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Small-molecule Structure Correctors Target Abnormal Protein Structure and Function: The Structure Corrector Rescue of Apolipoprotein E4–associated Neuropathology

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Abstract

An attractive strategy to treat proteinopathies—diseases caused by malformed or misfolded proteins—is to restore protein function by inducing proper three-dimensional structure. We hypothesized that this approach would be effective in reversing the detrimental effects of apolipoprotein (apo) E4, the major allele that significantly increases the risk of developing Alzheimer's disease and other neurodegenerative disorders. ApoE4's detrimental effects result from its altered protein conformation ("domain interaction"), making it highly susceptible to proteolytic cleavage and the generation of neurotoxic fragments. Here, we review apoE structure and function, how apoE4 causes neurotoxicity, and describe the identification of potent small-molecule-based "structure correctors" that induce proper apoE4 folding. SAR studies identified a series of small molecules that significantly reduced apoE4's neurotoxic effects in cultured neurons, and a series that reduced apoE4 fragment levels in vivo, providing proof-of-concept for our approach. Structure corrector–based therapies could prove highly effective for the treatment of many protein-misfolding diseases.

Keywords

Alzheimer's disease; apoE; cholesterol transport; neurotoxicity; ER stress; mitochondrial dysfunction; lysosomal storage diseases; cystic fibrosis; p53; neurotoxic fragments; proteinopathies

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INTRODUCTION

Proteinopathies, broadly defined as diseases associated with malformed or misfolded proteins, can be caused by very minor changes that profoundly alter protein structure and/or function, e.g., a single amino acid interchange. Such disorders are often related to neurologic diseases with the accumulation of protein deposits, but can also be related to the mishandling of structurally abnormal proteins in the endoplasmic reticulum (ER) or Golgi apparatus leading to cellular defects. Small-molecule structure correctors capable of reversing the mishandling, impaired intracellular sorting, and functional defects of misfolded proteins hold great promise for the treatment of several disorders, including the transmembrane conductance regulator (CFTR) related to cystic fibrosis,[1–5] lysosomal enzymes related to various lysosomal storage diseases,[6–12] and p53 in various cancers.[13–21] Restoring the normal trafficking of misfolded proteins by reducing ER/Golgi apparatus retention alleviates protein accumulation and, ultimately, cellular toxicity.[22, 23] In addition, as will be discussed, apolipoprotein (apo) E4 impacts neuropathology, at least in part, because of its abnormal structure and altered cellular transport and function.[24–29]

ApoE, and specifically the apoE4 isoform,[30–32] represents the major genetic risk factor for AD, which is the most common form of dementia. As our population continues to age, the number of patients with AD is going to double or triple over the next decades, and without new drugs that can cure or greatly retard the disease process, our healthcare system will be bankrupt. None of the drugs on the market are truly effective, at best only slowing the process in some patients. This article focuses on a new therapeutic approach targeting apoE4 with small-molecule structure correctors, which can prevent many of the detrimental effects of apoE4 in cultured neurons and in vivo in mouse models.

APOE: OVERVIEW AND GENERAL ROLE IN LIPID METABOLISM

ApoE, discovered in the early 1970s as a cholesterol-induced apoprotein on lipoproteins, functions as a lipid transport protein in the blood and interstitial fluids, redistributing cholesterol and phospholipids to cells for use in metabolic pathways or membrane repair after injury.[24, 25, 29] It is a 299-amino acid protein (34 kDa) with three common isoforms—apoE2, apoE3, and apoE4—encoded by a single gene on chromosome 19. The three alleles differ in their frequencies in the population, with ε2 expressed by 5–10%, ε3 by 70–78%, and ε4 by 14–20% of the population (Table 1), and give rise to three heterozygous and three homozygous phenotypes: apoE2/2, apoE3/2, apoE3/3, apoE4/3, apoE4/4, and apoE4/2. The apoE polymorphisms arise from single amino acid substitutions at residues 112 and 158, where apoE3 has Cys-112 and Arg-158, apoE4 has arginines at both sites, and apoE2 has cysteines at these sites. These single amino acid polymorphisms have a profound effect on both the structure and function of the apoE protein.[24–29, 33, 34]

ApoE is synthesized primarily by hepatocytes and circulates in the plasma on lipoprotein particles—including very-low-density lipoproteins, chylomicrons, and a subclass of HDL—at a typical plasma concentration of 40–70 μg/ml.[25, 28, 29] However, the second most common site of synthesis is the brain, primarily by astrocytes and to a lesser extent by

stressed or injured neurons.[24–26, 28] Macrophages throughout the body, including microglia in the brain, also synthesize apoE.[25, 28] In the cerebrospinal fluid, apoE represents the major lipid transport apoprotein and is associated with HDL-like particles and small phospholipid disks. The native apoE is always associated with lipids.

ApoE serves as a major ligand in the uptake of cholesterol- and triglyceride-rich lipoproteins through cell-surface LDL and LDL receptor–related proteins.[25, 28, 29, 35] The LDL receptor–binding region of apoE encompasses a highly positively charged region of the protein (amino acids 134–150) that is rich in arginine and lysine residues.[25, 28, 29, 33, 34] ApoE3 and apoE4 bind equally well to the receptors; however, apoE2 is defective in receptor binding and is associated with a lipid disorder called type III hyperlipoproteinemia.[28, 36]

The lipid-binding region of apoE is in the carboxyl-terminal domain encompassing amino acid residues 244–272, which is predicted to form an amphipathic α-helix.[25, 33, 34] ApoE3 and apoE2 preferentially bind to small, phospholipid-rich HDL, whereas apoE4 binds to larger, triglyceride-rich lipoproteins in the plasma.

APOE: STRUCTURAL DETERMINANTS OF FUNCTION

ApoE has two structural domains: the amino-terminal domain (residues 1–191), which contains the LDL receptor–binding region (residues 134–150), and the carboxyl-terminal domain, which contains the lipid-binding region (residues 244–272). The two domains appear to be connected by a hinge region[24, 25, 28, 33, 34] (Figure 1A).

X-ray crystallographic analyses of the amino-terminal domains of apoE3 and apoE4 reveal a marked difference in orientation of the side chains of critical residues.[37] Most importantly, the side chain of Arg-61 in apoE4 extends away from the 4-helical amino-terminal bundle and is available to interact with Glu-255. However, in apoE3, the side chain of Arg-61 is less available to interact with Glu-255. The arginine–glutamic acid interaction alters the structure of apoE4 and is responsible for a unique and abnormal structural feature called domain interaction.[25, 28, 37, 38] ApoE3, and especially apoE2, display less domain interaction and are visualized as having a more open structure. ApoE also displays varying degrees of biophysical instability (apoE4 > apoE3 > apoE2). The abnormal apoE4 domain interaction and instability are responsible for several of its neuropathological effects, and blocking apoE4 domain interaction has been shown to abolish these detrimental effects.

ApoE4 domain interaction has been extensively investigated by site-directed mutagenesis, as well as by fluorescence resonance energy transfer (FRET) and electron paramagnetic distance measurements.[33, 39–41] For example, mutation of Arg-61 to threonine or Glu-255 to alanine blocks apoE4 domain interaction, which converts apoE4 to an apoE3-like molecule —both structurally and functionally—and abolishes several of apoE4's detrimental effects.[22, 24–26] In addition, apoE4 domain interaction occurs in neurons, as demonstrated by studies showing that apoE3 or apoE4 constructs conjugated to yellow fluorescent protein on the amino terminus and cyan fluorescent protein on the carboxyl terminus can produce a FRET signal when transfected in Neuro-2a cells. Interestingly, the apoE3-transfected cells give a low FRET signal, whereas the apoE4-transfected cells give a much higher signal,[41]

indicating that the amino-terminal and carboxyl-terminal domains of apoE4 are in closer proximity than in apoE3.

THE ROLE OF APOE4 IN NEUROPATHOLOGY, INCLUDING ALZHEIMER'S DISEASE, TRAUMATIC BRAIN INJURY, AND OTHER NEUROPATHOLOGICAL DISORDERS

ApoE genotype has a major impact on AD in two ways.[30–32] First, expression of the apoE4 allele increases the risk of developing the disease during one's lifetime, and second, apoE4 expression decreases the age-of-onset, resulting in the disease's cognitive effects occurring 7–8 years earlier in individuals carrying one apoE4 allele and 15–16 years earlier in those carrying two apoE4 alleles. Importantly, 65–80% of all AD patients have at least one apoE4 allele[42] (Table 1). In contrast, only about 25% of individuals in the general population are carriers of apoE4; thus, apoE4 is greatly enriched in patients with AD.

ApoE4 expression also impacts the clinical outcome from traumatic brain injury,[43–49] whereby those with at least one apoE4 allele display poor recovery 3–6 months following the injury. Furthermore, apoE4 has been associated with the pathogenesis of multiple sclerosis,[50, 51] frontotemporal dementia,[52] stroke,[53, 54] and in some studies in Parkinson's disease patients.[55–58] It appears that apoE4 *sets the stage* for neuropathology and that a variety of *second hits*, which stress or injure neurons, determine the class of neurons affected and the nature of the neurological defect. Our studies indicate that injured or stressed neurons turn on the synthesis of apoE, and in the context of apoE4, neuropathology occurs secondary to the generation of apoE toxic fragments—with apoE4 being much more likely than apoE3 (apoE4 > apoE3) to generate neurotoxic species (Figure 2).[24–27]

MECHANISMS RESPONSIBLE FOR APOE4-INDUCED NEUROPATHOLOGY

It is now recognized that multiple factors acting through various pathways are involved in AD pathogenesis. The amyloid hypothesis focuses on the effect of Aβ peptide accumulation in causing neurotoxicity, disrupting synaptic connections and causing plaque formation (for review, see refs. 27, 59). Clearly, there are apoE isoform–specific effects on the amyloid pathway, whereby apoE4 expression is linked to defective clearance of Aβ[60–62] and increased Aβ synthesis.[63] In addition, apoE (apoE4 > apoE3) can facilitate the fibrillization of Aβ and enhance the formation of toxic Aβ species.[27, 64] However, it is also abundantly clear that apoE (apoE4 > apoE3) can have a direct effect on neurons leading to neuropathology, including AD. Furthermore, because injured or stressed neurons turn on apoE synthesis, in the context of apoE4, increased apoE expression following injury also promotes apoE4-mediated neuropathology by increasing neurotoxic fragment levels. ApoE4 alone affects neuronal cells in vitro and in vivo, impairing synaptic connections as well as learning and memory, and causing neuronal cell loss.[24–27]

Our working hypothesis—and the basis upon which the structure corrector studies were undertaken—arose from understanding the structural difference that distinguishes apoE4 from apoE3.[24–28] Because of the unique domain interaction between Arg-61 and Glu-255,

apoE4 assumes an abnormal conformation that is highly susceptible to neuron-specific proteolysis, generating neurotoxic fragments that escape the secretory pathway and enter the cytosol (Figure 2). These fragments are associated with a variety of detrimental effects, including 1) mitochondrial dysfunction in neurons, including altered mitochondrial membrane potential[65] and decreased levels and activity of mitochondrial electron transport enzymes [e.g., a 35–40% reduction in mitochondrial cytochrome c oxidase (mtCOX1) and ATP synthase];[66] 2) impaired neuronal mitochondrial motility;[67] 3) impaired synaptogenesis and reduced synaptic spine density in mice both in vitro and in vivo;[67, 68] 4) altered cytoskeletal profiles, including microtubule disruption,[69–71] tau hyperphosphorylation, and neurofibrillary tangle–like intracellular inclusions;[72, 73] and 5) CNS neuropathology,[74] including loss of GABAergic hippocampal interneurons[75] and impaired learning and memory behaviors in transgenic mice.[62, 76, 77] ApoE4 expression is also associated with impaired neurite outgrowth[69, 70, 78] and increased Aβ production,[63] lysosomal disruption, Aβ-associated apoptosis, and neuronal cell death.[79] *Our data indicate that most of these detrimental effects can be reversed when apoE4's domain interaction is blocked by site-directed mutagenesis (i.e., arginine-61 to threonine) or by small-molecule structure correctors.*[24–28, 63, 67, 68, 79–81]

IDENTIFICATION OF SMALL-MOLECULE STRUCTURE CORRECTORS AND THEIR MECHANISM OF ACTION

Using the DOCK virtual screening approach based on the x-ray crystallographic structure of apoE4, we identified a few dozen small molecules—out of 200,000 structural motifs in the library—that were predicted to bind preferentially to apoE4 and disrupt domain interaction.[63] Two compounds, GIND-25 and GIND-105, converted apoE4 into an apoE3-like molecule with respect to lipid-binding characteristics and effects on cultured neurons.[63] In addition, we screened a ChemBridge library enriched in drug-like small molecules and demonstrated that a phthalazinone **1** (CB9032258)[81] and a pyrazoline disrupted domain interaction using a FRET assay and restored mtCOX1 levels in cultured neurons (assays described below). These observations formed the basis for a collaboration with Merck Research Laboratories to synthesize small-molecule analogs of these initial hits and establish SARs of specific chemical series in an effort to identify potent (i.e., low nanomolar) apoE4 structure correctors (apoE4SCs) (Figure 1B).

To identify apoE4SCs that disrupt apoE4 domain interaction, we developed an intracellular FRET assay to biophysically measure the apoE4 intramolecular interaction.[81] GFP was fused to the amino terminus and an *Escherichia coli* dihydrofolate reductase (eDHFR) was fused to the carboxyl terminus of apoE4 and apoE3 (to yield GFP-apoE4-eDHFR and GFP-apoE3-eDHFR, respectively). The constructs were then stably expressed in Neuro-2a cells. An eDHFR high-affinity ligand, trimethoprim, was conjugated with hexachlorofluorescein to serve as the acceptor fluorophore (Figure 3A). Neuro-2a cells expressing GFP-apoE4-eDHFR had a significantly higher FRET signal (~40%) than those expressing GFP-apoE3-eDHFR.[81] The higher signal reflects the closer proximity of the amino- and carboxyl-terminal domains in apoE4, and establishes a readout that can be used to screen for

apoE4SCs that disrupt domain interaction by reducing the FRET signal to one resembling apoE3.

We also developed a secondary in-cell western assay in Neuro-2a cells to measure the downstream functional effects of the FRET assay–positive apoE4SCs, and found that structure correctors that block apoE4 domain interaction restored COX1 levels to those seen in apoE3-expressing cells.[81] Additional assays included studies of the effects of apoE4SCs on neurite outgrowth, mitochondrial motility, synaptic spine formation, and intracellular trafficking of apoE4 by fluorescence recovery after photobleaching.[67]

APOE4 STRUCTURE CORRECTOR PROOF-OF-CONCEPT IN VITRO

The phthalazinones represent a chemical series of highly active apoE4SCs that block domain interaction in apoE4-expressing cells without affecting apoE3-expressing cells. The initial phthalazinone, **1**, displayed specificity for inhibiting apoE4 FRET intensity (Figure 3B) and more potent phthalazinones [**2** (PH-001) and **3** (PH-002)][81] showed low nanomolar activity in inhibiting the FRET signal in apoE4-expressing Neuro-2a cells (Figure 3C). A good SAR was established by the small-molecule analogs provided by Merck Research Laboratories. Representative structures of active and inactive structure correctors are shown in Figure 4 and are more extensively described in ref. 81.

Domain interaction also modulates the detrimental effects of apoE4 on mitochondrial activity and levels.[81] In Neuro-2a cells expressing a site-directed mutant that blocks domain interaction, apoE4-R61T, COX1 levels were similar to those of apoE3-expressing cells (Figure 5A). Similarly, the structure corrector **1** increased COX1 levels specifically in apoE4-expressing cells, without affecting their apoE3-expressing counterparts (Figure 5B). **2** and **3** demonstrated nanomolar potency with respect to increasing COX1 levels, and **3** not only increased COX1 levels in Neuro-2a cells expressing apoE4 (Figure 5C), but also increased COX1 levels in primary cortical and hippocampal neurons from NSE-apoE4 transgenic mice.[81] Furthermore, both apoE4-R61T-expressing neuronal cells (Figure 6A, C, and D), as well as apoE4-expressing neuronal cells treated with **2** or **3** (Figure 6B–E), displayed mitochondrial motility, synaptic spine formation, and neurite outgrowth similar to neuronal cells expressing apoE3.[81]

Using EPR, we demonstrated selective target engagement for **3**, whereby **3** affected the mobility of a Cys-76 spin label on the apoE4 amino-terminal domain in a dose-dependent manner. A six-featured pharmacophore model, based on the SAR of the active phthalazinones, was consistent with the EPR data and confirmed that residues in the vicinity of Arg-61 were essential for structure-corrector activity (Figure 7).[81] Thus, the phthalazinone class of structure correctors provides proof-of-concept that blocking domain interaction abolishes or significantly retards the detrimental effects of apoE4 in vitro. However, to date the most potent phthalazinone apoE4SCs caused significant toxicity in mice. Therefore, we have expanded our validation studies to other apoE4SCs.

A series of pyrazoline analogs were prepared by Merck Research Laboratories and used to establish SARs. This series was highly effective in blocking apoE4 domain interaction as

determined by FRET, and demonstrated parallel effects in enhancing COX1 levels and reducing mitochondrial toxicity in apoE4-expressing neuronal cells. For example, the pyrazoline **4** (PY-101)[81] (structure not disclosed), which presently is our lead structure corrector, displayed an EC_{50} in the FRET, COX1, and mitochondrial motility assays of 1200, 687, and 60 nM, respectively. Additional pyrazoline analogs (PY-103, PY-104, and PY-106; structures not disclosed)[81] had EC_{50}'s in the FRET assay of 45, 132, and 496 nM and in the COX1 assay of 48, 125, and 215 nM, respectively. In vivo data, established using **4**, demonstrate that it achieves excellent brain penetration following 10 consecutive days of daily intraperitoneal dosing of NSEapoE4 mice (30 mg/kg: 7.9 ± 1.7 μM plasma; 35.1 ± 7.5 μM brain) and no in vivo toxicity during this treatment period. Furthermore, there was a 20–25% decrease in the levels of apoE4 fragments in the brain and hippocampus. In addition, COX1 levels increased in the hippocampi by about 55% (unpublished data). These data demonstrate that potent apoE4SCs have therapeutic effects in vivo, at least in mice with the human apoE4 isoform, and suggest that apoE4 is a promising drug target in patients with neuropathology associated with this apoE isoform.

APOE4 STRUCTURE CORRECTORS TARGET THE NEURONAL SECRETORY PATHWAY AND RESTORE APOE4 TRANSIT IN THE ENDOPLASMIC RETICULUM AND GOLGI APPARATUS

As discussed, stressed or injured neurons turn on the synthesis of apoE, but the induction of apoE4 expression uniquely causes pathology (Figure 2). Using our cell-based fluorescence recovery after photobleaching assay, we found that intracellular trafficking of apoE4 in the ER and Golgi apparatus was significantly retarded compared with apoE3 (Figure 8). In contrast, the time spent by the apoE4-R61T mutant in transit in the secretory pathway was restored to apoE3 levels, both with respect to the extent and rate of recovery after photobleaching.[67] Furthermore, transit secretory time for apoE4 was also restored to apoE3 levels in apoE4-expressing cells treated with **3** (Figure 8).[67]

These data provide several insights into the mechanisms underlying apoE4's neurotoxic effects and how they can be modified. 1) ApoE4, because of domain interaction, is mishandled and possibly misfolded as it is being synthesized or soon after entering the ER lumen, disrupting normal trafficking through the secretory pathway. 2) ApoE4 in the secretory pathway is likely recognized as "abnormal" and undergoes partial proteolytic cleavage, generating neurotoxic fragments, while secretion of intact apoE4 is reduced. 3) ApoE4-R61T, which lacks domain interaction, as well as apoE4 treated with a structure corrector (**3**), behave like apoE3 in transfected cells, displaying a normal transit time through the ER and Golgi apparatus and a resistance to proteolytic cleavage. ApoE4 is partially proteolyzed by a unique chymotrypsin serine protease, generating neurotoxic fragments that can escape the ER and enter the cytosol.[73, 82, 83] However, despite the fact that apoE4 assumes an abnormal structure in neurons, apoE4 does not activate the ER stress pathway.[67]

Thus, it appears that the active *structure correctors target the newly synthesized apoE4* to restore its normal trafficking in neuronal cells. Newly synthesized apoE4 is likely to be the apoE4SC target because apoE only displays domain interaction when it is non- or poorly

lipidated, as would occur during or soon after synthesis, whereas the helical structure of apoE is reorganized when it is fully lipidated.[33]

STRUCTURE CORRECTORS AS DRUGS: LESSONS LEARNED FROM OTHER DISEASES

Cystic Fibrosis and the Cystic Fibrosis Transmembrane Conductance Regulator

Cystic fibrosis is a common, frequently lethal genetic disease that affects 1 in 2500 individuals.[1–5] The morbidity is associated with chronic lung infections and deterioration of lung function caused by mutations in the chloride channel CFTR. Numerous mutations in this channel have been described;[84] however, the most common of these is the F508 deletion mutation, which occurs in about 90% of patients.[85] This mutation impairs transit of the ion channel through the ER, leading to its targeting for proteosomal degradation, and ultimately, reduced expression on the apical membrane of pulmonary epithelial cells. The deletion of phenylalanine at residue 508 causes the CFTR to misfold, although targeted degradation is not 100% efficient and many individuals still express some CFTR. The three-dimensional structure of the CFTR identifies a potential site responsible for the F508 deletion dysfunction.[86]

A promising therapeutic approach is to stabilize the structure and enhance the transit of the mutated CFTR to the cell surface with small-molecule structure correctors. A recent article by Pedemonte et al.[5] compared the efficacy and potency of several structure correctors reported in the literature. Van Goor et al.[87] provided specific information on VX-809. In addition, Vertex Pharmaceuticals has obtained results from a Phase 2 trial on VX-809, a lead F508 deletion CFTR structure corrector that, encouragingly, led to an improvement in sweat chloride levels compared with placebo.[88] Studies are under way to determine if lung function also improved.

Vertex has also developed a drug that serves as a potentiator of CFTR activity.[89] About 4% of cystic fibrosis patients have a G551D mutation that causes a gating abnormality in chloride ion transport.[90] In January 2012, the FDA approved the use of Kalydeco (invacafor; VX-770) as a CFTR potentiatior in cystic fibrosis patients 6 years of age and older (http://www.vrtx.com/). Vertex also has a Phase 2 study of VX-809 alone or in combination with Kalydeco, to determine if the structure corrector and potentiator improve the outcome in patients with the F508 deletion. An interim analysis of this study shows that the combination improves lung function in patients homozygous for the F508 deletion.[91] These studies lend support to the concept of structure correctors as a promising approach to treat diseases caused by protein misfolding, such as in cystic fibrosis.

Lysosomal Storage Diseases: Fabry, Gaucher, and Pompe Diseases

Lysosomal storage diseases are caused, at least in part, by defects in the transit of mature functional enzymes from the ER to the lysosome.[22, 92] Small-molecule structure correctors, referred to as pharmacological chaperones, have been shown to promote normal trafficking by stabilizing enzyme structure and protecting the enzymes from proteolysis.

Fabry Disease

Fabry disease is a lipid storage disorder associated with mutations in α- galactosidase A (α-GAL-A). Normally, this enzyme resides in lysosomes where it catabolizes the complex lipid globotriaoylceramide, but mutated α-GAL-A (commonly by missense mutation) is retained in the ER and prematurely degraded. The consequent accumulation of globotriaoylceramide results in an array of problems, including kidney failure, increased risk of myocardial infarction and stroke, and vision problems (for review, see refs. 6, 7, 93).

Amicus Therapeutics has developed a small-molecule pharmacological chaperone that interacts with the mutated enzyme in the ER, stabilizes its structure, and allows it to traffic normally to lysosomes.[8–12] A Phase 3 clinical trial was undertaken in 2011 to determine whether migalastat (DGJ, AT-1001), which affects the α-GAL-A structure (http://www.amicustherapeutics.com/), improves disease outcomes in 140 Fabry disease patients, the majority of whom have missense mutations. The x-ray crystallographic structure of α-GALA, as well as a model for how DGJ increases the stability of the enzyme, have been reported.[94]

Gaucher Disease

Gaucher disease, a lysosomal lipid storage disorder characterized by the deposition of glucocerebrosides primarily in macrophages, is caused by mutations in the β-glucocerebrosidase (GCase) enzyme.[95–97] These mutations lead to protein misfolding, causing the mutant enzyme to poorly traffic from the ER and Golgi apparatus to lysosomes, and ultimately targets it for degradation. As a result, the abnormal accumulation of lipids is observed in the spleen, liver, lungs, bone marrow, and brain. In addition, mutations in GCase represent a genetic risk factor for Parkinson's disease, and Gaucher disease patients have an estimated 10–20-fold increased risk for developing Parkinson's disease, as GCase is involved in the catabolism of α-synuclein in the brain.[98, 99]

Structure-corrector molecules have been identified that allow for normal GCase trafficking to the lysosome, which would benefit Gaucher patients as well as potentially reduce the risk for developing Parkinson's disease in these patients.[97, 100–102] Amicus Therapeutics has performed preclinical studies on AT3375, a more potent pharmacologic chaperone of the first-generation lead AT2101 (isofagomine) (http://www.amicustherapeutics.com/). The three-dimensional structural interaction of isofagomine with GCase, and the mechanism by which it stabilizes GCase structure and improves export from the ER, have been described.[94, 103]

Pompe Disease

Pompe disease is a lysosomal glycogen storage disease caused by mutations in the acid alpha-glucosidase enzyme.[104, 105] Excessive amounts of glycogen accumulate in the heart and skeletal muscles, and the severity of the disease is related to the degree of enzyme deficiency. Here again, this disease is targeted for treatment using a structure corrector,[106–108] and Amicus Therapeutics has a Phase 2 study under way using AT2220 (duvoglystat). This drug is being used in combination with enzyme replacement therapy (http://www.amicustherapeutics.com/).

Cancer and p53

A central role for p53 as a tumor suppressor and the importance of p53 mutations in ~50% of all human tumors[109, 110] have resulted in significant interest in the development of treatment strategies to restore normal p53 function, including a structure-corrector approach.[111, 112] Active p53 arrests cell-cycle pathways and induces apoptosis,[13, 14] and mutant p53 prevents apoptosis and sustains tumor growth by impairing the ability of p53 to bind DNA and regulate gene transcription. Mutant p53 is unstable and misfolded, thereby preventing normal target interactions and leading to its high accumulation in tumor cells (for reviews, see refs. 109, 110).

The majority of point mutations in p53 occur in the DNA-binding domain, changing the conformation of the protein and/or abolishing its DNA contacts.[13, 111] Small-molecule structure correctors reactivate and rescue mutant p53 by stabilizing the DNA-binding domain and changing the conformation of p53 from a mutant to a wildtype form.

The first small molecule identified to promote and stabilize a wildtype-like structure for mutant p53 is CP-31398; however, the detailed mechanism of action of this compound is elusive.[15] Various other compounds capable of restoring folding, DNA binding, and mutant p53 reactivating capacity have been identified.[14–21] Two interesting compounds, called PRIMA-1 and APR-246, restore DNA binding and stabilize the folding structure of mutant p53. Recent pharmacodynamic studies showed that PRIMA-1 and APR-246 are in fact pro-drugs that give rise to MQ, a thiol-reactive compound that binds covalently to mutant p53 and enhances its activity.[20, 21] A structural analog of PRIMA-1 is in Phase 1/2 clinical trials in patients with hematologic and prostate cancers (Aprea, Solna, Sweden).[112]

STRUCTURE CORRECTORS TO TARGET DISEASE

Structural correction of misfolded protein variants to wildtype forms using small molecules represents a novel therapeutic approach to restore normal intracellular handling and transport and protect the protein from intracellular proteolysis and degradation. ApoE4 represents a critical target for this approach, because of the magnitude of the impact that this apoE isoform has on a variety of disease states and the sheer magnitude of the number of individuals worldwide who carry this allele (one in four). As discussed, apoE4 expression has an incredibly strong effect on AD risk as well as the risk for developing other neurodegenerative diseases. In addition, apoE4 is associated with an increased prevalence of cardiovascular disease because of its impact on plasma lipids and lipoproteins, which explains about 10% of the variance in plasma cholesterol levels.[25, 28] Furthermore, apoE4 is associated with pathology related to infectious and autoimmune diseases, whereby homozygosity for apoE4 accelerates the progression to death in HIV/AIDS patients.[113] Autoimmune diseases, such as multiple sclerosis, and systemic diseases, such as lupus erythematosus, may reflect an impaired clearance of apoptotic bodies, cellular debris, and a proinflammatory state, all of which would inevitably involve apoE, given the fundamental role of this apoprotein in lipid metabolism.[114–116]

The detailed understanding of the structure and function of apoE provides an ideal base upon which to undertake this therapeutic approach. However, only time will tell if such a therapeutic approach will benefit patients.

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ABBREVIATIONS USED

α-GAL-A	α-galactosidase A
apoE4SC	apoE4 structure corrector
CFTR	cystic fibrosis transmembrane conductance regulator
COX1	cytochrome c oxidase
eDHFR	*Escherichia coli* dihydrofolate reductase
ER	endoplasmic reticulum
FRET	fluorescence resonance energy transfer
GCase	β-glucocerebrosidase

Biographies

Robert W. Mahley is senior investigator/president emeritus at The J. David Gladstone Institutes and a professor of medicine and pathology at the University of California, San Francisco. He completed his medical and doctorate degrees at Vanderbilt University in 1970 and a pathology internship in 1971. He is an internationally known expert on heart disease, cholesterol metabolism and, more recently, Alzheimer's disease. He has made fundamental contributions to our understanding of the role of apoE in the cardiovascular and nervous systems. These findings laid the groundwork for the explosion of research linking apoE4, a variant of apoE, to the pathogenesis of Alzheimer's disease and neurodegeneration. He is a member of the National Academy of Sciences, the Institute of Medicine, and the American Academy of Arts & Sciences.

Yadong Huang is an associate investigator in the Gladstone Institute of Neurological Disease and an associate professor of neurology and pathology at the University of California, San Francisco. He completed his medical and doctorate degrees at Qingdao Medical University and Peking Union Medical College, P. R. China. He identified apoE4 proteolysis as a major contributor to the pathogenesis of Alzheimer's disease, and has become an internationally recognized expert on Alzheimer's disease. His research interests include the development of therapeutic approaches to prevent apoE4 proteolysis or to convert apoE4 to an apoE3-like molecule. Dr. Huang has published numerous scientific

papers in the field of apoE research. He is a member of several scientific societies, including the Society for Neuroscience and the Alzheimer's Association.

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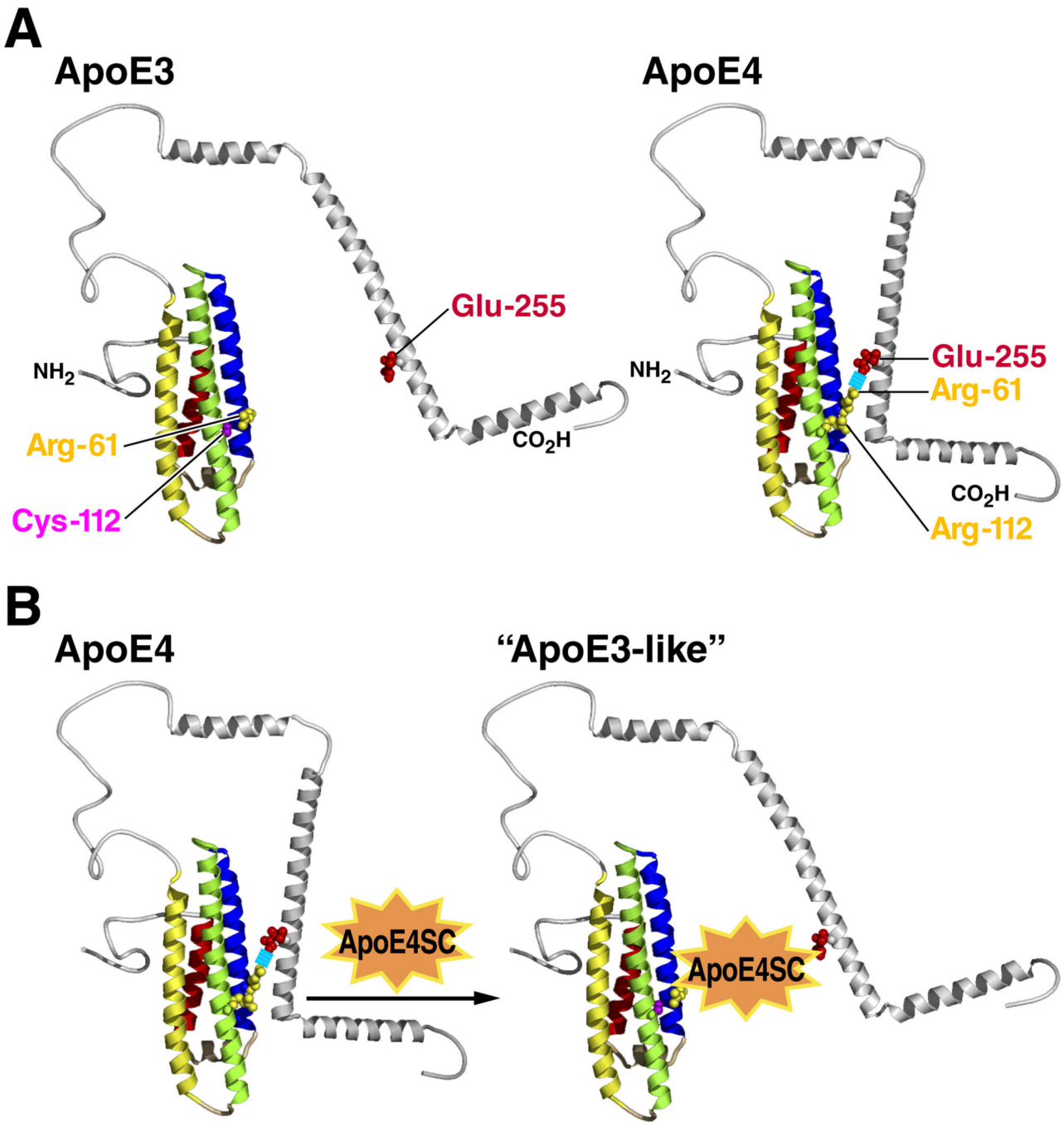

Figure 1.

Models of apoE3 and apoE4. (A) ApoE4 displays a unique property called domain interaction caused by the ionic interaction between Arg-61 in the amino-terminal domain with Glu-255 in the carboxyl-terminal domain. ApoE3 is also a dynamic structure and undergoes domain interaction to a significantly less degree than apoE4. (B) ApoE4 domain interaction can be blocked by a small-molecule apoE4SC that disrupts the ionic interaction between Arg-61 and Glu-255. This converts apoE4 to an apoE3-like molecule both structurally and functionally.

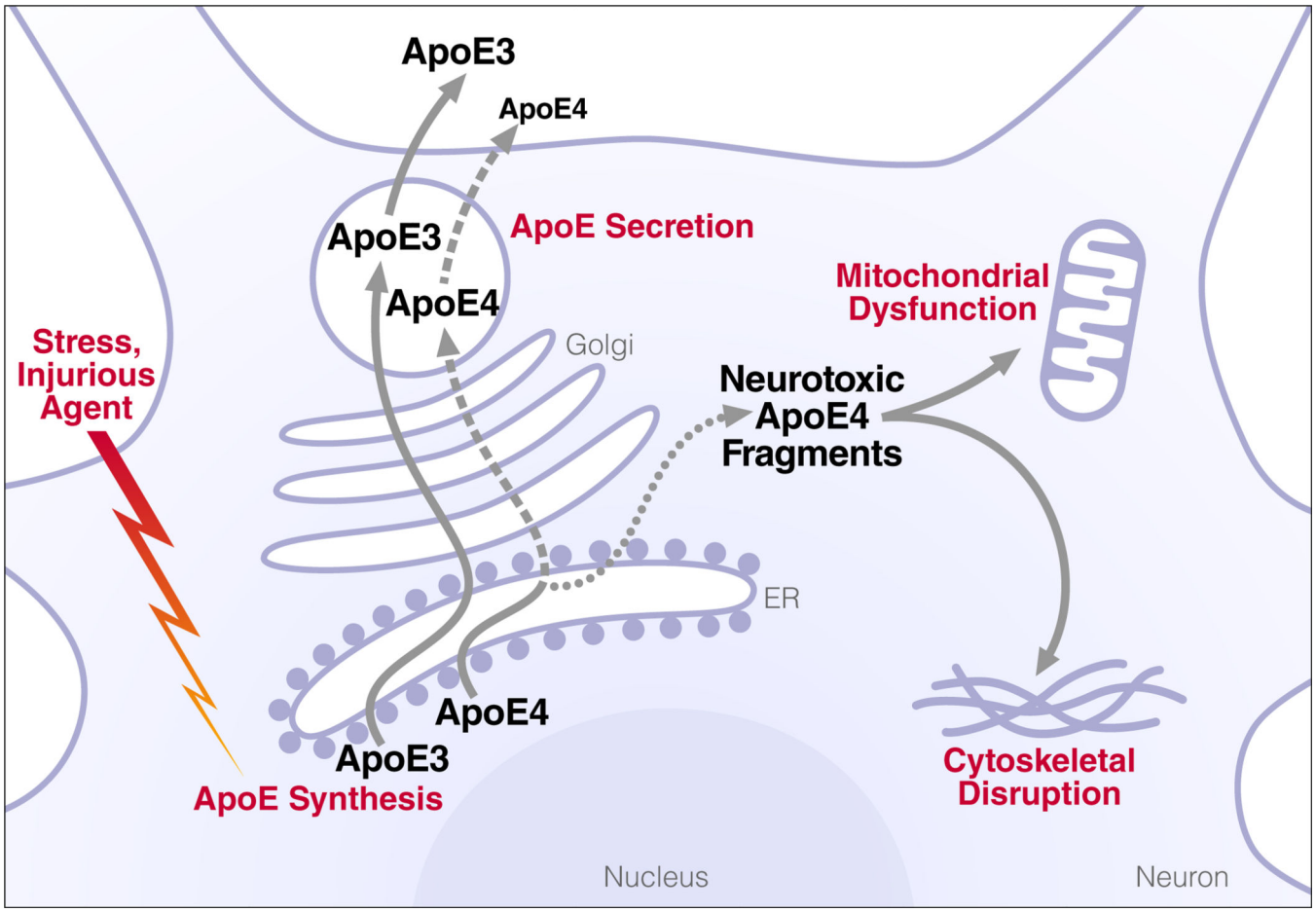

Figure 2.

ApoE synthesis is induced by injurious agents that stress or damage CNS neurons to participate in the transport of cholesterol and other lipids for membrane repair and synapse formation. ApoE3 progresses through the secretory pathway; however, apoE4, because of domain interaction, displays impaired trafficking through the ER and Golgi apparatus and is targeted to a neuron-specific protease that initially clips off the carboxyl-terminal 27–30 amino acids, generating neurotoxic fragments. These fragments escape the secretory pathway and enter the cytosol, where they target the mitochondria, resulting in mitochondrial dysfunction, and alter the cytoskeleton, enhancing tau phosphorylation and forming neurofibrillary-like tangles.

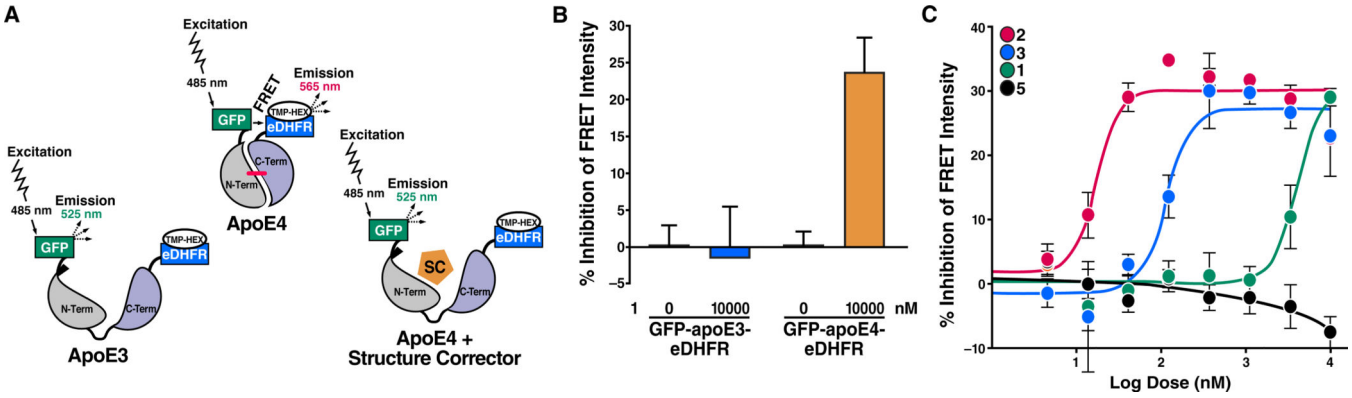

Figure 3.

(A) Model describing the FRET construct. The FRET signal is generated between the donor and acceptor fluorophores. ApoE4 gives a higher FRET signal because of the closeness of the amino- and carboxyl-terminal domains. The apoE4 signal is reduced by the small-molecule apoE structure corrector (SC) through the disruption of domain interaction. (B) A small-molecule apoE4SC (**1**) inhibits the FRET signal in GFP-apoE4-eDHFR-expressing Neuro-2a cells, but has little effect on GFP-apoE3-eDHFR-expressing cells. (C) Dose-response analysis reveals the relative potencies of three active phthalazinones (**2**, **3**, and **1**) and an inactive one [**5** (PH-008)] (see ref. 81 for details). Modified from figure originally published in Chen H-K *et al.* Small molecule structure correctors abolish detrimental effects of apolipoprotein E4 in cultured neurons. *J. Biol. Chem.* 2012, 287:5253–5266. © the American Society for Biochemistry and Molecular Biology.

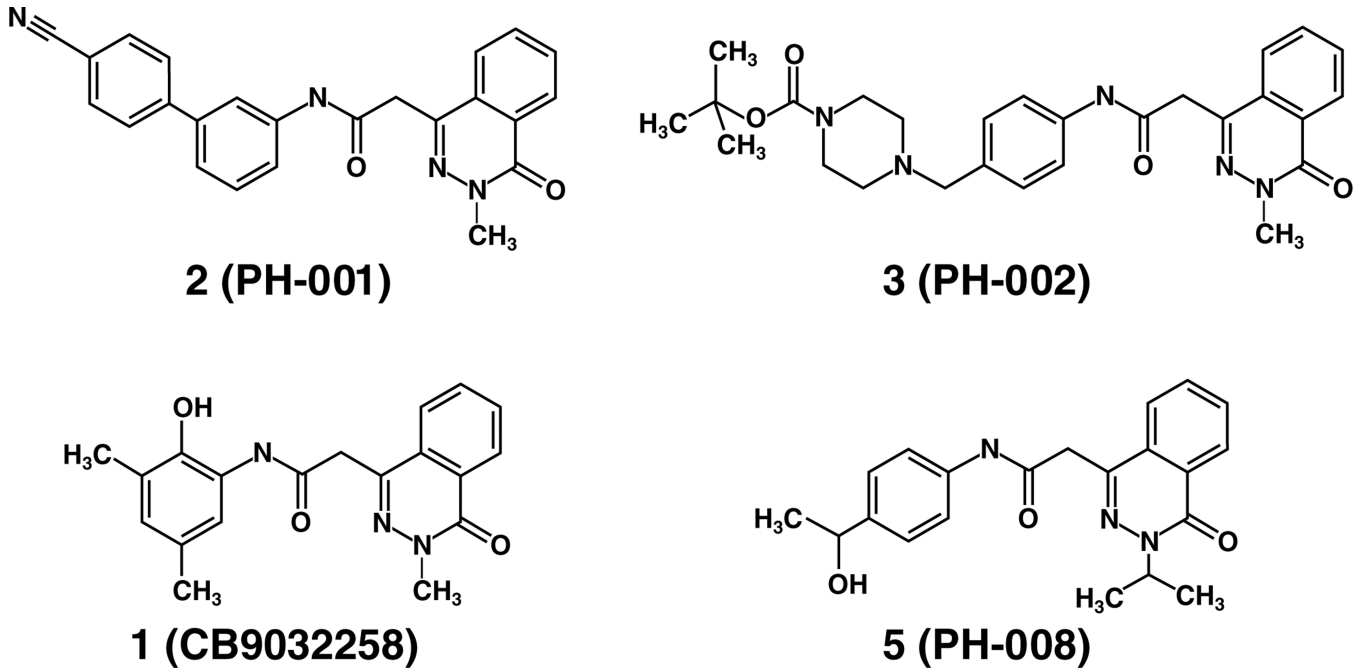

Figure 4.

Structure of phthalazinones, displaying high potency (nanomolar) to low potency and inactivity with respect to the ability to block apoE4 domain interaction and to rescue apoE4 detrimental effects on neurons. Modified from figure originally published in Chen H-K *et al.* Small molecule structure correctors abolish detrimental effects of apolipoprotein E4 in cultured neurons. *J. Biol. Chem.* 2012, 287:5253–5266. © the American Society for Biochemistry and Molecular Biology.

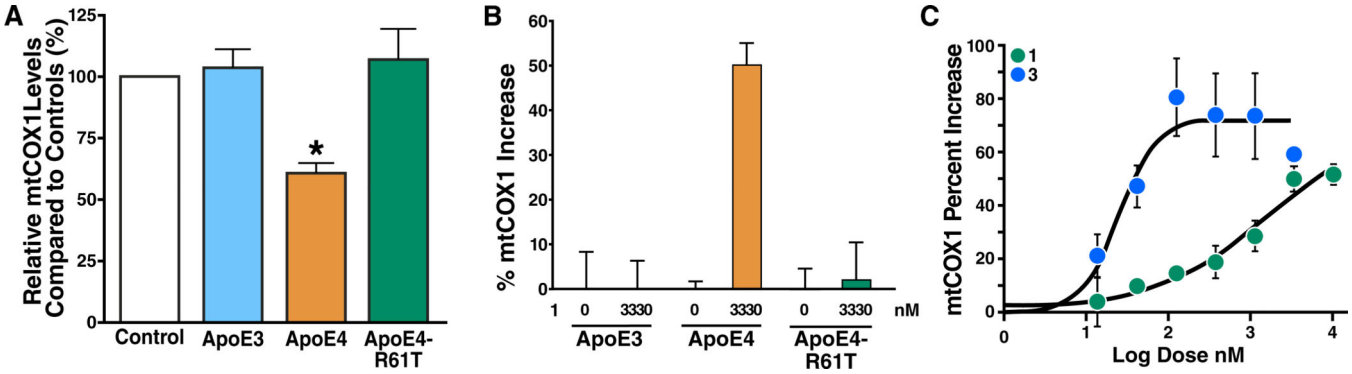

Figure 5.

MtCOX1 levels determined by the in-cell western assay demonstrated apoE4 is associated with decreased COX1 levels, which can be restored in apoE4-expressing Neuro-2a cells by apoE structure correctors. (A) Neuro-2a cells expressing apoE3 or apoE4-R61T, which lacks domain interaction, display mtCOX1 levels similar to control cells, whereas apoE4-expressing cells have significantly lower levels (mean ± SD in three separate experiments; $p < 0.001$). (B) The small-molecule structure corrector **1** increased mtCOX1 levels in apoE4-expressing cells but not in the apoE3- or apoE4-R61T-expressing cells. (C) Dose-response curves for phthalazinones **3** and **1** in Neuro-2a cells (expressed as a percent increase in mtCOX1 levels at nanomolar concentrations). **3** displayed an IC_{50} of 41 nM (see ref. 81 for details). Modified from figure originally published in Chen H-K *et al.* Small molecule structure correctors abolish detrimental effects of apolipoprotein E4 in cultured neurons. *J. Biol. Chem.* 2012, 287:5253–5266. © the American Society for Biochemistry and Molecular Biology.

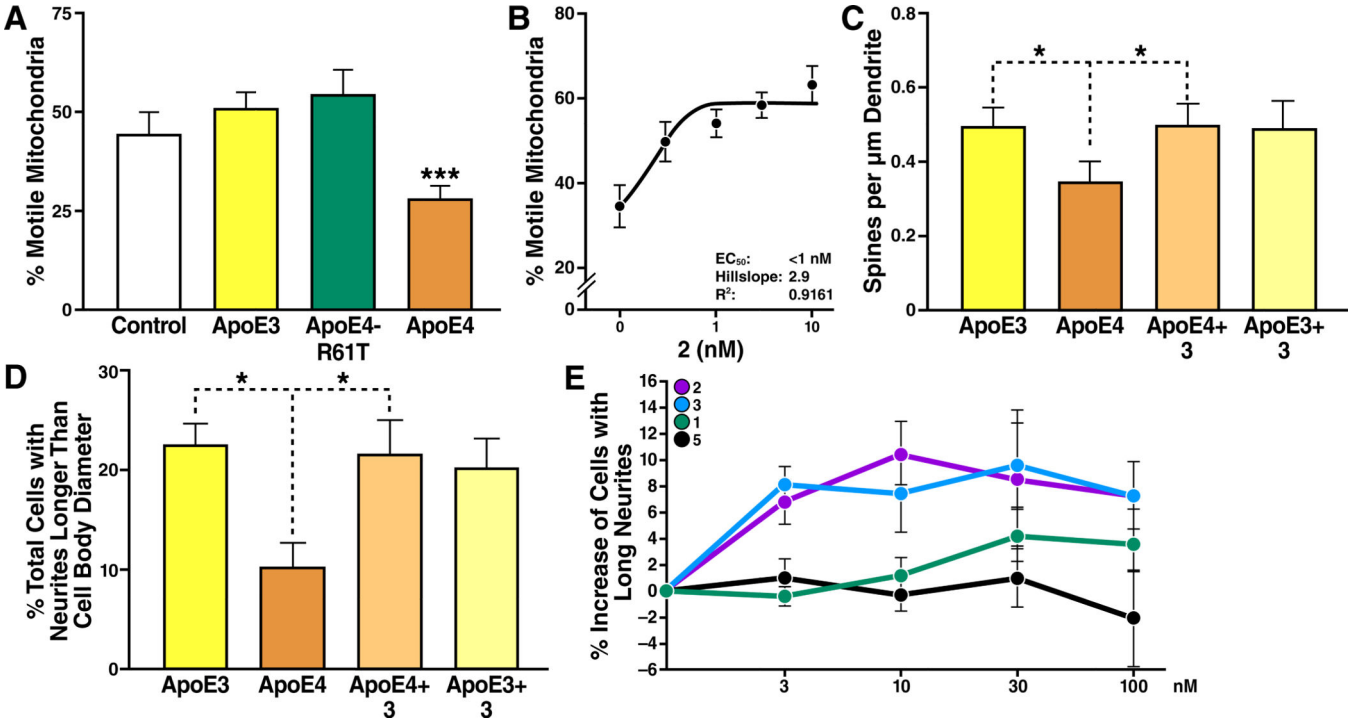

Figure 6.

ApoE4SCs rescue apoE4-associated impairment of mitochondrial motility, dendritic spine formation, and neurite outgrowth in cultured neurons. (A) Mitochondrial motility was retarded in PC12 cells incubated with apoE4, whereas cells incubated with apoE3 or apoE4-R61T behaved like control cells (mean ± SE expressed as a percentage of motile mitochondria; $p < 0.001$ for apoE4 versus control cells). (B) Dose-response analysis reveals that mitochondrial motility was restored in apoE4-expressing PC12 cells treated with apoE4SC **2** (see ref. 81 for details). (C) Dendritic spine density (spines per µm dendrite) was measured in hippocampal primary neurons from mice expressing apoE3 or apoE4 and transiently transfected with EGFP-β-actin to highlight the spines. ApoE4 expression resulted in a significant reduction in spines ($p < 0.01$). Spine density was restored in apoE4-expressing neurons following treatment with **3** (100 nM) without affecting apoE3-expressing cells (see ref. 67 for details). Modified from figure originally published in Brodbeck J *et al.* Structure-dependent impairment of intracellular apolipoprotein E4 trafficking and its detrimental effects are rescued by small-molecule structure correctors. *J. Biol. Chem.* 2011, 286:17217–17226. © the American Society for Biochemistry and Molecular Biology. (D) ApoE4-expressing Neuro-2a cells demonstrated a 50–60% decrease in cells displaying neurites longer than the cell body diameter compared with apoE3-expressing cells. **3** restored neurite outgrowth in apoE4-expressing cells (100 nM; $p < 0.01$) (see ref. 81 for details). (E) Active phthalazinones (**2**, **3**, and **1**) reverse the impaired neurite outgrowth seen in apoE4-expressing Neuro-2a cells in vitro (**2** and **3** at 3 nM; $p < 0.0005$) compared with no addition control cells, and **1** at 30 nM ($p < 0.02$) compared with no addition control cells (see ref. 81 for details). Modified from figure originally published in Chen H-K *et al.* Small molecule structure correctors abolish detrimental effects of apolipoprotein E4 in cultured

neurons. *J. Biol. Chem.* 2012, 287:5253–5266. © the American Society for Biochemistry and Molecular Biology.

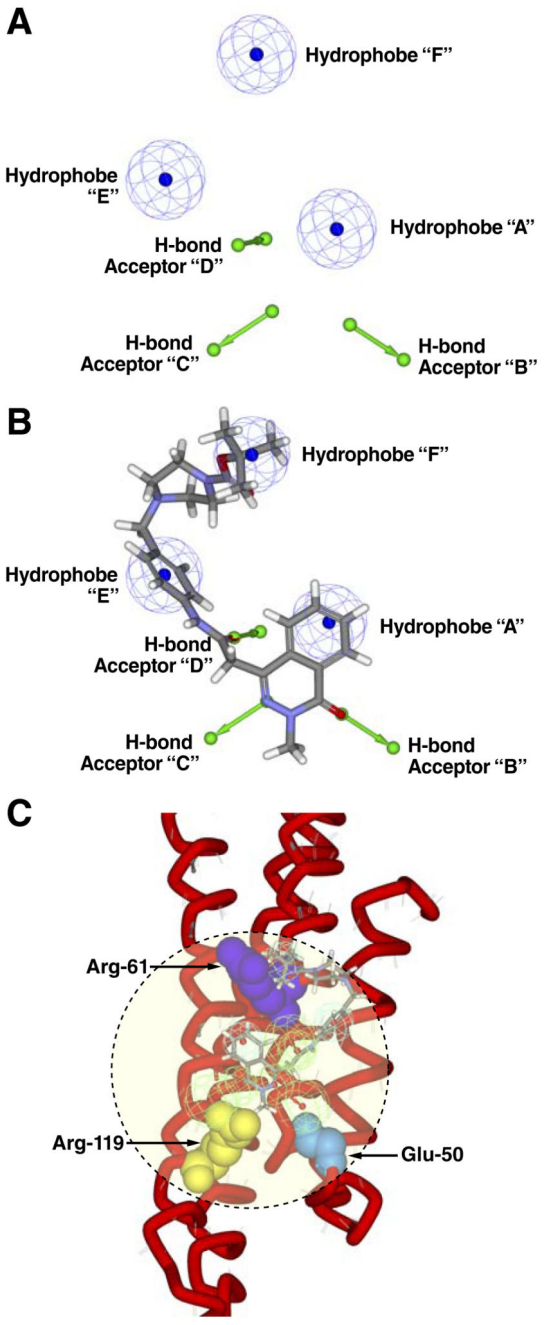

Figure 7.

(A) The six-featured pharmacophore model possesses 3 hydrophobic spheres (blue: "A," "E," and "F") and 3 hydrogen bond acceptors (green: "B," "C," and "D"). (B) The **3** phthalazinone is fitted to the six-featured pharmacophore model. (C) Predicted model of how the active **3** docks within the amino-terminal domain of apoE4. In this model, hydrophobe "F" interacts with Arg-61 (purple), hydrogen bond acceptor "B" interacts with Arg-119 (yellow), and hydrogen bond acceptor "C" interacts with Glu-50 (light blue). Modified from figure originally published in Chen H-K *et al.* Small molecule structure

correctors abolish detrimental effects of apolipoprotein E4 in cultured neurons. *J. Biol. Chem.* 2012, 287:5253–5266. © the American Society for Biochemistry and Molecular Biology.

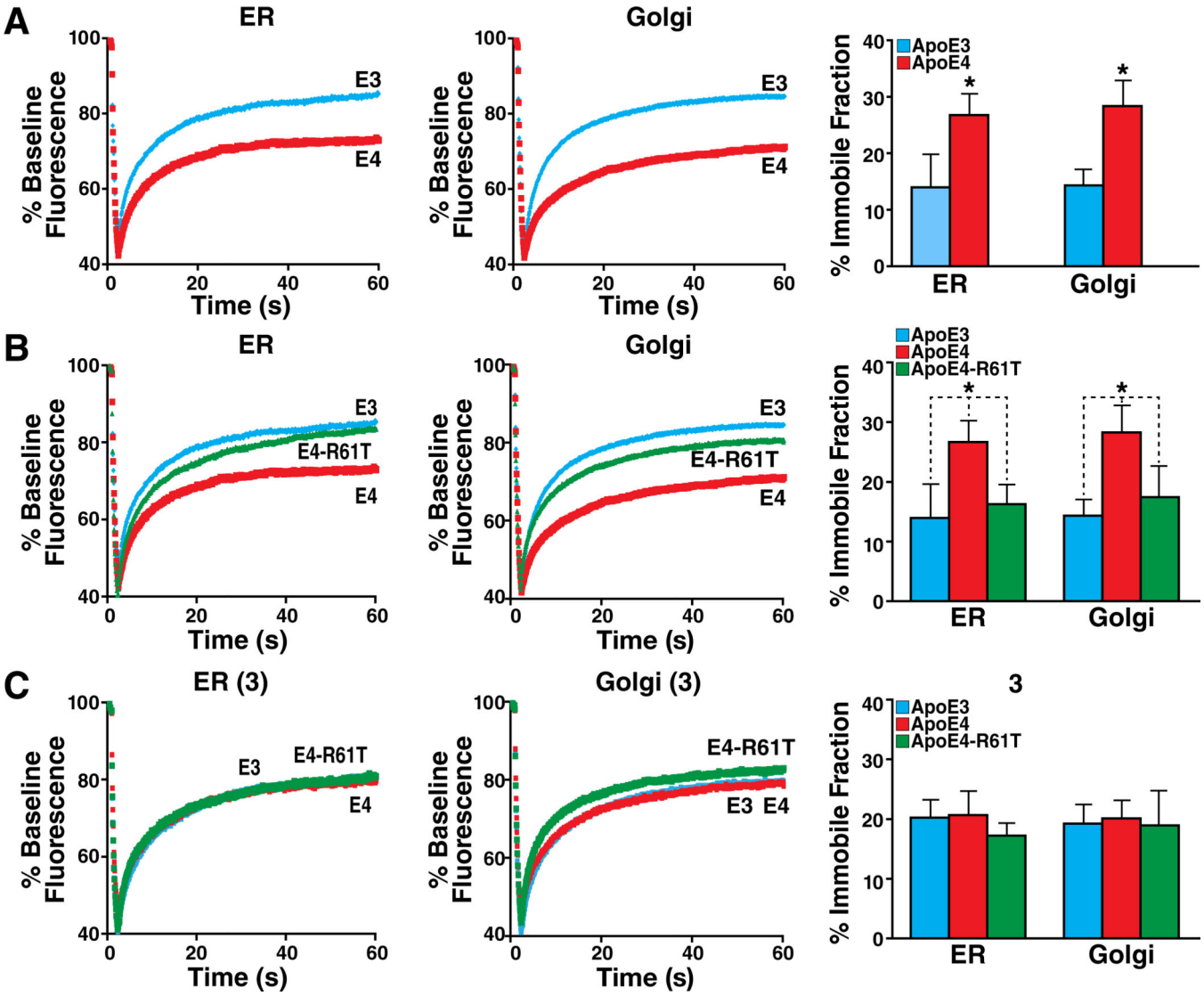

Figure 8.

ApoE4 impairs fluorescence recovery in the ER and Golgi apparatus in Neuro-2a cells as determined by fluorescence recovery after photobleaching. Cells expressing EGFP-apoE3 or EGFP-apoE4 were exposed to a laser to photobleach the fusion proteins in the ER or Golgi apparatus and then followed for recovery over a period of 60 s. (A) Fluorescence recovery is decreased in the EGFP-apoE4-expressing cells compared with EGFP-apoE3-expressing cells. The bar graph reports data as a percent of the immobile fraction, which reflects the impaired trafficking of apoE4 (mean ± SD; $p < 0.001$). (B) The impaired trafficking of apoE4 is reversed in the apoE4-R61T mutant, which lacks domain interaction. ApoE4-R61T behaves like apoE3 (mean ± SD; $p < 0.001$). (C) Treatment of apoE4-expressing cells with **3** (100 nM) restored the trafficking of apoE4 through the ER and Golgi apparatus. **3** does not affect the trafficking of apoE3 or apoE4-R61T (see ref. 67 for details). Modified from figure originally published in Brodbeck J *et al.* Structure-dependent impairment of intracellular apolipoprotein E4 trafficking and its detrimental effects are rescued by small-molecule

structure correctors. *J. Biol. Chem.* 2011, 286:17217–17226. © the American Society for Biochemistry and Molecular Biology.

Table 1

ApoE Allele Frequency in the General Population and in Patients with AD

Allelic frequency		
Control	**Sporadic AD**	**Familial AD**
ε2 = 8%	ε2 = 4%	ε2 = 2%
ε3 =78%	ε3 = 59%	ε3 = 48%
ε4 = 14%	ε4 = 37%	ε4 = 50%
ApoE4 carriers = 26%	ApoE4 carriers = 64%	ApoE4 carriers = 80%

ApoE4 carrier frequency derived from 5103 subjects.[42]