Screening of Promising Chemotherapeutic Candidates from Plants against Human Adult T-Cell Leukemia/Lymphoma (VI): Cardenolides from Asclepias curassavica

Daisuke Nakano, Kenji Ishitsuka, Maya Takashima, Rie Arima, Aya Satou, Ryota Tsuchihashi, Masafumi Okawa, Kazuo Tamura, and Junei Kinjo

*Faculty of Pharmaceutical Sciences, Fukuoka University; 8–19–1 Nanakuma, Jonan-ku, Fukuoka 814–0180, Japan: 1Division of Hematology and Immunology, Center for Chronic Viral Diseases, Graduate School of Medical and Dental Sciences, Kagoshima University; 8–35–1 Sakuragaoka, Kagoshima 890–8544, Japan: and 2Department of Internal Medicine, Division of Medical Oncology, Hematology and Infectious Disease, Fukuoka University; 7–45–1 Nanakuma, Jonan-ku, Fukuoka 814–0180, Japan.

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In the course of our screening program for novel chemotherapeutic candidates from plants against adult T-cell leukemia/lymphoma, the extracts of Asclepias curassavica L. showed potent activity against MT-1 and MT-2 cells. Therefore, we attempted to isolate their active components. We identified a new cardenolide, 19-dihydrocalactinic acid methyl ester (1), along with 16 known cardenolides (2–17). Their structures were determined on the basis of spectroscopic data. Almost all of the isolated cardenolides inhibited the growth of both tumor cell lines. All the doubly linked cardenolides (11–17) except for 14 showed more potent activity than the other cardenolides. A comparison of the activities of 11, 14, and 16 revealed that the presence of hydroxy or acetoxy functional groups at C-16 led to a decrease in the activity. The 50% effective concentration (EC50) value of calotropin (11) against MT-2 cells was comparable to the potency of the clinical antineoplastic drug doxorubicin. The cytotoxic effect of 11 toward normal mononuclear cells obtained from the peripheral blood (PB-MNCs) was observed at a concentration 6 to 12 times higher than that used to induce growth inhibition against MT-1 and MT-2 cells. The proportions of annexin V-positive cells after 72 h of treatment with 11 were increased, indicating that it significantly induced apoptosis in MT-1 and MT-2 cells in a concentration-dependent manner. Cell cycle experiments demonstrated that 11 arrested MT-1 and MT-2 cells at the G2/M phase. Therefore, compound 11 may be a promising candidate for the treatment of adult T-cell leukemia/lymphoma.

Key words screening; adult T-cell leukemia/lymphoma; Asclepias curassavica; cardenolide

INTRODUCTION

Adult T-cell leukemia/lymphoma (ATL) is a malignancy of mature peripheral T-lymphocytes associated with human T-cell lymphotropic virus type I (HTLV-I). Combinations of conventional chemotherapeutic agents used against other types of malignant lymphomas have been administered to patients with acute- and lymphoma-type ATL, but the therapeutic outcomes remain very poor. Therefore, we conducted a search for novel chemotherapeutic candidates against ATL from plants using two cell lines. MT-1 cells were established from the peripheral blood (PB) tumor cells of ATL patients and MT-2 cells were established from cord blood T-cells by cocultivation of normal human cord lymphocytes and PB tumor cells of an ATL patient.

We previously reported the screening results of 459 extracts and the isolation of active constituents (withanolides, aporphine alkaloids, phenanthroidolizidine alkaloids, quinolone alkaloids and coumarins) against MT-1 and MT-2 cell lines. Herein, following from the results of our previous screening program, we report the isolation of cardenolides as active components from Asclepias curassavica. That belongs to the family Apocynaceae and includes cardenolides. The plants from Apocynaceae are well known for their folkloric use in the treatment of many cancerous conditions.

MATERIALS AND METHODS

General Experimental Procedures Optical rotation was measured using an MCP 150 module type small polarimeter (Anton Paar). The 1H- and 13C-NMR spectra were measured using a JNM-ECZ600R spectrometer (JEOL) at 600 and 150 MHz, respectively, and the chemical shifts are reported on the δ (ppm) scale. The high resolution (HR)-FAB-MS were measured using a JEOL JMS-HX110 mass spectrometer and acquired in a glycerol matrix. HPLC was conducted using a Waters machine equipped with a 1525 binary pump and a 2489 UV/Vis detector. Separation was carried out using a Cosmosil 5C18 MS-II column (20.0 × 250 mm, octadecyl silica (ODS), 5 μm, Nacalai Tesque, Kyoto, Japan). Apoptosis and cell cycle analyses were carried out using a Muse Cell Analyzer (Merck KGaA, Darmstadt, Germany).

Plant Materials The plant materials used in this study were taken from the same place as reported previously. Voucher specimens were deposited in the Laboratory of Pharmacognosy at Fukuoka University (FUN 090232).

Extraction and Isolation The samples were powdered and extracted according to the procedure described in a previous paper. The aerial parts of Asclepias curassavica (1370 g), were extracted with MeOH under reflux. The extract (70.2 g) was initially partitioned between n-hexane and 80% MeOH.
The 80% MeOH phase was partitioned between EtOAc and 40% MeOH. The EtOAc layer was subjected to a MCI gel column using 50, 60, 70, and 80% MeOH. The 80% MeOH fraction was subjected to a silica gel column using CHCl₃:MeOH:H₂O = 9:1:0.1 to give fr. AE-1. Fraction AE-1 was subjected to a silica gel column using n-hexane:acetone = 3:2 to give fr. AE-1-1. Fraction AE-1-1 was subjected to a silica gel column using CHCl₃:MeOH = 20:1 to give compound 1 (2.8 mg, 0.00020%).

The extract of the aerial parts was subjected to several chromatographic purification steps to obtain compounds 2–8 and 11–14. Compounds 9–10 and 15–17 were obtained from the root extracts.

**Table 1. **¹H- and ¹³C-NMR Chemical Shift Data of Compound 1

| Position | δC | δH                  |
|----------|----|---------------------|
| 1        | 41.0 | 3.11 (1H, dd, 13.1, 4.8), 1.11 (1H, m) |
| 2        | 70.9 | 4.28 (1H, m)        |
| 3        | 85.7 | 3.78 (1H, m)        |
| 4        | 34.3 | 1.76 (1H, m), 1.70 (1H, m) |
| 5        | 44.6 | 2.00 (1H, m)        |
| 6        | 29.9 | 1.32 (1H, m), 1.28 (1H, m) |
| 7        | 23.3 | 1.96 (1H, m), 1.84 (1H, m) |
| 8        | 41.7 | 2.02 (1H, m)        |
| 9        | 50.5 | 1.21 (1H, m)        |
| 10       | 40.8 |                |
| 11       | 27.9 | 2.37 (1H, m), 1.27 (1H, m) |
| 12       | 40.2 | 1.41 (1H, m), 1.26 (1H, m) |
| 13       | 50.1 |                |
| 14       | 84.7 |                |
| 15       | 33.0 | 2.08 (1H, m), 1.86 (1H, m) |
| 16       | 27.3 | 2.09 (1H, m), 1.96 (1H, m) |
| 17       | 51.4 | 2.76 (1H, m)        |
| 18       | 16.2 | 1.04 (3H, s)        |
| 19       | 59.6 | 4.06 (1H, dd, 11.4, 4.5), 3.85 (1H, dd, 11.4, 3.8) |
| 20       | 175.9 |                |
| 21       | 73.6 | 5.28 (1H, dd, 18.3, 1.8), 5.00 (1H, dd, 18.3, 1.8) |
| 22       | 117.5 | 6.13 (1H, s)       |
| 23       | 174.4 |                |
| 1’       | 108.6 | 5.65 (1H, s)       |
| 2’       | 85.3 |                |
| 3’       | 172.1 |                |
| 4’       | 41.4 | 2.70 (1H, dd, 12.7, 10.0), 2.50 (1H, dd, 12.7, 6.2) |
| 5’       | 76.5 | 4.85 (1H, m)        |
| 6’       | 22.5 | 1.46 (3H, d, 6.2)   |
| Me       | 51.8 | 3.79 (3H, s)        |
| 19-OH    | 5.94 | (1H, t, 4.1)       |

Cell Culture

Two HTLV-1-infected T-cell lines, MT-1 and MT-2, were kindly provided by Dr. I. Miyoshi of Kochi University in Nangoku, Japan. Culture conditions were as previously described. The cells were cultured in RPMI-1640 medium containing fetal bovine serum and 1% kanamycin. Cells were cultured at 37 °C in humidified 5% CO₂/95% air. Normal mononuclear cells obtained from the peripheral blood (PB-MNCs) of healthy individuals after informed consent were separated by Ficoll-Hypaque density sedimentation.

**Measurement of Antiproliferative Effects against MT-1 and MT-2 Cells**

Cellular growth was determined using the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The MT-1 and MT-2 cells were maintained in RPMI-1640 medium containing fetal bovine serum (15%). A 50 µL aliquot of the cell suspension (5000 cells per well) and 50 µL of the test sample solution or suspension were plated in flat-bottomed microtiter wells (extract final concentration: 100, 10, 1, 0.1 µg/mL and control, compound final concentration: 100 µg/mL–10 pg/mL and control) and incubated for 72 h at 37 °C in humidified atmosphere of 5% CO₂/95% air. After incubation, 10 µL isopropanol was added to solubilize the MTT-formazan product. The absorbance at 450 nm was measured with a microplate reader.

**Measurement of Cytotoxicity toward Normal Cells**

The PB-MNC cells were maintained in RPMI-1640 medium containing fetal bovine serum (15%). A 50 µL aliquot of the cell suspension (5000 cells per well) and 50 µL of the test sample solution or suspension were plated in flat-bottomed microtiter wells (extract final concentration: 100, 10, 1, 0.1 µg/mL and control, compound final concentration: 100 µg/mL–10 pg/mL and control) and incubated for 72 h at 37 °C in humidified atmosphere of 5% CO₂/95% air. After incubation, 10 µL MTT reagent solution was added to the microtiter wells. After incubation for 4 h at 37 °C, 100 µL isopropanol was added to solubilize the MTT-formazan product. The absorbance at 450 nm was measured with a microplate reader.
A 50 µL aliquot of the cell suspension (10000 cells per well) and 50 µL of the test sample solution were plated in flat-bottomed microtiter wells (final concentration: 100, 10, 1, 0.1 µg/mL and control) and incubated for 72 h at 37 °C in a humidified atmosphere of 5% CO₂ in air. After cultivation, 10 µL of the MTT reagent solution was added to the microtiter wells. After incubation for 4 h at 37 °C, Fig. 2. Key HMBC Correlations of Compound 1

Table 2. The Antiproliferative Activity of Compounds 1–17

| Compound | EC₅₀ (µM) | MT-1 | MT-2 | PB-MNCs |
|----------|-----------|------|------|---------|
| 1        | 0.94      | 1.00 | N.T. |         |
| 2        | 18.0      | 24.5 | N.T. |         |
| 3        | 126       | 90.1 | N.T. |         |
| 4        | 1.99      | 1.10 | N.T. |         |
| 5        | 3.1       | 3.1  | N.T. |         |
| 6        | 2.67      | 3.47 | N.T. |         |
| 7        | 6.19      | 5.72 | N.T. |         |
| 8        | 7.25      | 8.01 | N.T. |         |
| 9        | 1.11      | 0.140| N.T. |         |
| 10       | 0.290     | 0.140| N.T. |         |
| 11       | 0.038     | 0.019| 0.226|         |
| 12       | 0.035     | 0.052| N.T. |         |
| 13       | 0.030     | 0.028| N.T. |         |
| 14       | 3.83      | 1.69 | N.T. |         |
| 15       | 0.220     | 0.160| N.T. |         |
| 16       | 0.220     | 0.160| N.T. |         |
| 17       | 0.158     | 0.158| N.T. |         |
|          | 0.015     | 0.013| N.T. |         |

Doxorubicin 0.015 0.013 N.T.

N.T.: not tested.

Fig. 3. Calotropin (11) Induces Cell Apoptosis in MT-1 and MT-2 Cells
100 µL isopropanol was added to solubilize the MTT-formazan product. The absorbance at 450 nm was measured with a microplate reader.

**Apoptosis Analysis** Detection of apoptosis was performed using The Muse Annexin V & Dead Cell Assay Kit according to the manufacturer’s protocols. MT-1 and MT-2 cells incubated in the presence or absence of compound 11 for 72 h were collected by centrifugation (310 × g at 4 °C for 10 min), suspended in 100 µL of RPMI 1640 medium and incubated with 100 µL of Annexin V reagent at room temperature for 20 min. Cells were measured by a Muse Cell Analyzer.

**Cell Cycle Analysis** MT-1 and MT-2 cells incubated in the presence or absence of compound 11 for 72 h were collected by centrifugation (310 × g at room temperature for 10 min), suspended in 50 µL of phosphate buffered saline (PBS) and fixed with 1 mL of 70% ethanol for over 3 h at −20 °C. After fixation, cell pellets obtained by centrifugation (310 × g, 10 min) were suspended in 1 mL of PBS, incubated with 200 µL of Muse Cell Cycle Reagents in the dark for 30 min and measured by a Muse Cell Analyzer.

**RESULTS AND DISCUSSION**

As we reported previously, the extracts of *A. curassavica* showed potent activity against MT-1 (50% effective concentration (EC$_{50}$), aerial parts: 11.5 µg/mL, roots: 4.2 µg/mL) and MT-2 (EC$_{50}$, aerial parts: 10.1 µg/mL, roots: 4.1 µg/mL) cells. Therefore, we attempted to isolate their active components.
The extracts were subjected to several chromatographic purification steps to obtain compounds 1–8 and 11–14 from the aerial parts, whereas compounds 9–10 and 15–17 were obtained from the roots (Fig. 1). Compound 1 was a new compound, and compounds 2–17 were previously identified as shown by comparison of their physical data with those reported in the literature.9,11–18

The negative HR-FAB-MS of compound 1 displayed a peak for [M−H]− at m/z 563.2871 suggesting that its molecular formula was C34H42O6.

The 13C-NMR displayed 30 carbon signals (Table 1). Two carbonyl carbons resonated at δ 174.4 (C) and 172.1 (C), and two olefin carbons were located at δ 175.9 (C) and 117.5 (CH). Five signals for carbons bearing oxygen were observed at δ 73.6 (CH2), 85.7 (CH), 84.7 (C), 70.9 (CH), and 59.6 (CH2) and one methyl carbon was located at δ 16.2. In addition, one methoxy methyl carbon at δ 51.8 and two oxygenated carbon signals at δ 85.3 and 76.5 were observed. The 1H-NMR spectra showed one methyl singlet (δ 1.04) and one additional methyl doublet at δ 1.46 (J = 6.2 Hz). The connectivity of the protonated carbons was determined from the correlation spectroscopy (COSY) and heteronuclear multiple bond connectivity (HMBC) spectra (Fig. 2). The HMBC correlation for δH 5.65 (H-1'1) with δC 85.7 (C-3) suggested a furan ring moiety at C-3 and the correlation for δH 3.79 (OMe) with δC 172.1 (C-3') indicated that a methyl ester existed at C-3'. The correlation for δH 4.06 (H-19) with δC 40.8 (C-10) and δC 50.5 (C-9) indicated that a hydroxymethyl existed at C-19. Additionally, the 13C-NMR spectrum of 1 was in agreement with that of 2 except that the C-19 of 1 was a hydroxymethyl group.21 Thus, the structure of 1 was determined to be 19-dihydroxylactonic acid methyl ester, a new compound.

The anti-proliferative effects of 1–17 are listed in Table 2. Almost all of the isolated cardenolides inhibited the growth of both tumor cell lines. Except for 14, the doubly linked cardenolides30 (11–17) showed more potent activity than the other cardenolides.

Compounds 11–13 were the most potent. A comparison of the activities of compounds 11, 14 and 16 revealed that the presence of a hydroxy or acetoxy group at C-16 led to a decrease in the activity. Similar results were obtained for 12, 15 and 17. Furthermore, the hydroxy function at C-16 in 5 slightly decreased the activity compared with 4. The presence of a 3-hydroxy-5-methoxylactone-3-carboxylate function at C-3 in compound 2 seemed to enhance the activity compared with compound 3. Additionally, glycosylation of an allomethylose moiety in 10 seemed to enhance the activity compared with compound 8. A comparison of the activities of compounds 4 and 8 revealed that the presence of a glucose moiety at C-3 led to a decrease in the activity. Similar results were obtained for 6 and 7.

The EC50 value of 11 against MT-2 cells was comparable to the potency of the clinically used antineoplastic drug doxorubicin. Furthermore, we examined its cytotoxicity, and analyzed its ability to induce apoptosis and arrest the cell cycle.

The cytotoxic effect of compound 11 toward normal PB-MNCs was observed at a concentration 6 to 12 times higher than that required to induce growth inhibition against MT-1 and MT-2 cells (Table 1).

Annexin V-positive cells were used to investigate the effects of 11 on apoptosis. The proportion of annexin V-positive cells after 72 h of treatment with 11 is shown in Fig. 3. The distribution of apoptotic cells, which were located in the upper-right (late apoptotic/dead cells) and lower-right (early apoptotic cells) quadrants, was increased in cells treated with compound 11. Therefore, compound 11 significantly induced apoptosis in MT-1 and MT-2 cells in a concentration-dependent manner.

The percentage of cells in the G0/G1, S and G2/M phases of the cell cycle was quantitatively measured using the Muse Cell Cycle Reagent. The reagent includes the nuclear DNA intercalating stain propidium iodide and ribonuclease (RNase). Propidium iodide discriminates cells at different stages of the cell cycle based on their differential DNA content in the presence of RNase to increase the specificity of the DNA staining. The cell cycle experiments demonstrated that 11 arrested MT-1 and MT-2 cells at the G2/M phase (Fig. 4).

Recently, it was shown that calotropin (11) inhibits Wt signaling by increasing casein kinase 1a in colon cancer cells39 and 11 induces cell cycle arrest and apoptosis in cisplatin-resistant lung cancer cells.20

In conclusion, as part of our investigations of Asclepiadaceae plants, extracts of A. curassavica showed potent inhibitory effects against MT-1 and MT-2 cell lines. We isolated 17 cardenolides from the extract and examined their structure—activity relationships. Almost all of the isolated cardenolides inhibited the growth of both tumor cell lines. The most active component (11) had lower toxicity toward normal PB-MNCs than HTLV-1-infected T-cell lines. We demonstrated that 11 enhanced apoptosis and arrested MT-1 and MT-2 cells at the G2/M phase. Therefore, cardenolides are promising candidates for the treatment of ATL, similar to withanolides.21

Conflict of Interest The authors declare no conflict of interest.

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