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Short communication

Structure–activity relationship of seco-tanapartholides isolated from *Achillea falcata* for inhibition of HaCaT cell growth

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ABSTRACT

Four sesquiterpene lactones were isolated from *Achillea falcata*, through bioassay-guided fractionation, based on their differential ability to affect HaCaT cell growth. Identified seco-tanapartholides: 3-β-methoxy-iso-seco-tanapartholide (**1**), tanaphillin (**2**), iso-seco-tanapartholide (**3**), and 8-hydroxy-3-methoxy-iso-seco-tanaparatholide (**4**) were found to differentially decrease keratinocyte cell viability. In addition, the stereoselectivity, lipophilicity, and number and position of hydroxyl groups present in these compounds were correlated with their biological activities for HaCaT cell growth inhibition. Statistical analyses confirmed an enhanced potency of the β-OH iso-seco-tanapartholide over the α:β-OH diastereoisomeric mixture. The highest potency, however, was mainly the function of the enhanced lipophilicity of the molecule.

1. Introduction

The genus *Achillea* comprises over hundred species worldwide most of which are found in Europe, Asia, and North America [1,2]. This genus, a member of the Asteraceae (Compositae) family, has been extensively used in folk medicine for the treatment of fever, cough, bronchitis, asthma, skin inflammation, and liver ailments [1,3–5]. In particular, *Achillea falcata* has been reported to have beneficial effects on internal hemorrhage, uterine hemorrhoid, stomach ailment, gastritis, and bladder stones [6].

Biological activities of plant-derived extracts are attributed to the presence of secondary metabolites, such as sesquiterpene lactones that are abundant mainly in the Asteraceae family. Biological activities of sesquiterpene lactones are mediated by a Michael-type addition reaction of their α,β-unsaturated carbonyl electrophilic groups (α-methylene-γ-lactone or an α,β-unsaturated cyclopentenone) with thiol-containing enzymes and proteins [7,8]. This interaction adversely affects cellular function and depends on the conformation, geometry, lipophilicity, chemical environment, and accessibility of the sesquiterpene molecule to the target proteins [9–11].

Using a bioassay-guided fractionation of the indigenous Lebanese plant *A. falcata* (*demascena*), we were able to extract and identify four sesquiterpene lactones with open guaianolide structures belonging to the seco-guaianolide family (seco-tanapartholide). Many studies have compared the effects of different functional groups on the cytotoxicty and/or specificity of sesquiterpene lactones on a particular enzyme [12–14]. In this study, the stereoselectivity, lipophilicity, and the position of the hydroxyl groups of these isolated seco-tanapartholides are correlated with their differential abilities to reduce HaCaT cell viability. These cells are representative of an immortalized, yet highly differentiated cell line and a well accepted model for early stage skin carcinogenesis [15].

2. Results and discussion

2.1. Bioactivity of isolated seco-tanapartholides

Fig. 1 displays the structure of four 1,10-seco-guaianolide derivatives that were isolated and identified from the indigenous Lebanese wild plant, *A. falcata*. They are: 3-β-methoxy-iso-seco-tanapartholide (**1**), tanaphillin (**2**), iso-seco-tanapartholide (**3**), and 8-hydroxy-3-methoxy-iso-seco-tanaparatholide (**4**) [16–21].

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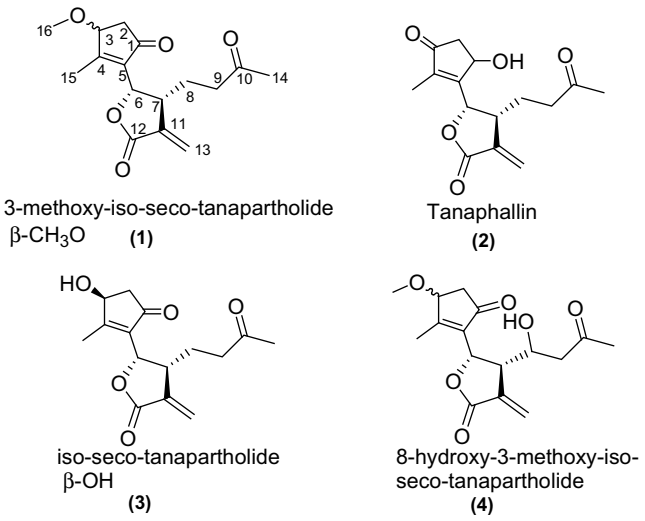

Fig. 1. Seco-tanapartholides (**1**), (**2**), (**3**), and (**4**) isolated from the indigenous Lebanese plant *A. falcata*.

The iso-seco-tanapartholide molecule was isolated in two forms: the conformer β-OH (**3a**), and the mixture of α- and β-OH diastereoisomers (**3b**). Compound (**2**) was unstable and a photochemical activation of a 3-H substitution in tanaphillin led to its conversion into a diastereoisomeric mixture of iso-seco-tanpartholide. Hence, the bioactivity of tanaphillin was similar to that of compound (**3b**) throughout the text ($p > 0.05$; *Independent Sample t-test assuming equal variances*). The ability of (**1**) to inhibit lipopolysaccharide (LPS)-induced Nuclear factor-κB (NF-κB) activation, tumor necrosis factor-α (TNF-α) production in RAW264.7 macrophages, and nitrogen oxide (NO) production has been previously assessed [22]. However, the bioactivities of the other isolated seco-tanapartholides, compounds (**2**), (**3**), and (**4**), have not yet been investigated. Here, the chemical structures of the isolated seco-tanapartholides are correlated with their biological activities for inhibition of HaCaT cell growth.

As measured by lactate dehydrogenase (LDH) release, none of the tested compounds showed greater than 18% cytotoxicity on the HaCaT cells at 6 h treatment with concentrations ranging from 5 to 100 μg/ml (Table 1A). This reflects a non-acute effect of the tested seco-tanapartholides on HaCaT cells. Triton-X 100 was used as a positive control with a 50%-inhibitory concentration (IC_{50}) of $(0.8 \pm 0.2) \times 10^{-2}$% (Table 1B). However, treatment of these cells for 24 h with compounds (**1**), (**3a**), (**3b**), and (**4**) at increasing concentrations up to 100 μg/ml showed a dose-dependent growth inhibition, as measured by the ability of cells to metabolize tetrazolium salt (MTT) (Fig. 2). Among all, compound (**1**) showed the most potent growth-inhibitory effect on the HaCaT cells (Fig. 2), but at the concentration of 25 μg/ml, it significantly affected the viability of primary human keratinocytes (unpublished data). A promising anti-tumor drug should possess selective growth-inhibitory properties against tumor cells relative to normal counterparts. In fact, such properties could be attributed to compound (**1**) at concentrations lower than 25 μg/ml. At 5 and 10 μg/ml, compound (**1**) decreased the viability of HaCaT cells by 44% and 66%, respectively, (Fig. 2) while reducing that of primary keratinocytes by less than 10% (unpublished data). All other seco-tanapartholides did not result in more than 11% and 32% decrease of control values at these two concentrations, respectively. The IC_{50} values of the different tested compounds were calculated using the best-fit regression model and are shown in Table 2. As expected, the IC_{50} of compound (**1**) was significantly less than that of any other tested seco-tanapartholide, followed by that of compound (**3a**) ($p < 0.05$; ANOVA: Dunnet's *t*-test). Interestingly, compound (**1**) was found to cause G_2/M cell cycle arrest at 24 h without evident signs of apoptosis (unpublished data).

2.2. Structure–activity relationships

The bioactivity of the tested seco-tanapartholides is primarily attributed to the presence of the α-methylene-γ-lactone functional group, as is the case for most sesquiterpene lactones [23,24]. However, the differences in the calculated IC_{50} values could be attributed either to the presence of different functional groups or to conformational changes. In fact, the diastereoisomer mixture (β and α) of the iso-seco-tanpartholide (**3b**) and the pure β-conformer (**3a**) are shown to have different potencies at equivalent concentrations with more pronounced differences with increasing concentrations up to 50 μg/ml. As expected, the IC_{50} of compound (**3a**) (13.0 ± 0.44 μg/ml) is significantly less than that of compound (**3b**) (19.1 ± 0.83 μg/ml) ($p < 0.05$; *Independent Sample t-test assuming equal variances*). Hence, an optical–biological activity dependency between diastereoisomers of sesquiterpene lactones is reported, to our knowledge, for the first time. Stereoselectivity studies conducted on sesquiterpene lactones was mostly related to the position of cyclopentanone or γ-lactone rings [25]. However, other bioactive families of molecules, such as enantiomers of Baogongteng-A and its analogs and enantiomers of Schweinfurthin F, elicited different responses towards a variety of bioassays [26,27]. In addition, comparing the β-OCH_3 of (**1**) to the β-OH conformation of (**3**) at C-3, we notice that the IC_{50} of compound (**1**) (7.1 ± 1.19 μg/ml) is almost two folds smaller than that of compound (**3a**) (13.0 ± 0.44 μg/ml) (Table 2). Increased potency of (**1**) over (**3**) could be attributed to the enhanced lipophilicity of the former, hence, its increased ability to cross the plasma membrane. Similarly, the increased potency of compound (**1**) over compound (**4**) (a mixture of two diastereoisomers) may be attributed to the presence of an additional hydroxyl group at C-8 in compound (**4**), and thus, its decreased lipophilicity. In some systems, compounds with oxygen-bearing functional groups were found to have enhanced activities through forming hydrogen bonds with amino acid

Table 1A
Cytotoxicities at 6 h of tested seco-tanapartholides (**1**), (**3a**), (**3b**) and (**4**) on HaCaT cells. Data of compound (**2**) was omitted as this compound was unstable and irreversibly converting into compound (**3b**). Results are tabulated as mean ± SD, as described in "Experimental section".

Concentrations (μg/ml)									
Compounds	0	5	10	15	20	25	50	75	100
	Cytotoxicity at 6 h (% Control) ± SD								
1	0 ± 2.7	7 ± 9.7	7 ± 9.4	7 ± 15.9	5 ± 11.5	7 ± 14.6	0 ± 2.1	0 ± 1.1	0 ± 2.8
3a	0 ± 2.6	4 ± 5.1	18 ± 2.7	0 ± 0.3	11 ± 1.6	11 ± 11.7	0 ± 0.3	2 ± 0.3	nd
3b	0 ± 2.7	2 ± 10.0	3 ± 7.7	2 ± 10.1	5 ± 15.3	3 ± 10.6	0 ± 3.1	0 ± 1.9	0 ± 0.3
4	0 ± 2.6	4 ± 4.3	4 ± 3.3	4 ± 3.9	4 ± 5.4	5 ± 6.6	2 ± 1.0	1 ± 1.5	0 ± 0.7

nd: Not determined.

Table 1B
Cytotoxicity at 6 h of Triton-X 100 on HaCaT cells. Results are tabulated as mean ± SD, as described in "Experimental section".

Concentrations (% of cell culture media)						
	0	1×10^{-4}	1×10^{-3}	1×10^{-2}	1×10^{-1}	1
Positive Control	Cytotoxicity at 6 h (% control) ± SD					
Triton-X 100	0 ± 0.6	0 ± 0.9	1 ± 1.6	51 ± 3.7	77 ± 1.4	78 ± 5.1

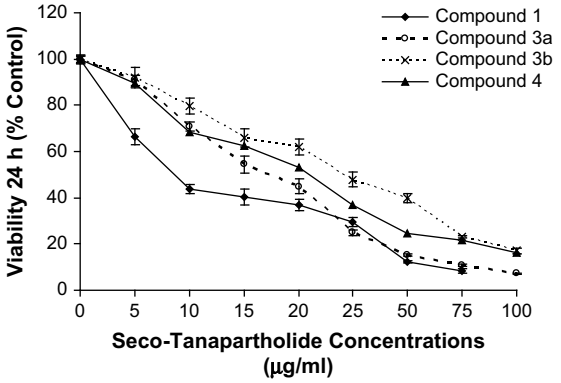

Fig. 2. Effects of seco-tanapartholides on HaCaT cell viability. HaCaT cells were plated in 96-well plates at a density of 1×10^5 cells/ml. At 50–60% confluency, cells were treated with vehicle ethanol, as control, or with different concentrations up to 100 µg/ml of seco-tanapartholides. The "Viability 24 h" at 100 µg/ml of compound (**1**) was not determined, and data of compound (**2**) was omitted as this compound was unstable and irreversibly converting into compound (**3b**). Cell viability was determined at 24 h using the CellTiter 96 non-radioactive cell proliferation kit, expressed as percentage of control-treated cells for each group, and plotted as the mean ± SE, as described in "Experimental section".

Table 2
Average IC_{50} values of seco-tanapartholides (**1**), (**3a**), (**3b**), and (**4**) for inhibition of HaCaT cell viability.

Molecule	Average IC_{50} (µg/ml)	±SE
Compound (**1**)	7.1	1.19
Compound (**3a**) (β-conformer)	13.0	0.44
Compound (**3b**) (β-OH:α-OH)	19.1	0.83
Compound (**4**)	16.7	0.86

residues adjacent to the active centre in the target protein [23,28]. However, this generalization does not apply to the open guaianolide structure of compound (**4**) since the OH group at the C-8 position does not seem appropriate for hydrogen bonding with the target protein. This open structure could be involved in enolization by intramolecular hydrogen bonding with the ketone moiety at C-10 forming an unstrained six-membered ring. This is in conformity with another study that emphasized the number of the hydroxyl groups rather than the position as the important criterion for biological activities [29]. Nonetheless, the importance of the position of hydrogen bond acceptors in determining the biological activity was also reported [23,30]. It would be interesting to test in keratinocytes whether changes in the chemistry of the compounds affect NF-κB and activator protein-1 (AP-1) activities, oxidant properties, as well as cell cycle and cell death regulation.

3. Conclusion

The inhibitory effects of four 1,10-seco-tanapartholides on HaCaT cell viability were investigated in relation with their chemical structures. The potency of the tested compounds varied depending on the nature of their substituents with the lowest IC_{50} values corresponding to the highest lipophilicity of the seco-tanapartholide derivatives. The stereoselectivity of the molecule was shown to exhibit unequal potency towards HaCaT cells. Furthermore, the presence of an additional hydroxyl group in one of the molecules at the C-8 position has reduced the potency of the derivative due to possible intramolecular enolization. Our results provide a rationale for the design of plant-derived anti-cancer drugs from sesquiterpene lactones.

4. Experimental section

4.1. Cell culture and treatment

The HaCaT cell line was cultured in DMEM containing 10% heat-inactivated fetal bovine serum, 1% sodium pyruvate, 1% penicillin–streptomycin and 1% kanamycin antibiotics with normal calcium concentration in the medium (2 mM). Cells were grown at 37 °C, 95% air, and 5% CO_2 and seeded into 96-well plates at a density of 5000 cells per well. When 50–60% confluent, keratinocytes were treated with vehicle solvent, as control, or with different concentrations of the tested seco-tanapartholides and of Triton-X 100. The controls contained 0.15% ethanol in fresh culture media, which was equal to the highest ethanol concentration obtained at the tested concentration of 25 µg/ml of each compound. When concentrations higher than 25 µg/ml were mandatory for the calculation of IC_{50} values, 1% ethanol was used instead of control. These ethanol concentrations had no effect on HaCaT cell viability (data not shown).

4.2. Cytotoxicity and cell viability assays

Drug cytotoxicity was assayed after 6 h of treatment using CytoTox 96® assay; whereas, cell viability was assayed after 24 h of treatment, using CellTiter 96® assay, according to manufacturer's instructions (Promega Corp., Madison, WI). Triton-X 100, with concentrations up to 1% of cell culture media was used as positive control in CytoTox 96® assays. The CytoTox 96® assay quantitatively measures the activity of LDH, a stable cytosolic enzyme that is released upon cell lysis. Released LDH in culture supernatants is measured with a coupled enzymatic assay which results in the conversion of a tetrazolium salt into a red formazan product, the absorbance of which is recorded at 490 nm using an ELISA microplate reader. The cytotoxicities of the tested compounds are then normalized relative to the maximum toxicity of a Lysis Buffer and tabulated as the mean ± standard deviation (SD) of at least three measurements per condition. Cytotoxicity results were reproduced in two independent experiments using concentrations up to 25 µg/ml. The CytoTox 96® assay, when performed for short time points, such as 6 h, allows testing for an acute drug effect which is typically manifested by cell bursting and LDH release. Lysed cells quantified at later time points, such as 24 h, of drug exposure do not necessarily result from an acute response as most types of cell death also eventually lead to cell lysis after prolonged exposure to a drug. Hence, "Viability 24 h" was assayed using the CellTiter 96® non-radioactive cell proliferation assay kit. This assay is an MTT-based method which, instead of quantifying lysed cells, measures the ability of metabolically active cells to convert tetrazolium salt into a blue formazan product, the absorbance of which is recorded at 570 nm using an ELISA microplate reader. Viability results were

expressed as percentage of control and plotted as the mean ± standard error (SE) of at least three measurements per condition. These results were reproduced in at least three independent experiments using concentrations up to 25 μg/ml.

4.3. Statistical analyses

SPSS Version 16.0 and Microsoft Office Excel 2007 were used to calculate the best-fit regression model, the IC_{50} values, the Independent Sample *t*-test and ANOVA with its associated *post-hoc* tests. The model regression function for compounds (**1**) and (**2**) was best estimated as 'Growth' while that for compounds (**3a**), (**3b**), and (**4**) was 'Power'. Statistical significance is claimed when the *P*-value is different from 0.05.

4.4. Plant material

The plant material of *A. falcata* was collected from the Bcharre Cedars area in Lebanon at an altitude of 1330 m during the flowering stage in July. Voucher specimens were deposited in the herbarium of the Faculty of Agriculture and Food Sciences, Department of Horticulture at the American University of Beirut (Beirut, Lebanon).

4.5. Extraction and isolation

The aerial parts (300 g for each trial) of *A. falcata* were collected, dried, grinded, and soaked in methanol for 16 h at room temperature. The crude methanol extract "I" was concentrated to 1/10 of its volume and then acidified to pH 2 using a concentrated H_2SO_4 solution. Following, a mixture of $CHCl_3$:H_2O (2:1 v/v) was added. The organic phase was collected and labeled as "I.2". Fraction I.2 was evaporated under reduced pressure to give 13 g of a very dark green extract. This extract was fractionated by column chromatography on silica gel (Silica gel 0.030–0.075 mm, 90 A, 1 kg) using gradient elution of $CHCl_3$:acetone (9:1), $CHCl_3$:acetone (2:1), followed by acetone and finally methanol. Thirty-two sub fractions were collected. Fractions I.2.25 and I.2.26 eluted using acetone were purified using solid phase extraction (SPE). The pure molecules, (**1**) (8.5 mg) and the two epimers of (**4**) (3.7 mg), were isolated from the I.2.25 fraction, while (**2**) (34.6 mg) and the β-OH epimer of (**3**) (1 mg) followed by a mixture of the two epimers of (**3**) (4 mg) were isolated from the I.2.26 fraction.

4.6. Structure elucidation

Structural elucidation of the bioactive components was performed on a Nicolet AVATAR 360 FTIR spectrometer equipped with a KBr pellet cell holder. Spectrum was collected by averaging 128 scans at wave numbers ranging from 750 to 4000 cm^{-1} at a resolution of 1 cm^{-1}. All NMR data were obtained using a Bruker 300 MHz spectrometer where TMS is used as an internal standard. Samples were dissolved in deuterated chloroform ($CDCl_3$) and their spectra were obtained. Gas Chromatography (GC) analysis was performed using a Trace™ gas chromatograph equipped with HP-5 capillary column (30 m long, 250 μm i.d, and 0.25 μm film thickness) and Helium as a carrier at a flow rate of 1 ml/min. The maximum temperature was 350 °C. The column was heated from 35 °C to 290 °C. The injector temperature was set at 300 °C in a splitless mode. Results were recorded as percent of total peak areas. The mass spectrometer employed in the GC–MS analysis was a Polarization Q series mass selective detector in the electron impact (EI) ionization mode (70 eV).

Spectral data of the four seco-tanapartholides were in agreement with the data reported in the literature [16–21].

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