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The characterization of microtubule-stabilizing drugs as possible therapeutic agents for Alzheimer's disease and related tauopathies

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ABSTRACT

Tau, a protein that is enriched in neurons of the central nervous system (CNS), is thought to play a critical role in the stabilization of microtubules (MTs). Several neurodegenerative disorders referred to as tauopathies, including Alzheimer's disease and certain types of frontotemporal lobar degeneration, are characterized by the intracellular accumulation of hyperphosphorylated tau fibrils. Tau deposition into insoluble aggregates is believed to result in a loss of tau function that leads to MT destabilization, and this could cause neurodegeneration as intact MTs are required for axonal transport and normal neuron function. This tau loss-of-function hypothesis has been validated in a tau transgenic mouse model with spinal cord tau inclusions, where the MT-stabilizing agent, paclitaxel, increased spinal nerve MT density and improved motor function after drug absorption at neuromuscular junctions. Unfortunately, paclitaxel is a P-glycoprotein substrate and has poor blood–brain barrier permeability, making it unsuitable for the treatment of human tauopathies. We therefore examined several MT-stabilizing compounds from the taxane and epothilone natural product families to assess their membrane permeability and to determine whether they act as substrates or inhibitors of P-glycoprotein. Moreover, we compared brain and plasma levels of the compounds after administration to mice. Finally, we assessed whether brain-penetrant compounds could stabilize mouse CNS MTs. We found that several epothilones have significantly greater brain penetration than the taxanes. Furthermore, certain epothilones cause an increase in CNS MT stabilization, with epothilone D demonstrating a favorable pharmacokinetic and pharmacodynamic profile which suggests this agent merits further study as a potential tauopathy drug candidate.

1. Introduction

The MT-associated protein tau forms filamentous inclusions within neurons in several CNS disorders, including Alzheimer's disease (AD) and certain frontotemporal dementias [1–3]. Collectively, these diseases are referred to as tauopathies, and alterations in normal tau structure and/or function likely play a causative role in the neuropathology of these various conditions. Indeed, tau gene (*MAPT*) mutations cause frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) [4,5], and dementia ratings in AD are correlated with the extent of tau brain deposits [6,7].

Tau is highly expressed in neurons, where it plays a critical role in MT stabilization [8,9] and axonal transport. The tau hyperphosphorylation that occurs in all tauopathies [10,11] results in reduced tau binding to MTs and a decreased ability to promote MT assembly [12–16]. Moreover, tau phosphorylation has been reported to enhance its propensity to fibrillize [17,18] and this could cause destabilization of MTs due to the depletion of free tau.

The concept that neuropathology can result from tau loss-of-function is supported by studies that showed MT abnormalities and axonal transport deficits in motor axons of transgenic (Tg) mice that over-express human tau [19]. Importantly, impaired tau function was compensated for in these mice by treatment with the MT-stabilizing agent paclitaxel, as drug absorption by motor neurons at peripheral neuromuscular junctions resulted in increased MT density and marked improvement in motor function [20]. These data suggest that MT-stabilizing drugs that are utilized

Abbreviations: AD, Alzheimer's disease; BBB, blood–brain barrier; B/P, brain-to-plasma; CNS, central nervous system; FTDP-17, frontotemporal dementia with Parkinsonism linked to chromosome 17; MDCK-MDR, Madin–Darby canine kidney cells expressing MDR1; MT, microtubule; NF, neurofilament; NGF, nerve growth factor; ON, optic nerve; Pgp, P-glycoprotein; Tg, transgenic.

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in oncology may be potential therapeutics for AD and other tauopathies. However, paclitaxel and most related taxanes are thought to have poor blood–brain barrier (BBB) permeability and are thus likely unsuitable for the treatment of human tauopathies, where pathology is predominantly in the brain. Accordingly, compensation for tau loss-of-function in tauopathies will require brain-penetrant MT-stabilizing agents.

The relative inability of paclitaxel and analogues to enter the brain is believed to be due in part to these compounds acting as substrates for the P-glycoprotein (Pgp) transporter that resides in endothelial cells that form the BBB [21,22]. Taxane derivatives have been prepared that retain anti-mitotic activity in Pgp-expressing cells [23–25], but this activity appeared to result from the compounds inhibiting Pgp function [26,27]. Similarly, other taxanes such as TXD258 [28] and RPR-109881A [29] have been reported to accumulate in the brain, although the former is a modest Pgp substrate that may saturate the transporter at high blood drug levels [28]. Because Pgp plays an important role in shielding the brain from undesirable xenobiotics, inhibitors of Pgp or substrates that impede Pgp function could be detrimental as therapeutics for chronic disorders like AD and related tauopathies. Accordingly, we [30] and others [31] have reported on the synthesis of novel taxane analogues that are neither Pgp substrates nor inhibitors. Here we report on the further characterization of such molecules, as well as examples from the epothilone class of MT-stabilizing agents. The compounds were evaluated in cell-based assays to assess whether they interact with Pgp and to gauge their membrane permeability. Moreover, because there is little published information on the brain penetration of existing MT-stabilizing agents *in vivo*, we compared the brain and plasma levels of these compounds in mice after systemic administration. Finally, we determined whether these molecules can affect CNS MTs in mice, utilizing a marker of MT stabilization (i.e., tubulin acetylation). We find that members of the epothilone series of MT-stabilizing compounds have much greater BBB penetration than the tested taxanes. Moreover, certain of the epothilones cause a significant increase in MT stabilization within the CNS. Thus, we have identified candidate MT-stabilizing compounds that are suitable for testing in Tg mouse models of tauopathy, and certain of these may hold promise as possible therapeutic agents for AD and related tauopathies.

2. Materials and methods

2.1. Reagents

Tissue culture medium and penicillin/streptomycin were purchased from Mediatech (Manassas, VA). Fetal bovine serum was from Hyclone/Thermo Scientific (Waltham, MA), and horse serum was obtained from SAFC Biosciences/Sigma–Aldrich (St. Louis, MO). Mouse nerve growth factor (NGF) was purchased from Collaborative Biomedical Products (Bedford, MA). All protease inhibitors were from Sigma–Aldrich. Ixabepilone was obtained from the University of Pennsylvania Hospital pharmacy. All HPLC solvents were purchased from Fisher Scientific (Pittsburgh, PA). Acetyl-tubulin antibody (6-11B-1) was from Sigma–Aldrich, 12G10 α-tubulin antibody was purified from culture supernatant of hybridoma cells obtained from the Developmental Studies Hybridoma Bank at the University of Iowa, and RMO189 neurofilament (NF) medium chain antibody was developed in-house [32]. BlockAce was obtained from AbD Serotec (Oxford, UK). HRP-conjugated anti-mouse IgG was from Jackson Immunoresearch (West Grove, PA). Horse radish peroxidase labeling of antibodies was with a Peroxidase Labeling Kit (Roche Applied Science, Indianapolis, IN). TMB-peroxidase substrate was obtained from KPL (Gaithersburg, MD). Protein A/G plus-agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). BCA protein determination reagent was from Thermo Scientific.

2.2. Synthesis of epothilones

Epothilone D (CNDR-66) and its closely related congeners (CNDR-85–87), as well as 12,13-desoxy-epothilone F (CNDR-89) and its 9,10-dehydro synthetic precursor (CNDR-88), were prepared as previously described by Danishefsky and co-workers [33–35].

2.3. MDCK-MDR assays

Bi-directional permeability studies employing Madin–Darby canine kidney cells transfected with human MDR1 (MDCK-MDR) were conducted by Absorption Systems, Inc. (Exton, PA) to determine the membrane permeability and Pgp transport liability of test compounds. Briefly, MDCK-MDR monolayers were grown to confluence on collagen-coated, microporous, polycarbonate membranes in 12-well plates. The permeability assay buffer was Hanks Balanced Salt Solution containing 10 mM HEPES and 15 mM glucose at a pH of 7.4. Test compounds were added to the assay buffer at a concentration of 5 μM. For Pgp inhibition studies, 10 μM digoxin was incubated in the absence or presence of 5 μM test compound. Cell monolayers were dosed on the apical (A) or basolateral (B) compartment and incubated at 37 °C with 5% CO_2 in a humidified incubator. After 1 and 2 h, aliquots were taken from the receiver chambers and replaced with fresh assay buffer. Samples were taken from the donor chamber after 2 h. Each determination was performed in duplicate. The lucifer yellow flux was also measured for each monolayer to ensure no damage was inflicted to the cell monolayers during the measurement period. Compound concentrations in the samples were determined by LC–MS/MS using electrospray ionization. The apparent permeability coefficient, P_{app}, in both the A–B and the B–A directions, as well as percent recovery and efflux ratio, were calculated as follows:

$$P_{app} = \left(\frac{d\mathrm{Cr}}{dt}\right) \times \frac{\mathrm{Vr}}{A \times \mathrm{CN}}$$

$$\text{percent recovery} = 100 \times \frac{(\mathrm{Vr} \times \mathrm{Crfinal}) + (\mathrm{Vd} \times \mathrm{Cdfinal})}{\mathrm{Vd} \times \mathrm{CN}}$$

$$\text{efflux ratio} = \frac{P_{app\,B-A}}{P_{app\,A-B}}$$

where $d\mathrm{Cr}/dt$ is the slope of the cumulative concentration in the receiver compartment versus time in μM/s; Vr is the volume of the receiver compartment in cm^3; Vd is the volume of the donor compartment in cm^3; A is the area of the cell monolayer (1.13 cm^2 for 12-well plates); CN is the nominal concentration of the dosing solution in μM; Crfinal is the cumulative receiver concentration in μM at the end of the incubation period and; Cdfinal is the concentration of the donor in μM at the end of the incubation period.

2.4. Compound treatments

All compounds were dissolved in 100% DMSO. For the MDCK-MDR and PC12 cell culture studies, compounds were diluted at least 1:1000 into culture medium before addition to cells. All mice (B6C3F1) received test compounds dissolved in DMSO via intraperitoneal (i.p.) administration of volumes of ~40 μl, with volume adjusted to body weight to ensure the proper dosage. Young male B6C3F1 mice (2–3 months of age) were utilized in all studies, with the number of mice per treatment group indicated in the figure legends.