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# Glaziovianin A, a new isoflavone, from the leaves of *Ateleia glazioviana* and its cytotoxic activity against human cancer cells

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**Abstract**—A new isoflavone, named glaziovianin A (**1**), was isolated from the leaves of *Ateleia glazioviana* (Legminosae) by means of a cytotoxicity-guided fractionation procedure against HL-60 leukemia cells. The chemical structure of **1** was determined by analysis of its extensive spectroscopic data. Glaziovianin A (**1**) displayed differential cytotoxicities in the Japanese Foundation for Cancer Research 39 cell line panel assay. The pattern of the differential cytotoxicities of **1** was found to correlate to that shown by TZT-1027, suggesting that **1** inhibited tubulin polymerization as an action mechanism. Although **1** had little influence on microtubule networks in interphase cells, **1**-treated cells showed abnormal structures with unaligned chromosomes.


*Ateleia glazioviana* Baillon (Legminosae) is a native Brazilian tree whose leaves have been suggested to cause abortion, cardiac lesions, and central nervous system lesions in bovines.[1] Previously, we have isolated two novel triterpene saponins, enterolosaponins A and B, from the pericarps of the Brazilian medicinal plant *Enterolobium contrtisiliquum*, and enterolosaponin A exhibited a selective cytotoxicity against BAC1.2F5 mouse macrophages.[2] In our continuing studies of bioactive components from Brazilian plants, an EtOH extract of the leaves of *A. glazioviana* was found to show considerable cytotoxic activity against HL-60 human leukemia cells. Through a cytotoxicity-guided fractionation procedure, a new isoflavone, named glaziovianin A (**1**), and three known isoflavones (**2**–**4**) were isolated from the $CH_2Cl_2$-soluble portion responsible for the cytotoxic activity of the EtOH extract. This communication deals with the isolation and structural determination of **1** and with its cytotoxic activities against 39 tumor cell lines in the Japanese Foundation for Cancer Research human cancer cell line panel assay. An expected mode of action of **1** is also reported.

The leaves of *A. glazioviana* (1.3 kg)[3] were extracted with EtOH by Soxhlet apparatus. The EtOH extract was evaporated under reduced pressure to give a precipitate (2.75%) and crude EtOH extract. The precipitate, which showed cytotoxic activity against HL-60 cells with an $IC_{50}$ value of 4.3 μg/mL, was suspended with hexane to remove fatty substances. The defatted precipitate was extracted with EtOH to yield an ethanolic residue (19.0 g). This residue was dissolved in $CH_2Cl_2$ to give a $CH_2Cl_2$-soluble portion (3.1 g), which showed cytotoxic activity against HL-60 cells with an $IC_{50}$ value of 1.2 μg/mL. The $CH_2Cl_2$-soluble portion was applied on column chromatography using silica gel 60 H (Merck, Darmstadt, Germany) and eluted

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with hexane–$CH_2Cl_2$–*iso*–PrOH (15:9:1), giving five fractions (I–V). Fractions II and III were further purified by semi-preparative reversed-phase (C18) HPLC eluted with $CH_3CN$–$H_2O$ (1:1) to give glaziovianin A (**1**; 162 mg), along with three known isoflavones **2** (92 mg), **3** (4 mg), and **4** (16 mg).

Glaziovianin A (**1**) was isolated as an amorphous powder.[4] The molecular formula of **1** was determined to be $C_{20}H_{18}O_8$ by the HRESITOFMS data (*m*/*z* 387.1060). In the $^1H$ NMR spectrum of **1**, a characteristic resonance for H-2 of the isoflavone skeleton was observed at $\delta$ 7.90 (1H, s), which was associated with the one-bond coupled carbon signal at $\delta$ 153.5 by the HMQC spectrum. This assignment was supported by long-range correlations from the H-2 proton to the carbon resonances at $\delta$ 175.4 (C-4), 152.3 (C-9), 121.7 (C-3), and 118.1 (C-1′) in the HMBC spectrum. The $^1H$ and $^{13}C$ NMR spectra also included signals for three aromatic protons/carbons at $\delta_H$ 7.62/$\delta_c$ 104.9, $\delta_H$ 6.89/$\delta_c$ 99.6, and $\delta_H$ 6.53/$\delta_c$ 110.1, four methoxy protons/carbons at $\delta_H$ 4.00/$\delta_c$ 56.5, $\delta_H$ 3.99/$\delta_c$ 56.4, $\delta_H$ 3.87/$\delta_c$ 56.9, and $\delta_H$ 3.85/$\delta_c$ 60.2, and one methylenedioxy group at $\delta_H$ 6.02 (2H, s)/$\delta_c$ 101.8. In the HMBC spectrum of **1**, the aromatic proton at $\delta$ 7.62 showed long-range correlations with the carbons at $\delta_C$ 175.4 (C-4), 154.3 (C-7), 152.3 (C-9), 147.6 (C-6), and 117.9 (C-10), whereas that at $\delta$ 6.89 correlated with the carbons $\delta_C$ 154.3 (C-7), 152.3 (C-9), 147.6 (C-6), and 117.9 (C-10) (Fig. 1), allowing the $\delta$ 7.62 and 6.89 resonances to be assigned as H-5 and H-8, respectively. The remaining aromatic proton at $\delta$ 6.53, which exhibited HMBC correlations with the carbons at $\delta$ 121.7 (C-3 of the C-ring), 139.1 (C′-5), 139.0 (C-3′), 137.1 (C-4′), and 136.8 (C-2′), was assigned to H-6′ of the B-ring, and the other positions of the B-ring were revealed to be substituted. The positions of the one methylenedioxy group and the four methoxy groups were determined by analysis of the HMBC and NOE data. Long-range correlations between the methylenedioxy protons at $\delta$ 6.02 and the carbons at $\delta_c$ 139.0 (C-3′) and 137.1 (C-4′) allowed the methylenedioxy group to be deposited at C-3′ and C-4′ of the B-ring. NOE correlations between H-5 and the $\delta$ 3.99 (3H, s) signal, H-8 and the $\delta$ 4.00 (3H, s) signal, and between H-6′ and the $\delta$ 3.87 (3H, s) signal gave evidence of the presence of methoxy groups at C-6, C-7, and C-5′. Furthermore, an NOE correlation was observed between the H-2 proton of the C-ring part and the $\delta$ 3.85 (3H, s) signal, which showed the locus of the remaining methoxy group at C-2′. Thus, the structure of glaziovianin A (**1**) was established to be 6,7,2′,5′-tetramethoxy-3′,4′-methylenedioxyisoflavone. Compounds **2**, **3**, and **4** were identified as 6,7,2′-trimethoxy-4′,5′-methylenedioxyisoflavone, 6,7,3′,4′-tetramethoxyisoflavone, and 6,7,2′,4′,5′-pentamethoxyisoflavone, respectively.[5–8]

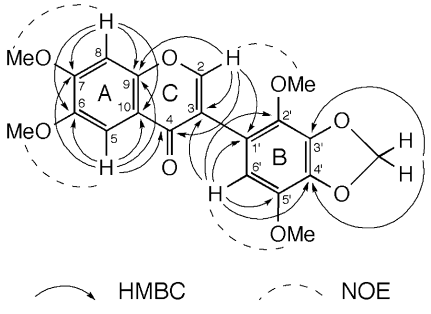
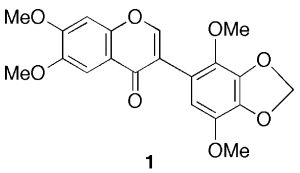



**Figure 1.** Important HMBC and NOE correlations of glaziovianin A (**1**).

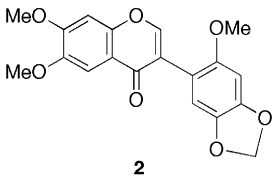
**Table 1.** $GI_{50}$ values of glaziovianin A (**1**) against 39 human cancer cell lines

| Type of cancer | Cell line | $GI_{50}$[a,b](μM) |
|---|---|---|
| Breast | HBC-4 | 1.95 |
| | BSY-1 | 0.05 |
| | HBC-5 | 0.47 |
| | MCF-7 | 0.39 |
| | MDA-MB-231 | 1.10 |
| Central nervous system | U251 | 0.79 |
| | SF-268 | 0.83 |
| | SF-295 | 0.34 |
| | SF-539 | 0.32 |
| | SNB-75 | 0.63 |
| | SNB-78 | 10.72 |
| Colon | HCC2998 | 3.02 |
| | KM-12 | 0.32 |
| | HT-29 | 0.46 |
| | HCT-15 | 0.29 |
| | HCT-116 | 0.56 |
| Lung | NCI-H23 | 5.13 |
| | HCI-H226 | 0.58 |
| | NCI-H522 | 0.23 |
| | NCI-H460 | 0.41 |
| | A549 | 0.83 |
| | DMS273 | 0.13 |
| | DMS114 | 0.20 |
| Melanoma | LOX-IMVI | 0.87 |
| Ovary | OVCAR-3 | 0.13 |
| | OVCAR-4 | 10.47 |
| | OVCAR-5 | 2.19 |
| | OVCAR-8 | 1.51 |
| | SK-OV-3 | 0.28 |
| Kidney | RXF-631L | 0.51 |
| | ACHN | 0.62 |
| Stomach | St-4 | 15.14 |
| | MKN1 | 0.69 |
| | MKN7 | 1.51 |
| | MKN28 | 0.45 |
| | MKN45 | 0.68 |
| | MKN74 | 0.39 |
| Prostate | DU-145 | 0.59 |
| | PC-3 | 0.10 |
| MG-MID[c] | | 0.66 |
| Delta[d] | | 1.17 |
| Range[e] | | 2.52 |

[a] Concentrations for inhibition of cell growth at 50% relative to control.
[b] The cell growth was determined according to the sulforhodamine B assay.
[c] Mean $GI_{50}$ value in all cell lines tested.
[d] Difference in the log $GI_{50}$ value between the most sensitive cells and the MG-MID value.
[e] Difference in the log $GI_{50}$ value between the most and least sensitive cells.



1



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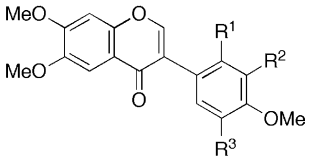

| | $R^1$ | $R^2$ | $R^3$ |
|---|---|---|---|
| **3** | H | OMe | H |
| **4** | OMe | H | OMe |

Glaziovianin A (**1**), **2**, **3**, and **4** showed cytotoxic activity against HL-60 cells with $IC_{50}$ values of 0.29 μM, 16 μM, 23 μM, and 8.5 μM, respectively.[9] Glaziovianin A (**1**) has two methoxy groups and a methylenedioxy group at the ring B part and was the most cytotoxic to HL-60 cells, whose $IC_{50}$ value was almost equal to that of etoposide ($IC_{50}$ 0.37 μM) used as a positive control. Consequently, **1** was evaluated in the Japanese Foundation for Cancer Research 39 cell line assay (Table 1).[10] As a result, **1** showed that the mean concentration required to achieve a $GI_{50}$ level against the panel cells tested was 0.66 μM, and exhibited differential cytotoxicities, with breast cancer BSY-1 ($GI_{50}$ 0.045 μM), prostate cancer PC-3 ($GI_{50}$ 0.095 μM), lung cancer DMS273 ($GI_{50}$ 0.13 μM), and ovarian cancer OVCAR-3 ($GI_{50}$ 0.13 μM) being especially sensitive to it. Furthermore, by analysis of the differential cellular sensitivity using the COMPARE program, the pattern of the differential cytotoxicities of **1** was found to correlate to that shown by TZT-1027,[11] one of the potent microtubule inhibitors, suggesting that **1** inhibited tubulin polymerization as a mechanism of action. To clarify the mechanism of **1**, we investigated the effects of **1** on

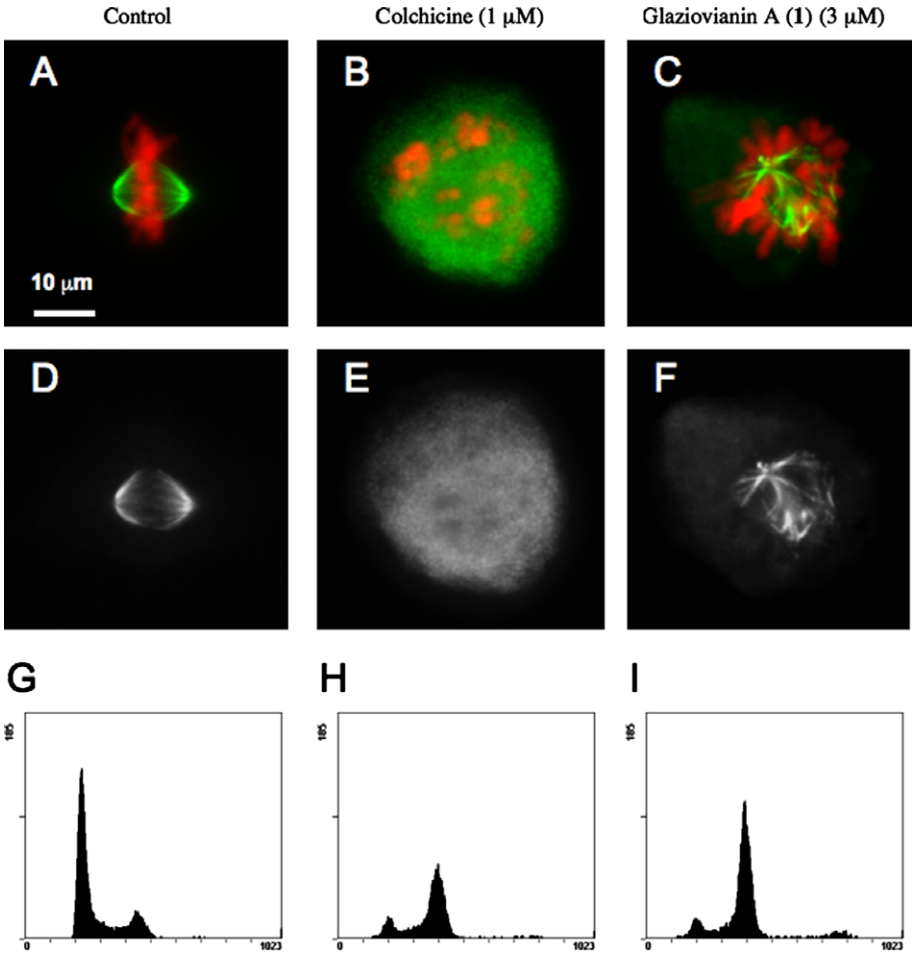


**Figure 2.** Effects of colchicine and glaziovianin A (**1**) on tubulin and cell cycle progression in 3Y1 cells. (A–F) Effects of colchicine and **1** on tubulin cytoskeletons. 3Y1 cells were treated with 1 μM of colchicine (B, E) and 3 μM of **1** (C, F) for 18 h. (A–C) Merged images with tubulin (green) and chromosomes (red) are shown. (D–F) Tubulin cytoskeletons stained with anti-α-tubulin antibodies. (G–I) Effects of colchicine and **1** on cell cycle progression in 3Y1 cells. 3Y1 cells were treated with 1 μM of colchicine (H) and 3 μM of **1** (I) for 18 h.

cell cycle progression and microtubule assembly in rat normal fibroblast 3Y1 cells (Fig. 2).[12] As predicted by the results of the panel screening, **1** arrested cell cycle progression at the M phase at the concentration of 3 μM after 18 h of treatment (Fig. 2I). Next, we observed the cytoplasmic microtubule network in situ by immunofluorescence microscopy.[13] When the cells were treated with colchicine, a potent inhibitor of microtubule assembly, at a concentration of 1 μM for 18 h, the microtubule network was completely disrupted. On the contrary, **1** had little influence on microtubule networks in interphase cells (data not shown). These results were unexpected, because it is known that antitumor drugs listed with high scores in panel screenings directly inhibit tubulin dynamics. Therefore, we then observed the M phase cells arrested by **1** in detail. Untreated control cells showed a typical bipolar mitotic spindle (Fig. 2A and D). In contrast, **1**-treated cells showed abnormal spindle structures with unaligned chromosomes (Fig. 2C and F). These results strongly suggest that **1** arrests cell cycle progression at the M phase by activating the spindle checkpoint through the inhibition of normal spindle formation. Further investigation into the detailed mechanisms on the activity of **1** is currently under way.

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3. The leaves of *A. glazioviana* were collected in Pelotas city, Rio Grande do Sul State, Brazil, in January of 2000. The plant was identified by a taxonomist from Universidade Federal de Pelotas – Rio Grande do Sul State and a voucher specimen was deposited at the Herbarium UFPel (Voucher No. 23124).
4. Glaziovianin A (**1**): $[\alpha]_D$ + 13.6° (*c* 0.10, $CHCl_3$); ESI-TOFMS *m/z* 387.1060 $[M + H]^+$ (calcd for $C_{20}H_{19}O_8$, 387.1080); UV (MeOH) $\lambda_{max}$ 315.8 (log ε 3.95), 256.4 (log ε 4.27); IR (film) $\nu_{max}$ 2930 (CH), 1637, 1607, 1502, 1455, 1227, 1033 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 500 MHz) δ 7.90 (1H, s, H-2), 7.62 (1H, s, H-5), 6.89 (1H, s, H-8), 6.53 (1H, s, H-6′), 6.02 (2H, s, $OCH_2$O-3′,4′), 4.00 (3H, s, $OCH_3$-7), 3.99 (3H, s, $OCH_3$-6), 3.87 (3H, s, $OCH_3$-5′), 3.85 (3H, s, $OCH_3$-2′); $^{13}C$ NMR ($CDCl_3$, 125 MHz) δ 175.4 (C-4), 154.3 (C-7), 153.5 (C-2), 152.3 (C-9), 147.6 (C-6), 139.1 (C-5′), 139.0 (C-3′), 137.1 (C-4′), 136.8 (C-2′), 121.7 (C-3), 118.1 (C-1′), 117.9 (C-10), 110.1 (C-6′), 104.9 (C-5), 101.8 ($OCH_2$O-3′,4′), 99.6 (C-8), 60.2 ($OCH_3$-2′), 56.9 ($OCH_3$-5′), 56.5 ($OCH_3$-7), 56.4 ($OCH_3$-6).
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9. HL-60 cells, which were obtained from the Human Science Research Resources Bank (JCRB 0085, Osaka, Japan), were maintained in RPMI 1640 medium (Sigma, St. Louis, MO, USA) containing heat-inactivated 10% (v/v) fetal bovine serum (FBS) supplemented with 100 U/mL penicillin G sodium and 100 μg/mL streptomycin sulfate (Gibco, Grand Island, NY, USA). For cytotoxicity assay, the leukemia cells were washed and resuspended in the above medium at $4 \times 10^4$ cells/mL, and 196 μL of this cell suspension was placed in each well of a 96-well flat-bottomed plate (Iwaki Glass, Chiba, Japan). The cells were incubated in a humidified air–$CO_2$ (19:1) atmosphere for 24 h at 37 °C. After incubation, 4 μL of EtOH–$H_2O$ (1:1) solution containing the sample was added to give the final concentrations of 0.1–20 μg/mL; 4 μL of EtOH–$H_2O$ (1:1) was added into control wells. The cells were further incubated for 72 h in the presence of each agent. Then cell growth was evaluated by the modified MTT assay procedure established by Sargent and Taylor (Sargent J. M.; Taylor C. G. *Br. J. Cancer* **1989**, *60*, 206) as follows. At the end of incubation, 10 μL of 5 mg/mL MTT in phosphate-buffered saline (PBS) was added to every well, and the plate was further incubated in a humidified air–$CO_2$ (19:1) atmosphere for 4 h at 37 °C. The plate was then centrifuged at 1500*g* for 5 min to precipitate cells and MTT formazan. An aliquot of 150 μL of the supernatant was removed from every well, and 175 μL DMSO was added to dissolve the MTT formazan. The plate was mixed on a microshaker for 10 min and then read on a microplate reader (Spectra Classic, Tecan, Salzburg, Austria) at 550 nm. Each assay was done in triplicate, and cytotoxicity was expressed as $IC_{50}$ value, which reduced the viable cell number by 50%.
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12. Rat normal fibroblast 3Y1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) in a humidified air–$CO_2$ (19:1) atmosphere. The distribution of DNA content in the cell populations was analyzed by flow cytometry. The cells were fixed with cold (−20 °C) 70% EtOH (v/v) and stained with propidium iodide (Sigma). Total fluorescence intensities were determined by quantitative flow cytometry with Cytomics FC500 (Beckman Coulter, Fullerton, CA, USA).
13. Immunofluorescence observation of tubulin was performed as described in a previous paper Usui, T.; Kondoh, M.; Cui, C. B.; Mayumi, T.; Osada, H. *Biochem. J.* **1998**, *333*, 543, The cytoskeletons were photographed with a cooled charge-coupled device camera (PROVIS AX70, Olympus, Tokyo, Japan).