Inhibition of the G_2 DNA Damage Checkpoint by Oliveroline Isolated from *Duguetia odorata*

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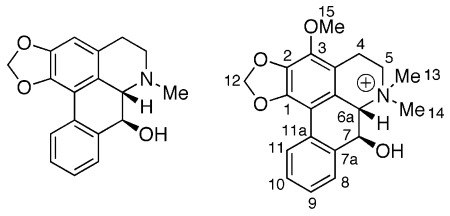
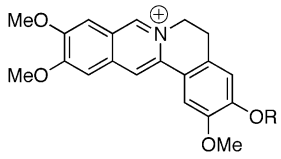
Plant extracts obtained from the U.S. National Cancer Institute's Open Repository collection have been screened for their ability to inhibit the G_2 DNA damage checkpoint. An extract of *Duguetia odorata* showed promising activity in the assay. Bioassay-guided fractionation revealed that oliveroline (**1**) was responsible for the G_2 checkpoint inhibitory activity of the *D. odorata* extract. The new alkaloid *N*-methylguatterine (**2**) was identified during this investigation.

Ionizing radiation and DNA-damaging drugs are widely used in the treatment of cancer. However, their efficacy can be limited by the activation of cell cycle checkpoints that temporarily halt cancer cell proliferation and allow increased time for DNA repair.[1] Chemical inhibition of the G_2 phase checkpoint has been proposed as a strategy to enhance the effectiveness of DNA-damaging therapy, and a number of checkpoint inhibitors have been discovered in recent years.[2] These include inhibitors of the checkpoint kinase Chk1 that are currently in clinical development.

Crude plant extracts obtained from the NCI Open Respository collection[3] in a 96-well plate format were screened in a cell-based assay[4] for their ability to inhibit the G_2 DNA damage checkpoint. The extract N075679-Z/3 from *Duguetia odorata* (MacBride 1929) (Annonaceae) showed promising activity in the assay. Bioassay-guided fractionation of the extract led to the isolation of oliveroline (**1**), *N*-methylguatterine (**2**), dehydrodiscretine (**3**), and pseudopalmatine (**4**). The structures of the known alkaloids oliveroline (**1**),[5] dehydrodiscretine (**3**),[6] and pseudopalmatine (**4**)[7] were confirmed by comparing their NMR and MS data with literature values.





3 R = H
4 R = Me

N-Methylguatterine (**2**), a new aporphine alkaloid, was isolated as an optically active colorless solid that gave a $[M]^+$ ion at *m*/*z* 340.1534 in the HRESIMS, consistent with a molecular formula of $C_{20}H_{22}NO_4$ (calcd for $C_{20}H_{22}NO_4$ 340.1549). The ^1H NMR spectrum of **2** showed a strong resemblance to the spectrum of oliveroline (**1**), indicating that the two molecules were closely related and facilitating the identification of several structural fragments in **2**. These included a 1,2-disubsituted benzene (δ 7.87, m, H-11; 7.65, m, H-8; 7.42, m, H-9/H-10), a methylene dioxy (6.25, s, H-12; 6.07, s, H-12′), an isolated spin system comprised of two adjacent methylenes (3.75, m, H-5; 3.61, m, H-5′; 2.93, m, H-4/H-4′), an isolated spin system comprised of an OH (6.95, d, J = 7 Hz, O*H*-7) and two adjacent methines (4.76, d, J = 12 Hz, H-6a; 5.02, dd, J = 7, 12 Hz, H-7), two N-methyls (3.17, s, Me-13; 3.61, s, Me-14), and a methyl ether (4.02, s, Me-15). HMBC correlations observed between the Me-13 proton resonance (δ 3.17) and the carbon resonances assigned to Me-14 (δ 56.8), C-5 (61.8), and C-6a (70.8) and between the Me-14 proton resonance (3.61) and the Me-13 (42.1), C-5, and C-6a carbon resonances confirmed that N-6 was dimethylated. The proton spectrum of **2** was missing a resonance that could be assigned to H-3, and the Me-15 proton resonance at δ 4.02 showed an HMBC correlation to a carbon resonance at δ 138.9 (C-3), demonstrating that C-3 was substituted with a methyl ether. Closely related N-6 dimethylated aporphine alkaloids with a methyl ether at C-3 show similar C-3 chemical shifts.[5b,8] Comparison of the H-6a to H-7 scalar coupling in **2** (J = 12 Hz) with the scalar coupling between H-6a and H-7 (J = 13 Hz) in oliveroline (**1**) showed that the relative configurations at C-6a and C-7 were identical in both molecules and their CD spectra were virtually superimposable, confirming that they had identical absolute configurations as well.

Oliveroline (**1**) was responsible for the G_2 checkpoint inhibitory activity of the *D. odorata* extract and was active at concentrations above 10 μM (Figure 1). Alkaloid **2** was isolated in insufficient amounts for biological testing, and alkaloids **3** and **4** were inactive in the assay (Figure 1). Compounds **1**, **3**, and **4** did not inhibit cell proliferation potently in the absence of DNA damage (IC_{50} = 45, 25, and 250 μM, respectively), but they were 2−3-fold more active toward cells irradiated with 6.5 Gy (IC_{50} = 20, 7, and 80 μM, respectively). Oliveroline (**1**) is an efficacious but not highly potent inhibitor of the G_2 checkpoint that shows little structural resemblance to other checkpoint inhibitors described to date. Unlike most other checkpoint inhibitors, oliveroline (**1**) does not inhibit Chk1 kinase activity *in vitro*, and, therefore, it has promise as a chemical biology tool to elucidate the complex mechanisms underlying the response of cells to DNA damage.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a JASCO P-1010 polarimeter. UV absorptions were recorded on a Waters 2487 dual λ absorbance detector. CD spectra were obtained using a JASCO J-700 spectropolarimeter. ^1H and ^{13}C NMR spectra were obtained using a Bruker AMX-500 spectrometer. High-resolution ESI mass spectra were obtained on a Macromass LCT mass spectrometer.

Plant Material and Isolation of Alkaloids. A sample of *D. odorata* stem bark was collected in Peru in February 1992 by the New York Botanical Gardens as part of a contract with NCI. A voucher specimen is located at the National Herbarium in Washington, D.C. under the

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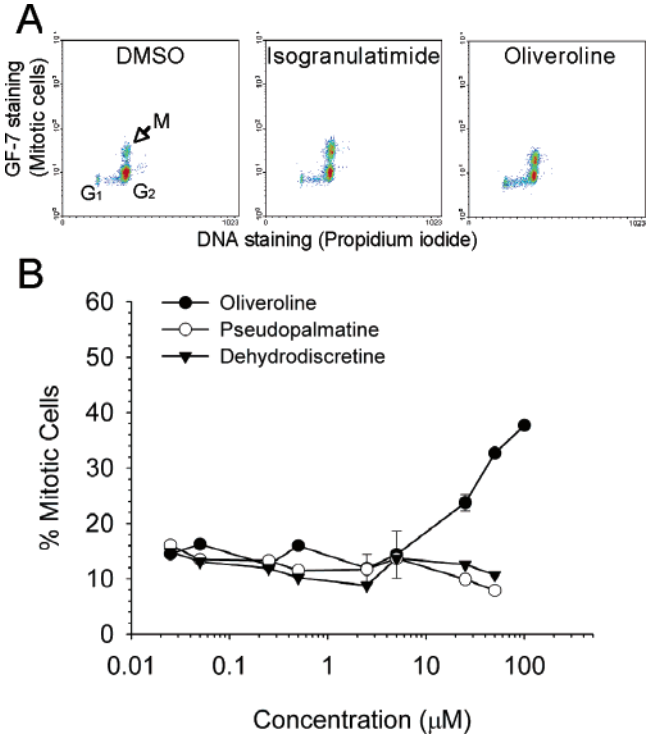

Figure 1. G_2 checkpoint inhibition by oliveroline (**1**). The G_2 checkpoint inhibitory activity of compounds **1**, **3**, and **4** was assessed by flow cytometry as described in the Experimental Section. (A) In the presence of the drug carrier DMSO, 16% cells escaped G_2 arrest, while exposure of cells to the Chk1 inhibitor isogranulatimide (10 μM) or oliveroline (50 μM) caused over 35% of the cells to escape G_2 arrest. (B) Concentration dependence of the checkpoint inhibitory activity of oliveroline (**1**). Pseudopalmatine (**4**) and dehydrodiscretine (**3**) were inactive at all concentrations tested.

code number 0CKH0164. The crude MeOH extract was prepared at NCI and sent to Vancouver as a gum. Four grams of the crude extract was suspended in 100 mL of H_2O, and the suspension was sequentially extracted with hexanes (3 × 50 mL), CH_2Cl_2 (3 × 50 mL), EtOAc (3 × 50 mL), and butanol (3 × 50 mL). The butanol-soluble material (400 mg), which was active in the assay, was subjected to Sephadex LH-20 chromatography eluting with 100% MeOH, followed by further purification on a reversed-phase Sep Pak (eluent: H_2O to MeOH step gradient) to yield one biologically active fraction. This fraction was subjected to repeated reversed-phase HPLC (C_{18}, eluent: 6 H_2O:4 MeOH:0.1 TFA) to yield oliveroline (**1**) (1.7 mg), *N*-methylguatterine (**2**) (1.3 mg), dehydrodiscretine (**3**) (3.3 mg), and pseudopalmatine (**4**) (2.6 mg).

(+)-*N*-Methylguatterine (2): brownish oil, $[\alpha]_D^{21}$ +6.2 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ϵ) 242 (3.48), 279 (3.42); CD (MeOH) λ_{max} ($\Delta\epsilon$) 239 nm (−162.82); ^{1}H NMR (500 MHz, DMSO) δ 7.87 (m, H-11), 7.65 (m, H-8), 7.42 (m, H-10), 7.41 (m, H-9), 6.95 (d, J = 6.81 Hz, 7-OH), 6.25 (s, H-12a or H-12b), 6.07 (s, H-12a or H-12b), 5.02 (dd, J = 6.82 Hz, J = 12.20 Hz, H-7), 4.76 (d, J = 12.2 Hz, H-6a), 4.02 (s, Me-15), 3.75 (m, H-5a or H-5b), 3.61 (m, H-5a or H-5b), 3.61 (s, Me-13), 3.17 (s, Me-14), 2.93 (m, H-4); ^{13}C NMR (125 MHz, $CDCl_3$) δ 145.1 (C-1), 138.9 (C-3), 136.9 (C-7a), 135.9 (C-2), 128.0 (C-9 or C-10), 127.9 (C-9 or C-10), 127.6 (C-11a), 125.4 (C-11), 123.9 (C-8), 119.2 (C-1b), 116.3 (C-3a), 109.5 (C-1a), 101.7 (C-12), 70.8 (C-6a), 68.2 (C-7), 61.8 (C-5), 59.4 (C-15), 56.8 (C-14), 42.1 (C-13), 18.6 (C-4); HRESIMS *m/z* 340.1534 [M^+] (calcd for $C_{20}H_{22}NO_4$ 340.1549).

Checkpoint Inhibitor Activity. MCF-7 mp53 cells were seeded at 2×10^5 cells/dish in 35 mm diameter dishes and subsequently cultured for 24 h. Cells were then irradiated with 6.5 Gy using a ^{60}Co source (1.2 Gy/min, Gammacell 220, Atomic Energy Commission of Canada). Sixteen hours later, when 90% of cells were arrested in G_2 phase,[3] drugs were added with 100 ng/mL nocodazole, and cells were cultured for another 8 h. Cells were then collected in SAB (phosphate-buffered saline with 1% fetal bovine serum and 0.1% sodium azide) and fixed in 10 volumes of 70% EtOH at 4 °C overnight. Cells were washed in 0.5% Tween-20 in SAB and incubated with a mitosis-specific antibody GF-7 for 1 h, washed twice, and suspended with 1:500 diluted Alexa 488-conjugated goat anti-rabbit antibody (Molecular Probes A-11029) for 30 min. Following two more washes, cells were suspended in RNase A (Roche Diagnostics, 500 units/mL in 4 mM sodium citrate buffer, pH 8.4) for 30 min at 37 °C. An equal volume of 50 μg/mL propidium iodide prepared in 4 mM sodium citrate pH 8.4 was added for an additional 20 min. Cells were resuspended at a final concentration of 1×10^6 cells/mL in 25 μg/mL propidium iodide solution and stored in the dark overnight. Cells were analyzed in a Becton-Dickson FACS-Calibur, collecting 20 000 events per sample. All data were analyzed using WinMDI freeware.

Cell Viability Assay. MCF-7 mp53 cells were seeded at 1000 cells/well in 96-well plates, grown overnight, exposed to different concentrations of compound for 24 h, and irradiated with 0 or 6.5 Gy immediately thereafter. DMSO carrier did not exceed 1% final concentration. Twenty-four hours later the compounds were washed away and cells were allowed to grow in fresh medium until those not treated with compound approached confluence, typically 4−6 days. Cell proliferation was measured as follows: 25 μL of a 5 mg/mL solution of 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in phosphate-buffered saline was added to cells in the presence of 100 μL of cell culture medium. After a 2 h incubation at 37 °C, 100 μL of 20% sodium dodecyl sulfate dissolved in dimethylformamide/water (1:1), pH 4.7, was added, and the absorbance at 570 nm was measured after overnight incubation.

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