

Inhibitory Effects of Cancer Cell Proliferation by Novel Histone Deacetylase Inhibitors Involve p21/WAF1 Induction and G₂/M Arrest

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Two compounds were synthesized which have a structural component other than those of our new series histone deacetylase (HDAC) inhibitors to determine the structure–activity relationship. It was also examined whether the inhibitory effects on cancer cell proliferation by HDAC inhibitors involve p21/WAF1 induction and G₁ or G₂/M arrest in p53-mutated MG63 human osteosarcoma cells as do other HDAC inhibitors. It was demonstrated that inhibitors with the 2-naphthylcarbonyl group and hydroxamic acid at both terminal sides as well as the phenylene component at the center of molecule markedly induce the p21/WAF1 protein by stimulating p21/WAF1 gene promoter activity. Furthermore, cell cycle analysis revealed that these compounds arrest MG63 cells in the G₂/M phase.

Key words histone deacetylase inhibitor; p21/WAF1; MG63 cell; HCT116 cell; G₂/M phase; cell cycle arrest

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) play a crucial role in gene expression through reversible acetylation and deacetylation of histones. HAT-mediated hyperacetylation of lysine residues in the N-terminal tails of core histones loosens the histone–DNA binding and activates gene transcription. In contrast, HDAC-catalyzed deacetylation of acetylated lysine residues leads to tight histone–DNA binding, which restricts the access of transcriptional factors.^{1–3} These enzymes are known to correlate with cell cycle progression, and their deregulation is associated with tumorigenesis.^{4–6} HDAC inhibitors appear to be attractive new antitumor candidates because they are expected to suppress the cell cycle progression of human tumor cells and cause apoptosis by inducing the expression of cell cycle-arresting genes such as p21/WAF1⁷ and GADD45.⁸ Recently, X-ray crystallographic analysis has disclosed the interaction mode between the HDAC-like protein (HDLP) from the bacterium *Aquifex aeolicus* and the natural HDAC inhibitor TSA from actinomyces; TSA binds inside the pocket of HDLP, making tight contacts with residues at the rim, walls, and bottom of the pocket. The zinc ion near the bottom is coordinated by the hydroxamic acid of TSA.⁹ For the design of a new series HDAC inhibitors, we chose a benzyl component for interaction with the walls of a HDAC pocket, a bicyclic arylcarbonyl component for capping the pocket, and the hydroxamic acid for capture of the zinc ion at the bottom. Among the synthesized HDAC inhibitors, compound 1 showed promising inhibitory efficacies against the proliferation of HCT116 human colorectal carcinoma cells (IC₅₀ 0.7 μM) and HDACs activities (IC₅₀ 0.044 μM) (Table 1).¹⁰ Further improvement of the pharmacokinetic profile afforded the nitrogen-introduced compounds 2–4. These compounds were improved in water solubility. Compound 2 exhibited inhibitory activities stronger than those of suberoylanilide hydroxamic acid (SAHA) against a panel of cancer cells as well as the maximal 185% T/C of survival rate in the experiments with P388 cell-inoculated mice.¹¹

In the present study, we synthesized two derivatives 5 and 6 with a structural component (2,6-naphthylene or thiol

group) other than those of the HDAC inhibitors 1–4 to determine whether these groups can be substitutes for phenylene and hydroxamic acid groups in 1–4, making contacts with the walls and zinc ion of the HDAC pocket, respectively. We also examined whether the inhibitory effects on cancer cell proliferation by the novel histone deacetylase inhibitors involve p21/WAF1 induction and G₁ or G₂/M arrest as do other HDAC inhibitors through assessment of Western blot, luciferase, and cell cycle progression assays.

Chemistry Compounds 5 and 6 were synthesized in the following way (Chart 1): Condensation of methyl 6-amino-2-naphthoate hydrochloride and phenylacetyl chloride in the presence of Et₃N gave 5a, which, after conversion to carboxylic acid 5b, was reacted with BOP-Cl and *O*-benzylhydroxylamine hydrochloride to give 5c. It was then hydrogenated over 10% Pd-C, yielding the desired 5. Condensation

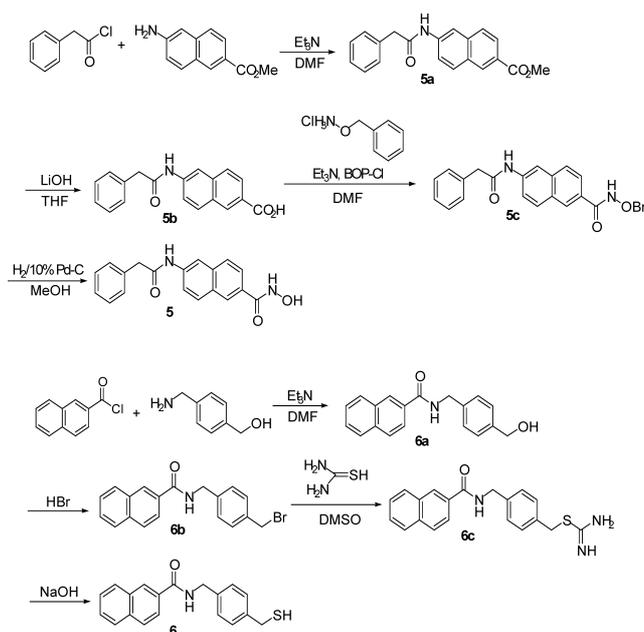


Chart 1. Synthetic Pathways of 5 and 6

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of [4-(aminomethyl)phenyl]methanol and phenylacetyl chloride in the presence of Et_3N gave **6a**, which was treated with HBr to give benzyl bromide **6b**. Replacement reaction of bromine (**6b**) with thio-ureido (**6c**) followed by alkaline treatment yielded the desired **6**.

MATERIAL AND METHODS

Melting points were determined on a Yanagimoto MP-32 micromelting point apparatus and are uncorrected. IR spectra were recorded on Shimadzu FTIR-8400 infrared spectrophotometer. Low-resolution (LR)-FAB-MS spectra were measured on a JEOL JMS-HX 100 instrument, whereas high-resolution (HR)- and LR-electron impact (EI)-MS spectra were measured on a JEOL The Tandem MStation JMS-700. $^1\text{H-NMR}$ spectra were recorded on JEOL EX-270 (270 MHz) and JEOL EX-400 (400 MHz) instruments using tetramethylsilane as an internal standard. Analytical TLC and PLC were performed using Silica gel 60 F_{254} (Merck, 0.25 and 0.5 mm, respectively) glass plates. Column chromatography was performed using Silica Gel 60 (70–230 mesh ASTM). TSA was purchased from Sigma-Aldrich Co.

Methyl 6-[(Phenylacetyl)amino]-2-naphthoate (5a) To a suspension of methyl 6-amino-2-naphthoate hydrochloride (3.0 g, 14.9 mmol) in absolute DMF (60 ml) were added Et_3N (8.3 ml, 60.0 mmol) and phenylacetyl chloride (2.16 ml, 16.4 mmol). The mixture was stirred at room temperature overnight. After being evaporated, the residue was dissolved in CHCl_3 (200 ml) and washed successively with 1 N HCl (3 × 50 ml), saturated NaHCO_3 (3 × 50 ml), and brine (3 × 50 ml). The organic layer was dried and concentrated to dryness. The resulting residue was recrystallized from CHCl_3 -*n*-hexane to give the pure compound **5a** (1.3 g, 4.3 mmol, 30.0% yield). mp 177.5–180.9 °C. IR (KBr) cm^{-1} : 3031, 1714, 1656, 1584, 1238, 1097, 775, 545. $^1\text{H-NMR}$ (CDCl_3) δ : 3.38 (2H, d, $J=5.9$ Hz, PhCH_2), 3.95 (3H, s, CO_2CH_3), 7.33–8.20 (11H, m, arom. H_{11}), 8.50 (1H, s, CH_2CONH); EI-MS m/z : 319 (M^+), 201, 170, 91; HR-EI-MS m/z : 319.1207 (Calcd for $\text{C}_{20}\text{H}_{17}\text{NO}_3$: 319.1208).

6-[(Phenylacetyl)amino]-2-naphthoic Acid (5b) LiOH 1 M (7.8 ml, 7.8 mmol) was added to a solution of **5a** (1.1 g, 3.5 mmol) in a mixture of water (0.5 ml) and THF (15.1 ml), and the mixture was stirred at room temperature overnight. After evaporation of THF, the residual water solution was adjusted to pH 3 with 1 N HCl. The resulting precipitate was filtered off and washed with water to give the pure compound **5b** (1.0 g, 3.3 mmol, 94% yield). mp 361.1–363.5 °C. IR (KBr) cm^{-1} : 3031, 1651, 1611, 1546, 1392, 1261, 925, 786. $^1\text{H-NMR}$ (CD_3OD) δ : 4.45 (2H, s, PhCH_2), 7.94–8.98 (11H, m, arom. H_{11}). EI-MS m/z : 305 (M^+), 187, 91, 44; HR-EI-MS m/z : 305.1025 (Calcd for $\text{C}_{19}\text{H}_{15}\text{NO}_3$: 305.1025).

***N*-(Benzyloxy)-6-[(phenylacetyl)amino]-2-naphthamide (5c)** To a suspension of *O*-benzylhydroxylamine hydrochloride (0.31 g, 1.97 mmol) in absolute DMF (8.2 ml) were added **5b** (0.50 g, 1.6 mmol), BOP-Cl (0.50 g, 1.96 mmol), and Et_3N (0.67 ml, 4.8 mmol). The mixture was stirred at room temperature overnight. After being evaporated, the residue was dissolved in CHCl_3 (100 ml) and washed successively with 1 N HCl (3 × 20 ml), saturated NaHCO_3 (3 × 20 ml), and brine (3 × 20 ml). The organic layer was dried over Na_2SO_4 and concentrated. The resulting

residue was recrystallized from EtOAc to give the pure compound **5c** (0.15 g, 0.37 mmol, 23% yield). mp 229.6–232.1 °C. IR (KBr) cm^{-1} : 3292, 1643, 1537, 1313, 1228, 1028, 812, 696. $^1\text{H-NMR}$ ($\text{DMSO}-d_6$) δ : 3.31 (2H, d, $J=5.9$ Hz, PhCH_2), 4.95 (2H, s, OCH_2), 7.22–8.36 (16H, m, arom. H_{16}), 10.55 (1H, t, $J=5.9$ Hz, NHOH), 11.85 (1H, s, CH_2CONH). EI-MS m/z : 410 (M^+), 395, 304, 186, 91. HR-EI-MS m/z : 410.1640 (Calcd for $\text{C}_{25}\text{H}_{22}\text{N}_2\text{O}_3$: 410.1630).

***N*-(Hydroxy)-6-[(phenylacetyl)amino]-2-naphthamide (5)** Ten percent Pd-C (77 mg) was added to a solution of **5c** (0.10 g, 0.24 mmol) in MeOH (30 ml), and the mixture was stirred under a H_2 atmosphere overnight. The catalyst was removed by filtration and washed successively with MeOH and CHCl_3 . The combined filtrate was evaporated to give pure compound **5** (67.4 mg, 0.21 mmol, 87.5% yield). mp 230.0–230.5 °C (dec.). IR (KBr) cm^{-1} : 3030, 1651, 1554, 1023, 898, 771. $^1\text{H-NMR}$ ($\text{DMSO}-d_6$) δ : 3.69 (2H, d, $J=5.9$ Hz, CH_2), 7.22–8.34 (11H, m, arom. H_{11}), 9.00 (1H, t, $J=5.9$ Hz, NHOH), 10.46 (1H, s, CH_2CONH). FAB-MS m/z : 321 ($\text{M}+\text{H}^+$), 277, 185, 93, 75; HR-FAB-MS m/z : 321.1245 (Calcd for $\text{C}_{19}\text{H}_{17}\text{N}_2\text{O}_3$: 321.1239).

***N*-[4-(Hydroxymethyl)benzyl]-2-naphthamide (6a)** 2-Naphthoyl chloride (4.73 g, 21.8 mmol) was reacted with [4-(aminomethyl)phenyl]methanol (3.02 g, 22.0 mmol) in the presence of Et_3N (9.0 ml, 88.3 mmol) in the same way as for **5a**, yielding **6a** (3.86 g, 13.2 mmol, 60.2% yield). mp 163.0–164.0 °C. IR (KBr) cm^{-1} : 2886, 1544, 954, 837, 781, 482. $^1\text{H-NMR}$ (CD_3OD) δ : 4.72 (2H, s, NHCH_2Phe), 4.80 (2H, s, PheCH_2OH), 6.54 (2H, m, arom. H_2), 7.36–7.39 (3H, m, arom. H_3), 7.50–7.64 (2H, m, arom. H_2), 7.82–7.86 (2H, m, arom. H_2), 7.88–7.92 (2H, m, arom. H_2). FAB-MS m/z : 292 ($\text{M}+\text{H}^+$), 277, 185. HR-FAB-MS m/z : 292.1340 (Calcd for $\text{C}_{19}\text{H}_{18}\text{NO}_2$: 292.1338).

***N*-[4-(Bromomethyl)benzyl]-2-naphthamide (6b)** A mixture of **6a** in 49% aqueous HBr was heated at 90 °C for 10 min. After cooling, the resulting precipitate was filtered off and washed with water to give pure compound **6b** (1.72 g, 4.9 mmol, 90.6% yield). mp 171.2–177.0 °C (dec.). IR (KBr) cm^{-1} : 1633, 1548, 1510, 1232, 871, 838, 742. $^1\text{H-NMR}$ ($\text{DMSO}-d_6$) δ : 4.46 (2H, s, NHCH_2), 4.56 (2H, s, CH_2Br), 6.90 (4H, m, arom. H_4), 7.3–8.4 (7H, m, arom. H_7). FAB-MS m/z : 354 ($\text{M}+\text{H}^+$), 297, 185, 171, 157, 155. HR-FAB-MS m/z : 354.0521 (Calcd for $\text{C}_{19}\text{H}_{17}\text{BrNO}$: 354.0494).

4-[(2-Naphthoylamino)methyl]benzyl Imidothiocarbamate (6c) To a suspension of **6b** in DMSO (10 ml) was added thiourea (123.8 g, 1.63 mmol), and the mixture was stirred at room temperature for 18 h and then lyophilized to remove the solvent. The resulting residue was recrystallized from CHCl_3 to give pure compound **6c** (1.3 g, 4.3 mmol, 30.0% yield). mp 217.3–218.7 °C. IR (KBr) cm^{-1} : 3855, 3845, 3726, 3622, 3008, 1537, 783. $^1\text{H-NMR}$ ($\text{DMSO}-d_6$) δ : 3.51 (2H, s, $\text{CH}_2\text{-S}$), 4.53 (2H, d, $J=5.6$, CH_2Br), 7.3–9.2 (11H, m, arom. H_{11}). FAB-MS m/z : 350 ($\text{M}+\text{H}^+$). HR-FAB-MS m/z : 350.1342 (Calcd for $\text{C}_{20}\text{H}_{20}\text{N}_3\text{OS}$: 350.1327).

***N*-[4-(Sulfanylmethyl)benzyl]-2-naphthamide (6)** A suspension of **6c** in aqueous 6% NaOH was refluxed for 8 h. After cooling, the solution was adjusted to pH 3 with HCl 6 M. The resulting precipitate was filtered off and washed with water to give pure compound **6** (51 mg, 0.17 mmol, 63.4% yield). mp 213.4–216.3 °C. IR (KBr) cm^{-1} : 3303, 1695, 1639, 1546, 1508, 1417, 1355, 1313, 831, 781, 738.

$^1\text{H-NMR}$ ($\text{DMSO-}d_6$) δ : 3.76 (2H, s, CH_2SH), 4.51 (2H, d, $J=6.0\text{ Hz}$, NHCH_2), 7.2–9.3 (11H, m, arom. H_{11}). FAB-MS m/z : 308 ($\text{M}+\text{H}^+$); HR-FAB-MS m/z : 308.1090 (Calcd for $\text{C}_{19}\text{H}_{18}\text{NOS}$: 308.1109).

Evaluation of Histone Deacetylase Inhibitory Activities

Partial purification of HDACs and measurement of inhibitory activities of test compounds against HDACs using [^3H]-acetylated histones were performed according to the procedure by Mori *et al.*¹² IC_{50} values in Table 1 represent the molar concentrations (μM) required to inhibit the HDACs by 50%.

Cell Culture HCT116 cells (purchased from the ATCC) were cultured in McCoy's 5a medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), streptomycin 2.5 $\mu\text{g/ml}$, and penicillin 2.5 units/ml at 37 °C in a humidified atmosphere of 5% CO_2 . Cells were passaged every 2 d into 60-mm dishes by washing twice with phosphate-buffered saline (PBS) and incubated with 0.25% trypsin and 0.03% EDTA for 5 min at 37 °C prior to resuspension in growth medium. MG63 human osteosarcoma cells (kindly provided by Dr. Y. Yanase, Wakayama Medical College) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% FBS, streptomycin 10 $\mu\text{g/ml}$, and penicillin 10 units/ml at 37 °C in a humidified atmosphere of 5% CO_2 . Cells were passaged every 3 d into 100-mm dishes by washing twice with PBS and incubated with 0.05% trypsin and 0.02% EDTA for 3 min at 37 °C prior to resuspension in growth medium.

Evaluation of Growth Inhibition against HCT116 Cells

Cells were plated in 96-well plates at densities of $1.0 \times 10^5/\text{ml}$. On the same day, compounds were added, and the cells were cultured for 2 d. After addition of WST-1 and 1-methoxy-5-methyl-phenazinium methylsulfate, the OD_{450} and OD_{630} were measured in the conventional way.¹¹

Analysis of Cell Cycle Progression Cells were removed from culture dishes by trypsinization and collected by centrifugation. After washing with PBS, cells were suspended in PBS containing 0.1% Triton X-100 to prepare nuclei. After the suspension was filtered through 50-nm nylon mesh, 0.1% RNase and 50 mg/ml propidium iodide were added. DNA contents in stained nuclei were analyzed with FACSCalibur (Becton Dickinson). The suspension of cells was analyzed for each DNA histogram. The number of stained nuclei in each phase was measured according to the Mod FitLT program in the FACSCalibur system.

Plasmid Transfection an Assessment of Luciferase Assay

The human wild-type p21/WAF1 promoter, pWWP-

Luc, was a kind gift from Dr. B. Vogelstein. Transient transfection was performed at 60% confluency in 12-well titer plates with the calcium phosphate technique using Cellfect transfecting agent (Amersham-Pharmacia). Luciferase activities were measured using a luminometer, and were normalized for the amount of the protein in cell lysates. All experiments were carried out in triplicate.

Western Blot Analysis MG63 cells in 100-mm plates were treated with **1–6** at the various concentrations indicated in DMEM for 24 h. After the medium was aspirated, the cells were washed with cold PBS and ice-cold radioimmunoprecipitation assay (RIPA) buffer. Western blot analysis was performed using mouse monoclonal antibody recognizing p21 (PM15091A from Pharmingen) as the primary antibody. The signal was detected with a chemiluminescence system (ECL, Amersham-Pharmacia). Protein concentration was determined using the Bio-Rad Protein Assay Kit.

RESULTS AND DISCUSSION

Table 1 shows the inhibitory activities of **1–6** against the proliferation of HCT116 cells as well as HDACs. Compounds **1–4** had comparatively low IC_{50} values (HCT116, 0.7–3.9 μM ; HDAC, 0.026–0.044 μM). However, replace-

Table 1. Inhibition against HCT116 Cell Growth and HDACs

Compd.	Structure	HCT116	HDACs
		IC_{50} (μM)	IC_{50} (μM)
1		0.7	0.044
2		3.9	0.039
3		3.6	0.043
4		3.8	0.026
5		80.4	7.7
6		85.8	>10
TSA		0.5	0.003

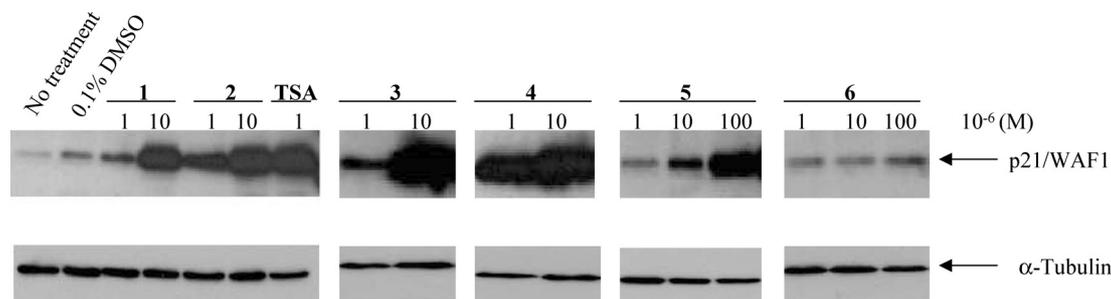


Fig. 1. Compound **1**, **2**, **3**, or **4** Stimulates Endogenous p21/WAF1 Protein Expression

Lysates (30 μg) of MG63 cells exposed to various concentrations of **1**, **2**, **3**, **4**, **5**, or **6** as indicated or 0.1% DMSO for 24 h were examined using SDS-polyacrylamide gel electrophoresis and analyzed with immunoblotting using antibodies for p21/WAF1 and α -tubulin.

ment of the 1,4-phenylene and 2-naphthylcarbonyl group in **1** with the 2,6-naphthylene and phenylcarbonyl group in **5** as well as of hydroxamic acid in **1** with thiol in **6** resulted in much greater IC_{50} values.

We analyzed the effects of **1–6** and TSA on p21/WAF1 protein expression in MG63 cells. As shown in Fig. 1, treatment with **1–6** induced p21/WAF1 protein expression in a dose-dependent manner in Western blot analysis, although the protein levels induced by **5** and **6** were much lower than those by **1–4**, in consonance with the low HDAC and HCT116 inhibition of **5** and **6**. In contrast, the level of alpha-tubulin was unaltered by each treatment (Fig. 1). We thus investigated whether these compounds could stimulate the activity of the p21/WAF1 gene promoter in cells transiently transfected with the 2.4-kb wild-type p21/WAF1 promoter-luciferase fusion plasmid pWWP-Luc. The p21/WAF1 gene promoter activity was markedly stimulated by **1–4**, in line with the enhancement of the protein expression, as illustrated in Fig. 2. Further, the effects of these compounds on cell cycle progression of MG63 cells were investigated by measuring the DNA content of nuclei of MG63 cells with flow cytometry. Exposure to $1 \mu\text{M}$ of **4** or to $10 \mu\text{M}$ each of **1**, **2**, and **3** for 24 h apparently decreased S-phase cells, whereas it caused accumulation of cells in the G_2/M phase (Fig. 3). Interestingly, the levels of p21/WAF1 gene expression and G_2/M phase accumulation were mutually correlated. We have so far shown that p21/WAF1 is upregulated by HDAC inhibitors including TSA and sodium butyrate in MG63 cells; this was associated with G_2/M arrest⁷ It is also known that enhancement of p21/WAF1 causes G_1 arrest.¹³ p21/WAF1 inhibits cyclin-dependent kinase (CDK) activities such as CDK2, CDK4, or CDK6 promoting G_1/S -phase transition.¹³ In addition to its role in G_1 transition, p21/WAF1 reaccumulates in nuclei near the G_2/M boundary and causes a transient block in the late G_2 phase.¹⁴ In addition to CDK regulation, p21/WAF1 binds directly to PCNA, which is associated with

DNA replication and cell proliferation.¹⁵

The observed increase in the p21/WAF1 level as well as the cell cycle arrest in the G_2/M phase strongly suggest that **1–4** are potent inhibitors of cell cycle progression. Although the p21/WAF1 gene is a p53-regulated one,¹⁶ it was upregulated by **1**, **2**, **3**, or **4** in the p53-mutated MG63 cells. Since the p53 gene is frequently mutated,¹⁷ clarification of the p53-independent activating pathway of the p21/WAF1 gene might contribute to cancer therapy.

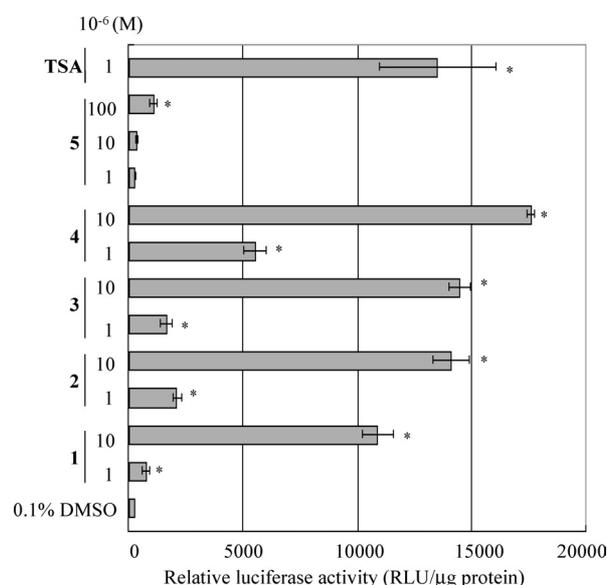


Fig. 2. Compound **1**, **2**, **3**, or **4** Activates p21/WAF1 Promoter Activity

MG63 cells were transiently transfected with the pWWP-Luc reporter plasmid, and luciferase activity was measured after incubation with or without various concentrations of **1**, **2**, **3**, **4** or **5**. Relative luciferase activity is shown as raw light units (RLU) in cell lysates/ μg of protein. All results are the mean \pm S.E. of at least three independent experiments, and the significant differences from the control (0.1% DMSO) were determined by Student's test and are indicated by an asterisk ($p < 0.05$).

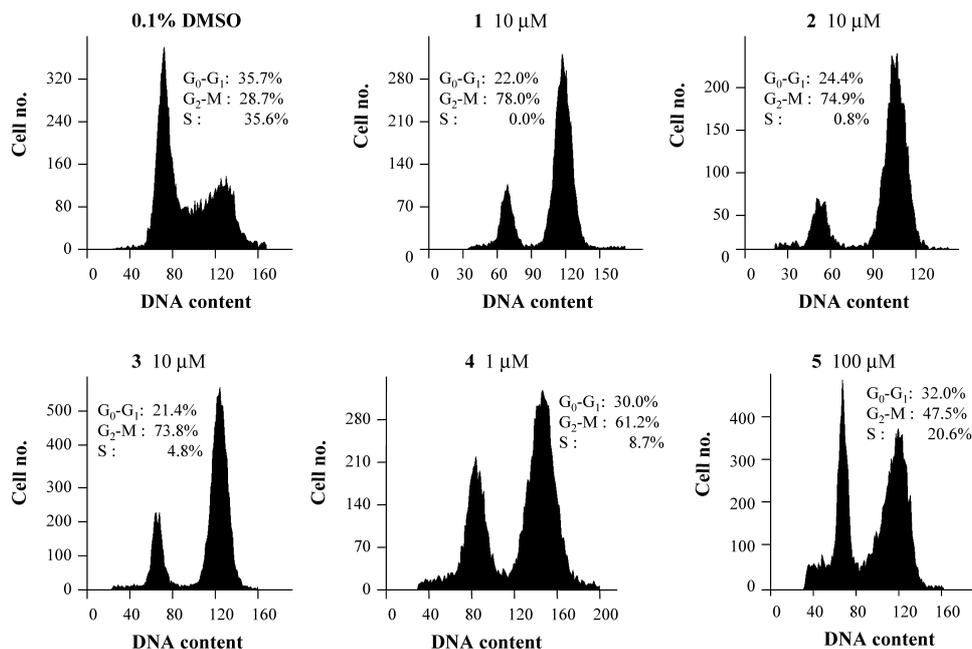


Fig. 3. Treatment with **1**, **2**, **3**, or **4** Causes G_2/M Arrest

MG63 cells were treated with or without various concentrations of **1**, **2**, **3**, **4**, or **5** as indicated for 24 h, and their DNA content was analyzed using fluorescence flow cytometry. The percentages of the population in the G_0/G_1 , G_2/M , and S phase are indicated. The experiment was repeated three times and representative histograms are shown.

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