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Structural requirements for the neuroprotective effects of aspirin analogues against *N*-methyl-D-aspartate and zinc ion neurotoxicity

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Abstract

In order to elucidate the structural requirements for the dual neuroprotective activity of aspirin against *N*-methyl-D-aspartate (NMDA) and zinc ion neurotoxicity, various aspirin analogues and derivatives, modified at the carboxylic group, the acetyl group, and the chain length between the carboxylic acid moiety and phenyl ring, were synthesized. Replacement of the carboxylic acid group with alkyl groups (compounds **2c** and **2d**) resulted in a dramatic increase in neuroprotective activity against NMDA neurotoxicity, while reduction of the carboxylic acid group to the alcohol (compound **2g**) completely abolished this activity. In contrast to NMDA neurotoxicity, compounds that are devoid of the carboxylic acid group did not show any activity against zinc ion neurotoxicity. Replacement of the acetyl group with a propionyl (compound **5a**) or butyryl group (compound **5b**) did not significantly change the activity against NMDA neurotoxicity, but replacement of the acetyl group with a propionyl group (compound **5a**) resulted in a slight decrease in activity against zinc ion neurotoxicity. Compound 12, which has ethylene units between the carboxylic acid moiety and phenyl ring in the structure of aspirin, exhibited greater neuroprotective activity against NMDA neurotoxicity than the compared compounds (aspirin, compound **9** and compound **17**), which have different chain lengths. A similar trend was also observed in the neuroprotective activity against zinc ion neurotoxicity. These results indicate that the carboxylic acid group in aspirin is not indispensable for the inhibitory effect against NMDA neurotoxicity, but is essential for the inhibitory effect against zinc ion neurotoxicity. The acetyl group and ethylene unit's distance are favourable for the inhibitory effect against NMDA neurotoxicity as well as zinc ion neurotoxicity.

Introduction

It is well documented that blockade of *N*-methyl-D-aspartate (NMDA) receptor can protect neurons against ischaemic brain damage and traumatic brain injury because excessive stimulation of NMDA receptors has been implicated in neuronal cell death (Simon et al 1984; Greenmayre 1986; Faden et al 1989; Knopfel et al 1995). In addition to the important role of the NMDA receptor in neuronal cell death, extensive evidence has recently emerged demonstrating a detrimental role of Zn^{2+} in triggering neuronal cell death under various pathological conditions. For example, large amounts of Zn^{2+}, which are stored specifically in the presynaptic vesicles of glutamatergic neurons in the brain and are co-released with glutamate in an activity-dependent manner (Frederickson & Moncrieff 1994), have been known to influence neuronal activity via modulation of excitatory or inhibitory synaptic transmission. Furthermore, Zn^{2+} translocation was observed in degenerating neurons after transient forebrain ischaemia or traumatic brain injury (Rich et al 1995). Thus, neuronal cell death from ischaemia or trauma was prevented by the blockade of Zn^{2+} translocation with Ca-EDTA (Suh et al 2000).

Aspirin, an anti-inflammatory drug, which is widely used to treat stroke patients in the emergency room, was shown to protect neurons against hypoxic-ischaemia or neurotoxins such as NMDA or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Patel et al 1993; Rich et al 1995; Ridker et al 1997). These neuroprotective effects of aspirin have

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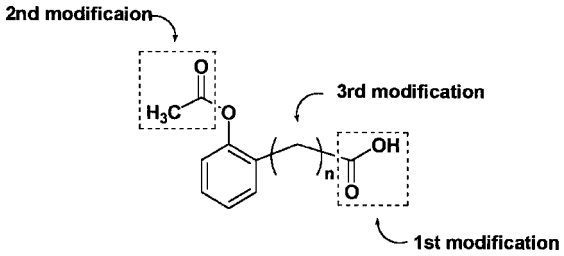

Figure 1 Direction of modifications.

been attributed to the blockade of NF-j B and c-Jun N-terminal kinase or oxidative stress (Grilli et al 1996; Ko et al 1998). In the course of our search for new anti-ischaemic agents, we recently observed that aspirin protects cortical neurons from NMDA and zinc ion neurotoxicity in the millimolar concentration range (Kim et al 2001). This observation prompted us to investigate the structural requirements of aspirin for its neuroprotective effects against NMDA and zinc ion neurotoxicity because the results could provide a clue for the development of an anti-ischaemic agent that has a dual neuroprotective activity against NMDA and zinc ion neurotoxicity.

In the structure of aspirin, there are two distinctive functional groups: the carboxylic acid group and the acetyl group. In order to investigate the role of each functional group on neuroprotective activity, we modified the carboxylic acid group with various alkyl groups, ester and other functional groups, while the acetyl group was replaced with various alkanoyl groups. In addition, we investigated the importance of the chain length between the carboxylic acid and the phenyl ring on the activity, with the insertion of methylene units (Figure 1). We describe here the syntheses and the in-vitro neuroprotective activity of various aspirin analogues and derivatives against NMDA and zinc ion neurotoxicity on ICR mice cerebral cortical cell cultures.

Materials and Methods

Chemicals and instruments

All starting materials were commercially available research grade chemicals and were used without further purification. All solvents were dried and redistilled before use by standard purification methods. Melting points were determined on a Fisher-Johns melting point apparatus and were uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Gemini 200 spectrometer at 200 MHz and 50 MHz, respectively. Chemical shifts are reported in ppm (d) from tetramethylsilane as internal standard. IR spectra were recorded on a Nicolet FT-IR 550 spectrometer. Elemental analyses were performed using a Fisons Eager 200 instrument (Italy). Thin-layer chromatography was performed on precoated silica gel F254 plates (Merck). Flash column chromatography was performed by using Merck silica gel 60 (15–40 l m). For the biological evaluation, aspirin was purchased from Sigma, NMDA was purchased from RBI (Natick, MA), and the cell culture media and serum were purchased from Gibco BRL (Rockville, MD).

General procedure for the *O*-acetylation of 2-substituted phenols

To a solution of 2-substituted phenol (20 mmol) in acetic anhydride (50 mL), concentrated H_2SO_4 (1 mL) at 0°C was carefully added under a nitrogen atmosphere. The reaction mixture was stirred for 30 min at −10°C. Ice chips were then slowly added to the reaction mixture and the solvent was removed in-vacuo. The residue was partitioned between ethyl acetate (100 mL) and H_2O (100 mL). The organic layer was washed with H_2O (30 mL), 10% $NaHCO_3$ solution (3×30 mL), 5% HCl solution (2× 30 mL) and brine (30 mL), and then dried over anhydrous $MgSO_4$. After evaporation of the solvent, the residue was purified by column chromatography to give the desired product.

Methyl 2-acetoxybenzoate (2a)

To a solution of salicylic acid (7.00 g, 50.6 mmol) in methanol (25 mL), concentrated H_2SO_4 (2.5 mL) at 0°C was carefully added under a nitrogen atmosphere. The reaction mixture was stirred for 6 h at 100°C, cooled to room temperature and the solvent removed in-vacuo. The reaction mixture was partitioned between ethyl acetate (200 mL) and H_2O (200 mL). The organic layer was washed with H_2O (100 mL), 10% $NaHCO_3$ solution (100 mL), 5% HCl solution (100 mL) and brine (100 mL), and then dried over anhydrous $MgSO_4$. After filtering, the organic layer was concentrated to give the residue, which was purified by column chromatography to give 5.4 g (69% yield) of methyl 2-hydroxybenzoate as a colourless oil. This was *O*-acetylated as described above to give 3.02 g (67% yield) of methyl 2-acetoxybenzoate (**2a**) as a white solid: mp 46.5°C (lit. 50°C; Parman et al 1997); IR (KBr, cm^{-1}) 3010, 2950, 1756, 1724; ^{1}H NMR ($CDCl_3$) d 2.32 (s, 3H, $COCH_3$), 3.83 (s, 3H, -CO_2CH_3), 7.08 (dd, 1H, J = 1.1, 8.1 Hz, -Ph), 7.27 (m, 1H, -Ph), 7.52 (m, 1H, -Ph), 8.03 (dd, 1H, J = 1.3, 8.0 Hz, -Ph); ^{13}C NMR ($CDCl_3$) d 20.83, 52.02, 123.03, 123.77, 125.90, 131.63, 133.71, 150.63, 164.75, 169.51; elemental analysis for $C_{10}H_{10}O_4$: calculated, C 61.85, H 5.19; found, C 62.08, H 5.36.

Ethyl 2-acetoxybenzoate (2b)

Salicylic acid (7.00 g, 50.6 mmol) in ethanol (33 mL) was subjected to the same reaction described for the synthesis of **2a** to give 5.56 g (86% yield) of ethyl 2-acetoxybenzoate (**2b**) as a colourless oil: IR (neat, cm^{-1}) 3079, 2983, 1767, 1714; ^{1}H NMR ($CDCl_3$) d 1.34 (t, 3H, J = 7.1 Hz, -CH_3), 4.31 (q, 2H, J = 7.1 Hz, -CO_2CH_2-), 7.07 (dd, 1H, J = 1.2, 7.6 Hz, -Ph), 7.29 (dd, 1H, J = 1.2, 7.5 Hz, -Ph), 7.52 (dd, 1H, J = 1.8, 7.9 Hz, -Ph), 8.01 (dd, 1H, J = 1.8, 7.7 Hz, -Ph); ^{13}C NMR ($CDCl_3$) d 13.91, 20.72, 60.81, 123.42, 123.51, 125.74, 131.50, 135.54, 164.33, 169.31; elemental analysis for $C_{11}H_{12}O_4$: calculated, C 63.45, H 5.81; found, C 63.63, H 6.07.

2-Acetoxytoluene (2c)

o-Cresol (5.41 g, 50.0 mmol) was subjected to the *O*-acetylation reaction described above to give 5.8 g (77% yield) of 2-acetoxytoluene (**2c**) as a colourless liquid: IR (neat, cm^{-1}) 3046, 2950, 1769, 1330; ^1H NMR ($CDCl_3$) d 2.19 (s, 3H, $-CH_3$), 2.31 (s, 3H, $-COCH_3$), 7.01 (m, 1H, -Ph), 7.21 (m, 3H, -Ph); ^{13}C NMR ($CDCl_3$) d 15.91, 20.50, 121.73, 125.81, 126.72, 129.93, 130.91, 149.23, 169.01; elemental analysis for $C_9H_{10}O_2$: calculated, C 71.98, H 6.71; found, C 71.85, H 6.67.

2-Acetoxyethylbenzene (2d)

2-Ethylphenol (2.0 g, 16 mmol) was subjected to the *O*-acetylation reaction described above to give 1.2 g (78% yield) of 2-acetoxyethylbenzene (**2d**) as a colourless oil: IR (neat, cm^{-1}) 3072, 3046, 2973, 2940, 1769; ^1H NMR ($CDCl_3$) d 1.35 (t, 3H, J = 7.7 Hz, $-CH_3$), 2.24 (s, 3H, $-COCH_3$), 2.47 (q, 2H, J = 7.3 Hz, $-CH_2-$), 6.95 (m, 1H, -Ph), 7.23 (m, 3H, -Ph); ^{13}C NMR ($CDCl_3$) d 13.87, 20.48, 22.91, 121.94, 125.91, 126.58, 129.14, 135.50, 148.64, 169.11; elemental analysis for $C_{10}H_{12}O_2$: calculated, C 73.15, H 7.37; found, C 73.30, H 7.14.

2-Acetoxypropylbenzene (2e)

2-Propylphenol (2.00 g, 14.7 mmol) was subjected to the *O*-acetylation reaction described above to give 1.8 g (70% yield) of 2-acetoxypropylbenzene (**2e**) as a colourless oil: IR (neat, cm^{-1}) 3072, 3046, 2973, 2940, 1769; ^1H NMR ($CDCl_3$) d 0.94 (t, 3H, J = 7.8 Hz, $-CH_3$), 1.58 (m, 2H, $-CH_2-$), 2.31 (s, 3H, $-COCH_3$), 2.50 (t, 2H, J = 7.5 Hz, $-CH_2-$), 7.03 (m, 1H, -Ph), 7.22 (m, 3H, -Ph); ^{13}C NMR ($CDCl_3$) d 13.81, 20.65, 22.92, 32.01, 122.04, 125.82, 126.68, 130.06, 134.05, 148.77, 169.32; elemental analysis for $C_{11}H_{14}O_2$: calculated, C 74.13, H 7.92; found, C 74.06, H 7.79.

2-Fluorophenyl acetate (2f)

2-Fluorophenol (1.5 g, 13 mmol) was subjected to the *O*-acetylation reaction described above to give 1.62 g (91% yield) of 2-fluorophenyl acetate (**2f**) as a colourless liquid: IR (neat, cm^{-1}) 3080, 2950, 2830, 1775; ^1H NMR ($CDCl_3$) d 2.28 (s, 3H, $-CH_3$), 7.21 (m, 4H, -Ph); ^{13}C NMR ($CDCl_3$) d 19.92, 116.15, 116.51, 123.32, 124.27, 126.88, 138.04, 151.43, 156.37, 167.99; elemental analysis for $C_8H_7FO_4$: calculated, C 62.34, H 4.58; found, C 62.09, H 4.87.

2-Acetoxyphenylmethanol (2g)

To a solution of 2-acetoxysalicylic acid (2.00 g, 11.1 mmol) in dry tetrahydrofuran (THF; 30 mL), B_2H_6 (1.0 M solution in THF, 22 mL) at $-10°C$ was slowly added under a nitrogen atmosphere. The reaction mixture was stirred for 3 h at $-10°C$ and the solvent was removed in-vacuo. The residue was partitioned between ethyl acetate (50 mL) and H_2O (50 mL). The organic layer was washed with H_2O (30 mL), 10% $NaHCO_3$ solution (30 mL), 5% HCl solution (30 mL) and brine (30 mL), and dried over anhydrous $MgSO_4$. After evaporation of the solvent, the residue was purified by column chromatography to give 2.11 g (85% yield) of 2-acetoxyphenylmethanol (**2g**) as a colourless oil: IR (neat, cm^{-1}) 3410, 3045, 2960, 1710; ^1H NMR ($CDCl_3$) d 2.08 (s, 3H, $-CH_3$), 5.11 (s, 2H, $-CH_2-$), 6.90 (m, 2H, -Ph), 7.25 (m, 2H, -Ph), 7.79 (bs, 1H, -OH); ^{13}C NMR ($CDCl_3$) d 20.69, 62.84, 116.82, 120.29, 121.57, 130.57, 131.49, 154.99, 173.28; elemental analysis for $C_9H_{10}O_3$: calculated, C 65.05, H 6.07; found, C 65.27, H 6.02.

2-Propionoxybenzoic acid (5a)

To a solution of salicylic acid (2.00 g, 14.4 mmol) in dry pyridine (40 mL), propionyl chloride (2.68 g, 2.50 mL, 28.9 mmol) at 0°C was slowly added. The reaction mixture was stirred for 30 min at $-10°C$ and then quenched with ice. After the solvent was removed in-vacuo, the reaction mixture was extracted with ethyl acetate (2×50 mL). The organic layer was washed with H_2O (30 mL), 10% HCl solution (3×30 mL), 10% $NaHCO_3$ solution (2×10 mL) and brine, and then dried over anhydrous Na_2SO_4. The crude product was purified by column chromatography followed by recrystallization from ethyl acetate/ether/hexane (1:1:1) to give 1.4 g (93% yield) of 2-propionoxybenzoic acid (**5a**) as a white solid: mp 97°C; IR (KBr, cm^{-1}) 3400, 3036, 2950, 1760, 1330; ^1H NMR ($CDCl_3$) d 1.28 (t, 3H, J = 7.6 Hz, $-CH_3$), 2.65 (q, 2H, J = 7.6 Hz, $-CH_2-$), 7.11 (dd, 1H, J = 1.1, 8.1 Hz, -Ph), 7.35 (dd, 1H, J = 1.1, 7.7 Hz, -Ph), 7.65 (dd, 1H, J = 1.7, 7.8 Hz, -Ph), 8.12 (dd, 1H, J = 1.6, 7.9 Hz, -Ph), 10.65 (bs, 1H, -COOH); ^{13}C NMR ($CDCl_3$) d 8.67, 27.54, 122.19, 123.89, 125.92, 132.33, 134.72, 151.25, 170.21, 173.00; elemental analysis for $C_{10}H_{10}O_4$: calculated, C 62.85, H 5.19; found, C 62.80, H 5.36.

2-Butyroxybenzoic acid (5b)

Salicylic acid (1.00 g, 7.24 mmol) and butyryl chloride (930 mg, 8.69 mmol) were subjected to the same reaction described for the synthesis of **5a** to give 1.40 g (93% yield) of 2-butyroxybenzoic acid (**5b**) as a white solid: mp 81.5°C; IR (KBr, cm^{-1}) 3330, 3020, 2973, 1768, 1683; ^1H NMR ($CDCl_3$) d 1.06 (t, 3H, J = 7.3 Hz, $-CH_3$), 1.82 (m, 2H, $-CH_2-$), 2.61 (t, 2H, J = 7.4 Hz, $-COCH_2-$), 7.12 (d, 1H, J = 8.0 Hz, -Ph), 7.33 (t, 1H, J = 7.7 Hz, -Ph), 7.59 (t, 1H, J = 7.8 Hz, -Ph), 8.12 (d, 1H, J = 7.9 Hz, -Ph), 11.72 (bs, 1H, -COOH); ^{13}C NMR ($CDCl_3$) d 13.58, 17.97, 36.00, 122.39, 123.96, 125.91, 132.34, 134.65, 151.24, 170.20, 172.08; elemental analysis for $C_{11}H_{12}O_4$: calculated, C 63.45, H 5.81, found; C 63.37, H 5.97.

2-Propionoxytoluene (5c)

o-Cresol (2.00 g, 18.5 mmol) and propionyl chloride (3.42 g, 36.9 mmol) were subjected to the same reaction described for the synthesis of **5a** to give 2.21 g (73% yield) of 2-propionoxytoluene (**5c**) as a colourless liquid: IR

(neat, cm^{-1}) 3032, 2980, 2880, 1762, 1130; ^1H NMR ($CDCl_3$) d 1.25 (t, 3H, J = 7.5 Hz, -CH_3), 2.15 (s, 3H, -CH_3), 2.57 (q, 2H, J = 7.6 Hz, -CH_2-), 6.99 (d, 1H, J = 8.0 Hz, -Ph), 7.15 (m, 3H, -Ph); ^{13}C NMR ($CDCl_3$) d 8.99, 15.86, 27.28, 121.63, 125.66, 126.64, 129.82, 130.83, 149.15, 172.34; elemental analysis for $C_{10}H_{12}O_2$: calculated, C 73.15, H 7.37; found, C 73.31, H 7.08.

2-Butyroxytoluene (5d)

o-Cresol (2.00 g, 18.5 mmol) and butyryl chloride (3.94 g, 36.9 mmol) were subjected to the same reaction described for the synthesis of **5a** to give 2.62 g (79% yield) of 2-butyroxytoluene (**5d**) as a colourless liquid: IR (neat, cm^{-1}) 3033, 2973, 2875, 1762; ^1H NMR ($CDCl_3$) d 1.09 (t, 3H, J = 7.5 Hz, -CH_3), 1.77 (m, 2H, -CH_2-), 2.15 (s, 3H, -CH_3), 2.53 (q, 2H, J = 7.6 Hz, -CH_2-), 6.99 (d, 1H, J = 8.0 Hz, -Ph), 7.16 (m, 3H, -Ph); ^{13}C NMR ($CDCl_3$) d 13.46, 15.94, 18.32, 35.78, 121.66, 125.66, 126.64, 129.82, 130.83, 149.17, 171.49; elemental analysis for $C_{11}H_{14}O_2$: calculated, C 74.13, H 7.92; found, C 74.31, H 7.73.

Benzyl 2-acetoxyphenylacetate (8)

To a solution of 2-hydroxyphenylacetic acid (5.00 g, 32.8 mmol) in dry CH_2Cl_2 (100 mL) and triethylamine (4.6 mL), benzyl bromide (6.70 g, 39.1 mmol) at 0°C was slowly added. The reaction mixture was refluxed for 3 h, cooled to room temperature, and then quenched with 1 M HCl solution (10 mL). The solvent was removed in-vacuo. The residue was extracted with ethyl acetate (50 mL) and the organic layer was washed with H_2O (50 mL), 10% HCl solution (3×50 mL), 10% $NaHCO_3$ solution (2×30 mL) and brine, and then dried over anhydrous Na_2SO_4. After evaporation of the solvent, the crude product was isolated by column chromatography and then recrystallized from ethyl acetate/ether/hexane (2:1:1) to give 7.81 g (82% yield) of analytically pure benzyl 2-hydroxyphenylacetate as a white solid: mp 101–102°C; IR (KBr, cm^{-1}) 3410, 3065, 2960, 1723; ^1H NMR ($CDCl_3$) d 3.68 (s, 2H, -CH_2-), 5.11 (s, 2H, -CO_2CH_2-), 6.81 (m, 2H, -Ph), 7.08 (m, 2H, -Ph), 7.33 (s, 5H, -Ph).

To a solution of benzyl 2-hydroxyphenylacetate (5.0 g, 20 mmol) in acetic anhydride (50 mL), concentrated H_2SO_4 (1 mL) at 0°C was carefully added under a nitrogen atmosphere. The reaction mixture was stirred for 30 min at −10°C, ice chips were slowly added, and then the solvent was removed in-vacuo. The residue was partitioned between ethyl acetate (100 mL) and H_2O (100 mL). The organic layer was washed with H_2O (30 mL), 10% $NaHCO_3$ solution (3×30 mL), 5% HCl solution (2×30 mL) and brine (30 mL), and then dried over anhydrous $MgSO_4$. After evaporation of the solvent, the residue was purified by column chromatography to give 5.13 g (87% yield) of benzyl 2-acetoxyphenylacetate (**8**) as a colourless oil: IR (neat, cm^{-1}) 3072, 3039, 2953, 1769; ^1H NMR ($CDCl_3$) d 2.21 (s, 3H, -$COCH_3$), 3.19 (s, 2H, -CH_2-), 5.20 (s, 2H, -CO_2CH_2-), 7.28 (m, 2H, -Ph), 7.31 (m, 2H, -Ph), 7.38 (s, 5H, -Ph).

2-Acetoxyphenylacetic acid (9)

The mixture of **8** (4.00 g, 14.0 mmol) and 10% Pd-C (1.0 g) in THF (90 mL) and MeOH (10 mL) was hydrogenated under 30 psi for 6 h at room temperature. The reaction mixture was filtered and the filtrate was evaporated in-vacuo. The crude product was purified by column chromatography and recrystallized from ethyl acetate/ether/hexane (2:1:1) to give 1.8 g (66% yield) of 2-acetoxyphenylacetic acid (**9**) as a white solid: mp 71.5–72°C; IR (KBr, cm^{-1}) 3300, 3065, 2960, 1763, 1710; ^1H NMR ($CDCl_3$) d 2.30 (s, 3H, -$COCH_3$), 3.58 (s, 2H, -CH_2-), 7.06 (d, 1H, J = 7.9 Hz, -Ph), 7.32 (m, 3H, -Ph), 11.35 (s, 1H, -COOH); ^{13}C NMR ($CDCl_3$) d 20.07, 36.09, 122.53, 125.82, 126.19, 128.69, 131.35, 149.03, 169.15, 176.71; elemental analysis for $C_{10}H_{10}O_4$: calculated, C 61.85, H 5.19; found, C 62.03, H 5.30.

2-Acetoxycinnamic acid (11)

2-Hydroxycinnamic acid (6.00 g, 36.5 mmol) was subjected to the *O*-acetylation reaction described above to give 6.12 g (81% yield) of 2-acetoxycinnamic acid (**11**) as a white solid: mp 157.5–158°C; IR (KBr, cm^{-1}) 3400, 3053, 2960, 2825, 1773, 1680; ^1H NMR ($CDCl_3$) d 2.39 (s, 3H, -$COCH_3$), 6.48 (d, 1H, J = 16.1 Hz, vinyl-H), 7.15 (d, 1H, J = 8.0 Hz, -Ph), 7.28 (t, 1H, J = 7.8 Hz, -Ph), 7.46 (t, 1H, J = 7.7 Hz, -Ph), 7.67 (d, 1H, J = 7.7 Hz, -Ph), 7.86 (d, 1H, J = 16.1 Hz, vinyl-H), 11.45 (bs, 1H, -COOH); ^{13}C NMR ($CDCl_3$) d 20.93, 119.36, 123.15, 126.83, 126.68, 127.83, 131.57, 140.29, 149.40, 169.15, 172.15; elemental analysis for $C_{11}H_{10}O_4$: calculated, C 64.07, H 4.89; found, C 64.01, H 5.08.

3-(2-Acetoxyphenyl)propionic acid (12)

A mixture of **11** (2.00 g, 9.70 mmol) and 10% Pd-C (0.2 g) dissolved in THF (90 mL) and MeOH (10 mL) was hydrogenated under 20 psi for 5 h at room temperature. The reaction mixture was filtered and the filtrate was evaporated in-vacuo. The crude product was obtained by column chromatography and was recrystallized from ethyl acetate/ether/hexane (2:1:1) to give 1.94 g (94% yield) of 3-(2-acetoxyphenyl)propionic acid (**7**) as a white solid: mp 54–54.5°C; IR (KBr, cm^{-1}) 3430, 3048, 2940, 1769, 1703; ^1H NMR ($CDCl_3$) d 2.32 (s, 3H, -$COCH_3$), 2.65 (t, 2H, J = 7.8 Hz , -CH_2-), 2.89 (t, 2H, J = 7.8 Hz , -CH_2-), 7.02 (d, 1H, J = 8.0 Hz, -Ph), 7.35 (m, 3H, -Ph); ^{13}C NMR ($CDCl_3$) d 20.81, 25.10, 34.12, 122.39, 126.24, 127.65, 129.96, 131.88, 148.83, 169.53, 179.02; elemental analysis for $C_{11}H_{12}O_4$: calculated, C 63.45, H 5.81; found, C 63.45, H 6.00.

Ethyl 2-*O*-methoxymethylphenylacetate (13)

2-Hydroxyphenylacetic acid (8.00 g, 52.5 mmol) in ethanol (25 mL), concentrated H_2SO_4 (2.5 mL) at 0°C was carefully added under a nitrogen atmosphere. The reaction mixture was stirred for 6 h at 100°C, cooled to room temperature, and the solvent removed in-vacuo. The reaction mixture was partitioned between ethyl acetate (200 mL) and H_2O

(200 mL). The organic layer was washed with H_2O (100 mL), 10% $NaHCO_3$ solution (100 mL), 5% HCl solution (100 mL) and brine (100 mL), and then dried over anhydrous $MgSO_4$. After filtering, the organic layer was concentrated to give the residue, which was purified by column chromatography to give 8.53 g (90% yield) of ethyl 2-hydroxyphenylacetate as a pale yellow oil: IR (neat, cm^{-1}) 3400, 3030, 2950, 1720; ^1H NMR ($CDCl_3$) d 1.98 (t, 3H, J = 7.3 Hz, -CH_3), 3.62 (s, 2H, -CH_2-), 4.14 (q, 2H, J = 7.3 Hz, -CO_2CH_2-), 6.82 (m, 2H, -Ph), 7.06 (m, 2H, -Ph), 7.65 (s, 1H, -OH). To a suspension of ethyl 2-hydroxyphenylacetate (5.00 g, 27.7 mmol) and 95% NaH (0.87 g, 36.0 mmol) in dry THF (100 mL), chloromethylmethyl ether (2.68 g, 33.2 mmol) was added under a nitrogen atmosphere. The reaction mixture was stirred for 5 h at room temperature. The solvent was removed in-vacuo and the residue was dissolved in ethyl acetate (100 mL). The organic layer was washed with H_2O (50 mL), 10% $NaHCO_3$ solution (50 mL), 5% HCl solution (50 mL) and brine (50 mL), and then dried over anhydrous $MgSO_4$. After evaporation of the solvent, the residue was purified by column chromatography to give 5.31 g (90% yield) of ethyl 2-*O*-methoxymethylphenylacetate (**13**) as a pale yellow oil: IR (neat, cm^{-1}) 3078, 3010, 2980, 1743; ^1H NMR ($CDCl_3$) d 1.25 (t, 3H, J = 7.2 Hz, -CH_3), 3.46 (s, 3H, -OCH_3), 3.66 (s, 2H, -CH_2-), 4.18 (q, 2H, J = 7.2 Hz, -CO_2CH_2-), 5.18 (s, 2H, -OCH_2O-), 7.01 (d, 1H, J = 7.2 Hz, -Ph), 7.16 (d, 1H, J = 7.9 Hz, -Ph), 7.24 (m, 2H, -Ph); ^{13}C NMR ($CDCl_3$) d 13.19, 35.82, 55.57, 60.24, 93.98, 113.63, 121.34, 123.53, 128.19, 130.67, 154.94, 173.55; elemental analysis for $C_{12}H_{16}O_4$: calculated, C 64.27, H 7.19; found, C 64.21, H 7.02.

2-(2-*O*-Methoxymethylphenyl)acetaldehyde (14)

To a solution of **13** (3.00 g, 13.3 mmol) in dry THF (50 mL), DIBAL-H (1.0 M in hexane, 29.5 mL, 29.5 mmol) was carefully added using a syringe at −10°C under a nitrogen atmosphere. The reaction mixture was stirred for 1 h at −10°C and then quenched with 1 M HCl solution (20 mL). The solvent was removed in-vacuo and the residue was dissolved in ethyl acetate (100 mL). The organic layer was washed with H_2O (50 mL), 10% $NaHCO_3$ solution (50 mL), 5% HCl solution (50 mL) and brine (50 mL), and then dried over anhydrous $MgSO_4$. After evaporation of the solvent, the product was purified by column chromatography to give 2.12 g (85% yield) of 2-(2-*O*-methoxymethylphenyl)ethanol as a colourless oil: IR (neat, cm^{-1}) 3380, 3072, 2940, 2830; ^1H NMR ($CDCl_3$) d 2.46 (bs, 1H, -OH), 2.92 (t, 2H, J = 7.3 Hz, -CH_2-), 3.49 (s, 3H, -OCH_3), 3.85 (t, 2H, J = 7.3 Hz, -CH_2O-), 5.18 (s, 2H, -OCH_2O-), 6.99 (m, 1H, -Ph), 7.19 (m, 3H, -Ph). To a solution of 2-(2-*O*-methoxymethylphenyl)ethanol (1.82 g, 10.0 mmol) in dry CH_2Cl_2 (100 mL), PCC (4.31 g, 20.0 mmol) at 0°C was slowly added under a nitrogen atmosphere. The reaction mixture was stirred for 30 min at room temperature and then filtered through celite. The solvent was removed in-vacuo and the residue was washed with H_2O (50 mL), 5% NaOH solution (2×50 mL), 10% HCl solution (2×30 mL) and brine, and then dried over anhydrous Na_2SO_4. After evaporation of the solvent, the residue was purified by column chromatography to give 2.11 g (85% yield) of 2-(2-*O*-methoxymethylphenyl)acetaldehyde (**14**) as a colourless oil: IR (neat, cm^{-1}) 3065, 2950, 2830, 2720, 1730; ^1H NMR ($CDCl_3$) d 3.45 (s, 3H, -OCH_3), 3.67 (d, 2H, J = 2.0 Hz, -CH_2-), 5.18 (s, 2H, -OCH_2O-), 6.99 (m, 1H, -Ph), 7.15 (m, 3H, -Ph), 9.70 (t, 1H, J = 2.0 Hz, -CHO); ^{13}C NMR ($CDCl_3$) d 45.39, 55.88, 94.00, 113.65, 121.62, 128.76, 131.18, 155.16, 199.61; elemental analysis for $C_{10}H_{12}O_3$: calculated, C 66.65, H 6.71; found, C 66.85, H 6.96.

Ethyl 4-(2-hydroxyphenyl)-2-butenoate (15)

To a solution of **14** (1.20 g, 6.66 mmol) and 4 Å molecular sieve (10.3 g) in dry THF (50 mL), triethyl phosphonoacetate (1.79 g, 7.99 mmol) and LiOH.H_2O (335 mg, 7.99 mmol) at room temperature were added under a nitrogen atmosphere. The reaction mixture was stirred for 5 h at 40°C and then filtered through celite. The solvent was removed in-vacuo and the residue was dissolved in ethyl acetate (100 mL). The organic layer was washed with H_2O (50 mL), 10% $NaHCO_3$ solution (50 mL), 5% HCl solution (50 mL) and brine, and then dried over anhydrous $MgSO_4$. After evaporation of the solvent, the crude product was purified by column chromatography to give 1.47 g (88% yield) of ethyl 4-(2-*O*-methoxymethylphenyl)-2-butenoate as a colourless oil: ^1H NMR ($CDCl_3$) d 1.24 (t, 3H, J = 7.0 Hz, -CH_3), 3.43 (s, 3H, -OCH_3), 3.51 (d, 2H, J = 6.6 Hz, -CH_2-), 4.17 (q, 2H, J = 7.0 Hz, -CO_2CH_2-), 5.13 (s, 2H, -OCH_2O-), 5.88 (d, 1H, J = 16.4 Hz, =CHCOO-), 6.86 (m, 3H, -Ph), 7.17 (m, 2H, vinyl-H, -Ph). The solution of ethyl 4-(2-*O*-methoxymethylphenyl)-2-butenoate (1.40 g, 5.57 mmol) dissolved in acetic acid (25 mL) and H_2SO_4 (25 mL) was stirred for 3 h at 80°C and then cooled to room temperature. Ice chips (30 g) were added to the solution and the resulting reaction mixture was extracted with ethyl acetate (2×50 mL). The organic layer was washed with H_2O (50 mL), 5% NaOH solution (2×50 mL), 10% HCl solution (2×30 mL) and brine, and then dried over anhydrous Na_2SO_4. After evaporation of the solvent, the product was purified by column chromatography to give 1.05 g (77% yield) of ethyl 4-(2-hydroxyphenyl)-2-butenoate (**15**) as a colourless oil: IR (neat, cm^{-1}) 3390, 3030, 2980, 1720, 1705; ^1H NMR ($CDCl_3$) d 1.26 (t, 3H, J = 7.0 Hz, -CH_3), 3.51 (d, 2H, J = 6.6 Hz, -CH_2-), 4.15 (q, 2H, J = 7.0 Hz, -CO_2CH_2-), 5.82 (d, 1H, J = 16.4 Hz, =CHCOO-), 6.84 (m, 3H, -Ph), 7.18 (m, 3H, -OH, vinyl-H, -Ph); ^{13}C NMR ($CDCl_3$) d 13.92, 32.87, 115.21, 120.16, 121.15, 123.89, 130.25, 148.36, 154.02, 167.77.

4-(2-Acetoxyphenyl)-2-butenoic acid (16)

A solution of **15** (1.0 g, 4.8 mmol) in acetonitrile (10 mL), THF (10 mL) and 1.5 M NaOH solution (10 mL) was stirred for 30 min at room temperature. The reaction mixture was acidified with 1 M HCl solution to give a white precipitate. The precipitate was filtered and then recrystallized from ethyl acetate/ether/hexane (1:1:1) to give 0.78 g (90%

yield) of 4-(2-hydroxyphenyl)-2-butenoic acid as a white solid: mp 109.5°C; IR (KBr, cm^{-1}) 3410, 3072, 2933, 1690, 1650; ^1H NMR ($CDCl_3$) d 3.43 (d, 2H, J = 6.6 Hz, -CH_2-), 5.72 (d, 1H, J = 15.6 Hz, =CHCOO-), 6.87 (m, 2H, -Ph), 7.06 (m, 3H, vinyl-H, -Ph), 9.52 (bs, 1H, -COOH). 4-(2-Hydroxyphenyl)-2-butenoic acid (750 mg, 16.3 mmol) was subjected to the *O*-acetylation reaction as described above to give 840 mg (91% yield) of 4-(2-acetoxyphenyl)-2-butenoic acid (**16**) as a white solid: mp 75.5–76.0°C; IR (KBr, cm^{-1}) 3200, 3050, 2920, 2830, 1755, 1680; ^1H NMR ($CDCl_3$) d 2.31 (s, 3H, -$COCH_3$), 3.46 (d, 2H, J = 6.7 Hz, -CH_2-), 5.78 (d, 1H, J = 15.6 Hz, =CHCOO-), 7.11 (m, 2H, -Ph), 7.23 (m, 3H, vinyl-H, -Ph), 9.52 (bs, 1H, -COOH); ^{13}C NMR ($CDCl_3$) d 20.84, 33.10, 121.94, 122.58, 126.36, 128.17, 129.26, 130.51, 148.59, 148.85, 169.24, 171.75; elemental analysis for $C_{12}H_{12}O_4$: calculated, C 65.45, H 5.49; found, C 65.43, H 5.47.

4-(2-Acetoxyphenyl)butanoic acid (17)

In the presence of 10% Pd-C (100 mg), a solution of 4-(2-acetoxyphenyl)-2-butenoic acid (0.50 g, 2.27 mmol) in THF (36 mL) and MeOH (4 mL) was hydrogenated under 20 psi for 6 h at room temperature. The reaction mixture was filtered and the filtrate was evaporated in-vacuo. The residue was subjected to column chromatography to give the crude product, which was recrystallized from ethyl acetate/hexane (1:1) to give 0.45 g (90% yield) of 4-(2-acetoxyphenyl)butanoic acid (**17**) as a white solid: mp 42.0–42.5°C; IR (KBr, cm^{-1}) 3350, 3065, 2960, 1716; ^1H NMR ($CDCl_3$) d 1.93 (m, 2H, -CH_2-), 2.33 (s, 3H, -$COCH_3$), 2.41 (m, 2H, -CH_2-), 2.63 (m, 2H, -CH_2-), 7.07 (d, 1H, J = 7.4 Hz, -Ph), 7.26 (m, 3H, -Ph), 11.72 (bs, 1H, -COOH); ^{13}C NMR ($CDCl_3$) d 20.45, 24.51, 29.00, 32.89, 122.12, 125.90, 127.04, 130.02, 132.80, 148.68, 169.49, 179.49; elemental analysis for $C_{12}H_{14}O_4$: calculated, C 65.85, H 6.35; found, C 65.69, H 6.65.

Primary cortical cell culture

By following the reported method (Freund & Reddig 1994; Sensi et al 1997; Kim et al 1999), cerebral cortices were removed from the brains of 15-day-old fetal ICR mice in accordance with a protocol approved by our institutional animal care committee. The neocortices were gently triturated and plated on 24-well plates (with 5 hemispheres per plate) precoated with 100 l g mL^{-1} of poly-D-lysine and 4 l g mL^{-1} of laminine. The plating media consisted of Eagle's minimal essential media (MEM, Earle's salts, supplied glutamine-free) supplemented with horse serum (5%), fetal bovine serum (5%), 2 mM glutamine and 21 mM glucose. Cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2. After 7 days in-vitro, the cultures were transferred into a growth medium identical to the plating medium, but lacking the fetal bovine serum. At 7–9 days in-vitro, 10 l M cytosine arabinofuranoside was included to halt the overgrowth of glia. Mixed cultures of neurons and glia were then fed twice a week.

Neurotoxicity experiment

By following the reported method (Freund & Reddig 1994; Sensi et al 1997; Kim et al 1999), brief exposures to 300 l M NMDA for 10 min and 300 l M $ZnCl_2$ for 30 min were carried out in a HEPES-buffered control salt solution (HCSS) consisting of 120 mM NaCl, 5 mM KCl, 1.6 mM $MgCl_2$, 2.3 mM $CaCl_2$, 15 mM glucose, 20 mM HEPES and 10 mM NaOH. After exposure, cultures were exchanged with MEM that was supplemented with glucose (final concentration 25 mM) and sodium bicarbonate (final concentration 26.2 mM). The resulting neuronal cell death was analysed 24 h later by measuring the amount of lactate dehydrogenase (LDH) released into the bathing medium as previously described (Koh & Choi 1987). The percentage neuronal death was normalized to the mean LDH value released 24 h after continuous exposure to 500 l M NMDA (100%) or a sham control (0%). The synthesized drug concentration was fixed at 300 l M in 0.1% DMSO and concurrently added during exposure to NMDA or $ZnCl_2$. Statistical significance was evaluated using the Student–Neuman–Keuls test.

Results and Discussion

Synthesis

Carboxylic acid ester derivatives of aspirin, methyl 2-acetoxybenzoate (**2a**) and ethyl 2-acetoxybenzoate (**2b**), were synthesized by the esterification of salicylic acid with

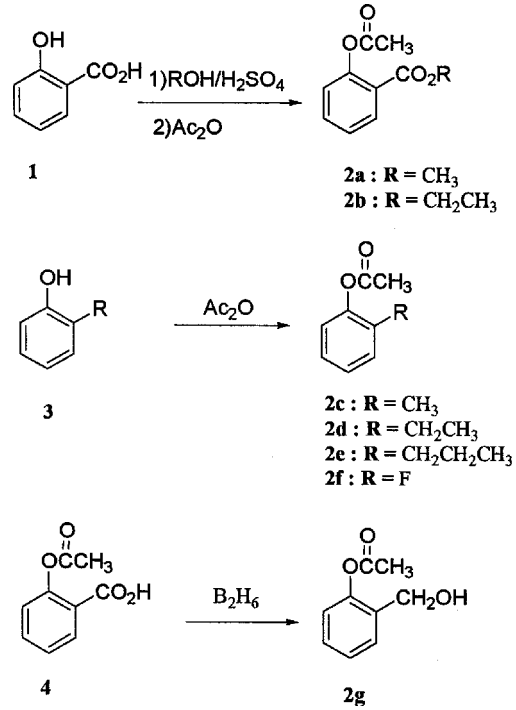

Figure 2 Synthesis of aspirin analogues modified at the carboxylic acid group.

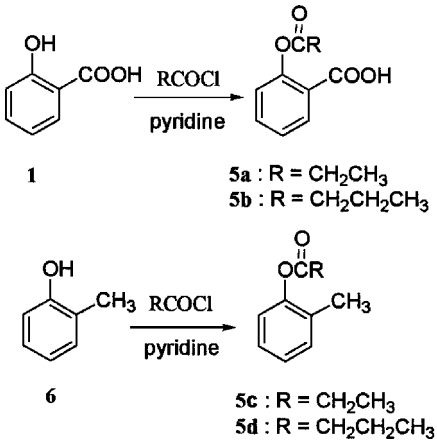

Figure 3 Synthesis of the alkanoyl derivatives of salicylic acid and cresol.

methanol and ethanol followed by *O*-acetylation with acetic anhydride (Figure 2).

Various 2-substituted aspirin derivatives such as 2-acetoxytoluene (**2c**), 2-acetoxyethylbenzene (**2d**), 2-acetoxypropylbenzene (**2e**), and 2-fluorophenyl acetate (**2f**) were prepared from acetylation of the corresponding *o*-cresol, 2-ethylphenol, 2-propylphenol and 2-fluorophenol, using acetic anhydride and H_2SO_4 at $-10°C$. Reduction of 2-acetoxybenzoic acid with B_2H_6 (Brown & Heim 1973) gave 2-acetoxyphenylmethanol (**2g**) in 76% yield.

Alkanoyl derivatives, 2-*O*-propionylbenzoic acid (**5a**) and 2-*O*-butyrylbenzoic acid (**5b**), were prepared by the *O*-acylation of salicylic acid with propionyl chloride and butanoyl chloride in pyridine, respectively. Alkanoyl derivatives of cresol, 2-*O*-propionylcresol (**5c**) and 2-*O*-butyrylcresol (**5d**), were similarly prepared from acylation of *o*-cresol (Figure 3).

The synthesis of an aspirin analogue that has one more methylene unit between the carboxylic acid moiety and the ring in aspirin is outlined in Figure 4. Since there is a possibility of intramolecular cyclization during the acetylation, the carboxylic acid group in 2-hydroxyphenylacetic acid was first protected. Benzylation of 2-hydroxyphenylacetic acid with benzyl bromide/triethylamine in CH_2Cl_2 (Kim et al 1985) followed by the *O*-acetylation gave benzyl 2-acetoxyphenylacetate (**8**) in good yield. Hydrogenolysis of **8** using 10% Pd-C with THF/MeOH (9:1) co-solvent at 30 psi for 6 h removed the benzyl group to afford 2-acetoxyphenylacetic acid (**9**).

3-(2-Acetoxyphenyl)propionic acid (**12**), which has two methylene units between the carboxylic acid moiety and the ring in aspirin, was synthesized from *O*-acetylation of

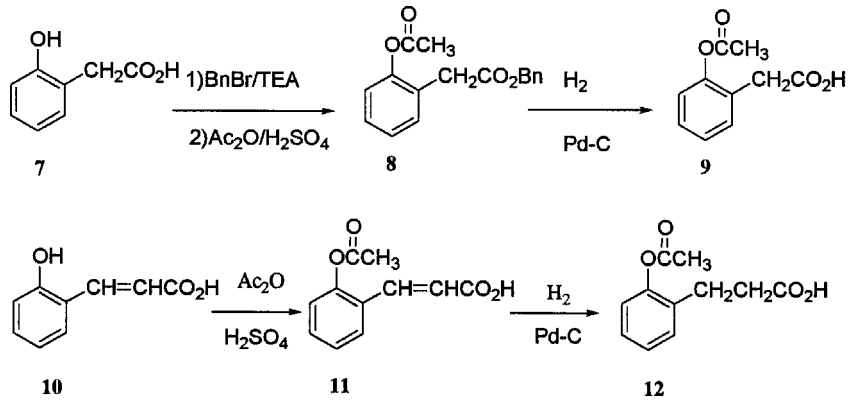

Figure 4 Synthesis of 2-acetoxyphenylacetic acid and 3-(2-acetoxyphenyl)propionic acid.

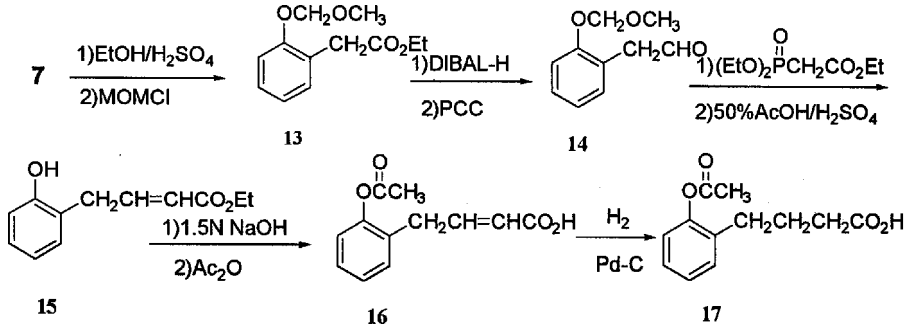

Figure 5 Synthesis of 4-(2-acetoxyphenyl)butanoic acid.

2-hydroxycinnamic acid (**10**) followed by hydrogenation (Vitali et al 1972) with 10% Pd-C in THF/MeOH (9:1) co-solvent (Figure 4).

2-Hydroxyphenylacetic acid (**7**) was used as starting material for the synthesis of 4-(2-acetoxyphenyl)butanoic acid (**17**), which has three methylene units (Figure 5). The esterification of the carboxylic acid group in 2-hydroxyphenylacetic acid (**7**) with ethanol followed by protection of the hydroxyl group with chloromethyl methyl ether (MOM-chloride) gave ethyl 2-*O*-methoxymethylphenylacetate (**13**). Reduction of the ester compound **13** with DIBAL-H in THF (Yoon & Gyoung 1985) gave 2-(2-*O*-methoxymethylphenyl)ethanol, which was subsequently oxidized with pyridinium chlorochromate (PCC) in methylene chloride to afford 2-(2-*O*-methoxy-2-methylphenyl)-acetaldehyde (**14**). Horner–Wadsworth–Emmons type reaction of **14** using triethyl phosphonoacetate, LiOH, and 4 Å molecular sieve in THF at 40°C, followed by the acid-catalysed deprotection of the MOM group, gave **15**. Base-catalysed hydrolysis of the ester group in **15** followed by *O*-acetylation afforded 4-(2-hydroxyphenyl)-2-butenoic acid (**16**). Finally, hydrogenation of the double bond in **16** gave 4-(2-acetoxyphenyl)butanoic acid (**17**).

Biological evaluation

Aspirin analogues were tested for in-vitro inhibitory activity against NMDA and zinc neurotoxicity in cortical cell cultures by measuring LDH release. The results are summarized in Tables 1 and 2.

We initially examined the function of the carboxylic acid group on neuroprotective activity by esterification of the carboxylic acid group with alkyl groups (methyl: **2a**; ethyl: **2b**), and found that these derivatives exhibited significantly improved inhibitory activity against NMDA neurotoxicity compared with aspirin. Since simple transformation of the carboxylic acid group into the lipophilic ester group improved the activity significantly, we further examined whether the carboxylic acid group is necessary for the neuroprotective activity by replacement of the carboxylic acid group with simple alkyl groups. Replacement of the carboxylic acid group with either a methyl group (**2c**) or an ethyl group (**2d**) also exhibited potent inhibitory activity, while the propyl compound **2e** showed higher inhibitory activity than aspirin, but less active than methyl and ethyl derivatives. The introduction of the fluorine group (**2f**), which is known as an isostere of the methyl group, but is less lipophilic than the methyl group (Friedman 1951), at the carboxylic acid group site, resulted in a modest reduction of activity compared with the methyl analogue (compound **2c**). However, reduction of the carboxylic acid group to the alcohol (**2g**) completely abolished neuroprotective activity. These results indicate that the carboxylic acid group is not indispensable for the inhibitory effect against NMDA neurotoxicity, and can be replaced with the appropriate alkyl groups for better activity.

The importance of the acetyl group in aspirin was investigated by replacing the acetyl functional group with the related alkanoyl groups. Thus, when the acetyl group in aspirin was replaced with either a propionyl group or a butanoyl group, the propionyl derivative (**5a**) showed slightly lower activity than aspirin, while the butyryl derivative (**5b**) was equipotent to aspirin. However, in the case where the acetyl group in cresol derivatives was replaced with either a propionyl group (**5c**) or a butyryl group (**5d**), the derivatives showed significantly lower activity than *O*-acetylcresol (**2c**). This result indicates that the acetyl group is favoured for the NMDA activity.

The effects of the chain length between the carboxylic acid group and phenyl ring moiety on inhibitory activity against NMDA neurotoxicity were investigated with compounds **9**, **12** and **17**, and it was observed that the chain length played an important role in the inhibitory activity. Apparently, when the chain length was increased, the activity gradually increased up to the ethylene unit's compound (compound **12**, n = 2) and then decreased dramatically with the propylene unit's compound (compound **17**, n = 3). It is of interest that the flexibility affects the activity significantly. Thus, reduction of the flexibility in the ethylene unit by the introduction of a double bond (compound **11**) exhibited significantly increased activity compared with the flexible compound **12**. This trend of inhibitory effects was also observed in the propylene unit's analogues (compound **16**). These results suggested that the rigid ethylene unit's compound is a more favourable conformation for the NMDA activity than other compounds (n = 0, 1, 3).

Table 1 The effects of aspirin analogues on NMDA neurotoxicity.[a]

Compounds	N[b]	LDH release (%)[c]
NMDA (300 *I* M)	8	100±17.56
Aspirin+NMDA	4	83.38±7.62
2a+NMDA	8	46.45±15.92*
2b+NMDA	8	14.13±23.11*
2c+NMDA	8	11.55±14.70*
2d+NMDA	7	12.89±9.78*
2e+NMDA	7	39.39±9.79*
2f+NMDA	4	70.85±14.2
2g+NMDA	4	128.86±28.22
5a+NMDA	8	87.85±4.24
5b+NMDA	8	82.76±13.86
5c+NMDA	4	82.26±7.64
5d+NMDA	4	92.02±7.44
9+NMDA	8	74.94±22.34
11+NMDA	8	41.99±10.18*
12+NMDA	8	58.43±9.33*
16+NMDA	8	84.51±11.31
17+NMDA	4	96.72±8.22

[a]Neuronal cell death was measured by lactate dehydrogenase (LDH) release after mixed cultures of cortical neurons and glia were fed twice a week until experiments at 13 days in-vitro. [b]Experimental number. [c]Drug concentration was fixed at 300 *I* M (in 1% DMSO). The measured values are expressed as LDH±s.d. (The LDH release of NMDA set a standard for 100%) after the exposure of drugs for 10 min. *$P < 0.05$, significant difference compared with the group treated with aspirin+NMDA (analysis of variance and Student–Neuman–Keuls test).

Table 2 The effects of aspirin analogues on zinc ion neurotoxicity.[a]

Compounds	N[b]	LDH release (%)[c]
Zn^{2+} (300 *I* M)	4	100±16.44
Aspirin+Zn^{2+}	3	28.61±4.33
2a+Zn^{2+}	4	100 >*
2b+Zn^{2+}	4	100 >*
2c+Zn^{2+}	4	100 >*
2d+Zn^{2+}	4	100 >*
2e+Zn^{2+}	4	100 >*
2f+Zn^{2+}	4	93.75±13.44*
2g+Zn^{2+}	4	100 >*
5a+Zn^{2+}	4	35.38±9.26
5b+Zn^{2+}	3	90.88±10.04*
5c+Zn^{2+}	4	97.11±11.66*
5d+Zn^{2+}	4	100 >*
9+Zn^{2+}	4	49.67±6.24*
11+Zn^{2+}	4	86.19±8.98*
12+Zn^{2+}	4	16.87±1.02
16+Zn^{2+}	4	86.92±9.36*
17+Zn^{2+}	4	89.06±17.06*

[a]Neuronal cell death was measured by lactate dehydrogenase (LDH) release after mixed cultures of cortical neurons and glia were fed twice a week until experiments at 13 days in-vitro. [b]Experimental number. [c]Drug concentration was fixed at 300 *I* M. The measured values are expressed with LDH±s.d. after the exposure of drugs for 30 min. *$P < 0.05$, significant difference compared with the group treated with aspirin+Zn^{2+} (analysis of variance and Student–Neuman–Keuls test).

With respect to the inhibitory effect against zinc ion neurotoxicity, it was a surprise to find that most of the tested compounds showed lower activity than aspirin, except compound **12** (Table 2). In contrast to NMDA neurotoxicity, compounds that are devoid of the carboxylic acid group did not show any inhibitory activity against zinc ion neurotoxicity. Furthermore, replacement of the acetyl group in aspirin with a propionyl group (**5a**) resulted in a slight decrease in activity, but replacement of the acetyl group with a butyryl group (**5b**) decreased activity significantly. The distance between the carboxylic acid and the phenyl ring was also very important for the inhibitory effect against zinc ion neurotoxicity and it revealed a similar trend as in NMDA activity. Thus, compound **12**, which has a flexible ethylene unit between the carboxylic acid and the phenyl ring showed a slight increase in activity compared with aspirin, while the propylene unit's compounds (compound **17**) significantly lost the activity. It is of interest to note that the reduction of flexibility resulted in a significant loss of the activity against zinc ion neurotoxicity, although it improved the activity against NMDA neurotoxicity. These results suggest that the carboxylic acid moiety is essential for the inhibitory activity against zinc ion toxicity, but the acetyl group can be modified with a propionyl group without significant loss of activity. In addition, the flexible ethylene group may have a more favourable conformation than aspirin for the inhibitory activity against zinc ion neurotoxicity.

Conclusions

The structure–activity relationship study of aspirin analogues reveals the unique features of aspirin that are responsible for the neuroprotective activity against NMDA and zinc ion neurotoxicity. The carboxylic group is essential for the inhibitory effect against zinc ion neurotoxicity. However, it is not necessary to work against NMDA neurotoxicity. The acetyl group is more favourable than either a propionyl or butyryl group for the inhibitory activity against NMDA and zinc ion neurotoxicity, but can be replaced with a propionyl group without a significant loss of activity.

The distance and flexibility between the carboxylic acid and the phenyl ring are also important for the neuroprotective activity. The results obtained in this study confirm that the rigid ethylene unit between the carboxylic acid and the phenyl ring is desirable for the improved activity against NMDA neurotoxicity, but the flexible ethylene unit's compound is more favourable for the activity against zinc ion neurotoxicity. Therefore, additional structural elaboration of compound **12** may provide improved dual neuroprotective activity against NMDA and zinc ion neurotoxicity.

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