Isolation, Structures, and Structure−Cytotoxic Activity Relationships of Withanolides and Physalins from *Physalis angulata*

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Phytochemical investigation of *Physalis angulata* was initiated following primary biological screening. Fractionation of $CHCl_3$ and *n*-BuOH solubles of the MeOH extract from the whole plant was guided by *in vitro* cytotoxic activity assay using cultured HONE-1 and NUGC cells and led to the isolation of seven new withanolides, withangulatins B−H (**1**−**7**), and a new minor physalin, physalin W (**8**), along with 14 known compounds, including physaprun A, withaphysanolide, dihydrowithanolide E, physanolide A, withaphysalin A, and physalins B, D, F, G, I, J, T, U, and V. New compounds (**1**−**8**) were fully characterized by a combination of spectroscopic methods (1D and 2D NMR and MS) and the relative stereochemical assignments based on NOESY correlations and analysis of coupling constants. Biological evaluation of these compounds against a panel of human cancer cell lines showed broad cytotoxic activity. Withangulatin B (**1**) and physalins D (**10**) and F (**11**) displayed potent cytotoxic activity against a panel of human cancer cell lines with EC_{50} values ranging from 0.2 to 1.6 μg/mL. Structure−activity relationship analysis indicated that withanolides and physalins with 4β-hydroxy-2-en-1-one and 5β,6β-epoxy moieties are potential cytotoxic agents.

The genus *Physalis* (Solanaceae) includes about 90 species that are widely distributed throughout tropical and subtropical regions of the world. Species such as *P. philadelphica*, *P. peruviana*, *P. grisea*, *P. chenopodifolia*, *P. coztomatl*, and *P. angulata* are cultivated or gathered from wild populations for their edible fruits. *Physalis* species, like other withasteroid-containing plants, are integrated in traditional systems of medicine worldwide.[1] *Physalis angulata*, known in Chinese as "Kuzhi", is a branched annual shrub that has been used as a popular medicine in various countries to treat different illnesses, such as malaria, asthma, hepatitis, dermatitis, liver disorders, and rheumatism, and as a diuretic, anticancer, antimycobacterial, antileukemic, antipyretic, and immuno-modulatory agent.[2] It is also used widely for dietary purposes, for instance, for the preparation of jams from the fruits. Prior phytochemical examination of this plant led to the isolation of a wide spectrum of physiologically active compounds, mainly withasteroids.[3]

Withasteroids are structurally diverse steroidal compounds with an ergosterol skeleton in which C-22 and C-26 are oxidized to form a δ-lactone. These compounds are generally polyoxygenated, and this profusion of oxygen functions has led to several natural modifications of the carbocyclic skeleton, as well as of the side chain, resulting in compounds with complex structural features classified as withaphysalins, physalins, ixocarpalactones, perulactones, and acnistins. This structural variation is responsible for wide-ranging pharmacological activities.[4−6] Withanolides have been studied for their antibacterial, antileishmanial, antitrypanosomal, anti-inflammatory, antitumor, antiulcer, antistress, cytotoxic, immunosuppressive, and immunomodulating effects, as well as for induction of quinone reductase activity and protection against CCl_4-induced hepatotoxicity.[7−10] They are also reported to induce phase II enzymes in animal models, which is considered to be one mechanism for cancer chemoprevention.[11,12] Ecologically, the withanolides exhibit activity as feeding deterrents, insecticides, and ecdysteroid antagonists and appear to have a significant role in the chemical defense armamentarium of Solanaceous plants.[13]

As a part of our program aimed at the discovery of novel withanolides from Taiwanese *Physalis* species, we previously reported the isolation of two new physalins and a novel ergostane derivative closely related to withanolides from *Physalis angulata*.[3] In the current work, cytotoxicity-monitored fractionation of $CHCl_3$ and *n*-BuOH solubles of a MeOH extract from the whole plant of *P. angulata* led to the isolation of seven new withanolides, withangulatins B−H (**1**−**7**), and a new minor physalin, physalin W (**8**), together with 14 known compounds. The structural elucidation of new compounds was carried out through extensive spectroscopic data interpretation, and the isolates were evaluated for cytotoxic activity. The structural determination and cytotoxicity as well as structure−activity relationships of isolates are reported herein.

Results and Discussion

Withangulatin B (**1**) was isolated as a colorless gum, and its molecular formula was determined to be $C_{28}H_{38}O_9$ by HRFABMS (*m*/*z* 519.2514 $[M + H]^+$). An UV absorption maximum at 222 nm indicated the presence of an α,β-unsaturated ketone or unsaturated lactone chromophore. IR absorption bands at 3402, 1715, and 1679 cm^{-1} were assignable to hydroxyl, α,β-unsaturated δ-lactone, and α,β-unsaturated ketone moieties, respectively. The ^1H NMR spectrum of **1** displayed characteristic signals for five methyl groups (δ_H 1.07, 1.29, 1.41, 1.88, and 1.94) and two α,β-unsaturated olefinic protons [δ_H 6.22 (1H, d, J = 10.0 Hz, H-2) and 6.91 (1H, dd, J = 10.0, 6.0 Hz, H-3)]. In the $^1H-^1H$ COSY spectrum of **1**, H-3 correlated with both H-2 and the oxygenated methine proton at δ_H 3.73 (1H, dd, J = 6.0, 2.5 Hz, H-4). These data indicated the presence of a 4β-hydroxy-2-en-1-one unit in the molecule.[11] A ^1H signal at δ_H 3.28 (1H, br s, H-6) together with ^{13}C signals for oxygenated quaternary and oxymethine carbons at δ 64.0 (C-5) and 62.9 (C-6) suggested a 5β,6β-epoxy group in **1**.[11] The ^{13}C NMR and DEPT spectra of **1** disclosed 28 carbons: an α,β-unsaturated

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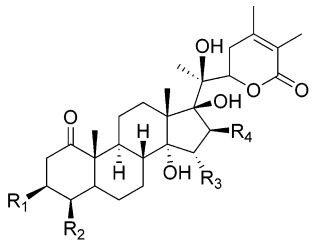
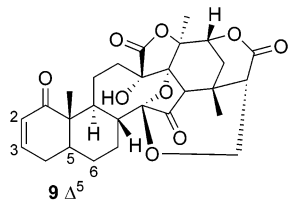
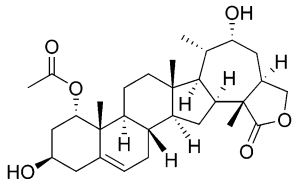
Chart 1



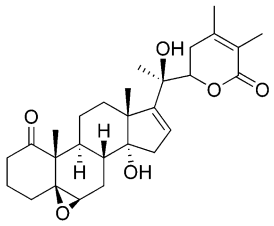
1 Δ^2; 5β, 6β-epoxy; R_1 = H, R_2 = OH, R_3 = OH R_4 = H
2 5β, 6β-epoxy; R_1 = H, R_2 = OH, R_3 = OCH_3, R_4 = H
3 5α-OH, 6β-OH; R_1 = H, R_2 = OH, R_3 = OCH_3, R_4 = H
4 5β, 6β-epoxy; R_1 = H, R_2 = OCH_3, R_3 = H, R_4 = H
6 Δ^2; 5α-OH, 6β-OH; R_1 = H, R_2 = OH, R_3 = H R_4 = OH
7 5β, 6β-epoxy; R_1 = OCH_3, R_2 = H, R_3 = H, R_4 = OH
17 Δ^2; 5β, 6β-epoxy; R_1 = H, R_2 = OH, R_3 = H, R_4 = H
18 Δ^2; 5β, 6β-epoxy; R_1 = H, R_2 = H, R_3 = H, R_4 = H
19 Δ^2; 5α-OH, 6β-OH; R_1 = H, R_2 = OH, R_3 = H R_4 = H



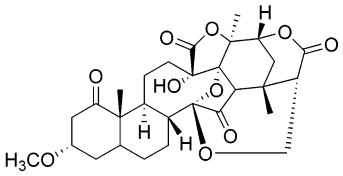
9 Δ^5
10 5α-OH, 6β-OH
11 5β, 6β-epoxy
12 Δ^4; 6α-OH
13 5α-OCH_3, 6β-OH
14 5α, 6α-epoxy



16



5



8 5αH
15 5β, 6β-epoxy

ketone (δ_C 201.6, C-1; δ_C 133.0, C-2; δ_C 141.2, C-3), an α,*β*-unsaturated *δ*-lactone (δ_C 150.5, C-24; δ_C 121.4, C-25; δ_C 165.7, C-26), three oxygenated methines (δ_C 70.3, C-4; δ_C 77.4, C-15; δ_C 79.4, C-22), an epoxide (δ_C 64.0, C-5; δ_C 62.9, C-6), and three oxygenated quaternary carbons (δ_C 81.7, C-14; δ_C 87.6, C-17; δ_C 79.0, C-20), in addition to five methyls, five methylenes, two additional methines, and two additional quaternary carbons. These NMR data were closely comparable to those of the known withanolide 4*β*-hydroxywithanolide E (**17**), suggesting that **1** is also a withanolide.[14] Comparison of the ^{1}H and ^{13}C NMR spectral data of these two compounds indicated that they have the same substituent patterns and relative configurations in rings A−C and in their respective lactone ring. This structural similarity was confirmed by correlations observed in their 2D NMR spectra. The ^{13}C NMR and DEPT spectra of **1** and 4*β*-hydroxywithanolide E (**17**)[14,15] suggested that these compounds vary at C-15, with an oxygenated methine carbon at C-15 in **1** rather than the methylene in 4*β*-hydroxywithanolide E (**17**). For **1**, HMBC correlations of both C-16 methylene protons [δ_H 2.77 (1H, dd, J = 15.0, 6.7 Hz) and 2.63 (1H, dd, J = 15.0, 3.2 Hz)] with an oxygenated methine (C-15) and three oxygenated quaternary (C-14, C-17, and C-20) carbons indicated that a hydroxy group is attached to C-15. This assignment was supported by the molecular formula, $C_{28}H_{38}O_9$, which is 16 units larger than that of 4*β*-hydroxywithanolide E (**17**). The relative stereochemistry at C-15 was deduced from the NOESY spectrum of **1**, in which correlations between protons H-18/H-15, H-16*β* and H-21/H-16α indicated that the 15-hydroxy and 18-methyl groups were on opposite sides of ring D and allowed unambiguous assignment of α-stereochemistry to the 15-hydroxy group (18*β*-CH_3). On the basis of the foregoing spectroscopic studies, the structure of **1** was fully established, and **1** was given the trivial name withangulatin B, following a prior convention for withanolide constituents of *P. angulata*.[16]

Withangulatin C (**2**) was obtained as a colorless gum. The molecular formula of $C_{29}H_{42}O_9$, 16 mass units higher than that obtained for **1**, was determined on the basis of its HRFABMS data (*m*/*z* 535.2832 [M + H]$^+$). The ^{1}H and ^{13}C NMR data of **2** (Tables 1 and 2) exhibited characteristic signals for a 5*β*,6*β*-epoxy-4*β*-hydroxy-1-one withanolide skeleton.[17] The ^{1}H NMR data for **1** and **2** were almost identical, with the exceptions that signals were found for two pairs of methylene protons [δ_H 2.43 (2H, m, H-2) and 2.13 (2H, m, H-3)] in **2** rather than the two olefinic protons for H-2 and H-3 of the α,*β*-unsaturated ketone of ring A in **1**, and an additional methoxy singlet at δ_H 3.32 was present in **2**. Accordingly, the ^{13}C NMR spectrum of **2** exhibited additional signals for a methoxy group at δ_C 56.7 and two secondary carbons at δ_C 33.8 and 25.7, but lacked resonances for a C-2,3 double bond compared to **1**. The methoxy group in **2** was located at C-15 as a result of the observed HMBC correlations from the methoxy protons to the C-15 carbon (δ_C 77.4). NOESY data of **1** and **2** were analogous and indicated that OCH_3-15 also has an α-orientation. Hence, compound **2** was determined to be 2,3-dihydro-15-methoxywithangulatin B and given the trivial name withangulatin C.

Withangulatin D (**3**) displayed a pseudomolecular ion peak at *m*/*z* 553.2936 from HRFABMS analysis, 29 carbon signals in its ^{13}C NMR spectrum, and IR absorptions for hydroxyl (3526 cm^{-1}), saturated ketone (1687 cm^{-1}), and α,*β*-unsaturated *δ*-lactone (1706 cm^{-1}) functions. These data agree with a withanolide structure with a molecular formula of $C_{29}H_{44}O_{10}$. Comparison of ^{1}H and ^{13}C NMR data (Tables 1 and 2) of **3** with those of **2** revealed that these compounds have the same substitution pattern and stereochemistry, with the sole difference that **3** possesses a 5α,6*β*-diol rather than the 5*β*,6*β*-epoxy group of **2**. This structural difference was evident from the substantial downfield shifts of the H-6 signal to δ_H 5.28 (1H, br s) as well as those of C-5 to δ_C 81.9 and C-6 to δ_C 76.6. Accordingly, the structure of **3** was concluded to be 5α,6*β*-dihydroxywithangulatin C and **3** was named withangulatin D.

HRFABMS analysis gave a molecular formula of $C_{29}H_{42}O_8$ for withangulatin E (**4**). The presence of five methyl groups, a 5*β*,6*β*-epoxy moiety, and the normal withanolide side chain was suggested from the ^{1}H and ^{13}C NMR data (Tables 1 and 2). Both the ^{1}H and ^{13}C NMR data of **4** were very similar to those of 4*β*-hydroxywithanolide E (**17**),[14,15] with certain differences evident in the ring A substitutions. In the ^{1}H NMR and COSY spectra of **4**, a methoxy group at δ_H 3.27 (3H, s), four mutually coupled methylene protons at δ_H 2.47 and 2.16 (each 2H, m, H-2 and H-3), and an oxygenated

Table 1. ^{1}H NMR Data of Compounds **1**−**7**

position	**1**	**2**	**3**	**4**	**5**	**6**	**7**
2	6.22 d (10)	2.43 m	2.45 m	2.47 m	2.32 m	6.26 d (10.3)	3.12 m
3	6.91 dd (10, 6)	2.13 m	2.16 m	2.16 m	1.86 m; 1.48 m	6.83 dd (10.3,4.4)	3.93 m
4	3.73 dd (6, 2.5)	3.48 br t (2.7)	3.44 dd (14.2,5.4)	3.72 dd (8.2,2.5)	2.31 m; 2.13 m	5.54 d (4.4)	3.10 m; 2.34 dt (12.8,2.3)
6	3.28 br s	3.20 br s	5.28 br s	3.30 br s	3.20 br s	4.78 dd (12.5,3.6)	3.56 br s
7	2.25 m; 1.25 m	2.25 m; 1.24 m	2.22 m; 1.29 m	2.26 m; 1.28 m	2.27 m; 1.25 m	2.54 td (15.3,4.8); 1.38 m	2.63 m; 2.31 dd (18.4,5.6)
8	1.98 m	1.98 m	1.95 m	1.64 m	1.76 m	2.17 dt (12.8,2.8)	1.78 m
9	2.75 m	1.59 m	2.58 m	1.96 m	1.80 m	2.68 m	2.06 dt (12.8,2.3)
11	1.73 m; 1.53 m	1.61 m; 1.44 m	1.67 m; 1.46 m	1.60 m; 1.44 m	1.68 m; 1.43 m	1.56 m; 1.42 m	1.66 m; 1.41 m
12	2.55 m; 1.75 m	2.65 m; 1.32 m	2.69 m; 1.30 m	2.69 m; 1.34 m	2.72 m; 1.38 m	2.77 br d (12.4); 1.38 m	2.60 m; 1.33 m
15	4.88 d (5.7)	3.64 m	4.35 m	1.52 m; 1.74 m	2.77 dd (14.2,5.9); 2.32 dd (14.2, 1.8)	3.04 dd (15.8,8.8); 2.01 d (15.8)	3.08 m; 1.99 m
16	2.77 dd (15, 6.7); 2.63 dd (15, 3.2)	2.84 dd (15,4.3); 2.68 dd (15,4.3)	2.81 m; 2.72 m	2.69 m; 1.57 m	5.81 dd (3.1, 1.8)	5.19 (dd, 12.6,2.5)	3.94 d (6.4)
18	1.07 s	1.04 s	1.04 s	1.03 s	1.08 s	1.36 s	1.30 s
19	1.29 s	1.28 s	1.24 s	1.14 s	1.15 s	1.65 s	1.78 s
21	1.41 s	1.41 s	1.42 s	1.41 s	1.29 s	1.75 s	1.76 s
22	4.86 dd (11, 5.2)	4.85 dd (10.3,6.2)	4.79 t (7.2)	4.82 dd (10.8,5.2)	4.39 dd (13.1,3.6)	5.17 dd (12.3,3.7)	5.22 dd (13.2,2.8)
23	2.52 m	2.48 m	2.52 m	2.80 m; 2.51 br d (16.5)	2.38 br dd (17.5, 5)	2.92 br d (15.8); 2.66 m	2.90 dd (17,13.2); 2.66 m
27	1.94 s	1.93 s	1.93 s	1.92 s	1.96 s	1.95 s	1.91 s
28	1.88 s	1.86 s 3.32 s (OCH_3-15)	1.86 s 3.50 s (OCH_3-15)	1.86 s 3.27 s (OCH_3-4)	1.89 s	1.77 s	1.76 s 3.25 s (OCH_3-3)

Table 2. ^{13}C NMR Data of Compounds **1**−**7**

position	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	201.6	209.6	207.1	210.7	213.4	202.1	209.9
2	133.0	33.8	37.3	33.5	30.6	126.3	41.4
3	141.2	25.7	25.0	26.1	22.6	147.3	78.0
4	70.3	75.6	63.7	72.4	34.5	64.9	37.1
5	64.0	64.8	81.9	62.1	64.4	79.7	65.4
6	62.9	60.4	76.6	61.7	60.7	66.7	60.2
7	29.6	29.3	29.3	29.4	26.2	30.8	26.9
8	32.3	32.2	32.0	32.2	27.8	39.7	33.2
9	36.6	35.7	37.5	36.4	35.7	39.4	35.3
10	47.7	50.3	54.8	52.2	52.3	57.9	51.3
11	21.4	20.9	22.2	21.3	22.0	23.6	21.9
12	34.1	37.8	37.8	37.8	29.9	36.8	30.5
13	54.5	54.5	58.3	54.6	52.6	55.2	55.2
14	81.7	81.6	81.4	81.6	84.1	81.9	81.9
15	77.4	77.4	77.7	35.2	39.8	37.2	37.2
16	37.9	40.0	41.2	42.6	124.4	81.5	75.1
17	87.6	87.6	87.4	87.6	156.1	88.0	88.5
18	19.6	19.6	19.6	19.6	17.6	21.3	21.0
19	16.7	14.8	11.2	12.7	12.4	10.1	15.3
20	79.0	79.0	78.9	79.0	74.7	75.0	79.5
21	20.6	20.1	20.2	20.2	20.4	19.6	20.4
22	79.4	79.5	79.2	79.5	79.4	81.4	81.8
23	34.3	34.2	34.2	34.2	30.1	35.1	35.4
24	150.5	150.6	150.8	150.6	149.5	151.3	151.2
25	121.4	121.4	121.3	121.4	121.5	121.4	121.7
26	165.7	165.8	165.8	165.8	165.3	167.0	167.1
27	12.3	12.3	12.3	12.3	12.1	12.5	12.7
28	20.6	20.6	20.6	20.6	20.6	20.2	20.4
29		56.7	59.4	55.9			56.9

methine proton at δ_H 3.72 (1H, dd, J = 8.2, 2.5 Hz, H-4) were apparent. The ^{13}C NMR spectrum of **4** exhibited signals for two secondary carbons [δ_C 33.5 (C-2) and 26.1 (C-3)] rather than resonances for a double bond at C-2 and C-3, an additional methoxy group (δ_C 55.9), and an oxygenated tertiary carbon at δ_C 72.4 (C-4). Thus, compound **4** has a nonconjugated ketone (δ_C 210.7, C-1) with a methoxy group at C-4 in ring A. Thus, **4** was concluded to be 2,3-dihydro-4β-methoxywithanolide E and named withangulatin E.

Withangulatin F (**5**) was obtained as a colorless gum. Its IR absorptions were indicative of hydroxyl (3463 cm^{-1}), saturated ketone (1684 cm^{-1}), and α,β-unsaturated δ-lactone (1715 cm^{-1}) groups. The HRFABMS of **5** displayed a pseudomolecular ion peak at m/z 471.2668, which together with the ^{13}C NMR data suggested a molecular formula of $C_{28}H_{38}O_6$. Comparison of the spectroscopic data of **5** (Tables 1 and 2) and **4** revealed that withangulatin F is the 4-desmethoxy-16-dehydroderivative of **4**. In addition to characteristic withanolide signals, its ^{1}H NMR and COSY spectra showed signals for three mutually coupled methylene groups at δ_H 2.32 (2H, m), 1.86 and 1.48 (each 1H, m), and 2.31 and 2.13 (each 1H, m) assignable to H-2, -3, and -4, respectively. A vinylic proton at δ_H 5.81 (1H, dd, J = 3.1, 1.8 Hz) was assigned to H-16 on the basis of its COSY correlations with H-15 methylene protons at δ_H 2.77 (1H, dd, J = 14.2, 5.9 Hz) and 2.32 (1H, dd, J = 14.2, 1.8 Hz). Signals for the C-16 and C-17 trisubstituted double bond were observed at δ_C 124.4 (C-16) and 156.1 (C-17), respectively. The position of the double bond was confirmed by the long-range correlations of the proton at H-16 with the carbons at C-13 (δ_C 52.6), C-14 (δ_C 84.1), and C-20 (δ_C 74.7), as well as those of H-15 with C-13 and C-17. Thus, **5** was concluded to be 4-desmethoxy-16-dehydrowithangulatin E and named withangulatin F.

Withangulatin G (**6**) has a molecular formula of $C_{28}H_{40}O_{10}$ and, thus, has one more oxygen atom than found in the known withanolide withaperuvin (**19**),[18,19] based on HREIMS data (m/z 536.2623 [M]$^+$). The NMR spectra showed signals due to five tertiary methyl groups (δ_H 1.36, 1.65, 1.75, 1.77, and 1.95; δ_C 10.1, 12.5, 19.6, 20.2, and 21.3), a 4β-hydroxy-2-en-1-one system in ring A [δ_H 6.26 (1H, d, J = 10.3 Hz, H-2), 6.83 (1H, dd, J = 10.3, 4.4 Hz, H-3), and 5.54 (1H, d, J = 4.4 Hz, H-4); δ_C 202.1 (C-1), 147.3 (C-3), 126.3 (C-2), and 64.9 (C-4)], and a 5α,6β-diol moiety in ring B [δ_H 4.78 (1H, dd, J = 12.5, 3.6 Hz, H-6); δ_C 66.7 (C-6) and

Table 3. EC_{50} Values of Tested Compounds[a] against a Human Tumor Cell Line Panel

cmpd	DU-145	1A9	HCT116	LNCAP	KB	KB-VIN	A431	A549	HCT-8	PC-3	ZR751
1	1.3	0.2	0.4	0.2	ND[b]	ND	ND	ND	ND	ND	ND
2	–[c]	6.5	11.4	13.9	ND	ND	ND	ND	ND	ND	ND
6	–	18.8	–	13.4	ND	ND	ND	ND	ND	ND	ND
7	–	3.7	9.2	9.6	ND	ND	ND	ND	ND	ND	ND
8	–	9.2	–	–	ND	ND	ND	ND	ND	ND	ND
9	ND	ND	ND	5.3	3.0	1.3	1.8	5.9	1.5	0.9	2.6
10	ND	ND	ND	1.3	1.2	7.0	1.4	1.6	1.2	1.6	0.4
11	ND	ND	ND	1.0	0.9	1.9	1.1	1.3	1.0	1.1	0.3
14	ND	ND	ND	–	–	–	17.0	–	–	–	–
15	ND	ND	ND	15.9	11.9	20.0	13.4	16.3	11.1	18.1	8.2

[a] Compounds **3**, **12**, **13**, and **16** did not show significant cytotoxic activity. [b] ND: not determined. [c] "–": did not reach 50% inhibition at 20 μg/mL.

79.7 (C-5)], in addition to signals for a typical α,β-unsaturated δ-lactone in the side chain. The ^{1}H and ^{13}C NMR data (Tables 1 and 2) of **6** in pyridine-d_5 were quite close to those of **19**,[18,19] but differences were evident between these compounds in terms of the splitting pattern and the chemical shift of H-16. In **6**, both the proton signal at δ_H 5.19 (1H, dd, J = 12.6, 2.5 Hz, H-16) and the corresponding oxygenated methine carbon at δ_C 81.5 (C-16) were shifted downfield relative to those in **19**. Coupled with the molecular formula, these data confirmed the presence of a secondary hydroxy group at C-16, in addition to the 4β and 6β hydroxy groups. Further confirmation was found in a HMBC experiment, which showed correlations between the H-16 proton and the C-13 (δ_C 55.2), C-14 (δ_C 81.9), and C-20 (δ_C 75.0) carbons. The orientation of the hydroxy group was deduced as β on the basis of NOE correlations between H-16 and CH_3-21. Hence, withangulatin G (**6**) was elucidated as 16β-hydroxywithaperuvin.

Withangulatin H (**7**), obtained as a colorless gum, is a withanolide with a molecular formula of $C_{29}H_{42}O_9$, as indicated by its HREIMS (m/z 534.2827 $[M]^+$) data and the presence of 29 signals in the ^{13}C NMR spectrum. This compound showed IR bands for hydroxy (3372 cm^{-1}), cyclohexanone (1692 cm^{-1}), and α,β-unsaturated δ-lactone (1715 cm^{-1}) functionalities. Comparison of ^{1}H and ^{13}C NMR data of **7** with analogous data for withanolide E (**18**)[20] indicated that these two compounds have the same substituent patterns and relative configurations in rings B and C and in the lactone ring, but differences in the substituents in rings A and D. In the ^{1}H NMR spectrum of **7**, the H-3 signal was a multiplet at δ_H 3.93 and the H-2 methylene protons appeared as a multiplet at δ_H 3.12. A methoxy singlet was found at δ_H 3.25. ^{13}C NMR signals at δ_C 209.9 (C-1), 41.4 (C-2), 78.0 (C-3), and 56.9 (OCH_3) confirmed saturation of the C-2,3 bond and the presence of a methoxy group at C-3. The location of the methoxy group was also confirmed by the observed proton/carbon HMBC correlations of OCH_3/C-3; H-2/C-1, C-3, C-10; and H-3/C-2, C-1. In addition, the ^{1}H and ^{13}C NMR spectra of **7** also exhibited signals at δ_H 3.94 (1H, d, J = 6.4 Hz) and δ_C 75.1. This proton and carbon showed HMQC coupling, and all data indicated a hydroxylated carbon in ring D. The HMBC experiment displayed correlations between the H-16 proton and carbons at δ_C 55.2 (C-13), 81.9 (C-14), and 79.5 (C-20), which were in agreement with the presence of a 16-hydroxy group in ring D.[11,12,17] Similar NOESY correlations were found with **6** and **7** and indicated that OH-16 also has a β-orientation. Hence, withangulatin H (**7**) was elucidated as 2,3-dihydro-16-hydroxy-3-methoxywithanolide E.

Physalin W (**8**) was shown to have the molecular formula $C_{29}H_{36}O_{10}$ from a molecular ion peak at m/z 544.2308 in the HREIMS analysis. The IR spectrum displayed absorption bands at 3375, 1774, 1763, 1739, and 1715 cm^{-1}, which were compatible with the presence of hydroxy, γ-lactone, five-membered ring ketone, δ-lactone, and cyclohexanone functionalities, respectively. Twenty-nine carbon signals, including three methyl groups (δ_C 12.0, 22.4, and 25.6), one methoxy group (δ_C 55.7), eight methylenes (one oxygenated at δ_C 62.3), seven methines (two oxygenated at δ_C 72.7 and 79.4), and 10 quaternary carbons (two carbonyls at δ_C 210.5 and 209.0 and two lactone carbonyls at δ_C 167.5 and 174.8), were evident from the ^{13}C and DEPT spectra of **8**. The most notable feature in the ^{13}C NMR spectrum of **8** was the observation of a signal for a doubly oxygenated quaternary carbon at δ_C 107.2, which suggested the presence of a hemiketal functionality found in the basic physalin skeleton.[3] Consistent with the ^{13}C NMR data, the ^{1}H NMR spectrum of **8** displayed signals for three methyls at δ 1.19 (3H, s, CH_3-19), 1.28 (3H, s, CH_3-28), and 2.30 (3H, s, CH_3-21) and methylene signals at δ 3.94 (1H, d, J = 13.3 Hz, H-27) and 4.80 (1H, dd, J = 13.3, 4.6 Hz, H-27) characteristic of a CH_2-(27)–O–C–O(14) bridge commonly present in physalin B (**9**) and related physalins.[21] Detailed ^{1}H and ^{13}C NMR analyses using 2D correlational techniques were performed. From these data, **8** differs from physalin U (**15**)[3] only in the B ring substitution. While the latter compound contains a C-5,6 epoxy group, the former compound is unsubstituted at these positions on the basis of the following data. The 400 MHz ^{1}H NMR spectrum of **8**, taken in pyridine-d_5 solution, was characterized by the absence of an epoxide proton signal and the presence of mutually coupled methylene and methine protons. The relatively high-field shifts of these protons [δ_H 2.83 (1H, dd, J = 14.1, 5.6 Hz, H-6), 2.25 (1H, dd, J = 14.1, 1.6 Hz, H-6), and 1.62 (1H, m, H-5)] indicated the absence of oxygenation at C-5 and C-6. The HMBC long-range ^{1}H–^{13}C connectivities between H-5/C-7, 9, 10, 19; OCH_3-3/C-3; H-16/C-15, 25, 28; CH_3-19/C-1, 9, 10; CH_3-21/C-17, 22; H-22/C-24, 26; H-27/C-26; and CH_3-28/C-16, 23, 25 established the structural fragments of all five rings, as well as the positions of the quaternary carbons, tertiary methyls, and methoxy group. The relative configuration of the stereogenic centers was determined by NOE effects observed for H-3/H-5; H-5/H-7, 9; CH_3-19/H-2, 4, 8, 11; and H-27/H-21, 23, 25 in a NOESY experiment. Thus the structure of **8** has been assigned as shown.

In addition, 14 known compounds were identified as physaprun A, withaphysanolide, dihydrowithanolide E, withaphysalin A, physalins B (**9**), D (**10**), F (**11**), G (**12**), I (**13**), J (**14**), T, U (**15**), and V, and physanolide A (**16**) by spectroscopic data comparison with published values.[3,15,20–27]

Withanolides **1**–**3**, **6**, and **7**,[28] physalins **8**–**15**, and physanolide A (**16**) were assayed for cytotoxic activity against DU-45, 1A9, HCT116, LNCAP, KB, KB-VIN, A431, A549, HCT-8, PC-3, and ZR751 human tumor cell lines, as described previously,[13] and the EC_{50} values obtained are summarized in Table 3. Compound **1** showed the highest cytotoxic activity against the tested tumor cell lines with EC_{50} values ranging from 0.2 to 1.3 μg/mL. Physalins **9**, **10**, and **11** also displayed strong cytotoxicity against most of the cell lines with EC_{50} values less than 3.0 μg/mL. However, physalin B (**9**) showed decreased activity against LNCAP and A549, and physalin D (**10**) against KB-VIN tumor cell lines.

It was previously proposed that 4β-hydroxy-2-en-1-one and 5β,6β-epoxy units were necessary for bioactivities of withanolides[9–11] on the basis of bioassay evaluation results of withanolides isolated from several plants in the Solanaceae family. Recently, it was further

verified that an α,β-unsaturated ketone unit in ring A appears to be necessary for significant activity of withanolides.[12] In the present study, only withanolide **1** had both of these essential functionalities, and correspondingly, it displayed strong cytotoxicity toward DU-145, 1A9, HCT116, and LNCAP tumor cell lines with EC_{50} values of 1.3, 0.2, 0.4, and 0.2 μg/mL, respectively. Withanolides **2** and **7**, which contain the 5,6-epoxide but lack the 2-en-1-one system in ring A, exhibited moderate cytotoxicity against 1A9, HCT116, and LNCAP tumor cell lines. Cleavage of the epoxide ring to give a 5α,6β-diol as in **6** resulted in decreased cytotoxicity. Elimination of both essential groups as in **3** abolished cytotoxicity.

The effect of an α,β-unsaturated ketone and 5,6-epoxide was extended in the present study to compounds with a basic physalin skeleton. Among **9**−**15**, physalin **11**, with a 2-en-1-one system in ring A and a β-epoxide at the 5,6-positions, showed the highest cytotoxicity toward all tested tumor cell lines with EC_{50} values ranging from 0.9 to 1.9 μg/mL. However, physalin **14**, which differs from the active compound **11** only in the stereochemistry of the 5,6-epoxide ring, displayed no cytotoxicity at the highest concentration tested. This difference clearly indicates that the orientation, not only the presence, of the epoxide ring in ring B is of great importance to the activity. In addition, the structural differences between **9**, **10**, and **11** are confined to the C-5 and C-6 substitutions. Compared with **11**, physalin **9** (C-5,6 double bond) showed decreased potency against all cell lines but PC-3, while **10** (5α,6β-diol) showed comparable or slightly decreased potency. Interestingly, the identity or size of the C-5 oxygenated substituent appears to affect the cytotoxic activity, as **10** (5α-OH) was active, while **13** (5α-OCH_3) was completely inactive. In addition, **12** (C-4,5 double bond, no C-5 or C-6 substituent) was also inactive. This compound would have a significantly changed conformation of the A−B ring system due to the extended double bonds in ring A. Finally, physalin **15**, with only a 5β,6β-epoxide ring, but no double bond at C-2, exhibited only weak cytotoxicity toward the examined tumor cell lines with EC_{50} values ranging from 8.2 to 20.0 μg/mL.

Experimental Section

General Experimental Procedures. Melting points were determined using a Yanagimoto MP-S3 micro melting point apparatus without correction. Optical rotations were measured using a Jasco DIP-370 digital polarimeter. UV spectra were obtained on a Hitachi UV-3210 spectrophotometer, and IR spectra were recorded on a Shimadzu FT-IR DR-8011 spectrophotometer. ^{1}H NMR (300 and 400 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on Bruker AMX-400, AVANCE-300, and Varian Unity Plus 400 spectrometers using $CDCl_3$ and pyridine-d_5 as the solvents. Chemical shifts are shown in δ values (ppm) with tetramethylsilane as an internal standard. Low and high EI and FAB (positive-ion mode) mass spectra were measured on a JEOL JMS-700 spectrometer with samples being dispersed in glycerol and bombarded with a beam of Xe atoms with an acceleration of 8 kV. Reversed-phase column chromatography was accomplished with Diaion HP-20 and Sephadex LH-20 columns. Silica gel column chromatography was carried out using Kieselgel 60 (70−230 and 230−400 mesh, Merck). Thin-layer chromatography (TLC) was executed on precoated Kieselgel 60 F_{254} plates (Merck), with compounds visualized by UV light or spraying with 10% (v/v) H_2SO_4 followed by charring at 110 °C for 10 min.

Plant Material. The whole plants of *P. angulata* were collected in Tainan Hsien, Taiwan, Republic of China, in August 2002. The plant material was identified taxonomically by Prof. C. S. Kuoh, Department of Life Science, National Cheng Kung University at Tainan, Taiwan. A voucher specimen has been deposited at the Herbarium of National Cheng Kung University, Tainan, Taiwan, under the acquisition number Wu 200200020.

Extraction and Isolation. The dried and milled whole plants of *P. angulata* (6 kg) were extracted with MeOH six times (6 × 20 L) under reflux, for 8 h each. After filtration and evaporation of the solvent under reduced pressure, the combined crude methanolic extract (600 g) was suspended in distilled H_2O and then partitioned successively with $CHCl_3$ and *n*-BuOH to afford dried $CHCl_3$-soluble (190 g), *n*-BuOH-soluble (190 g), and H_2O-soluble (220 g) residues, respectively. The $CHCl_3$-soluble extract was defatted with *n*-hexane to yield *n*-hexane solubles (45 g) and a residue (145 g). This residue showed significant cytotoxicity (88% and 98% inhibition at 50 μg/mL) against HONE-1 and NUGC cell lines in a preliminary cytotoxicity screening assay. Accordingly, this residue was subjected to column chromatography over silica gel and eluted using a step gradient of a $CHCl_3$−MeOH solvent system (100:0, 80:1, 60:1, 40:1, 20:1, 10:1, 5:1, 1:1, 100:0) to obtain eight fractions (F1−F8) based on TLC profile. Each fraction was concentrated *in vacuo* and monitored by an *in vitro* cytotoxicity assay. Active fractions F1, F2, and F4 showed inhibitory percentage values of 99% and 91%; 98% and 95%; and 57% and 97%, respectively, against HONE-1 and NUGC cell lines at 50 μg/mL concentration. Fraction 1 gave four subfractions (F1-1 to F1-4) after column chromatography over silica gel with *n*-hexane−$CHCl_3$ (99:1 to 1:9). Subfraction F1-1 was further chromatographed over silica gel using *n*-hexane−EtOAc (from 9:1 to 1:9) and afforded **9** (168.8 mg), **10** (62.0 mg), **11** (17.2 mg), **14** (16.2 mg), and physalin T (2.7 mg). Further purification of subfraction F1-2 by repeated column chromatography followed by thin-layer chromatography gave compounds withaphysalin A (17.6 mg), **15** (6.0 mg), and physalin V (3.0 mg). Similarly, repeated column chromatography and preparative thin-layer chromatography purification of subfraction F1-3 yielded dihydrowithanolide E (20.5 mg) and **3** (3.7 mg). Subfraction F1-4 was purified by preparative TLC twice, developed sequentially with *n*-hexane−EtOAc (3:1) and *n*-hexane−$CHCl_3$−MeOH (10:8:1), leading to the isolation of compounds **4** (5.0 mg), **5** (8.3 mg), and physaprun A (5.0 mg).

The second active fraction, F2, was subjected to repeated silica gel column chromatography using gradient mixtures of *n*-hexane−acetone (from 5:1 to 1:1) followed by preparative TLC with *n*-hexane−$CHCl_3$−MeOH (10:8:1) to afford **16** (5.0 mg), **10** (62.0 mg), **12** (3.9 mg), **13** (8.9 mg), and **15** (4.0 mg). The third active fraction, F4, was further chromatographed over silica gel eluted with $CHCl_3$−acetone (from 9:1 to 2:1) and then purified by preparative TLC with *n*-hexane−$CHCl_3$−MeOH (10:8:1) to yield **1** (307.9 mg), **2** (4.1 mg), **5** (20.0 mg), and withaphysanolide (11.3 mg).

The *n*-BuOH solubles were passed through a column of Diaion HP-20 using a H_2O−MeOH gradient (9:1 to 1:9, then pure MeOH) to give eight fractions (Fb1−Fb8). The eight fractions were again evaluated in the cytotoxicity assay. The most active fraction Fb8 (35% and 94% inhibition at 50 μg/mL) was selected for further purification. Fraction Fb8 was chromatographed successively on silica gel ($CHCl_3$−MeOH−H_2O, 9:1:0.1) and Sephadex LH-20 (MeOH−H_2O) followed by preparative TLC with $CHCl_3$−MeOH−H_2O (10:1:0.1) to afford **10** (15.4 mg), **6** (2.7 mg), **7** (3.0 mg), and **8** (2.5 mg).

Withangulatin B (1): colorless gum; $[\alpha]^{25}_D$ +197.2 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 222 (4.13) nm; IR ($CHCl_3$) ν_{max} 3402, 2980, 2953, 2875, 1715, 1679, 1383, 1248, 1137, 1017, 757 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) and ^{13}C NMR ($CDCl_3$, 75 MHz), see Tables 1 and 2; FABMS *m*/*z* 519 $[M + H]^+$ (5), 485 (3), 460 (8), 359 (2), 307 (24), 154 (100), 136 (67); HRFABMS *m*/*z* 519.2514 $[M + H]^+$ (calcd for $C_{28}H_{39}O_9$,519.2516).

Withangulatin C (2): colorless gum; $[\alpha]^{25}_D$ −69.2 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 231 (3.60) nm; IR ($CHCl_3$) ν_{max} 3369, 2950, 1691, 1455, 1382, 1248, 1137, 1094, 915, 732 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) and ^{13}C NMR ($CDCl_3$, 75 MHz), see Tables 1 and 2; FABMS *m*/*z* 535 $[M + H]^+$ (2), 517 (18), 507 (3), 499 (4), 481 (2), 463 (3), 443 (100), 399 (96), 154 (79), 136 (87); HRFABMS *m*/*z* 535.2832 $[M + H]^+$ (calcd for $C_{29}H_{43}O_9$, 535.2829).

Withangulatin D (3): colorless gum; $[\alpha]^{25}_D$ −12.8 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 202 (3.62) nm; IR ($CHCl_3$) ν_{max} 3526, 2924, 1706, 1687, 1457, 1382, 1242, 1135, 1083, 975, 759 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) and ^{13}C NMR ($CDCl_3$, 75 MHz), see Tables 1 and 2; FABMS *m*/*z* 553 $[M + H]^+$ (2), 535 (20), 517 (9), 499 (4), 481 (5), 443 (30), 307 (61), 154 (100), 136 (97); HRFABMS *m*/*z* 553.2936 $[M + H]^+$ (calcd for $C_{29}H_{45}O_{10}$, 553.2934).

Withangulatin E (4): colorless gum; $[\alpha]^{25}_D$ +127.5 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 226 (3.95) nm; IR ($CHCl_3$) ν_{max} 3563, 2924, 1687, 1456, 1379, 1210, 1136, 1085, 974 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) and ^{13}C NMR ($CDCl_3$, 75 MHz), see Tables 1 and 2; FABMS *m*/*z* 519 $[M + H]^+$ (8), 501 (7), 483 (6), 473 (9), 443 (37), 399 (26),

307 (12), 154 (100), 136 (77); HRFABMS *m/z* 519.2879 $[M + H]^+$ (calcd for $C_{29}H_{43}O_8$, 519.2880).

Withangulatin F (5): colorless gum; $[\alpha]^{25}_D$ +18.5 (*c* 0.02, MeOH); UV (MeOH) λ_{max} (log ϵ) 212 (3.31) nm; IR ($CHCl_3$) ν_{max} 3463, 2928, 1715, 1684, 1452, 1376, 1215, 1134, 1082, 976 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) and ^{13}C NMR ($CDCl_3$, 75 MHz), see Tables 1 and 2; FABMS *m/z* 471 $[M + H]^+$ (9), 453 (9), 443 (37), 399 (26), 307 (12), 154 (100), 136 (77); HRFABMS *m/z* 471.2668 $[M + H]^+$ (calcd for $C_{28}H_{39}O_6$, 471.2670).

Withangulatin G (6): colorless gum; $[\alpha]^{25}_D$ +8.6 (*c* 0.02, MeOH); UV (MeOH) λ_{max} (log ϵ) 220 (4.22), 296 (3.08) nm; IR ($CHCl_3$) ν_{max} 3393, 2931, 1680, 1459, 1381, 1253, 1138, 1083, 967, 754 cm^{-1}; ^1H NMR (pyridine-d_5, 300 MHz) and ^{13}C NMR (pyridine-d_5, 75 MHz), see Tables 1 and 2; EIMS *m/z* 536 $[M]^+$ (2), 518 (2), 500 (7), 482 (12), 464 (10), 446 (9), 428 (8), 410 (9), 367 (2), 313 (30), 277 (31), 259 (35) 152 (39), 125 (35), 109 (28); HREIMS *m/z* 536.2623 $[M]^+$ (calcd for $C_{28}H_{40}O_{10}$, 536.2621).

Withangulatin H (7): colorless gum; $[\alpha]^{25}_D$ −4.1 (*c* 0.03, MeOH); UV (MeOH) λ_{max} (log ϵ) 219 (4.26), 297 (3.07) nm; IR ($CHCl_3$) ν_{max} 3372, 2927, 1715, 1692, 1445, 1383, 1248, 1137, 1090, 972, 754 cm^{-1}; ^1H NMR (pyridine-d_5, 400 MHz) and ^{13}C NMR (pyridine-d_5, 100 MHz), see Tables 1 and 2; EIMS *m/z* 534 $[M]^+$ (3), 516 (10), 506 (2), 503 (4), 498 (16) 488 (4), 485 (15), 480 (14), 470 (16), 466 (21), 462 (3), 452 (2), 448 (9), 424 (3), 213 (31), 169 (53) 152 (74), 125 (100), 109 (59); HREIMS *m/z* 534.2827 $[M]^+$ (calcd for $C_{29}H_{42}O_9$,534.2829).

Physalin W (8): colorless gum; $[\alpha]^{25}_D$ +6.8 (*c* 0.02, MeOH); UV (MeOH) λ_{max} (log ϵ) 211 (4.11), 296 (3.23) nm; IR ($CHCl_3$) ν_{max} 3375, 2925, 1774, 1763, 1739, 1715, 1456, 1377, 1234, 1169, 1063, 992, 752 cm^{-1}; ^1H NMR (pyridine-d_5, 400 MHz) δ 4.80 (1H, dd, J = 13.3, 4.6 Hz, H-27), 4.68 (1H, dd, J = 13, 2.6 Hz, H-22), 3.94 (1H, d, J = 13.3 Hz, H-27′), 3.60 (1H, m, H-3), 3.28 (1H, br t, J = 12.8 Hz, H-9), 3.15 (1H, br s, H-16), 3.05 (3H, s, OCH_3-3), 2.99 (1H, m, H-2), 2.96 (1H, dd, J = 4.9 Hz, H-25), 2.83 (1H, dd, J = 14.1, 5.6 Hz, H-6), 2.65 (1H, m, H-2′), 2.48 (1H, m, H-12), 2.45 (1H, br t, J = 12.8 Hz, H-8), 2.30 (1H, s, CH_3-21), 2.25 (1H, dd, J = 14.1, 1.6 Hz, H-6′), 2.13 (1H, m, H-4), 2.06 (2H, m, H-23), 1.98 (1H, m, H-7), 1.76 (1H, m, H-12′), 1.62 (1H, m, H-5), 1.57 (1H, m, H-4′), 1.53 (1H, m, H-11), 1.46 (1H, m, H-11′), 1.37 (1H, m, H-7′), 1.28 (3H, s, CH_3-28), 1.19 (3H, s, CH_3-19); ^{13}C NMR (pyridine-d_5, 100 MHz) δ 210.5 (C-1), 209.0 (C-15), 174.8 (C-18), 167.5 (C-26), 107.2 (C-14), 81.5 (C-17), 81.3 (C-13), 79.4 (C-22), 77.4 (C-20), 72.7 (C-3), 62.3 (C-27), 55.9 (C-10), 55.7 (OCH_3-3), 54.4 (C-16), 50.8 (C-25), 42.5 (C-2), 37.4 (C-9), 36.4 (C-4), 34.7 (C-8), 32.7 (C-23), 30.0 (C-5), 29.6 (C-24), 26.3 (C-12), 26.0 (C-7), 25.6 (C-28), 22.4 (C-21), 21.9 (C-6), 20.0 (C-11), 12.0 (C-19); EIMS *m/z* 544 $[M]^+$ (8), 530 (64), 528 (11), 526 (21), 516 (8), 514 (12), 513 (8), 500 (9), 498 (32) 496 (15), 488 (6), 486 (13), 485-(11), 482 (24), 470 (13), 468 (14), 452 (12), 439 (14), 375 (100); HREIMS *m/z* 544.2308 $[M]^+$ (calcd for $C_{29}H_{36}O_{10}$, 544.2308).

Cytotoxicity Assay. Two human cancer cell lines, NUGC (gastric adenocarcinoma) and HONE-1 (nasopharyngeal carcinoma), were seeded in 96-well microliter plates at a density of 6000/well in 10 μL of culture medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and nonessential amino acid) and maintained at 37 °C in a humidified incubator with 5% CO_2. After an overnight adaptation period, test compounds (final concentration 50 μg/mL) in serum-free medium were added to individual wells. Cells were treated with test compounds for 3 days. Cell viability was determined by the 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl)tetrazolium salt (MTS) reduction assay.[29] Actinomycin D (5 μM, final concentration) and DMSO (0.3%, final concentration) were used as positive and vehicle controls. Results were expressed as percent of DMSO control.

***In Vitro* Cytotoxicity Assay.** All stock cultures were grown in T-25 flasks. Freshly trypsinized cell suspensions were seeded in 96-well microtiter plates at densities of 1500−7500 cells per well with compounds added from DMSO-diluted stock. After 3 days in culture, attached cells were fixed with cold 50% trichloroacetic acid and then stained with 0.4% sulforhodamine B (SRB). The absorbency at 562 nm was measured using a microplate reader after solubilizing the bound dye. The mean EC_{50} is the concentration of agent that reduces cell growth by 50% under the experimental conditions and is the average from at least three independent determinations that were reproducible and statistically significant. The following human tumor cell lines were used in the assay: A549 (non small cell lung cancer), ZR751 (estrogen receptor positive breast cancer), A431 (EGFR overexpressing skin cancer), 1A9 (ovarian), LNCAP (AR-dependent prostate cancer), HCT-8 (ileocecal), PC-3 (prostate cancer), KB (nasopharyngeal carcinoma), KB-VIN (vincristine-resistant KB subline), HCT116 (ileocecal), and DU-145. All cell lines were obtained from the Lineberger Comprehensive Cancer Center (UNC-CH) or from ATCC (Rockville, MD) and were cultured in RPMI-1640 medium supplemented with 25 μM HEPES, 0.25% sodium bicarbonate, 10% fetal bovine serum, and 100 μg/mL kanamycin.[30]

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