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Melodinine V, an antitumor bisindole alkaloid with selective cytotoxicity from *Melodinus henryi*

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ABSTRACT

Melodinine V (**1**) with a vincanol–eburenine skeleton, was isolated from *Melodinus henryi*. The structure was elucidated by extensive spectroscopic methods and further confirmed by the single crystal X-ray diffraction analysis. Melodinine V showed selective cytotoxic activities against human colon cancer cell line HT-29 and inhibited cell proliferation in a concentration-dependent manner. It induced cell cycle arrest at G1 phase and cellular apoptosis by increasing histone-associated DNA fragmentation in the treated HT-29 cells.

Since the structure of vinblastine, one of the most important bisindole alkaloids in pharmacy, was established in 1962,[1–3] bisindole alkaloids, monoterpenoid indole dimers, became attractive for their complicated structures as well as potent biological activities.[4–8] Then, a number of bisindole alkaloids have been isolated from different plants.[4,9–14] Some of them, known as *vinca* alkaloids, vincristine,[15] vindesine,[16] and vinblastine[17,18] are promising naturally occurring antitumor agents, and have been launched as commercial drugs in many countries.[19,20] Pharmacological investigations on the crude and purified alkaloids from *Melodinus* (Apocynaceae), which is rich in indole and bisindole alkaloids, revealed their significant antitumor[21–23] and antibacterial activities.[24] In our continuous investigation on plants belonging to this genus, many new and/or bioactive compounds were isolated,[25–35] in which some of indole and bisindole alkaloids showed significant cytotoxicity against human cancer cell lines. Now, a new bisindole alkaloid, melodinine V (**1**) from *Melodinushenryi* showed selective cytotoxicity in human colon cancer cell line (HT-29). Effects of melodinine V (**1**) on cell cycle phase distribution and cellular apoptosis were further investigated. Reported herein are the isolation,[36] structural elucidation, and antitumor bioactivity of it.

Melodinine V (**1**)[37] was obtained as crystal and gave a positive reaction with Dragendorff's reagent, characteristic of an alkaloid. A molecular formula of $C_{38}H_{46}N_4O$ was assigned for compound **1** on the basis of positive HRESIMS ($[M+H]^+$ at m/z 575.3734), indicating 18 degrees of unsaturation. Its UV spectrum showed absorption characteristic of an indolenine chromophore (292, 204 nm), which was supported by the resonance at δ_C 193.0 (s) in ^{13}C NMR attributable to an imine carbon.[38] In the ^1H NMR spectrum, two triplets at δ_H 0.67 (3H, t, J = 7.3 Hz, Me-18) and 0.76 (3H, t, J = 7.4 Hz, Me-18′) were assigned to the protons of two methyls connected to methenes. In the ^{13}C NMR spectrum, 38 carbons were observed (Table 1), including two methyls, 15 methylenes, nine methines, and 12 quaternary carbons. Thus, compound **1** was identified preferentially as a bisindole alkaloid with two eburnamenine-type units similar to vincanol (unit A) and eburenine (unit B)[39] (Fig. 1).

In the ^1H NMR spectrum, four aromatic protons at δ_H 7.67 (1H, d, J = 8.0 Hz), 7.21 (1H, t, J = 8.0 Hz), 7.10 (1H, t, J = 8.0 Hz) and 7.08 (1H, d, J = 8.0 Hz) were assigned to be the unsubstituted indole

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0960-894X/

Table 1
^{1}H and ^{13}C NMR data of melodinine V (**1**) in pyridine-d_5 (δ in ppm and J in Hz)

No.	δ_H	δ_C	No.	δ_H	δ_C
2		134.8 s	2′		193.0 s
3a	2.35 (1H, dd, 4.4, 13.0)	44.6 t	3′a	2.19 (1H)^{o3}	52.3 t
3b	2.44 (1H, d, 13.0)		3′b	3.08 (1H, dd, 2.6, 8.4)	
5a	3.22 (1H)^{o1}	51.3 t	5′	3.18 (1H)^{o1}	55.1 t
5b					
6a	2.51 (1H)^{o2}	17.5 t	6′a	2.19 (1H)^{o3}	32.4 t
6b	2.99 (1H, ddd, 2.0, 6.4, 13.1)		6′b	2.24 (1H)^{o3}	
7		104.7 s	7′		62.7 s
8		129.5 s	8′		131.2 s
9	7.67 (1H, d, 8.0)	118.3 d	9′		153.5 s
10	7.21 (1H, t, 8.0)	119.4 d	10′	6.95 (1H, d, 8.2)	114.3 d
11	7.10 (1H, t, 8.0)	120.5 d	11′	7.14 (1H, d, 8.2)	127.2 d
12	7.08 (1H, d, 8.0)	112.3 d	12′		126.2 s
13		137.2 s	13′		154.5 s
14a	1.16 (1H)^{o6}	21.1 t	14′a	1.41 (1H)^{o5}	22.2 t
14b	1.64 (1H, m)		14′b	1.81 (1H, m)	
15a	1.04 (1H)^{o7}	24.4 t	15′a	1.06 (1H)^{o7}	33.3 t
15b	1.26 (1H)^{o4}		15′b	1.27 (1H)^{o4}	
16	6.38 (1H, d, 6.0)	50.1 d	16′a	1.56 (1H, m)	29.6 t
			16′b	2.48 (1H)^{o2}	
17a	1.94 (1H, t, 12.2)	44.4 t	17′a	2.88 (1H, t, 11.1)	24.1 t
17b	2.27 (1H)^{o3}		17′b	3.29 (1H, m)	
18	0.67 (1H, t, 7.3)	7.5 q	18′	0.76 (1H, t, 7.4)	8.1 q
19a	1.39 (1H)^{o5}	29.1 t	19′	1.19 (2H)^{o6}	30.8 t
19b	2.22 (1H)^{o3}				
20		35.2 s	20′		36.6 s
21	4.02 (1H, s)	59.8 d	21′	3.25 (1H, s)	75.2 d

$^{o1-o7}$Overlapped signals.

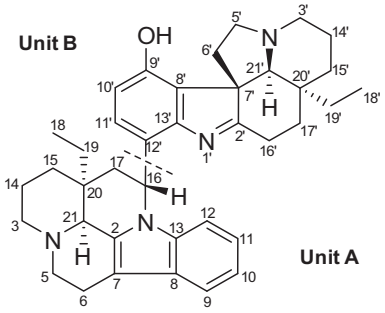

Figure 1. The structure of melodinine V (**1**).

moiety in unit A, which were further supported by ^{1}H–^{1}H COSY and HMBC correlations (Fig. 2). The NMR spectra also displayed an indolenine moiety in unit B [eight carbon signals δ_C 193.0 (s), 154.5 (s), 153.5 (s), 131.2 (s), 127.2 (d), 126.2 (s), 114.3 (d), 62.7 (s); two doublet aromatic protons signals δ_H 7.14 (1H, d, J = 8.2 Hz) and 6.95 (1H, d, J = 8.2 Hz)],[38] and the substituents should be in *para*-position of aromatic ring in unit B, indicated by the electron-withdrawing inductive effect of the hydroxyl and chemical shift of the indolenine moiety. Furthermore, the hydroxyl was placed at C-9′, instead of C-12′, as evidenced by HMBC correlations of δ_H 6.95 (1H, d, J = 8.2 Hz, H-10′) with δ_C 153.5 (s, C-9′), 131.2 (s, C-8′), 126.2 (s, C-12′) and 62.7 (s, C-7′), and of δ_H 7.14 (1H, d, J = 8.2 Hz, H-11′) with δ_C 154.5 (s, C-13′) and 50.1 (d, C-16). Moreover, the linkage of units A and B by C-16/C-12′ was established by the HMBC correlation of H-11′ (δ_H 7.14, 1H, d, J = 8.2 Hz) with δ_C 50.1 (d, C-16) (Fig. 2), which also indicated the placement for the 9′-OH unambiguously. The NMR spectral data of compound **1** were similar to those of bisleuconothine A,[40] except for an imine appeared at *N*1′–C2′ in unit B of compound **1**, which was supported by HMBC correlations of δ_H 3.25 (1H, s, H-21′) and 2.48 (1H, overlap, H-16′b) with δ_C 193.0 (s, C-2′). Thus, the

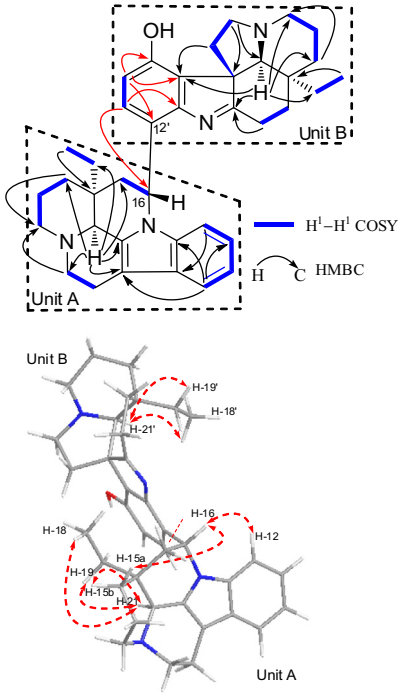

Figure 2. Selected HMBC (↷), ^{1}H–^{1}H COSY (▬) and ROESY (↷) correlations of melodinine V (**1**).

planar structure of compound **1** was elucidated to possess a vincanol–eburenine skeleton.

In the ROESY spectrum, NOE correlations of H-21/Me-18, and H-21′/Me-18′ suggested that the relative configurations of units A and B in compound **1** were identical to those of vincanol and eburenine,[39] respectively. Besides, H-21 showed NOE correlation to one proton of H_2-15 (H-15b), while H-16 showed NOE correlation to another proton of H_2-15 (H-15a), which suggested H-21

and H-16 were on the opposite sides. Then, H-16 of compound **1** was assigned to be β orientation, the same as that of bisleuconothine A. Finally, the single-crystal X-ray[41] further supported the assigned structure, and the absolute configuration of melodinine V should be identical to that of bisleuconothine A by comparison of specific rotations of two compounds (Fig. 3).

Melodinine V was evaluated for its cytotoxicity against three human cancer cell lines using MTT method.[42,43] It showed cytotoxicity against HT-29, PANC-1 and MCF-7 cells, with IC_{50} values of 4.9, 28.1, and 54.5 μM, respectively (Fig. 4A). However, it (up to 100 μM, data not shown) did not cause cytotoxic effect on human normal skin fibroblasts, suggesting its selective cytotoxicity against cancer cells. Furthermore, the cell proliferation of HT-29, which was evaluated using thymidine incorporation assay, was inhibited by melodinine V in a concentration-dependent manner, with IC_{50} value of 2.3 μM (Fig. 4B). It induced cell cycle arrest (7.4 and 9.8 μM) at G1 phase after 24 h incubation (p <0.01, Fig. 5A). To further determine the induction of apoptosis by melodinine V, quantification of DNA fragmentation of apoptotic cells was analyzed by the Cell Death Detection ELISA.[44,45] Results showed that cellular apoptosis as indicated histone-associated DNA fragmentation was significantly increased in melodinine V treated HT-29 cells in a concentration-dependent manner (p <0.01, Fig. 5B).

Similar to bisleuconothine A, another bisindole with an eburnane-aspidosperma skeleton firstly found in the bark of *Leuconotis griffithii*,[40] melodinine V induced apoptosis in human colon cancer cells. Bisleuconothine A was shown to be a selective Wnt signaling inhibitor and inhibited cell proliferation through induction of apoptosis by increasing the cleavage of caspases,[46] while the apoptosis induced by melodinine V was demonstrated here to be related to DNA fragmentation. Bisleuconothine A induced weak G0/G1 cell cycle arrest in colon cancer cells whereas melodinine V caused significant increases in the cell proportion in G1 phase. In addition, melodinine V (up to 100 μM) was not cytotoxic towards human normal fibroblasts. Last but not least, its inhibitory effect on cell proliferation in HT-29 cells was stronger than that of cisplatin (IC_{50} values: 2.3 μM vs 26.2 μM). These findings suggested that melodinine V might have potential to be developed as an anti-tumor agent.

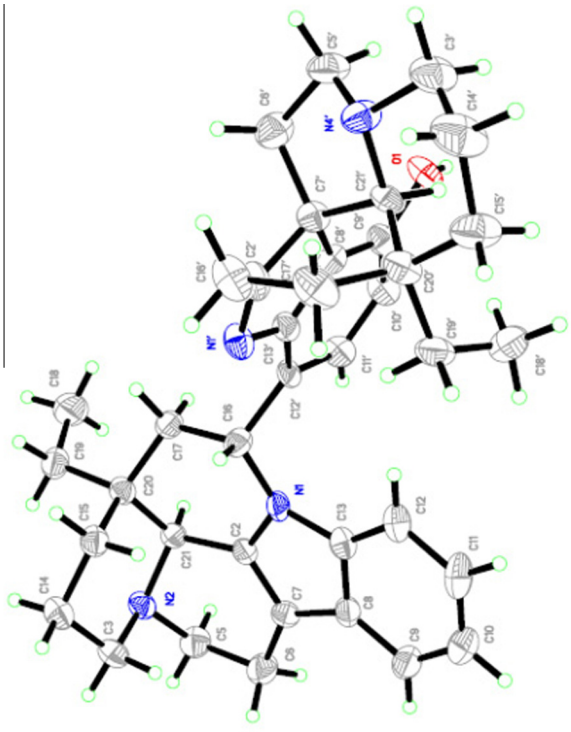

Figure 3. X-ray structure of melodinine V (**1**).

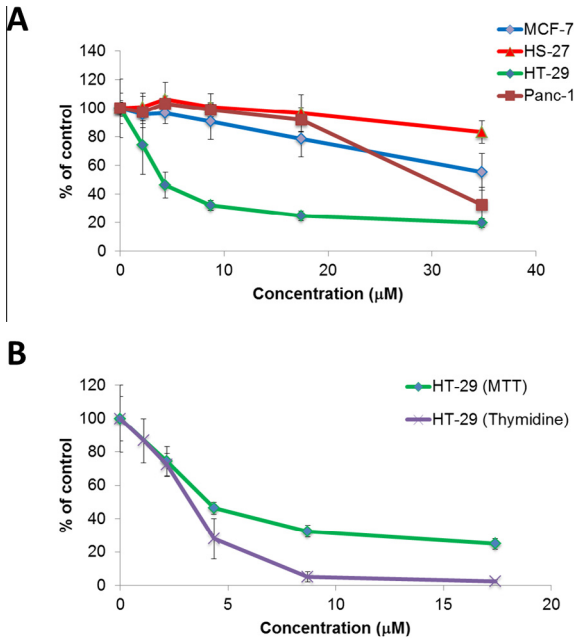

Figure 4. Effects of melodinine V (**1**) on cell viabilities of different cell lines (A) and on cell proliferation (B) of HT-29 cells. Cells were treated with increasing concentrations of melodinine V for 48 h, and cell viability and cell proliferation were determined by the MTT and thymidine incorporation assays, respectively. Results were expressed as percentages of MTT absorbance or count per minute with respect to the untreated vehicle control wells (mean ± SD of 3 independent experiments with 4 wells each).

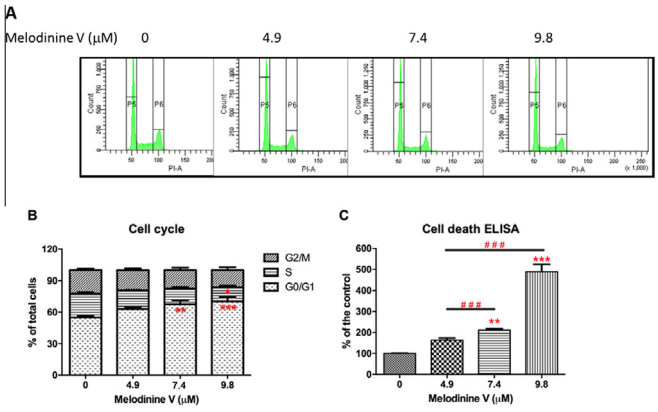

Figure 5. Effects of melodinine V (**1**) on cell cycle phase distribution and apoptosis induction in HT-29 cells. (A) Cell cycle analysis of HT-29 cells treated with vehicle or melodinine V for 24 h. After treatment, cells were analyzed by flow cytometry. (B) The fractions of cells in each cell cycle phase were summarized in the bar chart. (C) Melodinine V increased the apoptosis activity of HT-29 cells as quantified of histone-complexed DNA fragments by the Cell Death ELISA assay. Data were expressed as means + SD from 2 independent experiments with 3 wells each. Differences between the treated and untreated control groups were determined by one-way ANOVA followed by *post-hoc* Tukey's multiple comparison test. $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001 as compared to the control group. $^{\#\#\#}p$ <0.001 as compared among treatment groups.

Acknowledgment

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Supplementary data

Supplementary data (1D, 2D NMR and MS spectra of melodinine V) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.09.023. These data include MOL files and InChiKeys of the most important compounds described in this article.

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36. *Plant material: M. henryi* was collected from Mengna County, Yunnan province, P.R. China, and identified by Mr. Jing-Yun Cui, Xishuangbanna Tropical Plant Garden. A voucher specimen (No. Cui20081128) has been deposited at the Kunming Institute of Botany, Chinese Academy of Sciences. *Extraction and isolation:* Air-dried and powdered plant material (12 kg) was extracted with 90% EtOH (24 h × 3) to afford a crude extract (950 g). The extract was partitioned between EtOAc and 0.5% HCl solution. The acidic water soluble material was adjusted to pH 9–10 with 10% ammonia solution and then extracted with EtOAc to give an alkaloidal extract (77 g). The alkaloidal extract was subjected to silica gel column chromatography ($CHCl_3$/MeOH, 1:0 to 0:1) to afford fractions A–H. Fraction E (7 g) was subjected to silica gel column chromatography ($CHCl_3$/MeOH, 10:1) to afford Subfractions E1-E4. Subfraction E2 was separated further on RP-18 (MeOH/H_2O, 4:6) and Sephadex LH-20 (MeOH) column to yield compound **1** (13 mg).
37. *Melodinine V (**1**):* white bulk crystals (MeOH); $[\alpha]_D^{25.5}$ +177.8 (*c* 0.20, MeOH); UV (MeOH) λ_{max} (log ε) 292 (3.57), 284 (3.60), 204 (4.40) nm; IR (KBr) ν_{max} 3421, 2927, 1585,1454 cm^{-1}; ^1H (400 MHz) and ^{13}C NMR (100 MHz) data (pyridine-d_5) see Table 1; positive ion HRESIMS *m*/*z*: 575.3734 (calcd for $C_{38}H_{47}N_4O$ $[M+H]^+$, 575.3749).
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41. *Crystal data for melodinine V (**1**):* $C_{38}H_{46}N_4O$ MW = 574; monoclinic, space group $P2_1$; a = 8.307(3) Å, b = 9.459(7) Å, c = 12.480(6) Å, α = 80.200, β = 72.656, γ = 65.332, V = 849.5(3) $Å^3$, Z = 1, d = 1.186 g/cm^3, crystal dimensions 0.19 × 0.13 × 0.08 mm was used for measurement on a SHELXL-97 with a graphite monochromator, Mo Kα radiation. The total number of reflections measured was 6953, of which 2860, were observed, $I > 2\sigma(I)$. Final indices: R_1 = 0.0801, wR_2 = 0.1725. The crystal structure of compound **1** was solved by direct method SHLXS-97 (Sheldrick, 1990) and expanded using difference Fourier technique, refined by the program SHLXL-97 (Sheldrick, 1997) and the full-matrix least-squares calculations. Crystallographic data for the structure of compound **1** have been deposited in the Cambridge Crystallographic Data Centre (deposition number: CCDC 1432170). Copies of these data can be obtained free of charge from the Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB21EZ, UK; fax: +44 1223 336 033; or deposit@ccdc.cam.ac.uk).
42. *Cell proliferation and cytotoxicity assays:* The human cancer cells [colon cancer (HT-29), pancreas cancer (PANC-1), breast cancer (MCF-7)] or skin fibroblast (Hs-27) were obtained from American Type Culture Collection (Manassas, VA, USA) and were maintained in McCoy's 5A medium or RPMI 1640 medium, containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. Other culture supplements were obtained from Life Technologies (Grand Island, NY, USA). Cells (5×10^4/ml) were seeded in 96-well flat-bottom culture plates with 100 μL culture medium and incubated overnight. Subsequently, 100 μL culture media containing various concentrations of melodinine V were added into the wells, with cisplatin (Sigma, USA) as a positive control. Then the plates were incubated at 37 °C for 48 h. Plain medium containing vehicle solvent was added to the control wells. The cytotoxicities of test compound in cells were assessed by MTT assay. The effects of melodinine V on the proliferation were determined by [methyl-^{3}H]-thymidine incorporation. The procedures of MTT and thymidine incorporation assays were described in details in our previous study.
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44. *Cell cycle analysis and cell death ELISA:* Human colon cancer cells HT-29 (3×10^5/well) were seeded at 6-well culture plates and incubated overnight. The cells were treated with melodinine V at various concentrations for 24 or 48 h, with cisplatin (Sigma, USA) as a positive control. Cells were harvested after incubation and subjected to propidium iodide flow cytometry analysis as described in previous study. Human colon cancer cells were also collected for apoptosis analysis using the Cell Death Detection ELISA PLUS kit (Roche Applied Science, Basel, Switzerland), according to the manufacturer's instructions. Briefly, cancer cells (3×10^4/well in 24-well culture plate) treated with melodinine V for 48 h were collected and lysed with 200 μL lysis buffer. The intact nuclei after lysis process were pelleted by centrifugation at 200×*g* for 10 min. Then, the supernatant (20 μL) were transferred to the streptavidin-coated wells with immunoreagent (biotinized anti-histone and peroxidase-conjugated anti-DNA) for 2 h at room temperature. Each well was then washed three times with incubation buffer and incubated with ABTS (2,2′-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) solution for 5 min. ABTS stop solution was then added to the wells immediately. Finally, absorbance was measured at 405 and 490 nm using a microplate spectrophotometer to determine the amount of colored product.
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