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Synthesis and Evaluation of Brain-targeted Chemical Delivery Systems for the Neurotrophomodulator 4-Methylcatechol

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Abstract

Since various 4-alkylcatechols stimulate nerve growth factor (NGF) biosynthesis both in-vitro and in-vivo, delivery of these agents to the brain may provide beneficial effect for the treatment of neurodegenerative diseases such as Alzheimer's.

Several dihydropyridine-pyridinium salt type redox chemical delivery systems (CDS) of 4-methylcatechol (4-methylcatechol) were prepared as potential brain selective targetry forms for 4-methylcatechol. After preliminary evaluation by in-vitro stability studies in various buffer solutions and biological media, a selected CDS was further investigated in the rat to determine its in-vivo distribution. Selective and sustained delivery of the compound of interest to the rat brain was achieved. Furthermore, the NGF stimulatory activity in the rat brain after peripheral administration of the selected CDS was evaluated by measuring the levels of pre-pro-NGF mRNA in the rat hippocampus and frontal cortex, by dot blot hybridization and analysis.

Results showed the peripheral administration of the CDS to achieve a 1·7-fold increase in NGF mRNA compared to control in the rat hippocampus, and an approximately 1·4-fold increase in the frontal cortex.

Although evidence indicates that nerve growth factor (NGF) may have promise as a therapeutic agent for neurodegenerative diseases like Alzheimer's (Lapchak 1993; Olson 1993), its potential use in such a CNS disorder is prevented by its inability to cross the blood-brain barrier. An alternative, the use of pharmacological agents that enhance NGF biosynthesis and release in the CNS, has been the focus of several studies in the past few years (De Bernardi et al 1991; Mocchetti 1991; Thoenen et al 1991). The inducibility of NGF has been demonstrated in a number of in-vivo and in-vitro systems by a variety of compounds (Schwartz 1988; Furukawa et al 1989; Follesa & Mocchetti 1992; Carswell 1993). Among them, 4-methylcatechol is a potent NGF stimulator both in-vitro (mouse fibroblast L929 cells and L-M cells) (Furukawa et al 1991; Carswell et al 1992) and in-vivo, in the rat peripheral nervous system (Kaechi et al 1993; Hanaoka et al 1994) and in the rat brain after intracerebroventricular injection (Saporito et al 1993). Peripheral administration of 4-methylcatechol results in minimal penetration to the brain, since the morphological and enzymatic components of the blood-brain barrier prohibits not only large molecules such as peptides (e.g. NGF) from entering the brain but also small molecules lacking adequate lipophilicity. Efficient and sustained delivery of 4-methylcatechol to the brain could be achieved by means of an appropriate brain-targeted chemical delivery system (CDS) such as the one based on the dihydropyridine/pyridinium salt redox reaction (Bodor & Brewster 1983; Bodor & Farag 1983; Brewster et al 1989). In this redox-delivery system (Scheme 1), the lipoidal dihydropyridine moiety is covalently attached to the drug or active agent, thus increasing its lipophilicity and its blood-brain barrier permeability. Upon systemic administration of the complex, after extensive distribution, the dihydropyridine is oxidized to the charged pyridinium ion in the brain and in systemic tissues by the same means as the ubiquitous NADPH/NADP redox system. The charged pyridinium-drug complex is thus retained, or locked, in the brain since the blood-brain barrier prevents rapid re-equilibration of polar species, and enzymatic hydrolysis of the drug-carrier complex results in a sustained release of active drug. Meanwhile, in the periphery, the ionized pyridinium-drug moiety can be rapidly cleared by renal or biliary excretion due to its increased hydrophilicity. The overall result is that peripheral (side) effects or toxicity should be reduced by preventing significant accumulation of the parent active agent in the periphery, while it is specifically delivered to its site of action (CNS). Toxicity within the CNS is also attenuated since the majority of the active species is present in the form of an inactive carrier complex.

Several CDSs were designed and synthesized as potential brain-selective targetry forms for 4-methylcatechol (Scheme 2). Thus, one of the catecholic hydroxyls is esterified with the dihydropyridine targetor while the other is masked with another lipophilic function, or both catechol hydroxyls are esterified with the dihydropyridine moiety.

In-vitro stability studies of the CDSs and their quaternary metabolites in various buffer systems and biological media were performed in order to determine the feasibility of delivery of 4-methylcatechol by this method. Subsequently, in-vivo studies (in rat) of a selected CDS were performed to demonstrate oxidation of the CDS, rapid peripheral elimination of the pyridinium salt and sustained central delivery of 4-methylcatechol, as well as NGF stimulatory activity in the rat hippocampus and cerebral cortex (brain regions where NGF is predominantly expressed).

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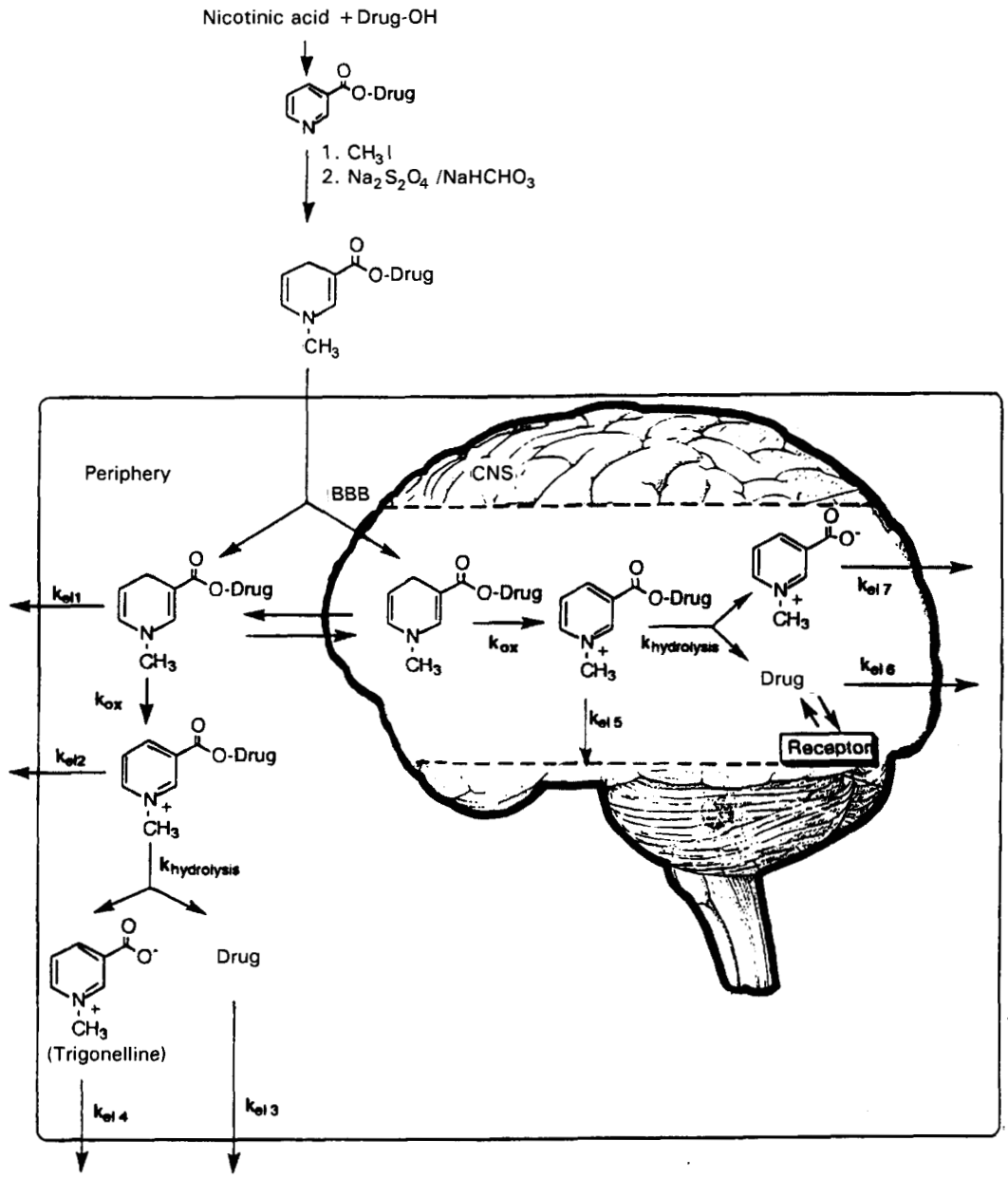
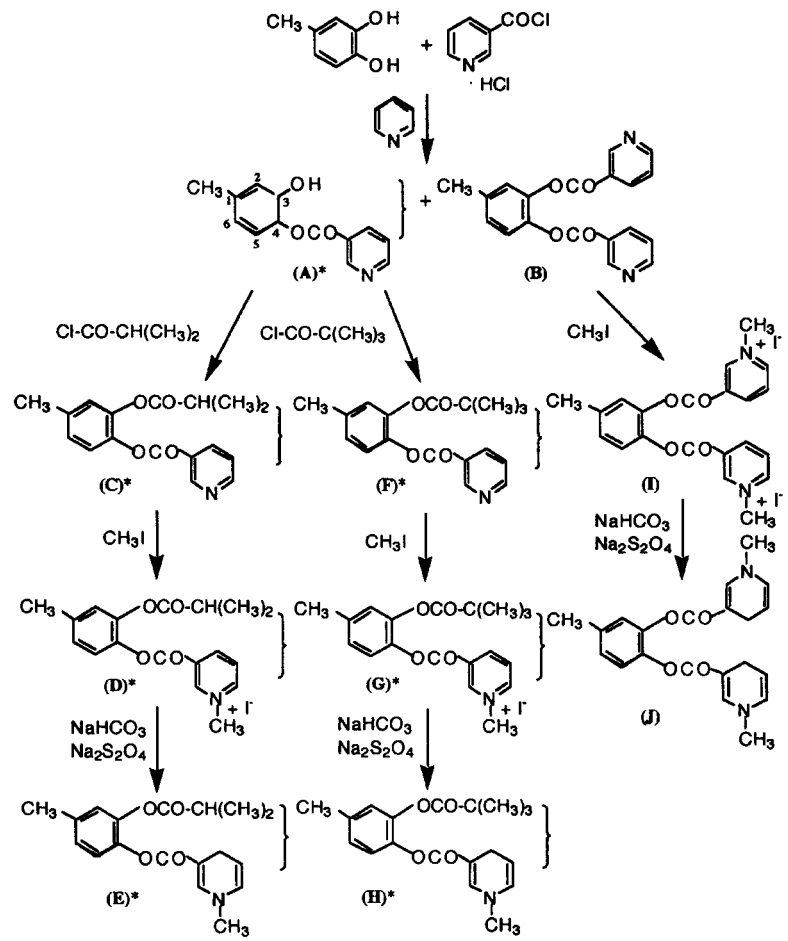


Scheme 1. Representation of the drug-targetor complex that promotes CNS retention and accelerated peripheral elimination.

Materials and Methods

Synthesis

All chemicals used were reagent grade obtained from Aldrich Company and solvents from Fisher Scientific. All melting points were recorded using Fisher-Johns melting point apparatus and are uncorrected. NMR data were recorded with Varian T-90, QE-300, or Varian Unity-300 spectrometers and are reported in parts per million (δ) relative to tetramethylsilane. The elemental analyses were carried out either at Atlantic Microlab. Inc., Atlanta, Ga, or at the Analytical Services of the Department of Chemistry of the University of Florida. Mass spectrometry was performed with a Kratos MFC 500 instrument (Center for Drug Discovery, University of Florida, Gainesville, Florida). Fast atom bombardment (FAB) ionization was performed by a 6-keV xenon beam and dissolving the samples in either glycerol, 3-nitrobenzyl alcohol or 3-nitrobenzyl alcohol + sodium matrix. Ultra violet spectra were obtained with a Perkin Elmer UV/VIS spectrometer Lambda II. Thin layer chromatography was carried out using Merck DC-aluminium foil plates coated to a thickness of 0·2 mm with silica gel 60 containing Florescent (254) indicator. The high performance liquid chromatography (HPLC) system consisted of a SP 8810 precision isocratic pump, SP4290 injector, Waters RCM C-18 column, SP 8450 UV/visible detector and SP4290 integrator. The mobile phase consisted of acetonitrile-water or methanol-water in different proportions.



Scheme 2. Synthetic scheme for the preparation of the 4-methylcatechol CDSs. *Consists of the mixture of the two isomers i.e. the 3 and 4 nicotinoyl derivative (see Synthesis in Results and Discussion).

Synthesis of 3(or 4)-hydroxy-4(or 3)-nicotinoyloxytoluene (A) and 3,4-dinicotinoyloxytoluene (B). 4-Methylcatechol (4·3 g, 34·7 mmol) was dissolved in pyridine (90 mL) and nicotinoyl chloride hydrochloride (9·27 g, 52·1 mmol) was added. After heating under reflux for 48 h, the pyridine was distilled under vacuum at 60°C, and dichloromethane added. The mixture was washed with ice-cold water, dried over Na_2SO_4 and the mixture of products (A) and (B) were separated by flash chromatography (silica gel, 2% methanol in chloroform) to afford 2·88 g, 12·6 mmol (36·3%) of (A) and 2·97 g, 8·9 mmol (25·7%) of (B). (A): white solid, mp 176–178°C. ^{1}H-NMR (DMSO-d_6) δ (ppm): 9·61 (bs, 1H, for Ph-OH) 9·21 (t, 1H, for pyrid. H-2, $J_{2,4\text{-}6} = 1{\cdot}2\text{–}0{\cdot}8$ Hz) 8·87 (dt, 1H, for pyrid. H-6, $J_{6,5} = 4{\cdot}5$ Hz, $J_{6,4\text{-}2} = 2{\cdot}1$, 0·8 Hz) 8·43 (dt, 1H, for pyrid. H-4 $J_{4,5} = 8{\cdot}1$ Hz, $J_{4,6\text{-}2} = 2{\cdot}1$, 1·5 Hz) 7·63 (dd, 1H, for pyrid. H-5, $J_{5,6} = 4{\cdot}8$ Hz, $J_{5,4} = 7{\cdot}5$ Hz) 7·02 (d, for Ph-H-5—*para* esterified molecule—$J_{5,6} = 8{\cdot}1$ Hz) 6·97 (s, for Ph-H-2′—*meta* esterified isomer) 6·925 (d, for Ph-H-5′, $J_{5',6'} = 9$ Hz) 6·85 (d for Ph-H-6′, $J_{6',5'} = 8{\cdot}1$ Hz) 6·77 (s, for Ph-H-2) 6·64 (dd for Ph-H-6, $J_{6,5} = 8{\cdot}1$ Hz, $J_{6,2} = 1{\cdot}2$ Hz) (total integration for all phenylic protons, range 7·02–6·64 ppm, is 3 H; the integration ratio of the specific H peaks of the phenyl ring are: H2-H2′ = 6:4, H6-H6′ = 6:4, and H5-H5′ = 6:4) 2·23 (d, total 3H, for Ph-CH_3, ratio of two isomers -ratio of the 2 peaks-6:4). Elemental analysis for $C_{13}H_{11}NO_3$: theory: C 68·12, H 4·80, N 6·11; found: C 68·02, H 4·82, N 6·03 (MW = 229) MS (FAB) 230 ($M+H^+$,100). HPLC: single peak of retention time 1·77 min (60% acetonitrile in water) and 1·76 min (80% methanol in water). (B): white solid, mp 91–92°C. ^{1}H-NMR ($CDCl_3$) δ (ppm): 9·2 (t, 2H, for 2 pyrid. H-2, $J_{2,4\text{-}6} = 1{\cdot}8$ Hz) 8·72 (dt, 2H, for 2 pyrid. H-6, $J_{6,5} = 5{\cdot}1$ Hz, $J_{6,4\text{-}2} = 1{\cdot}8$, 1·2 Hz) 8·25 (dt, 2H, for 2 pyrid· H-4, $J_{4,5} = 8{\cdot}1$ Hz, $J_{4,2\text{-}6} = 1{\cdot}8$ Hz) 7·3 (dd, 2H, for 2 pyrid. H-5, $J_{5,6} = 5{\cdot}1$ Hz, $J_{5,4} = 7{\cdot}8$ Hz) 7·29 (s, 1H, for Ph-H-2) 7·2 (d, for Ph-H-5, $J_{5,6} = 7{\cdot}5$ Hz) 7·12 (d, for Ph-H-6, $J_{6,5} = 8{\cdot}7$ Hz) 2·38 (s, 3H, for Ph-CH_3). Elemental analysis for $C_{19}H_{14}N_2O_4$: theory: C 68·26, H 4·19, N 8·38; found: C 68·35, H 4·19, N 8·34. (MW = 334) MS (FAB) 335 ($M+H^+$,98), 357 ($M+Na^+$,100).

Synthesis of 3-isobutyryloxy-4-nicotinoyloxytoluene (C). In a suspension of (A) (2 g, 9 mmol) in $CHCl_3$ (20 mL), isobutyryl chloride (2 mL, 18 mmol) in $CHCl_3$ (15 mL) was added dropwise (1:2) followed by 5 mL, 0·062 mol, of pyridine. The mixture was refluxed for 48 h and after evaporating the

solvents, the viscous liquid residue was purified by column chromatography (silica gel, 10% methanol in chloroform) to afford 1·5 g (60%) of (C): ^{1}H-NMR ($CDCl_3$) δ (ppm): 9·36 (d, 1H, for pyrid. H-2, $J_{2,4} = 1·7$ Hz) 8·85 (dt, 1H, for pyrid. H-6, $J_{6,5} = 4·8$ Hz, $J_{6,4} = 1·3$ Hz) 8·42 (dt, 1H, for pyrid. H-4, $J_{4,5} = 8$ Hz, $J_{4,2} = 1·95$ Hz) 7·46 (qt, 1H, for pyrid. H-5, $J_{5,4} = 7·9$ Hz, $J_{5,6} = 4·86$ Hz, $J_{5,2} = 1$ Hz) 7·19–7·04 (m, 3H, for 3 Ph-H) 2·68 (septet of d, 1H, for isopropyl CH, J = 7 Hz, J = 2·58 Hz) 2·38 (s, 3H, for Ph-CH_3) 1·15–1·12 (dd, 6H, for isopropyl 2 CH_3, J = 1·95 Hz, J = 7 Hz). Elemental analysis for $C_{17}H_{17}NO_4$: theory: C 68·22, H 5·68, N 4·68; found: C 68·57, H 5·92, N 4·63.

*Synthesis of 3-isobutyryloxy-4-(*N*-methylnicotinoyloxy) toluene iodide (D).* In a solution of (C) (1·2 g, 4 mmol) in anhydrous acetone (30 mL), an excess of methyl iodide (2 mL) was added and the mixture heated at 50°C for 8 h. After distilling part of the solvent, ether was added and the semi-solid precipitate was triturated to give a yellow crystalline mass that was filtered off and washed with ether to afford 1·24 g (71%) of (D): bright yellow solid, mp 131–133°C. ^{1}H-NMR (DMSO-d_6) δ (ppm): 9·7 (s, 1H, for pyridinium H-2) 9·2 (d, 1H, for pyrid. H-6) 9·0 (d, 1H, for pyrid. H-4) 8·2 (t, 1H, for pyrid. H-5) 7·2 (m, 3H, for 3Ph-H) 4·4 (s, 3H, for N-CH_3) 2·8–2·6 (m, 1H, for isopropyl CH) 2·3 (s, 3H, for Ph-CH_3) 1·1 (d, 6H, for isopropyl 2 CH_3). Elemental analysis for $C_{18}H_{20}INO_4$: theory: C 48·97, H 4·53, N 3·17, I 28·79; found: C 49·02, H 4·61, N 3·13, I 28·83. (MW = 441) MS (FAB) 314 (M-I-, 100).

*Synthesis of 3-isobutyryloxy-4-(*N*-methyl-1,4-dihydronicotinoyloxy) toluene (E).* Compound (D) (0·882 g, 2 mmol) was dissolved in de-aerated water, peroxide-free ether was added, the mixture cooled, and $NaHCO_3$ (0·336 g, 4 mmol) was added slowly followed by $Na_2S_2O_4$ (0·696 g, 4 mmol). The reaction mixture was stirred in an ice bath, under N_2, for 2 h after which the layers were separated, washed, and the combined ether solutions dried and evaporated to afford 0·2 g (30%) of (E): Yellow viscous liquid that reduced rapidly methanolic silver nitrate. ^{1}H-NMR ($CDCl_3$) δ (ppm): 7–6·8 (m, 3H, for Ph-H) 6·7 (dd, 1H, for dihydropyr. H-2) 5·65 (dt, 1H, dihydropyr. H-6) 4·85 (m, 1H, dihydropyr. H-5) 3·15 (bs, 1H, dihydropyr. H-4) 2·95 (s, 3H, for N-CH_3) 2·8 (m, 1H, for isopropyl CH) 2·3 (3H, for Ph-CH_3) 1·3 (m, 6H, for isopropyl 2 CH_3). (MW = 315) MS (FAB) 338 ($M+Na^+$,100). UV (in MeOH) λ_{max} 270, 345 nm.

Synthesis of 3-pivaloyloxy-4-nicotinoyloxytoluene (F). Compound (A) (0·641 g, 2·8 mmol) was dissolved in $CHCl_3$ (15 mL) and excess of pivaloyl chloride (1·5 mL, 12 mmol) in $CHCl_3$ (10 mL) was added dropwise. After the addition of pyridine (5 mL) the reaction mixture was stirred at room temperature for 10 h. The solvents were evaporated, toluene added, the white undissolved solid was filtered off and the filtrate was condensed and purified by flash chromatography (silica gel, 2% methanol in chloroform) to afford 0·61 g (68·7%) of (F): viscous liquid. ^{1}H-NMR ($CDCl_3$) δ (ppm): 9·36 (d, 1H, for pyrid. H-2, $J_{2,4} = 2$) 8·86 (dt, 1H, for pyrid. H-6, $J_{6,5} = 4·5$ Hz, $J_{6,4} = 2$ Hz) 8·42 (dt, 1H, for pyrid. H-4, $J_{4,5} = 8$ Hz, $J_{4,2\text{-}6} = 2$ Hz) 7·47 (dd, 1H, for pyrid. H-5, $J_{5,4} = 8$ Hz, $J_{5,6} = 4·5$ Hz) 7·2–7·0 2 (m, 3H, for 3 Ph-H) 2·4 (s, 3H, for Ph-CH_3) 1·2 (s, 9H, for t-butyl 3 CH_3). (MW = 313) MS (FAB) 336 ($M+Na^+$,100), 314 ($M+H^+$,75).

*Synthesis of 3-pivaloyloxy-4-(*N*-methylnicotinoyloxy)toluene iodide (G).* Compound (F) (0·5 g, 1·6 mmol) was dissolved in anhydrous acetone (20 mL) and iodomethane (5 L) was added. The mixture was refluxed for 24 h after which ether was added. The yellow precipitate that was filtered off was recrystallized by acetone/ether to afford 0·36 g (50%) of (G): bright yellow solid, mp 140–142°C. ^{1}H-NMR (DMSO-d_6) δ (ppm): 9·7 (s, 1H, for pyrid. H-2) 9·3 (d, 1H, for pyrid. H-6, $J_{6,5} = 4·8$ Hz) 9·14 (d, 1H, for pyrid. H-4, $J_{4,5} = 6·4$ Hz) 8·35 (t, 1H, for pyrid. H-5, $J_{5,4\text{-}6} = 7·6$ Hz) 7·4–7·2 (m, 3H, for 3 Ph-H) 4·5 (s, 3H, for N-CH_3) 2·4 (s, 3H, for Ph-CH_3) 1·15 (s, 9H, for t-butyl 3 CH_3). Elemental analysis for $C_{19}H_{22}INO_4$: theory: C 50·10, H 4·83, N 3·07, I 27·91; found: C 50·21, H 4·86, N 3·04. (MW = 455) MS (FAB) 328 (M-I-,100).

*Synthesis of 3-pivaloyloxy-4-(*N*-methyl-1,4-dihydronicotinoyloxy)toluene (H).* Compound (G) (0·059 g, 0·13 mmol) was dissolved in deaerated water (8 mL) and peroxide-free ether (5 mL) was added. $NaHCO_3$ (0·043 g, 0·52 mmol) was slowly added while stirring in an ice bath followed by $Na_2S_2O_4$ (0·09 g, 0·52 mmol). The reaction was stirred for 1 h under N_2, the layers separated, washed and the ether layers dried over Na_2SO_4, filtered, evaporated under vacuum to afford 0·021 g (50%) of (H): yellow viscous liquid that reduces instantly methanolic silver nitrate. ^{1}H-NMR ($CDCl_3$) δ (ppm): 7–6·8 (3H, for 3 Ph-H) 6·65 (1H, for dihydro H-2) 5·65 (d, 1H, for dihydro H-6) 4·84 (1H, for dihydro H-5, $J_{5,4} = 8$ Hz) 3·15 (m, 2H, for pyrid. 2 H-4) 2·42 (s, 3H, for N-CH_3) 2·35 (s, 3H, for Ph-CH_3) 1·4 (s, 9H, for t-butyl 3 CH_3). (MW = 329) MS (FAB) 352 ($M+Na^+$,100). UV (MeOH) λmax 268, 345 nm.

*Synthesis of 3,4-(*N*-methylnicotinoyloxy)toluene iodide (I).* To a solution of (B) (0·65 g, 1·9 mmol) in MeOH, an excess of iodomethane (2 mL) was added and the mixture was heated at 40°C for 2 h. The solvent was evaporated, acetone was added, and stirred for 1 h. The yellow precipitate was filtered off and recrystallized from methanol/ether to afford 0·74 g (61·6%) of (I): yellow solid, mp 204–206°C. ^{1}H-NMR (DMSO-d_6) δ (ppm): 9·7 (2H, for 2 pyrid. H-2) 9·2 (d,2H, for 2 pyrid. H-6) 9·1 (d, 2H, for 2 pyrid. H-4) 8·4 (m, 2H, for 2 pyrid. H-5) 7·5 (m, 3H, for 3 Ph-H) 4·5 (s, 6H, for 2 N-CH_3) 2·5 (s, 3H, for Ph-CH_3). Elemental analysis for $C_{21}H_{20}I_2N_2O_4$: theory: C 40·77, H 3·23, N 4·53, I 41·10; found: C 40·83, H 3·25, N 4·56, I 41·13 (MW = 618).

*Synthesis of 3,4-(*N*-methyl-1,4-dihydronicotinoyloxy)toluene (J).* Compound (I) (0·741 g, 1·2 mmol) was dissolved in deaerated water, ethyl acetate was added and the mixture cooled in an ice-bath. $NaHCO_3$ (0·8 g, 9·6 mmol) was added very slowly followed by $Na_2S_2O_4$ (1·6 g, 9·6 mmol). The whole procedure was conducted under N_2 and the mixture was stirred for 1 h 45 min. Subsequently, the two layers were separated, washed and the combined ethyl acetate extracts dried over Na_2SO_4. After filtration and evaporation they afforded 0·197 g (45%) of (I): yellow liquid that reduced instantly methanolic silver nitrate. ^{1}H-NMR ($CDCl_3$) δ (ppm): 6·95–6·67 (m, 5H, for 3 Ph-H and 2 dihydropyridine H-2) 5·7 (d, 2H, for 2 dihydropyridine H-6) 4·9 (m, 2H, for 2 dihydropyridine H-5) 3·2 (bs, 4H,

for 2 dihydropyridine H-4) 3·02 (s, 3H, for Ph-CH_3) 2·3 (d, 6H, for 2 N-CH_3). (MW = 366) MS (FAB) 389 ($M+Na^+$,100). UV (MeOH) λmax 280 and 353 nm.

In-vitro *stability*

Analytical method. A high performance liquid chromatographic (HPLC) method was developed to assay the CDSs and their metabolites. The HPLC system consisted of an Autochrom M500 pump, Rheodyne injector, Waters RCM C-18 column, Spectroflow 757 absorbance detector and a Fisher Recordall series 5000. The mobile phase consisted of aqueous phosphate buffer solution (0·037 M, pH 6·5) and acetonitrile in different proportions. HPLC peaks were integrated and used as a measure of the concentration of the compounds (assay detection limit 1 mg mL^{-1}) and were plotted against time to evaluate the disappearance rates of the compounds. The stabilities were determined by measuring the pseudo-first order rate constant (k_{obs}, min^{-1}) or the half-life ($t_{\frac{1}{2}}$, min) of disappearance of the compound in the solution. k_{obs} is determined from the slope of the log of the disappearance curve (k_{obs} = slope × 2·303) and the $t_{\frac{1}{2}}$ of the compounds is calculated from the formula $t_{\frac{1}{2}} = 0{\cdot}693/k_{obs}$.

Stability in buffers. USP standard phosphate buffer solutions (0·2 M) (USP XXI, 1985) in the pH range of 4·5 to 9·5 were used in this study. Solutions of D and E in buffers were made at a concentration of 1 mg mL^{-1}. The solutions were kept at 37°C and aliquots were taken at frequent time intervals and injected in the HPLC. The study was repeated 3 times, and the half life at each pH was calculated from the average of the values obtained.

Stability in biological media. A 0·3-mL aliquot of stock solution (3 mg mL^{-1} DMSO) of the CDS compounds or their quaternary metabolites were added, respectively, to 3 mL of biological medium (whole heparinized rat blood, 20% rat brain homogenate, or 20% rat liver homogenate, in isotonic phosphate buffer of pH 7·4), which was kept in a 37°C water bath, to obtain a final concentration of 0·33 mg mL^{-1} biological media. Samples of 0·1 mL were taken at appropriate time intervals and were mixed with 0·2 mL acetonitrile containing 5% DMSO. The mixtures were centrifuged and the supernatants injected in the HPLC. The experiment was repeated once more and the average half lives calculated.

In-vivo *distribution*

Analytical method. The HPLC system consisted of an SP 8810 precision isocratic pump, SP4290 injector, Waters RCM C-18 column, SP 8450 UV/visible detector and SP4290 integrator. The mobile phase consisted of aqueous phosphate buffer solution (0·05 M, pH 6·5) and acetonitrile in different proportions for the detection of the dihydro compounds, quaternary metabolites and final metabolites. The area under the peak was used as a measure of the concentration of the compound. Detection was made at 345 nm for the dihydro compounds and at 254 nm for metabolites.

Experimental procedure. Male Sprague–Dawley rats (200–220 g) were injected intravenously (tail vein) with compound J (50 mg kg^{-1} body weight) in 1:1 DMSO and 50% 2-hydroxypropyl-β-cyclodextrin in water. Animals were killed at the appropriate time points and trunk blood and brain collected. Samples were mixed or homogenised with 2 vol of acetonitrile containing 5% DMSO, centrifuged, and supernatants analysed by HPLC. Calibration curves were used to determine the concentrations of the CDSs and their metabolites in blood or brain, respectively.

In-vivo *NGF-stimulatory activity*

The effect of the 4-methylcatechol chemical delivery system on the biosynthesis of NGF in the specific brain regions, hippocampus and frontal cortex, was determined by measuring the levels of NGF mRNA. For this assay, 3 groups of animals were used. Male SpragueDawley rats (320–360 g) were injected intravenously (tail vein) with: 4-methylcatechol-CDS (J) (50 mg kg^{-1} body weight, drug vehicle consisted of 1:1 DMSO and 50% 2-hydroxypropyl-β-cyclodextrin in water, total injected volume 1·8 mL kg^{-1}); drug vehicle only (1·8 mL kg^{-1}); or 4-methylcatechol (dose of 4-methylcatechol was 17 mg kg^{-1} equimolar with 4-methylcatechol-CDS, in same amount of drug vehicle). After 8 h animals were killed by decapitation, and the brains removed and placed on ice-cooled surface. The hippocampus and frontal cortex were rapidly dissected out and separately placed in volume of lysis solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0·5% *N*-lauryl sarcosine, and 0·1 M 2-mercaptoethanol, total volume 1 mL (100 mg tissue)$^{-1}$ and immediately homogenized. RNA isolation: total RNA was isolated using the acid guanidinium isothiocyanate method followed by the phenol/chloroform method of purification and isopropanol precipitation (Chomczynski & Sacchi 1987; Sambrook et al 1989). The concentration of the RNA was evaluated spectrophotometrically at a wavelength of 260 nm. The purity of RNA was assessed by calculating the ratio of absorbance at 260/280 nm, and only ratios greater than 1·8 were analyzed further. Pre-pro NGF mRNA was measured by dot blot analysis utilizing the rat pre-pro NGF probe of 771 bp (provided by Drs S. Whittemore and P. Isackson and purified by Dr. S. Singh) (Whittemore et al 1988). Blotting of total RNA: two concentrations (5 and 15 μg) of RNA from each sample (to ascertain linearity of hybridization vs RNA loaded) were filtered though a nylon membrane held in a Hybri-Dot manifold (Bethesda Research Laboratories) and UV crosslinked (using a UV Crosslinker). The blot was prehybridized (5 × SSPE, 5 × Denhardts solution, 50% formamide, 0·5% SDS, and 100 μg mL^{-1} denatured salmon sperm DNA) for at least 2 h at 42°C, and then hybridized under the same conditions for 22 h with the final hybridization buffer containing 1–2 × 10^6 counts min^{-1} mL^{-1} of [a-^{32}P]-dCTP-labelled pre-pro NGF probe: labelling was performed with [a-^{32}P]-dCTP (specific activity: 3000 Ci mM^{-1}, 10 μCi mL^{-1}, DuPont, New England Nuclear, Cambridge, MA) using the Random primer probe labelling kit (Gibco-BRL) and purification with a Sephadex-50 column. The dot blot was quantitated by computer image analysis with the Betascope Blot Analyser, Model 603 (Betagen Corp., Waltham, MA). The data was obtained as the counts of [^{32}P]dCTP-NGF per dot band of blotted RNA collected over a 16-h time period. Following this, the NGF probe was stripped from the membrane which was then rehybridized with the actin probe. Since the amount of actin mRNA is considered an indicator of total RNA per sample, the hybridization with the actin probe was done in order to normalize the amount of NGF mRNA measured per

total amount of RNA loaded for each sample. Thus, an increased NGF signal for a sample would represent, after normalizing, true stimulation of the specific mRNA synthesis and could not be attributed to variation of the total RNA loaded for that sample. The radiolabelling of the actin probe, subsequent hybridization of the membrane with it, and the quantitation of the actin mRNA signal was performed using the same procedure described previously for the NGF probe and mRNA. Results were expressed as counts min^{-1} for the NGF signal per counts min^{-1} for the actin signal (NGF mRNA units/actin mRNA units). Statistical analysis was performed using the ANOVA followed by Fisher PLSD test for determination of group differences.

Results and Discussion

Synthesis

The mono esterification reaction of 4-methylcatechol with nicotinoyl chloride produced an interesting result: compound A, as deduced from the NMR spectral data, was a mixture of two isomers: the *para* esterified isomer (*para* to the methyl group of the catechol ring) appeared in slightly higher amounts (60:40) than the *meta*, as derived from the proton NMR integration ratios of the phenyl hydrogen peaks or the 4-methyl hydrogen peaks. The two isomers were indistinguishable by all other analytical methods used. They had identical Rf values on TLC using different solvent systems, and yielded a single peak on HPLC using two different mobile phases (60% acetonitrile/water and 80% methanol/water). Thus, resolution of the two isomers could not be achieved and subsequent reactions using A as a starting material yielded inseparable isomeric products. The second esterification of compound A with isobutyryl chloride (or pivaloyl chloride) yielded the two corresponding isomers in almost equal proportions (53:47). Compounds C, D, E, F, G, and H were all approximately 1:1 mixtures of the two isomers, as derived from the corresponding NMR data. All gave a single peak in diverse HPLC systems, and a single spot on various TLC systems.

In-vitro *stability studies*

Stability in buffers. Theoretically, compounds with potential biological activity, apart from potency, should exhibit some stability in aqueous solution, to be useful for therapeutic purposes, for example to make stable pharmaceutical formulations. The in-vitro stability study conducted herein consisted of an approach to investigate these properties.

The stability of E (chosen as a representative among the three 4-methylcatechol CDSs) was studied in USP Buffers of pH range 5–9 at 37°C, and it exhibited a bell shaped pH profile (Fig. 1). The optimum stability was observed at around pH 6. Instability in more acidic conditions (pH 5, $t_{\frac{1}{2}} = 28$ min) is due to the hydration of the dihydro ring (Scheme 3), while in alkaline conditions instability is attributed to rapid hydrolysis of the esters (in pH 8, $t_{\frac{1}{2}} = 3$ min).

The corresponding quaternary compound, D, appears relatively more stable in acidic conditions while it also degrades rapidly in alkaline conditions due to facilitated ester hydrolysis.

Stability in biological media. In-vitro stability studies in biological matrices may not result in absolute values but give a relative picture of the stability profile of the compounds under investigation. They also give indications of the possible metabolic profile in in-vivo conditions.

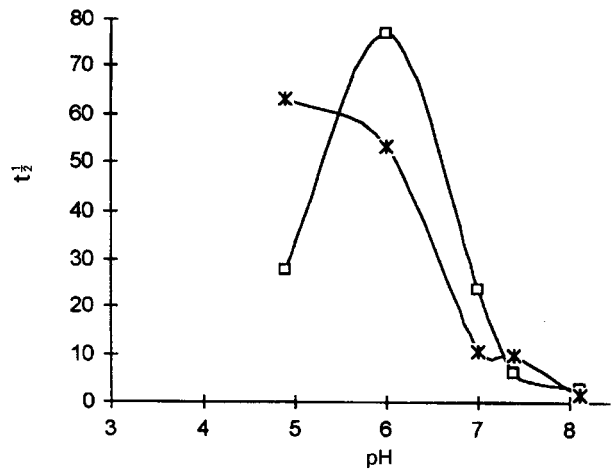


FIG. 1. pH profile of 4-methylcatechol CDS (E; □) and its quaternary metabolite (D; ∗). Each value is the mean of three independent determinations.

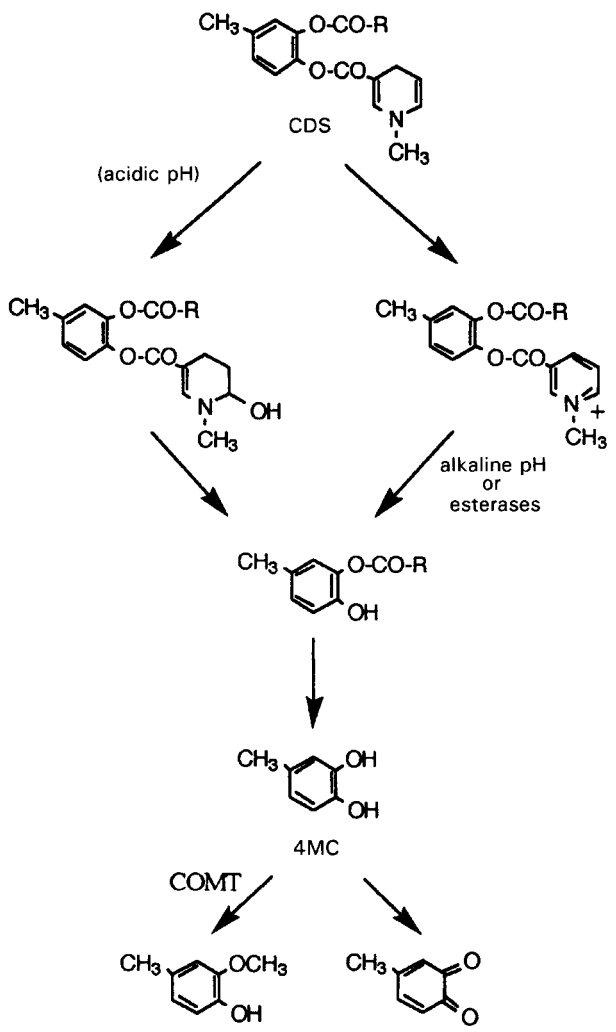
Stability in the rat brain homogenate. Dihydro compounds E, H, and J exhibited half lives over the range 2–50 min in the rat brain homogenate (Table 1). Degradation pathways, as shown in Scheme 3, consisted of oxidation to the quaternary and sequential hydrolysis of the two ester bonds.

The corresponding quaternary compounds (with the exception of I) showed even lower half-lives suggesting that the oxidation of the dihydro (or else the produced trigonelline group) facilitates ester bond cleavage. In other words, the rate limiting step for the hydrolysis of the dihydro group is oxidation to the corresponding pyridinium. The surprisingly long half-life of the quaternary I is probably due to the fact that access to esterases may be prohibited by steric and electrostatic interference (both ester groups are bulky and charged).

Stability in whole rat blood. Shorter half lives, as expected due to the abundance of esterases in the blood compared to the brain, were obtained in whole rat blood (Table 2) for all dihydro compounds and most of the quaternary metabolites. Comparing the half-lives of the dihydro compounds E, H and J, the di-dihydrotrigonelline ester J exhibited the highest stability in rat whole blood.

In-vivo *distribution studies*

In view of the relative stability in-vitro, in both whole rat blood and brain homogenate (Tables 1 and 2), of the dihydro J as compared to the other dihydro compounds, it was selected as the most promising delivery system for 4-methylcatechol, and was further investigated in-vivo. The CDS of 4-methylcatechol (J) rapidly enters the rat brain, after intravenous administration, and is therein converted rapidly to the quaternary metabolite I which is sustained for long periods, releasing 4-methylcatechol (Fig. 2A). Initial high levels of 4-methylcatechol may be attributed to its direct release from the CDS which evidently occurs to some extent, while the decrease of 4-methylcatechol



Scheme 3. Degradation and metabolic pathways of 4-methylcatechol CDSs and their quaternary metabolites in-vitro and/or in-vivo.

levels with time is due to the extensive metabolism of the catechol moiety. Nevertheless, a small amount of 4-methylcatechol (concentration range 0·2–0·02 mmol (mg brain tissue)$^{-1}$), provided by the slow but constant hydrolysis of the quaternary in the brain, is maintained for a long period of time.

Neither the dihydro (J) nor the catechol (4-methylcatechol) were detected in the blood even at early times. The dihydro that remained in the circulation was rapidly converted to the quaternary that was eliminated eventually or gave small amounts of 4-methylcatechol that were however rapidly eliminated before detection. Ratio of the quaternary metabolite in blood/brain is represented in Fig. 2B.

It should be noted that after peripheral administration (i.v. injection) of equimolar amounts (to the CDS J) of 4-methylcatechol and in the same drug vehicle, small amounts of 4-methylcatechol were detected in the brain, that rapidly disappeared within the first few minutes (results not shown).

In-vivo *(CNS) NGF-stimulatory activity*

The in-vivo (CNS) NGF-stimulatory study of the brain-targeted CDS of 4-methylcatechol (J) showed, after a single bolus intravenous administration, a 1·76-fold increase in NGF mRNA compared to control in the rat hippocampus (1·3-fold increase compared to the 4-methylcatechol-control) (Table 3). Administration of 4-methylcatechol itself demonstrated a small increase (1·3-fold) in NGF mRNA compared to control. In the frontal cortex a 1·32-fold increase was observed by the CDS (1·22-fold increase compared to 4-methylcatechol-control) (Table 3), while 4-methylcatechol minimally increased (1·1-fold) the NGF mRNA content in this region compared to control.

The small induction of 4-methylcatechol itself (4-methylcatechol-control) on brain NGF mRNA levels (1·3-fold increase in the hippocampus and 1·1-fold in the frontal cortex) may be attributed to its peripheral effects that perhaps may indirectly influence CNS levels, or due to the minimal amounts capable of crossing the blood-brain barrier in order to induce NGF locally (in the CNS).

Table 1. In-vitro stability in rat brain 20% homogenate of CDSs (E, H, and J) and their quaternary metabolites (D, G, and I).

	D	E	G	H	I	J
r	0·997	0·999	0·995	0·986	0·979	0·98
k	0·666	0·071	0·326	0·014	0·003	0·013
$t_{\frac{1}{2}}$ (min)	1	9·8	2·1	49·5	231	53·3

Each value is the mean of two independent determinations. r is the regression coefficient, k is the rate of disappearance, and $t_{\frac{1}{2}}$ is the half-life.

Table 2. In-vitro stability in rat blood of CDSs (E, H, and J) and their quaternary metabolites (D, G, and I).

	D	E	G	H	I	J
r	0·99	0·984	0·95	0·972	0·991	0·991
k	0·037	2·23	0·642	0·633	0·012	0·014
$t_{\frac{1}{2}}$ (min)	18·7	0·3	1·1	1·1	57·7	49·5

Each value is the mean of two independent determinations. r is the regression coefficient, k is the rate of disappearance, and $t_{\frac{1}{2}}$ is the half-life.

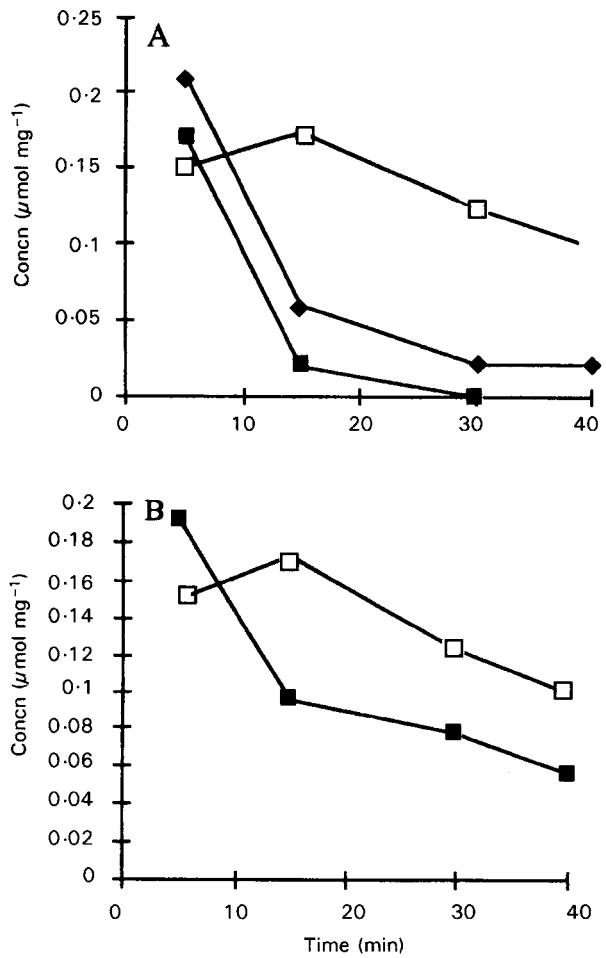


FIG. 2. A. In-vivo brain concentration of the CDS J (■), its quaternary metabolite I (□), and final metabolite, 4-methylcatechol (4-methylcatechol, ◆). B. In-vivo blood (■)/brain (□) distribution of quaternary metabolite I. Each value is the mean of two independent determinations.

The concentration of 4-methylcatechol achieved in the brain with the CDS (J) is comparable with the dose used in the study of direct intracerebroventricular injection of 4-methylcatechol (0·1–0·02 mmol) (Saporito et al 1993). Furthermore, comparable is the effect of 4-methylcatechol which in that case (Sapotito et al 1993) increased hippocampal NGF mRNA levels approximately 165% relative to control at 8 h post injection time.

The hippocampus and cerebral cortex are the primary target sites for the basal forebrain-originating cholinergic neurons which undergo degeneration in Alzheimer's, and are the brain regions that contain the highest NGF mRNA levels (Whittemore & Seiger 1987). Deductions about corresponding NGF protein increases paralleling the induction of mRNA cannot be made directly from this study, although 4-methylcatechol induces both NGF mRNA and protein in-vitro (Furukawa et al 1989, 1991; Carswell et al 1992) suggesting that there is no interference with protein translation mechanisms.

The difference of induction in the hippocampus and cortex (NGF seems more inducible in the hippocampus than in the cortex by 4-methylcatechol or its CDS) could be attributed to different mechanisms of NGF-induction by the compounds

Table 3. Effect of 4-methylcatechol-CDS (J) on hippocampal and frontal cortical NGF mRNA levels.

	NGF mRNA (units per actin mRNA units)	
	Hippocampus	Frontal cortex
Control	0·264 ± 0·053	0·331 ± 0·053
4-Methylcatechol	0·341 ± 0·051	0·351 ± 0·052
4-Methylcatechol-CDS (J)	0·462 ± 0·031*	0·438 ± 0·028*

Each value is the mean ± s.e. of 4 to 6 animals. *$P < 0·05$ relative to control according to Fisher PLSD.

under investigation in these two regions via different cell types. In addition to neuronal cells, glial cells have also been shown in-vitro and in-vivo (Bakhit et al 1991) to be not only capable of producing basal NGF levels but also being stimulated by agents to induce their production, and therefore these cells can provide an additional source of NGF in that region. In view of the fact that the mechanism(s) of NGF induction are not well elucidated as yet, differential NGF induction of various CNS cell types as well as differential receptor distribution and sensitivity requires further investigation.

Conclusion

Overall, significant levels of 4-methylcatechol, the compound of interest, were attained within the rat brain with the corresponding CDS, while levels of this compound in the periphery were not only very low, but also disappeared rapidly. Thus, selective and sustained delivery of the compound of interest to the brain was achieved. Furthermore, the in-vivo increase in NGF mRNA in the hippocampal and cortical region of the rat brain by the 4-methylcatechol CDS, demonstrates the feasibility of NGF-induction within the CNS by peripheral administration of a 4-methylcatechol brain-targeted CDS, and lends credit towards the development of dihydropyridine CDS derivatives for other established NGF inducers. Apart from the potential therapeutic application of such derivatives, this peripheral (non-invasive and not damaging to the CNS) administration of efficient NGF-inducers would provide perhaps a better means to study the in-vivo mechanism of NGF stimulation, as well as consequent effects of NGF, with a controlled dosage and treatment schedule of administration of the NGF-stimulating compounds. Moreover, site-specific brain targeting provides, possibly, the only means for peripheral administration of NGF-stimulating agents for CNS application, since the well documented peripheral NGF-induction (in the peripheral nervous system) would be a serious, indiscriminate, and persistent undesired side-effect.

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