JM-2007-01358v

Supporting Information

Acylpyrogallols as Inhibitors of Anti-Apoptotic Bcl-2 Proteins*

*Guozhi Tang, Zaneta Nikolovska-Coleska, Su Qiu, Chao-Yie Yang, Jie Guo, and Shaomeng Wang**

[+]Comprehensive Cancer Center and Departments of Internal Medicine, Pharmacology and Medicinal Chemistry, University of Michigan, 1500 E. Medical Center Drive, Ann Arbor, MI 48109, USA

I. Chemistry

Elemental analyses of compounds **5-9**.

Comp.	Formula	C%		H%		N%	
		Calc.	**Found**	**Calc.**	**Found**	**Calc.**	**Found**
5	$C_{16}H_{16}O_4$	70.57	70.12	5.92	5.81		undetectable
6	$C_{22}H_{20}O_5$	72.51	72.44	5.53	5.29		ibid.
7	$C_{23}H_{20}O_6$	70.40	70.11	5.14	5.20		ibid.
8	$C_{30}H_{20}O_6$	75.62	75.58	4.23	4.33		ibid.
9	$C_{27}H_{17}NO_6S$	67.07	66.91	3.54	3.51	2.90	2.82

Solvents and reagents were obtained commercially and were used without further purification. All reactions were carried out under inert atmosphere (N_2) and at room temperature unless otherwise noted. Anhydrous CH_2Cl_2 was distilled from calcium hydride. BBr_3 solution (1M) in CH_2Cl_2 was fresh prepared in lab and used in several days. All reported yields are of isolated products. Reactions were monitored by TLC carried out on 250 μm E. Merck silica gel plates (60F-254) using UV light as visualizing agent. E. Merck silica gel (60, particle size 15-40 μm) was used for flash column chromatography. NMR spectra were recorded on a Bruker Avance300 spectrometer (300 MHz). Chemical shifts (δ) are reported as δ values (ppm) downfield relative to TMS as an internal standard, with multiplicities reported in the usual manner. High resolution electrospray ionization mass spectra (MS) were run on a Micromass AutoSpec Ultima mass spectrometer. Elemental analysis (EA) was performed by the Department of Chemistry, University of Michigan (Ann Arbor) using a Perkin-Elmer 2400 Series II Analyzer.

Selected experimental procedures and chemical data for compounds **5-18**:

5-isopropyl-2,3,4-trimethoxybenzaldehyde (10). To a stirring solution of 1-isopropyl-2,3,4-trimethoxybenzene (4.21 g, 20.0 mmol) and 15 mL of HMPA in 60 mL of anhydrous THF was added *t*-BuLi (1.7M in pentane, 17.6 mL, 30.0 mmol) dropwise at -78 ^{o}C. After stirred at -40 ^{o}C for 30 min, the reactants were brought to -78 ^{o}C. 2 mL of

anhydrous DMF (25.8 mmol) was added dropwise into the flask. The mixture was gradually warmed up to 0 ^{o}C over 4h, before quenched with aqueous NH_4Cl solution and extracted with EtOAc. The combined organic phase was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The crude product was purified by flash chromatography with hexane/EtOAc (8:1) to give 4.34 g of **10** as liquid (91% yield). ^{1}H NMR ($CDCl_3$) δ 7.36-7.30 (m, 4H), 7.25-7.19 (m, 1H), 6.93 (s, 1H), 5.90 (d, *J* = 6.5 Hz, 1H), 3.86 (s, 3H), 3.85 (s, 3H), 3.56 (s, 3H), 3.25 (hept, *J* = 6.9 Hz, 1H), 3.05 (d, *J* = 6.5 Hz, 1H), 1.19 (d, *J* = 6.9 Hz, 6H); ^{13}C NMR ($CDCl_3$) δ 150.79, 148.95, 146.03, 144.09, 137.40, 132.23, 128.49, 127.56, 126.30, 119.46, 73.09, 61.14, 60.56, 60.45, 26.86, 23.48.

(5-Isopropyl-2,3,4-trimethoxy-phenyl)-phenyl-methanol (11a). A diethyl ether solution of phenylmagnesium bromide (3M, 2.5 mL, 7.5 mmol) was added dropwise into a solution of **10** (1.19 g, 5.0 mmol) in 40 mL of anhydrous THF at 0 ^{o}C. After stirring at room temperature for 2h, the reaction was quenched with aqueous NH_4Cl solution and extracted with EtOAc. The combined organic extracts were washed with brine (2X), dried over Na_2SO_4, and evaporated. The residue was purified by flash chromatography with hexane/EtOAc (5:1) to give 1.52 g of **11a** (96% yield). ^{1}H NMR ($CDCl_3$) δ 7.36-7.30 (m, 4H), 7.25-7.19 (m, 1H), 6.93 (s, 1H), 5.90 (d, *J* = 6.5 Hz, 1H), 3.86 (s, 3H), 3.85 (s, 3H), 3.56 (s, 3H), 3.25 (hept, *J* = 6.9 Hz, 1H), 3.05 (d, *J* = 6.5 Hz, 1H), 1.19 (d, *J* = 6.9 Hz, 6H); ^{13}C NMR ($CDCl_3$) δ 150.79, 148.95, 146.03, 144.09, 137.40, 132.23, 128.49, 127.56, 126.30, 119.46, 73.09, 61.14, 60.56, 60.45, 26.86, 23.48.

(5-Isopropyl-2,3,4-trimethoxy-phenyl)-phenyl-methanone (12a). To a solution of **11a** (1.0 g, 3.2 mmol) in CH_2Cl_2 (30 mL) was added Dess-Martin periodinane (2.0, 4.7 mmol) at 0 ^{o}C. After stirring for 30 min, the reaction mixture was filtered through Celite, washed with saturated $Na_2S_2O_3$ solution, followed by $NaHCO_3$ solution and brine. The organic phase was dried over Na_2SO_4 and concentrated. Chromatography purification of residue gave 0.8 of **12a** with 80% yield. ^{1}H NMR ($CDCl_3$) δ 7.83 (m, 2H), 7.57 (m, 1H), 7.48-7.43 (m, 2H), 6.98 (s, 1H), 3.95 (s, 3H), 3.91 (s, 3H), 3.71 (s, 3H), 3.28 (hept, *J* = 6.9 Hz, 1H), 1.19 (d, *J* = 6.9 Hz, 6H); ^{13}C NMR ($CDCl_3$) δ 196.09, 153.66, 150.39, 145.90,

138.07, 137.56, 132.89, 129.84, 128.75, 128.22, 121.36, 61.76, 61.19, 60.77, 26.93, 23.32.

(5-Isopropyl-2,3,4-trimethoxy-phenyl)-(4-phenoxy-phenyl)-methanone (12b). ^{1}H NMR ($CDCl_3$) δ 7.81 (m, 2H), 7.39 (m, 2H), 7.19 (m, 1H), 7.07 (m, 2H), 6.98 (m, 2H), 6.94 (s, 1H), 3.92 (s, 3H), 3.91 (s, 3H), 3.74 (s, 3H), 3.26 (hept, *J* = 6.9 Hz, 1H), 1.18 (d, *J* = 6.9 Hz, 6H); ^{13}C NMR ($CDCl_3$) δ 194.80, 162.16, 155.58, 153.51, 150.18, 146.07, 137.78, 132.53, 132.45, 130.20, 129.14, 124.77, 121.15, 120.40, 117.14, 62.04, 61.34, 60.95, 27.08, 23.50.

Phenyl-(2,3,4-trihydroxy-5-isopropyl-phenyl)-methanone (5). A CH_2Cl_2 solution of BBr_3 (1M, 6.0 mL) was added dropwise to a stirring solution of **12a** (314 mg, 1.0 mmol) in 20 mL of anhydrous CH_2Cl_2 at -78 ^{o}C. The mixture was allowed to warm up and stirred at -10 ^{o}C for 1h. 2 mL of methanol was added into the flask at -78 ^{o}C to quench the reaction. The solution was washed with water and brine, dried over Na_2SO_4, and concentrated. The residue was purified by chromatography (Hexane/EtOAc, 2:1) to give **5** (220 mg, 81% yield). ^{1}H NMR ($CDCl_3$) δ 12.63 (s, 1H), 7.67 (d, *J* = 7.0 Hz, 2H), 7.60-7.47 (m, 3H), 7.06 (s, 1H), 6.47 (br, 1H), 6.22 (br, 1H), 3.19 (hept, *J* = 6.8 Hz, 1H), 1.15 (d, *J* = 6.8 Hz, 6H); ^{13}C NMR ($CDCl_3$) δ 200.58, 149.39, 148.39, 138.03, 131.65, 130.80, 129.10, 128.21, 126.68, 122.90, 112.42, 26.85, 22.35; ESI (Na^+ added): calcd. for $C_{16}H_{16}O_4Na^+$: 295.0946; Found: 295.0948; EA calcd C, 70.57, H, 5.92; found C, 70.12, H, 5.81.

(4-Phenoxy-phenyl)-(2,3,4-trihydroxy-5-isopropyl-phenyl)-methanone (6). ^{1}H NMR ($CDCl_3$) δ 12.60 (s, 1H), 7.69 (d, *J* = 8.7 Hz, 2H), 7.43-7.38 (m, 2H), 7.24-7.17 (m, 1H), 7.12-7.04 (m, 5H), 6.47 (br, 1H), 6.24 (br, 1H), 3.21 (hept, *J* = 6.9 Hz, 1H), 1.17 (d, *J* = 6.9 Hz, 6H); ^{13}C NMR ($CDCl_3$) δ 199.34, 161.22, 155.68, 149.44, 148.40, 132.50, 131.72, 131.06, 130.23, 126.79, 124.74, 122.77, 120.36, 117.29, 112.64, 27.06, 22.60. ESI (Na^+ added) calcd. for $C_{22}H_{20}O_5Na^+$: 387.1208; Found: 387.1209; EA calcd C, 72.51, H, 5.53; found C, 72.44, H, 5.29.

5-Bromo-2,3,4-trimethoxy-benzaldehyde (13). ^{1}H NMR ($CDCl_3$) δ 10.23 (s, 1H), 7.79 (s, 1H), 4.04 (s, 3H), 4.00 (s, 3H), 3.93(s, 3H); ^{13}C NMR ($CDCl_3$) δ 187.74, 156.88, 156.60, 146.99, 126.46, 126.00, 112.48, 62.44, 61.20, 61.17

5-[1,3]Dioxolan-2-yl-2,3,4-trimethoxy-benzaldehyde (14). A solution of **9** (5.50 g, 20.0 mmol), ethylene glycol (1.86 g, 30 mmol), and TsOH (0.1 g) in 100 mL of toluene was refluxed overnight in a flask equipped with Dean-Stark water separator. The mixture was concentrated and the residue was partitioned between water and EtOAc. The organic phase was washed with water (2X) and brine, dried over Na_2SO_4 and evaporated. The crude product and 20 mL of HMPA were dissolved in 100 mL of anhydrous THF. To this solution was added *n*-BuLi (2M in cyclohexane, 12.0 mL, 24.0 mmol) dropwise at -78 ^{o}C. After stirring at this temperature for 40min, 2.5 mL of DMF was added into the solution. The mixture was gradually warmed up to 0 ^{o}C over 4h, before quenched with aqueous NH_4Cl solution and extracted with EtOAc. The combined organic phase was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The crude product was purified by flash chromatography with hexane/EtOAc (3:1) to give 4.35 g of **14** as liquid (81% yield). ^{1}H NMR ($CDCl_3$) δ 10.28 (s, 1H), 7.79 (s, 1H), 6.05 (s, 1H), 4.15-4.12 (m, 2H), 4.08-4.03 (m, 8H), 3.91 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 188.80, 158.19, 158.17, 145.60, 127.54, 125.06, 121.53, 99.12, 65.42, 62.38, 61.58, 60.95.

(5-[1,3]Dioxolan-2-yl-2,3,4-trimethoxy-phenyl)-(4-phenoxy-phenyl)-methanol (15). ^{1}H NMR ($CDCl_3$) δ 7.35-7.29 (m, 5H), 7.08 (t, J = 7.3 Hz, 1H), 6.99-6.95 (m, 4H), 6.04 (s, 1H), 5.92 (d, J = 5.9 Hz, 1H), 4.15-4.08 (m, 2H), 4.07-3.99 (m, 2H), 3.92 (s, 3H), 3.87 (s, 3H), 3.64 (s, 3H), 2.83 (d, J = 5.9 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 157.30, 156.24, 152.28, 151.93, 146.06, 138.80, 132.59, 129.69, 127.99, 126.39, 123.13, 119.74, 118.73, 118.66, 99.59, 72.12, 65.33, 61.57, 60.65, 60.60.

5-[Hydroxy-(4-phenoxy-phenyl)-methyl]-2,3,4-trimethoxy-benzaldehyde (16). 150 mg of TsOH was added into a solution of **11** (2.0 g, 4.6 mmol) in 20 mL of acetone. After stirred at room temperature for 30min, the reaction mixture was poured into saturated $NaHCO_3$ solution. The aqueous phase was extracted with EtOAc. The combined organic

phase was washed with water (2X) and brine, dried over Na_2SO_4 and concentrated. Chromatography (hexane/EtOAc, 2:1) gave 1.55 g of **16** with a yield of 86%. ^{1}H NMR ($CDCl_3$) δ 10.29 (s, 1H), 7.71 (s, 1H), 7.36-7.30 (m, 4H), 7.10 (t, *J* = 7.3 Hz, 1H), 7.00 (d, *J* = 7.6 Hz, 2H), 6.97 (d, *J* = 8.6 Hz, 2H), 5.94 (d, *J* = 5.7 Hz, 1H), 4.02 (s, 3H), 3.88 (s, 3H), 3.76 (s, 3H), 2.63 (d, *J* = 5.7 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 188.90, 157.30, 157.04, 156.86, 156.66, 145.65, 138.08, 133.29, 129.75, 127.93, 124.95, 123.35, 121.39, 118.90, 118.68, 71.64, 62.42, 60.83, 60.78.

2-Methyl-1-[2,3,4-trimethoxy-5-(4-phenoxy-benzoyl)-phenyl]-propan-1-one (18a). ^{1}H NMR ($CDCl_3$) δ 7.80 (m, 2H), 7.40 (m, 2H), 7.28 (s, 1H), 7.21 (m, 1H), 7.10-7.07 (m, 2H), 7.01-6.98 (m, 2H), 4.01 (s, 3H), 3.92 (s, 3H), 3.84 (s, 3H), 3.38 (hept, *J* = 6.9 Hz, 1H), 1.16 (d, *J* = 6.9 Hz, 6H); ^{13}C NMR ($CDCl_3$) δ 206.11, 193.33, 162.32, 155.29, 154.98, 154.67, 145.98, 132.28, 131.79, 130.07, 128.92, 128.72, 124.72, 124.38, 120.30, 117.08, 61.84, 61.83, 61.00, 39.84, 18.67.

(4-Phenoxy-phenyl)-[2,3,4-trimethoxy-5-(naphthalene-1-carbonyl)-phenyl]-methanone (18b). ^{1}H NMR ($CDCl_3$) δ 8.26 (s, 1H), 7.98 (dd, *J1* = 8.6 Hz, *J2* = 1.5 Hz, 1H), 7.92-7.88 (m, 3H), 7.84 (d, *J* = 8.8 Hz, 2H), 7.61 (m, 1H), 7.54 (m, 1H), 7.39 (t, *J* = 7.8 Hz, 2H), 7.20 (m, 1H), 7.19 (s, 1H), 7.07 (d, *J* = 7.7 Hz, 2H), 6.99 (d, *J* = 8.8 Hz, 2H), 3.97 (s, 3H), 3.91 (s, 3H), 3.82 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 194.75, 193.28, 162.37, 155.26, 154.56, 146.13, 135.70, 134.82, 132.36, 132.27, 131.74, 130.07, 129.66, 128.76, 128.66, 128.37, 127.82, 126.81, 124.91, 124.74, 124.38, 120.30, 117.08, 62.02, 61.91, 61.14.

[5-(Benzothiazole-2-carbonyl)-2,3,4-trimethoxy-phenyl]-(4-phenoxy-phenyl)-methanone (18c). ^{1}H NMR ($CDCl_3$) δ 8.14-8.11 (m, 1H), 8.02-7.99 (m, 1H), 7.90 (d, *J* = 8.8 Hz, 2H), 7.61 (s, 1H), 7.56-7.53 (m, 2H), 7.41 (t, *J* = 7.8 Hz, 2H), 7.21 (t, *J* = 7.4 Hz, 1H), 7.08 (d, *J* = 7.6 Hz, 2H), 7.01 (d, *J* = 8.8 Hz, 2H), 3.97 (s, 3H), 3.94 (s, 3H), 3.93 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 192.73, 186.28, 166.87, 162.30, 156.26, 156.04, 155.29, 153.48, 146.30, 137.19, 132.45, 131.77, 130.07, 128.25, 127.67, 127.00, 126.70, 125.73,

125.50, 124.73, 122.35, 120.29, 117.06, 62.01, 60.99; ESI (H^+ added) calcd. for $C_{30}H_{23}NO_6SH^+$: 526.1324; Found: 526.1313.

2-Methyl-1-[2,3,4-trihydroxy-5-(4-phenoxy-benzoyl)-phenyl]-propan-1-one (7). ^{1}H NMR ($CDCl_3$) δ 13.34 (s, 1H), 12.70 (s, 1H), 7.84 (s, 1H), 7.70 (d, *J* = 8.7 Hz, 2H), 7.44 (m, 2H), 7.24 (m, 1H), 7.15-7.08 (m, 4H), 5.59 (br, 1H), 3.34 (hept, *J* = 6.8 Hz, 1H), 1.20 (d, *J* = 6.8 Hz, 6H); ^{13}C NMR ($CDCl_3$) δ 209.68, 198.78, 161.68, 155.38, 155.15, 154.86, 132.90, 131.56, 131.39, 130.12, 128.33, 124.84, 120.23, 117.22, 112.34, 111.14, 34.84, 19.30; ESI (H^+ added) calcd. for $C_{23}H_{20}O_6H^+$: 393.1338; Found: 393.1349; EA calcd C, 70.40; H, 5.14; found C, 70.11, H, 5.20.

(4-Phenoxy-phenyl)-[2,3,4-trihydroxy-5-(naphthalene-1-carbonyl)-phenyl]-methanone (8). ^{1}H NMR ($CDCl_3$) δ 12.95 (s, 1H), 12.89 (s, 1H), 8.12 (s, 1H), 7.87 (m, 3H), 7.76 (s, 1H), 7.70 (dd, *J1* = 8.5 Hz, *J2* = 1.3 Hz, 1H), 7.64-7.62 (m, 3H), 7.48 (t, *J* = 6.9 Hz, 1H), 7.34 (m, 2H), 7.22 (m, 1H), 6.88-6.85 (m, 4H), 5.84 (b, 1H); ^{13}C NMR ($CDCl_3$) δ 200.12, 198.80, 161.53, 155.49, 155.35, 155.02, 134.91, 134.30, 133.07, 132.64, 131.95, 131.32, 131.08, 130.45, 130.10, 128.84, 128.52, 128.48, 127.88, 127.28, 125.00, 124.75, 120.26, 116.97, 112.43, 112.32. ESI (H^+ added) calcd. for $C_{30}H_{20}O_6H^+$: 477.1338; Found: 477.1341; EA calcd C, 75.62; H, 4.23; found C, 75.58, H, 4.33.

[5-(Benzothiazole-2-carbonyl)-2,3,4-trihydroxy-phenyl]-(4-phenoxy-phenyl)-methanone (9). Two rotamers exit in $CDCl_3$. From 1: ^{1}H NMR ($CDCl_3$) δ13.16 (s, 1H), 12.97 (s, 1H), 9.68 (s, 1H), 8.02(d, *J* = 7.2 Hz, 1H), 7.90 (d, *J* = 8.6 Hz, 3H), 7.62-7.42 (m, 4H), 7.17 (d, *J* = 8.6 Hz, 3H), 7.03 (d, *J* = 8.7 Hz, 2H), 5.60 (s, 1H); From 2: ^{1}H NMR ($CDCl_3$) δ13.20 (s, 1H), 12.94 (s, 1H), 9.72 (s, 1H), 8.00 (dd, *J1* = 6.3 Hz, *J2* = 2.4 Hz, 1H), 7.93-7.87 (m, 3H), 7.58-7.52 (m, 2H), 7.45 (t, *J* = 7.8 Hz, 2H), 7.26 (m, 1H), 7.16 (m, 3H), 7.03 (m, 1H), 5.70 (br, 1H); ^{13}C NMR ($CDCl_3$) δ199.11, 185.50, 166.90, 161.76, 156.34, 155.77, 155.39, 153.38, 136.66, 134.05, 132.74, 132.18, 131.48, 130.22, 128.01, 127.24, 125.27, 124.87, 122.22, 120.38, 117.22, 113.26, 111.20; EA calcd 67.07; H, 3.54; N, 2.90; found C, 66.91, H, 3.51, N, 2.82.

II. Competitive binding assays for Bcl-2, Mcl-1 and Bcl-xL proteins

Protein expression and purification

Human Bcl-2 protein. The isoform 2 construct of the human Bcl-2 with an N-terminal 6xHis tag was used. Bcl-2 protein was produced in *E. coli* BL21(DE3) cells. Cells were grown at 37°C in 2xYT containing antibiotics to an OD_{600} of 0.6. Protein expression was induced by 0.4 mM IPTG at 20°C for 20 h. Cells were lysed in 50mM Tris pH 8.0 buffer containing 500 mM NaCl, 0.1%bME and 40 μl of Leupectin/Aprotin. The protein was purified from the soluble fraction using Ni-NTA resin (QIAGEN), following the manufacturer's instructions. The protein was further purified on a Superdex75 column (Amersham Biosciences) in 25mM Tris pH 8.0 buffer containing 150 mM NaCl and 2 mM DTT.

Human Bcl-xL protein. Human Bcl-xL protein, which has an internal deletion for the 45-85 amino acid residues and a C-terminal truncation for the amino acid residues 212-233, was cloned into the pHis-TEV vector (a modified pET vector). Protein was produced in *E. coli* BL21(DE3) cells with an N-terminal 8xHis tag. Cells were grown at 37°C in 2xYT containing antibiotics to an OD_{600} of 0.6. Protein expression was induced by 0.4 mM IPTG at 25°C for 16 h. Cells were lysed in 50mM Tris, pH7.5 buffer containing 200mM NaCl, 0.1%bME and Leupectin/Aprotin. His-TEV-Bcl-xL protein was purified from the soluble fraction using Ni-NTA resin (QIAGEN), following the manufacturer's instructions. The His-TEV-Bcl-xL protein was further purified on a Superdex75 column (Amersham Biosciences) in 20mM Tris pH7.5 buffer containing 150mM NaCl and 5mM DTT.

Human Mcl-1 protein. Human Mcl-1 cDNA was purchased from Origene. The Mcl-1 fragment, amino acid residues 171-327, was cloned into the pHis-TEV vector (a modified pET vector) through BamHI and EcoRI sites, using the oligonucleotides: 5'-CGGGATCCGAGGACGAGTTGTACCG-GCAG-3' and 5'-GGAATTCCTAGCCACCTTCTAGGTCCTCTAC-3'. Mcl-1 protein with a N-terminal 8xHis tag was produced in *E. coli* BL21(DE3) cells. Cells were grown at 37°C in 2xYT

containing antibiotics to an OD_{600} of 0.6. Protein expression was induced by 0.4 mM IPTG at 37°C for 4 h. Cells were lysed in 50 mM Tris pH 8.0 buffer containing 500 mM NaCl, 0.1%bME and 40 μl of Leupectin/Aprotin. Mcl-1 protein was purified from the soluble fraction using Ni-NTA resin (QIAGEN), following the manufacturer's instructions. The protein was further purified on a Source Q15 column (resin and column are from Amersham Biosciences) in 25 mM Tris pH 8.0 buffer, with NaCl gradient.

An enzyme-linked immunosorbent assay (ELISA) for Bcl-2 protein

Biotinylated Bim peptide (residues 81-106, biotin-(β)A-(β)ADMRPEIWIAQELRRIGDEFNAYYARR-amide, (hereafter called biotin-Bim) was diluted to 0.09 μg/ml in SuperBlock blocking buffer in PBS (Pierce Biotechnology, Inc, Rockford, IL, catalog # 37515) and incubated for 1.5 h in 96-well microtiter plates previously coated with streptavidin (Qiagen, Catalog # 15500) to allow the formation of the complex between Biotin-Bim and streptavidin. All incubations were performed at r.t. unless otherwise noted. Each test compound was first dissolved in pure DMSO to obtain a 20 mM stock solution. For each compound, different concentrations were incubated with 20 nM His-tagged Mcl-1 protein in PBS for 1 h with a final DMSO concentration of 4%. The plates were washed three times with PBS containing 0.05% Tween-20. The inhibitor and protein mixture (100 μl) were transferred to the plate containing the biotin-Bim/streptavidin complex and incubated for 2 hours. The plate was then washed as before and mouse anti-His antibody that conjugated with horseradish peroxidase (Qiagen, Catalog # 34460) was added into the wells and incubated for 1 h. The plate was then washed with PBS containing 0.05% Tween-20. o-phenylenediamine (20 μM, Sigma, Catalog #P5412) in phosphate-citrate buffer (0.2M Na_2HPO_4, 0.1M citric acid, pH 5.0) was used to detect the enzymatic activity of the horseradish peroxidase. The reaction was followed kinetically at 450 nm for 15 min on a spectrophotometer (Molecular Devices-VersaMAX) and Vmax was generated by SoftMax Pro (Molecular Devices). Vmax values were plotted against inhibitor concentrations and IC_{50} values and Hill slopes were calculated using GraphPad Prizm 4 software (GraphPad Software, San Diego, CA). Three to five independent experiments were performed with each inhibitor to calculate average IC_{50} value and standard deviation (SD).

Fluorescence polarization-based binding assay for Mcl-1 protein

FAM-Bid peptide and human Mcl-1 protein were used. It was determined that FAM-Bid peptide binds to human Mcl-1 protein with a K_d value of 1.71 nM. The competitive binding assays for Mcl-1 were performed in the same manner as that for Bcl-2 with the following exceptions: 5 nM Mcl-1 and 1 nM FAM-Bid peptide in an assay buffer of 25 mM Tris, pH 8.0; 150 mM NaCl and 0.05% Pluronic acid 68.

Fluorescence polarization-based binding assay for Bcl-xL protein

For this assay, we have employed the Bak BH3 peptide labeled with 6-carboxyfluorescein succinimidyl ester (FAM-Bak) instead of the FAM-Bim to maximize the signal. It was determined that FAM-Bak has a K_d value of 6 nM to Bcl-xL protein. The competitive binding assay for Bcl-xL was same as that for Bcl-2 with the following exceptions. 30 nM of Bcl-xL protein and 2.5 nM of FAM-Bak peptide were used in the assay buffer, which consisted of 50 mM Tris-Bis, pH 7.4 and 0.01% bovine gamma globulin.

IV. Docking simulations of the designed inhibitors with the Mcl-1 and Bcl-2 proteins

In the binding model prediction between the compounds with Bcl-2, the same protocol used in our previous study was employed.[1]

The three-dimensional structure of Mcl-1 used for docking simulation was taken from the complex structure between human Mcl-1 and mNoxa (PDB entry: 2NLA).[2] Although the complex structure between human Mcl-1 and human Bim was available (PDB entry: 2NL9), one MET was replaced with MSE for crystallographic purpose and the structure of human Mcl-1 is incomplete compared with that in 2NLA. For the purpose of our molecular dynamics (MD) simulations, we used the Mcl-1 structure from 2NLA in our modeling studies.

All the docking simulations were performed using GOLD (version 2.2.).[3] The designed compounds were drawn and energy-minimized using the Sybyl program.[4] The center of the binding site of Mcl-1 was set at Phe228 and the radius of the binding site was defined as 13 Å, large enough to cover the putative binding pocket. For each genetic algorithm (GA) run, a maximum number of 200,000 operations were performed on a population of 5 islands of 100 individuals. Operator weights for crossover, mutation and

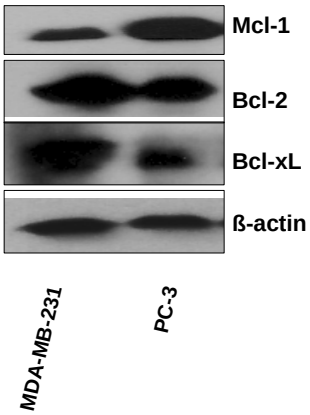
migration were set to 95, 95 and 10 respectively. The docking was terminated after 20 runs for each inhibitor. ChemScore, implemented in the Gold program, was used as the fitness function to evaluate the docked conformations. Twenty highest ranked conformations by the fitness function were saved as predicted docking modes for analysis. The highest ranked docked conformations of the compounds **4**-**9** were shown in Figure 3 in the main body of the manuscript as the predicted binding models.

V. Western blot analysis and biological assays

Western blot analysis

The levels of Bcl-2, Bcl-xL and Mcl-1 proteins were probed with Western blot using specific antibody against Bcl-2, Bcl-xL and Mcl-1 proteins. The protein levels of Bcl-2, Bcl-xL and Mcl-1 in the MDA-MB-231 (2LMP) human breast cancer cell line and the PC-3 human prostate cancer cell lines are shown in ***Figure S1***.

Figure S1. Western blot analysis of Bcl-2, Bcl-xL, Mcl-1 proteins in the MDA-MB-231 (2LMP) breast cancer and PC-3 prostate cancer cell lines.



Trypan blue exclusion assay for cell viability

Trypan blue exclusion assay was used for determination of cell viability. Cells were treated with compound 5 and 9 for different time points. Cell viability was determined using the Trypan blue exclusion assay. Blue cells or morphologically unhealthy cells were scored as dead cells. At least 50 cells from each treatment, performed in triplicate, were counted. The data obtained with treatment of compounds **5** and **9** for 48 and 96

hours in the MDA-MB-231 (2LMP) and PC-3 human cancer cell lines are shown in ***Figures S2*** and ***S3***, respectively.

Figure S2. Induction of cell death by compounds **5** and **9** in the MDA-MB-231 (2LMP) breast cancer cell line.

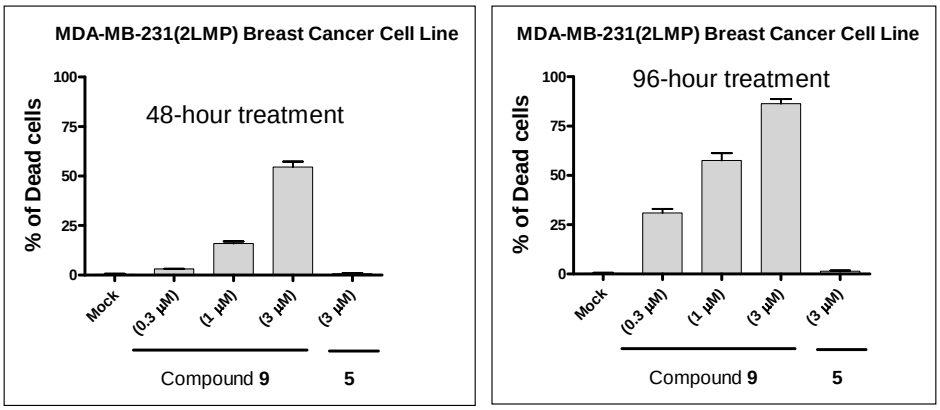
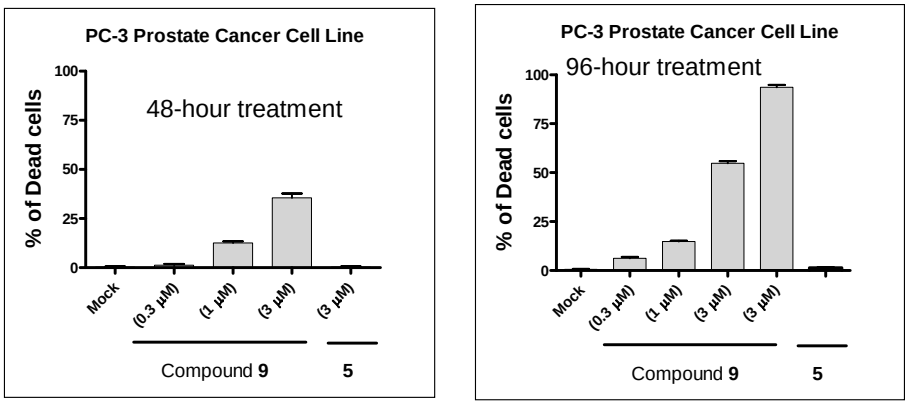


Figure S3. Induction of cell death by compounds **5** and **9** in the PC-3 human prostate cancer cell line.



WST-8 cell growth assay

Cells were seeded in 96-well flat bottom cell culture plates at a density of 3-4×10^3 cells/well with compounds and incubated for 4 days. The rate of cell growth inhibition after treatment with different concentrations of the inhibitors was determined by WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (Dojindo Molecular Technologies Inc., Gaithersburg, Maryland). WST-8 was added at a final concentration of 10% to each well, and then the plates were incubated at 37°C for 2-3 hrs. The absorbance of the samples was measured at 450 nm

using a TECAN ULTRA Reader. Concentration of the compounds that inhibited cell growth by 50% (IC_{50}) was calculated by comparing absorbance in the untreated cells and the cells treated with the compounds.

Annexin-V/propidium iodide Assay

Apoptosis analysis was performed using an annexin-V/propidium iodide (PI) apoptosis detection kit (Roche, Indianapolis, IN) according to manufacturer's instructions. Briefly, **c**ells treated with inhibitors at different concentrations and time-points. Cells were harvested, washed with ice-cold PBS and then stained with annexin-V-FITC and PI for 15 minutes at room temperature in the dark. Stained cells were analyzed in a FACS calibur flow cytometer. Annexin-V (+) and PI (−) cells were considered early-stage apoptotic cells, while annexin V (+) and PI (+) cells were measured as late-stage apoptotic cells.

References

1. Wang, G.; Nikolovska-Coleska, Z.; Yang, C.-Y.; Wang, R.; Tang, G.; Guo, J.; Shangary, S.; Qiu, S.; Gao, W.; Yang, D.; Meagher, J.; Stuckey, J.; Krajewski, K.; Jiang, S.; Roller, P. P.; Abaan, H. O.; Tomita, Y.; Wang, S. Structure-Based Design of Potent Small-Molecule Inhibitors of Anti-Apoptotic Bcl-2 Proteins *J. Med. Chem.*, **2006**, *49*, 6139-6142.
2. Berman, H. M.; Westbrook, J.; Feng, Z.; Gilliland, G; Bhat, T. N.; Weissig, H.; Shindyalov, I. N.; Bourne, P. E. The Protein Data Bank. *Nucleic Acids Res.* **2002,** *28*, 235-242. (www.rcsb.org/pdb/).
3. Jones, G.; Willett, P.; Glen, R. C.; Leach, A. R.; Talylor, R., Development and validation of a genetic algorithm for flexible docking. *J. Mol. Biol.* **1997**, 267, 727-748.
4. Sybyl, a molecular modeling system, is supplied by Tripos, Inc., St. Louis, MO 63144.