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ORIGINAL PAPER

Structure–activity relationships and the cytotoxic effects of novel diterpenoid alkaloid derivatives against A549 human lung carcinoma cells

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Abstract The cytotoxicity of three alkaloids from the roots of *Aconitum yesoense* var. *macroyesoense* as well as 36 semi-synthetic C_{20}-diterpenoid atisine-type alkaloid derivatives against A549 human lung carcinoma cells was examined. Ten acylated alkaloid derivatives, pseudokobusine 11-veratroate (**9**), 11-anisoate (**12**), 6,11-dianisoate (**14**), 11-*p*-nitrobenzoate (**18**), 11,15-di-*p*-nitrobenzoate (**22**), 11-cinnamate (**25**) and 11-*m*-trifluoromethylbenzoate (**27**), and kobusine 11-*p*-trifluoromethylbenzoate (**35**), 11-*m*-trifluoromethylbenzoate (**36**) and 11,15-di-*p*-nitrobenzoate (**39**), exhibited cytotoxic activity, and 11,15-dianisoylpseudokobusine (**16**) was found to be the most potent cytotoxic agent. Their IC_{50} values against A549 cells ranged from 1.72 to 5.44 μM. In the occurrence of cytotoxic effects of atisine-type alkaloids, replacement by an acyl group at both C-11 and C-15 resulted in the enhancement of activity of the parent alkaloids compared to that from having hydroxy groups at this position, and the presence of a hydroxy group at the C-6 position was required for the cytotoxic effects. These acylated alkaloid derivatives inhibit cell growth through G1 arrest.

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Keywords Diterpenoid alkaloids · Pseudokobusine · Kobusine · Cytotoxic agents · A549 human lung carcinoma cells · Structure–activity relationship

Introduction

Diterpenoid alkaloids are classified according to their chemical structure as C_{19}-norditerpenoid alkaloids, which consist of an aconitine or a lycoctonine skeleton, and C_{20}-diterpenoid alkaloids, consisting of an atisine or a veatchine skeleton. A large number of diterpenoid alkaloids have been isolated from various species of *Aconitum* and *Delphinium* (Ranunculaceae) [1, 2]. The pharmacological properties of C_{19}-norditerpenoid alkaloids, including aconitine, mesaconitine, hypaconitine and jesaconitine, have been studied extensively and reviewed [1, 2]. Aconitine and mesaconitine are representative toxins that exhibit activity both centrally and peripherally, with predominant effects on the cardiovascular and respiratory systems, by preventing the normal closing of sodium channels [3, 4]. In contrast, there is little information regarding the pharmacological properties of C_{20}-diterpenoid alkaloids and their chemically transformed products. Kobusine (**1**) and pseudokobusine (**2**), the major alkaloid constituents of *Aconitum yesoense* var. *macroyesoense*, and certain semi-synthetic derivatives of diterpenoid alkaloids have been shown by using a Doppler-type laser blood flow meter to significantly increase cutaneous blood flow in the hind feet of anaesthetized mice [5–7].

The majority of drugs used in cancer chemotherapy can be divided into alkylating agents, anti-metabolites, antibiotics, plant alkaloids, topoisomerase inhibitors, monoclonal antibodies and other antitumor agents [8–16]. However, little information on the cytotoxic properties of *Aconitum*

alkaloids has been reported, despite their intense toxicities. Two reports on the effects of C_{19}-norditerpenoid alkaloids on cancer cells have appeared in recent years. 8-*O*-Azeloyl-14-benzoylaconine, an aconitine-type C_{19}-norditerpenoid alkaloid, exhibited anti-proliferative activity [17] and the cytotoxic effects of various C_{19}-norditerpenoid alkaloids against tumor cell lines have been reported [18]. Our previous study demonstrated the effects of various naturally occurring and semi-synthetic diterpenoid alkaloids on the growth of the A172 human malignant glioma cell line [19]. The results of previous studies showed that seven acylated alkaloid derivatives, 12-acetylluciculine, pseudokobusine 11-veratroate (**9**), 11-anisoate (**12**), 11-*p*-nitrobenzoate (**18**), 11-cinnamate (**25**) and 11-*m*-trifluoromethylbenzoate (**27**), and 11-(*m*-trifluoromethylbenzoyl)kobusine (**36**), had significant cytotoxic effects on the growth of A172 cells. Esterification of the hydroxyl group at C-11 may, thus, contribute to the enhancement of activity of the parent alkaloids more than that of the OH group at C-11. Cytotoxic properties and radiation-sensitizing effects of various types of novel derivatives prepared from *Aconitum* alkaloids have also been investigated [20]. 11-Anisoylpseudokobusine (**12**) and 11-*m*-trifluoromethylbenzoyl pseudokobusine (**36**) showed significant suppressive effects against the non-Hodgkin's lymphoma Raji cell line [21]. 11-*m*-Trifluoromethylbenzoylpseudokobusine (**36**) clearly inhibited the phosphorylation of extracellular signal-regulated kinase, induced enhanced phosphoinositide 3-kinase phosphorylation and led to the subsequent accumulation of G1 and/or sub-G1 phase in Raji cells. In addition, suppressive effects of 11-anisoylpseudokobusine (**12**) and 11-*m*-trifluoromethylbenzoylpseudokobusine (**36**) on the growth of human $CD34^+$ hematopoietic stem/progenitor cells were observed.

In the present study, the effects of various semi-synthetic novel C_{20}-diterpenoid alkaloids on the growth of the A549 human lung cancer cell line were examined. Twenty novel derivatives were prepared from natural compounds. In order to carry out structure–activity relationship studies of the anti-proliferative effect against A549 cells, three natural and 36 semi-synthetic diterpenoid alkaloids were tested.

Materials and methods

General experimental procedures

Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. ^{1}H-NMR spectra in $CDCl_3$ were recorded on JEOL GX-270 and AL-400 spectrometers using tetramethylsilane as an internal standard. Chemical shifts are given in ppm. Electron impact (EI) mass spectra were measured on Hitachi M-2000 and JEOL JMS-700 spectrometers. IR spectra were recorded with an IR spectrophotometer, Perkin-Elmer Spectrum 100. All products reported showed ^{1}H-NMR spectra and mass spectra in agreement with the assigned structures. Reactions were carried out under an inert atmosphere of dry nitrogen or argon, unless otherwise described. Standard syringe techniques were used for transferring dry solvents. Reaction courses and product mixtures were monitored routinely by TLC on silica gel (precoated Merck F_{254} plates) and visualized with Dragendorff reagent. Chromatography was performed using silica gel and the indicated solvent system. All other chemicals used were of analytical grade.

Alkaloids

The diterpenoid alkaloids kobusine (**1**), pseudokobusine (**2**) and 15-veratroylpseudokobusine (**10**) were used after extraction from the roots of *A. yesoense* var. *macroyesoense*, followed by purification and identification by methods described previously [22, 23]. Thirty acyl derivatives, *N*-benzyl-*N*,6-seco-6-dehydropseudokobusine (**3**) [19], *N*,15-dibenzyl-*N*,6-seco-6-dehydropseudokobusine (**4**) [19], 6-benzoylpseudokobusine (**5**) [23], 6,11-dibenzoylpseudokobusine (**6**) [23], 15-benzoyl-6,11-di-*p*-nitrobenzoylpseudokobusine (**7**) [19], 6-veratroylpseudokobusine (**8**) [7], 11-veratroylpseudokobusine (**9**) [7], 6-anisoylpseudokobusine (**11**) [7], 11-anisoylpseudokobusine (**12**) [7], 15-anisoylpseudokobusine (**13**) [7], 6,11-dianisoylpseudokobusine (**14**) [7], 6,15-dianisoylpseudokobusine (**15**) [7], 11,15-dianisoylpseudokobusine (**16**) [7], 6-*p*-nitrobenzoylpseudokobusine (**17**) [23], 11-*p*-nitrobenzoylpseudokobusine (**18**) [7], 15-*p*-nitrobenzoylpseudokobusine (**19**) [24], 6,11-di-*p*-nitrobenzoylpseudokobusine (**20**) [24], 6,15-di-*p*-nitrobenzoylpseudokobusine (**21**) [24], 11,15-di-*p*-nitrobenzoylpseudokobusine (**22**) [7], 6,11,15-tri-*p*-nitrobenzoyl pseudokobusine (**23**) [24], 6-cinnamoylpseudokobusine (**24**) [6], 11-cinnamoylpseudokobusine (**25**) [6], 6-(*m*-trifluoromethylbenzoyl)pseudokobusine (**26**) [19], 11-(*m*-trifluoromethylbenzoyl)pseudokobusine (**27**) [19], 11-benzoyl kobusine (**30**) [6], 11-anisoylkobusine (**31**) [7], 11-veratroylkobusine (**32**) [7], dihydrokobusine (**33**) [7], 11-cinnamoylkobusine (**34**) [6] and 11-(*m*-trifluoromethylbenzoyl)kobusine (**36**) [19], were prepared by methods described previously. Six semi-synthetic derivatives, 6-(*p*-trifluoromethylbenzoyl)pseudokobusine (**28**), 11-(*p*-trifluoromethylbenzoyl)pseudokobusine (**29**), 11-(*p*-trifluoromethylbenzoyl)kobusine (**35**), 11-*p*-nitrobenzoylkobusine (**37**), 15-*p*-nitrobenzoylkobusine (**38**) and 11,15-di-*p*-nitrobenzoylkobusine (**39**), were prepared from kobusine (**1**) and pseudokobusine (**2**). These semi-synthetic alkaloids were synthesized at controlled reaction times and temperatures.

Synthesis of 6-(**28**) and 11-(*p*-trifluoromethylbenzoyl) pseudokobusine (**29**)

A solution of **2** (0.111 g, 0.34 mmol) and *p*-trifluoromethylbenzoyl chloride (0.1 ml, 0.67 mmol) in pyridine (2 ml) was stirred for 30 min at ambient temperature. After adding water, the reaction mixture was extracted with chloroform after the addition of aqueous NH_4OH. The organic layer was washed with aqueous saturated $NaHCO_3$ and brine, and then dried over anhydrous $MgSO_4$. The solvent was evaporated under reduced pressure and the resulting residue was purified by silica gel column chromatography eluting with 1% methanol–NH_4OH–saturated $CHCl_3$ to give **28** (19 mg, 11%), **29** (34 mg, 20%) and **2** (14 mg). 6-(*p*-Trifluoromethylbenzoyl)pseudokobusine (**28**): amorphous. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.98 (3H, s, H-18), 3.96 (1H, s, H-15), 4.08 (1H, d, $J = 4.8$ Hz, H-11), 5.14 and 5.24 (each 1H, s, H-17), 7.71 (2H, d, $J = 8.0$ Hz, H–Ar), 8.16 (2H, d, $J = 8.0$ Hz, H–Ar). IR (ATR) cm^{-1}: 3316, 1726, 1562, 1322, 1259, 1163, 896. EIMS *m*/*z*: 501 (M^+), 429, 329, 190, 173. HREIMS *m*/*z*: 501.2130 (calcd. for $C_{28}H_{30}F_3NO_4$: 501.2127). 11-(*p*-Trifluoromethylbenzoyl)pseudokobusine (**29**): colorless crystals (acetone–hexane), mp 293°C (dec.). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 1.18 (3H, s, H-18), 4.00 (1H, s, H-15), 5.06 and 5.23 (each 1H, s, H-17), 5.32 (1H, d, $J = 4.8$ Hz, H-11), 7.64 (1H, d, $J = 8.3$ Hz, H–Ar), 8.00 (1H, d, $J = 8.3$ Hz, H–Ar). IR (ATR) cm^{-1}: 3291, 1717, 1557, 1323, 1267, 1166, 901. EIMS *m*/*z*: 501 (M^+), 328, 173. HREIMS *m*/*z*: 501.2125 (calcd. for $C_{28}H_{30}F_3NO_4$: 501.2127).

Synthesis of 11-(*p*-trifluoromethylbenzoyl)kobusine (**35**)

A solution of **1** (0.041 g, 0.13 mmol) and *p*-trifluoromethylbenzoyl chloride (0.04 ml, 0.26 mmol) in pyridine (1 ml) was stirred for 1.5 h at 0°C (ice bath). After adding water, the reaction mixture was extracted with chloroform after the addition of aqueous NH_4OH. The organic layer was washed with aqueous saturated $NaHCO_3$ and brine, and then dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure, and the resulting residue was purified by silica gel column chromatography eluting with 2% methanol–NH_4OH–saturated $CHCl_3$ to give **35** (16 mg, 26%). 11-(*p*-Trifluoromethylbenzoyl)kobusine (**35**): white crystals (acetone–hexane), mp 213–216°C. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.98 (3H, s, H-18), 4.03 (1H, s, H-15), 5.08 and 5.25 (each 1H, s, H-17), 5.41 (1H, d, $J = 4.8$ Hz, H-11), 7.69 (2H, d, $J = 8.3$ Hz, H–Ar), 8.07 (2H, d, $J = 8.3$ Hz, H–Ar). IR (ATR) cm^{-1}: 3361, 1718, 1555, 1323, 1275, 1164, 895. EIMS *m*/*z*: 485 (M^+), 312, 296, 173. HREIMS *m*/*z*: 485.2157 (calcd. for $C_{28}H_{30}F_3NO_3$: 485.5376).

Synthesis of 11-(**37**), 15- (**38**) and 11,15-di-*p*-nitrobenzoylkobusine (**39**)

A solution of **1** (0.106 g, 0.34 mmol) and *p*-nitrobenzoyl chloride (0.252 mg, 1.36 mmol) in pyridine (2 ml) was stirred for 2 h at 0°C (ice bath). After adding water, the reaction mixture was extracted with chloroform after the addition of aqueous NH_4OH. The organic layer was washed with aqueous saturated $NaHCO_3$ and brine, and then dried over anhydrous $MgSO_4$. The solvent was evaporated under reduced pressure, and the resulting residue was purified by silica gel column chromatography eluting with 0.5% methanol–NH_4OH–saturated $CHCl_3$ to give **37** (55 mg, 35%), **38** (14 mg, 9%), **39** (52 mg, 25%) and **1** (4 mg). 11-*p*-Nitrobenzoylkobusine (**37**): amorphous. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.99 (3H, s, H-18), 4.05 (1H, d, $J = 5.6$ Hz, H-15), 5.08 and 5.25 (each 1H, s, H-17), 5.42 (1H, d, $J = 4.9$ Hz, H-11), 8.13 (2H, d, $J = 8.5$ Hz, H–Ar), 8.28 (2H, d, $J = 8.5$ Hz, H–Ar). IR (ATR) cm^{-1}: 3377, 1717, 1605, 1525, 1279, 1103, 900. EIMS *m*/*z*: 462 (M^+), 433, 312, 295. HREIMS *m*/*z*: 462.2179 (calcd. for $C_{27}H_{30}N_2O_5$: 462.2153). 15-*p*-Nitrobenzoylkobusine (**38**): colorless crystals (acetone–hexane), mp 214–216°C. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.96 (3H, s, H-18), 4.14 (1H, d, $J = 4.6$ Hz, H-11), 5.25 and 5.39 (each 1H, s, H-17), 5.73 (1H, s, H-15), 8.21 (2H, d, $J = 8.9$ Hz, H–Ar), 8.30 (2H, d, $J = 8.9$ Hz, H–Ar). IR (ATR) cm^{-1}: 3055, 1710, 1606, 1524, 1269, 1104, 901. EIMS *m*/*z*: 462 (M^+), 432, 312, 296. HREIMS *m*/*z*: 462.2131 (calcd. for $C_{27}H_{30}N_2O_5$: 462.2153). 11,15-*p*-Nitrobenzoylkobusine (**39**): colorless crystals (acetone–hexane), mp 232–233°C. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.97 (3H, s, H-18), 5.19 and 5.41 (each 1H, s, H-17), 5.51 (1H, d, $J = 4.6$ Hz, H-11), 5.81 (1H, s, H-15), 8.02 (2H, d, $J = 8.9$ Hz, H–Ar), 8.05 (4H, d, $J = 8.9$ Hz, H–Ar), 8.07 (2H, d, $J = 8.3$ Hz, H–Ar). IR (ATR) cm^{-1}: 3073, 1711, 1606, 1524, 1260, 1102, 902. EIMS *m*/*z*: 611 (M^+), 581, 461, 432. HREIMS *m*/*z*: 611.2265 (calcd. for $C_{34}H_{33}N_3O_8$: 611.2265).

Inhibition of growth of the human lung cancer cell line A549

All test alkaloids were dissolved in dimethyl sulfoxide (DMSO) at 1 or 5 mg/ml immediately before use and diluted in the medium before addition to the cells. Cells were cultured in a DMEM medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics [penicillin (100 UI/ml) and streptomycin (100 UI/ml)].

To determine the effects of alkaloids on cell growth, exponentially growing A549 cells (4×10^3 cells/well) were seeded in 24-well plates (Falcon, Becton–Dickinson Biosciences, Franklin Lakes, NJ, USA) with 500 ml of medium, and each alkaloid (final concentration: 1 or 5 μg/ml) was added to each plate 24 h later. The total cell numbers were determined after 3 days using a particle counter (model Z^{TM} series, Coulter Electronics, Hialeah, FL, USA). The results are expressed as inhibition values in comparison to untreated controls and as IC_{50} values (concentration causing 50% inhibition relative to untreated controls). All experiments were repeated at least three times. Excluding the possible anti-proliferative effects of DMSO, the maximum concentration of DMSO (0.5%) was used in control culture and did not affect the growth of A549 cells. Pemetrexed (Eli Lily and Company, Indianapolis, IN, USA) was used as the reference control.

Cell cycle analysis by flow cytometry

A549 cells were treated with each compound at doses of its IC_{50} and double IC_{50} values and incubated for 24 h. The harvested cells were treated with PBS containing 0.1% Triton X-100 (Wako) and were stained with propidium iodide (50 μg/ml, Sigma). Analysis of cell cycle distribution was performed using a flow cytometer (Beckman–Coulter, Cell Lab Quanta^{TM} SC MPL, Fullerton, CA, USA).

Statistical analysis

The data are expressed as the mean ± SD of 3 cultures of a group, and the significance of differences between the control and experimental groups were determined using either Student's *t* test or Mann–Whitney's *U*-test, depending on the data distribution. Statistical analysis was performed using the Excel 2003 software package (Microsoft, Redmond, WA, USA) with the add-in software Statcel 2 (OMS, Saitama, Japan).

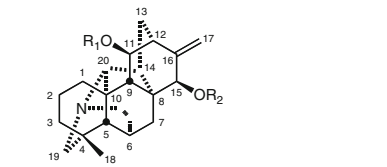
Results and discussion

Aconitum diterpenoid alkaloids and their novel derivatives were examined for the suppressive effects on the growth of the A549 human lung cancer cell line [20]. C_{19}-norditerpenoid aconitine-type alkaloids (five alkaloids) and lycoctonine-type alkaloids (seven alkaloids) were found to be inactive. Among the seven C_{20}-diterpenoid veatchine-type alkaloids tested, 12-acetylluciculine and 12-benzoylluciculine showed slight inhibitory activities against growth.

Alkaloids **1** and **2** contain two and three hydroxy groups, respectively, in the common basic structure of the atisine skeleton, being devoid of any other substituents. In the molecules of **1** and **2** (Fig. 1), semi-synthetic derivatives were tested for suppressive effects at 1 μg/ml additions and IC_{50} values against the growth of A549 cells were examined (Table 1). *N*-Benzyl-*N*,6-seco-6-dehydropseudokobusine (**3**) and *N*,15-dibenzyl-*N*,6-seco-6-dehydropseudokobusine (**4**) were inactive. Among the benzoyl derivatives (**5**–**7**) of **2**, 6-benzoylpseudokobusine (**5**) and 15-benzoyl-6,11-di-*p*-nitrobenzoylpseudokobusine (**7**) were inactive. 6,11-Dibenzoylpseudokobusine (**6**) had a weak cytotoxic effect, which was altered by an aryl substituent at C-11 or by a hydroxy group at C-15. Among the veratroyl derivatives (**8**–**10**) of **2**, 6-veratroylpseudokobusine (**8**) was inactive. 15-Veratroylpseudokobusine (**10**) displayed little cytotoxic effect. In contrast, 11-veratroylpseudokobusine (**9**) had a significant cytotoxic effect. Therefore, the suppressive effects were elicited by the presence of an acyl substituent at C-11. Among the anisoyl (**11**–**16**) and *p*-nitrobenzoyl (**17**–**23**) derivatives of **2**, 6-anisoylpseudokobusine (**11**), 6,15-dianisoylpseudokobusine (**15**), 6,15-di-*p*-nitrobenzoylpseudokobusine (**21**) and 6,11,15-tri-*p*-nitrobenzoylpseudokobusine (**23**) were inactive. 6-*p*-Nitrobenzoylpseudokobusine (**17**) and 15-*p*-nitrobenzoylpseudokobusine (**19**) displayed little cytotoxic effect, and 15-anisoylpseudokobusine (**13**) and 6,11-di-*p*-nitrobenzoylpseudokobusine (**20**) showed only weak cytotoxic effects. 11-Anisoylpseudokobusine (**12**), 6,11-dianisoylpseudokobusine (**14**), 11,15-dianisoylpseudokobusine (**16**), 11-*p*-nitrobenzoylpseudokobusine (**18**) and 11,15-di-*p*-nitrobenzoylpseudokobusine (**22**) had significant cytotoxic effects. Accordingly, the cytotoxic effects of 6-substrates (**11**, **17**) were weaker than those of 6,11-disubstrates (**14**, **20**), and 11-substrates (**12**, **18**) had more potent cytotoxic effects than those of 6,11-disubstrates (**14**, **20**). In fact, 11-acyl derivatives (**25**, **27**) exhibited more potent cytotoxic effects than those of 6-substrates (**24**, **26**), but *p*-trifluoromethylbenzoyl derivatives (**28**, **29**) were inactive. In addition 11,15-dianisoylpseudokobusine (**16**) and 11,15-di-*p*-nitrobenzoylpseudokobusine (**22**) were found to be about 1.3-fold and 2-fold more potent than 11-anisoylpseudokobusine (**12**) and 11-*p*-nitrobenzoylpseudokobusine (**18**), respectively. Substitution of the hydroxy group at C-11 of pseudokobusine had variable effects. Benzoate (**6**) and *p*-trifluoromethylbenzoate (**29**) were in active. Veratroate (**9**, $IC_{50} = 4.07$ μM), *p*-nitrobenzoate (**18**, $IC_{50} = 5.08$ μM), cinnamate (**25**, $IC_{50} = 4.24$ μM) and *m*-trifluoromethylbenzoate (**27**, $IC_{50} = 4.67$ μM) showed significant cytotoxic effects. *p*-Trifluoromethylbenzoate (**29**) had little effect at 5 μg/ml, whereas the effect of *m*-trifluoromethylbenzoate (**27**) was more potent than that of **29**. As to the effects of the substitution position by these benzoyl groups, *m*-position gave good result. Anisoate

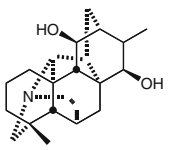
(**12**, IC_{50} = 2.20 μM) was found to be about 2-fold more potent than these substrates. Consequently, in the occurrence of cytotoxic effects of atisine-type alkaloids, replacement by an acyl group at C-11 resulted in the enhancement of activity of the parent alkaloids more than when a hydroxy group was present at this position, and the presence of a hydroxy group at the C-6 position was required for the cytotoxic effects. Furthermore, replacement by an acyl group at both C-11 and C-15 [e.g., 11,15-dianisoylpseudokobusine (**16**) and 11,15-di-*p*-nitrobenzoylpseudokobusine (**22**)] was required for the enhancement of the cytotoxic effect of 11-substrates (**12**, **18**).

Similarly, the suppressive effects of kobusine derivatives (**30**–**39**) at 1 μg/ml additions and IC_{50} values against the growth of A549 cells were examined (Table 1). 11-Benzoylkobusine (**30**), 11-veratroylkobusine (**32**) and dihydrokobusine (**33**) were inactive. 11-Anisoylkobusine (**31**) and 11-cinnamoylkobusine (**34**) displayed little cytotoxic effects. 11-(*p*-Trifluoromethylbenzoyl)kobusine (**35**) and 11-(*m*-trifluoromethylbenzoyl)kobusine (**36**) had significant cytotoxic effects. Among the *p*-nitrobenzoyl derivatives (**37**–**39**) of **1**, 11-*p*-nitrobenzoylkobusine (**37**) and 15-*p*-nitrobenzoylkobusine (**38**) had little cytotoxic effects, and the effect of **37** was slightly more potent than that of **38**. However, 11,15-di-*p*-nitrobenzoylkobusine (**39**) had a significant cytotoxic effect and showed an IC_{50} value against A549 cells of 3.02 ± 0.47 μM. The cytotoxic effects of kobusine derivatives were weak compared with

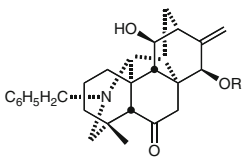


	R_1	R_2
1	H	H
30	Bz	H
31	As	H
32	Vr	H
34	Cm	H
35	PB	H
36	MB	H
37	NB	H
38	H	NB
39	NB	NB

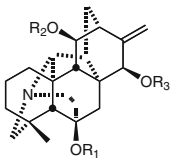
Ac = $COCH_3$
As = $COC_6H_4OCH_3$ (*p*)
Bz = COC_6H_5
Cm = $COCH{=}CHC_6H_5$
MB = $COC_6H_4CF_3$ (*m*)
NB = $COC_6H_4NO_2$ (*p*)
PB = $COC_6H_4CF_3$ (*p*)
Vr = $COC_6H_3(OCH_3)_2$ (3,4)



33



3: R = H
4: R = $CH_2C_6H_5$



	R_1	R_2	R_3
2	H	H	H
5	Bz	H	H
6	Bz	Bz	H
7	NB	NB	Bz
8	Vr	H	H
9	H	Vr	H
10	H	H	Vr
11	As	H	H
12	H	As	H

	R_1	R_2	R_3
13	H	H	As
14	As	As	H
15	As	H	As
16	H	As	As
17	NB	H	H
18	H	NB	H
19	H	H	NB
20	NB	NB	H
21	NB	H	NB

	R_1	R_2	R_3
22	H	NB	NB
23	NB	NB	NB
24	Cm	H	H
25	H	Cm	H
26	MB	H	H
27	H	MB	H
28	PB	H	H
29	H	PB	H

Fig. 1 Structure of C_{20}-diterpenoid alkaloids and their derivatives

Table 1 Cytotoxic effects of atisine-type C_{20}-diterpenoid alkaloids against A549 cell lines

Compound	1 μg/ml[a]	5 μg/ml[a]	IC_{50} (μM)
Control	1.00	1.00	–
1	1.07 ± 0.16	1.00 ± 0.05	ND
2	1.12 ± 0.16	0.93 ± 0.04	ND
3	1.01 ± 0.03	ND	ND
4	0.98 ± 0.02	ND	ND
5	1.02 ± 0.01	ND	ND
6	0.88 ± 0.06*	ND	ND
7	0.96 ± 0.06	ND	ND
8	0.98 ± 0.06	0.86 ± 0.03	ND
9	0.65 ± 0.06*	ND	4.07 ± 0.00
10	0.94 ± 0.01	ND	ND
11	1.02 ± 0.01	1.05 ± 0.04	ND
12	0.65 ± 0.08*	ND	2.20 ± 0.11
13	0.89 ± 0.03	0.56 ± 0.04*	ND
14	0.66 ± 0.04*	0.15 ± 0.04*	3.68 ± 0.30
15	1.04 ± 0.14	0.25 ± 0.06*	ND
16	0.54 ± 0.14*	0.03 ± 0.01*	1.72 ± 0.03
17	0.93 ± 0.04	0.83 ± 0.03	ND
18	0.75 ± 0.08*	ND	5.08 ± 0.15
19	0.93 ± 0.05	0.47 ± 0.04*	ND
20	0.86 ± 0.11	0.11 ± 0.08*	ND
21	0.98 ± 0.11	0.42 ± 0.10*	ND
22	0.74 ± 0.14*	0.03 ± 0.01*	2.66 ± 0.21
23	1.05 ± 0.06	0.91 ± 0.05	ND
24	0.95 ± 0.04	0.90 ± 0.02	ND
25	0.59 ± 0.10*	ND	4.24 ± 0.00
26	1.05 ± 0.06	ND	ND
27	0.69 ± 0.09*	ND	4.67 ± 0.08
28	1.03 ± 0.05	1.17 ± 0.31	ND
29	1.09 ± 0.19	0.72 ± 0.07*	ND
30	1.06 ± 0.19	0.64 ± 0.11*	ND
31	0.98 ± 0.14	0.35 ± 0.04*	11.42 ± 0.71
32	1.04 ± 0.25	0.50 ± 0.06*	ND
33	1.09 ± 0.06	1.04 ± 0.05	ND
34	0.93 ± 0.19	0.44 ± 0.11*	ND
35	0.72 ± 0.11*	0.21 ± 0.04*	5.44 ± 0.41
36	0.64 ± 0.06*	ND	3.75 ± 0.14
37	0.91 ± 0.19	0.37 ± 0.10*	ND
38	0.92 ± 0.23	0.89 ± 0.04*	ND
39	0.75 ± 0.22	0.27 ± 0.24*	3.02 ± 0.47
Pemetrexed	0.14 ± 0.05*	0.13 ± 0.04*	0.48 ± 0.07

ND not determined

[a] Each value is the mean ± SD of three determinations

* $p < 0.05$, indicating significant difference from the control value

the cytotoxic effects of pseudokobusine derivatives, except trifluoromethylbenzoate (**35**, **36**). Hence, the presence of a hydroxy group at the C-6 position enhanced the suppressive effects against the growth of A549 cells.

In a test at 5 μg/ml additions against the growth of A549 cells, 26 alkaloids (**1**, **2**, **8**, **11**, **13**–**17**, **19**–**24**, **28**–**35**, **37**–**39**) were examined. Pseudokobusine (**2**) and alkaloids **8**, **17**, **23**, **24** and **38** displayed weak cytotoxic effects. Alkaloids **13**, **19**, **21**, **29**, **30**, **32** and **34** had significant cytotoxic effects and were found to be about 1.4- to 2.4-fold more potent than 1 μg/ml addition. In contrast, alkaloids **14**, **15**, **20**, **31**, **35**, **37** and **39** had significantly more potent cytotoxic effects and were found to be about 3- to 8-fold more potent than 1 μg/ml addition. Alkaloids **16** and **22** showed the strongest cytotoxic activities against A549 cells.

The cell cycle distribution of A549 cells at 24 and 48 h after treatment was analyzed by a fluorescence cell analyzer. Compounds **14**, **16** and **35** showed remarkable enhancement at the G1 phase for up to double IC_{50} dose at 24 h (Fig. 2), and these compounds increased the G1 phase population of A549 cells in a time-dependent manner (Fig. 3). These results suggest that cytotoxic derivatives can disturb G1 to S phase entrance [25–27]. It is well established that cell cycle progression is highly dependent on cyclins, Cdks (cyclin-dependent kinases) and Cdk inhibitors [28]. Raf-MEK-Erk signaling and/or PTEN-PI3K-AKT signaling contributes to G1 to S transition via subsequent cyclin production and the inhibition of MEK and PI3K activity induced complete G1 phase arrest [29].

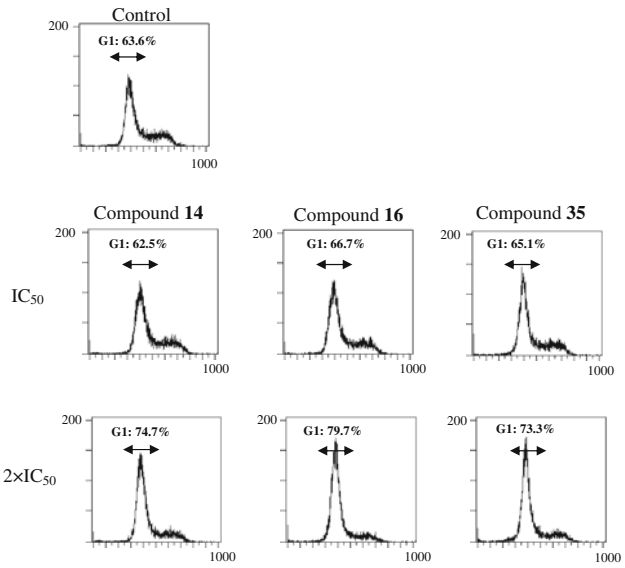


Fig. 2 The effects of compounds **14**, **16** and **35** on the cell cycle distribution of A549 cells. A549 cells treated with vehicle (DMSO) alone and each compound at doses of its IC_{50} and double IC_{50} values for 24 h were fixed, and the cell cycle distribution was then analyzed by flow cytometry. Representative cytograms are shown

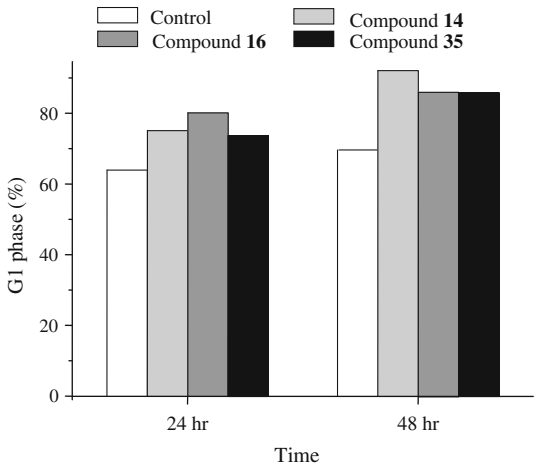


Fig. 3 The effects of compounds **14**, **16** and **35** on the G1 phase population of A549 cells. A549 cells following treatment with vehicle alone and each compound at doses of double IC_{50} values for 24 and 48 h

Moreover, in a previous study, a C-11 acyl derivative showed an inhibitory effect on the growth of A549 cells without enhancement of apoptosis or DNA damage [20]. Thus, it appears that these diterpenoid alkaloid derivatives do not induce genotoxic stress and inhibit cell growth through G1 arrest.

The results of this study suggest that C-6 and C-15 hydroxyl groups in pseudokobusine are necessary for a cytotoxic effect. Esterification of the hydroxyl group at C-11 may, thus, contribute to the enhancement of activity of the parent alkaloids more than that of the OH group at C-11. Furthermore, replacement by an acyl group at C-15 in 11-substrates, such as 11,15-dianisoylpseudokobusine (**16**), 11,15-di-*p*-nitrobenzoylpseudokobusine (**22**) and 11,15-di-*p*-nitrobenzoylkobusine (**39**), was involved in the activation of the cytotoxic effect. These three compounds incorporated all of the favorable modifications identified to date. They possess a novel structure and show remarkable IC_{50} value in the sub-micromolar range. Substitution of the hydroxyl group had variable effects. Benzoyl and benzyl substitutions were inactive. Cinnamoyl, *p*-nitrobenzoyl, *m*-trifluoromethylbenzoyl and veratroyl substitutions were effective. Anisoate was found to be about 2-fold more potent than these substrates. Current studies are focused on the use of semi-synthetic analogues of diterpenoid alkaloids to further probe the mechanisms of the cytotoxic effect on the growth of the A549 human lung cancer cell line. The present results suggested that novel alkaloid derivatives affect the metabolism of tumor cells as a part of the anti-proliferative activities. The suppressive effects of these alkaloids on the growth of human $CD34^+$ hematopoietic stem/progenitor cells will be examined in the future.

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