis, topoisomerase II inhibition, and molecular docking. Meanwhile, for determining its safety, the acute toxicity on mice was also tested.

Materials and methods

Materials and reagents

Aconitine linoleate (1) (isolated from the Aconitum sin-chiangense W. T. Wang, plants were collected from Yili Kazakh Autonomous Prefecture, Xinjiang Uygur Autonomous Region, China, in 2016, and was identified by Dr. Lixia Li. The plants are Stored in the Natural Medicine Research Center of Sichuan Agricultural University (the voucher specimen No. 20140616–1); MCF-7 cell line (Breast cancer cell MCF-7), MCF-7/ADR (Adriamycin-resistant subline), and NIH3T3 cell line (Mouse fibroblast cells) (Cell Resource Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences); Kunming mice (CHENGDU DOSSY EXPERIMENTAL ANIMALS), PBS (HyClone); DMEM high glucose (HyClone); fetal bovine serum (FBS) (Gibco); propidium iodide (PI) (Yeasen Biotech); Adriamycin (RYON); etoposide and
mal cells, we tested the NIH3T3 cell line (mouse fibroblast) concentration of 5
them with trypsin, prepare a cell suspension with a concentration 37.5, 18.75, and 9.375 μg/mL). We take logarithmic growth
configured into a series of drug concentrations (150, 75, and adjusted the pH value to 6–7 with 1 mol/L NaOH, then
the cytotoxic activities of aconitine linoleate (1) were meas-
Taking Adriamycin and etoposide as the positive controls,
ysis (Promega); paraffin wax (Shanghai Hualing Kangfu Machinery Factory); Ehrlich hematoxylin stain and
Eosin Solution (Solarbio); LDH Cytotoxicity Assay Kit (solarbio); Prestained Protein Ladder, 10 to 180 kDa and
10–170 kDa). RIPA Lysis Buffer, Topoisomerase II alpha Rabbit Monoclonal Antibody, Topoisomerase I Rabbit
Monoclonal Antibody, BeyoECL Moon, SDS-PAGE Sample Loading Buffer (5 ×), TOP2B Rabbit Polyclonal antibody (PROTEINTECH GROUP); Goat Anti-Rabbit IgG, HRP Conjugated (Wuhan Saiweier Biological Technology); pBR322 DNA, DNA Topoisomerase I (Takara Bio); PVDF Membrane(Biotopped); and DNA Topoisomerase IIA (TopoGEN).

All animal experiments were approved by the guidelines of the Animal Ethical and Welfare Committee (#20,190,036) in Sichuan Agricultural University. And, the animals were treated according to the Guide for the Care and Use of Laboratory Animals (Eighth Edition, 2011, The National Academies Press).

In vitro anti-proliferative activity

Taking Adriamycin and etoposide as the positive controls, the cytotoxic activities of aconitine linoleate (1) were measured in vitro on MCF-7 cell line (Breast cancer cell MCF-7) and MCF-7/ADR (Adriamycin-resistant subline) using MTS Cell Proliferation Colorimetric Assay Kit (Abdelhaleem et al. 2018; Luan et al. 2021). The cytotoxicity test sample (purity greater than 95%) was dissolved in 1% HCl and adjusted the pH value to 6–7 with 1 mol/L NaOH, then configured into a series of drug concentrations (150, 75, 37.5, 18.75, and 9.375 μg/mL). We take logarithmic growth phase MCF-7 cells or MCF-7/ADR cells, digest and count them with trypsin, prepare a cell suspension with a concentration of 5 × 10^4 cells/mL with the culture medium, and add 100 μL of the cell suspension to each well of a 96-well cell culture plate (approximately 5 × 10^3 cells per well). We place the 96-well cell culture plate in a 37 °C, 5% CO₂ incubator for 24 h. Then, culturing these cells for 48 h with the prepared drugs at various concentrations, MTS solution (20 μL, 2.5 mg/mL in PBS), and 180 μL of culture medium was added for continuous incubation for 2.5 h, then measured at 490 nm for the light absorption value. The cell growth inhibition rate was calculated with the equation: survival fraction = O.D. (treated cells)/O.D. (control cells). The relation between surviving fraction and compound concentration was plotted and IC₅₀ (the concentration required for 50% inhibition of cell viability) was calculated for each test compound. In order to further explore the selectivity between 1 and normal cells, we tested the NIH3T3 cell line (mouse fibroblast cells) in the same way (except that the drug concentration was set to 380, 190, 95, 47.5, 23.75 μg/mL).

In vitro cell cycle analysis

After treating with compound 1 for 48 h, the MCF-7 cells were washed twice with ice-cold phosphate buffer saline (PBS). The cells were obtained after centrifugation for 5 min (1000 r/min) and fixed with 70% ethanol for 6 h at 4 °C, then washed with PBS, resuspended with 0.1 mg/mL RNase, stained with 40 mg/mL propidium iodide (PI), and analyzed by flow cytometry using FACS Calibur (Becton Dickinson) (Schmitta et al. 2019). The cell cycle distributions were calculated using Flowjo.7.6. and SPSS 12.0 software (Becton Dickinson). The interference with the normal cell cycle distribution was indicated after exposure of MCF-7 cells to this compound.

Topo inhibition assay

Topo I inhibition assay

Taking hydroxy camptothecin as a positive control, reaction mixtures (20 μL) with supercoile pBR322 DNA (0.25 μg in the 2 μL 10 × Tris/glycine/SDS (TGS) buffer), 1 unit topo I, 2 μL 0.1% BSA, and 0.2 μL aconitine linoleate (1) with different concentrations and appropriate distilled deionized water were prepared. After incubating at 37 °C for 30 min, 10% SDS (1 μL) was added to stop the reaction. A 20 μL aliquot was electrophoresed in 1.2% agarose gel at 80 V for 20 min in 0.5 × Tris–acetaate (TBE) buffer (20 mM Tris–acetate, 0.5 mM EDTA, pH 8.3), then at 130 V for 25 min. After electrophoresis, DNA bands were stained in ethidium bromide (0.5 μg/mL) and visualized by the Bio-Rad gel imaging system.

Topo IIα inhibition assay

Taking etoposide as a positive control, reaction mixtures (20 μL), containing 0.15 μg of supercoile pBR322 DNA in the 4 μL 10 × Tris/glycine/SDS (TGS) buffer (50 mM spermidine, 720 mM KCl, 350 mM Tris–HCl pH8.0, 50 mM MgCl₂, 50 mM DTT). 5 units topo IIα, 0.2 μL aconitine linoleate (1) with different concentrations, and appropriate distilled water, were obtained. After incubating at 37 °C for 30 min, 10% SDS (1 μL) was added to stop the reaction. An aliquot (20 μL) was electrophoresed in 1.2% agarose gel at 80 V for 20 min in 0.5 × Tris–acetaate (TBE) buffer (20 mM Tris–acetate, 0.5 mM EDTA, pH 8.3), then at 130 V for 25 min. After electrophoresis, DNA bands were stained in ethidium bromide (0.5 μg/mL) and visualized by the Bio-Rad gel imaging system.
Compared with positive control group (Adriamycin, etoposide), MCF-7/ADR, and NIH3T3.

Then, an MD study was performed to revise the docking accuracy. For Vina docking, the exhaustive value was set to 20 for improving docking accuracy. For Vina docking, the exhaustive value was set to 20 and center_z: -60.34 with dimensions size_x: 15, size_y: 15, and size_z: 15. The exhaustive value was set to 20 for improving docking accuracy. For Vina docking, the default parameters were used if it was not mentioned. Then, an MD study was performed to revise the docking result.

### Molecular dynamics simulation

The AmberTools 15 programs and Amber 14 (Götz et al. 2012; Pierce et al. 2012; Salomon-Ferrer et al. 2013) were used for MD simulations of the selected docked pose. The compound was first prepared by the tool, ACPYPE (da Silva and Vranken 2012), basing on ANTECHAMBER (Wang et al. 2004, 2006) for generating automatic topologies and parameters in different formats for different molecular mechanics programs, such as calculation of partial charges. Then, the forcefield “leaprc.gaff” (generalized amber forcefield) was used for preparing the ligand, while “leaprc.ff14SB” was used for the receptor. The system was placed in a rectangular box (with a 10.0 Å boundary) of TIP3P water using the “SolvateOct” command with the minimum distance between any solute atoms. PMEMD (Particle Mesh Ewald Molecular Dynamics) module was accelerated by GPU (NVIDIA® Tesla K20c) for a short-term minimization (500 steps per steepest descent and conjugate gradient method), 500 ps heating and 50 ps of density equilibration with weak restraints to reach the equilibrium of solvated complexes. At last, 20 ns of MD simulations were carried out. All the molecular dynamics were performed on the Dell Precision T5500 workstation. The Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) method, implemented in AmberTools 15, was used for calculating the binding free energies (ΔGbind in kcal/mol). Moreover, to identify the key protein residues responsible for the ligands binding process, the binding free energy was decomposed on a per-residue basis. The binding free energy of MM/GBSA for each complex was estimated by following equations: ΔGbind = Gcomplex − Gprotein − Gligand (1); ΔGbind = ΔH − TΔS ≈ ΔGsol – TΔS; (2) ΔGgas = ΔEele + ΔEvdw (3); ΔGsol = ΔEGB + ΔO SA; (4) (where ΔGbind is the binding free energy and Gcomplex, Gprotein, and Gligand are the free energies of complex, protein, and ligand, respectively. The sum of entropy contribution (TΔS) and enthalpy (ΔH) is for the binding free energy. The enthalpy (ΔH) includes the free energy of solvation (ΔGsol) and free energy of the gas phase (ΔGgas). ΔGsol is composed of ΔGSA, the nonelectrostatic

### Molecular modeling

**Molecular docking**

DS BIOVIA Discovery Studio 3.5 and Autodock vina 1.1.2 were used to conduct the molecular docking studies for the binding mode between compound 1 and topo IIα (Trott and Olson 2010). The three-dimensional (3D) structure of topo IIα (PDB ID: 5GWK) was obtained from the protein database (http://www.rcsb.org/pdb/home/home.do). The 3D structure of the compound was drawn by ChemBioDraw Ultra 14.0 and ChemBio3D Ultra 14.0 software. The docking input files were generated by the AutoDockTools 1.5.6 package (Sanner 1999; Morris et al. 2009). Ligand structures were prepared for docking by merging non-polar hydrogen atoms and defining rotatable bonds. The search grid of the topo IIα was identified as center_x: 23.7, center_y: -38.695, and center_z: -60.34 with dimensions size_x: 15, size_y: 15, and size_z: 15. The exhaustive value was set to 20 for improving docking accuracy. For Vina docking, the default parameters were used if it was not mentioned. Then, an MD study was performed to revise the docking result.

### Table 1

The IC50 (μM) results of compounds 1 and 4 against MCF-7, MCF-7/ADR, and NIH3T3

| No | MCF-7  | MCF-7/ADR | NIH3T3 |
|----|--------|-----------|--------|
| 1  | 7.58±0.06a,b | 7.02±0.12a,b | 72.08±0.15 |
| 4  | >400   | 86.35±0.32a,b,c | -     |
| Adriamycin | 1.22±0.43a,b,c | 67.61±6.50b,c | -     |
| Etoposide   | 18.01±1.64a,b,c | 35.48±0.29a-c | -     |
| SI          | 9.51   | -         | 10.26  |

Compared with positive control group (Adriamycin, etoposide). aP<0.05, bP<0.01; compared with 1 group, cP<0.05; -, means no data; SI, selectivity of compound 1 between MCF-7 or MCF-7/ADR and NIH3T3 cells

**Fig. 2** The in vitro anti-proliferative activity of Aconitine linoleate (1), adriamycin and etoposide examined against MCF-7 (Breast Cancer) and MCF-7/ADR cell lines. (Low: 5 μM, Medium: 10 μM, High: 15 μM)
solvation component (non-polar contribution) and ΔG_{GB}, and the electrostatic solvation energy (polar contribution). ΔG_{gas} includes ΔE_{ele} (electrostatic) and ΔE_{vdw} (van der Waals) energies, which exhibits the gas-phase interaction energy between protein and ligand.

### Acute toxicity

The acute toxicity of the lipo-diterpenoid alkaloids to animal subjects was investigated on the normal mice by using aconitine linoleate (1). Briefly, 110 Kunming mice (aged 4–5 weeks, weighed 18–22 g, sex in half) were divided

**Fig. 3** Effect of aconitine linoleate (1) on the proliferation cycle of MCF-7/ADR cell

| Receptor protein | PDB number | LibDockScore | Poses |
|------------------|------------|--------------|-------|
| Topo II| 5bTc | 324.932 | 26 |
| Topo I | 1nh3 | 83.3349 | 1 |
| Epidermal growth factor HER2 | 3WSQ | 147 | 23 |
| Epidermal growth factor HER3 | 5O4O | 142 | 93 |
| Matrix metalloproteinases | 1bqo | 109.144 | 1 |
| Human basic fibroblast growth factor | 1bfb | 0 | 0 |
| Human vascular endothelial growth factor | 1vpo | 0 | 0 |
| Protein kinase C | 2k9 | 0 | 0 |
| Insulin receptor | 3ekk | 0 | 0 |
| Platelet-derived growth factor rece | 3mjj | 144.6 | 56 |

LibDockScore indicates the degree of docking between receptors and ligands; Poses represents the way ligands bind to proteins; if the score and poses are 0, the docking failed

| Table 3 | Comparison of the docking results of between aconitine linoleate (1) and aconitine (4) with receptor protein topo IIα and topo IIβ |
|------------------|------------|--------------|-------|
| Compound | Receptor protein | PDB number | LibDockScore | Poses |
| 1 | Topo IIα | 4fm9 | 102.535 | 13 |
| | Topo IIβ | 4g0w | 0 | 0 |
| 4 | Topo IIα | 4fm9 | 0 | 0 |
| | Topo IIβ | 4g0w | 0 | 0 |

LibDockScore indicates the degree of docking between receptors and ligands; Poses represents the way ligands bind to proteins; if the score and poses are 0, the docking failed
A

B

C

D

\( \Delta G_{\text{bind}} \) (kcal/mol)

\( \Delta E_{\text{vdw}} \)

\[ \Delta E_{\text{ele}} \]

\[ \Delta E_{\text{disp}} \]

\[ \Delta E_{\text{int}} \]

\[ \Delta E_{\text{total}} \]

\( \text{i} \) - \( \text{j} \)

\( \text{Time (ns)} \)

\( \text{Residue number (Chain A)} \)

\( \text{Residue number (Chain B)} \)
randomly into 11 groups, including one negative control group (dilute hydrochloric acid solution, pH 5.4), five dose groups (220, 210, 200, 190, 180 mg/kg) of aconitine linoleate, and five dose groups (0.6, 0.55, 0.5, 0.4, 0.3 mg/kg) for the positive control aconitine. All the groups were given the samples by intraperitoneal injection once time. The animals were observed for toxic signs for 4 h after the drug injection. And, the changes in physical appearance, injury, pain, and signs of illness were recorded daily for 14 days. Finally, the mice were sacrificed after 14 days and the major tissues, including the heart, spleen, kidney, liver, lung, and thymus, were excised for the analysis of macroscopic pathological anatomy. Then, the tissues, fixed in formaldehyde 37%, were taken for microscopic evaluation using hematoxylin and eosin staining in the Pathology Laboratory of College of veterinary medicine, Sichuan Agricultural University. The pathological changes of the tissues were assessed and compared.

Results

Biology

Taking Adriamycin and etoposide as a positive control, the in vitro anti-proliferative activity of aconitine linoleate (1) was examined against MCF-7 and MCF-7/ADR cell lines by the MTS method (Ahmed et al. 2017). The results were expressed as IC_{50} (median growth inhibitory concentration) values, representing the concentrations needed to produce a 50% inhibition of cell growth after incubation for 48 h (Table 1 and Fig. 2) (Luan et al. 2021). In order to further explore the selectivity between 1 and normal cells, we used the NIH3T3 cell line as normal cells for comparison and found that the IC_{50} was 72.08 ± 0.15 μM, and selective index (SI) = 9.51 and 10.26 respectively compared with MCF-7 and MCF-7/ADR (Table 1).

To explore the effect of the aconitine linoleate on cell proliferation of MCF-7/ADR cells, we analyzed the cell cycle distribution of aconitine linoleate (1) at its IC_{50} concentration (Table 1) using flow cytometry assay. Our data in Fig. 3 shows that aconitine linoleate (1), containing 8-linoleic acid side chain, 3-OH, and N-ethyl group, exhibits the potent anti-proliferative effect on MCF-7/ADR cells by increasing the percentage of cells of the G_{0}/G_{1} phase, decreasing the same in the S phase.

Molecular docking

To better understand the potential binding targets of lipo-diterpenoid alkaloids, the molecular docking of aconitine linoleate (1) with 18 kinds of common antitumor targets was first screened by Discovery Studio 3.5.

The docking results in Table 2 indicated the most favorable target would be topo II for the highest LibDockScore (324.932). Interestingly, topo IIα may be the selective target (LibDockScore of 102.535), rather than topo IIβ, which has been failed in the docking process. In contrast, aconitine (4) could not successfully dock with those two targets (Table 3), relating to the bad anti-proliferative activity of aconitine against those two cancer cells.

The potential binding mode between compound 1 and topo IIα has been further explored by molecular docking and molecular dynamic simulations, using the AutoDock vina 1.1.2 and Amber14 software. Bonding models of 1 and topo IIα were depicted in Fig. 4, showing the residue A/Arg-487 close to the phenyl group of the compound 1 by cation-π interaction (Fig. 4A). To gain more information about the residues surrounding the binding site and their contribution to the whole system, the MMGBSA approach was used to calculate the contribution of the electrostatic, solvation, Van der Waals force, and residues to the free energy of binding. Each residue interaction free energy was presented as electrostatic (ΔE_{ele}), solvation (ΔE_{solv}), Van der Waals (ΔE_{vdw}), and total contribution (ΔE_{total}). In the topo IIα-I complex, the residue A/Arg-487 has a strong electrostatic (ΔE_{ele}) contribution, with the ΔE_{ele} of < −11.0 kcal/mol (Fig. 4B). In addition, due to the close proximity between the nucleotides and compound 1, strong Van der Waals interactions (−4.5 kcal/mol) between nucleotides C/DC-8, D/DT-9, F/DA-12, and F/DG-13, and the ligand has been detected. Overall, the majority of the decomposed energy interaction originated from Van der Waals interactions, mainly through hydrophobic interactions, such as A/Met-762, A/Met-766, and B/Ala-801. It referred that the introduction of hydrophobic groups, such as halogen, nitro, and benzene ring, into the molecule, may increase the binding force with topo IIα, thereby enhancing its anti-proliferative activity. Furthermore, the calculation of the total binding free energy for the topo IIα-I complex led to an estimated ΔG_{bind} of −9.1 kcal/mol for 1 (Table 4), indicating the strongly bind of 1 to the binding site of the topo IIα. This suggests that the anti-proliferative activity of 1 is very likely to take effect through the complex with topo IIα.

The docking results of the 20-ns molecular dynamics simulation confirmed the preferential binding mechanism of topo IIα and 1. The root mean square
deviation (RMSD) values of the protein skeleton basing on the initial structure with the simulation time was performed to figure out the dynamic stability of the models and ensure the rationality of the sampling strategy (Fig. 4C), which proved the stability of the protein in two systems under the simulation. The flexibility of the whole protein residues in topo IIα-1 complex and free topo IIα was detected by the root mean square fluctuations (RMSF) values (Fig. 4D), presenting the difference in flexibility between the binding site of topo IIα with 1. The results presented a small degree of flexibility with a RMSF of less than 3 Å at the binding site, referring to these residues seems to be more rigid as a result of binding to 1. In summary, the rational explanation of the interactions between 1 and topo IIα by the molecular dynamic simulations provided valuable information for further development of the topo IIα inhibitors.

In order to prove the molecular docking results, the activity inhibition of topoisomerase enzyme with compound 1 was suspected as an expecting mechanism for the action of lipo-diterpenoid alkaloids. The assay was performed to evaluate the topo I, IIα inhibitory activity (Fig. 5). Aconitine linoleate (1) exhibited promising inhibition activity below 200 μM, comparable to the known potent topo IIα inhibitor, etoposide, whereas it exhibited no obvious inhibition against topo I, even at the concentration of 200 μM. Thus, it was proved that compound 1 exerted its anti-proliferative activity through selective topo IIα inhibition, basing the results of both molecular docking and the inhibition of topoisomerase activity. However, in the topo IIα inhibition assay, the IC₅₀ values were much higher than in the cytotoxicity assays. It can be concluded that compound 1 possessed the anti-proliferative activity partially dependent on the topo IIα inhibition.

**Acute toxicity**

For further application, the toxicity evaluation should be explored as an important factor. To investigate the toxicity of lipo-diterpenoid alkaloids, the acute toxicity test of aconitine linoleate (1) was carried out in Kunming mice for the LD₅₀ (half-lethal dose) value. The results showed that the LD₅₀ value of aconitine linoleate (1) was 2.2 × 10⁵ nmol/Kg. Compared with aconitine (LD₅₀ = 4.5 × 10² nmol/kg), the safe dosage of aconitine linoleate to mice is 489 times higher than aconitine, suggesting the obvious toxicity reduction.

### Table 4 Average binding free energies (kcal mol⁻¹) for the Topo2α-1 complex along with different energy contributions

| Compd | ΔEvdw | ΔEele | ΔGgas | ΔEGB | ΔGSA | ΔGsolv | ΔH | TΔS | ΔGbind |
|-------|-------|-------|-------|------|------|--------|----|-----|--------|
| L29   | −99.8 | −45.6 | −145.4| 88.7 | −11.0| 77.7   | −67.7| −58.6| −9.1   |

### Table 5 Acute toxicity test results of compounds 1 and 4 in mice through celiac injection

| Compound | Concentration (mg/kg) | Number of deaths | LD₅₀ (nmol/kg) |
|----------|-----------------------|------------------|----------------|
| 1        | 180                   | 3                |                |
|          | 190                   | 4                |                |
|          | 200                   | 6                |                |
|          | 210                   | 7                |                |
|          | 220                   | 9                | 2.2×10⁵        |
| 4        | 0.3                   | 1                | 4.5×10²        |
|          | 0.4                   | 2                |                |
|          | 0.5                   | 3                |                |
|          | 0.55                  | 6                |                |
|          | 0.6                   | 9                |                |

A total of 11 groups including the negative control group (dilute hydrochloric acid solution, pH 5.4), each with 10 mice; there was no death in the negative control group.
for lipo-diterpenoids (Table 5). Furthermore, as shown in Fig. 6, after aconitine linoleate treatment, no obvious pathological lesions appear in the main tissues, including the heart, spleen, kidney, liver, lung, and thymus. The pathological morphology in mice tissue with/without compound 1 (2.2 × 10^5 nmol/kg) or aconitine (4.5 × 10^2 nmol/kg) was also conducted by the H&E staining method. Compared with the blank group, only liver and lung tissue in the aconitine linoleate group showed slight pathological changes, which are much milder than the aconitine group. For liver tissue, the obvious damage is found in the aconitine group with moderate diffuse vacuolar degeneration and punctate necrosis foci, which are occasionally seen in the aconitine linoleate group. For lung tissue, the alveolar septum is significantly thicker and the number of alveoli is decreased in the aconitine group, whereas the alveolar septum in the aconitine linoleate group is slightly thicker, significantly better than that of the aconitine group.

**Discussion**

Firstly, compared with aconitine (4), aconitine linoleate (1) exhibited high in vitro cytotoxic activities against both MCF-7 and MCF-7/ADR cell lines and displayed much lower toxicity in the in vivo acute toxicity evaluation in mice. Based on the results of molecular docking, aconitine linoleate (1) was selectively well docked with topo IIα, while aconitine (4) failed. Considering the tumor-targeting effect of long-chain fatty acids (Sauer et al. 2000), it is concluded that the introduction of the 8-lipo group significantly enhanced its affinity and selectivity for topo IIα, as well as increasing the tumor targeting which led to the low toxicity.

Secondly, according to the data of flow cytometry assay, aconitine linoleate (1) exhibits a cytotoxic effect on MCF-7/ADR cells by increasing the percentage of cells of the G0/G1 phase, decreasing the same in the S phase. As everyone knows, topo IIα and IIβ are two types of topo II
in humans. The expression level of topo IIα is the highest in the G2/M phase, followed by the S phase, and lowest in the G0/G1 phase, but that of topo IIβ is always constant (Jun et al. 2015). Therefore, due to the selective inhibition of topo IIα, aconitine linoleate (I) targeted the tumor cells in the S phase.

Thirdly, as a widely used chemotherapeutic agent, doxorubicin, mainly targeting topo IIα (Du et al. 2011), has caused drug resistance during the clinical application, which is a complex process (Ganapath and Ganapathi 2013). Different resistant mechanisms have been recognized, including the enhanced expression of the P-glycoprotein, ABCB1, ABCG2, the down expression level of topo IIα, overexpression of multidrug resistance protein 1(MDR1), and even the activation of autophagy (Mechteher et al. 1998, Latorre et al. 2012, Shatha AbuHammad and Zihlif 2013, Guo et al. 2016). Although targeted to the same enzyme, aconitine linoleate (I) is mainly a topo catalytic inhibitor, which could selectively inhibit topo IIα, rather than a topo poison like doxorubicin. They may have a different topo-inhibit mechanism. Moreover, the reversal mechanism of tumor drug resistance of aconitine linoleate (I) should be a multi-targeted way, which need further exploration in the next step.

Conclusions

We conclude that aconitine linoleate (I) not only selectively inhibits the expression and activity of topo IIα, but also induces a significant increase in the percentage of cells at the G0/G1 phase, leading to its excellent anti-proliferative activity against MCF-7 and MCF-7/ADR in vitro. Since the introduction of the 8-lipo group, I is much less toxic than aconitine. Lipo-alkaloid would be a powerful and potentially antitumor compound against drug-resistant breast cancer, bringing more possibilities for fighting breast cancer in the future.

Accession codes

The PDB access code for the structure of I bond to topoisomerase IIα is SGWK. The authors will release the atomic coordinates and experimental data upon article publication.

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Data availability As supplemental material and upon request.

Declarations

Ethics approval and consent to participate The animal use protocol listed below has been reviewed and approved by the Sichuan Agricultural University Animal Ethical and Welfare Committee.

Consent for publication All authors approved the manuscript to be published.

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