Preparation of a Series of Novel Bichalcones Linked with a 1,4-Dimethylenepiperazine Moiety and Examination of Their Cytotoxicity

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The chalcone basic skeleton is a unique template which is associated with widespread biological activities. In the present study, a series of novel bichalcones linked with a 1,4-dimethylenepiperazine moiety was prepared through Mannich reaction and Clasien–Schmidt condensation. The synthetic analogs 2—16 were subjected into the cytotoxicity examinations using a panel of 25 human tumor cell lines. Among the tested compounds, 3 and 4 which possessed the 3-pyridyl and phenyl groups as the substructure, respectively, displayed significant cytotoxicity against all the tumor cell lines. The results suggested that these bichalcones were potential to be the anticancer lead drugs.

Key words bichalcone; 1,4-dimethylenepiperazine; cytotoxicity; human tumor cell line

Chalcones possess a 1,3-diphenyl-2-propen-1-one basic skeleton in which two aromatic rings are connected by a three carbon α,β-unsaturated carbonyl system. They are the intermediates in the biosynthesis of flavonoids which are substances widespread in plants and with an array of biological activities; however, their structure differs considerably from the other members of the flavonoids family.[1)] Chalcones are reported with diverse biological activities including antihyperglycemic,[2)] antibacterial,[3)] antiplatelet,[4)] antiulcerative,[5)] antimalarial,[6)] antiviral,[7)] antileishmanial,[8)] antioxidant,[9)] antitubercular,[10)] tyrosinase inhibiting,[11)] anti-inflammatory,[12)] and analgesic activities.[13)] In addition, chalcones exhibited various effects of inhibition on cell proliferation and demonstrated anticancer activities.[14—19)] The bichalcones are well represented in the Anacardiacea family.[20)] In general, naturally occurring bichalcones carry either C–O–C or C–C linkage between the two chalcone units. The *Rhus* genus is also a rich source for bisflavonoids and bichalcones.[21,22)] For examples, natural bichalcones from *Rhus pyroides* demonstrated various degrees of cytotoxicity, however, they also showed more selectivity toward colon cancer cell lines, especially the HT29 and HCT-116 cell lines.[23)] Thus for the development of anticancer lead drugs, the bichalcones were selected as the synthetic targets for preparation of cytotoxic agents.

The Mannich reaction is an important carbon–carbon bond-forming step in organic synthesis, and it has been widely utilized in the synthesis of nitrogen-containing compounds, including natural products and biologically active principles.[24,25)] Mannich bases of chalcones and related compounds displayed significant cytotoxicity towards murine P388 and L1210 leukemia cancer cell lines, as well as a number of other tumor cell lines.[26)] The Mannich base group was accompanied by enhancing the bioactivity both *in vitro* and *in vivo*.[27)] Therefore, introducing a Mannich base group into the bichalcone may enhance the bioactivity. In addition, the extra nitrogen atom may increase the hydrophilicity of the resulted compounds and thus improve their potentials as lead drugs. Considering their pharmacological importance,[28—30)] a series of bichalcones linked with a 1,4-dimethylenepiperazine moiety possessed different substituents in the B-ring of the chalcone basic skeleton were synthesized in the present study. Furthermore, the synthesized analogs (**2**—**16**) were tested for cytotoxicity against 25 human tumor cell lines, including GBM-8401, M059K (human glioblastoma cell lines), FaDu (human pharyngeal squamous carcinoma cell line), CE146T/VGH (human esophageal carcinoma cell line), HSC-3 (human oral squamous carcinoma cell line), CAL-27 and SAS (human tongue squamous carcinoma cell lines), A549 and H-460 (human lung carcinoma cell lines), SK1—CP1, Huh7, HepG2 and Hep3B (human hepatoma cell lines), MDA-MB-231 and MCF-7 (human breast adenocarcinoma cell lines), A375 (human melanoma cell line), HeLa (human cervical carcinoma cell line), MG-63 and U-2 OS (human osteosarcoma cell lines), HT-29 and Colo 25 (human colon carcinoma cell lines), NPC-039 and NPC-076 (human nasopharyngeal carcinoma cell lines), and NT-2 and SH-SY5Y (human neuroblastoma cell lines), to construct the preliminary biological profiles of bichalcones. These data could be provided as the bases for further design and development of bichalcones linked with the Mannich base group.

Results and Discussion

The template precursor **1a** was prepared by the Mannich reaction among piperazine, paraformaldehyde, and two equivalents of 4-hydroxy-3-methoxyacetophenone (**1**). The reaction mixture in ethanol was stirred at 120 °C and monitored by TLC. After 20 h the reaction was completed and acetophenone dimer linked with 1,4-dimethylpiperazine (**1a**) was afforded in a good yield (Fig. 1a). In the ^{1}H-NMR spectrum of **1a**, two singlets at δ 7.46 (2H) and 7.28 (2H) were indicative of tetra-substituted aromatic basic skeleton. In the upfield region, one methoxy singlet and one methylene singlet were located at δ 3.92 (6H) and 3.80 (4H), respectively. In addition, one broad singlet at δ 3.00—2.50 (8H, br s) and

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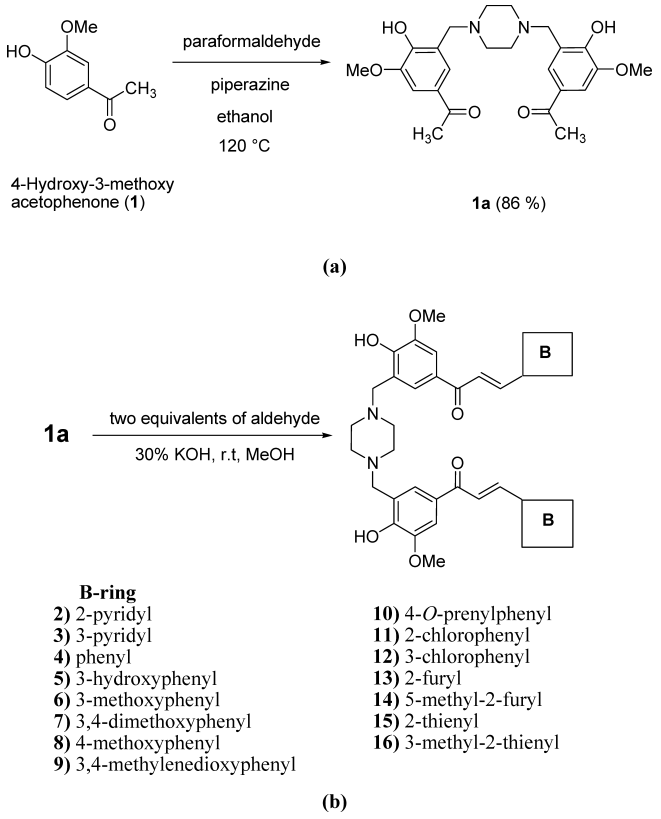

Fig. 1. (a) Synthesis of Precursor **1a**; and (b) Target Compounds **2**—**16**

one singlet resonated at δ 2.53 (6H) indicated the presences of nitrogenated methylene groups and acetyl methyls, respectively. The carbon signal at δ 196.5 in its ^{13}C-NMR spectrum also supported the appearance of acetyl group. On the basis of the above spectroscopic evidences, the structure of this colorless solid was deduced as shown in Fig. 1a.

The structural characteristic of **1a** was two acetyl methyl groups on the benzene ring which was ready for the following Claisen–Schmidt condensation with the corresponding aldehydes. In the basic methanol solution, different substituted aromatic or heteroaromatic aldehydes were reacted with **1a** to result in the formation of the target compounds **2**—**16**. In the ^{1}H-NMR spectrum of **2**, two doublets at δ 8.05 (2H, J=15.0 Hz) and 7.70 (2H, J=15.0 Hz) were characteristic for the *trans*-double bond of the chalcone basic skeleton. There were also a set of four mutually coupled signals at δ 8.62, 7.63, 7.39, and 7.21 characteristic of the four heteroaromatic protons of the 2-pyridyl moiety. In addition, one singlet at δ 7.55 (2H) and one multiplet at δ 7.39 (2H) indicated the presence of tetra-substituted aromatic ring. In the upfield region, two methoxys, two methylenes, and four nitrogenated methylenes were located at δ 3.89 (6H), 3.75 (4H), and 2.46 (8H), respectively. According to the spectroscopic data described above, the structure of this yellow solid was concluded as **2**. With the similar procedures, the chemical structures of **3**—**16** were also identified as shown in Fig. 1b.

The cytotoxicity data of **1a** and **2**—**16** towards 25 human tumor cell lines were summarized in Table 1 and aloe-emodin was used as the reference compound.[31,32] Comparison of the cytotoxicity of **2** and **3**, which have 2-pyridyl and 3-pyridyl groups in the B-ring, respectively, displayed the structure effect on the bioactivity. Compound **3** exhibited more significant cytotoxicity against all tested tumor cell lines with IC_{50} values ranging from 2.1±0.08 to 9.8±0.20 μM (Table 1), however **2** showed selective cytotoxic activity on CAL-27 and FaDu cell lines with IC_{50} values 2.0±0.05 and 9.8±0.29 μM, respectively. These results indicate that 3-pyridyl group as B-ring was responsible for potent cytotoxicity in bichalcones linked with 1,4-dimethylenepiperazine. Compound **4**, which has phenyl group as B-ring, displayed the most significant cytotoxicity towards all 25 human tumor cell lines with IC_{50} values ranges from 2.0±0.05 to 9.9±0.10 μM. Introducing any substituents into the phenyl ring would reduce the cytotoxicity. Insertion of hydroxyl group at C-3 position of the phenyl moiety in B-ring as in **5**, would result in similar or some weaker cytotoxicity. Only for A375 melanoma cells **5** would show more significant cytotoxicity than **4**. If the C-3 hydroxyl group was protected with methyl group as in **6**, it would decrease the cytotoxicity against all the tested cell lines. It indicated that the C-3 hydroxy group would be a factor on the cytotoxicity. Another comparison of the structure–activity relationship was found between **6** and **8**, which have 3-methoxyphenyl and 4-methoxyphenyl groups as B-ring, respectively. Compound **6** displayed significant cytotoxicity on NPC076 (IC_{50} 9.9±0.12 μM), FaDu (IC_{50} 9.9±0.15 μM), HSC-3 (IC_{50} 9.7±0.10 μM), CAL-27 (IC_{50} 8.0±0.08 μM), H-460 (IC_{50} 8.0±0.05 μM), A375 (IC_{50} 8.1±0.08 μM) and MG-63 (IC_{50} 7.9±0.10 μM) tumor cell lines, however **8** did not show any cytotoxicity at concentration of 10 μM. These results inferred that methoxy group at C-4 position in B-ring was responsible for weaker cytotoxicity compared with that at C-3. It could be further confirmed through the weaker cytotoxicity of compounds **7**, **9**, and **10** since **7** and **9** both possessed the di-substitutions at C-3 and C-4 positions and **10** had similar functional pattern as **8**.

Compounds **11** and **12** are positional isomers in B-ring, while **11** showed moderate cytotoxicity on CAL-27, A375 and MG-63 cell lines with IC_{50} values 6.2±0.10, 9.9±0.12, and 8.1±0.12 μM, respectively, and **12** was potent on NPC076, FaDu, CAL-27, MDA-MB-231, A375 and MG-63 cell lines with IC_{50} values 4.0±0.15, 6.0±0.06, 2.0±0.05, 6.1±0.10, 8.0±0.06, and 6.1±0.06 μM, respectively. From the results for **4**—**12**, the structure–activity relationships could be generalized as follows. The phenyl group as B-ring in the bichalcones would be the most cytotoxic. Insertion of hydroxyl group in the C-3 position of the phenyl ring would decrease the cytotoxicity, however, protection of this hydroxyl group or shift of this substituent to C-4 would reduce the cytotoxicity significantly. Similar results would be found for the chloro substituent. Another heterocyclic five membered ring furan as B-ring in **13**, showed significant cytotoxicity on NPC076, CAL-27, FaDu and A375 cell lines with IC_{50} values 4.0±0.06, 9.7±0.15, 6.1±0.12 and 5.9±0.10 μM, respectively. As the B-ring furan group was replaced with 5-methylfuran as in **14**, no cytotoxicity would be observed in **14**. If the furan group was changed to thiophene as in **15**, it displayed selectively cytotoxicity towards CAL-27 cell lines with IC_{50} value 2.1±0.10 μM. But no cytotoxicity would be found if methyl group was introduced into the 3-position as in **16**. Consequently, introducing heteroatoms into

Table 1. Anti-cancer Activity of Synthesized Novel Bichalcone Analogs (**1a**, **2**—**16**)

Cpds / Cell lines	IC_{50} (μM)																
	1a	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	Aloe-emodin
GBM-8401	>10	>10	2.1±0.08	2.0±0.05	4.0±0.10	>10	>10	>10	>10	>10	>10	9.9±0.12	>10	>10	>10	>10	>100.0
MO59K	>10	>10	—	2.0±0.06	—	—	—	—	—	—	—	—	—	—	—	—	—
SH-SY5Y	>10	>10	6.0±0.10	>10	—	—	—	—	—	—	—	—	—	—	—	—	>100.0
NT-2	>10	>10	—	8.0±0.03	—	—	—	—	—	—	—	—	—	—	—	—	15.0±3.0
NPC 039	>10	>10	6.2±0.03	2.1±0.08	9.9±0.10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	60.0±2.0
NPC 076	>10	>10	6.1±0.04	1.0±0.03	9.7±0.06	9.9±0.12	>10	>10	>10	>10	>10	4.0±0.15	4.0±0..06	>10	>10	>10	60.0±2.0
FaDu	>10	9.8±0.29	2.1±0.08	2.2±0.05	4.0±0.10	9.9±0.15	>10	>10	>10	>10	>10	6.0±0.06	9.7±0.15	>10	>10	>10	60.0±4.0
CE146T/VGH	>10	>10	6.0±0.06	8.1±0.05	8.1±0.10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>100.0
HSC-3	>10	>10	6.1±0.08	>10	>10	9.7±0.10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	15.0±3.0
CAL-27	>10	2.0±0.05	6.1±0.10	2.0±0.08	4.0±0.06	8.0±0.08	>10	>10	>10	>10	6.2±0.10	2.0±0.05	6.1±0.12	>10	2.1±0.10	10	>100.0
SAS	>10	>10	—	2.0±0.03	—	—	—	—	—	—	—	—	—	—	—	—	—
A549	>10	>10	9.8±0.20	4.1±0.05	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	40.0±2.0
H-460	>10	>10	8.1±0.15	6.2±0.20	6.1±0.05	8.0±0.05	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	40.0±5.0
SK1-CP1	>10	>10	9.9±0.21	6.1±0.15	8.0±0.10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	—
Huh-7	—	—	—	6.0±0.07	—	—	—	—	—	—	—	—	—	—	—	—	80.0±5.0
HepG2	>10	>10	8.2±0.06	8.1±0.13	6.0±0.06	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	75.0±5.0
Hep3B	—	—	—	9.9±0.10	—	—	—	—	—	—	—	—	—	—	—	—	60.0±3.0
MDA-MB-231	>10	>10	6.1±0.06	4.0±0.10	6.0±0.05	>10	>10	>10	>10	>10	>10	6.1±0.10	>10	>10	>10	>10	40.0±3.0
MCF-7	>10	>10	5.9±0.08	6.0±0.08	—	>10	>10	>10	>10	>10	>10	—	>10	>10	>10	>10	80.0±5.0
A375	>10	>10	3.9±0.10	4.0±0.08	2.0±0.05	8.1±0.08	>10	>10	>10	>10	9.9±0.12	8.0±0.06	5.9±0.10	>10	>10	>10	>100.0
HeLa	>10	>10	6.1±0.10	3.9±0.06	—	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	45.0±2.0
MG-63	>10	>10	6.0±0.05	4.1±0.05	6.0±0.08	7.9±0.10	>10	>10	>10	>10	8.1±0.12	6.1±0.06	>10	>10	>10	>10	60.0±2.0
U-2-OS	>10	>10	>10	10	9.8±0.20	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	—
HT-29	>10	>10	—	6.0±0.12	—	—	>10	>10	>10	>10	>10	—	>10	>10	>10	>10	10.0±2.0
Colo-25	>10	>10	—	6.1±0.06	—	—	>10	>10	>10	>10	>10	—	>10	>10	>10	>10	>100.0

Cells were incubated with various concentrations of compounds, or vehicle solvent (0.01% DMSO) for 36 h, and the cell viability was examined by MTT assay. The IC_{50} values of different cell lines were examined. Compounds giving less than 50% inhibition at 10 μM are described with IC_{50} >10 μM. —: not determined.

the B-ring would not enhance the cytotoxic bioactivity of the bichalcones.

In the present study, fifteen novel bichalcones linked with 1,4-dimethylenepiperazine **2**—**16** were synthesized through Mannich reaction and Clasien–Schmidt condensation. Among these synthetic compounds, **4** was highly active and displayed the most significant cytotoxicity,[33] with IC_{50} values lower than 4.0 μM towards GBM-8401, MO59K, NPC 039, NPC 076, FaDu, CAL-27, SAS, MDA-MB-231, A375, and HeLa tumor cell lines. Comparison of the cytotoxicity of **4** with those reported in the previous research results published by our group,[34] the substituents on the aromatic ring significantly reduced the anti-cancer potentials. However, these results still indicated that **4** could be a good candidate for the development of anticancer lead drugs.

Experimental

General The chemicals were acquired from commercial sources and used without further purification unless stated otherwise. The melting points were determined by a Yanagimoto MP-S3 apparatus without correction. The IR spectra were measured on a Shimadzu Fourier transform (FT)-IR Prestige 21 spectrophotometer. ^{1}H and ^{13}C-NMR spectra were recorded on a Bruker Avance-300 spectrometer, using tetramethylsilane (TMS) as internal standard; all chemical shifts are reported in parts per million (ppm, δ). FAB-MS and high resolution (HR)-FAB-MS spectra were obtained on a JEOL JMS-700 mass spectrometer. Column chromatography was performed on silica gel (70—230 mesh, 230—400 mesh). TLC was conducted on precoated Kieselgel 60 F254 plates (Merck), and the spots were visualized by UV.

Synthesis of 1a To a solution of 4-hydroxy-3-methoxyacetophenone (**1**) (16.6 g, 100 mmol), paraformaldehyde (3.0 g, 100 mmol), and piperazine (4.3 g, 50 mmol) were added in EtOH (75 ml) at room temperature as previously reported.[25,35—38] Then the resulting mixture was heated to reflux for 20 h at 120 °C. After completion, the reaction mixture was concentrated under reduced pressure and the crude product was purified by column chromatography to yield a 1,4-dimethylenepiperazine linked acetophenone dimer (**1a**, 86%). **1a**: white solid, mp 246—248 °C (95% $CHCl_3$–MeOH); IR (neat) ν_{max}: 2830, 1654, 1581, 1450, 1408, 1354, 1211, 1180, 1080, 999, 948, 894 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.46 (2H, s), 7.28 (2H, s), 3.92 (6H, s), 3.80 (4H, s), 3.00—2.50 (8H, br s), 2.53 (6H, s); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 196.5, 151.9, 147.9, 128.9, 122.6, 119.7, 110.4, 60.7, 55.9, 52.1, 26.1; FAB-MS *m/z* (rel. int. %): 443 ([M+H]$^+$, 5), 307 (19), 289 (10), 155 (24), 154 (100), 149 (13), 138 (27), 137 (55), 136 (68), 120 (11), 107 (20), 90 (12), 89 (19), 77 (12); HR-FAB-MS *m/z* 443.2182 [M+H]$^+$ (Calcd for $C_{24}H_{31}O_6N_2$, 443.2182).

General Procedure for the Synthesis of 1,4-Dimethylenepiperazine Linked Bichalcones The general synthetic strategy employed to prepare the bichalcones linked with a 1,4-dimethylenepiperazine moiety was based on the Claisen–Schmidt condensation. As shown in Fig. 1b, a series of 15 derivatives were prepared by base-catalyzed condensation of **1a** with appropriate aldehydes in MeOH. To a stirred reaction mixture at 0 °C was added a 30% solution of KOH (40 ml) drop wise over 30 min. The reaction mixture was kept at room temperature for 24—36 h, then diluted with water and extracted with EtOAc. Pure target compounds were obtained by silica gel column chromatography (CC) of the residue eluting with various solvent mixtures as indicated below. The chemical structures of all the 15 bichalcones were established on the basis of IR, ^{1}H-, ^{13}C-NMR, FAB-MS and HR-FAB-MS analyses.

(2*E*,2′*E*)-1,1′-(5,5′-(Piperazine-1,4-diylbis(methylene))bis(4-hydroxy-3-methoxy-5,1-phenylene))bis(3-(pyridin-2-yl)prop-2-en-1-one) (2) Compound **1a** (1.105 g, 2.5 mmol) and 2-pyridinecarboxaldehyde (0.535 g, 5.0 mmol) were treated as described above and the title compound was isolated as yellow solid in 77% yield. (**2**): mp 214—216 °C (90% benzene–acetone); IR (neat) ν_{max}: 2823, 1654, 1577, 1462, 1342, 1311, 1288, 1230, 1165, 1091, 1002, 752 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 8.62 (2H, br s), 8.05 (2H, d, *J*=15.0 Hz), 7.70 (2H, d, *J*=15.0 Hz), 7.63 (2H, br s), 7.55 (2H, s), 7.39 (4H, m), 7.21 (2H, d, *J*=4.8 Hz), 3.89 (6H, s), 3.75 (4H, s), 2.46 (8H, br s); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 188.0, 153.2, 152.2, 149.9, 148.2, 141.7, 136.8, 129.3, 125.4, 124.9, 124.2, 123.0, 119.8, 110.8, 60.5, 55.9, 52.0; FAB-MS *m/z* (rel. int. %): 621 ([M+H]$^+$, 15), 307 (19), 289 (10), 269 (9), 268 (25), 155 (23), 154 (100), 138 (26), 137 (52), 136 (71), 132 (12), 120 (11), 107 (22), 106 (11), 89 (21), 77 (14); HR-FAB-MS *m/z* 621.2711 [M+H]$^+$ (Calcd for $C_{36}H_{37}O_6N_4$ 621.2713).

(2*E*,2′*E*)-1,1′-(5,5′-(Piperazine-1,4-diylbis(methylene))bis(4-hydroxy-3-methoxy-5,1-phenylene))bis(3-(pyridin-3-yl)prop-2-en-1-one) (3) Compound **1a** (1.105 g, 2.5 mmol) and 3-pyridinecarboxaldehyde (0.535 g, 5.0 mmol) were treated as described above and the title compound was isolated as yellow solid in 87% yield. (**3**): mp 233—235 °C (80% benzene–acetone); IR (neat) ν_{max}: 2831, 1658, 1589, 1458, 1415, 1338, 1307, 1219, 999 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 8.83 (2H, s), 8.58 (2H, d, *J*=4.5 Hz), 7.92 (2H, d, *J*=6.0 Hz), 7.74 (2H, d, *J*=15.6 Hz), 7.62 (2H, d, *J*=15.6 Hz), 7.54 (2H, s), 7.38 (2H, s), 7.33 (2H, m), 3.93 (6H, s), 3.82 (4H, s), 2.71 (8H, br s); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 187.3, 152.2, 150.7, 149.6, 148.2, 139.8, 134.4, 130.7, 129.1, 123.6, 123.2, 122.6, 119.8, 110.8, 60.5, 55.9, 52.0; FAB-MS *m/z* (rel. int. %): 621 ([M+H]$^+$, 18), 391 (11), 354 (11), 352 (12), 307 (14), 269 (12), 268 (41), 155 (22), 154 (100), 149 (35), 139 (10), 138 (27), 137 (56), 136 (78), 132 (15), 120 (13), 107 (27), 89 (27), 77 (18); HR-FAB-MS *m/z* 621.2714 [M+H]$^+$ (Calcd for $C_{36}H_{37}O_6N_4$ 621.2713).

(2*E*,2′*E*)-1,1′-(5,5′-(Piperazine-1,4-diylbis(methylene))bis(4-hydroxy-3-methoxy-5,1-phenylene))bis(3-phenylprop-2-en-1-one) (4) Compound **1a** (1.105 g, 2.5 mmol) and benzaldehyde (0.53 g, 5.0 mmol) were treated as described above and the title compound was isolated as yellow solid in 74% yield. (**4**): mp 213—215 °C (95% benzene–acetone); IR (neat) ν_{max}: 2827, 1654, 1597, 1489, 1338, 1265, 1161, 1087, 975, 937 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.73 (2H, d, *J*=15.6 Hz), 7.57 (4H, m), 7.49 (2H, s), 7.45 (2H, d, *J*=15.6 Hz), 7.33 (8H, m), 3.88 (6H, s), 3.76 (4H, s), 2.46 (8H, br s); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 188.2, 151.9, 148.2, 143.8, 135.0, 130.3, 129.7, 128.8, 128.3, 122.5, 121.4, 119.8, 110.9, 60.7, 56.0, 52.1; FAB-MS *m/z* (rel. int. %): 619 ([M+H]$^+$, 35), 618 (18), 351 (15), 307 (15), 268 (13), 267 (50), 165 (12), 155 (23), 154 (100), 138 (27), 137 (54), 136 (78), 131 (25), 17 (25), 77 (18); HR-FAB-MS *m/z* 619.2806 [M+H]$^+$ (Calcd for $C_{38}H_{39}O_6N_2$ 619.2808).

(2*E*,2′*E*)-1,1′-(5,5′-(Piperazine-1,4-diylbis(methylene))bis(4-hydroxy-3-methoxy-5,1-phenylene))bis(3-(3-hydroxyphenyl)prop-2-en-1-one) (5) Compound **1a** (1.105 g, 2.5 mmol) and 3-hydroxybenzaldehyde (0.61 g, 5.0 mmol) were treated as described above and the title compound was isolated as yellow solid in 39% yield. (**5**): mp 203—205 °C (80% benzene–acetone); IR (neat) ν_{max}: 2839, 1647, 1585, 1450, 1284, 1122, 1083, 995, 952 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 8.24 (2H, d, *J*=15.6 Hz), 8.13 (2H, s), 8.02 (2H, d, *J*=15.6 Hz), 7.98 (2H, s), 7.67 (6H, dd, *J*=9.0, 1.5 Hz), 7.28 (2H, d, *J*=7.2 Hz), 4.28 (6H, s), 4.18 (4H, s), 2.91 (8H, s); ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 187.0, 157.6, 151.8, 147.6, 142.9, 129.8, 123.8, 122.0, 121.8, 119.7, 117.5, 115.0, 110.5, 58.0, 55.7, 51.9; FAB-MS *m/z* (rel. int. %): 651 ([M+H]$^+$, 8), 393 (5), 391 (8), 349 (7), 307 (14), 289 (10), 283 (15), 176 (11), 155 (23), 154 (100), 149 (29), 139 (11), 138 (27), 137 (55), 136 (75), 120 (13), 107 (26), 89 (24), 77 (18); HR-FAB-MS *m/z* 651.2708 [M+H]$^+$ (Calcd for $C_{38}H_{39}O_8N_2$ 651.2706).

(2*E*,2′*E*)-1,1′-(5,5′-(Piperazine-1,4-diylbis(methylene))bis(4-hydroxy-3-methoxy-5,1-phenylene))bis(3-(3-methoxyphenyl)prop-2-en-1-one) (6) Compound **1a** (1.105 g, 2.5 mmol) and 3-methoxybenzaldehyde (0.68 g, 5.0 mmol) were treated as described above and the title compound was isolated as pale yellow solid in 58% yield. (**6**): mp 197—199 °C (95% benzene–acetone); IR (neat) ν_{max}: 2831, 1654, 1597, 1342, 1276, 1049, 756 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.76 (2H, d, *J*=15.6 Hz), 7.56 (2H, s), 7.50 (2H, d, *J*=15.6 Hz), 7.39 (2H, s), 7.32 (2H, d, *J*=8.4 Hz), 7.16 (2H, s), 6.96 (2H, d, *J*=7.8 Hz), 3.96 (6H, s), 3.85 (4H, s), 2.74 (8H, br s); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 188.1, 159.8, 151.9, 148.2, 143.7, 136.4, 129.8, 129.6, 122.5, 121.8, 120.8, 119.8, 115.7, 113.5, 110.9, 60.7, 56.0, 55.3, 52.2; FAB-MS *m/z* (rel. int. %): 679 ([M+H]$^+$, 22), 678 (12), 307 (18), 297 (32), 161 (16), 155 (22), 154 (100), 138 (25), 137 (51), 136 (74), 107 (24), 89 (25), 77 (22); HR-FAB-MS *m/z* 679.3019 [M+H]$^+$ (Calcd for $C_{40}H_{43}O_8N_2$ 679.3019).

(2*E*,2′*E*)-1,1′-(5,5′-(Piperazine-1,4-diylbis(methylene))bis(4-hydroxy-3-methoxy-5,1-phenylene))bis(3-(3,4-dimethoxyphenyl)prop-2-en-1-one) (7) Compound **1a** (1.105 g, 2.5 mmol) and 3,4-dimethoxybenzaldehyde (0.83 g, 5.0 mmol) were treated as described above and the title compound was isolated as yellow solid in 60% yield. (**7**): mp 195—197 °C (95% benzene–acetone); IR (neat) ν_{max}: 2831, 1652, 1585, 1492, 1415, 1350, 1261, 1138 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.67 (2H, d, *J*=15.6 Hz), 7.47 (2H, s), 7.35 (2H, d, *J*=15.6 Hz), 7.30 (2H, br s), 7.17 (2H, dd, *J*=9.0, 3.0 Hz), 7.06 (2H, s), 6.80 (2H, d, *J*=9.0 Hz), 3.87 (6H, s), 3.86 (6H, s), 3.83 (6H, s), 3.75 (4H, s), 2.65 (8H, br s); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 188.3, 151.7, 151.2, 149.1, 148.1, 144.1, 129.9, 128.0, 122.7, 122.4, 119.8, 119.4, 111.1, 111.0, 110.3, 60.7, 56.1, 56.0, 55.9, 52.1; FAB-MS *m/z* (rel. int. %):

739 ([M+H]$^+$, 1), 391 (30), 307 (6), 279 (6), 179 (5), 167 (20), 155 (13), 265 (59), 150 (14), 149 (100), 138 (17), 137 (37), 136 (46), 113 (20), 107 (18), 91 (12), 90 (11), 77 (13), 71 (20); HR-FAB-MS *m/z* 739.3233 [M+H]$^+$ (Calcd for $C_{42}H_{47}O_{10}N_2$ 739.3231).

(2*E*,2′*E*)-1,1′-(5,5′-(Piperazine-1,4-diylbis(methylene))bis(4-hydroxy-3-methoxy-5,1-phenylene))bis(3-(4-methoxyphenyl)prop-2-en-1-one) (8) Compound **1a** (1.105 g, 2.5 mmol) and 4-methoxybenzaldehyde (0.68 g, 5.0 mmol) were treated as described above and the title compound was isolated as yellow solid in 74% yield. (**8**): mp 235—237 °C (95% benzene–acetone); IR (neat) ν_{max}: 2835, 1651, 1593, 1508, 1458, 1342, 1296, 1161, 1056, 999, 979, 825 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.70 (2H, d, J=15.6 Hz), 7.52 (4H, d, J=9.0 Hz), 7.49 (2H, s), 7.34 (2H, d, J=15.6 Hz), 7.29 (2H, s), 6.85 (4H, d, J=9.0 Hz), 3.84 (6H, s), 3.76 (10H, s), 2.46 (8H, br s); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 188.2, 161.4, 151.7, 148.1, 143.7, 130.0, 129.9, 127.7, 122.3, 119.8, 119.1, 114.3, 110.9, 60.7, 56.0, 55.3, 52.1; FAB-MS *m/z* (rel. int. %): 679 ([M+H]$^+$, 6), 308 (4), 307 (19), 289 (11), 155 (23), 154 (100), 138 (26), 137 (52), 136 (70), 120 (11), 107 (21), 89 (20), 77 (13); HR-FAB-MS *m/z* 679.3019 [M+H]$^+$ (Calcd for $C_{40}H_{43}O_8N_2$ 679.3019).

(2*E*,2′*E*)-1,1′-(5,5′-(Piperazine-1,4-diylbis(methylene))bis(4-hydroxy-3-methoxy-5,1-phenylene))bis(3-(benzo[*d*][1,3]dioxol-5-yl)prop-2-en-1-one) (9) Compound **1a** (1.105 g, 2.5 mmol) and 3,4-methylenedioxybenzaldehyde (0.75 g, 5.0 mmol) were treated as described above and the title compound was isolated as yellow solid in 61% yield. (**9**): mp 246—248 °C (90% benzene–acetone); IR (neat) ν_{max}: 2821, 1652, 1462, 1280, 1230 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.74 (2H, d, J=15.3 Hz), 7.57 (2H, s), 7.39 (2H, br s), 7.36 (2H, d, J=15.3 Hz), 7.19 (2H, s), 7.14 (2H, d, J=8.1 Hz), 6.86 (2H, d, J=8.1 Hz), 6.04 (4H, s), 3.97 (6H, s), 3.86 (4H, s), 2.75 (8H, br s); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 188.2, 151.9, 149.7, 148.4, 148.2, 143.8, 129.9, 129.5, 125.0, 122.4, 119.9, 119.5, 111.0, 108.6, 106.6, 101.6, 60.8, 56.1, 52.2; FAB-MS *m/z* (rel. int. %): 707 ([M+H]$^+$, 3), 391 (21), 307 (15), 289 (10), 167 (12), 155 (23), 154 (100), 149 (55), 138 (27), 137 (54), 136 (74), 120 (12), 113 (11), 107 (25), 91 (14), 90 (17), 89 (25), 77 (19), 71 (12); HR-FAB-MS *m/z* 707.2603 [M+H]$^+$ (Calcd for $C_{40}H_{39}O_{10}N_2$ 707.2605).

(2*E*,2′*E*)-1,1′-(5,5′-(Piperazine-1,4-diylbis(methylene))bis(4-hydroxy-3-methoxy-5,1-phenylene))bis(3-(4-(3-methylbut-2-enyloxy)phenyl)prop-2-en-1-one) (10) Compound **1a** (1.105 g, 2.5 mmol) and 4-*O*-prenylbenzaldehyde (0.95 g, 5.0 mmol) were treated as described above and the title compound was isolated as pale yellow solid in 50% yield. (**10**): mp 196—198 °C (95% benzene–acetone); IR (neat) ν_{max}: 2835, 1647, 1585, 1454, 1161, 1087, 941, 752 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.70 (2H, d, J=15.6 Hz), 7.51 (2H, d, J=15.6 Hz), 7.50 (4H, s), 7.33 (4H, d, J=9.0 Hz), 6.86 (4H, d, J=9.0 Hz), 5.41 (2H, t, J=6.3 Hz), 4.48 (4H, d, J=6.3 Hz), 3.88 (6H, s), 3.76 (4H, s), 2.62 (8H, br s), 1.72 (6H, s), 1.68 (6H, s); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 188.3, 160.8, 151.7, 148.1, 143.8, 138.7, 130.0, 127.6, 122.3, 119.8, 119.1, 119.0, 115.0, 111.0, 64.9, 60.8, 56.0, 52.2, 25.8, 18.2; FAB-MS *m/z* (rel. int. %): 707 ([M+H]$^+$, 42), 435 (21), 351 (29), 283 (57), 163 (25), 154 (100), 152 (16), 137 (56), 136 (88), 121 (17), 107 (42), 90 (26), 89 (39), 85 (44), 77 (39); HR-FAB-MS *m/z* 787.3961 [M+H]$^+$ (Calcd for $C_{48}H_{55}O_8N_2$ 787.3958).

(2*E*,2′*E*)-1,1′-(5,5′-(Piperazine-1,4-diylbis(methylene))bis(4-hydroxy-3-methoxy-5,1-phenylene))bis(3-(2-chlorophenyl)prop-2-en-1-one) (11) Compound **1a** (1.105 g, 2.5 mmol) and 2-chlorobenzaldehyde (0.7 g, 5.0 mmol) were treated as described above and the title compound was isolated as yellow solid in 70% yield. (**11**): mp 228—230 °C (95% benzene–acetone); IR (neat) ν_{max}: 2837, 1647, 1585, 1450, 1346, 1284, 1122, 1083, 995, 952 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 8.06 (2H, d, J=15.6 Hz), 7.67 (2H, dd, J=9.0, 3.0 Hz), 7.48 (2H, s), 7.39 (2H, d, J=15.6 Hz), 7.38 (4H, m), 7.24 (4H, m), 3.84 (6H, s), 3.76 (4H, s), 2.46 (8H, br s); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 188.3, 152.0, 148.2, 139.7, 135.3, 133.4, 130.9, 130.2, 129.4, 127.7, 127.0, 124.5, 122.7, 119.8, 111.1, 60.7, 56.0, 52.1; FAB-MS *m/z* (rel. int. %): 687 ([M+H]$^+$, 3), 307 (19), 289 (11), 155 (23), 154 (100), 139 (9), 138 (26), 137 (52), 136 (69), 120 (11), 107 (21), 91 (10), 89 (20), 77 (13); HR-FAB-MS *m/z* 687.2031 [M+H]$^+$ (Calcd for $C_{38}H_{37}O_6N_2Cl_2$ 687.2029).

(2*E*,2′*E*)-1,1′-(5,5′-(Piperazine-1,4-diylbis(methylene))bis(4-hydroxy-3-methoxy-5,1-phenylene))bis(3-(3-chlorophenyl)prop-2-en-1-one) (12) Compound **1a** (1.105 g, 2.5 mmol) and 3-chlorobenzaldehyde (0.7 g, 5.0 mmol) were treated as described above and the title compound was isolated as yellow solid in 63% yield. (**12**): mp 248—250 °C (90% benzene–acetone); IR (neat) ν_{max}: 2830, 1647, 1597, 1458, 1311, 1276, 1083, 1056, 960, 837 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.74 (2H, d, J=15.6 Hz), 7.66 (2H, s), 7.58 (2H, d, J=1.0 Hz), 7.53 (2H, d, J=15.6 Hz), 7.49 (2H, d, J=6.0 Hz), 7.38 (6H, m), 3.98 (6H, s), 3.92 (4H, s), 2.77 (8H, s); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 188.2, 152.1, 148.1, 148.0, 142.2, 136.6, 134.6, 130.4, 129.1, 127.5, 126.6, 122.8, 122.5, 119.9, 110.7, 60.2, 55.8, 51.9; FAB-MS *m/z* (rel. int. %): 687 ([M+H]$^+$, 6), 391 (17), 307 (14), 289 (10), 167 (13), 154 (100), 149 (58), 138 (28), 137 (56), 136 (76), 120 (12), 113 (12), 107 (26), 89 (27), 77 (20); HR-FAB-MS *m/z* 687.2028 [M+H]$^+$ (Calcd for $C_{38}H_{37}O_6N_2Cl_2$ 687.2029).

(2*E*,2′*E*)-1,1′-(5,5′-(Piperazine-1,4-diylbis(methylene))bis(4-hydroxy-3-methoxy-5,1-phenylene))bis(3-(furan-2-yl)prop-2-en-1-one) (13) Compound **1a** (1.105 g, 2.5 mmol) and 2-furaldehyde (0.48 g, 5.0 mmol) were treated as described above and the title compound was isolated as yellow solid in 77% yield. (**13**): mp 230—232 °C (95% benzene–aetone); IR (neat) ν_{max}: 2832, 1651, 1593, 1458, 1323, 1207, 1083, 960, 748 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.51 (2H, d, J=15.3 Hz), 7.48 (2H, m), 7.46 (2H, s), 7.37 (2H, d, J=15.3 Hz), 7.26 (2H, s), 6.61 (2H, d, J=3.3 Hz), 6.42 (2H, dd, J=3.3, 3.0 Hz), 3.87 (6H, s), 3.75 (4H, s), 2.68 (8H, br s); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 187.5, 151.9, 151.7, 148.1, 144.5, 129.8, 129.6, 122.4, 119.8, 118.7, 115.7, 112.5, 110.8, 60.7, 56.0, 52.1; FAB-MS *m/z* (rel. int. %): 599 ([M+H]$^+$, 12), 307 (17), 289 (10), 257 (12), 155 (22), 154 (100), 139 (10), 138 (27), 136 (73), 121 (10), 107 (23), 90 (15), 89 (23), 77 (15); HR-FAB-MS *m/z* 599.2394 [M+H]$^+$ (Calcd for $C_{34}H_{35}O_8N_2$ 599.2393).

(2*E*,2′*E*)-1,1′-(5,5′-(Piperazine-1,4-diylbis(methylene))bis(4-hydroxy-3-methoxy-5,1-phenylene))bis(3-(5-methylfuran-2-yl)prop-2-en-1-one) (14) Compound **1a** (1.105 g, 2.5 mmol) and 5-methyl-2-furaldehyde (0.55 g, 5.0 mmol) were treated as described above and the title compound was isolated as yellow solid in 63% yield. (**14**): mp 174—176 °C (90% benzene–acetone); IR (neat) ν_{max}: 2831, 1651, 1570, 1450, 1346, 1157, 1083, 995, 952, 779 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.42 (8H, m, including *trans* coupled protons), 6.52 (2H, s), 6.03 (2H, s), 3.87 (6H, s), 3.74 (4H, s), 2.31 (8H, br s), 2.27 (6H, s); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 187.6, 155.4, 151.6, 150.3, 148.0, 129.9, 129.8, 122.3, 119.7, 117.7, 116.9, 110.7, 109.1, 60.6, 55.9, 52.0, 13.9; FAB-MS *m/z* (rel. int. %): 627 ([M+H]$^+$, 38), 355 (15), 307 (14), 271 (35), 154 (100), 149 (21), 137 (53), 136 (81), 120 (13), 107 (30), 89 (28), 77 (21); HR-FAB-MS *m/z* 627.2706 [M+H]$^+$ (Calcd for $C_{36}H_{39}O_8N_2$ 627.2706).

(2*E*,2′*E*)-1,1′-(5,5′-(Piperazine-1,4-diylbis(methylene))bis(4-hydroxy-3-methoxy-5,1-phenylene))bis(3-(thiophen-2-yl)prop-2-en-1-one) (15) Compound **1a** (1.105 g, 2.5 mmol) and thiophene-2-carboxaldehyde (0.56 g, 5.0 mmol) were treated as described above and the title compound was isolated as pale yellow solid in 90% yield. (**15**): mp 213—215 °C (90% benzene–acetone); IR (neat) ν_{max}: 2829, 1647, 1589, 1458, 1365, 1288, 1199, 752 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.84 (2H, d, J=15.3 Hz), 7.47 (2H, s), 7.32 (2H, d, J=5.1 Hz), 7.28 (2H, br s), 7.24 (2H, d, J=15.3 Hz), 7.19 (2H, d, J=5.1 Hz), 7.00 (2H, t, J=4.2 Hz), 3.87 (6H, s), 3.77 (4H, s), 2.45 (8H, br s); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 187.6, 151.9, 148.1, 140.4, 136.3, 131.7, 129.6, 128.3, 128.2, 122.4, 120.2, 119.8, 110.8, 60.7, 56.0, 52.1; FAB-MS *m/z* (rel. int. %): 631 ([M+H]$^+$, 14), 307 (16), 273 (19), 154 (100), 149 (15), 137 (58), 136 (73), 120 (12), 107 (23), 89 (23), 77 (15); HR-FAB-MS *m/z* 631.1938 [M+H]$^+$ (Calcd for $C_{34}H_{35}O_6N_2S_2$ 631.1937).

(2*E*,2′*E*)-1,1′-(5,5′-(Piperazine-1,4-diylbis(methylene))bis(4-hydroxy-3-methoxy-5,1-phenylene))bis(3-(3-methylthiophen-2-yl)prop-2-en-1-one) (16) Compound **1a** (1.105 g, 2.5 mmol) and 3-methyl-2-thiophenecarboxaldehyde (0.63 g, 5.0 mmol) were treated as described above and the title compound was isolated as yellow solid in 72% yield. (**16**): mp 216—218 °C (95% benzene–acetone); IR (neat) ν_{max}: 2827, 1647, 1585, 1492, 1458, 1292, 1161, 1056, 833, 713 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 8.01 (2H, d, J=15.0 Hz), 7.56 (2H), 7.36 (2H, d, J=6.0 Hz), 7.28 (4H, m), 6.90 (2H, d, J=6.0 Hz), 3.96 (6H, s), 3.85 (4H, s), 2.69 (8H, br s), 2.39 (6H, s); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 187.6, 151.8, 148.1, 142.3, 134.7, 134.6, 131.3, 129.8, 126.8, 122.3, 119.8, 119.3, 110.9, 60.7, 56.0, 52.1, 14.2; FAB-MS *m/z* (rel. int. %): 659 ([M+H]$^+$, 10), 307 (18), 289 (12), 287 (11), 155(23), 154 (100), 138 (27), 137 (53), 136 (77), 120 (12), 107 (24), 90 (17), 89 (25), 77 (21); HR-FAB-MS *m/z* 659.2248 [M+H]$^+$ (Calcd for $C_{36}H_{39}O_6N_2S_2$ 659.2250).

Cell Culture The human glioblastoma cell lines (GBM-8401 and M059K), human pharyngeal squamous carcinoma cell line (FaDu), human esophageal carcinoma cell line (CE146T/VGH), human oral squamous carcinoma cell line (HSC-3), human tongue squamous carcinoma cell lines (CAL-27 and SAS), human lung carcinoma cell lines (A549 and H-460), human hepatoma cell lines (SK1—CP1, Huh7, HepG2 and Hep3B), human breast adenocarcinoma cell lines (MDA-MB-231 and MCF7), human melanoma cell line (A375), human cervical carcinoma cell line (HeLa), human osteosarcoma cell lines (MG-63 and U-2 OS), and the human colon carcinoma cell lines (HT-29 and Colo 25) were obtained from the Food In-

dustry Research and Development Institute (Hsinchu, Taiwan). The human nasopharyngeal carcinoma cell lines (NPC-TW 039 and NPC-TW 076) were provided by Dr. C. Y. Yang (Institute of Molecular Biology, National Chung Hsing University, Taichung, Taiwan). The human neuroblastoma cell line (NT-2) was kindly provided by Dr. C. L. Liao (Department of Microbiology and Immunology, National Defense Medical Center, Taichung, Taiwan). The human neuroblastoma cell line (SH-SY5Y) was provided by J. G. Chung (Department of Biological Science and Technology, China Medical University, Taichung, Taiwan). The CAL-27, FaDu, Huh7, HeLa, and MCF-7 cell lines were cultured in MEM supplemented with 5% fetal bovine serum (FBS). The SH-SY5Y, NPC-39, NPC-076, A375, A549, Colo 25, CE146T/VGH, HSC-3, HT-29, MDA-MB-231, SAS, SK1-CP1, HepG2, Hep3B, and MG-63 cell lines were cultured routinely in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FBS. The NT-2 cell line was cultured in OPTI-MEM supplemented with 5% FBS. The GBM-8401 and H-460 cell lines were grown in RPMI 1640 medium containing 5% FBS. The U-2 OS cell line was grown in McCoy's medium supplemented with 5% FBS. All cell lines were grown in 10-cm tissue culture dishes at 37 °C in a humidified incubator containing 5% CO_2.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) Assay The cells were seeded at a density of 0.5—1×10^4 cells per well in to 96-well plates. After 16 h of incubation, cells were grown to *ca.* 60% confluence and treated with either vehicle or various concentrations of compounds at 37 °C for 36 h before being harvested. The treated cells were washed once with phosphate buffered saline (PBS) and incubated with 0.5 mg/ml MTT for 5 h. The resulting formazan precipitate was dissolved in 100 μl of dimethyl sulfoxide (DMSO) and the optical density (OD) of formazan was determined using an enzyme-linked immunosorbent assay (ELISA) reader (Thermo Labsstems Multiskan Spectrum, Frankin, MA, U.S.A.) at 570 nm. IC_{50} values were calculated according to the Logit method.

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