Supporting Information

Synthesis and Evaluation of 3-Aroylindoles as Anticancer Agents: Metabolite Approach

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1. Chemistry

1.1. General methods

All commercial chemicals and solvents are reagent grade and were used without further treatment unless otherwise noted. All reactions were carried out under an atmosphere of dry nitrogen. Reactions were monitored by TLC using Merck 60 F_{254} silica gel glass backed plates (5 × 10 cm); zones were detected visually under ultraviolet irradiation (254 nm) or by spraying with phosphomolybdic acid reagent (Aldrich) followed by heating at 80°C. Flash column chromatography was done using silica gel (Merck Kieselgel 60, No. 9385, 230-400 mesh ASTM). ^{1}H NMR spectra were obtained with a Varian Mercury-300 spectrometer operating at 300 MHz. Chemical shifts were recorded in parts per million (ppm, δ) and were reported relative to the solvent peak or TMS. LCMS data were measured on an Agilent MSD-1100 ESI-MS/MS System. Purity of the final compounds were determined using an Agilent 1100 series HPLC system using C-18 column (Agilent ZORBAX Eclipse XDB-C18 5 μm. 4.6 mm × 150 mm) and were found to be ≥ 95%, unless otherwise stated.

1.2. Synthesis

(4-Hydroxy-6-methoxy-1*H*-indol-3-yl)-(3,4,5-trimethoxyphenyl)methanone (3). Friedel-Crafts acylation step: 4-Benzyloxy-6-methoxy-1*H*-indole[1] (**17**) (0.07 g, 0.26 mmol) and $ZnCl_2$ (0.07 g, 0.52 mmol) were pre-dried and dissolved in methylene chloride (20 mL) under nitrogen with stirring. To the reaction mixture, ethyl magnesium bromide in ether (3 M solution in ether, 0.13 mL, 0.39 mmol) was added slowly over 2 h. 3, 4, 5-Trimethoxybenzoyl chloride (0.07 g, 0.31 mmol) in methylene chloride (10 mL) was then added into the reaction mixture and left to stir for an hour. $AlCl_3$ (0.04 g, 0.30 mmol) was added to the reaction mixture and left to react for 17 h. The solvent was evaporated under reduced pressure, and the residue was purified by silica gel column chromatography (EtOAc:*n*-hexane=1:2→1:1) to give (4-benzyloxy-6-methoxy-1*H*-indol-3-yl)-(3,4,5-trimethoxyphenyl)methanone (**18**; 30 mg, 26%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.69 (s, 3H), 3.73 (s, 6H), 3.87 (s, 3H), 4.96 (s, 2H), 6.33 (d, J = 2.1 Hz, 1H), 6.44 (s, 1H), 7.11 (s, 2H), 7.15-7.33 (m, 5H), 9.28 (brs, 1H). LCMS $(M+H)^+$ 448.2.

Debenzylation step: To compound **18** (0.01 g, 0.03 mmol) in ethyl acetate (3 mL), 10% palladium on charcoal (2.0 mg) was added; the mixture was hydrogenated in a Parr apparatus at 45 psi for 16 h. Then the reaction mixture was filtered through celite before washing with ethyl acetate (10 mL). The solvent was evaporated under reduced pressure, yielding yellow product **3** (10 mg, 89%). ^{1}H NMR (300 MHz, CD_3OD) δ 3.81 (s, 3H), 3.86 (s, 3H), 3.92 (s, 6H), 6.26 (d, J = 1.8 Hz, 1H), 6.50 (d, J = 1.8 Hz, 1H), 7.12 (s, 2H), 7.80 (s, 1H). LCMS $(M+H)^+$ 358.2.

(7-Hydroxy-6-methoxy-1*H*-indol-3-yl)-(3,4,5-trimethoxyphenyl)methanone (5). Acetic acid 6-methoxy-1*H*-indol-7-yl ester[2] (**24**) (0.06 g, 0.27 mmol) and $ZnCl_2$ (0.07 g, 0.52 mmol) were pre-dried and dissolved in methylene chloride (20 mL) under nitrogen with stirring. To the reaction mixture, ethyl magnesium bromide in ether (3 M solution in ether, 0.14 mL, 0.42 mmol) was added slowly over 2 h. 3,4,5-Trimethoxybenzoyl chloride (0.08 g, 0.32 mmol) in methylene chloride (10

mL) was then added into the reaction mixture and left to stir for an hour. $AlCl_3$ (0.04 g, 0.30 mmol) was added to the reaction mixture and left to react for 17 h. The solvent was evaporated under reduced pressure, and the residue was purified by silica gel column chromatography (EtOAc:*n*-hexane=1:2→1:1) followed by preparative thin layer chromatography (MeOH:CH_2Cl_2=1:20) to give **5** (45 mg, 45%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.90 (s, 6H), 3.93 (s, 3H), 3.98 (s, 3H), 7.02 (d, *J* = 8.7 Hz, 1H), 7.11 (s, 2H), 7.68 (d, *J* = 1.5 Hz, 1H), 7.87 (d, *J* = 8.7 Hz, 1H), 8.86 (brs, 1H). LCMS $(M+H)^+$ 358.1.

(3-Hydroxy-4,5-dimethoxy-phenyl)-(6-methoxy-1*H*-indol-3-yl)methanone (6). 3-Hydroxy-4,5-dimethoxy-benzoic acid methyl ester (**25**) (0.23 g, 1.06 mmol), K_2CO_3 (0.53 g, 3.83 mmol), and benzyl bromide (0.25 mL, 2.10 mmol) in EtOH (40 mL) were refluxed overnight. The reaction mixture was filtered; solvents were removed under reduced pressure; and the residue obtained was dissolved in MeOH/H_2O (4:1; 5 mL) mixture and stirred overnight at RT with LiOH (0.04 g, 1.55 mmol). The solvents were removed under reduced pressure, dissolved the residue in water, acidified with dil. HCl and extracted with ethyl acetate. The solvent was removed under reduced pressure and the residue purified by silica gel column chromatography (CH_2Cl_2:MeOH = 95:5) to give 3-benzyloxy-4,5-dimethoxy-benzoic acid (**26**) (27 mg, 88%). ^{1}H NMR (300 MHz, CD_3OD) δ 3.80 (s, 3H), 3.87 (s, 3H), 5.13 (s, 2H), 7.29-7.40 (m, 5H), 7.48 (d, *J* = 7.5 Hz, 2H); LCMS $(M+H)^+$ 289.2. Thionyl chloride (0.20 mL, 2.74 mmol) was added to **26** (0.06 g, 0.21 mmol) in methylene chloride (5 mL) and stirred for 30 min. The solvent was removed under reduced pressure; the residue obtained (acid chloride) was reacted immediately without purification with 6-methoxyindole (0.03 g, 0.18 mmol) using Friedel-Crafts acylation condition described above for compound **3** to give (3-benzyloxy-4,5-dimethoxyphenyl)-(6-methoxy-1*H*-indol-3-yl)methanone (**27**). Compound **27** obtained from previous step was debenzylated using the conditions reported for compound **3** to give **6** (15 mg, 22%). ^{1}H NMR (300 MHz, CD_3OD) δ 3.85 (s, 3H), 3.87 (s, 3H), 3.89 (s, 3H), 6.89 (dd, *J* = 8.7, 2.1 Hz, 1H), 6.94-6.97 (m, 2H), 7.00 (d, *J* = 2.1 Hz, 1H), 7.75 (s, 1H), 8.10 (d, *J* = 8.7 Hz, 1H). LCMS $(M+H)^+$ 328.1. HPLC purity: 91.25%.

(4-Hydroxy-3,5-dimethoxy-phenyl)-(6-methoxy-1*H*-indol-3-yl)methanone (7). Thionyl chloride (0.50 mL, 6.85 mmol) was added to a solution of 4-hydroxy-3,5-dimethoxy-benzoic acid (**28**) (0.50 g, 2.52 mmol) in THF (5 mL) maintained at 0 °C, then stirred for 30 min. The solvent was removed under reduced pressure; the residue obtained (acid chloride) was reacted immediately without purification with 6-methoxy indole (0.50 g, 3.40 mmol) using Friedel-Crafts acylation conditions described above for compound **3** to give **7** (21 mg, 25%). ^{1}H NMR (300 MHz, CD_3OD) δ 3.88 (s, 3H), 3.91 (s, 6H), 6.89 (dd, *J* = 8.7, 2.1 Hz, 1H), 7.01 (d, *J* = 2.1 Hz, 1H), 7.16 (s, 2H), 7.79 (s, 1H), 8.08 (d, *J* = 8.7 Hz, 1H). LCMS $(M+H)^+$ 328.1.

(7-Chloro-6-methoxy-1*H*-indol-3-yl)-(3,4,5-trimethoxyphenyl)methanone (9). (6-Methoxy-1*H*-indol-3-yl)-(3,4,5-trimethoxyphenyl)methanone (**2**)[3] (1.0 g, 2.93 mmol) was dissolved in *t*-butanol (96 mL) under nitrogen at 60 °C with stirring. To the cooled reaction mixture (30–40 °C), NCS (0.39 g, 2.98 mmol) was added in aliquots over a period of 0.5 h in the dark. The

reaction was allowed to proceed for 16 h at room temperature. The solvent was removed under reduced pressure and the residue was purified by flash column chromatography over silica gel (EtOAc: *n*-hexane = 1:2) to give **9** (58 mg, 53%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.91 (s, 6H), 3.96 (s, 3H), 4.01 (s, 3H), 7.06 (d, *J* = 8.7 Hz, 1H), 7.12 (s, 2H), 7.71 (d, *J* = 2.7 Hz, 1H), 8.22 (d, *J* = 8.7 Hz, 1H), 8.69 (brs, 1H). LCMS $(M+H)^+$ 376.0.

(6-Methoxy-7-methyl-1*H*-indol-3-yl)-(3,4,5-trimethoxyphenyl)methanone (11). To a solution of (7-iodo-6-methoxy-1*H*-indol-3-yl)-(3,4,5-trimethoxyphenyl)methanone (**10**) (0.064 g, 0.14 mmol) in THF (3 mL), $Fe(acac)_3$ (0.001 g, 0.002 mmol), N-methyl pyrrolidinone (0.1 mL), and methyl magnesium bromide (3 M solution in ether, 0.06 mL, 0.18 mmol) were added under nitrogen. The reaction was allowed to proceed for 16 h; the solvent was removed under reduced pressure. The residue was purified by flash column chromatography over silica gel (EtOAc: *n*-hexane = 1:2 → 1:1) to give **11** (18 mg, 37%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.39 (s, 3H), 3.90 (s, 6H), 3.92 (s, 3H), 3.93 (s, 3H), 7.01 (d, *J* = 8.7 Hz, 1H), 7.11 (s, 2H), 7.66 (d, *J* = 3.0 Hz, 1H), 8.17 (d, *J* = 8.7 Hz, 1H), 8.46 (s, br, 1H). LC-MS $(M+H)^+$ 356.1.

(7-Ethyl-6-methoxy-1*H*-indol-3-yl)-(3,4,5-trimethoxyphenyl)methanone (12). (7-Iodo-6-methoxy-1*H*-indol-3-yl)-(3,4,5-trimethoxyphenyl)methanone (**10**) (0.02 g, 0.04 mmol) was reacted with ethyl magnesium bromide (3 M solution in ether, 0.02 mL, 0.05 mmol) in a manner similar to that for compound **11** to give **12** (4 mg, 28%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.25 (t, *J* = 7.5 Hz, 3H), 2.88 (q, *J* = 7.5 Hz, 2H) 3.90 (s, 6H), 3.93 (s, 3H), 3.94 (s, 3H), 7.02 (d, *J* = 8.7 Hz, 1H), 7.12 (s, 2H), 7.66 (d, *J* = 3.0 Hz, 1H), 8.18 (d, *J* = 8.7 Hz, 1H), 8.46 (s, br, 1H). LCMS $(M+H)^+$ 370.2. HPLC purity: 91.94%.

1.3. HPLC purity determination

The percentage purity of compounds were determined by an Agilent 1100 series HPLC system using C18 column.

Elution conditions: Mobile phase A-Acetonitrile; Mobile phase B-Water containing 0.1% formic acid + 10 mmol NH_4OAc. The flow-rate was 0.2 ml/min and the injection volume was 5 µl. The system operated at 25 °C. Peaks were detected at 210 nm.

Elution condition

Time (min)	Mobile Phase A (ratio)	Mobile Phase B (ratio)
0	10	90
45	90	10
50	10	90
60	10	90

C-18 column: Agilent ZORBAX Eclipse XDB-C18 5 µm. 4.6 mm × 150 mm column.

2. Colchicine binding and tubulin polymerization inhibition

Table 1s. Inhibition of colchicine binding and tubulin polymerization by compounds **3–5**, **10**, and **12**.

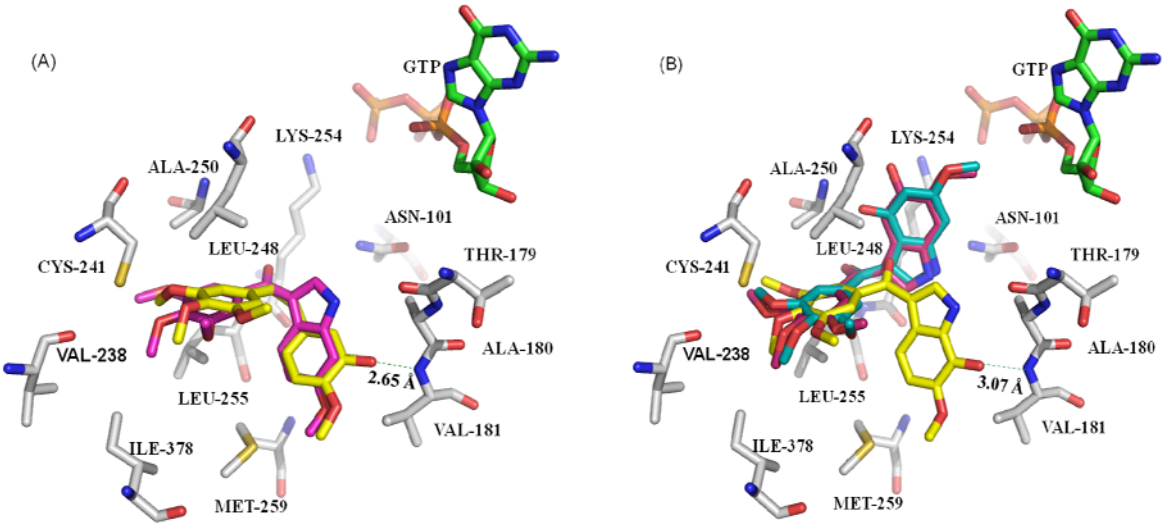
Compounds	Colchicine binding[a]			Inhibition of tubulin polymerization[a]
	% inhibition ± SD[b]		K_i	
	1 μM inhibitor	5 μM inhibitor	(μM± SD)	IC_{50} (μM± SD)
3	13.90 ± 8.20	33.09 ± 3.01	2.180 ± 1.700	> 10
4	0.51 ± 0.70	20.82 ± 3.63	1.409 ± 1.535	-
5	96.50 ± 1.80	99.45 ± 0.30	0.030 ± 0.018	2.0 ± 0.9
10	89.89 ± 1.99	96.04 ± 0.12	0.029 ± 0.023	3.5 ± 0.4
12	30.05 ± 11.03	60.73 ± 5.52	0.651 ± 0.454	> 10
Colchicine	42.83 ± 10.96	76.95 ± 1.12	0.977 ± 0.171	3.2 ± 0.3

[a] Values are expressed as the mean of at least three independent experiments.

[b] Percentage inhibition of [^{3}H] colchicine binding; [^{3}H] colchicine concentration was at 5 μM.

3. Molecular modeling

Figure 1s. Docking of compounds **1**-**5** to colchicine binding site of tubulin (PDB ID: 1SA0) using Gold. (A) Docking pose of compound **1** (yellow) and **2** (pink) in the colchicine binding site. 3-OH group of **1** is within hydrogen bonding distance of Val181 backbone NH. (B) Docking pose of compounds **3** (pink), **4** (cyan) and **5** (yellow) in the colchicine binding site. 7-OH group of **5** is within hydrogen bonding distance of Val181 backbone NH.



4. Biology

a. Reagents

Colchicine was purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]colchicine (specific activity, 60–87 Ci/mmol) reagent was purchased from Perkin-Elmer Life Sciences (Boston, MA).

b. Growth inhibition assay

Human cervical carcinoma KB cells (this cell line was originally believed to be derived from an epidermal carcinoma of the mouth but has now been shown with HeLa characteristics), colorectal carcinoma HT29 cells, non small cell lung carcinoma H460 cells were maintained in RPMI 1640 medium supplied with 5% fetal bovine serum. Cells in logarithmic growth phase were cultured at a density of 5000 cells/ml/well in a 24-well plate. The cells were exposed to various concentrations of the test drugs for 72 h. The methylene blue dye assay was used to evaluate the effect of test drugs on cell growth, as has been described previously.[4] The IC_{50} value resulting from 50% inhibition of cell growth was calculated graphically as a comparison with control growth.

c. Colchicine binding assay

The assay was basically performed according to the method of Lambeir and Engelborghs.[5] MAP-rich tubulin (1 μM) was incubated with 5.0 μM of [^{3}H]-colchicine at either 1.0 or 5.0 μM concentrations of test compounds in a buffer containing 0.05 M PIPES (pH 6.9), 1 mmol $MgCl_2$, and 1 mmol GTP. After incubating at room temperature for 1 h, the samples were centrifuged through Sephadex G-50 columns (Amersham Biosciences, Piscataway, NJ). The eluates in the flow-through were analyzed for radioactivity by scintillation counting.

d. *In vitro* tubulin polymerization assay[6-8]

In brief, microtubule-associated protein-rich tubulin in 100 μl buffer containing 100 mmol PIPES (pH 6.9), 2 mmol $MgCl_2$, 1 mmol GTP, and 2% (v/v) DMSO was placed in 96-well microtiter plates in the presence of test agents. The increase in absorbance was measured at 350 nm in a PowerWave X Microplate Reader (Bio-Tek Instruments, Winooski, VT) at 37 °C and recorded every 30 s for 30

min. The area under the curve was used to determine the concentration that inhibited tubulin polymerization by 50% (IC_{50}). The area under the curve of the untreated control was set to 100% polymerization, and the IC_{50} was calculated by nonlinear regression.

e. *In vitro* metabolic stability testing

The incubation mixture, in 74 mmol potassium phosphate buffer (pH 7.4), contained: microsomal proteins, 0.5 mg/mL; NADPH, 3 mmol; $MgCl_2$, 3 mmol; **2** or **29**, 1 μM. The final volume of incubation was 250 μL. Incubation was carried out, in triplicate, aerobically at 37 °C with constant shaking on a temperature-controlled heating block. Reaction was started by the addition of NADPH after pre-incubating the reaction mixture (without NADPH) for 10 min at 37 °C. Control incubation without NADPH was performed as described above. At 0, 5, 10, 20 and 30 min after the start of reaction, an aliquot (30 μL) of the incubation mixture was taken from each incubation, mixed with 100 μL of ice-cold acetonitrile to terminate the reaction. Before analysis, the sample was precipitated by centrifugation at room temperature. The remaining supernatant was analyzed for the concentration of each compound to determine the metabolic rate.

f. *In vivo* pharmacokinetic evaluation

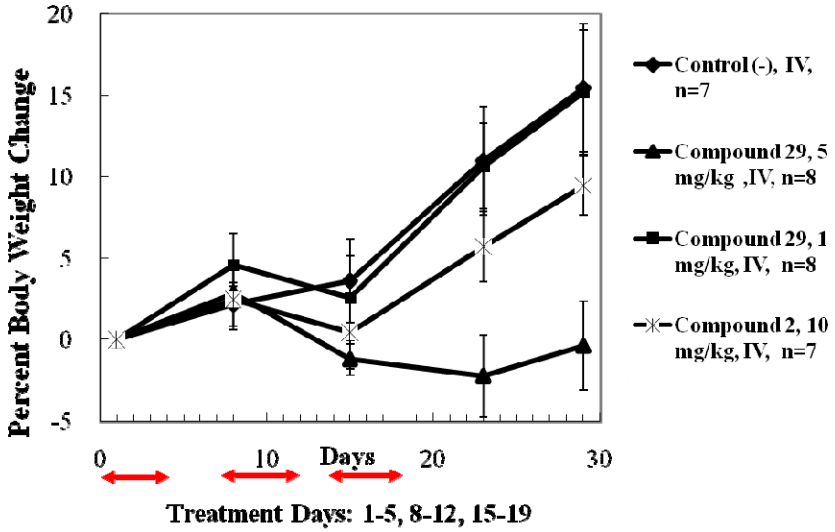
This study was approved by Institutional Animal Care and Use Committee of National Health Research Institutes. A solution of test compound (10 mg/mL) was prepared by dissolving appropriate amount compound in a mixture of PEG 400/dehydrated ethanol/Solutol (20:30:50, v/v/v) and was diluted with two parts of physiological saline to make the dosing solution of 3.3 mg/mL before dosing. Male Sprague-Dawley rats, weighing 250–350 g each (8–10 weeks old), were obtained from BioLASCO, Ilan, Taiwan. Single 5 mg/kg dose of **2** or **29** was separately administered to group of three male rats each intravenously by a bolus injection to the tail vein. The volume of dosing solution administered was adjusted according to the body weight recorded before dose administration. At 0 (prior to dosing), 2, 5, 15, and 30 min and at 1, 2, 4, 6, 8, 12, 24, 30, and 48 h after dosing, a blood sample (~150_L) was collected from each animal via the jugular-vein cannula and stored in ice (0–4 °C). Plasma was separated from the blood by centrifugation (14,000×*g* for 15 min at 4 °C in a Beckman Model AllegraTM 6R centrifuge) and stored in a freezer (−60 °C). All samples were analyzed for the test compound by LC-MS/MS. Data were acquired via multiple reactions monitoring. The limit of quantitation in current PK study is 0.1 ng/mL and the limit of quantitation in our previous reported method[9] is 0.5 ng/mL. Plasma concentration data were analyzed with standard non-compartmental method with WinNonLin software program (version 3.1, Pharsight Corp, CA, USA).

g. *In vivo* antitumor evaluation

Adult male nude mice were purchased from The National Laboratory Animal Center, Taipei, Taiwan. Animals had access to food and water *ad libitum*. Experimental procedures using animals were approved by the Institutional Animal Care and Use Committees of The National Health Research Institutes. Human KB cells were cultured in RPMI-1640 supplemented with 10% heat-inactived bovine serum (FBS) and 1% *L*-glutamine and incubated at 37 °C in humidified

atmosphere consisting 5% CO_2. Eight-week-old nude mice were inoculated with KB cells subcutaneouosly at 1×106 KB cells per mouse mixed with equal volume of Matrigel (Becton Dickinson) in 0.1 ml via 24–gauge needle. Tumor volume was measured by using a electronic caliper and calculated with the formula length×width^2×0.5 twice a week. When the size of a growing tumor > 100 mm^3, the KB tumor bearing mice were administered compound **29** (dissolved in 5% DMSO + 20% Cremophor EL + normal saline) i.v. via the tail veins for 5 days per week for 3 consecutive weeks at 1 and 5 mg/kg, respectively. The reference compound **2** (dissolved in 5% DMSO + 20% Cremophor EL + normal saline) at 10 mg/kg, i.v. was included for comparison. Tumor size and animal body weight were measured twice a week after drug treatments. At the end of the experiments, animals were euthanized with carbon dioxide followed by cervical dislocation.

Figure 2s. Percentage body weight change in nude mice bearing KB xenografts tumors, treated with compounds **2** and **29**.



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