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Olipa Ngassapa, Djaja D. Soejarto, John M. Pezzuto, Norman R. Farnsworth, and Chun-Tao Che

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FURTHER LUPANE LACTONES FROM *KOKOONA OCHRACEA*[1]

OLIPA NGASSAPA, DJAJA D. SOEJARTO, JOHN M. PEZZUTO, NORMAN R. FARNSWORTH,*

Program for Collaborative Research in the Pharmaceutical Sciences, Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, PO Box 6998, Chicago, Illinois 60680

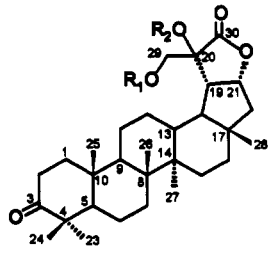
and CHUN-TAO CHE

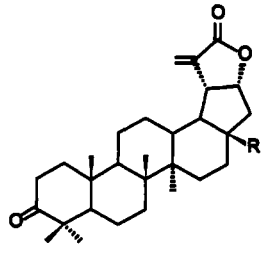
Department of Chemistry, Hong Kong University of Science and Technology, Hong Kong

ABSTRACT.—Additional new lupane lactones were isolated from the stem bark of *Kokoona ochracea* (Celastraceae). Their structures have been elucidated, through the application of 1D and 2D nmr spectroscopic methods, as 20,29-dihydroxy-3-oxolupan-30,21α-olide (ochraceolide D) [**1**] and 28-hydroxy-3-oxolup-20(29)-en-30,21α-olide (ochraceolide E) [**2**]. These compounds and the mono- and di-acetates of ochraceolide D (**4** and **5**, respectively) were evaluated for in vitro cytotoxic activity against P-388 murine lymphocytic leukemia cells and a panel of human cancer cell systems. Ochraceolide D [**1**] was significantly cytotoxic (ED_{50}, 3.9 μg/ml) against human glioblastoma (U373) cells. Other compounds (**4**, **5**, and **2**) exhibited only a weak cytotoxic response in certain cancer cell lines.

Previously we reported the isolation of cytotoxic lupane lactones, ochraceolides A–C, from the non-polar extracts of the stembark of *Kokoona ochracea* (Elm.) Merrill (Celastraceae) (1). Among these, ochraceolide A [**3**] was obtained as the most abundant compound (0.3% yield). These compounds were the first example of lupanes possessing a γ-lactone located between C-30 and C-21 of the lupane skeleton. As a continuation of our previous work, we have isolated additional new lupane lactones, ochraceolides D and E, and we report here the structure elucidation and the cytotoxic activity of these compounds.

Ochraceolide D [**1**] was obtained as a colorless crystalline compound ($CHCl_3$/MeOH) which gave a molecular ion peak at *m/z* 486.3341 in its hreims. This corresponds to a molecular formula of $C_{30}H_{46}O_5$, indicating that the molecule has eight double bond equivalents. The ir spectrum of **1** revealed the presence of a ketone (1700 cm^{-1}), a γ-lactone (1748 cm^{-1}), and hydroxyl groups (3486 cm^{-1}). Indeed, acetylation of **1** (Ac_2O/pyridine) afforded a monoacetate **4** and a diacetate **5**. The ir spectrum of **5** did not reveal the presence of any additional free hydroxyl groups, indicating that compound **1** possessed only two hydroxyl groups. Analysis of the ^1H- and ^{13}C-nmr spectra (Table 1)





1 $R_1=R_2=H$
4 $R_1=Ac$, $R_2=H$
5 $R_1=R_2=Ac$

2 $R=CH_2OH$
3 $R=Me$

[1]Parts of this study were derived from the Ph.D. Thesis of Olipa Ngassapa, University of Illinois at Chicago (1992).

TABLE 1. ^{13}C-nmr Spectral Data (δ, $CDCl_3$) for Compounds **1**, **4**, **5**, and **2**.

Carbon	Compound			
	1[a]	**4**	**5**	**2**
C-1	39.73 t	39.46 t	39.56 t	39.44 t
C-2	34.30 t	34.01 t	34.04 t	33.97 t
C-3	216.55 s	218.23 s	217.86 s	218.00 s
C-4	47.31 s	47.19 s	47.29 s	47.20 s
C-5	54.82 d	54.63 d	54.85 d	54.63 d
C-6	19.82 t	19.52 t	19.54 t	19.54 t
C-7	33.73 t	33.42 t	33.42 t	33.36 t
C-8	41.13 s	40.86 s	40.82 s	40.80 s
C-9	49.97 d	49.58 d	49.76 d	49.12 d
C-10	36.95 s	36.75 s	36.90 s	36.75 s
C-11	21.80 t	21.57 t	21.51 t	21.10 t
C-12	27.25 t	26.75 t	26.55 t	26.67 t
C-13	37.78 d	37.25 d	37.00 d	37.05 d
C-14	43.07 s	42.81 s	43.02 s	42.95 s
C-15	28.31 t	27.76 t	27.77 t	27.64 t
C-16	34.89 t	34.39 t	34.22 t	30.20 t
C-17	43.23 s	42.92 s	43.02 s	48.75 s
C-18	46.36 d	45.87 d	47.51 d	51.78 d
C-19	46.90 d	46.53 d	45.54 d	45.68 d
C-20	77.52 s	74.59 s	79.09 s	139.44 s
C-21	82.31 d	82.56 d	82.24 d	82.63 d
C-22	48.59 t	47.97 t	45.86 t	42.72 t
C-23	26.76 q	26.62 q	26.59 q	26.67 q
C-24	21.18 q	20.94 q	20.68 q	20.97 q
C-25	16.12 q	15.98 q	16.04 q	15.87 q
C-26	15.96 q	15.79 q	15.82 q	15.70 q
C-27	14.93 q	14.50 q	14.30 q	14.32 q
C-28	19.20 q	19.19 q	18.77 q	62.85 t
C-29	67.85 t	67.90 t	66.62 t	124.68 t
C-30	181.24 s	177.90 s	173.49 s	171.27 s
CH_3CO		20.65 q	20.99, 20.91 q	
CH_3CO		170.25 s	169.85, 169.61 s	

[a]The spectrum was recorded in pyridine-d_5.

of **1** revealed that it was a pentacyclic triterpene closely related to ochraceolides A [**3**], B, and C. ^{1}H- and ^{13}C-nmr assignments for **1** were made through the application of ^{1}H-^{1}H DQCOSY, ^{1}H-^{13}C HETCOR, APT, and selective INEPT nmr experiments, and by comparison with the nmr data for ochraceolides A–C (1) and other related compounds (2,3). An intense peak at *m/z* 205 in the eims of **1** was indicative of a fragment composed of rings A and B with a C-3 ketone function (4). Thus, a carbonyl signal at 216.55 ppm was assigned to C-3. The remaining carbonyl signal at 181.24 ppm was assignable to C-30, which was involved in lactone formation. The rather downfield resonance of C-30 at 181.24 ppm, when compared with ochraceolides A–C (170–174 ppm) (1), suggested the presence of a free carboxyl group. However, this was ruled out based on the ir spectrum, which showed the presence of a γ-lactone (1748 cm^{-1}). Furthermore, selective INEPT experiments (5) confirmed the location of the γ-lactone to be between C-30 and C-21. Thus, irradiation of H-21 at 5.21 ppm ($^3J_{CH}$=5 Hz) enhanced the signals of C-30 (181.24 ppm) and C-18 (46.36 ppm), which are 3 bonds away from H-21. In the ^{13}C-nmr spectra, signals of hydroxylated carbons appeared at 67.85 and 77.52 ppm. The former was correlated to a pair of doublets (J=9.6 Hz) at 4.19 and 4.50 ppm and was assigned to C-29. The remaining signal at 77.52 ppm was for a quaternary carbon and was assigned

to C-20, based on the downfield shift of C-30 to 181.24 ppm. These assignments were confirmed through selective INEPT experiments, when irradiation of the C-29 protons ($^3J_{CH}$=4 Hz) enhanced the signals of C-19 (46.90 ppm), C-20 (77.52 ppm), and C-30 (181.24 ppm). Additionally, irradiation of H-18 ($^3J_{CH}$=5 Hz) enhanced the signal of C-20, as well as the signals of C-13 (37.78 ppm), C-14 (43.07 ppm), and C-17 (43.23 ppm). The signals of C-20, C-29, and C-30 were also enhanced when H-19 (3.16 ppm) was irradiated ($^3J_{CH}$=5 Hz), further confirming location of the γ-lactone and the two hydroxyl groups. Based on ^{1}H-^{1}H NOESY experiment, the γ-lactone was determined to be α-oriented. However, the orientation of the C-20 hydroxyl group remains to be determined. Thus, the structure of ochraceolide D was established as 20,29-dihydroxy-3-oxolupan-30,21α-olide [**1**].

Ochraceolide E [**2**] was also obtained as colorless crystals from $CHCl_3$, mp 233–235°. The molecular formula of **2** was determined as $C_{30}H_{44}O_4$ based on hreims, which gave a molecular ion at *m/z* 468.3243 (calcd 468.3240). Its ir spectrum revealed the presence of a six-membered cyclic ketone (1700 cm^{-1}), an α,β-unsaturated γ-lactone (1730 cm^{-1}), and a hydroxyl group (3439 cm^{-1}). Evidence from nmr data revealed that compound **2** was a lupene triterpene and was closely related to ochraceolide A [**3**]. However, unlike ochraceolide A, compound **2** had only five tertiary methyl groups, not six, indicating that one methyl was converted to another functional group. Indeed, ^{1}H nmr showed a pair of AB doublets at 3.51 and 3.68 ppm (J=10.8 Hz), which were correlated to a carbon at 62.85 ppm in the ^{1}H-^{13}C HETCOR spectrum of **2**. This clearly showed that one group was a primary alcohol; the ir spectrum also exhibited a band at 3439 cm^{-1}. The eims of **2** showed a fragment ion peak at *m/z* 437, resulting from the loss of an angular CH_2OH group (4). A missing carbon signal for C-28 in the ^{13}C-nmr spectra of **2** at 19 ppm suggested that the primary alcohol was located at C-28. This was confirmed by selective INEPT experiments, when the carbon signal at 62.85 ppm (C-28) was enhanced after irradiation of the downfield H-22, at 2.61 ppm ($^3J_{CH}$=5 Hz). In the same experiment, the signals of C-17 (48.75 ppm), C-18 (51.78 ppm), and C-19 (45.68 ppm) were also enhanced when H-22 was irradiated.

The locations of the γ-lactone and exomethylene were confirmed by selective INEPT experiments, through irradiations involving H-18, H-19, H-21, and H-29. A ^{1}H-^{1}H NOESY experiment showed that the γ-lactone was α-oriented, based on the correlations observed between H_2-28 and H-19, H-21 and H-22β. Thus the structure of ochraceolide E was determined as the new compound 28-hydroxyl-3-oxolup-20(29)-en-30,21α-olide [**2**].

Ochraceolide D [**1**], its derivatives **4** and **5**, and ochraceolide E [**2**] were evaluated for in vitro cytotoxicity against P-388 murine lymphocytic leukemia and twelve human cancer cell lines (BC-1 breast cancer, HT-1080 fibrosarcoma, LU-1 lung cancer, MEL-2 melanoma, COL-2 colon cancer, KB oral epidermoid carcinoma, KB-V1 multidrug-resistant KB, A431 epidermoid carcinoma, LNCaP hormone-dependent prostate cancer, ZR-75-1 hormone-dependent breast cancer, and U373 glioblastoma). The results in Table 2 indicate that all four compounds are cytotoxic against certain cancer cell lines. However, the activity was weak (ED_{50} >4 μg/ml) in most instances. Only ochraceolide D [**1**] was significantly active against human glioblastoma cells, exhibiting an ED_{50} value of 3.9 μg/ml. Interestingly, its derivatives **4** and **5** were inactive in the U373 cell line, but exhibited a weak activity against other cell systems, including the multidrug-resistant cell line KB-V1. It is also notable that the activity of ochraceolide D 20,29-diacetate [**5**] was more pronounced on KB-V1 in the presence of vinblastine (+VLB), than on KB-V1 in the absence of vinblastine (−VLB) or the parent cell line, KB. These observations indicate that compound **5** enhances the cytotoxic effect of vinblastine.

TABLE 2. In vitro Cytotoxic Activity of Compounds **1**, **4**, **5**, and **2**.

Cell line	Compound tested (ED_{50}, μg/ml)			
	1	**4**	**5**	**2**
BC-1	>20	>20	>20	>20
HT-1080	14.7	>20	>20	12.2
LU-1	>20	>20	>20	>20
MEL-2	>20	20.0	6.2	11.9
COL-2	>20	>20	>20	>20
KB	>20	16.7	16.8	>20
KB-V1 (+VLB)	>20	11.7	6.7	>20
KB-V1 (−VLB)	>20	14.6	18.3	>20
P-388	>5	4.6	4.8	>5
A431	9.5	>20	>20	>20
LNCaP	15.5	>20	>20	>20
ZR-75-1	>20	>20	11.5	18.8
U373	3.9	>20	>20	8.6

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Nmr spectra were obtained in $CDCl_3$ or pyridine-d_5 with TMS as an internal standard. ^{1}H-nmr spectra were recorded on a Varian XL-300 spectrometer, operating at 300 MHz. ^{13}C-nmr spectra were obtained on Varian XL-300 (75.4 MHz) or Nicolet NMC-360 (90.8 MHz) spectrometers. Low resolution eims and hreims were obtained on Varian MAT-112S and Finnigan MAT-90 spectrometers, respectively. Ir and uv spectra were recorded with Nicolet MX-1 FT-IR and Beckman DU-7 spectrophotometers, respectively. Cc was performed on Si gel 60 (70–230 mesh, E. Merck, Darmstadt, Germany). All solvents were spectral grade or redistilled before use.

COLLECTION AND EXTRACTION OF PLANT MATERIAL.—The stem bark of *K. ochracea* was collected in July 1988 on Palawan Island, Philippines, and identified by one of us (D.D.S.) and Dr. D.A. Madulid (Philippine National Herbarium, Manila, Philippines). Voucher specimens (Soejarto and Madulid 6098) are deposited in the John G. Searle Herbarium of the Field Museum of Natural History, Chicago, and the Philippine National Herbarium, Manila.

Air-dried powdered stem bark (5 kg) of *K. ochracea* was extracted and fractionated as previously described (1). Briefly, the plant material was extracted with petroleum ether, then MeOH, to afford a petroleum ether extract (35.8 g) and an MeOH extract (320 g). Partitioning of the latter (310 g) between H_2O and $CHCl_3$, followed by drying, afforded $CHCl_3$-soluble (155 g), interphase (93.2 g), and H_2O-soluble (59.1 g) materials. The petroleum ether extract and the $CHCl_3$-soluble material were found to possess in vitro cytotoxic activity against P-388 (murine lymphocytic leukemia) and various human cancer cell lines. These extracts had similar tlc profiles; therefore they were combined (188 g) and fractionated further by Si gel cc [petroleum ether/EtOAc; EtOAc/MeOH) to afford eight major fractions (F1–F8). Cytotoxicity was retained in fractions F3–F6. The major compound, ochraceolide A, was obtained from fraction F3. Ochraceolide D (513 mg) was obtained from F4 as a precipitate formed on concentration of F4. It was crystallized from $CHCl_3$/MeOH. Further purification of fraction F4 by cc (petroleum ether/$CHCl_3$; $CHCl_3$/MeOH) afforded six major fractions (F4-I–F4-VI). Ochraceolides B and C were obtained on further purification of fraction F4-II. Further purification of fraction F4-IV, by flash chromatography (EtOAc/toluene), gravity cc ($CHCl_3$/MeOH), and gel filtration (Sephadex LH-20, MeOH), followed by crystallization ($CHCl_3$), afforded ochraceolide E (76.3 mg).

Ochraceolide D [**1**].—Colorless crystals ($CHCl_3$/MeOH): mp 253–256°; $[\alpha]^{25}D$ +10° (c=0.1, MeOH); uv (MeOH) λ max 206 nm (end absorption); ir (KBr) ν max 3486 (OH), 2980–2884 (CH, aliphatic), 1748 (C=O, γ-lactone), 1700 (C=O, ketone), 1462, 1386, 1256, 1208, 1167, 1084, 1040 cm^{-1}; ^{1}H nmr (pyridine-d_5, 300 MHz) δ 5.21 (1H, ddd, J=8.7, 8.1, 7.5 Hz, H-21), 4.50 (1H, d, J=9.6 Hz, H_a-29), 4.19 (1H, d, J=9.7 Hz, H_b-29), 3.16 (1H, dd, J=10.7, 9.2 Hz, H-19), 2.49 (2H, m, H-2), 2.28 (1H, dd, J=10.7, 11.2 Hz, H-18), 2.13 (1H, dd, J=12.0, 7.1 Hz, H-22β), 1.14 (3H, s, Me-23), 1.06 (3H, s, Me-24), 1.05

(3H, s, Me-26), 1.03 (3H, s, Me-27), 0.90 (3H, s, Me-25), 0.82 (3H, s, Me-28); ^{13}C nmr see Table 1; eims (70 eV) *m/z* (% rel. int.) $[M]^+$ 486 (28), $[M-Me]^+$ 471 (8), 381 (14), 220 (10), 206 (22), 205 (73), 187 (14), 161 (29), 147 (39), 107 (55), 95 (68), 81 (88), 55 (98), 41 (100); hreims *m/z* $[M]^+$ 486.3341 ($C_{30}H_{46}O_5$ requires 486.3345).

ACETYLATION OF OCHRACEOLIDE D [**1**].—Ocharceolide D (75 mg) was acetylated with a mixture of Ac_2O (2 ml) and pyridine (1 ml) at room temperature for 24 h. The reaction mixture was dried in vacuo, and the residue was purified by Si gel cc (petroleum ether/$CHCl_3$) to afford two amorphous derivatives, ochraceolide D 29-acetate [**4**] (68.2 mg) and ochraceolide D 20,29-diacetate [**5**] (10.5 mg).

Ochraceolide D 29-acetate [**4**].—Uv (MeOH) λ max 204 nm (end absorption); ir (dry film) ν max 3470 (OH), 2967–2878 (CH, aliphatic), 1777, 1757 (C=O, γ-lactone and acetate), 1703 (C=O, ketone), 1460, 1385, 1373, 1232, 1163, 1144, 1046, 752 cm^{-1}; ^{1}H-nmr ($CDCl_3$, 300 MHz) δ 4.97 (1H, ddd, *J*=8.1, 7.7, 7.3 Hz, H-21), 4.27 (1H, d, *J*=10.9 Hz, H_a-29), 4.08 (1H, d, *J*=10.9 Hz, H_b-29), 3.13 (1H, br s, 20-OH), 2.45 (3H, m, H_2-2, H-19), 2.19 (1H, dd, *J*=12.0, 7.3 Hz, H-22β), 2.09 (3H, s, Ac), 1.93 (2H, m, H-1, H-18), 1.08 (3H, s, Me-23), 1.08 (3H, s Me-26), 1.03 (3H, s, Me-24), 0.97 (3H, s, Me-27), 0.94 (3H, s, Me-25), 0.83 (3H, s, Me-28); ^{13}C nmr see Table 1; eims (70 eV) *m/z* (% rel. int.) $[M]^+$ 528 (4), $[M-Me]^+$ 513 (2), 443 (3), 381 (4), 205 (12), 161 (10), 147 (13), 107 (19), 43 (100).

Ochraceolide D 20,29-diacetate [**5**].—Uv (MeOH) λ max 205 nm (end absorption); ir (dry film) ν max 2967–2868 (CH, aliphatic), 1779, 1747 (C=O, γ-lactone and acetate), 1703 (C=O, ketone), 1462, 1385, 1372, 1223, 1055, 756 cm^{-1}; ^{1}H nmr ($CDCl_3$, 300 MHz) δ 4.97 (1H, ddd, *J*=8.5, 8.0, 7.5 Hz, H-21), 4.38 (1H, d, *J*=10.4 Hz, H_a-29), 4.21 (1H, d, *J*=10.4 Hz, H_b-29), 2.47 (3H, m, H_2-2 and H-19), 2.17 (3H, s, Ac), 2.10 (3H, s, Ac), 2.10 (1H, dd, H-22β), 1.92 (1H, m, H-1), 1.08 (3H, s, Me-23), 1.08 (3H, s, Me-26), 1.03 (3H, s, Me-24), 0.96 (3H, s, Me-27), 0.90 (3H, s, Me-25), 0.82 (3H, s, Me-28); ^{13}C nmr see Table 1; eims (70 eV) *m/z* (% rel. int.) $[M]^+$ 570 (0.5), 509 (2), 450 (1), 381 (4), 305 (1), 205 (9), 189 (3), 187 (4), 121 (10), 106 (12), 43 (100).

Ochraceolide E [**2**].—Colorless crystals ($CHCl_3$); mp 233–235°; $[\alpha]^{25}D$ +29° (*c*=0.1, MeOH); uv (MeOH) λ max 214 nm (ε=6524); (KBr) ν max 3439 (OH), 2984–2875 (C-H, aliphatic), 1730 (C=O, γ-lactone), 1700 (C=O, ketone), 1653, 1457, 1013 cm^{-1}; ^{1}H nmr ($CDCl_3$, 300 MHz) δ 6.31 (1H, d, *J*=1.7 Hz, H_a-29), 5.69 (1H, br s, H_b-29), 5.01 (1H, ddd, *J*=7.4, 6.7, 6.0 Hz, H-21), 3.68 (1H, d, *J*=10.9 Hz, H_a-28), 3.51 (1H, d, *J*=10.7 Hz, H_b-28), 3.29 (1H, m, H-19), 2.61 (1H, dd, *J*=13.4, 7.5 Hz, H-22β), 2.46 (2H, m, H_2-2), 1.09 (3H, s, Me-26), 1.08 (3H, s, Me-23), 1.03 (3H, s, Me-24), 0.95 (3H, s, Me-25), 0.93 (3H, s, Me-27); ^{13}C nmr see Table 1; eims (70 eV) *m/z* (% rel. int.) $[M]^+$ 468 (47), $[M-Me]^+$ 451 (15), $[M-CH_2OH]^+$ 437 (15), 382 (22), 245 (25), 231 (34), 219 (39), 217 (42), 205 (100), 185 (35); hreims *m/z* $[M]^+$ 468.3243 ($C_{30}H_{44}O_4$ requires 468.3240).

CYTOTOXICITY ASSAYS.—P-388, HT-1080, KB, A431, and U373 cell lines were purchased from the American Type Culture Collection, Rockville, Maryland. Other human cancer cell types, including BC-1, COL-2, LU-1, and MEL-2 were established from primary human tumors in the Specialized Cancer Center, University of Illinois College of Medicine at Chicago. The multidrug-resistant cell line, KB-V1, was supplied by Dr. Igor B. Roninson, Department of Genetics, University of Illinois College of Medicine at Chicago. It was developed from KB cells by treatment of the latter with sublethal doses of vinblastine over an extended period of time (6). The ZR-75-1 and LNCaP cell lines were supplied by Glaxo Group Research, Greenford, United Kingdom.

All media were supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), 0.25 μg/ml of fungizone (except media for P-388, and U373), and 1.5–3% of 7.5% $NaHCO_3$ solution, and the pH values were adjusted to 7.1–7.2. All cell lines, except MEL-2, were maintained at 37° in a humidified atmosphere containing 5% CO_2 in air. Melanoma (MEL-2) cells were maintained in a closed tissue culture container.

Cytotoxicity assays were performed in 96-well microtiter plates, basically by a procedure described previously (7). Briefly, compounds were dissolved initially in DMSO, and serial dilutions were made so that the final concentration of DMSO in each well was 0.5% v/v. Cultured cells were treated, in triplicate, with four concentrations (0.16–20 μg/ml) of test compounds. Treated cell cultures were incubated at 37° in a humidified atmosphere of 5% CO_2 in air (except MEL-2) for 72 h, or 48 h in the case of P-388 and HT-1080, and 96 h for U373 cells. Melanoma (MEL-2) cells were incubated at 37° for 72 h, in the absence of CO_2. After the incubation period, the cells were fixed with cold 50% trichloroacetic acid (TCA), or 20% TCA for P-388 cells. Then the cells were stained with 0.4% (w/v) sulforhodamine B dye in 1% HOAc. Unbound dye was rinsed off with 1% HOAc, and protein-bound dye was solubilized with 10 mM unbuffered Tris base [tris(hydroxymethyl)aminomethane]. Protein (cell quantity) was measured by the determination of optical density at 515 nm with a BT 2000 MicroKinetics Reader (Bio-Tek Instruments, Inc.). The averaged data were expressed as percent relative to controls treated only with DMSO. The dose that inhibited cell growth by 50% (ED_{50}) was then determined, and the results are presented in Table 2.

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