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(3*R*,5*S*,7a*s*)-(3,5-Bis(4-fluorophenyl)tetrahydro-1*H*-oxazolo[3,4-*c*]oxazol-7a-yl)methanol, a Novel Neuroprotective Agent[⊥]

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Compounds that interact with microtubules, such as paclitaxel, have been shown to possess protective properties against β-amyloid (Aβ) induced neurodegeneration associated with Alzheimer's disease. In this work, the novel agent (3*R*,5*S*,7a*s*)-(3,5-bis(4-fluorophenyl)tetrahydro-1*H*-oxazolo[3,4-*c*]oxazol-7a-yl)methanol was investigated for effectiveness in protecting neurons against several toxic stimuli and its interaction with the microtubule network. Exposure of neuronal cultures to Aβ peptide in the presence of 5 nM (3*R*,5*S*,7a*s*)-(3,5-bis(4-fluorophenyl)tetrahydro-1*H*-oxazolo[3,4-*c*]oxazol-7a-yl)methanol resulted in a 50% increase in survival. Neuronal cultures treated with other toxic stimuli such as staurosporine, thapsigargin, paraquat, and H_2O_2 showed significantly enhanced survival in the presence of (3*R*,5*S*,7a*s*)-(3,5-bis(4-fluorophenyl)tetrahydro-1*H*-oxazolo[3,4-*c*]oxazol-7a-yl)methanol. Microtubule binding and tubulin assembly studies revealed differences compared to paclitaxel but confirmed the interaction of (3*R*,5*S*,7a*s*)-(3,5-bis(4-fluorophenyl)tetrahydro-1*H*-oxazolo[3,4-*c*]oxazol-7a-yl)methanol with microtubules. Furthermore, in vitro studies using bovine brain microvessel endothelial cells experiments suggest that (3*R*,5*S*,7a*s*)-(3,5-bis(4-fluorophenyl)tetrahydro-1*H*-oxazolo[3,4-*c*]oxazol-7a-yl)methanol can readily cross the blood−brain barrier in a passive manner.

Introduction

It had previously been suggested that stabilization of microtubules by antimitotic drugs could protect neurons from the toxic effects of β-amyloid (Aβ^a) peptide, a peptide believed to play a major role in the pathogenesis of Alzheimer's disease.[1] Indeed, we reported a study in which we demonstrated that microtubule stabilizing drugs such as taxanes, epothilones, and discodermolide had a protective effect against Aβ-induced degeneration of primary neurons in culture.[2] However, the success of a neuroprotective agent in vivo as a pharmaceutical product depends on the ability of the compound to reach the site of action within the brain. There are a variety of barriers to brain penetration including anatomical barriers, i.e., tight junctions, reduced pinocytosis, and glycocalyx,[3,4] metabolic barriers, i.e., peptidases, and phase I and phase II enzymes,[5] and an electrostatic barrier due to sulfated glycoproteins at the cell surface. Another major barrier to brain penetration is the existence of efflux transporters expressed at the brain endothelium.[6] If a drug is recognized as a substrate for one of these efflux transporters, the permeation across the blood−brain barrier can be significantly compromised.[7]

In 1997, Shintani et al. reported that a small synthetic compound named GS-164 stimulated microtubule assembly in vitro by a mechanism similar to that of paclitaxel.[8] Under the conditions used by us to prepare a sample of GS-164, a mixture of two diastereoisomers was obtained consisting of meso compound (3*R*,5*S*,7a*s*)-(3,5-bis(4-fluorophenyl)tetrahydro-1*H*-oxazolo[3,4-*c*]oxazol-7a-yl)methanol (**1**), racemate (3*R**,5*R**)-(3,5-bis(4-fluorophenyl)tetrahydro-1*H*-oxazolo-[3,4-*c*]oxazol-7a-yl)methanol (**2**), and (2-(4-fluorophenyl)oxazolidine-4,4-diyl)dimethanol (**3**), an incomplete condensation product (Figure 1). On the basis of modeling studies, the authors had proposed that the *R*,*R*-enantiomer of compound **2** mimics parts of the paclitaxel structure and is responsible for its paclitaxel-like activity. Our laboratory has now separated the diastereoisomers present in GS-164 and has investigated the properties of the (3*R*,5*S*,7a*s*)-isomer **1** (Figure 1). The compound exhibits neuroprotective properties when

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[a] Abbreviations: Aβ, β-amyloid; BBMEC, bovine brain microvessel endothelial cells; CsA, cyclosporine A; DAPI, 4′,6′-diamino-2-phenylindole; DMEM/F12 Dulbecco's modified Eagle's medium F12, DMSO, dimethyl sulfoxide; GTP, guanosine triphosphate; MAPs microtubule-associated proteins; PBS, phosphate buffered saline; PBSA phosphate buffered saline with ascorbic acid; PIPES, piperazine-1,4-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PEM, buffer containing piperazine-1,4-bis(2-ethanesulfonic acid), ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, and $MgSO_4$; P-gp, P-glycoprotein; PI, propidium iodide; Tris/Cl, tris(hydroxymethyl)aminomethane HCl.

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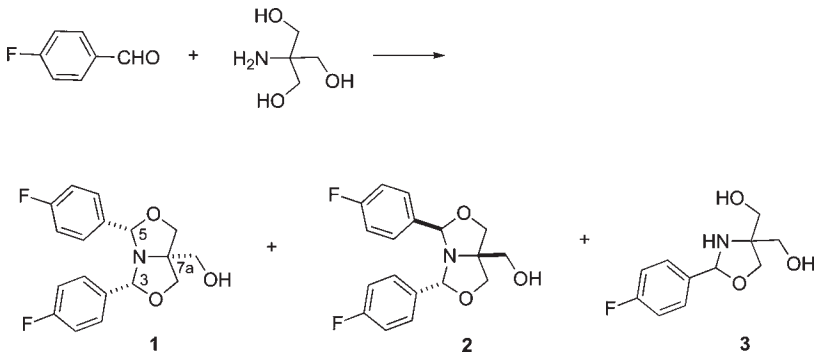

Figure 1. Reaction scheme for the synthesis **1**. Additional products are **2** and **3**.

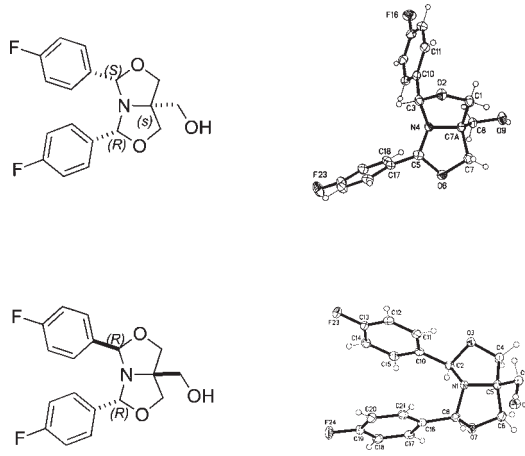

Figure 2. Structures and ellipsoid drawings of meso diastereoisomer (3*R*,5*S*,7a*s*)-**1** and (3*R**,5*R**)-**2**) from X-ray diffraction.

tested with cultured neurons and passively permeates the blood−brain barrier. Interestingly, although this compound binds weakly to microtubules, it does not show paclitaxel-like microtubule stabilizing activity in vitro.

Results

Synthesis of 1. The reaction of *p*-fluorobenzaldehyde with tris(hydroxymethyl)aminomethane led to three products (Figure 1) that were separable by flash chromatography. Compounds **1**, **2**, and **3** were produced in yields of 66%, 5%, and 21%, respectively. Single crystal X-ray diffraction was used to determine the stereochemistry of **1** and **2** (Figure 2). Thus, the major product was the 3*R*,5*S*,7a*s* meso compound **1**. The 3*R*,5*S*,7a*r* meso compound, although a potential product, was not formed. This is consistent with energy calculations.[9]

Microtubule Assembly and Stability. In the original article on GS-164 the authors concluded that the compound acted on tubulin in a manner similar to that of paclitaxel.[8] In addition, the authors used modeling to suggest that the 3*R*,5*R* isomer was responsible for the paclitaxel-like activity. We have found that this isomer is a minor product in the synthesis. We sought to determine whether the major product, the 3*R*,5*S*,7a*s* isomer **1**, had paclitaxel-like activity. We first tested the ability of **1** to stimulate tubulin assembly using microtubule protein (tubulin containing microtubule associated proteins) and pure tubulin. Microtubule assembly was measured in two different ways, using a fluorescence assay with microtubule protein without GTP (guanosine triphosphate), and a centrifugation assay with pure tubulin plus GTP and microtubule protein without GTP. Shown in

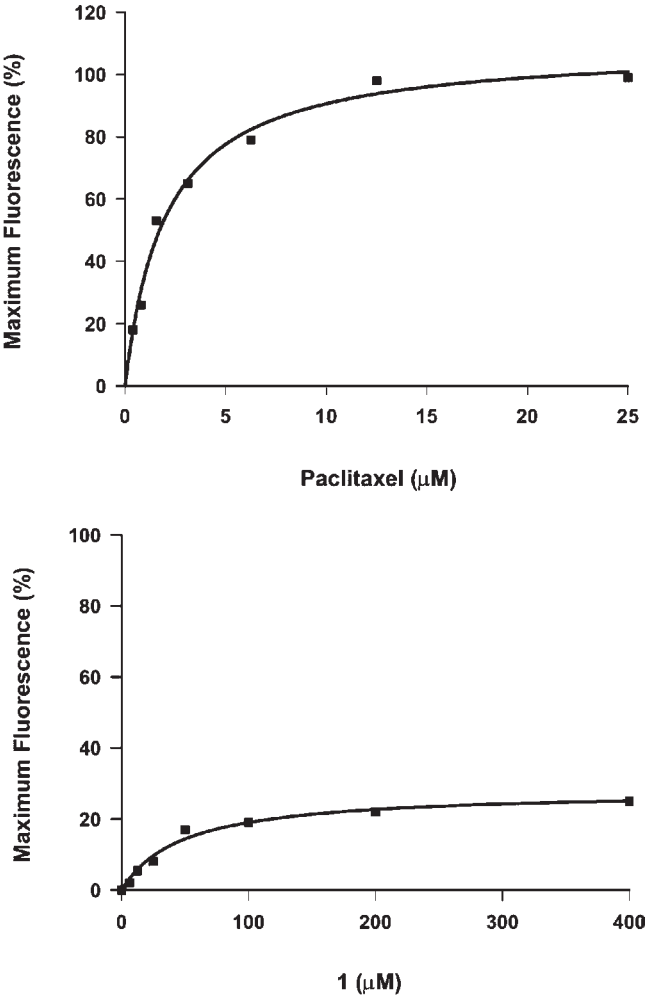

Figure 3. Stimulation of microtubule protein assembly by paclitaxel (top) and **1** (bottom) as determined by monitoring the increase in DAPI fluorescence. Microtubule protein (2 mg/mL) was incubated in PEM buffer containing 10 μM DAPI and varying concentrations of paclitaxel or **1** in 96-well plates for 30 min at 37 °C. Maximum fluorescence is that which is achieved by 25 μM paclitaxel.

Figure 3 are the results of the fluorescence assay. Compound **1** produced a small positive response (apparent microtubule formation) at low concentrations, but the response did not increase with increasing concentrations of **1**. The maximum increase in fluorescence was about 20% of that produced by paclitaxel. In the centrifugation assays, the amount of pelleted protein in the presence of **1** ranged from 10% to 20% of that formed in the presence of paclitaxel. Electron

microscopic examination indicated some fibrous material in the presence of **1**, indicating that the compound induces aggregation of tubulin but not microtubule formation. Similar results were obtained with **2** and **3** (data not presented).

Paclitaxel is known to stabilize microtubules against cold-induced depolymerization. As another means to compare the taxane and **1**, we studied the cold stability of microtubules formed in vitro. Microtubules were formed from pure tubulin at 37 °C. Upon centrifugation, 81% of the protein was found in the pellet. When the sample was placed on ice for 15 min before centrifugation, only 8% of the protein was in the pellet fraction. The addition of 20 μM paclitaxel to the microtubule suspension before the cold treatment stabilized the microtubules; the pellet contained 79% of the protein. On the other hand, the addition of 400 μM **1** had no effect on cold stability; 9% of the protein was found in the pellet. The results clearly demonstrate that **1** does not possess the paclitaxel-like property of stabilizing microtubules against cold-induced depolymerization.

Binding of 1 to Microtubules. With the use of tritiated compounds, the binding of **1** and paclitaxel to microtubules formed from microtubule protein was measured (Figure 4). Both compounds bound to microtubules in a ratio of 7 to 8 nmol/mg protein. Since microtubule protein is about 70% tubulin, 1 mg represents 0.7 mg of tubulin or 7 nmol. Thus, both compounds bind in about a 1 to 1 molar ratio, but about 10-fold more **1** than paclitaxel was required to achieve maximum binding. To determine whether the two compounds bind to the same site in tubulin, competitive binding experiments were performed. Microtubules were formed in the presence of 100 μM [^{3}H]**1** and 0−40 μM paclitaxel and in the presence of 20 μM [^{3}H]paclitaxel and 0−400 μM **1**. Binding of 20 μM paclitaxel was reduced 40% by 400 μM **1**. Binding of 100 μM **1** was reduced 42% by 40 μM paclitaxel. Thus, the two compounds appear to be weakly competitive with each other.

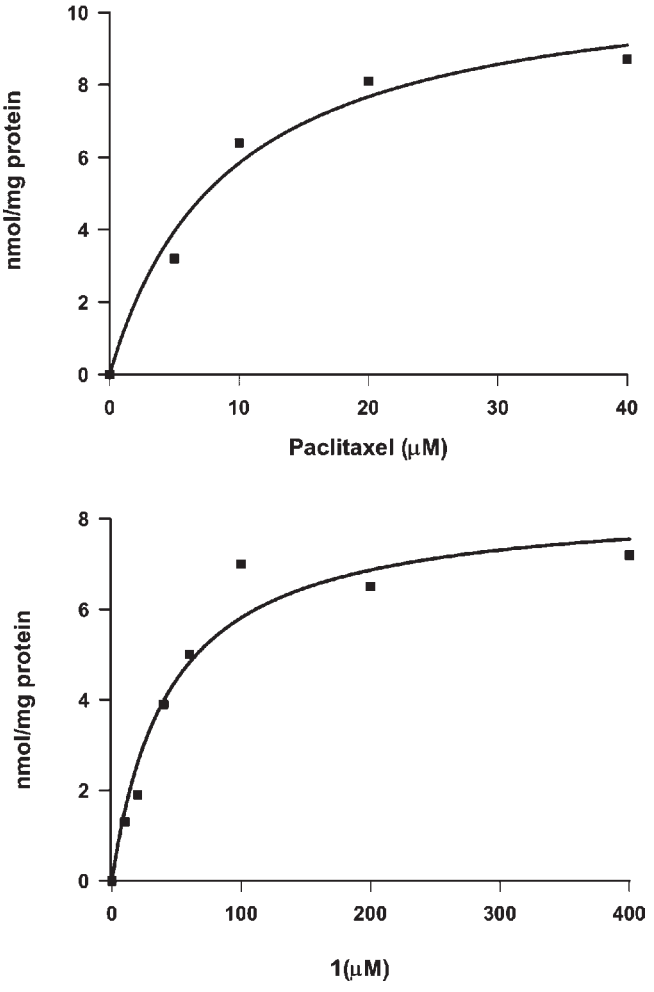

Figure 4. Binding of paclitaxel (top) and **1** (bottom) to microtubules. Microtubules were formed from microtubule protein (2 mg/mL) and incubated with different concentrations of [^{3}H]paclitaxel or [^{3}H]**1** for 10 min at 37 °C. The microtubules were collected by centrifugation and dissolved in 0.1 M NaOH. Protein concentrations and radioactivity measurements were then determined.

Protection of Primary Cortical Neurons against Aβ Peptide and Cell-Death Initiators. We and others have shown that exposure to Aβ induces beading, shortening, and eventual disappearance of neuritic processes presumably due to disruption of the cytoskeletal network, and we have also found that paclitaxel protects against Aβ toxicity.[10−12] Although **1** did not have paclitaxel activity in vitro, it did protect primary cortical neurons exposed to toxic agents. Protection against Aβ25−35 peptide is shown in Figure 5. The EC_{50} for **1** (concentration that leads to a 50% increase in neuronal survival in the presence of the Aβ peptide) was 5 nM. The racemic mixture **2** and the incomplete condensation product **3** had EC_{50} values of 50 and 40 nM, respectively. Although the small toxic Aβ25−35 peptide was used in the majority of studies, the observations were confirmed with exposure to Aβ1−42 peptide. Because **1** was the most effective of the three compounds and was the major product in the synthesis, further studies were restricted to this compound.

The effectiveness of **1** in protecting primary neurons against diverse toxic stimuli that lead to cell death via various pathways was also investigated. **1** was added at a concentration of 100 nM approximately 2 h before the toxic stimuli, and cell viability was determined. As shown in Figure 6, exposure of the neurons to the protein kinase C inhibitor staurosporine, the sarcoplasmic−endoplasmic Ca^{2+}-ATPase inhibitor thapsigargin, and the oxidative stress-inducing agents paraquat and hydrogen peroxide all led to a 50% loss in neurons within 24 h. In cultures treated with **1** before addition of the toxic stimuli, neuronal survival was

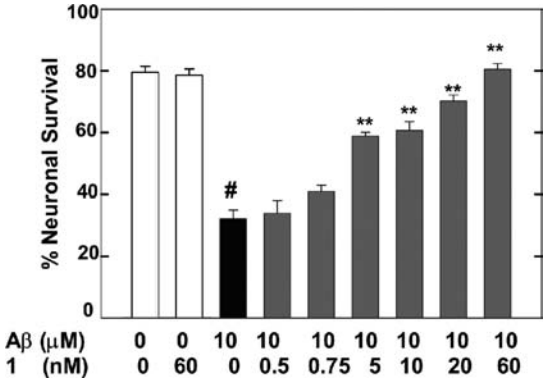

Figure 5. Dose dependent effects of **1** on survival of primary cortical neurons exposed to the toxic β-amyloid (Aβ) peptide for 48 h. The percentage of neurons surviving was determined by the live/dead assay. Data are from six randomly selected fields from two neuronal preparations ($n \approx 1500$ cells/condition). Statistical significance was determined by Students t test: (#) $p < 0.001$ for controls versus Aβ only; (**) $p < 0.001$ for Aβ only versus Aβ + drug.

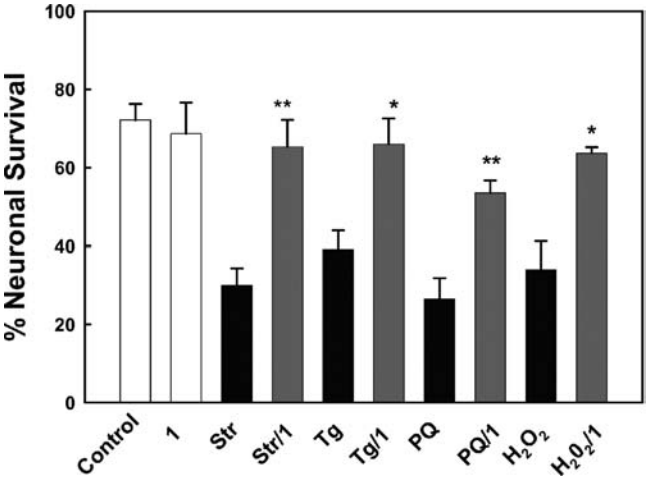

Figure 6. Effects of **1** on toxicity induced by diverse stimuli. Primary neurons were exposed to 100 nM staurosporine (Str), 100 nM thapsigargin (Tg), 25 μM paraquat (PQ), or 25 μM H_2O_2, ± 100 nM **1**. Mean percent survival was determined 24 h later, and statistical significance was assessed using Student's *t* test: (*) $p < 0.05$ and (**) $p < 0.005$ for each toxic stimulus alone versus the toxic stimulus plus the drug. Data are from ~1000 cells per condition.

significantly enhanced and, in most cases, the percentage of live cells was nearly at control levels. Thus, the protective effect of **1** is not selective for the cell death pathway induced by Aβ peptides but seems to be due to a more generalized cellular response that enables neurons to withstand stressful conditions.

Effects of 1 on Rhodamine 123 Uptake. Given the promising observations seen in the neuroprotective assays, additional studies were performed to investigate the ability of **1** to cross the blood−brain barrier. The rhodamine 123 assay assesses the interaction of a compound with the efflux transporter P-glycoprotein, which is expressed at the brain endothelium and can present a barrier to brain penetration. Paclitaxel is a known substrate of P-glycoprotein (P-gp) and consequently has very minimal brain penetration.[13] Compound **1** was tested in the rhodamine 123 uptake assay using bovine brain microvessel endothelial cells (BBMECs) grown in a 24-well plate. The uptake of rhodamine 123 was monitored in the presence of **1** at concentrations ranging from 1 to 50 μM. No significant increase in rhodamine 123 was observed except for the positive control, cyclosporine A (CsA), a known substrate for P-gp (data not shown). These data suggest that **1** is not recognized as a substrate for the P-gp efflux transporter.

Permeability of 1. Bidirectional permeability studies were used to predict the brain permeation of **1** and to determine if **1** permeates via an active or passive process. Compound **1** (10 μM) with trace levels of [^{3}H]**1** was added to the luminal or abluminal side of the BBMEC monolayer, and permeation was monitored for 1.5 h. During this time, permeation across the monolayer was linear ($r^2 = 0.99$) and all experiments were performed with $n = 4$. In the luminal to abluminal direction, $P_{app} = 2.86 \times 10^{-4}$ cm/s. In the abluminal to luminal direction, $P_{app} = 2.44 \times 10^{-4}$ cm/s. This indicates that there is no statistical difference in permeability as a result of direction. These data suggest that the permeability of **1** is a passive process, since permeation via an active process would yield directional differences in permeability.

To confirm that permeation of **1** is indeed a passive process, the permeability of **1** (10 μM) with trace levels of [^{3}H]**1** was investigated in the luminal to abluminal direction at temperatures ranging from 4 to 37 °C over 1.5 h to generate an Arrhenius plot (Figure 7). The activation energy calculated from the slope of the Arrhenius plot was 26.3 kJ/mol, which is suggestive of a passive process, since carrier-mediated processes typically fall within a range of 29−105 kJ/mol.[14]

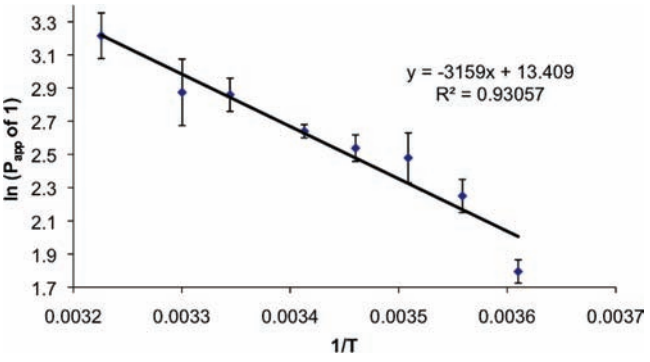

Figure 7. Arrhenius plot of the apparent permeability coefficients (P_{app}) of **1** in bovine brain microvessel endothelial cells at temperatures ranging from 4 to 37 °C over 1.5 h: data ± SD, $n = 4$.

Discussion

Following the report by Shantani et al.[8] that GS-164 promoted the assembly of tubulin and stabilized microtubules in vitro, we sought to test the ability of the compound as a neuroprotective agent. When we tested the three products of GS-164 synthesis, we could not find evidence that any of them promoted the assembly of tubulin. We did find that **1**, the *R*,*S* stereoisomer of GS-164, binds to microtubules in a molar ratio of about one **1**/tubulin molecule. However, it binds with an apparent affinity that is about 10% that of the affinity of paclitaxel and appears to be only partially competitive with the taxane. Moreover, **1** did not stabilize microtubules in vitro. In spite of these differences, **1**, like paclitaxel, has excellent neuroprotective properties against Aβ and other toxic stimuli. Since **1** must be able to cross the blood−brain barrier to exert its neuroprotective effects in neurodegenerative diseases, the potential for **1** to penetrate the blood−brain barrier was thoroughly investigated using in vitro methodologies. In vitro studies performed using BBMECs as a model of the blood−brain barrier indicated that **1** had excellent potential for brain penetration. The physiochemical properties of **1** make it a potential candidate for use as a CNS drug. Compared to paclitaxel, **1** has a much lower molecular weight, possesses halogen groups that promote passive diffusion,[15] and is not a substrate for any of the efflux transporters expressed at the brain endothelium. All of these features are important in determining whether a drug will be able to cross the blood−brain barrier and reach a site of action within the brain.[7,15,16]

The observation that **1** protects neurons against Aβ toxicity is similar to what we have found with known microtubule-stabilizing drugs such as paclitaxel.[2,10,17] The in vitro studies suggest that **1** interacts with microtubules differently than paclitaxel does. Exposure to **1** led to the preservation of the cytoskeletal integrity in primary neurons exposed to Aβ. Our previously published work using calcein labeling demonstrated that Aβ leads to the actual loss of neurites and not just a loss of tubulin labeling.[2,10,17] The neuritic dystrophy attests to the complexity of the signaling events involved in the

neuronal response to Aβ and suggests that **1** may be acting on a different pathway than paclitaxel in leading to preservation of the neurites. Furthermore, though it is reasonable to assume that paclitaxel ultimately protects neurons by stabilizing microtubules, the cellular mechanism may be more complex, possibly involving unique effects on specific signaling pathways. For example, we have found that paclitaxel prevents the Aβ-induced activation of Cdk5, one of the proline kinases strongly implicated in the abnormal phosphorylation of the τ protein found in tangles in Alzheimer's disease brain.[18]

Diverse experimental approaches have provided strong evidence that dysregulation of Ca^{2+} homeostasis is a driving force for neuronal death in neurodegenerative diseases,[19] and paclitaxel was shown to protect neurons against Ca^{2+} influx,[20] against glutamate-induced excitotoxicity,[21] and against thapsigargin-induced endoplasmic reticulum stress and dysfunction,[12] among other toxic insults. It is not yet known how this occurs, but specific Ca^{2+} signaling pathways may be sensitive to the state of the cytoskeletal network in neurons. Agents as structurally different as paclitaxel and **1** could modulate very different Ca^{2+} signaling cascades in intact cells. Although **1** does not mimic paclitaxel in vitro, this agent apparently activates unique protein interactions and signaling cascades, which ultimately leads to the preservation of neuritic processes. As previously suggested,[22] the state of the neuronal cytoskeleton may serve as a surveillance system that transduces multiple types of stress signals. Agents that invoke mechanisms that slow or prevent the neuritic dystrophy initiated by such signals give the neurons a chance to mobilize other defenses and thereby enhance their survival. Experimental strategies designed to delineate, at the molecular level, the mechanisms through which agents such as **1** and paclitaxel activate protective signaling cascades may well provide opportunities for innovative therapeutic interventions that capitalize on these targets.

Experimental Section

Chemistry. Analytical Characterization. ^{1}H and ^{13}C NMR spectra were recorded on a 400 MHz NMR instrument (400 and 100 MHz respectively). High-resolution mass spectra (HRMS) were obtained on a ZAB double-focusing mass spectrometer. IR spectra were recorded on a FTIR instrument. Melting points are uncorrected. Column chromatography was performed employing silica gel (230−400 mesh). The purities of **1** and **2** were determined by HPLC and were found to be >95% pure. X-ray diffraction data were collected on a CCD area detector using graphite-monochromated Mo Kα radiation (λ = 0.710 73 Å). CCDC 706431 (**1**) and 706432 (**2**) contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif).

Synthesis of 1. A suspension of *p*-fluorobenzaldehyde (27 g, 0.22 mol) and tris(hydroxymethyl)aminomethane (13 g, 0.11 mol) in toluene (350 mL) was heated at reflux temperature for 12 h with azeotropic removal of water. The reaction mixture was concentrated and stirred at room temperature, and the precipitated unreacted *p*-fluorobenzaldehyde was removed by filtration. The residue obtained after removal of solvent was subjected to flash chromatography on silica gel using hexane/ethyl acetate (4:1 and 1:1). Crystallization of the respective fractions afforded pure compounds (**1**, **2**, and **3**) in yields of 66%, 5%, and 21%, respectively. The structures of the products were assigned by spectral data, and the relative stereochemistry of **1** and **2** was assigned from single crystal X-ray diffraction data.

(3*R*,5*S*,7a*s*)-(3,5-Bis(4-fluorophenyl)tetrahydro-1*H*-oxazolo-[3,4-*c*]oxazol-7a-yl)methanol (1). 1 was crystallized from hexane as a colorless crystalline solid: mp 90 °C; IR ν cm^{-1} 3444, 2873, 1606, 1504, 1222, 1153, 1078, 1006, 837; ^{1}H NMR ($CDCl_3$) δ 3.50 (br s, 2H), 3.94 (d, J = 8.9 Hz, 2H), 4.05 (d, J = 8.9 Hz, 2H), 5.55 (s, 2H), 7.07 (m, 4H), 7.44 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 65.63, 72.66, 74.89, 96.65, 115.41, 115.58, 128.55, 128.62, 135.26, 135.29, 161.95, 163.91; HRMS (FAB+) m/z calculated for $C_{18}H_{18}NO_3F_2$ [M + H] 334.1255, found 334.1230.

(3*R,5*R**)-(3,5-Bis(4-fluorophenyl)tetrahydro-1*H*-oxazolo[3,4-*c*]oxazol-7a-yl)methanol (2).** was crystallized from hexanes/CH_2Cl_2 as a colorless crystalline solid: mp 115 °C; IR ν cm^{-1} 3440, 2870, 1606, 1512, 1427, 1384, 1226, 1155, 1076, 837; ^{1}H NMR ($CDCl_3$) δ 3.75 (m, 2H), 3.84 (d, J = 8.8 Hz, 1H), 3.88 (d, J = 8.9 Hz, 1H), 4.10 (d, J = 8.9 Hz, 1H), 4.20 (d, J = 8.8 Hz, 1H), 5.16 (s, 1H), 5.51 (s, 1H), 6.89 (m, 6H), 7.27 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 65.60, 72.12, 74.87, 93.03, 93.91, 115.14, 115.31, 115.35, 115.52, 115.78, 129.21, 129.29, 129.34, 129.42, 130.21, 130.24, 135.87, 135.90, 161.89, 164.34; HRMS (FAB+) m/z calculated for $C_{18}H_{18}NO_3F_2$ [M + H] 334.1255, found 334.1237.

(2-(4-Fluorophenyl)oxazolidine-4,4-diyl)dimethanol (3). 3 was crystallized from isopropanol as a colorless crystalline solid: mp 89 °C; IR ν cm^{-1} 3450, 2877, 1604, 1512, 1420, 1296, 1228, 1157, 1047, 837; ^{1}H NMR ($CDCl_3$) δ 3.58 (m, 3H), 3.71 (m, 2H), 3.85 (d, J = 8.6 Hz, 1H), 5.41 (s, 1H), 7.06 (m, 2H), 7.44 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 64.57, 64.98, 67.62, 70.73, 91.79, 115.81, 116.02, 128.29, 128.37, 134.91, 162.17, 164.63; HRMS (FAB+) m/z calculated for $C_{11}H_{15}NO_3F$ [M + H] 228.1036, found 228.1036.

Microtubule Assembly Assay. Microtubule protein (tubulin preparation containing microtubule-associated proteins (MAPs)) was obtained from bovine brain.[23] Pure tubulin was isolated from microtubule protein by a previously described procedure.[24] Microtubule assembly in vitro was measured using two different assays. One assay takes advantage of the fact that 4′,6-diamidino-2-phenylindole (DAPI) binds to microtubules, producing an increase in fluorescence.[25] The reactions were performed in 96-well plates in a volume of 120 μL per well. The wells contained PEM buffer (0.1 M PIPES, 1 mM $MgSO_4$, 1 mM EGTA, pH 6.9), 4% DMSO, 10 μM DAPI, 2 mg/mL microtubule protein, and varying concentrations of paclitaxel or **1**. The plates were incubated at 37 °C for 30 min, after which the fluorescence was measured in a multiplate reader. The readings were corrected for a control lacking either compound. A centrifugation assay was also employed in which microtubules are pelleted after the assembly reaction (data not shown).[26]

Microtubule Binding Assay. Binding of **1** and paclitaxel to microtubules was measured using tritiated compounds. [^{3}H]Paclitaxel was purchased from Moraveck Biochemicals, Inc., Brea, CA, and **1** was tritiated by ViTrax Co., Placentia, CA. Solutions (100 μL) of 2 mg/mL microtubule protein in PEM buffer containing 0.5 mM GTP and various concentrations of [^{3}H]**1** (1×10^5 cpm/nmol) or [^{3}H]paclitaxel (9×10^4 cpm/nmol) in Beckman TLA-100 centrifuge tubes were incubated at 37 °C for 10 min. Microtubule pellets were collected by centrifugation at 100000*g* for 4 min. The pellets were dissolved in 0.1 M NaOH, and radioactivity and protein concentrations were determined.

Neuronal Cell Culture. To prepare the primary neurons, dissociated cortical cell cultures were established from embryonic day 18 rat fetuses recovered from pregnant Sprague−Dawley rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN).[27] After the final precipitation step, neurons were suspended in fresh DMEM/F12 (Sigma Chemical Co., St. Louis, MO) with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA) and plated at a density of 2.5×10^5 cells in 35 mm glass-bottom microwell dishes (Mat-Tek Co., Ashland, MA), coated with poly-D-lysine. Serum-containing medium was removed after 24 h, and the cells were maintained in serum-free DMEM/F12 containing the N2 supplements. Cultures were grown at 37 °C in 5% CO_2 and 97% humidity as described.[27]

Treatment with Aβ Peptide and Cell-Death Initiators. After 5 days in culture, the primary neurons were exposed to either

Aβ25−35 or Aβ1−42 in the presence or absence of concentrations of **1** ranging from 0.5 to 60 nM. **1** was added approximately 2 h before the Aβ peptides. The Aβ25−35 was synthesized and purified in the Biochemical Research Services Lab at The University of Kansas. The reverse sequence peptide used as a control, Aβ35−25, and the Aβ1−42 peptide used in confirmatory experiments were purchased from Bachem (Torrance, CA). Prior to the addition of the peptides to the cultures, the Aβ peptide stocks (1.3 mg/mL) were diluted into 10 mM Tris/Cl, pH 7.4, and maintained at 37 °C for 24 h. Each batch of Aβ peptide was analyzed for β-sheet formation by circular dichroism, but no attempt was made to separate oligomers from fibrils. The peptides were added directly to the culture medium, at a final concentration of 10 μM. Control cultures received the DMSO vehicle alone, and the final concentration of DMSO never exceeded 0.04%. Assays were carried out 48 h following Aβ peptide addition. The effects of neurotoxic stimuli other than Aβ peptides were also used to assess the protective effect of **1** against various cell-death initiators. Neurotoxic stimuli included staurosporine (100 nM) to induce apoptosis, thapsigargin (100 nM) to cause ER stress, and paraquat (25 μM) or hydrogen peroxide (25 μM) to induce oxidative stress. All reagents were obtained from Sigma Chemical Co., St Louis, MO.

Measurement of Cell Viability. The effects of the Aβ peptides and **1** were determined by monitoring neuronal cell survival using the live/dead assay as previously described.[2,10] Following exposure to the peptides and other compounds, cells were labeled with 20 μM propidium iodide (PI) and 150 nM calcein acetoxy methyl ester (Molecular Probes, Eugene, OR) for 30 min at 37 °C, rinsed with phosphate buffered saline (PBS), and visualized under a Nikon inverted microscope (Nikon Eclipse TE200, Japan) with filters for fluorescein isothiocyanate and Texas red. Digital images were captured, and the number of viable (green) and dead (red) neurons was determined by counting the cells in 6−12 microscopic fields per culture dish in duplicate dishes for each treatment. All experimental treatments were carried out on at least two separate embryonic neuronal preparations with approximately 1500 neurons scored under each treatment condition. The raw data from each experiment were combined, and the significance of differences between cultures exposed to various treatments was determined as described using Student's t test.[2] Neuronal survival in the untreated control samples was considered to represent maximal viability, and survival in the Aβ-only samples represents minimal viability.

Isolation and Cell Culture of Brain Endothelial Cells. BBMECs were isolated from the gray matter of bovine cerebral cortices by enzymatic digestion followed by centrifugation as described.[28] Isolated BBMECs were seeded at a density of approximately 50 000 cells/cm^2 onto 24-well culture plates or 100 mm culture dishes as previously described.[29]

Rhodamine 123 Uptake Assay. In this assay, rhodamine 123 is used as a surrogate P-glycoprotein (P-gp) substrate.[30] The effect of the test compound on rhodamine 123 is determined by monitoring intracellular fluorescence. If the test compound is a substrate for P-gp, then addition of the compound will increase rhodamine 123 uptake relative to the negative control. BBMECs were seeded onto 24-well culture plates and used to assay for P-gp interaction.[29] Briefly, BBMECs were rinsed with warm PBSA (phosphate buffered saline containing $CaCl_2$, $MgSO_4$, glucose, and L-ascorbic acid) and allowed to acclimate for 10 min. The PBSA was removed, and solutions of **1** (1 μM- 50 μM) were added to the wells and allowed to incubate for 30 min at 37 °C. Rhodamine 123 was then added to all wells at a concentration of 5 μM, and cells were incubated for an additional 2 h. After the incubation, cells were quickly rinsed with ice cold PBS several times. Lysis buffer (0.5% v/v Triton X-100 in 0.2 M NaOH) was then added to all wells and allowed to solubilize cells for at least 30 min. Aliquots (200 μL) were taken from all wells to determine rhodamine 123 concentration, and 10 μL aliquots were removed from all wells to determine the protein concentration using a Pierce BCA protein assay kit (Pierce, Rockford, IL).

BBMEC Permeability Assays. BBMECs were grown on 0.4 μm Nucleopore polycarbonate membranes in a 100 mm culture dish coated with rat tail collagen and fibronectin. Once cells had formed a confluent monolayer as determined by light microscopy, the membranes were transferred to Side-Bi-Side diffusion chambers as previously described.[31,32] Briefly, each chamber was filled with 3 mL of PBSA and either the luminal (blood) or abluminal (brain) donor chambers contained 10 μM unlabeled **1** and a trace amount of [^{3}H]**1**. The temperature was maintained at 37 °C within the chamber with an external circulating water bath, and chamber contents were stirred with Teflon coated magnetic stir bars driven by an external console. At various time points up to 90 min, 200 μL aliquots were removed from the receiver side and 20 μL aliquots of the donor solution were taken at time zero. The integrity of the cell monolayer was tested after experiment by monitoring the permeability of [^{14}C]sucrose, a low permeability paracellular marker that should not readily cross the cell monolayer. All samples were analyzed by liquid scintillation counting. For temperature dependent permeability studies BBMECs were grown on 0.4 μm Nucleopore polycarbonate membranes and then transferred to Side-Bi-Side diffusion chambers as described above. **1** (10 μM) with trace amounts of [^{3}H]**1** was added to the luminal chambers, and 200 μL samples were taken from the abluminal chamber at various time points up to 90 min. The apparent permeability of **1** was calculated at a range of temperatures from 4 to 37 °C. An Arrhenius plot was generated to determine the activation energy of transport. The integrity of the cell monolayer was tested after experiment by monitoring the permeability of [^{14}C]sucrose, and samples were analyzed by liquid scintillation counting.

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