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Research Article

Synthesis and Evaluation of a Series of 3,4,5-Trimethoxycinnamic Acid Derivatives as Potential Antinarcotic Agents

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A series of 3,4,5-trimethoxycinnamic acid derivatives was prepared and evaluated for antinarcotic effects on morphine dependence in mice and binding affinities on serotonergic receptors. The key synthetic strategies involve generation of ketones 6–7, esters 9–12 through condensation reaction, and amides 13–19 via coupling reaction using 1-hydroxybenzotriazole/ethyl(dimethylaminopropryl)carbodiimide system in high yield. We found that the naloxone-induced morphine withdrawal syndrome was significantly suppressed by new synthetic 3,4,5-trimethoxycinnamic acid derivatives (20 mg/kg/day). Most of 3,4,5-trimethoxycinnamic acid derivatives were found to have high affinity to 5-HT_{1A} receptor. The naloxone-induced morphine withdrawal syndrome was attenuated by (+)8-OH-DPAT (0.1 mg/kg/day, i.p.), a 5-HT_{1A} receptor agonist. In cortical neuronal cells, (+)8-OH-DPAT (1 μM) produced an elevation of the pERK 1/2 expression, and the elevated pERK levels were inhibited by WAY 100635, a 5-HT_{1A} receptor-specific antagonist. Interestingly, the pERK levels were increased by the 3,4,5-trimethoxycinnamic acid derivatives and the derivatives-mediated changes in pERK levels were blocked by the WAY 100635. These results suggested that new synthetic 3,4,5-trimethoxycinnamic acid derivatives have a potential antinarcotic effect through acting as a 5-HT_{1A} receptor agonist in mice.

Key words: binding affinity, coupling reaction, morphine, pERK, 3,4,5-trimethoxycinnamic acids, 5-$HT1_A$ receptor

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Methoxycinnamic acids or hydroxycinnamic acids are an important substance for the remediation of serious disease, many of which possess widespread pharmacological properties related to various therapeutic fields such as oncology (1), osteoporosis (2), arthritis (3), anti-inflammatory (4), and inhibition of enzymes (5). Current research priorities are to better characterize the biological roles and biochemical features of *trans*-cinnamic acid derivatives (Figure 1).

In particular, 3,4,5-trimethoxycinnamic acid (TMCA) and its derivatives were recently focused on the role mechanism of anti-oxidative processes (6) and directed to the anti-oxidant activity as well as neurological disorders (7). Accumulated publications have focused on the stress induced with repeated cold exposure or intracerebroventricular injection of corticotrophin-releasing hormone (8). Recently, the Kawashima group (9) reported TMCAs were having significant anti-stress effects by suppressing norepinephrine (NE) content in locus coeruleus (LC). Interestingly, it has been known that elevation of NE in LC was shown in morphine withdrawal syndrome. The Geraci group (10) developed the hydroxycinnamic acid clustered by a calixarene platform for the radical scavenging and anti-oxidant activity. The Keung group (11) described the eugenol and its structural analogues exhibit antidepressant-like activity.

Morphine is most frequently used as analgesics in clinical treatment for reducing the sustaining pain. Chronic opioids exposure induces a strong dependence state and induce withdrawal syndrome when it was stopped abruptly. Injection of a general opioid receptor antagonist naloxone after a continuous opioid treatment leads to a withdrawal syndrome as characteristic behavior of opioid dependence (12). It has been reported that opioids have their dependence as well as analgesic effect through several pathways such as the serotonergic (13), glutamatergic (14), dopaminergic (15), GABAergic (16), and noradrenergic pathways (17). The accumulation of 5-hydroxytryptophan (5-HTP) and 3,4-dihydroxyphenylalanine (DOPA), a precursor of serotonin and dopamine, was increased by chronic morphine treatment and during morphine withdrawal syndrome precipitated by naloxone in various brain regions (18).

Hyperactivity of LC neuron is an important state of opiate withdrawal. LC hyperactivity by opiate withdrawal is medi-

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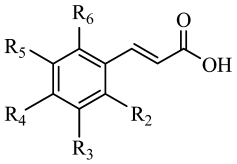
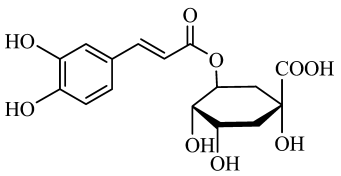


1 Cinnamic acid: R_2=R_3=R_4=R_5=R_6=H
2 *p*-Coumaric acid: R_2=R_3=R_5=R_6=H, R_4=OH
3 3,4,5-Trimethoxycinnamic acid: R_2=R_6=H, R_3=R_4=R_5=OMe



4 Chlorogenic acid

Figure 1: Structures of various cinnamic acids **1**–**4**.

ated by an excitatory amino acid release to the LC. 5-HT selectively blocks the way of excitatory amino acid release to the LC during opiate withdrawal (19). In addition, it has been reported the role of 5-HT (5-hydroxytryptamine) in the development of dependence on opiates (20), these studies indicated that 5-HT system may be related with opiate pathway in central nervous system (CNS) and hyperactivity of LC neuron induced by opiate withdrawal was deceased by activation of serotonergic neurotransmission. Several selective 5-HT_{1A} agonists include (+)8-OH-DPAT [8-hydroxy-2-(di-n-propylamino)tertraline] attenuate the antinociception of μ-opioid agonist, morphine in mice, but κ-opioid agonist action was not antagonized by 5-HT_{1A} agonist (21,22). 5-HT_{1A} receptor agonist induced activation of extracellular signal-regulated kinase (ERK1/2) and Akt for survival. (+)8-OH-DPAT stimulated ERK1/2 in hypothalamus of rat in time-dependent manner (23).

In the context of our medicinal chemistry program dealing with the development of TMCA and its derivatives having remarkable bioactivities for the neurological disorders, we have prepared fourteen TMCA derivatives. Previously, we have reported that 3,4,5-trimethoxyphenyl acrylamides showed good antinarcotic effect in mice (24). We wish to report herein a simple synthesis and evaluation of biological activity of TMCA derivatives **6–19** during morphine withdrawal *in vivo*, starting from TMCA via chlorination, esterification, and global coupling reaction. It was determined whether TMCA derivatives can attenuate the naloxone-induced jumping behavior in morphine-dependent mice, and to identify what is the main target among several neurotransmitter pathways and receptors being mediated in CNS.

Experimental Section

Chemistry

All other commercial reagents and solvents were used as received without further purification. Reaction solvents were distilled from calcium hydride for dichloromethane and from sodium metal and benzophenone for tetrahydrofuran. The reactions were monitored and the R_f values determined using analytical thin layer chromatography (TLC) with Merck silica gel 60, F-254 precoated plates (0.25 mm thickness). Spots on the TLC plates were visualized using ultraviolet light (254 nm), a cerium sulfate/ammonium dimolybdate/sulfuric acid solution followed by heating on a hot plate. Flash column chromatography was performed with Merck silica gel 60 (230–400 mesh) or purification of reaction mixture was recrystallized by diethyl ether in dichloromethane. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker DRX 250 at 250 and 63 MHz or 400 at 400 and 100 MHz, respectively. Proton chemical shifts are reported in ppm (δ) relative to internal tetramethylsilane (TMS, δ 0.00) or with the solvent reference relative to TMS employed as the internal standard ($CDCl_3$, δ 7.26 ppm). Data are reported as follows: chemical shift {multiplicity [singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m)], coupling constants [Hz], integration}. Carbon chemical shifts are reported in ppm (δ) relative to TMS with the respective solvent resonance as the internal standard ($CDCl_3$, δ 77.16 ppm). Infrared (IR) spectra were recorded on a JASCO FT/IR-430 spectrometer. Data are reported in wave numbers (per cm). ESI-LC-MS was recorded on a Waters ZQ 4000 LC-MS spectrometer. Melting Points were determined on a BIBBY Stuart Scientific MELTING POINT APPRATUS SMP3. Elemental analyses were performed on a CE instrument EA1110 (25).

(E)-*N*-methoxy-*N*-methyl 3,4,5-trimethoxycinnamamide (6)

To a stirred solution of TMCA (**3**, 1.0 g, 4.2 mmol) in dry dichloromethane (30 mL) was added ethyl(dimethylaminopropryl)carbodiimide (EDCI, 0.97 g, 5.0 mmol), 1-hydroxybenzotriazole (HOBt, 0.68 g, 5.0 mmol), and triethylamine (TEA, 0.70 mL, 5.0 mmol) and then the mixture was stirred at room temperature for 30 min. The *N*,*O*-dimethylhydroxyamine (0.49 g, 5.0 mmol) was added to the reaction at room temperature and the resulting mixture was stirred at room temperature for 24 h. The reaction mixture was diluted with dichloromethane (10 mL) and washed with saturated aqueous NH_4Cl solution (30 mL) and brine (30 mL). The organic layer was separated, dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (silica gel, ethyl acetate/*n*-hexanes = 2:1, v/v) to give pure **6** as white solid (0.89 g, 75%). White solid. mp 106–107 °C (lit (26). mp 102–104 °C); R_f = 0.4 (ethyl acetate/*n*-hexanes = 2:1, v/v); IR ν_{max} ($CHCl_3$, KBr) 3466, 2945, 1650, 1613, 1583, 1505, 1459, 1420, 1385, 1333, 1246, 1153, 1127, 1005 per cm; ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.66 (d, 1H, J = 15.7),

6.92 (s, 2H), 6.79 (d, 1H, J = 15.7), 3.91 (s, 6H), 3.88 (s, 3H), 3.78 (s, 3H), 3.32 (s, 3H); ^{13}C NMR (63 MHz, $CDCl_3$) δ 166.9 153.4 143.7 139.8 130.8 115.0 105.3 62.0 61.0 56.3 32.6; LC-MS (ESI+) m/z 321.47 [M+Na].

Preparation of compounds 7 and 8

To a solution of **6** (0.5 g, 1.8 mmol) in dry THF (20 mL) was added dropwise Et-MgBr (0.48 g, 3.6 mmol) or Ph-MgBr (0.65 g, 3.6 mmol) at −78 °C and the mixture was warmed to room temperature for 2.5 h. The reaction mixture was quenched by slowly addition of 10% aqueous HCl solution (two drops) and washed with brine (15 mL). The organic layer was separated, dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (silica gel, ethyl acetate/*n*-hexanes = 2:1, v/v) to give pure **7** and **8**.

(E)-Ethyl 3,4,5-trimethoxycinnamyl ketone (7)

Pale beige solid. mp 110–112 °C; R_f = 0.7 (ethyl acetate/*n*-hexanes = 2:1, v/v); IR ν_{max} ($CHCl_3$, KBr) 2933, 1661, 1625, 1614, 1581, 1504, 1461, 1420, 1336, 1245, 1194, 1156, 1127 per cm; ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.49 (d, 1H, J = 16.1), 6.78 (s, 2H), 6.66 (d, 1H, J = 16.1), 3.91 (s, 6H), 3.89 (s, 3H), 2.72 (q, 2H), 1.18 (t, 3H); ^{13}C NMR (63 MHz, $CDCl_3$) δ 201.0 153.6 142.4 140.4 130.2 125.5 105.6 61.1 56.3 34.0 8.4; LC-MS (ESI+) m/z 273.22 [M+Na]. Anal. Calcd for ($C_{14}H_{18}O_4$): C, 67.18; H, 7.25. Found: C, 67.18; H, 7.26.

(E)-Phenyl 3,4,5-trimethoxycinnamyl ketone (8)

Pale yellow solid. mp 138–139 °C (lit (27). mp 135–136 °C); R_f = 0.8 (ethyl acetate/*n*-hexanes = 2:1, v/v); IR ν_{max} ($CHCl_3$, KBr) 2923, 1603, 1450, 1413, 1385, 1124, 1096, 1080, 1041 per cm; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00–8.02 (m, 2H) 7.72 (d, 1H, J = 15.6), 7.58–7.62 (m, 1H), 7.50–7.54 (m, 2H), 7.40 (d, 1H, J = 15.6), 6.87 (s, 2H), 3.93 (s, 6H), 3.91 (s, 3H); ^{13}C NMR (63 MHz, $CDCl_3$) δ 190.7 153.5 145.2 138.3 132.9 130.4 128.8 128.6 121.5 105.5 61.2 56.3; LC-MS (ESI+) m/z 321.28 [M+Na].

Preparation of compounds 9 and 10

The solution of TMCA (**9**, 1.0 g, 4.2 mmol) and H_2SO_4 (2–3 drops) in methanol (15 mL) or ethanol (15 mL) was refluxed for 24 h. The mixture was cooled to room temperature and the solid was filtered. The crystal was washed with ether (5 mL) and dried under reduced pressure to generate **9** and **10**.

(E)-Methyl 3,4,5-trimethoxycinnamate (9)

White solid. mp 101–102 °C (lit (28). mp 91.5–92 °C); R_f = 0.9 (ethyl acetate/*n*-hexanes = 2:1, v/v); IR ν_{max} ($CHCl_3$, KBr) 3444, 3026, 1708, 1636, 1584, 1504, 1416, 1311, 1279, 1245, 1127, 1003 per cm; ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.61 (d, 1H, J = 15.9), 6.75 (s, 2H), 6.36 (d, 1H, J = 15.9), 3.88 (s, 9H), 3.80 (s, 3H); ^{13}C NMR (63 MHz, $CDCl_3$) δ 167.3 153.3 144.8 140.0 129.8 116.9 105.1 60.9 56.0 51.6; LC-MS (ESI+) m/z 275.02 [M+Na].

(E)-Ethyl 3,4,5-trimethoxycinnamate (10)

White solid. mp 69–70 °C (lit (29). mp 68–69 °C); R_f = 0.7 (ethyl acetate/*n*-hexanes = 2:1, v/v); IR ν_{max} ($CHCl_3$, KBr) 3464, 2353, 1710, 1635, 1582, 1506, 1456, 1416, 1275, 1152, 1126, 1003 per cm; ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.61 (d, 1H, J = 15.9), 6.76 (s, 2H), 6.35 (d, 1H, J = 15.9), 4.27 (q, 2H), 3.89 (s, 6H), 3.88 (s, 3H), 1.35 (t, 3H); ^{13}C NMR (63 MHz, $CDCl_3$) δ 167.1 153.6 144.7 142.0 130.1 117.6 105.3 61.1 60.7 56.3 14.5; LC-MS (ESI+) m/z 289.07 [M+Na].

(E)-Phenyl 3,4,5-trimethoxycinnamate (11)

To a solution of TMCA (**4**, 1.0 g, 4.2 mmol) was added thionyl chloride (0.37 mL, 5.0 mmol) and the mixture was heated at 65 °C on a water bath, until hydrogen chloride ceases to be evolved (about 1 h). The generated acid chloride **5** was used without purification. The phenol (0.44 mL 5.0 mmol) was added to reaction mixture at 65 °C and the resulting mixture was heated at 65 °C on a water bath until the hydrogen chloride was not evolved for approximately 1 h. The reaction mixture was allowed to cool at room temperature and distilled under reduced pressure. The residue was by flash column chromatography (silica gel, ethyl acetate/*n*-hexanes = 1:6, v/v) to give pure **11**. White solid. mp 103–104 °C; R_f = 0.2 (ethyl acetate/*n*-hexanes = 1:6, v/v); IR ν_{max} ($CHCl_3$, KBr) 3468, 2842, 1726, 1635, 1582, 1503, 1455, 1420, 1272, 1244, 1194, 1131, 1005 per cm; ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.79 (d, 1H, J = 15.9), 7.15–7.44 (m, 5H), 6.82 (s, 2H), 6.55 (d, 1H, J = 15.9), 3.90 (s, 9H); ^{13}C NMR (63 MHz, $CDCl_3$) δ 165.5 153.6 150.9 146.6 140.5 129.7 129.5 125.9 121.7 116.6 105.5 61.1 56.3; LC-MS (ESI+) m/z 337.02 [M+Na].

(E)-Benzyl 3,4,5-trimethoxycinnamate (12)

To a solution of TMCA (**3**, 1.0 g, 4.2 mmol) and benzyl bromide (0.60 mL, 5.0 mmol) in 30ml of DMF/1,4-Dioxane (1:1), $NaHCO_3$ (0.42 g, 5.0 mmol) was added at room temperature. The reaction mixture was heated and stirred at 90 °C for 24 h. The mixture was cooled to room temperature and diluted with ethyl acetate. And then it was washed with brine and water. The organic layer was dried over anhydrous $MgSO_4$, filtered, and the solvent was removed under reduced pressure. The residue was purified by flash column chromatography (silica gel, ethyl acetate/*n*-hexanes = 2:1, v/v) to give pure **12**. White solid. mp 92–93 °C; R_f = 0.7 (ethyl acetate/*n*-hexanes = 2:1, v/v); IR ν_{max} ($CHCl_3$, KBr) 3436, 2945, 1713, 1638, 1583, 1506, 1455, 1417, 1276, 1244, 1149, 1126, 1002 per

cm; ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.65 (d, 1H, J = 15.9), 7.32–7.47 (m, 5H), 6.75 (s, 2H), 6.40 (d, 1H, J = 15.9), 5.25 (s, 2H), 3.88 (s, 9H); ^{13}C NMR (63 MHz, $CDCl_3$) δ 166.9 153.5 145.2 140.2 136.1 129.9 128.7 128.4 117.2 105.3 66.5 61.1 56.2; LC-MS (ESI+) m/z 351.20 [M+Na].

General procedure for the preparation of amides 13–19

To a stirred solution of TMCA (9, 1.0 g, 4.2 mmol) in dry dichloromethane (30 mL) was added EDCI (0.97 g, 5.0 mmol), HOBt (0.68 g, 5.0 mmol), and triethylamine (TEA, 0.70 mL, 5.0 mmol) and then the reaction mixture was stirred at room temperature for 30 min. Each amine was then added into the reaction mixture and the resulting mixture was stirred at room temperature for 24 h. The reaction mixture was washed with brine (20 mL) and water (20 mL). The organic layer was separated, dried over anhydrous MgSO4, filtered, and the solvent was removed under reduced pressure. The residue was purified by flash column chromatography (silica gel, ethyl acetate/hexanes = 2:1, v/v).

(E)-1-(Pyrrolidin-1-yl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (13)

White solid. mp 159–160 °C (lit (30). mp 148–150 °C); R_f = 0.2 (ethyl acetate/hexanes = 2:1, v/v); IR ν_{max} ($CHCl_3$, KBr) 3469, 2970, 1646, 1584, 1507, 1415, 1332, 1243, 1128, 1004 per cm; ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.62 (d, 1H, J = 15.4), 6.75 (s, 2H), 6.62 (d, 1H, J = 15.4), 3.90 (s, 6H), 3.88 (s, 3H), 3.58–3.69 (m, 4H), 1.68–2.05 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.8 153.5 142.0 131.1 118.2 105.2 61.0 56.4 46.8 46.2 26.3 24.5; LC-MS (ESI+) m/z 314.09 [M+Na].

(E)-N-(Tetrahydrofuran-2-yl)methyl 3,4,5-trimethoxycinnamamide (14)

White solid. mp 98–99 °C; R_f = 0.2 (ethyl acetate/hexanes = 2:1, v/v); IR ν_{max} ($CHCl_3$, KBr) 3428, 1631, 1545, 1505, 1419, 1321, 1281, 1125 per cm; ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.51 (d, 1H, J = 15.5), 6.85–6.67 (m, 3H), 6.45 (d, 1H, J = 15.5), 4.27–3.95 (m, 2H), 3.85 (s, 9H), 3.80–3.59 (m, 2H), 3.37–3.20 (m, 1H), 2.11–1.81 (m, 3H), 1.70–1.55 (m, 1H); ^{13}C NMR (63 MHz, $CDCl_3$) δ 166.2 153.2 140.8 139.2 130.4 120.0 104.7 77.68 67.9 60.8 55.9 43.4 28.6 25.7; LC-MS (ESI+) m/z 344.15 [M+Na].

(E)-N-(Furan-2-ylmethyl) 3,4,5-trimethoxycinnamamide (15)

Pale yellow solid. mp 133–134 °C; R_f = 0.6 (ethyl acetate/hexanes = 2:1, v/v); IR ν_{max} ($CHCl_3$, KBr) 3449, 2945, 1661, 1620, 1583, 1503, 1458, 1421, 1322, 1281, 1151, 1124 per cm; ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.56 (d, 1H, J = 15.5), 7.34 (s, 1H), 6.70 (s, 2H), 6.38 (d, 1H, J = 15.5), 6.25–6.32 (m, 3H), 4.52 (d, 2H, J = 13.9), 3.85 (s, 6H), 3.83 (s, 3H); ^{13}C NMR (63 MHz, $CDCl_3$) δ 165.7 153.4 151.3 142.3 141.4 139.6 130.4 119.8 110.6 107.7 105.0 61.0 56.1 36.7; LC-MS (ESI+) m/z 340.01 [M+Na].

(E)-1-Morpholin-4-yl(3,4,5-trimethoxyphenyl)prop-2-en-1-one (16)

White solid. mp 134–135 °C (lit (31). mp 129–131 °C); R_f = 0.3 (ethyl acetate/hexanes = 2:1, v/v); IR ν_{max} ($CHCl_3$, KBr) 3448, 2852, 1645, 1587, 1505, 1459, 1341, 1273, 1228, 1152, 1127, 1046 per cm; ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.62 (d, 1H, J = 15.3), 6.74 (d, 1H, J = 15.3), 6.75 (s, 2H) 3.90 (s, 6H), 3.88 (s, 3H), 3.73 (s, 8H); ^{13}C NMR (63 MHz, $CDCl_3$) δ 165.5 153.4 143.3 139.6 130.7 115.7 105.0 100.0 66.9 61.0 56.2; LC-MS (ESI+) m/z 330.17 [M+Na].

(E)-1-[4-(3,5-Dimethoxyphenyl)piperazin-1-yl]-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (17)

Yellow solid. mp 168–170 °C; R_f = 0.5 (ethyl acetate/hexanes = 2:1, v/v); IR ν_{max} ($CHCl_3$, KBr) 3466, 2943, 1646, 1504, 1463, 1199, 1150, 1127, 1041 per cm; ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.62 (d, 1H, J = 15.3) 6.82 (d, 1H, J = 15.4) 6.76 (s, 2H) 6.10 (d, 2H, J = 1.9) 6.06 (d, 1H, J = 1.8) 3.90 (s, 6H) 3.88 (s, 3H) 3.83 (s, 4H) 3.78 (s, 6H) 3.22 (s, 4H); ^{13}C NMR (63 MHz, $CDCl_3$) δ 165.4 161.5 153.4 152.8 143.2 139.6 130.8 116.1 105.0 95.5 92.1 61.0 56.2 55.3 49.6; LC-MS (ESI+) m/z 465.17 [M+Na]. Anal. Calcd for ($C_{24}H_{30}N_2O_6$): C, 65.14; H, 6.83; N, 6.33. Found: C, 65.14; H, 6.83; N, 6.35.

(E)-1-(4-Benzylpiperidin-1-yl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (18)

White solid. mp 120–122 °C; R_f = 0.5 (ethyl acetate/hexanes = 2:1, v/v); IR ν_{max} ($CHCl_3$, KBr) 3466, 2209, 1645, 1456, 1125 per cm; ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.55 (d, 1H, J = 15.3), 7.32–7.21 (m, 5H), 6.78 (d, 1H, J = 15.3), 6.73 (s, 2H), 4.74–4.61 (m, 1H), 4.17–4.05 (m, 1H), 3.89 (s, 6H), 3.87 (s, 3H), 3.05 (t, 1H, J = 12.2), 2.63–2.51 (m, 2H), 1.74 (d, 3H, J = 12.4), 1.30–1.14 (m, 2H); ^{13}C NMR (63 MHz, $CDCl_3$) δ 165.3 153.4 142.4 139.9 139.4 131.1 129.1 128.3 126.1 116.9 104.9 61.0 56.2 46.3 43.0 38.4 31.9; LC-MS (ESI+) m/z 418.10 [M+Na].

(E)-N-4-ethylphenyl 3,4,5-trimethoxycinnamamide (19)

Pale yellow solid. mp 135–136 °C; R_f = 0.1 (ethyl acetate/hexanes = 2:1, v/v); IR ν_{max} ($CHCl_3$, KBr) 3247, 1661, 1585, 1504, 1462, 1415, 1330, 1276, 1129, 1007 per cm; ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.65 (d, 2H, J = 15.4), 7.54 (d, 2H, J = 8.0), 7.17 (d, 2H, J = 8.3), 6.73 (s, 2H), 6.51 (d, 1H, J = 15.4), 3.88 (s, 3H), 3.85 (s, 6H), 2.62 (q, 2H), 1.22 (t, 3H); ^{13}C NMR (63 MHz, $CDCl_3$) δ 165.7 153.1 141.4 140.3 139.2 136.1 130.3 128.2 120.9

120.1 104.8 60.8 55.7 28.2 15.5; LC-MS (ESI+) *m/z* 364.14 [M+Na].

Biological testing

Animals and drug administration

C57BL/6 mice were obtained from Daehan Biolink (Eumsung, Korea). It was housed on a 12 h light-dark cycle and maintained at 24 ± 3 °C. All animal procedures were in accordance with the Institutional Animal Care and Use Committee of Ewha Womans University. The mice (male, 20 ± 2 g) were randomly divided into each group and were given saline, morphine, or both morphine and TMCA analogues. The morphine chloride (10 mg/kg/day, Myungmun Pharm., Seoul) was dissolved in saline and TMCA analogues (20 mg/kg/day) were dissolved in 10% cremophor solution containing 2% dimethyl sulfoxide. Morphine and TMCA analogues were administered daily for 7 days intraperitoneally. TMCA analogues were administered 30 min prior to injection of morphine. Naloxone hydrochloride (5 mg/kg, i.p.) was injected 6 h after the final morphine injection for induction of morphine withdrawal syndrome in mice.

Measurement of morphine withdrawal syndrome

Morphine withdrawal syndrome was induced by the injection of naloxone (5 mg/kg), which is a competitive antagonist of opioid receptor. Immediately after the naloxone injection, mice were placed into the individual observation cylinders (25 cm in diameter and 50 cm in high) and the frequency of jumps of each mouse was observed for 30 min.

Receptor binding studies: serotonin receptor

For 5-HT_{1A} receptor binding, aliquots of frozen membrane (10 μg/well) from stable CHO-K1 cell line expressing the human recombinant 5-HT_{1A} receptor (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA), 0.25 nM [^{3}H]8-OH-DPAT (PerkinElmer) and appropriate concentrations of test compounds were added to 0.25 mL of 50 mM Tris–HCl (pH 7.4) buffer containing 10 mM $MgSO_4$, 0.5 mM EDTA, and 0.1% ascorbic acid. Incubations were carried out for 60 min at 27 °C, and these were terminated by rapid filtration using a microbeta filtermate-96 harvester (PerkinElmer) through filtermat A glass fiber filter presoaked in 0.3% polyethylenimine (PEI). The filter was washed with ice cold 50 mM Tris–HCl buffer solution (pH 7.4), and was then covered with MeltiLex, sealed in a sample bag, dried in an oven. The radioactivity retained in the filter was finally counted using MicroBeta Plus (Wallac, Finland). Non-specific binding was defined with 0.5 μM of methiothepin. The binding affinity (IC_{50}) of a compound was calculated by computerized non-linear regression analysis (GraphPad Prism Program, San Diego, CA, USA) using 7–8 varied concentrations of the compound run in duplicate tubes.

For serotonin 5-HT_{2A} receptor binding, an aliquot of frozen membrane from CHO-K1 cell line expressing the human recombinant 5-HT_{2A} receptor (PerkinElmer) and [^{3}H]Ketanserin (1 nM; PerkinElmer) were used in the presence of mianserin (20 μM) as non-specific. The reaction mixture was incubated for 60 min at 27 °C using 50 mM Tris–HCl (pH 7.4) buffer containin 4 mM $CaCl_2$ and 0.1% ascorbic acid, and harvested through filtermat A glass fiber filter presoaked in 0.5% PEI.

For serotonin 5-HT_{2C} receptor binding assays, frozen membranes from stable CHO-K1 cell line expressing the human recombinant 5-HT_{2C} receptor (PerkinElmer), 1 nM [^{3}H]Mesulergine (Amersham Biosciences, Buckinghamshire, UK) and test compounds were added into 50 mM Tris–HCl (pH 7.4) buffer containing 0.1% ascorbic acid and 4 mM $CaCl_2$. Non-specific binding was determined using 100 μM mianserin. The incubations were performed for 60 min at 27 °C, and these were terminated by rapid filtration through filtermat A glass fiber filter presoaked in 0.5% PEI.

For serotonin 5-HT_6 receptor binding, frozen membrane from stable CHO-K1 cell line expressing the human recombinant 5-HT_6 receptor (PerkinElmer), 1 nM [^{3}H]LSD (PerkinElmer), and appropriate concentrations of test compounds were added to 0.25 mL of 50 mM Tris–HCl (pH 7.4) buffer containing 0.1% ascorbic acid and 4 mM $CaCl_2$. Non-specific binding was determined using 10 μM methiothepin. The incubations were performed for 60 min at 27 °C, and these were terminated by rapid filtration through filtermat A glass fiber filter presoaked in 0.5% PEI.

For 5-HT_7 receptor binding, cell membrane from stable CHO-K1 cell line expressing the human recombinant 5-HT_7 receptor (PerkinElmer), 3 nM [^{3}H]LSD and appropriate concentrations of test compounds were added to 0.25 mL of 50 mM Tris–HCl (pH 7.4) buffer containing 10 mM $MgSO_4$ and 0.5 mM EDTA. The mixture was incubated for 120 min at 27 °C, and the reaction was terminated by rapid filtration through filtermat A glass fiber filter presoaked in 0.3% PEI. Non-specific binding was determined using 10 μM methiothepin.

Primary cortical cell culture

Cortical cell culture was prepared from embryo of the C57BL/6 mice gestational age of 15 days. The brain was dissected and kept in an ice-cold solution. The cortical tissues were dissociated to single cells by a gentle suspension. The cell suspension was centrifuged at 112 × *g* for 5 min, and the resulting pellets were suspended in the medium, MEM (Gibco, BRL, Rockville, MD, USA) supplemented with 5% heat-inactivated fetal calf serum and house serum (Gibco), described on the plate onto coated with Poly-D-Lysine (Sigma, St Louis, MO, USA). The cells were cultured in a CO_2 incubator (5% [v/v], 37 °C). Seven

days after plating, the cells were treated with 10 μM cytosine arabinofuranoside (Ara C) to reduce the growth of contaminating non-neuronal cells. The cortical neuronal cells were cultured in MEM supplemented with 10% horse serum, but without glutamine.

Western immunoblot analysis

(+)8-OH-DPAT (1 μM) was treated for various times (2, 5, 10, 30, 60 min) or was co-treated with WAY 100635 (1 μM), a selective antagonist of 5-HT_{1A} receptor, for 30 min in cultured cortical neuronal cell. TMCA analogues (10 μM) were treated with WAY 100635 (1 μM) for 30 min in cortical neuronal cells. The cortical neuronal samples were extracted with lysis buffer (150 mM NaCl, 50 mM Tris–Cl pH 8, 1 mM EDTA, 1% Triton-X 100, 1 mM PMSF, 1 mM Na_3Vo_4) and centrifuged at 16 300 × *g* for 15 min at 4 °C. Protein concentrations were determined using the protein assay kit (PIERCE Biotechnology, Rockford, IL, USA). 4× sample buffer was added to extract and boil for 4 min at 100 °C. Equal amount of cellular protein was separated by 10% polyacrylamide gels and was electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes. Transfer blots were blocked in 3% skim milk in TBS-Tween 0.1% for 1 h and incubated with antibodies specific to ERK1/2 and pERK1/2 (ERK1/2, 1:1000; and pERK1/2, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), GAPDH (1:1000; Cell signaling) at 4 °C overnight. Blots were washed three times for 30 min in 0.1% TBST, and incubated with an appropriate horseradish peroxidase-conjugated secondary antibody (Zymed Laboratiories, San Francisco, CA, USA). Bound antibodies were visualized following chemiluminescence detection (GE Healthcare Life Science, Pittsburg, PA, USA).

Statistical analysis

All values are expressed as mean ± standard error (SE). The results were subjected to an analysis of the variance (ANOVA) using the Newman–Keuls Multiple Comparison Test to analyze the difference.

Results and Discussion

Chemistry

A series of TMCA derivatives was prepared from commercially available TMCA (**3**) in high yield. The generation of ketones **7–8** was accomplished with 1.2 equivalents of *N*,*O*-dimethylhydroxyamine via global coupling method as EDCI and HOBt or 2-(7-aza-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HATU)/CH_2Cl_2 to produce key intermediate *N*-methoxy-*N*-methyl 3,4,5-trimethoxycinnamamide (**6**), which was smoothly condensed with freshly prepared Grignard Reagents (ethyl magnesium bromide or phenyl magnesium bromide) to give *(E)*-ethyl 3,4,5-trimethoxycinnamyl ketone (**7**) and *(E)*-phenyl 3,4,5-trimethoxycinnamyl ketone (**8**) in 27% or 63% yield, respectively (Scheme 1).

Treatment of acid **3** with 1.2 equivalents of thionyl chloride afforded 3,4,5-trimethoxycinnamic chloride (**5**), which was subsequently coupled with phenol to generate *(E)*-phenyl 3,4,5-trimethoxycinnamate (**11**) in 69% yield. Compound **3** was directly condensed with methanol or ethanol to give *(E)*-methyl 3,4,5-trimethoxycinnamate (**9**) or *(E)*-ethyl 3,4,5-trimethoxycinnamate (**10**) in 66% or 85% yield, respectively. Indeed, *(E)*-benzyl 3,4,5-trimethoxycinnamate (**12**) was obtained from acid **3** with treatment of 1.2 equivalents of sodium bicarbonate and 1.2 equivalents of benzyl bromide in 94% yield.

On the other hand, compound **3** was consequently coupled with various amines [pyrrolidine, tetrahydrofurfuryl amine, furfuryl amine, morpholine, 4-(3,5-dimethylphenyl)piperazine, 4-benzylpiperidine, 4-ethylaniline] in the presence of EDCI and HOBt in dichloromethane to give amides **13–19** in 65%–93% yields, respectively.

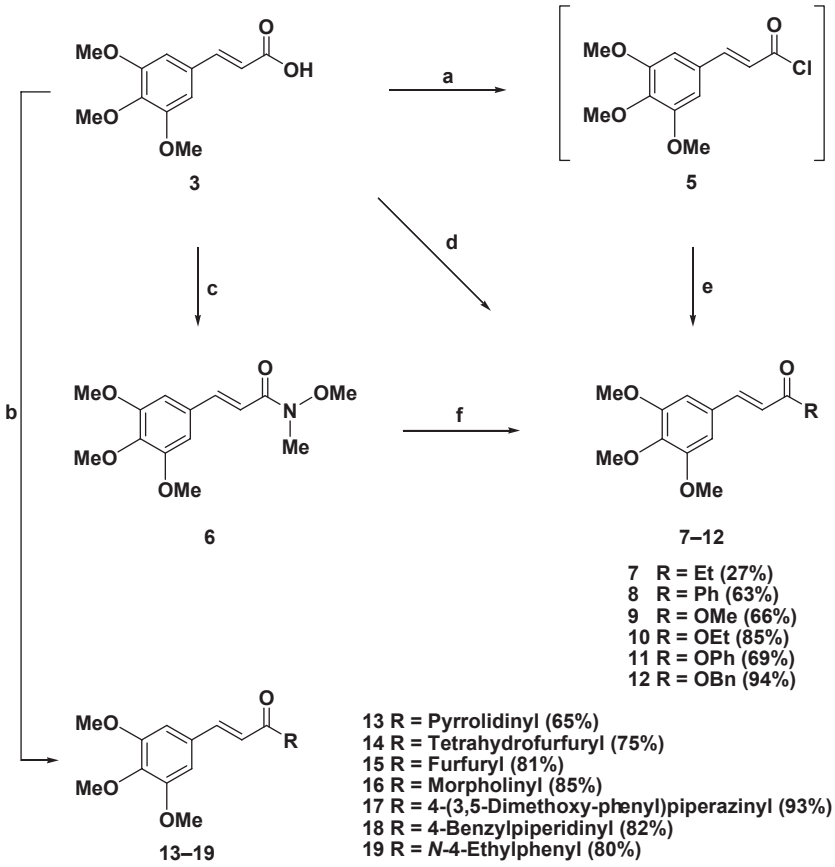
Biology

Effects of TMCA derivatives on naloxone-induced jumping behavior in morphine-dependent mice

The mice received morphine (10 mg/kg, i.p.) daily for 7 days to develop dependence on morphine and morphine-induced withdrawal state (jumping) was examined with TMCA analogues administration (20 mg/kg, i.p.). The naloxone-induced jumping frequency was decreased by treatment with TMCA analogues (Figure 2). Most of TMCA derivatives decreased the jumping frequency in morphine-dependent mice. Especially, TMCA analogues **14**, **16**, **17**, and **18** remarkably attenuated (about 90–98%) the naloxone-induced jumping frequency in morphine-dependent mice. As expected (+)8-OH-DPAT, which is known as a 5-HT_{1A} receptor agonist, strongly suppressed the jumping frequency (86%) in morphine-dependent mice. Of these TMCA analogues, morpholin moiety **16** exhibited potent inhibitory action to jumping frequencies which is indicator of morphine-induced withdrawal symptom, while compound **19** had not shown inhibitory action on jumping frequencies compared with other TMCA analogues. In addition, we found that the 1,4-diatomic moieties **14**, **16**, **17**, and **18** showed excellent activities on naloxone-induced jumping behavior in morphine-dependent mice.

Receptor binding assay of TMCA derivatives

It was examined to identify how to show the biological activity of TMCA derivatives in morphine withdrawal syndrome and in the receptor binding assay on serotonergic (5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_6, and 5-HT_7). Interestingly, all of tested TMCA derivatives showed high binding affinity to the 5-HT_{1A} receptor. The basal form of TMCA has a high affinity to 5-HT_{1A} and 5-HT2c receptor (Table 1).



Scheme 1: Synthesis of ketones **7–8**, esters **9–12**, and amides **13–19** from TMCA (**3**). Reagents and conditions: (a) $SOCl_2$ (1.2 equiv), reflux, 1 h; (b) various amines, HOBt, EDCI, TEA, CH_2Cl_2, rt, 24 h, 65–93%; (c) *N*,*O*-dimethylhydroxylamine, HOBt, EDCI, TEA, CH_2Cl_2, rt, 24 h (for **6**, 75%) or HATU/CH_2Cl_2, rt, 1 h, 80%; (d) MeOH or EtOH, H_2SO_4 (cat) (for **9**, 66%; for **10**, 85%) or BnBr, $NaHCO_2$ (1.2 equiv), DMF/1,4-dioxane (1:1), 90 °C, 24 h (for **12**, 94%); (e) Phenol, 1 h, 69%; (f) Et-MgBr or Ph-MgBr, THF, −78 °C → rt, 2.5 h (for **7**, 27%; for **8**, 63%).

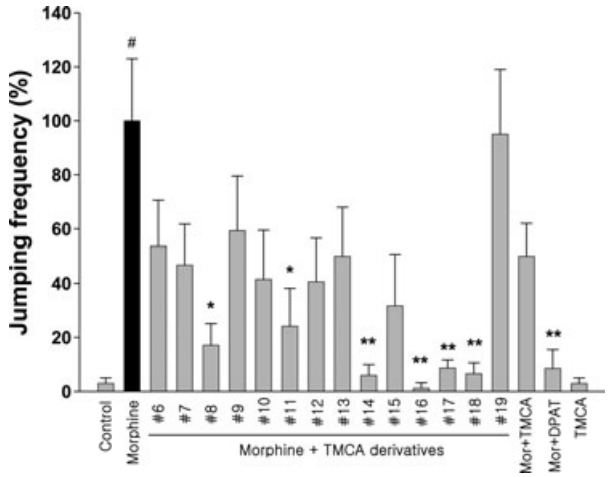


Figure 2: Effects of TMCA derivatives **6–19** on naloxone-induced jumping behavior in morphine-dependent mice. TMCA derivatives (20 mg/kg, i.p.) and (+)8-OH-DPAT (0.1 mg/kg, i.p.) were injected 30 min prior to the morphine injection (10 mg/kg, i.p.). All the mice were injected with the morphine or/and TMCA derivatives for 7 days. On last day, naloxone (5 mg/kg, i.p.) was injected 6 h after the final drug administration. Jumping frequency was observed for 30 min in transparent cylinder. Inhibition of jumping frequency (%) is compared with morphine-treated group. #$p < 0.01$ in comparison with control, *$p < 0.05$, **$p < 0.01$ in comparison with morphine.

(+)8-OH-DPAT induced elevation of extracellular signal-regulated kinase1/2 (ERK1/2) expression in cultured cortical neurons

We examined 5-HT_{1A} receptor activation by measuring the agonist-induced increase in pERK expression level in cultured cortical neurons. As shown in Figure 3A, levels of pERK were increased at 2, 5, 10, 30 min after treatment with (+)8-OH-DPAT (1 μM) in cultured cortical neurons, and pERK level was decreased to the basal level 60 min after treatment with (+)8-OH-DPAT. The pERK levels were highest at 30 min after treatment with (+)8-OH-DPAT, and its elevation was suppressed by the 5-HT_{1A} receptor-specific antagonist WAY 100635 (*N*-{2-[4-(2-methoxyphenyl)-1-piperazinyl] ethyl}-*N*-(2-pyridinyl)cyclohexanecarboxamide, 1 μM). However, WAY 100635 alone did not affect the level of pERK expression (Figure 3B).

Effect of 5-$HT1_A$ receptor-specific antagonist on TMCA derivatives-induced pERK expression in cultured cortical neurons

We determined all of new synthesized TMCA derivatives on 5-HT_{1A} receptor activities. We confirmed that the effect of TMCA derivatives on activation of ERK1/2 and its possible action as the 5-HT_{1A} receptor agonist using WAY 100635, a selective 5-HT_{1A} receptor antagonist. As shown in Fig-

Table 1: Receptor binding affinity of TMCA derivatives

Compound	Receptor binding affinity (IC_{50}, μM)[a]				
	5-HT_{1A}	5-HT_{2A}	5-HT_{2C}	5-HT_6	5-HT_7
6	2.7	>10	>10	>10	>10
7	7.4	>10	>10	>10	>10
8	9.4	>10	>10	>10	>10
9	4.1	>10	>10	>10	>10
10	4.8	>10	>10	>10	>10
11	1.1	>10	>10	>10	>10
12	2.4	>10	>10	>10	>10
13	1.3	>10	>10	>10	>10
14	1.1	>10	>10	>10	>10
15	4.5	>10	>10	>10	>10
16	8.5	>10	>10	>10	>10
17	1.5	>10	>10	>10	>10
18	2.0	>10	>10	>10	>10
19	2.5	>10	1.5	8.8	>10
TMCA	7.6	>10	2.5	>10	>10

TMCA, 3,4,5-trimethoxycinnamic acid.
[a]All compounds were tested at maximum 10 μM of concentration, and their binding affinities were calculated to IC_{50} (μM).

ure 4, when the cells were treated (10 μM) with TMCA derivatives **6**, **8**, **11**, **15**, and **18** for 30 min, derivatives stimulated the activation of ERK1/2 expression in cortical neuronal cells. However, other TMCA derivatives did not strongly stimulate the activation of ERK1/2 (Data not shown). The elevation of pERK expression by TMCA derivatives **6**, **8**, **11**, **15**, and **18** treatment was suppressed by WAY 100635 (1 μM), 5-HT_{1A} receptor-specific antagonist.

We here examined the role of new synthetic TMCA derivatives as antinarcotics, especially in morphine. Administration of the 5-HT_{1A} agonist, (+)8-OH-DPAT, inhibited naloxone-induced withdrawal syndrome in morphine-dependent mice. Interestingly, most of our synthesized TMCA derivatives effectively decreased jumping frequencies, which is an indication of grade of morphine withdrawal syndrome. In agreement with earlier findings (20), the treatment of (+)8-OH-DPAT attenuated the naloxone-induced jumping behavior in morphine-dependent mice. It was known that 5-HT selectively inhibits excitatory amino acid input to the LC during opiate withdrawal (19). Several selective 5-HT_{1A} agonists include (+)8-OH-DPAT that attenuate the antinociception in μ-opioid agonist morphine, not κ-opioid agonist (22). In our results, synthesized TMCA derivatives have high affinity on 5-HT_{1A} receptor. Our results suggest that the TMCA derivatives show antinarcotic effect possibly via activation of 5-HT_{1A} receptors. Using primary cortical neuronal cells expressing 5-HT_{1A} receptors as a model system, we confirmed that stimulation of the 5-HT_{1A} receptor was determined by the measurement of ERK1/2 expression. We observed the effect of 5-HT_{1A} receptors agonist, (+)8-OH-DPAT, on activation ERK1/2 in our cellular system. Synthesized TMCA derivatives induced elevation of ERK1/2 level and its elevation was blocked by the WAY 100635. It might be suggested that the role of antinarcotics effect of synthetic TMCA derivatives might be due to the agonistic action on 5-HT_{1A} receptor in morphine dependent mice.

Conclusion

A series of TMCA derivatives **6–19** was synthesized to elucidate properties of an antinarcotic effect on morphine dependence in mice and their binding affinities on dopaminergic and serotonergic receptors. The synthetic approach involves condensation and coupling reaction of acid **3**. We found that most of TMCA derivatives (20 mg/kg/day) have high affinity to 5-HT_{1A} receptor as well as strong suppressing effect on the naloxone-

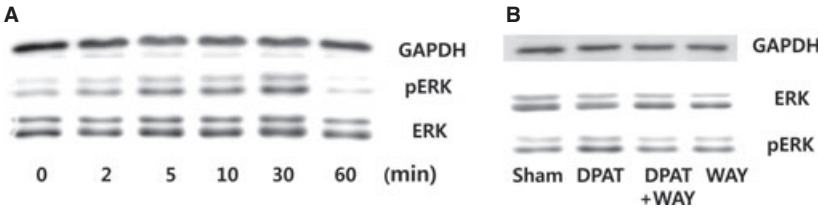


Figure 3: Elevation of pERK level by (+)8-OH-DPAT treatment in cultured cortical neurons. Cells were treated with (+)8-OH-DPAT and each cell was taken at variable time (2, 5, 10, 30, 60 min) (A). Cells were treated with (+)8-OH-DPAT (1 μM) and/or WAY 100635 (1 μM) for 30 min (B). The expression of pERK level was examined using Western blot analysis.

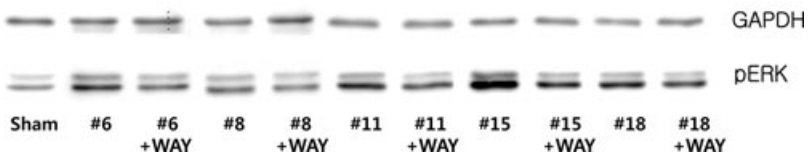


Figure 4: The effects of WAY 100635 on the TMCA derivatives-induced elevation of pERK levels. Cultured neuronal cells were treated with TMCA derivatives (10 μM) and/or WAY 100635 (1 μM) for 30 min. The expression of pERK levels was examined using Western blot analysis.

induced jumping behavior in morphine-dependent mice. It was found that the compound **16** exhibited strong inhibitory effect on the naloxone-induced jumping behavior in morphine-dependent mice among these analogues. In terms of structural characteristics, the presence of a morpholine group and a piperazine group at amide parts was important functionally for an antinarcotic efficacy and binding affinity. We have expected that simple synthesis of new TMCA derivatives and key fragments are useful for the modification of antinarcotic effect on morphine dependence in mice.

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