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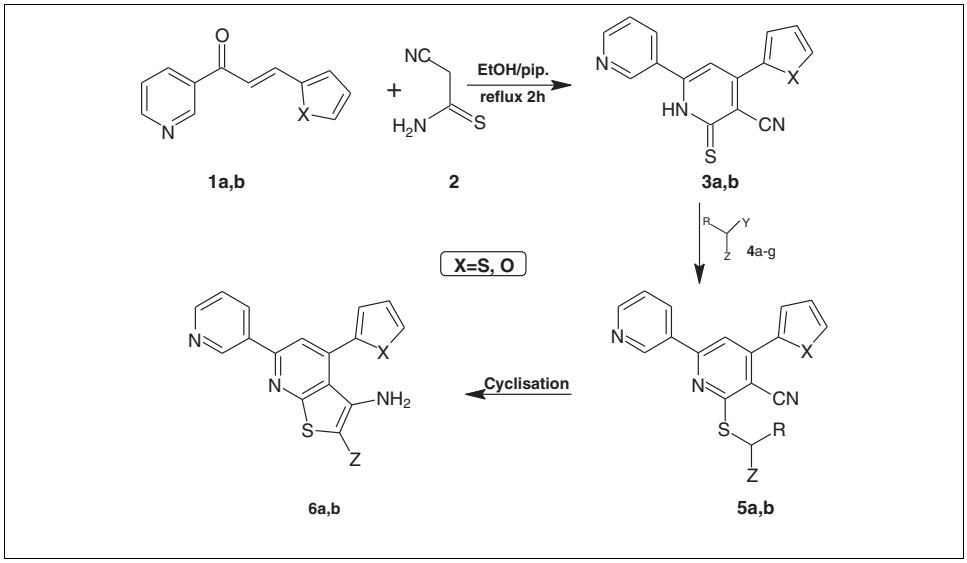

1-Pyridin-3-yl-3-(2-thienyl of 2-furyl)prop-2-en-1-ones **1a,b** reacted with 2-cyanoethanethioamide **(2)** to afford the corresponding 4-(thiophen-2-yl or furan-2-yl)-6-sulfanyl-2,3′-bipyridine-5-carbonitriles **3a,b**. The synthetic potentiality of compounds **3a,b** were investigated in the present study via their reactions with several active halogen containing compounds **4a–h**. Our aim here is the synthesis of 4-(2-thienyl or 2-furyl)-6-pyridin-3-ylthieno[2,3-*b*]pyridin-3-amines **6a–e**,**g–n** via 6-(alkyl-thio)-4-(2-thienyl or 2-furyl)-2,3′-bipyridine-5-carbonitriles **5a–e**,**i–m**. The structures of all newly synthesized heterocyclic compounds were elucidated by considering the data of IR, ^{1}H-NMR, mass spectra, as well as that of elemental analyses. Anti-cancer, anti-Alzheimer, and anti-COX-2 activities were investigated for all the newly synthesized heterocyclic compounds.

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INTRODUCTION

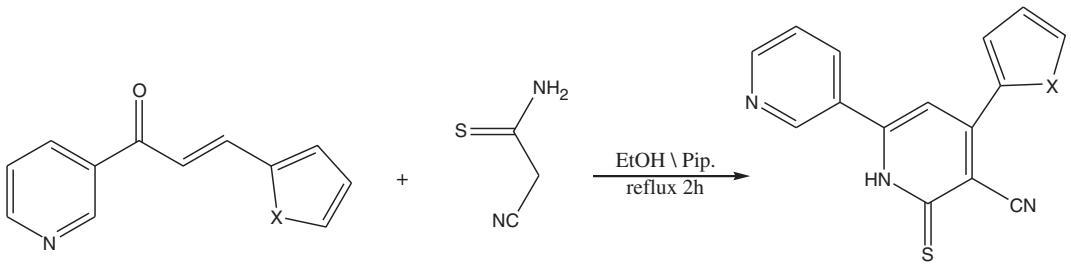
In conjunction to our previous recent work [1–14] and aiming to investigate and evaluate the biological activities of the newly synthesized heterocyclic compounds, we are interested here to use 3-(2-thienyl or 2-furyl)-1-pyridin-3-ylprop-2-en-1-ones **1a,b** as key compounds to synthesize 2,3′-bipyridine-5-carbonitriles **3a,b** required for several chemical transformations as well as our medicinal chemistry programs.

RESULTS AND DISCUSSION

1-Pyridin-3-yl-3-(2-thienyl)prop-2-en-1-one **(1a)** reacted with 2-cyanoethanethioamide **(2)** in absolute ethanol containing the catalytic amount of piperidine under reflux to afford the reaction product. Such reaction product formed via a *Michael addition* of $-CH_2-$ in **2** on $-CH{=}CH-$ of **1a** to give the reaction product **3a**. Considering the IR (cm^{-1}) data of this reaction product; SH (3216), CN (2210) groups and the mass spectrum which gave $m/z = 295$ (100%) that corresponds to the molecular weight of the assigned structure. Also, ^{1}H-NMR (δ ppm) spectrum revealed the presence of a broad signal of SH proton at 14.19 (cf. Scheme 1 and Experimental section).

In a similar manner, 1-pyridin-3-yl-3-(2-furyl)prop-2-en-1-one **(1b)** was reacted with 2-cyanoethanethioamide **(2)** under the same aforementioned experimental conditions to give the finally isolated **3b**. The chemical structure of **3b** was elucidated by considering the data of IR and mass spectra as well as that of elemental analyses (cf. Experimental section). A further confirmation of **3a,b** arose from their synthesis through an alternative pathway via the reaction of each of **1a,b**, malononitrile in a dispersed sulfur,

Scheme 1



1a,b **2** **3a,b**
X = S, O

morpholine, and ethanol under reflux for 2 h [15]. It is important to refer here that **3a,b** obtained by the two pathways are identical in all physical and chemical properties (cf. Scheme 1 and Experimental section).

The synthetic potentiality of each of **3a,b** was investigated through the electrophilic substitution reactions by using several electrophilic **C** species. Thus, it has been found that **3a** was reacted with ethyl chloroacetate **(4a)** in a stirred methanolic sodium methoxide solution at room temperature for 15 min to give the reaction product **5a**. The IR (cm^1) of this reaction product showed the bands of CN (2206) and CO (1736) of the newly introduced COOEt group. Its ^{1}H-NMR (δ ppm) spectrum revealed the signals of $-S\underline{CH_2}-$, $-COO\underline{CH_2}CH_3$, $-COOCH_2\underline{CH_3}$ protons. Depending on these spectral data, we concluded that the good nucleophilicity of **S** in **3a** facilitated the electrophilic attack of **4a** to afford **5a** in very pure state. Furthermore, structure **5a** was elucidated through its cyclization in ethanolic sodium ethoxide under reflux for 30 min to give the reaction product **6a**. The IR spectrum of **6a** showed no bands of CN group, and instead, the bands of the newly formed NH_2 group were detected. Thus, we concluded that both $-SCH_2-$ and CN groups in **5a** were involved in the intramolecular *Thrope-Zieglar* cyclization step to give the finally isolated **6a**. A further confirmation of **6a** structure was obtained through its preparation authentically via the reaction of **3a** with **4a** in ethanolic sodium ethoxide under reflux for 2 h (cf. Scheme 2 and Experimental section).

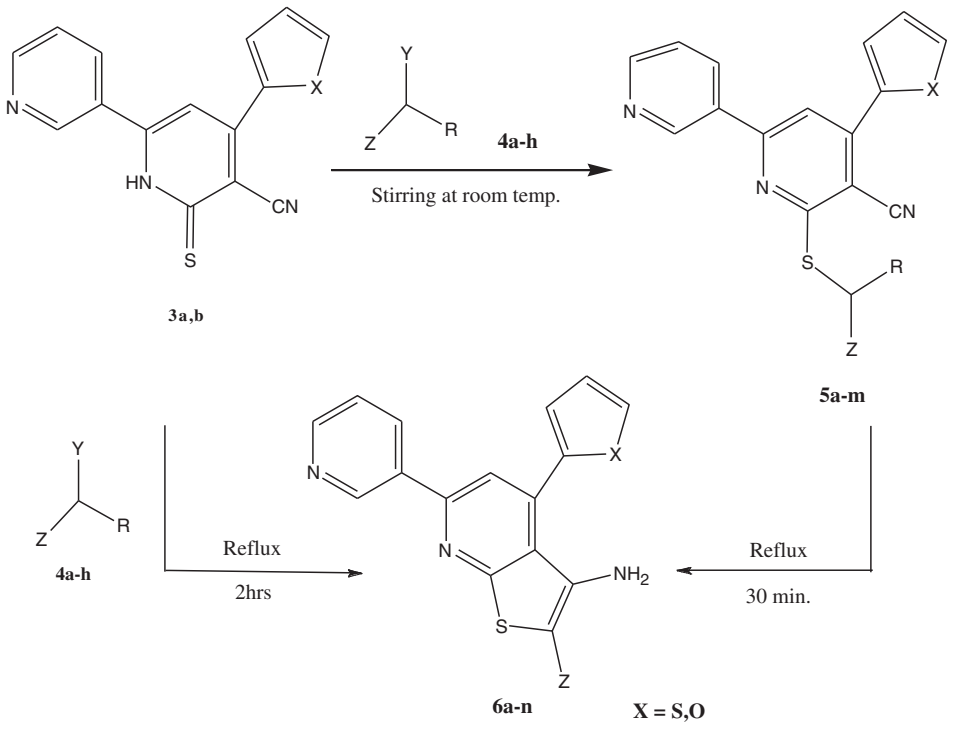
Similarly, **3a** was reacted with each of **4b–e** in a stirred methanolic sodium methoxide solution at room temperature to give the corresponding 2-alkylthio derivatives **5b–e** whose structures were elucidated by considering the data of IR, ^{1}H-NMR, and elemental analyses (cf. Scheme 2 and Experimental section). In a similar way, compounds **5b–e** were cyclized in ethanolic sodium ethoxide under reflux for 30 min and gave the corresponding thieno[2,3-b]pyridine derivatives **6b–e**, respectively, which were obtained also via refluxing of **3a** with each of **5b–e** in ethanolic sodium ethoxide for 2 h.

The structure of **6c** was further confirmed via its preparation through the reaction of **3a** with 3-chloropentan-2,4-dione **4f** (Y═Cl, R═Z═$COCH_3$) in methanolic sodium methoxide solution either under stirring at room temperature or refluxing for 2 h. The reaction seemed to proceed via the intermediate **I**, which converted to **II** that, in turn, released acetic acid molecule to give 2-acetyl-3-amino-4-(2-thienyl)-6-(3-pyridyl)thieno[2,3-b]pyridine **(6c)** (cf. Scheme 3).

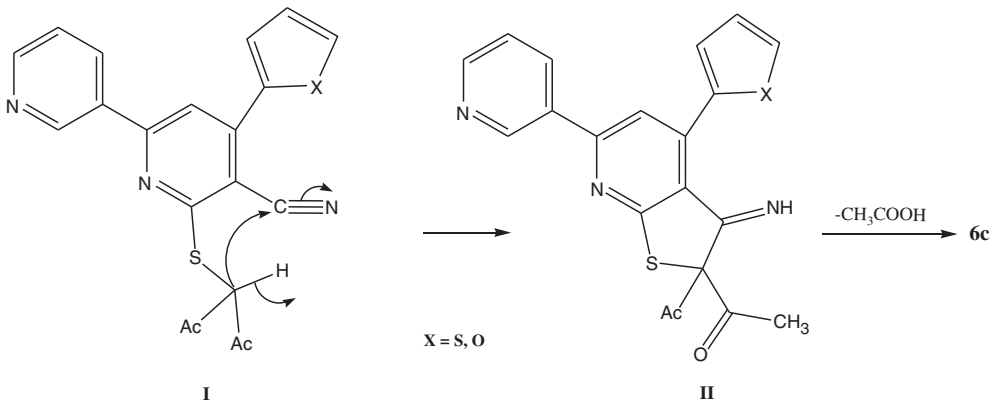
In contrast to the behavior of **3a** towards each of **4a–e**, it has been found that **3a** was reacted with each of **4g,h** either under stirring in methanolic sodium methoxide solution at room temperature or under reflux for 2 h in ethanolic sodium ethoxide to give the corresponding thieno[2,3-b] pyridine derivatives **6g,h**, respectively, whose structures elucidated by considering the data of IR, ^{1}H-NMR, mass spectra, as well as that of elemental analyses (cf. Scheme 2 and Experimental section).

In continuation to our effort for the investigation of the electrophilic substitution reaction along the SH group in each of **3a**,**b**, it has been found that compound **3b** was reacted with each of **4a,b,d,e,g** in a stirred methanolic sodium methoxide at room temperature for 15 min to afford the corresponding 2-alkylthio derivatives **5i–m**. The structures of these reaction products were elucidated by considering the data of their elemental analyses, IR, ^{1}H-NMR (cf. Experimental section). A further elucidation for these structures arose from their cyclization in ethanolic sodium ethoxide solution under reflux for 30 min to give the corresponding thieno[2,3-b]-pyridine derivatives **6i–m**, respectively. An authentic sample of each of **6i–m** were obtained via refluxing a mixture of **3b** and each of **4a**,**b**,**d**,**e**,**g** in ethanolic sodium ethoxide solution for 2 h. In contrast to this behavior, **3b** was reacted with either chloroacetone **4c** or 3-chloropentan-2,4-dione **4f** either under stirring in methanolic sodium methoxide solution at room temperature for 15 min or refluxing for 2 h in ethanolic sodium ethoxide solution to give the corresponding thieno[2,3-b]pyridine derivative **6n**. Unexpectedly, **5m** did not undergo cyclization

Scheme 2



Scheme 3



reaction under various experimental conditions to give the corresponding thieno[2,3-b]pyridine derivative.

BIOLOGICAL EVALUATION

Anti-Alzheimer activity. For compounds **2** and **1a,b**, their relative potency individually is high enough, whereas after their reactions to afford the corresponding bipyridine-5-carbonitriles **3a,b**, their relative potency decreased. For series **5**, the substituted pyridine derivatives have potent activities where the compounds arranged according to descending order of activity: **1a**, **5c**, **3a**, **5b**, **5d**, **5e**, **5a** (cf. Fig. 1). It is worth to mention that as the activity increases, both the pharmacokinetics and pharmacodynamics properties greatly improved to be directed towards a good bioavailability drug profiles (cf. Figs. 2 and 3).

For series **6** where pyridine is fused to another thiophene, all the tested compounds showed moderate potent activities, and the activity in descending order is **3a**, **6d**, **6g**, **6c**, **6h**, **6b**, **6a**, **1a**, **6e** (cf. Figs. 4 and 5). Generally, compounds **6**, with fused thiophene and pyridine rings, is less active than compounds **5**.

Structural activity relationship of anti-Alzheimer activity. Generally for compounds **5**, we note that the

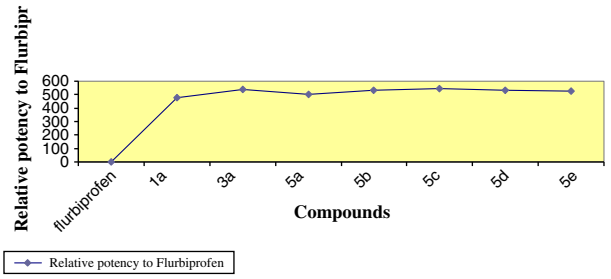


Figure 1. Anti-Alzheimer relative to flurbiprofen of 5 series.

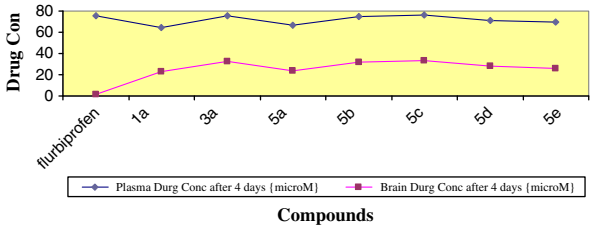


Figure 2. The *in vivo* pharmakinatic of 5 series.

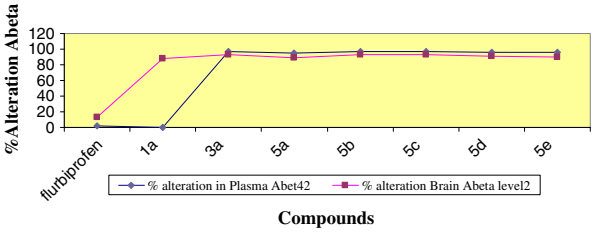


Figure 3. The *in vivo* pharmodynamic of 5 series.

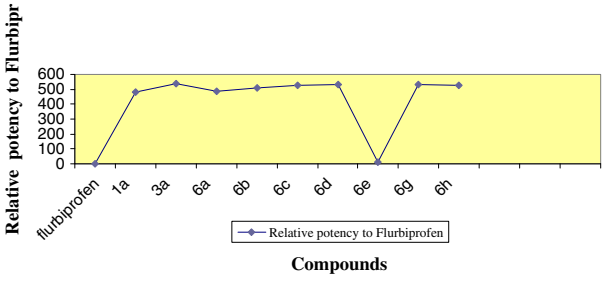


Figure 4. Anti-Alzheimer relative to flurbiprofen of 6 series.

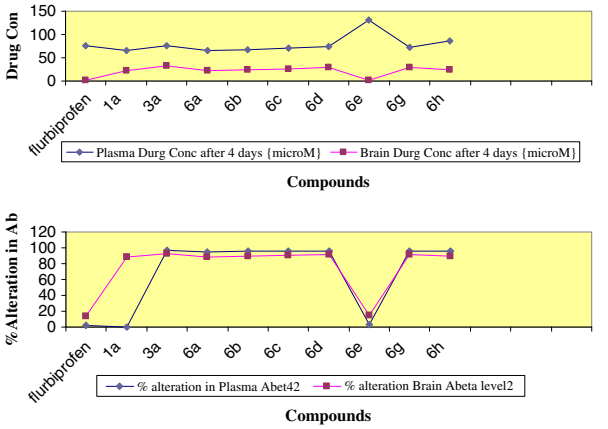


Figure 5. The *in vivo* pharmakinatic (top) and in vivo pharmacodynamic (bottom) of 6 series.

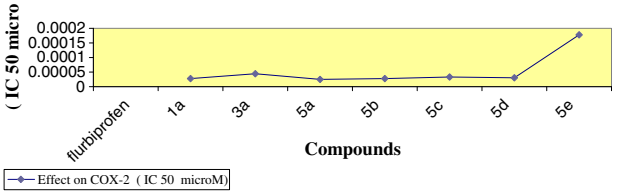


Figure 6. Effect on COX-2 of 5 series.

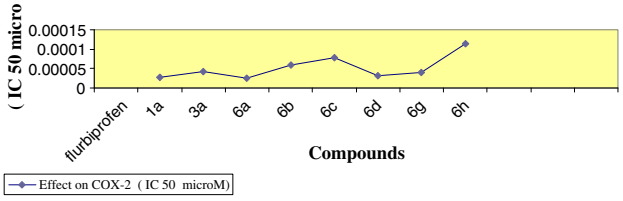


Figure 7. Effect on COX-2 of 6 series.

thienyl derivatives exhibit anti-Alzheimer activity, whereas their furyl analogs have no activity. The order of activity of relative potency for S-substitution in all compounds **5** with thienyl moiety is ketone > amide > acid > ester.

Anti-COX-2 activity. The potent activities of compounds **5** are arranged according to the following descending order: **5e**, **3a**, **5c**, **5d**, **1a**, **5b**, **5a** (cf. Fig. 6).

The relative potency of compounds **6** are found to be more than that of compounds **5**, noting that the relative potency of compounds **6** are arranged in the following descending order: **6h**, **6c**, **6b**, **3a**, **6g**, **6d**, **1a**, **6a** (cf. Fig. 7).

Structural activity relationship for anti-COX-2. Generally for compounds **5**, the thienyl moiety provides the highest activity, whereas furyl moiety is inactive as anti-COX-2 activity. Attaching of either –COOH or –COMe function to the SCH_2 provides the highest activity, whereas attaching of either $–CONH_2$ or COOEt functions provides moderate activity. Similarly, for compounds **6**, the thienyl moiety provides the highest activity.

Acute toxicity of both compounds **5** and **6** is illustrated in Figures 8 and 9.

EXPERIMENTAL

All melting points were uncorrected. IR (KBr discs) spectra were recorded on a FTIR-8201PC spectrophotometer (Shimadzu Corp., Japan). ^{1}H-NMR spectra were recorded on a Varian Mercury 300 MHz and Varian Gemini 200 MHz. spectrometers using TMS

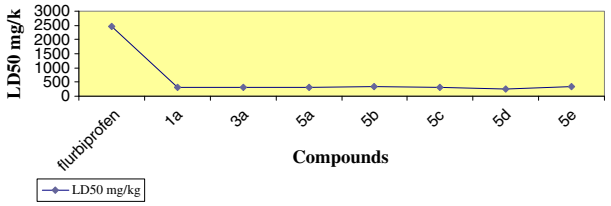


Figure 8. Acute toxicity of 5 series.

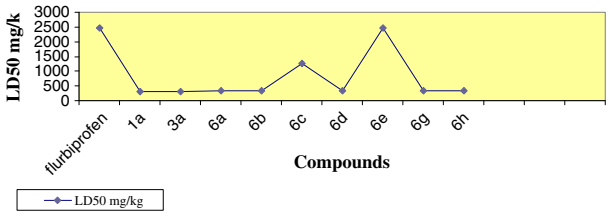


Figure 9. Acute toxicity of 6 series.

as an internal standard and DMSO-d_6 as solvent. Chemical shifts were expressed as δ (ppm) units. Mass spectra were recorded on Shimadzu GCMS-QP1000EX using an inlet type at 70 eV. The Micro analytical Center of Cairo University performed the microanalyses.

Synthesis of 3a,b (general method). A solution of each of **2** (0.1 g; 1 mmole) and **1a,b** (0.409 and 0.378 g; 1 mmole) in absolute ethanol (30 mL) containing the catalytic amount of piperidine (0.4 mL) heated under reflux for 5 h. The reaction mixture then evaporated, cooled, and triturated with ethanol. The products so formed were collected by filtration, washed with cold ethanol, and then crystallized from the dioxane to give the corresponding **3a,b**, respectively.

4-(2-Thienyl)-6-thioxo-1,6-dihydro-2,3′-bipyridine-5-carbonitrile (3a). Orange crystals (71%), mp 244°C, **IR** (ν cm^{-1}): 3216 (NH), 3086 (aromatic-CH) and 2210 (CN); **MS** (*m/z*) = 295 (M^+, 100% which is corresponding to the molecular weight), 294 (M^+–H, 32.3%); 267 (M^+–2H, CN, 15.1%); 262 (M^+–SH, 10.4%);**^{1}H-NMR** (DMSO-d_6) (δ ppm): 7.340–8.997 (m, 7H, Ar H's, pyridine H's, thiophene H's), 9.225 [s, 1H, pyridine C_5(H)] and 14.19 (s, br., 1H, NH); Anal. for $C_{15}H_9N_3S_2$ (295), Calcd./Found (%): C(60.99/61.23); H(3.07/3.32); N(14.23/14.00); S(21.71/21.54).

4-(2-Furyl)-6-thioxo-1,6-dihydro-2,3′-bipyridine-5-carbonitrile (3b). Orange crystals (65%), mp 244°C, **IR** (ν cm^{-1}): 3112 (NH), 3030 (aromatic-CH) and 2214 (CN); **MS** (*m/z*) = 279 (M^+, 100% which is corresponding to the molecular weight), 278 (M^+–H, 14.2%); 253 (M^+–CN, 1.3%); 252 (M^+–H, CN, 3.2%); 246 (M^+–SH, 3.2%); 246 (M^+–H_2S, CN 4.5%); Anal. for $C_{15}H_9N_3OS$ (279), Calcd./Found (%): C(64.50/64.76); H(3.25/3.65); N(15.04/15.31); S(11.48/11.18).

Synthesis of 5a–m (general procedure). A solution of each of **3a,b** (0.295 and 0.28 g; 1 mmole) and ethyl chloroacetate **(4a)**, 2-chloroacetamide **(4b)**, chloroacetone **(4c)**, 2-bromo-1-phenylethanone **(4d)**, chloroacetic acid **(4e)**, 3-chloropentane-2,4-dione **(4f)**, chloroacetonitrile **(4g)**, 2-bromo-1-(4-chlorophenyl) ethanone **(4h)** (0.122, 0.076, 0.093, 0.092, 0.134, 0.198, 0.233, and 0.094 g; 1 mmole) in sodium methoxide (prepared from 0.10 g of sodium and 25 mL methanol) was stirred at room temperature for 15 min. The formed precipitate was collected by filtration, washed with water, and dried then crystallized from ethanol to give **5a–m**, respectively.

Ethyl {[5-cyano-4-(2-thienyl)-2,3′-bipyridin-6-yl]thio}acetate (5a). Pale yellow crystals (88%), mp = 154°C; **IR** (ν cm^{-1}): 3079 (C–H, aromatic), 2206 (CN), 1736 (ester CO); Anal. for $C_{19}H_{15}N_3O_2S_2$ (381), Calcd./Found (%): C(59.82/59.64); H(3.96/4.15); N(11.02/10.87); S(16.81/16.65).

2-{[5-Cyano-4-(2-thienyl)-2,3′-bipyridin-6-yl]thio}acetamide (5b). White crystals (77%), mp = 270°C; **IR** (ν cm^{-1}): 3407, 3311 (NH_2) 3053 (C–H aromatic), 2217 (CN), 1670 (amide CO); Anal. for $C_{17}H_{12}N_4OS_2$ (352), Calcd./Found (%): C (57.93/57.76); H(3.43/3.75); N(15.90/15.73); S(18.20/18.54).

6-[(2-Oxopropyl)thio]-4-(2-thienyl)-2,3′-bipyridine-5-carbonitrile (5c). Pale yellow crystals (89%), mp = 170°C; **IR** (ν cm^{-1}): 3083 (C–H, aromatic), 2206 (CN), 1720 (CO); **^{1}H-NMR** (DMSO-d_6) (δ ppm): 2.288 (s, 3H, –CO$\underline{CH_3}$), 4.380 (s, 2H, –SCH_2–), 7.330–8.733 (m, 7H, pyridine H's, thiophene H's), 9.359 (s, 1H, pyridine C_5(H)); Anal. for $C_{18}H_{13}N_3OS_2$ (351), Calcd./Found (%): C(61.52/61.87); H(3.73/3.98); N(11.96/12.23); S (18.25/18.01).

6-[(2-Oxo-2-phenylethyl)thio]-4-(2-thienyl)-2,3′-bipyridine-5-carbonitrile (5d). Yellow crystals (91%), mp = 190°C; **IR** (ν cm^{-1}): 3062 (C–H, aromatic), 2211 (CN), 1698 (CO); Anal. for $C_{23}H_{15}N_3OS_2$ (413), Calcd./Found (%): C(66.80/66.64); H (3.66/3.97); N(10.16/10.31); S(15.51/15.87).

{[5-Cyano-4-(2-thienyl)-2,3′-bipyridin-6-yl]thio}acetic acid (5e). Orange crystals (80%), mp = 256°C; **IR** (ν cm^{-1}): 3446–2812 (acidic OH); 3024 (C–H, aromatic), 2213 (CN), 1612 (CO); Anal. for $C_{17}H_{11}N_3O_2S_2$ (353), Calcd./Found (%): C(57.77/57.43); H(3.14/3.53); N(11.89/11.65); S(18.15/18.65).

Ethyl {[5-cyano-4-(2-furyl)-2,3′-bipyridin-6-yl]thio}acetate (5i). Pale yellow crystals (80%), mp = 180°C; **IR** (ν cm^{-1}): 3058 (C–H aromatic), 2210 (CN), 1737 (ester CO); Anal. for $C_{19}H_{15}N_3O_3S$ (365), Calcd./Found (%): C(62.45/62.87); H(4.14/4.54); N(11.50/11.21); S(8.78/8.54).

6-[(Cyanomethyl)sulfanyl]-4-(2-furyl)-2,3′-bipyridine-5-carbonitrile (5j). Yellow crystals (84%), mp = 260°C; **IR** (ν cm^{-1}): 3053 (C–H aromatic), 2212, 2192 (CN); Anal. for $C_{16}H_8N_4OS$ (304), Calcd./Found (%): C(63.15/63.65); H (2.65/2.21); N(18.41/18.87); S(10.54/10.23).

2-{[5-Cyano-4-(2-furyl)-2,3′-bipyridin-6-yl]thio}acetamide (5k). White crystals (84%), mp = 260°C; **IR** (ν cm^{-1}): 3338, 3138 (NH_2), 3085 (C–H aromatic), 2213 (CN), 1741 (CO); **^{1}H-NMR** (DMSO-d_6) (δ ppm): 4.065 (s, 2H, –SCH_2–), 4.906 (br, s, 2H, –NH_2), 7.212–8.729 (m, 7H, pyridinyl H's, furyl H's), 9.458 [s, 1H, pyridinyl C_5(H)]; Anal. for $C_{17}H_{12}N_4O_2S$ (336), Calcd./Found (%): C(60.70/60.43); H(3.60/3.32); N(16.66/16.41); S(9.53/9.78).

4-(2-Furyl)-6-[(2-oxo-2-phenylethyl)thio]-2,3′-bipyridine-5-carbonitrile (5l). Orange crystals (91%), mp = 188°C; **IR** (ν cm^{-1}): 3046 (C–H aromatic), 2213 (CN), 1689 (CO); Anal. for $C_{23}H_{15}N_3O_2S$ (397), Calcd./Found (%): C(69.50/69.78); H(3.80/3.32); N(10.57/10.76); S(8.07/8.32).

{[5-Cyano-4-(2-furyl)-2,3′-bipyridin-6-yl]thio}acetic acid (5m). Orange crystals (80%), mp = 250°C; **IR** (ν cm^{-1}): 3226–2852 (acidic OH), 3027 (C–H aromatic), 2217 (CN), 1611 (CO); Anal. for $C_{17}H_{11}N_3O_3S$ (337), Calcd./Found (%): C(60.52/60.86); H(3.29/3.65); N(12.46/12.86); S(9.50/9.21).

The synthesis of 6a–n.

Method A. A solution of each of **5a–e,i–m** (0.38, 0.35, 0.35, 0.41, 0.35, 0.37, 0.30, 0.34, 0.40, and 0.34 g; 1 mmole) in sodium ethoxide solution (prepared from 0.10 g of sodium and 25 mL of ethanol)

heated under reflux for 30 min. The solid formed after cooling, collected by filtration, washed with water and ethanol, then crystallized from the proper solvent to give **6a–n**, respectively.

Method B. A solution of each of **3a,b** (0.295 and 0.28 g; 1 mmole) and ethyl chloroacetate **(4a)**, 2-chloroacetamide **(4b)**, chloroacetone **(4c)**, 2-bromo-1-phenylethanone **(4d)**, chloroacetic acid **(4e)**, 3-chloropentane-2,4-dione **(4f)**, chloroacetonitrile **(4g)**, 2-bromo-1-(4-chlorophenyl)-ethanone **(4h)** (0.122, 0.093, 0.092, 0.198, 0.094, 0.134, 0.75, and 0.233g; 1mmole) in sodium methoxide (prepared from 0.10 g of sodium and 25 mL of methanol) heated under reflux for 2 h. The solid products so formed after cooling, collected by filtration, washed with water and ethanol, and dried crystallized from the proper solvent to give **6a–n**, respectively.

Ethyl 3-amino-6-pyridin-3-yl-4-(2-thienyl)thieno[2,3-b]pyridine-2-carboxylate (6a). Yellow crystals crystallized from ethanol (90%); mp = 218°C; **IR** (ν cm^{-1}): 3480, 3346 (NH_2), 3038 (C–H aromatic), 1672 (CO); **MS** (*m/z*): 381 (M^+, 100% which is corresponding to the molecular weight), 380 (M^+–H, 5.2%); 336 (M^+–OEt, 15.0%); 334 (M^+–OEt, –2H, 74.8%); 308 (M^+–COOEt, 8.6%); Anal. for $C_{19}H_{15}N_3O_2S_2$ (381), Calcd./Found (%): C (59.82/59.45); H(3.96/4.32); N(11.02/11.43); S(16.81/16.97).

3-Amino-6-pyridin-3-yl-4-(2-thienyl)thieno[2,3-b]pyridine-2-carboxamide (6b). Yellow crystals crystallized from dioxane (81%); mp = 296 °C; **IR** (ν cm^{-1}) 3463, 3297, 3276, 3125 ($2NH_2$), 1666 (CO); **MS** (*m/z*): 352 (M^+, 100% which is corresponding to its molecular weight), 336 (M^+–NH_2, 10.0%); 334 (M^+–NH_2, –2H, 79.6%); 308 (M^+–$CONH_2$, 10.0%); Anal. for $C_{17}H_{12}N_4OS_2$ (352), Calcd./Found (%): C(57.93/57.95); H (3.43/3.45); N(15.90/15.95); S(18.20/18.24).

1-[3-Amino-6-pyridin-3-yl-4-(2-thienyl)thieno[2,3-b]pyridin-2-yl]ethanone (6c). Yellow crystals crystallized from dioxane (86%); mp = 250°C; **IR** (ν cm^{-1}) 3451, 3285 (NH_2), 3055 (C-H aromatic), 1622 (CO); **MS** (*m/z*): 351 (M^+, 100% which is corresponding to its molecular weight), 350 (M^+–H, 40.9%), 336 (M^+–CH_3 65.7%); 308 (M^+–$COCH_3$ 28.8%), Anal. for $C_{18}H_{13}N_3OS_2$ (351), Calcd./Found (%): C(61.52/61.55); H (3.73/3.77); N(11.96/11.99); S(18.25/18.29).

[3-Amino-6-pyridin-3-yl-4-(2-thienyl)thieno[2,3-b]pyridin-2-yl](phenyl)methanone (6d). Orange crystals crystallized from dioxane (86%); mp = 200 °C; **IR** (ν cm^{-1}): 3471, 3295 (NH_2), 3030 (C–H aromatic), 1691 (CO); **MS** (*m/z*): 413 (M^+, 75.2% which is corresponding to its molecular weight), 412 (M^+–H, 100%); 411 (M^+–2H, 14%); 308 (M^+–COPh, 24.1%); Anal. for $C_{23}H_{15}N_3OS_2$ (413), Calcd./Found (%): C(66.80/66.83); H(3.66/3.69); N(10.16/10.18); S(15.51/15.55).

3-Amino-6-pyridin-3-yl-4-(2-thienyl)thieno[2,3-b]pyridine-2-carboxylic acid (6e). Yellow crystals crystallized from dioxane (71%); mp = 210 °C; **IR** (ν cm^{-1}): 3473–3328 (NH_2, OH), 1665 (CO); **MS** (*m/z*) = 353 (M^+ 10.3% which is corresponding to its molecular weight), 334 (M^+–H, H_2O 4.5%); 309 (M^+–COOH, 100%); **^{1}H-NMR** (DMSO-d_6) (δ ppm): 6.531 (br, s, 2H, NH_2), 7.274–8.689 (m, 7H, pyridine H's, thiophene H's), 9.369 [s, 1H, pyridine C_5(H)] and 12.763 (s, br., 1H, COOH); Anal. for $C_{17}H_{11}N_3O_2S_2$ (353), Calcd./Found (%): C(57.77/57.90); H (3.14/3.38); N(11.89/11.62); S(18.15/18.30).

3-Amino-6-pyridin-3-yl-4-(2-thienyl)thieno[2,3-b]pyridine-2-carbonitrile (6g). Yellow crystals crystallized from dioxane (85%); mp = 280°C; **IR** (ν cm^{-1}): 3447, 3226 (NH_2) 3044 (C–H aromatic), 2197 (CN), **MS** (*m/z*) :334 (M^+, 100% which is corresponding to its molecular weight), 333 (M^+–H, 46.9%); 332 (M^+–2H, 5.0%); 308 (M^+–CN, 0.4%); 290 (M^+–2H, NH_2, CN, 17.7%); Anal. for $C_{17}H_{10}N_4S_2$ (334), Calcd./Found (%): C (61.06/61.09); H(3.01/3.06); N(16.75/16.80); S(19.18/19.20).

[3-Amino-6-pyridin-3-yl-4-(2-thienyl)thieno[2,3-b]pyridin-2-yl](4-chlorophenyl)-methanone (6h). Orange crystals crystallized from dioxane (90%); mp = 240 °C; **IR** (ν cm^{-1}): 3454, 3281 (NH_2), 3062 (C–H aromatic), 1675 (CO); **MS** (*m/z*):449 (M^++2, 32.0%); 448 (M^++1, 53.8%); 447 (M^+, 100% which is corresponding to its molecular weight), 446 (M^+–H, 88.7%); 412 (M^+–Cl, 0.1%); 336 (M^+–C_6H_4Cl, 5.9%); 308 (M^+–COC_6H_4Cl, 16.1%); Anal. for $C_{23}H_{14}ClN_3OS_2$ (447), Calcd./Found (%): C (61.67/61.70); H(3.15/3.19); Cl(7.91/7.95); N(9.38/9.42); S (14.32/14.36).

Ethyl 3-amino-4-(2-furyl)-6-pyridin-3-ylthieno[2,3-b]pyridine-2-carboxylate (6i). Yellow crystals crystallized from ethanol (90%); mp = 210°C; **IR** (ν cm^{-1}): 3499, 3377 (NH_2), 3037 (C–H aromatic), 1672 (CO); **MS** (*m/z*): 365 (M^+, 100% which is corresponding to its molecular weight), 320 (M^+–OEt, 21.3%); 292 (M^+–COOEt, 19.1%); Anal. for $C_{19}H_{15}N_3O_3S$ (365), Calcd./Found (%): C(62.45/62.21); H(4.14/4.32); N(11.50/11.76); S(8.78/8.98).

3-Amino-4-(2-furyl)-6-pyridin-3-ylthieno[2,3-b]pyridine-2-carbonitrile (6j). Yellow crystals crystallized from dioxane (85%); mp = 308°C; **IR** (ν cm^{-1}): 3482, 3207 (NH_2) 3057 (C–H aromatic), 2192 (CN); **MS** (*m/z*): 318 (M^+, 100% which is corresponding to its molecular weight), 317 (M^+–H, 43.8%); Anal. for $C_{17}H_{10}N_4OS$ (318), Calcd./Found (%): C(64.14/64.17) H(3.17/3.20); N(17.60/17.64); S(10.07/10.10).

3-Amino-4-(2-furyl)-6-pyridin-3-ylthieno[2,3-b]pyridine-2-carboxamide (6k). Yellow crystals crystallized from ethanol (80%); mp = 290°C; **IR** (ν cm^{-1}): 3494, 3467, 3330, 3133 ($2NH_2$) 1665 (amidic CO); **MS** (*m/z*): 336 (M^+, 100% which is corresponding to its molecular weight), 319 (M^+–H, NH_2, 30.6%); 318 (M^+–NH_2, –2H, 94.4%); 291 (M^+–H, $CONH_2$, 27.8%); 290 (M^+–2H, –$CONH_2$, 33.3%); Anal. for $C_{17}H_{12}N_4O_2S$ (336), Calcd./Found (%): C(60.70/60.73); H (3.60/3.63); N(16.66/16.69); S(9.53/9.56).

[3-Amino-4-(2-furyl)-6-pyridin-3-ylthieno[2,3-b]pyridin-2-yl](phenyl)methanone (6l). Orange crystals crystallized from ethanol (83%); mp = 220°C; **IR** (ν cm^{-1}): 3474, 3300 (NH_2), 3043 (C–H aromatic), 1602 (CO); **MS** (*m/z*): 397 (M^+, 22.5% which is corresponding to its molecular weight), 396 (M^+–H, 18.5%); 320 (M^+–Ph, 1.6%); 292 (M^+–COPh, 2.8%); Anal. for $C_{23}H_{15}N_3O_2S$ (397), Calcd./Found (%): C(69.50/69.54); H(3.80/3.84); N(10.57/10.60); S(8.07/8.11).

[3-Amino-4-(2-furyl)-6-pyridin-3-ylthieno[2,3-b]pyridin-2-yl](4-chlorophenyl)-methanone (6m). Orange crystals crystallized from ethanol (86%); mp = 235 °C; **IR** (ν cm^{-1}): 3478, 3279 (NH_2), 3048 (C–H aromatic); **MS** (*m/z*): 433 (M + 2 15.1%); 432 (M + 1 28.0%); 431 (M^+, 39.2% which is corresponding to its molecular weight), 430 (M^+–H, 43.5%); 396 (M^+–Cl, 4.8%); 292 (M^+–COC_6H_4Cl, 6.5%); Anal. for $C_{23}H_{14}ClN_3O_2S$ (431), Calcd./Found (%): C(63.96/64.00); H(3.27/3.30); Cl(8.21/8.24); N(9.73/9.77); S(7.42/7.45).

1-[3-Amino-4-(2-furyl)-6-pyridin-3-ylthieno[2,3-b]pyridin-2-yl]ethanone (6n). Yellow crystals crystallized from dioxane (83%); mp = 222°C; **IR** (ν cm^{-1}): 3478, 3289 (NH_2), 3066 (C-H aromatic), 1610 (CO); **MS** (*m/z*) = 335 (M^+, 100% which is corresponding to its molecular weight), 334 (M^+–H, 27.5%), 320 (M^+–CH_3 71.6%); 319 (M^+–NH_2, 11.9%); 292 (M^+–$COCH_3$ 22.9%); **^{1}H-NMR** (DMSO-d_6) (δ ppm): 2.408 (s, 3H, $COCH_3$), 6.842 (s, br., 2H, NH_2), 7.459–8.703 (m, 7H, pyridine H's, furan H's), 9.398 (s, 1H, pyridine C_5(H)); Anal. for $C_{18}H_{13}N_3O_2S$

(335), Calcd./Found (%): C(64.46/64.70); H(3.91/3.64); N(12.53/12.38); S(9.56/9.80).

MATERIALS AND METHODS

Aβ42 and Aβ40 assay. *Aβ42* and *Aβ40* were measured in the culture medium of H4 cells, a human neuroglioma cell line expressing the double Swedish mutation (K595N/M596L) of human amyloid precursor protein (APPsw). Cells were seeded onto 24-well plates (2×10^5 cell well^{-1}) and allowed to grow to confluence for 24 h, in 5% CO_2/95% air in a humidified atmosphere. Increasing concentrations (from 3 to 300–400 μ*M*) of the compounds were added to the cells overnight in a final volume of 0.5 mL. *R*-flurbiprofen was used as positive control (3–1000 μ*M*). DMSO (1%) was used as negative control. At the end of the incubation, 100 μL of supernatants were removed and treated with a biotinylated mouse monoclonal antibody (4G8, Signet Laboratories Inc., Dedham, MA, USA), specifically recognizing the 17–24 amino acid region of Aβ and two rabbit polyclonal antibodies (C-term 42 and C-term 40, BioSource International, Camarillo, CA, USA), specifically recognizing the C-terminus of Aβ42 and Aβ40, respectively. Antigen–antibody complexes were recognized by TAG-donkey anti-rabbit IgG (Jackson Immuno Research Laboratories, Soham, UK). Streptavidin coated magnetic beads captured the complexes, and the signals were read by an electrochemiluminescence instrument (Origen M8 Analyzer, BioVeris Corporation, Gaithersburg, MD, USA). The cytotoxicity potential of test compounds was assessed in the same cells of the Aβ assay (H4) with the 3-[4,5-dimethylthiazol-2-yl]-2,5-di-phenyltetrazol-ium bromide (MTT) assay. MTT is a soluble pale yellow salt that is reduced by mitochondrial succinate dehydrogenase to form an insoluble dark blue formazan product to which the cell membrane is impermeable. The ability of cells to reduce MTT provides an indication of mitochondria integrity and activity, and it may be interpreted as a measure of viability and/or cell number. After medium removal of Aβ 42 and Aβ 40 determination, cells were incubated for 3 h with 500 μL of culture medium containing 0.5 mg mL^{-1} of MTT, at 37°C, 5% CO2 and saturated humidity. After removal of the medium, 500 μL of 100% DMSO was added to each well. The amount of formed formazan was determined by reading the samples at 570 nm (background 630 nm) by using a microplater reader (model 450, Bio-Rad, Hercules, CA, USA).

COX-1 and COX-2 assay. The inhibition of the cyclooxygenase activity was estimated measuring prostaglandin E2 (PGE2) production from arachidonic acid according to a modified version of the method [16]. Recombinant human prostaglandin H2 synthase-1 (PGHS-1) and prostaglandin H2 synthase-2 (PGHS-2) were expressed in transfected Spodoptera frugiperda (Sf-9) cells (Invitrogen, San Diego, CA, USA). The microsomal fractions were prepared from the transfected cells and used to assay the enzymatic activities. Briefly, the enzymes (2g) reconstituted in a buffer (100 m*M* Tris–HCl, pH 8.0) containing 2 m*M* phenol were preincubated with vehicle (DMSO) or test compounds in DMSO (1%DMSO in the final assay) for 20 min at 22°C. The reaction mixture was completed with 1*M* hematin. The reaction was initiated adding arachidonic acid (4 and 2 μ for COX-1 and COX-2, respectively) and the mixture was incubated for 5 min at 22°C for COX-1 assay or for 10 min at 25°C for COX-2 assay. For control measurements, arachidonic acid was omitted from the reaction mixture. The reactions were stopped by the sequential addition of 1*M* HCl and 1*M* Tris–HCl (pH 8.0), followed by cooling to 4°C. The amount of PGE2 present in the reaction mixture was quantified using an enzyme immunoassay.

Studies in Tg2576 transgenic mice. Young male and female transgenic mice (Tg2576) expressing the human *APP* gene with the Swedish double mutation (K670N/M671L) under the transcriptional control of the hamster prion protein promoter [17] were used for the *in vivo* studies. Male animals were housed singly in individual cages, whereas female animals were placed in groups of 3–5 animals per cage. The experiments were performed in accordance with EEC Guidelines (86/609/ECC) for the use of laboratory animals.

Study 1. Groups of mole mice 4–5 months, each group composed of 21 male mice of 4–5 months of age, were given by oral *gavage* vehicle (Kool-Aid 7.5 mL kg^{-1}) or a suspension of each individual compound (100 or 300 mg kg^{-1} day^{-1} in Kool-Aid) once daily for 5 days. This vehicle was selected to replicate that reported with flurbiprofen in similar studies [17–19]. On day 5, mice were given a final dose of 100 or 300 mg kg^{-1} or vehicle and sacrificed 3 h later, as described in the succeeding text.

Study 2. Groups of female mice of 5–7 months of age, each group composed 17 female mice of 5–7 months of age, were given by oral *gavage* vehicle (Kool-Aid 7.5 mL kg^{-1}) or a suspension of individual compound (100 or 300 mg kg^{-1} day^{-1} in Kool-Aid) once daily for 4 days. On day 4, mice were given a final dose of 100 or 300 mg kg^{-1} or vehicle and sacrificed 3 h later, as described in the succeeding text.

Study 3. Groups of male and female mice of 4–5 months, each group composed of 33 male and female mice of 4–5 months were given vehicle or tested compounds or *R*-flurbiprofen-supplemented chow *ad libitum* for 4 weeks. There were 11 animals in each treatment group. *R*-flurbiprofen (Sigma, St. Louis, MO, USA), and the tested compounds were formulated into standard, color-coded, rodent diet by Charles River (Calco, Italy) at a final drug concentration of 375 ppm. The concentration of the drugs in the diet was the same as that used for flurbiprofen in previous studies [20–22].

Body weight and food consumption were monitored every 3–4 days.

Plasma and brain aβ measurements. Twenty-four hours before starting treatment, one blood sample was collected by means of retrobulbar puncture for measurement of baseline plasma, Aβ40, and Aβ42 concentrations. On the last day of treatment, mice were sacrificed by decapitation. Blood samples were collected in EDTA-coated tubes and centrifuged at 800 rpm for 20 min to separate the plasma. Plasma samples were divided into two aliquots of approximately 100 μL each and stored at −80°C until Aβ and drug assay. The brains were quickly removed and placed on an ice-cold plate. The cortex and hippocampus were dissected and immediately frozen on dry ice and stored at −80°C for Aβ assay. The remaining brain was immediately frozen on dry ice and stored at −80°C for drug level measurements. Plasma was diluted 1:4 for Aβ42 and 1:20 for Aβ40. For measurement of Aβ, brain tissue samples were homogenized in 70% formic acid at 1:10 (w/v). Homogenates were agitated at 4°C for 3 h and then centrifuged at 15,000 rpm for 25 min at 4°C. The supernatants were collected and neutralized with 1*M* Tris, pH 11 at 1:20 (w/v) dilution with 3× protease inhibitor mixtures (Boehringer Mannheim, Mannheim, Germany). Levels of Aβ40 and Aβ42 in plasma and in brain homogenate supernatants were measured with commercial ELISA kits (The Genetics Company, Zurich, Switzerland). The microtitre plates were coated with capturing purified monoclonal antibodies specifically recognizing the C-terminus of human Aβ40 (clone G2-10, reactive to amino acid residues 31–40, isotype IgG2b, kappa) or Aβ42 (clone G2-13, reactive to amino acid residues 33–42, isotype IgG1, kappa). As detection antibody, a monoclonal biotin conjugated antibody recognizing the N-terminus of human Aβ (clone W0-2, reactive to amino acid residues 4–10, isotype IgG2a, kappa) was used. The assay was linear in the range 25–500 pg mL^{-1}, and the detection limit was 25 pg mL^{-1}.

Plasma and brain drug measurements. Drugs levels in plasma and in brain samples were measured by liquid

Table 1

Antitumor activity and novel topoisomerase II inhibitors.

	Growth-inhibitory effect		The correlation coefficients with (topoisomerase I inhibitor), and cisplatin, calculated according to the NCI COMPARE analysis procedure using the tested compound as the benchmark				*In vivo* efficacy study	Inhibition of topoisomerase II activity
Compound	Average GI50	Relative potency to etoposide	Etoposide	Doxorubicin	SN-38	Cisplatin	Relative potency to etoposide	Relative potency to etoposide
1a	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
1b	377	124.41	4.35435	3.48348	2.86143	2.2393	128.1423	118.1895
3a	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
3b	381	125.73	4.40055	3.52044	2.89179	2.2631	129.5019	119.4435
5a	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
5b	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
5c	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
5d	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
5e	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
5i	355	117.15	4.10025	3.2802	2.69445	2.1087	120.6645	111.2925
5j	380	125.4	4.389	3.5112	2.8842	2.2572	129.162	119.13
5k	344	113.52	3.9732	3.17856	2.61096	2.0433	116.9256	107.844
5l	351	115.83	4.05405	3.24324	2.66409	2.0849	119.3049	110.0385
5m	388	128.04	4.4814	3.58512	2.94492	2.3047	131.8812	121.638
6a	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
6b	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
6c	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
6d	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
6e	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
6g	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
6h	3.49E-58	135.3	4.7355	3.7884	3.1119	2.4354	139.359	128.535
6i	341	112.53	3.93855	3.15084	2.58819	2.0255	115.9059	106.9035
6j	391	129.03	4.51605	3.61284	2.96769	2.3225	132.9009	122.5785
6k	337	111.21	3.89235	3.11388	2.55783	2.0017	114.5463	105.6495
6l	349	115.17	4.03095	3.22476	2.64891	2.0730	118.6251	109.4115
6m	366	120.78	4.2273	3.38184	2.77794	2.1740	124.4034	114.741
6n	370	122.1	4.2735	3.4188	2.8083	2.1978	125.763	115.995

chromatography as previously described [20]. Briefly, samples were prepared by adding 300 μL of acetonitrile and 40 μL of phosphoric acid 40% to 100 μL plasma or brain homogenate and placing the mixture in a vortex for 5 s. Plasma and brain samples were then centrifuged at 14,000 rpm for 5 min, and the supernatants (15 and 50 μL, respectively) were injected into the HPLC system. Equipment systems with fluorescence (Waters 474, Waters, Guyancourt, France) or mass spectrometry (API 2000, Applied Biosystems, Foster City, CA, USA) detectors were used. The chromatographic conditions were adapted to each compound to obtain good peak separation and detection sensitivity. A mixture of ammonium formate (20 m*M*) buffer–acetonitrile–methanol was used as mobile phase for the fluorescence detector. For drugs, the assay was linear in the range 20–4000 ng g^{-1} in the brain and 5–1000 ng mL^{-1} in plasma with limits of quantitation of 20 ng g^{-1} in the brain and 5 ng mL^{-1} in plasma. For drugs, the assay was linear between 400 and 20,000 ng g^{-1} in the brain and 100–8500 ng mL^{-1} in plasma with limits of quantitation of 400 ng g^{-1} in the brain and 100 ng mL^{-1} in plasma.

Antitumor activity and topoisomerase II inhibitors. DNA topoisomerase II has been shown to be an important therapeutic target in cancer chemotherapy. Here, we describe studies on the antitumor activity of novel topoisomerase II inhibitors. The tested compounds inhibited topoisomerase II activity at 10 times lower concentration than etoposide in relaxation assay and cells. Murine colon cancer Colon 38, murine leukemia P388, and human lung cancer LX-1 were obtained from the Cancer Chemotherapy Center, Japan Foundation for Cancer Research (Tokyo, Japan). Human lung cancer PC-1 and human gastric cancer MKN-1, MKN-7, MKN-28, and MKN-74 were obtained from Immuno Biology Laboratory (Gunma, Japan). Human lung cancer A549 and human colon cancer WiDr were obtained from Dainippon Pharmaceutical Co. Ltd (Osaka, Japan). Human gastric cancer HGC-27 and GT3TKB were obtained from Riken Cell Bank (Ibaraki, Japan). The other cell lines were purchased from American Type Culture Collection (Rockville, MD, USA) (Table 1).

Growth-inhibitory effect. Exponentially growing solid tumor cells in 0.1 mL of medium were seeded in 96-well plates on day 0. On day 1, 0.1 mL aliquots of medium containing graded concentrations of test drugs were added to the cell plates. After incubation at 37°C for 72 h, the cell number was estimated by sulforhodamine B assay; as described by Skehan *et al*. separately, the cell number on day 1 was also measured. GI50 values are the drug concentrations causing a 50% reduction in the net protein increase in control cells. The antitumor spectrum of ER-37328 was compared with those of other drugs by means of the NCI COMPARE analysis procedure. Exponentially growing P388 cells were seeded in 96-well plates on day 0. On day 1, 0.1 mL aliquots of medium containing graded concentrations of test drugs were added to the wells. After incubation at 37°C for 72 h, the cell number was determined by MTT assay .IC50 values were calculated as the drug concentrations that reduced the number of cells to 50% of the control number.

***In vivo* efficacy study.** Female C57BL/6 mice (6 weeks of age; Charles River, Atsugi, Japan) were housed in barrier facilities on a 12-h light/dark cycle, with food and water *ad libitum*. About 30 mg of Colon 38 tumor tissues was inoculated S. C. into the flank of animals. Mice were randomized and separated into control and treatment ($n = 5$ each) groups when the tumor volume reached approximately 300 mm^3 on day 1. The tested compound was dissolved in 5% glucose. Etoposide was diluted in saline, and doxorubicin was dissolved in saline. The tested compound was administrated on day 1 (single dose) by i.v. injection into the tail vein, and etoposide and doxorubicin were given at the maximum tolerated dose on the reported administration schedule. Control animals received 5% glucose. Tumor volume and body weight were measured on the days indicated in the figures. Tumor volume was calculated according to the following equation: tumor volume (mm^3) = (length − $width^2$)/2

Relaxation assay. Topoisomerase II was purified from P388 cells. One unit of the enzyme was defined as the activity to relax completely 0.125 μg of supercoiled pBR-322 DNA at 30°C for 1 h. For the assay, 0.125 μg of supercoiled pBR-322 DNA (Takara Shuzo Co. Ltd, Tokyo, Japan) was relaxed with one unit of topoisomerase II in 20 μL of the assay buffer [50 m*M* Tris–HCl(pH 8.0), 100 m*M* KCl, 10 m*M* MgCl2, 0.5 m*M* DTT, 0.5 μ*M* EDTA, 1 m*M* ATP, and 30 μg mL^{-1} BSA] in the presence or absence of an inhibitor at 30°C for 1 h. Samples were subjected to electrophoresis s in 0.7% agarose gels with tris-borate-EDTA (TBE) buffer [89 m*M* Tris, 89 m*M* boric acid, and 2 m*M* EDTA (pH 8.0)]. The DNA was stained with ethidium bromide and photographed under UV light. (pH8.0), 100 m*M* KCl, 10 m*M* $MgCl_2$, 0.5 m*M* DTT, 0.5 μ*M* EDTA, 1 m*M* ATP, and 30 μg mL^{-1} BSA] in the presence or absence of an inhibitor at 30°C for 1 h. Samples were subjected to electrophoresis s in 0.7% agarose gels with TBE buffer [89 m*M* Tris, 89 m*M* boric acid, and 2 m*M* EDTA (pH 8.0)]. The DNA was stained with ethidium bromide and photographed under UV light.

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