Both the D-(+) and L-(−) Enantiomers of Nicotine Inhibit Aβ Aggregation and Cytotoxicity†

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ABSTRACT: The underlying cause of Alzheimer's disease is thought to be the aggregation of monomeric β-amyloid (Aβ), through a series of toxic oligomers, which forms the mature amyloid fibrils that accumulate at the center of senile plaques. It has been reported that L-(−)-nicotine prevents Aβ aggregation and toxicity, and inhibits senile plaque formation. Previous NMR studies have suggested that this could be due to the specific binding of L-(−)-nicotine to histidine residues (His^6, His^{13}, and His^{14}) in the peptide. Here, we have looked at the effects of both of the L-(−) and D-(+) optical enantiomers of nicotine on the aggregation and cytotoxicity of Aβ(1−40). Surprisingly, both enantiomers inhibited aggregation of the peptide and reduced the toxic effects of the peptide on cells. In NMR studies with Aβ(1−40), both enantiomers of nicotine were seen to interact with the three histidine residues. Overall, our data indicate that nicotine can delay Aβ fibril formation and maintain a population of less toxic Aβ species. This effect cannot be due to a highly specific binding interaction between nicotine and Aβ, as previously thought, but could be due instead to weaker, relatively nonspecific binding, or to the antioxidant or metal chelating properties of nicotine. D-(+)-Nicotine, being biologically much less active than L-(−)-nicotine, might be a useful therapeutic agent.

Senile plaques and neurofibrillary tangles are the two major histopathological hallmarks of Alzheimer's disease (AD).[1] The senile plaques contain extracellular deposits of amyloid fibrils, which are composed of a 39−43-amino acid peptide termed Aβ. This peptide is derived by proteolytic cleavage from the β-amyloid precursor protein (βAPP). According to the "amyloid hypothesis" of AD, the deposition of Aβ in the brain is a central and seminal event in the pathogenesis of the disease (*1*, *2*). Aβ has neurotoxic properties *in vitro* and can induce neurofibrillary tangle formation in tau transgenic mice (*3*, *4*), but it is still not certain whether Aβ is directly responsible for neuronal cell death in AD.

The toxicity of Aβ *in vitro* appears to be related to its underlying protein conformation and state of aggregation (*5*, *6*). Synthetic Aβ becomes toxic on conversion from soluble, random coil/α-helical monomers to aggregated, β-pleated sheet oligomers or higher-order fibrillar structures (*7*, *8*). However, the precise molecular organization of the toxic form(s) of Aβ and the molecular mechanisms responsible for this toxicity are still not clear. In recent work, small nonfibrillar "oligomers" or larger "protofibrils" have been implicated in Aβ-induced cytotoxicity (*7*−*10*). The toxic effects of Aβ may be due to the metal-dependent formation of hydrogen peroxide and, subsequently, hydroxyl radicals from the peptide (*11*−*15*). Many alternative theories are also suggested, including an interaction of Aβ with membrane receptors (*16*−*18*), membrane insertion of Aβ in different lipid environments (*19*), and the formation by Aβ of Ca^{2+}-permeable membrane channels (*20*, *21*). Potential therapeutic strategies for AD include preventing or reversing the initial conformational change in Aβ to its pathological β-pleated sheet form, blocking or reversing the early stages of Aβ oligomerization, or inhibiting the direct or indirect cytotoxic effects of Aβ (e.g., with antioxidants or metal chelators).

Many compounds have now been identified that can block the aggregation and/or cytotoxic effects of Aβ (*22*). However, the precise molecular mechanisms by which these compounds achieve these effects are seldom clear. L-(−)-Nicotine is one compound that was reported some time ago to inhibit the conversion of the synthetic Aβ(1−42) peptide from its soluble, freshly dissolved form into insoluble, β-pleated sheet deposits (*23*). The interaction between this biologically active enantiomer of nicotine and a truncated, α-helical form of Aβ (residues 1−28) was examined by NMR spectroscopy. The resulting data suggested that L-(−)-nicotine binds specifically to histidine residues (His^6, His^{13}, and His^{14}) in the peptide molecule (*23*). Surprisingly, the D-(+) enantiomer of nicotine was not included in these studies, and there have

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[1] Abbreviations: Aβ, β-amyloid peptide; AD, Alzheimer's disease; βAPP, β-amyloid precursor protein; DETAPAC, diethylenetriaminepentaacetic acid; DELFIA, delayed enhanced lanthanide immunoassay (Wallac); DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; EM, electron microscopy; ESR, electron spin resonance; nAChRs, nicotinic acetylcholine receptors; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; TBS, Tris-buffered saline.

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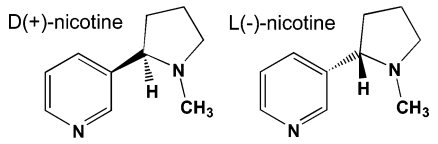

FIGURE 1: Chemical structures of the two nicotine enantiomers.

been no subsequent reports about the effects of this biologically less active enantiomer of nicotine on Aβ aggregation and fibril formation, although there has been one recent study on possible interactions between both nicotine enantiomers and Aβ(1−42) using NMR methods (*24*). Another recent study has demonstrated that L-(−)-nicotine not only inhibits aggregation of Aβ(1−40) and Aβ(1−42) but also can disaggregate fibrils preformed from both of these peptides (*25*). Renewed interest in the effects of nicotine on Aβ aggregation has also been stimulated by a report that L-(−)-nicotine significantly inhibits the accumulation of Aβ deposits in the brains of transgenic mice expressing a pathogenetic mutant form of βAPP (*26*). This could be due, at least in part, to the inhibition and/or reversal of Aβ aggregation, or alternatively, L-(−)-nicotine could influence Aβ deposition via the stimulation of nicotinic acetylcholine receptors (nAChRs), with consequent effects on the proteolytic processing of βAPP (*27*−*29*).

Nicotine can also protect against the cytotoxic effects of Aβ on cultured neuronal cells (*30*−*33*). This could be due to the effects on Aβ aggregation noted above. Alternatively, the α7 and α4β2 nAChRs have been implicated in the protection of rat hippocampal cultures from the toxic effects of Aβ(25−35) (*30*, *31*), and interestingly, a tight binding association has been demonstrated between Aβ(1−42) and α7 nAChRs (*18*). Thus, it is possible that nicotine could influence Aβ formation, aggregation, and toxicity through a number of different nAChR- and non-receptor-mediated mechanisms.

As part of our ongoing studies on the molecular mechanisms of Aβ aggregation and toxicity, and the effects of various types of inhibitors on these processes, we have found that, surprisingly, both the L-(−) and D-(+) enantiomers of nicotine are effective inhibitors of Aβ(1−40) aggregation and toxicity. Here, we report the results of these experiments.

MATERIALS AND METHODS

Peptide and Nicotine. The Aβ(1−40) peptide was synthesized on a Milligen 9050 peptide synthesizer starting from Val-PEG-PS resins and using Fmoc N-protection, as described in detail elsewhere (*34*). L-(−)-Nicotine was purchased as the free base, whereas D-(+)-nicotine (Figure 1) was purchased as the di-*p*-toluoyltartrate salt (both from Sigma). For some of the experiments, the D-(+) enantiomer was desalted by agitating a 10 mM stock solution in Milli Q water with Dowex SBR strong anionic exchange resin beads overnight at room temperature. This process was repeated twice with fresh beads before the solution was filtered through a washed 0.2 μM filter. The concentration of the resulting solution was determined by its absorbance at 260 nm. The purity of the desalted solution was assessed by NMR spectroscopy.

Aggregation-Dependent Aβ Immunoassay. This assay depends on the use of the same anti-Aβ monoclonal antibody for capture and detection (6E10 and biotinylated 6E10, respectively) in a sandwich format (*35*, *36*). Since both antibodies are directed at the same epitope on the peptide, monomeric forms of Aβ, once bound to the capture antibody on the plate, can offer no free binding site for detection antibodies. Only multimeric Aβ is detected by this assay because as the oligomers are assembled, increasing numbers of binding sites become available for the detection antibody, giving increasing signals over time. NUNC high binding microtiter plates (Nunc Maxisorb, Life Technologies, Paisley, Scotland) were coated overnight at 4 °C with 200 μL of 1 μg/mL 6E10 (Signet Pathology Systems Inc., Dedham, MA) in 10 mM PBS [150 mM NaCl (pH 7.4)] and blocked with assay buffer [50 mM Tris-HCl and 0.15 M NaCl (pH 7.6) containing 0.05% Tween 20, 0.5% γ-globulins (Sigma G-7516), and 1% gelatin (Sigma G-7765, from fish skin)] for 1 h at 37 °C. The capture plates were washed four times with washing buffer (PBS containing 0.05% Tween 20), blotted dry, sealed, and stored for up to 1 week at 4 °C. Aβ(1−40) peptide (50 μM) was incubated at 37 °C in 50 mM Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl (TBS) or PBS in the presence or absence of various concentrations of L-(−)-nicotine or D-(+)-nicotine. At appropriate time points, duplicate peptide samples were removed and diluted 1/50 into assay buffer. Each diluted sample was then pipetted into two wells of the capture plates (100 μL per well), giving four wells per time point. After being incubated for 2 h at 37 °C, the plates were washed and incubated for 2 h at 37 °C with biotinylated 6E10 (1 μg/mL in assay buffer, 200 μL per well). The plates were again washed, prior to the addition of streptavidin-europium (Wallac, Milton Keynes, Bucks, U.K.) diluted 1/500 in 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 0.5% bovine serum albumin, 0.05% γ-globulins, and 20 μM diethylenetriaminepentaacetic acid (DETAPAC), allowing quantification by the DELFIA method (Wallac). After being incubated for 1 h at room temperature, followed by washing and addition of DELFIA enhancer solution (Wallac), the plates were read in a Victor 1420 multifunction microtiter plate reader. The Aβ(1−40) peptide typically gave no signal above background (30000−40000 counts) when freshly dissolved, compared to >1 million counts after preincubation for 24−48 h at 37 °C. Results show the mean ± the standard deviation (SD) for one experiment carried out in quadruplicate.

Thioflavin T Assay. The presence of amyloid fibrils was monitored by thioflavin T fluorescence (*37*). Duplicate 15 μL samples of Aβ(1−40) [50 μM in 50 mM Tris-HCl buffer (pH 7.4)] were removed after incubation of the peptide solution for various periods of time at 37 °C in the presence or absence of L-(−)-nicotine or D-(+)-nicotine. These samples were each added to 2 mL of 10 μM thioflavin T in 50 mM glycine/NaOH (pH 9.0) before the characteristic change in fluorescence was monitored (excitation at 450 nm and emission at 482 nm) following binding of thioflavin T to the amyloid fibrils. Duplicate samples were scanned three times before and immediately after the addition of peptide. Results show the mean value of the duplicate samples ± the difference between those mean values.

Electron Microscopy. A 5 μL sample of Aβ(1−40) [50 μM in 50 mM Tris buffer (pH 7.4)] was removed after incubation at 37 °C and added to Formvar/carbon-coated grids for 10 min before excess fluid was blotted off. After the grids has been air-dried, 5 μL of 1% uranyl acetate was

added to the grid and the grid air-dried for 5−10 min before excess solution was blotted off. The grids were examined in a JEOL JEM-1010 transmission electron microscope.

NMR Spectroscopy. Peptide solutions were prepared in D_2O and diluted into phosphate-buffered D_2O solutions at pH 7.4. Final concentrations were 0.2 mM Aβ(1−40) with or without 0.4 mM L-(−)- or D-(+)-nicotine (desalted form) in 10 mM phosphate buffer containing internal reference TSP-d_4. NMR spectra were measured at 30 °C and 400 MHz using a JEOL GSX400 spectrometer. Typical two-dimensional COSY-45 spectra were determined using a spectral width of 3599.7 Hz, and 96 acquisitions for each of 256 increments were sampled into 2048 complex points. The arrays were zero-filled in the t_2 domain to 4096 complex points and then Fourier transformed using a sine-bell window offset of 5%. Prior to Fourier transformation in the t_1 dimension using a sine-bell window offset of 10%, data were extended to 1024 increments using order-16 Linear Prediction and zero-filled to 4096 points. Magnitude mode spectra were prepared for analysis and presentation using the software package NMRPipe (*38*).

Cell Toxicity Assays. SH-SY5Y human neuroblastoma cells were cultured in a 1/1 DMEM/Nutrient Mix F-12 mixture (Gibco BRL) containing 10 IU of penicillin/mL and 100 μg of streptomycin/mL, 15% fetal calf serum, 1% nonessential MEM amino acid supplement, and 2 mM glutamine at 37 °C in 5% CO_2 and 95% air. The cytotoxic effect of Aβ was assessed by measuring the cellular redox activity with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Cells were plated at a density of 7500 cells/well in 96-well plates in 100 μL of fresh culture medium. After 24 h, the medium was replaced with 200 μL of OPTI-MEM (Gibco BRL) serum-free medium, or OPTI-MEM containing 5 μM Aβ(1−40) which had been pre-incubated at 50 μM for 48 h at 37 °C in sterilized 50 mM Tris buffer (pH 7.4). The cells were incubated for a further 24 h, and then 20 μL of stock MTT in PBS was added to give a final concentration of 0.5 mg/mL in each well. After incubation at 37 °C for 4.5 h, the medium/MTT solution was removed and cell lysis buffer was added, followed by incubation at 37 °C. Absorbance readings were taken at 570 nm. Results show the mean ± SD for one experiment carried out in triplicate.

Effects of Nicotine on Fenton Chemistry. These were assessed by monitoring the ability of both isomers of nicotine to block the formation of hydroxyl radicals in the Fenton reaction:

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + {}^{\bullet}OH + {}^{-}OH$$

The formation of hydroxyl radicals was monitored by electron spin resonance (ESR) spectroscopy with 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) as a spin trap. To initiate the reaction, 25 μL of 1 mM H_2O_2, 25 μL of 0.05 M DMPO, and 25 μL of 1 mM DETAPAC were made up to 125 μL with Milli Q water, or with differing concentrations of each enantiomer of nicotine in Milli Q water, followed by the addition of 25 μL of 0.05 mM Fe(II) sulfate. DETAPAC enhances the formation efficiency of the hydroxyl radical adduct of DMPO (*14*). This 150 μL solution was transferred into a 1 mm internal diameter ESR sample tube, and spectra were recorded on a Bruker EMX X-band spectrometer operating with a magnetic field modulation of 100 kHz, with a modulation amplitude of 0.05 mT, a microwave power of 20 mW, and spectrum accumulation over 25 scans. In the absence of quenching, the reaction results in a clear four-line ESR spectrum characteristic of the hydroxyl radical adduct of DMPO (DMPO-OH) (*14*, *39*).

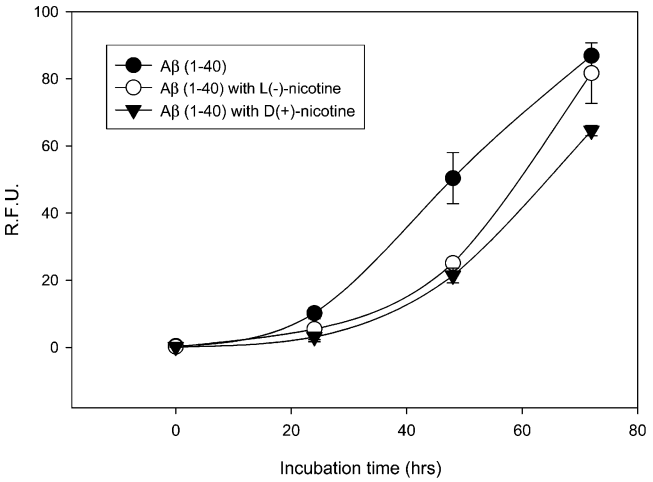

FIGURE 2: Effects of D-(+)- and L-(−)-nicotine on Aβ aggregation as determined with the thioflavin T assay. The Aβ(1−40) peptide (at 50 μM) was incubated at 37 °C on its own, and also in the presence of each enantiomer of nicotine (50 μM). All experiments were carried out in Tris-HCl buffer (pH 7.4). R.F.U. represents relative fluorescence units.

RESULTS

Aggregation Assays. These assays were carried out with the synthetic Aβ(1−40) peptide incubated with D-(+)- or L-(−)-nicotine (Figure 2), for up to 72 h, with aggregation being monitored by both the aggregation-dependent immunoassay and the thioflavin T methods. The immunoassay method detects earlier oligmeric forms of Aβ (*35*), whereas the thioflavin T method detects mainly mature β-pleated sheet amyloid fibrils (*37*). Both enantiomers of nicotine were effective inhibitors of Aβ(1−40) aggregation in these assays (see Figures 2 and 3). The D-(+) enantiomer was active as the free base or the di-*p*-toluoyltartrate salt, whereas the L-(−) enantiomer was purchased and tested only as the free base. Figure 2 shows data for the thioflavin T assay, with incubation of 50 μM peptide in 50 mM Tris-HCl buffer (pH 7.4) with or without an equimolar concentration of nicotine. After incubation for 48 h, both enantiomers gave approximately 50% inhibition of aggregation. In buffers containing physiological concentrations of saline (PBS or TBS), the concentration of nicotine required to give 50% inhibition of aggregation of 50 μM peptide, after incubation for 48 h, was increased to around 400 μM. This is illustrated in Figure 3, in this case using the immunoassay method. After incubation for 24 h, both enantiomers of nicotine significantly inhibited Aβ aggregation ($p < 0.005$, Student's *t* test) by approximately 50%. The effects of a wide range of different concentrations of nicotine on Aβ(1−40) aggregation were then tested. Both enantiomers of nicotine were shown to inhibit peptide aggregation in a clear, concentration-dependent manner (Figure 4). In these experiments, at 10 and 50 μM, D-(+)-nicotine was found to be a significantly better inhibitor than L-(−)-nicotine ($p < 0.05$, Student's *t* test).

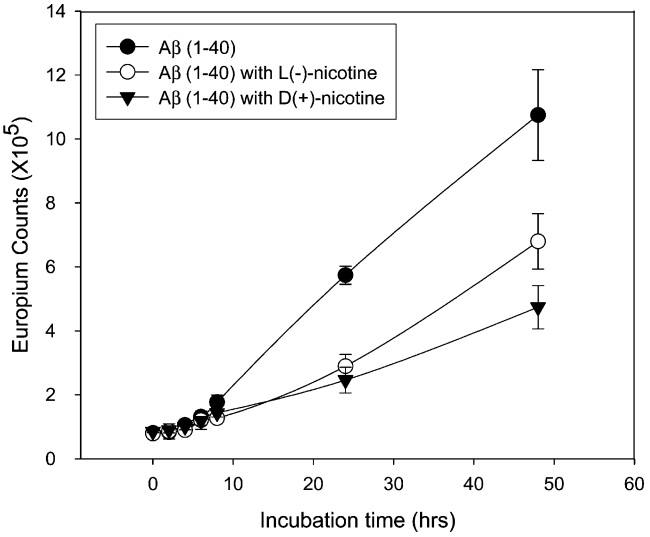

FIGURE 3: Effects of D-(+)- and L-(−)-nicotine on Aβ aggregation as determined with an immunoassay. The Aβ(1−40) peptide (at 50 μM) was incubated at 37 °C on its own, and also in the presence of each enantiomer of nicotine (400 μM). All experiments were carried out in PBS.

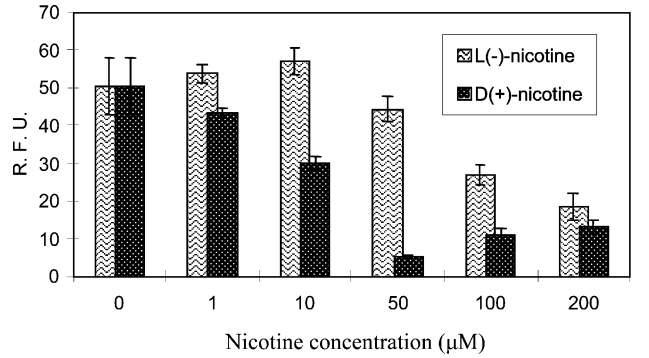

FIGURE 4: Dose-dependent effects of D-(+)- and L-(−)-nicotine on Aβ aggregation as determined with a thioflavin T assay. The Aβ(1−40) peptide (at 50 μM) was incubated in Tris-HCl buffer (pH 7.4) at 37 °C in the presence of the indicated concentrations of each enantiomer of nicotine.

Electron Microscopy. Both enantiomers of nicotine (at 800 μM) showed an inhibitory effect on the formation of amyloid-like fibrils from Aβ(1−40), when the peptide was incubated for 48 h at 50 μM in Tris-HCl buffer (pH 7.4). In the presence of either enantiomer of nicotine, the predominant form was a much shorter, more disrupted type of fibril (Figure 5).

NMR. The His signals of Aβ(1−40) in phosphate buffer (pH 7.4) were shifted equally *upfield* by either optical enantiomer of nicotine (Figure 6). This suggests that the interaction of nicotine is not due to specific binding with any individual locations on the peptide.

SDS (>8 mM) is commonly added to Aβ solutions under typical NMR conditions to hold the peptide in a predominantly α-helical conformation and slow peptide aggregation (*40*, *41*). However, when SDS was present in samples containing low concentrations (200 μM) of the Aβ(1−40) peptide, the histidine chemical shift perturbations seen with nicotine were virtually abolished. In control experiments, it was found that H(2)/(6), H(4), and H(5) signals from nicotine were displaced downfield in the presence of SDS (see Table 1), indicating that at these concentrations there was a

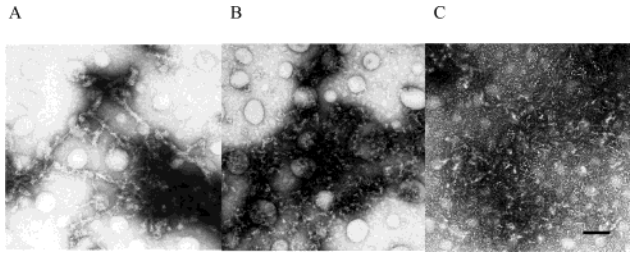

FIGURE 5: Effects of nicotine on Aβ aggregation as monitored by negative stain EM: (A) Aβ(1−40) control [50 μM peptide, incubated for 48 h in Tris-HCl buffer (pH 7.4) at 37 °C] without nicotine, (B) peptide with D-(+)-nicotine (800 μM), and (C) peptide with L-(−)-nicotine (800 μM). The scale bar is 100 nm.

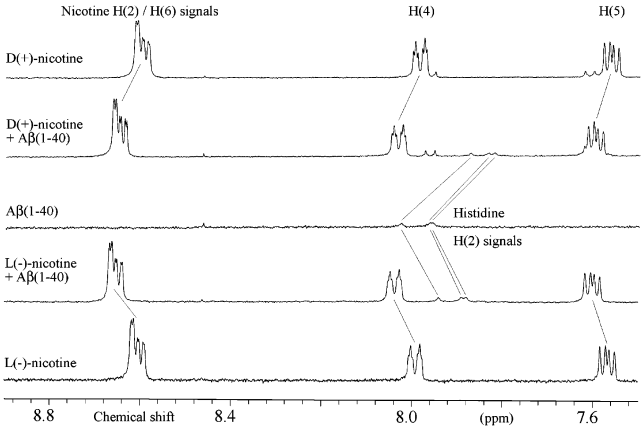

FIGURE 6: Effect of Aβ(1−40) on the chemical shift positions of pyridine protons contained in D-(+)- and L-(−)-nicotine.

significant interaction between these species. To avoid this problem, all NMR spectra were determined in the absence of SDS.

MTT Assays. The addition of pre-aggregated Aβ(1−40) to cultured SH-SY5Y human neuroblastoma cells resulted in a dramatic (approximately 50%) inhibition of their ability to metabolize MTT (Figure 7). However, if the peptide (at 50 μM) was preincubated in the presence of 10, 50, or 200 μM L-(−)- or D-(+)-nicotine prior to its addition to the cell culture medium, the ability of the cells to metabolize MTT was partially restored, in all cases to 70−80% of the controls without nicotine. Our results clearly show that both enantiomers of nicotine significantly and equally reduced the toxicity of the peptide. A further control was included to examine the possibility that the protective effects of nicotine were not manifested during the peptide preincubation step but were due to subsequent interactions of nicotine with the cells, for example, through binding to nAChRs. For this control, 50 or 200 μM L-(−)- or D-(+)-nicotine was added to the pre-aggregated peptide immediately before it was diluted into the medium of the cultured cells [i.e., the drugs were present when the cells were exposed to the toxic Aβ(1−40) aggregates but absent during preincubation of the peptide]. In this case, neither of the two enantiomers of nicotine demonstrated any significant protective effect on the cells (Figure 7).

Effects of Nicotine on Fenton Chemistry. A standard Fenton reaction was carried out in PBS, and the resulting hydroxyl radicals were detected using the spin trap DMPO in conjunction with ESR spectroscopy (*14*). Different concentrations of D-(+)- and L-(−)-nicotine were assessed for their ability to quench the formation of DMPO-OH. As

Table 1: Chemical Shift Data for Nicotine Proton Signals in Aβ(1−40) Solutions, with and without Added SDS

	δ		Δδ		
signal	0.4 mM L-(−)-nicotine in D_2O (pH 7.4)	0.4 mM L-(−)-nicotine in D_2O (pH 7.4) and 10 mM SDS	difference	with 0.2 mM Aβ(1−40)	difference
pyridine ring					
H(5)	7.568	7.596	0.028	7.601	0.033
H(4)	7.994	8.079	0.085	8.039	0.045
H(6)	8.598	8.691	0.093	8.647	0.049
H(2)	8.615	8.745	0.128	8.665	0.048
pyrrolidine ring					
part of H(3)	2.517	2.534	0.017	2.591[a]	0.074
NCH_3	2.579	2.785	0.206	2.753	0.174
part of H(5)	3.063	3.415	0.352	3.314[a]	0.251
part of H(5)	3.622	3.918	0.296	3.799[a]	0.180
H2	4.111	4.525	0.414	4.414[a]	0.303

[a] From COSY data.

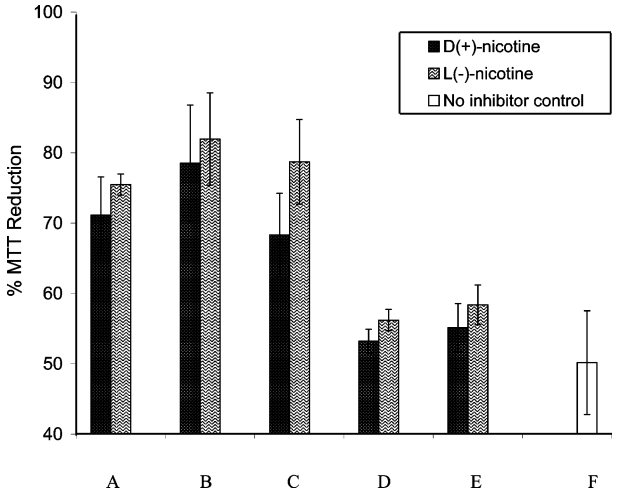

FIGURE 7: Effects of D-(+)- and L-(−)-nicotine on the toxicity of Aβ(1−40) to SH-SY5Y cells. Parts A−C show the effects of 200, 50, and 10 μM nicotine, respectively, when added *during* incubation of Aβ(1−40) at 50 μM for 48 h. In parts D and E, 200 and 50 μM nicotine, respectively, were added *after* peptide incubation. Part F is a control, without nicotine. The results are expressed relative to cells not exposed to Aβ (100% MTT reduction).

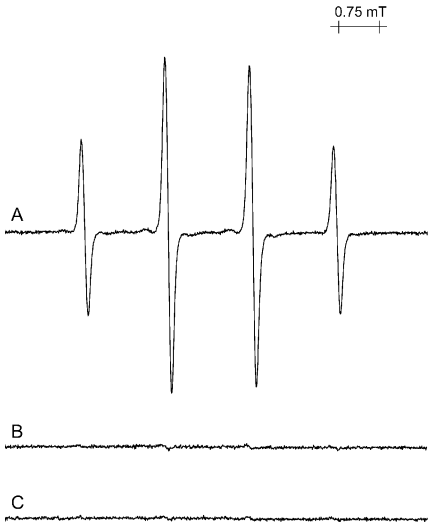

FIGURE 8: ESR spectra recorded when a solution of Fe(II) sulfate was added to a solution containing DMPO, hydrogen peroxide, and DETAPAC in the presence of (A) 50 μL of Milli Q water, (B) 50 μL of L-(−)-nicotine (1150 μM), and (C) 50 μL of D-(+)-nicotine (1150 μM). See the text for full details of experimental conditions and spectrometer settings.

might be expected, both enantiomers of nicotine were equally capable of inhibiting formation of the characteristic four-line DMPO-OH spectrum [a(N) = 1.50 mT and a(H) = 1.46 mT] (Figure 8) in a concentration-dependent manner.

DISCUSSION

Our data show clearly, in two different assay systems, that the biologically less active D-(+) optical enantiomer of nicotine is an equally effective or even better inhibitor of Aβ(1−40) aggregation than the biologically active L-(−) enantiomer. This surprising result, which was also confirmed by EM studies, indicates that the inhibitory effects of nicotine on Aβ aggregation cannot be due to highly specific, stereoselective binding to any form of soluble or aggregated Aβ, but could be due instead to weak, nonspecific binding to the peptide, or to some other property of nicotine and mechanism of action.

The immunoassay method detects the appearance of oligomeric Aβ formed during the very early stages of aggregation (*35*), suggesting that nicotine acts at an early step in the β-amyloid fibril assembly pathway. However, relatively high concentrations of nicotine were required to achieve this, with ∼50% inhibition being obtained at a 1/1 ratio of nicotine to peptide (or 4/1 in the presence of a physiological concentration of saline) after a 48 h aggregation period. The effects of salt in the incubation buffers are probably due to the enhanced rate of aggregation of Aβ in its presence (*42*) or to the mechanism of inhibition being disrupted by the presence of physiological saline. The thioflavin T data over 72 h (Figure 2) indicate that nicotine acts as a "kinetic" inhibitor; i.e., it delays rather than completely prevents the formation of amyloid fibrils. After long incubation periods in the presence of nicotine, Aβ eventually approaches the same state of aggregation as a noninhibitor control. This could explain a previous report of negative effects of nicotine on β-amyloid formation, where only a single, long incubation period was used (*43*).

In the only previous studies in the literature demonstrating inhibitory effects of nicotine on Aβ aggregation (*23*, *25*), only the L-(−) enantiomer was tested, and it was suggested that this binds specifically to an α-helical form of Aβ (*23*). This conclusion was based on NMR studies, carried out in the presence of SDS, which indicated that there is an interaction between L-(−)-nicotine and Aβ(1−28) which

involves a fast exchange and affects NMR signals from three histidine residues at positions 6, 13, and 14. With the addition of a 2-fold molar excess of L-(−)-nicotine, the H(2)−H(6) protons in this substrate were all displaced downfield to higher chemical shift values, while the three histidine H(2) proton lines all moved upfield (*23*).

Our own NMR measurements were performed using 0.2 mM Aβ(1−40), but SDS was *excluded* from the medium. We found that the His signals of the peptide were shifted upfield in the presence of either of the two enantiomers of nicotine (Figure 6). However, when SDS was present in our samples, these histidine chemical shift perturbations were virtually abolished. In control experiments, it was found that signals from nicotine were displaced strongly downfield in the presence of SDS, indicating that there was a significant interaction between these species (see Table 1). In a report which followed that of Salomon *et al.* (*23*), Zeng *et al.* (*24*) published some further NMR data, using Aβ(1−42), that appear to contradict their earlier findings. In this later report, the shift changes induced for nicotine in the presence of Aβ(1−42) were much smaller and nearly all in the opposite direction, i.e., upfield rather than downfield. It should be noted that the peptides were also different; the earlier work referred to the 1−28 species, whereas the later studies examined the 1−42 species. However, the interaction between the peptide and nicotine is known to involve the histidine residues, located at positions 6, 13, and 14 in both species. From the data in Table 1, it can be seen that, on the whole, the presence of SDS perturbs the nicotine resonances to a greater extent than does the presence of Aβ. Therefore, any comparison of nicotine resonances in SDS with those where Aβ is also present is invalid, since competing influences are present. The data of Zeng *et al.* (*24*) were obtained using a maximum of 2 mM nicotine, with 80 mM SDS; when Aβ was also present, its concentration was 0.5 mM. For their earlier work (*23*), these values were 4−10 mM nicotine, 10−20 mM SDS, and 2 mM Aβ; thus, the relative influence of SDS on nicotine was considerably reduced in these earlier measurements.

Our findings, as illustrated in Figure 6, indicate that the two nicotine isomers appear to exert similar influences on the Aβ system. It should be noted that ratios of nicotine to Aβ calculated from weights are not necessarily the same as those observed from spectral nicotine/histidine signal ratios because some of the Aβ has aggregated, disappearing from the solution and thus also from the spectra.

D-(+)-Nicotine, when used as the tartrate salt, precipitated Aβ from solution almost immediately. This was probably a salt effect as described by Hilbich *et al.* (*42*). Increases in the salt concentration are known to decrease the values of critical micellar concentrations (*41*). The presence of the tartrate would therefore enhance any latent ability of the Aβ to form micelles between unstructured random coil monomers, possibly leading to more rapid aggregation. Once the D-(+)-nicotine was desalted, it maintained Aβ solubility as for the L-(−) isomer.

In the absence of SDS, the probable solution structure of Aβ is a random coil (*44*) and the proton NMR data provide information about ionic interactions with the amino acid side chain substituents which occur under conditions of rapid exchange. This is in general agreement with the HSQC data of Zeng *et al.* (*24*), who described a narrow NH shift dispersion range for samples dissolved in water (in the absence of SDS) together with minimal chemical shift changes. These were stated to indicate that nothing was binding to the Aβ and therefore inducing changes in the backbone secondary structure about which these N−H correlations report.

One possible explanation for the NMR data obtained in the absence of SDS is that relatively nonspecific, charge-related interactions between either enantiomer of nicotine and histidine residues on Aβ slightly increase the average separations between peptide monomers in solution and so delay the onset of aggregation. This would be in keeping with the thioflavin T data suggesting that nicotine acts as a kinetic inhibitor and that the mechanism of inhibition may be disrupted by the presence of a physiological concentration of saline. However, there are some other plausible explanations. Zeng *et al.* (*24*) have suggested that nicotine might bind to a small oligomer, which cannot be viewed by NMR. However, since both nicotine enantiomers have the same effect, this would have to be due to relatively weak, nonspecific binding.

In the presence of either isomer of nicotine, samples retain their spectroscopic viability for significantly longer, making practicable overnight measurements that were not possible for the peptide in the absence of SDS. It therefore seems logical that nicotine is exerting an inhibitory effect that results in the maintenance of the soluble (monomeric) form. This activity cannot reside solely in the pyridine ring. Measurements using analogues of nicotine (unpublished work) containing alternate aliphatic heterocyclic ring structures show various reduced levels of activity relative to the nicotine enantiomers.

It is also possible that nicotine delays aggregation not through binding to Aβ but through some other chemical property shared by both enantiomers. We have shown recently that Aβ generates hydroxyl radicals during incubation in solution, in the presence of certain redox-active transition metal ions (*12*). These radicals, or other free radicals, could contribute to Aβ aggregation and toxicity (*12*, *15*). Hence, nicotine could affect Aβ aggregation or toxicity by virtue of its antioxidant properties. We have shown that both enantiomers of nicotine can quench the hydroxyl radicals produced by hydrogen peroxide in the Fenton reaction (see Figure 8). This is consistent with a previous report showing that nicotine has antioxidant activity (*45*). Interestingly, some other inhibitors of Aβ aggregation are also antioxidants. Rifampicin has hydroxyl radical scavenging ability and can inhibit Aβ(1−40) fibril formation (*46*). Naiki *et al.* (*47*) have demonstrated antioxidant-mediated inhibition of Aβ(1−40) and Aβ(1−42) fibril formation, and the antioxidant vitamin E exhibits neuroprotective properties *in vitro* against Aβ-induced toxicity in cell culture assays (*48*, *49*). However, in the case of nicotine, we consider this mechanism unlikely. Although hydroxyl radicals are generated from Aβ in phosphate buffers (*14*), we have failed to demonstrate hydroxyl radical production from Aβ in Tris-HCl buffers (S. Turnbull, unpublished observation). This is due to the fact that, at the concentrations usually employed, Tris-HCl itself has antioxidant properties and can quench the formation of hydroxyl radicals from the Fenton reaction. Nicotine inhibits the aggregation of Aβ(1−40) in both phosphate and Tris-HCl buffers (Figures 2 and 3), and it

seems unlikely that the addition of 50 μM nicotine could exert a sufficient additional antioxidant effect over and above that of 50 mM Tris-HCl to explain the inhibition of Aβ aggregation. One further possibility is that nicotine might influence Aβ aggregation and toxicity through acting as a metal (iron) chelator, which could again explain the equal effects of both enantiomers (*50*). Recently, it has been demonstrated (*51*) that nornicotine, a major nicotine metabolite in the central nervous system, has the capacity to react with glucose. This Amadori product can in turn covalently link to Lys^{16} in Aβ and thereby inhibit the ability of the peptide to aggregate.

In addition to their effects on aggregation, both D-(+)- and L-(−)-nicotine were equally good inhibitors of the toxic effects of Aβ(1−40) on SH-SY5Y cells, as assessed by the MTT assay (Figure 7). These effects on toxicity were evident even at 1/5 nicotine/Aβ concentration ratios. The nonincubated control, in which nicotine was added to the cultured cells at the same time as the preincubated Aβ(1−40), showed no neuroprotective effects. This demonstrates convincingly that the neuroprotective effects demonstrated here by either enantiomer of nicotine are not due to direct interactions with the cells, for example, through nAChRs [subunit types α3, α5, α7, β2, and β4 are expressed on SH-SY5Y cells (*52*)]. This conclusion is also supported by the positive effects of D-(+)-nicotine, which should not interact with nAChRs. Therefore, the reduction in the toxicity of Aβ(1−40) on the cells in this assay must be due to inhibitory mechanisms that take place during the peptide incubation stage. Although the appearance of fibrillar forms of Aβ is delayed by the presence of either enantiomer of nicotine during incubation, it is not clear from our data which forms of oligomers are responsible for the toxicity *and* are inhibited by nicotine. NMR evidence shows interactions between nicotine and monomeric Aβ, but there is no evidence that nicotine can interact with more mature forms of the aggregate. However, if the neuroprotection demonstrated by nicotine is mediated through interactions between oligomeric Aβ and membrane surfaces, then this protection might also be expected to be evident in the nonincubated controls. The only difference between the Aβ incubated with nicotine and the nonincubated control is the distribution of oligomeric species present in each sample. Although this difference is obvious from EM and is assumed to show a delayed appearance of fibrillar forms of Aβ (supported by aggregation assays), another possibility is that the morphology of the oligomers may be altered when they are formed in the presence of nicotine which may reduce their toxicity when they are added to the cells.

Overall, our data suggest that both enantiomers of nicotine can affect the early stages of Aβ aggregation to delay oligomerization and fibril formation to maintain a population of less toxic Aβ species. In terms of an effective treatment for AD, acetylcholinesterase inhibitors afford a limited symptomatic benefit to some patients, but they have little effect on the underlying disease process. Thus, it is important to develop new drug therapies for blocking the formation, deposition, and toxic effects of Aβ in the brain. In this respect, D-(+)-nicotine, being biologically much less active than L-(−)-nicotine, might be (or provide the starting point for development of) a useful therapeutic agent. The D-(+) enantiomer should have fewer peripheral side effects than the biologically active enantiomer. An additional advantage is that D-(+)-nicotine should not desensitize the nicotinic receptors over time as is often the case with agonists such as the L-(−) form (*53*, *54*).

Although not proven, smoking has been associated with a reduced risk of AD (*55*, *56*). Transgenic mouse models that develop AD plaques show greatly reduced plaque density in the neocortex and hippocampus after chronic nicotine treatment (*26*). In light of our results, it would be interesting to test D-(+)-nicotine in the transgenic model reported by Nordberg *et al*. (*26*).

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