AF710B, a Novel M1/σ1 Agonist with Therapeutic Efficacy in Animal Models of Alzheimer’s Disease

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Key Words
M1 muscarinic acetylcholine receptor · σ1 receptor · Alzheimer’s disease · AF710B · AF267B · AF series · Cognitive deficits · Memory · Disease modification · Amyloids · Tau protein · Neuroinflammation

Abstract
We previously developed orthosteric M1 muscarinic agonists (e.g. AF102B, AF267B and AF292), which act as cognitive enhancers and potential disease modifiers. We now report on a novel compound, AF710B, a highly potent and selective allosteric M1 muscarinic and σ1 receptor agonist. AF710B exhibits an allosteric agonistic profile on the M1 muscarinic receptor; very low concentrations of AF710B significantly potentiated the binding and efficacy of carbachol on M1 receptors and their downstream effects (p-ERK1/2, p-CREB). AF710B (1–30 μg/kg, p.o.) was a potent and safe cognitive enhancer in rats treated with the M1 antagonist trihexyphenidyl (passive avoidance impairment). These effects of AF710B involve σ1 receptor activation. In agreement with its antiamnesic properties, AF710B (at 30 nM), via activation of M1 and a possible involvement of σ1 receptors, rescued mushroom synapse loss in PS1-KI and APP-KI neuronal cultures, while AF267B (1 μM) was less potent in PS1-KI and ineffective in APP-KI models, respectively. In female 3xTg-AD mice, AF710B (10 μg/kg, i.p./daily/2 months) (i) mitigated cognitive impairments in the Morris water maze; (ii) decreased BACE1, GSK3β activity, p25/CDK5, neuroinflammation, soluble and insoluble Aβ\textsubscript{40}, Aβ\textsubscript{42}, plaques and tau pathologies. AF710B differs from conventional σ1 and M1 muscarinic (orthosteric, allosteric or bitopic) agonists. These results highlight AF710B as a potential treatment for Alzheimer’s disease (e.g. improving cognitive deficits, synaptic loss, amyloid and tau pathologies, and neuroinflammation) with a superior profile over a plethora of other therapeutic strategies.

Introduction
Stimulation of the M1 muscarinic acetylcholine receptor (M1 mAChR) is an attractive therapeutic approach for Alzheimer’s disease (AD), as reviewed in Fisher [1, 2]. Accordingly, we recently generated compelling preclinical data, both pharmacologically and genetically, that activation of M1 mAChR restores cognition and attenuates...
AD-like pathology in several animal models [3, 4]. Mechanistically, it was found that M1 mAChR targets crucial enzymes involved in the synthesis of β-amyloid (Aβ) and phosphorylation of tau [1–4].

The σ1 receptor (σ1R) is another potential target for drug development for AD, as it is considered to play a fundamental role in cognitive function [5, 6]. Notably, decreased levels of σ1R have been detected in early-stage AD patients [7], and that stimulation of σ1R alleviates the depressive symptoms, neuronal cell death and cognitive deficits in AD mouse models [8, 9]. Moreover, the mixed muscarinic/σ1R agonist ANAVEX2-73 resulted in significant inhibition of Aβ25–35-induced tau phosphorylation and Aβ1–42 seeding in mice [10] and was recently reported to exert positive cognitive effects in phase 2a clinical studies with AD patients (http://www.anavex.com/?post_type=news&p=1491).

σ1R is an endoplasmic reticulum (ER)-resident protein and a molecular chaperone, yet its functional role remains a mystery [5, 6]. Under homeostatic conditions, σ1R is concentrated at mitochondrion-associated membrane junctions – sites of contact between ER and mitochondria – where it regulates InsP3R-mediated calcium signaling and ER lipids [11]; however, agonists or ER stress can cause σ1R to exit the mitochondrion-associated membrane domain [6], allowing it to regulate the function of plasma membrane ion channels [5] and G-protein-coupled receptors (GPCRs) [6, 8, 12, 13]. Among other brain regions, the σ1R is expressed in hippocampal neurons with preferential localization to plasmalemmal, outer mitochondrial and ER cisternal membranes within somata, dendrites and postsynaptic elements [14]. Ultra-structural analysis also shows colocalization of σ1R with mAChR in postsynaptic densities [15]. However, the mechanistic nature of σ1R interactions with ion channels and GPCRs including mAChRs is poorly understood.

Over the past several years, our groups have focused on understanding the underlying molecular mechanism of M1 mAChR involvement in AD, and on the development of innovative therapeutics to treat this insidious disease [1–4]. Thus, we have created selective M1 mAChR orthosteric agonists in the ‘AF series’ of compounds (e.g. AF102B, AF267B, AF292) that are cognitive enhancers and disease modifiers (reviewed in Fisher [1, 2]; fig. 1).

In a further expansion of our rational drug discovery programs, we report here on AF710B (an active enantiomer of AF710; MW 357.5), which is a novel and potent M1 mAChR/σ1R agonist (fig. 2).

We report here results of the preclinical evaluation of AF710B in cellular and animal models of AD. Notably we found that AF710B showed exceptional efficacy in restoring cognitive decline associated with AD and with lessening BACE1, glycogen synthase kinase 3β (GSK3β) activity, p25/cyclin-dependent kinase 5 (CDK5), neuroinflammation, soluble and insoluble Aβ40, Aβ42, accumulation of amyloid plaques and neurofibrillary tangles. As the stabilization of postsynaptic dendritic spines in hippocampal neurons is important for learning and memory, we evaluated the potential role of M1 mAChR and σ1R in the regulation of synaptic morphology. We recently found that mushroom spines are preferentially lost in PS1-M146V-knockin (KI) mouse [16] and APP-KI mouse [17] models of AD. As mushroom spines are considered stable ‘memory spines’, their loss may underlie cognitive decline in AD [16]. We therefore examined the effects of AF710B versus AF267B on hippocampal spine stability and tested the involvement of M1 mAChR and σ1R in the mechanism of action of AF710B. The results of this study indicate that AF710B, but not AF267B, efficiently rescues mushroom spines and normalizes other pathological features in AD models.
AF710B, a Novel M1/σ1 Agonist for Treatment of Alzheimer’s Disease

Methods

Compounds
1-(2,8-Dimethyl-1-thia-3,8-diazaspiro[4.5]dec-3-yl)-3-(1H-indol-3-yl)propan-1-one (AF710; US Patent, 8,673,931B2) was synthesized at the Israel Institute for Biological Research (IIBR) and was separated by Chiral Technology Europe into two enantiomers, AF710A and AF710B. AF710B (>99.5% chemical purity; >99.5% enantiomeric excess; specific rotation α = –56° (C = 0.303, methanol)) was provided by IIBR. All other compounds were of analytical grade and were purchased from commercial sources.

Receptogram Profile in vitro
AF710B (10 μM) was tested in a high-throughput profiling that consists of a broad collection of 83 trans-membrane proteins (including GPCRs) and soluble receptors, sigma receptors, ion channels and monoamine transporters (CEREP, France; http://www.cerep.fr/cerep/users/pages/catalog/profiles/DetailProfile.asp?profile=2118). AF710B was further tested in binding studies at a wide concentration range on M1 mAChR (IIBR; see below), σ1R, o2R or α3β2, α4-nicotinic receptors as well as in cellular functional assays on histamine 2, 5-HT2A, 5-HT2C, 5-HT2B and μ receptors, and at 5-HT2B receptors in an in vitro rat stomach fundus bioassay, respectively (CEREP, France).

Animals (Rats and Mice)
All procedures in which animals were used were in accordance with: (i) the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals (1996) and were approved by the IIBR Institutional Animal Care and Use Committee; (ii) the NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center, and (iii) the Principles of Laboratory Animal Care from NIH publication 85–23 and were approved by the University of California, Irvine, Institutional Animal Care and Use Committee.

Binding Studies to M1 mAChR in Rat Cortical Homogenates
Male Sprague-Dawley rats, 250–300 g, were sacrificed by decapitation, and brains were removed. The cerebral cortex was dissected out and placed on ice, cleaned, weighed and transferred to 20 mM Tris-HCl buffer, 2 mM EDTA, pH 7.4. The tissue was homogenized in the buffer (1:10 weight/volume) using a polytron homogenizer, and after a –70°C freeze/thaw cycle, the homogenates were centrifuged at 35,000 g at 4°C for 10 min. The supernatants were removed, and the pellets were resuspended in Tris buffer at a ratio of 1:6 (weight/volume). The homogenates were divided into aliquots of 1 ml each and then stored at –70°C till use. The binding profile in rat cortical membranes of AF710B, carbachol and the effects of AF710B on carbachol binding to M1 mAChR, respectively, were studied in triplicates for each concentration of the tested compounds in displacement studies of the M1 selective antagonist [3H]pirenzepine (86 Ci/mmol, Perkin-Elmer, Mass., USA). Each such study was replicated in 4–6 separate experiments.

Retraction of the tested compounds in displacement studies of the M1 selective antagonist [3H]pirenzepine (86 Ci/mmol, Perkin-Elmer, Mass., USA). Each such study was replicated in 4–6 separate experiments. Competition curves, Ki, Kd and K2 values were derived using the GraphPad Prism software program, version 5.0.

Phospho-p44/42 MAPK (ERK1/2) and Phospho-CREB Detection Assay
Rat pheochromocytoma cells (PC12) stably transfected with rat M1 mAChR (PC12M1 cells) were seeded in 6-well plates at a density of 2 x 10⁴ cells/well. The following day, cells were washed twice with RPMI and returned to the incubator with serum-free media (starvation medium containing RPMI, 2 mM glutamine, 1% penicillin-streptomycin, 2.5 μg/ml amphotericin B, 0.1 mg/ml G418). On the next day, cells were pretreated for 3 h with AF710B at concentrations ranging from 0.1 to 100 nM, and then carbachol (10 nM) was added for 10 min. In each plate, one well served as a control (no treatment) and one well as a positive reference in which the cells were treated with carbachol (10 nM). Following this procedure, cells were collected and extracted with RIPA buffer (Sigma, R-0278, 200 μL) containing a protease inhibitor cocktail (Sigma, 1:100) and phenylmethylsulfonyl fluoride (Sigma, 1:1,000). Phospho-p44/42 mitogen-activated protein kinase [MAPK; phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2); 1:1,000, Cell Signaling] and phosphorylated cAMP response element-binding protein (p-CREB, Ser133; 1:1,000, Cell Signaling) were probed with anti-rabbit antibodies.

Passive Avoidance Studies in Rats
Naive Wistar rats, 225–250 g (3 months old), were trained and tested in a two-compartment box: a small illuminated compartment (21 x 23 x 22 cm³) with a lamp (60 W) at a height of 25 cm above the compartment, and a dark, large compartment (37 x 23 x 22 cm³) equipped with a grid floor. A door (7 x 5 cm) separated the two compartments. The passive avoidance (PA) task is comprised of a training (acquisition) phase and a retention phase. In the training procedure, each rat was individually placed in the small illuminated compartment, and after 60 s of familiarization/adaptation, the door to the large compartment was opened and the latency to enter was measured (initial latency). Immediately following entry into the dark compartment, the door was closed and an inescapable foot shock (0.6 mA for 3 s) was delivered through the grid floor. A cutoff point of 180 s was used for initial latency. Animals that failed to enter the dark compartment with all four paws within 180 s were excluded from the experiment. After the acquisition trial, the rat was returned to its home cage. Retention of the PA task was measured 24 h later, by again placing the rat in the light compartment, and after a 60-second adaptation interval, the door was opened and the latency to re-enter the dark compartment was measured. A cutoff point of either 300 (experiments 1 and 2) or 600 s (experiment 3) was used for retention latency. Animals that failed to step through within 300 or 600 s were removed from the apparatus and a 300- or 600-second latency was recorded for them.

In experiment 1, we tested the effects of AF710B (100, 30 and 10 μg/kg, p.o.) and in experiment 2 lower doses (1 and 3 μg/kg, p.o.). In both experiments the rats were divided into 2 groups. One main group was treated with trihexyphenidyl (5 mg/kg, s.c.), and the second main group was treated with vehicle (double-distilled water, DDW; 1 ml/kg, s.c.), 30 min before the shock. In each main group, rats were further divided into treatment subgroups (n = 9–11): one subgroup was treated with saline (10 ml/kg, p.o.), and the other subgroups were treated with AF710B, 60 min before the shock. In experiment 3, rats were divided into 2 groups. One group was treated with trihexyphenidyl (5 mg/kg, s.c.), and the second group was treated with vehicle (DDW 1 ml/kg, s.c.), 30 min before...
the shock. In each group, rats were divided into treatment subgroups (n = 9–10): one subgroup was treated with saline (10 mL/kg, p.o.), and the other subgroups were treated with AF710B (10 μg/kg, p.o.), with or without the σ1R antagonist NE-100 (1 mg/kg, p.o.), 60 min before the shock.

Statistical Analysis
Initial latency and retention latency were analyzed by a two-way MANOVA. Specific comparisons were made by simple main effect contrast analysis.

Hippocampal Primary Neuronal Cultures and Spine Morphology Analysis
PS1-M146V knockin (PS1-KI) [18] and APP-KI [19] homozygous mouse AD models were used in our studies. Wild-type (WT) nontransgenic (nTg) mice of the same strain (B6 strain) were used as a control. Primary hippocampal cultures from WT, PS1-KI and APP-KI mice were established from postnatal day P0–P1 pups and maintained in culture as previously described [16, 17, 20]. At day 7 in vitro (DIV7), hippocampal cultures were transfected with a tdTomato plasmid using a high calcium phosphate method [16]. At DIV16, neuronal cultures were treated with placebo (NBA medium), AF710B (30 nM) and AF267B (1 μM) for 16 h. Following drug treatment, the cultures were fixed (4% formaldehyde and 4% succrose in PBS, pH 7.4) for imaging. Z stacks of optical sections were captured using a x100 objective with a confocal microscope (Carl Zeiss Axiovert 100M with LSM510). Quantitative analysis of dendritic spines was performed by using the NeuronStudio software package (as in Sun et al. [16]). In brief, following classification of spine shapes (thin, stubby, mushroom), the percentage of mushroom spines was calculated for each neurite. For each genotype and drug combination, 12–20 cultured neurons were analyzed from at least 2 independent neuronal preparations. The data were pooled and averaged.

σ1R knockdown was achieved by lentivirus-mediated shRNA expression in hippocampal neurons. Bacteria containing plasmids encoding MISSION® shRNA targeting mouse σ1R (clone ID: NM_011014.2–1138s21c1; sequence: CCGGCGCTGTAGTAACTCTTGTTGACCTGAGTTCACCAGAGATTACTACAGGT TTGT) or scrambled shRNA were ordered from Sigma-Aldrich (St. Louis, Mo., USA). Plasmids were purified using a Maxi-prep (Nucleobond Xtra kit; Macherey-Nagel). After replacing culture media (DMEM + 10% FBS) with NBA, plasmids for shRNA along with plasmids for Δ8.9 and VSVG for lentiviral production and functional analysis in the spine loss assay and SDS-PAGE Western blot analysis of lysates from neuron cultures – using σ1R (SC-137075; Santa Cruz) and β-tubulin (E7; DSHB) antibodies and the immunoblotting protocol below – confirmed efficient knockdown by hippocampal neurons expressing small interfering RNA (siRNA) against σ1R.

Studies in 3xTg-AD Mice
Female 3xTg-AD mice harboring a presenilin-1 mutation (PS1M146V), the Swedish double mutation in APP (APPKM670/671ML) and a frontotemporal dementia mutation in tau (tauP301L) were used for all experiments [21]. Strain-matched nTg mice, 129/C57BL/6, were used as controls. AF710B was administered via intraperitoneal injection at a dose of 10 μg/kg/day. Mice were treated for 2 months, from 10 months of age to 12 months of age. No changes in body weight were found between vehicle- and AF710B-treated mice.

Morris Water Maze
Behavior paradigms to assess cognition were performed for all groups of mice 2 weeks before sacrifice as described previously [4]. Mice were trained to swim to a circular clear Plexiglas platform submerged 1.5 cm beneath the surface. Four trials were performed per day, for 60 s each with 5 min between trials. Mice were trained for as many days as needed for the group to reach the training criterion of 25 s. The probe test was assessed 24 h after the last trial, with the platform removed. Performance was monitored with the EthoVision XT video-tracking system (Noldus Information Technology, Leesburg, Va., USA).

Tissue Preparation
Mice were deeply anesthetized with sodium pentobarbital and killed by transcardial perfusion with 0.1 M PBS (pH 7.4) solution. The right brain hemispheres were fixed for 48 h in 4% paraformaldehyde and cryoprotected in 30% sucrose for immunohistochemical analysis. Frozen brains were sectioned coronally into 40-μm sections using a Leica SM2010R freezing microtome, serially collected in cold 0.02% sodium azide and stored at 4°C. The left hemispheres were snap-frozen on dry ice and subjected to protein extraction sequentially using T-PER tissue protein extraction reagent (Thermo Scientific, Rockford, Ill., USA) and 70% formic acid. The supernatant was aliquoted and stored at −80°C. The protein concentration in the supernatant was determined using the Bradford assay.

Immunoblotting
Equal protein amounts were separated on a 4–12% gradient SDS-PAGE, transferred to a nitrocellulose membrane and incubated overnight at 4°C with primary antibody. The following primary antibodies were used in this study: anti-Aβ (1-16) (6E10; Covance Research Products, Denver, Pa., USA), p-GSK3β (Ser9), human APP-CT20, ADAM10, ADAM17, BACE1, GSK3β, CDK5 (Calbiochem, San Diego, Calif., USA), human tau (HT7), phosphorylated tau (p-tau) AT180 (phospho-Thr231), p-tau AT270 (phospho-Thr181; Thermo Scientific), PP2A, p35, GAPDH (Sigma-Aldrich) and p-tau PHF-1 (phospho-S396/S404; Dr. Peter Davies, Albert Einstein College of Medicine, Manhasset, N.Y., USA). Following washing, the membranes were incubated with adjusted secondary antibodies coupled to horseradish peroxidase. The immunocomplexes were visualized using the SuperSignal West Pico Kit (Thermo Scientific). Band density measurements were made using ImageJ 1.36b imaging software (NIH, Bethesda, Md., USA).

Enzyme-Linked Immunosorobent Assay
For the determination of the Aβ levels, T-PER soluble fractions were loaded directly onto ELISA plates, whereas the formic acid supernatants (insoluble fractions) were diluted 1:20 in a neutralization buffer (1 M Tris base, 0.5 M NaH2PO4) before loading. MaxiSorp immunoplates (Nunc, Rochester, N.Y., USA) were coated with mAb20.1 antibody (Dr. William E. Van Nostrand, Stony Brook University, Stony Brook, N.Y., USA) at a concentration of...
25 μg/ml in coating buffer (0.1 M Na2CO3, pH 9.6) and blocked with 3% bovine serum albumin. Standard solutions for both Aβ40 and Aβ42 were made in the antigen capture buffer (20 mM NaH2PO4, 2 mM EDTA, 0.4 M NaCl, 0.05% 3-(3-cholamidopropyl)dimethylammonio)propane sulfonate, and 1% bovine serum albumin, pH 7.0) and loaded onto ELISA plates in duplicate. Samples were then loaded (also in duplicate) and incubated overnight at 4°C. Plates were then washed and probed with either horse-radish peroxidase-conjugated anti-Aβ40 (C99) or anti-Aβ42 (D32; Dr. Vitaly Vasilievko and Dr. David H. Cribbs, University of California, Irvine, Calif., USA) overnight at 4°C. The chromogen was 3,3′,5,5′-tetramethylbenzidine, and the reaction was stopped by 30% phosphoric acid. The plates were read at 450 nm using a plate reader (Molecular Dynamics, Sunnyvale, Calif., USA). The readings were then normalized to protein concentrations of the samples.

**Immunofluorescence**

Free-floating sections were pretreated with 3% hydrogen peroxide and 10% methanol in Tris-buffered saline (TBS) for 30 min to block endogenous peroxidase activity. After a TBS wash, sections were incubated once in 0.1% Triton X-100 in TBS for 15 min and once with 2% bovine serum albumin in TBS + Triton X-100 for 30 min. Sections were then incubated overnight at 4°C with p-tau AT8 (p-Ser212/Thr214, Thermo Scientific) with 5% normal serum in TBS. After the appropriate biotinylated secondary antibody (1:200 in TBS + 2% bovine serum albumin + 5% normal serum), sections were processed using the Vectastain Elite ABC reagent and 3,3′-diaminobenzidine (Vector Laboratories, Burlingame, Calif., USA) according to the manufacturer’s instructions. Sections were then mounted on gelatin-coated slides, dehydrated in graded ethanol, cleared in xylene and coverslipped with DPX mounting medium (BDH Laboratory Supplies, Poole, UK).

The immunostaining was assessed at 6 brain coronal levels. Specifically, 6 alternate 40-μm sections of the brain with an individual distance of ~160 μm were obtained between 1.34 and 2.54 mm posterior to the bregma. Images of stained hippocampal area were acquired using an AxioCam digital camera and AxioVision 4.6 software connected to an Axioskop 50 microscope (Carl Zeiss Microimaging, Thornwood, N.Y., USA). A threshold optical density that best discriminated staining from the background was obtained using the ImageJ 1.36b imaging software (NIH). All histological assessments were made by an examiner blinded to sample identities.

**Immunofluorescence**

Sections were first blocked with 3% normal serum, 2% bovine serum albumin and 0.1% Triton X-100 in TBS for 1 h at room temperature. Using the same buffer solution, sections were incubated overnight at 4°C with the following primary antibodies: anti-Aβ1-16 (6E10; Covance Research Products), anti-Iba-1, anti-GFAP (Millipore, Billerica, Mass., USA), HT7 and/or p-tau AT8 (p-Ser212/Thr214; Thermo Scientific). Sections were then rinsed and incubated for 1 h with secondary Alexa Fluor-conjugated antibodies (Invitrogen, Carlsbad, Calif., USA) at room temperature. Finally, sections were mounted onto gelatin-coated slides in Fluoro-gel (Invitrogen, Carlsbad, Calif., USA) at room temperature. Using the same buffer solution, sections were incubated at 4°C. Plates were then washed and probed with either horseradish peroxidase-conjugated anti-Aβ40 (C99) or anti-Aβ42 (D32; Dr. Vitaly Vasilievko and Dr. David H. Cribbs, University of California, Irvine, Calif., USA) overnight at 4°C. The chromogen was 3,3′,5,5′-tetramethylbenzidine, and the reaction was stopped by 30% phosphoric acid. The plates were read at 450 nm using a plate reader (Molecular Dynamics, Sunnyvale, Calif., USA). The readings were then normalized to protein concentrations of the samples.

**Thioflavin S Staining**

Sections were incubated in 0.5% thioflavin S in 50% ethanol for 10 min, differentiated twice in 50% ethanol, and washed in PBS solution. Staining was visualized under a confocal microscope. Volumetric imaging and image measurements were made using Imaris software (Bitplane Inc., South Windsor, Conn., USA). Thioflavin S levels represent the average value obtained by the analysis of images of the hippocampus.

**Statistical Analysis**

All data are expressed as means ± SEM. The statistical evaluation of the results was carried out using one- or two-way analysis of variance (ANOVA). Following significant ANOVAs, multiple post hoc comparisons were performed using Bonferroni’s test. Some data were analyzed using the unpaired t test. The accepted level of significance for the tests was p < 0.05. All tests were performed using the StatSoft software package (StatSoft Inc., Tulsa, Okla., USA).

**Results**

**AF710B Shows High Potency and Selectivity to M1 mAChR and σ1R**

In high-throughput receptogram profiling, AF710B emerged as a potent ligand for M1 mAChR and σ1R, respectively, as shown by displacement of an M1 muscarinic antagonist (tritiated pirenzepine, an M1 mAChR radioligand) from rat cerebral cortex and of a σ1R agonist (tritiated pentazocine, a σ1R radioligand) from guinea pig cerebral cortex with a 6- and 2-fold order of magnitude interval between two binding sites (K_H vs. K_L), respectively (fig. 3a). Furthermore, AF710B showed both high potency and selectivity for the M1 mAChR and σ1R and very clean additional pharmacological profiles with regard to off-target activity (fig. 3a). Thus, AF710B was found to display no significant off-target activity when screened at 10 μM in a radioligand displacement screen at 83 other GPCRs, ion channels and transporters known to mediate human side effects (fig. 3a). AF710B (10 nM to 100 μM) was inactive as an agonist or an antagonist on histamine 2 and 5-HT2A and 5-HT2C receptors while on the 5-HT2B receptor it is a very weak partial agonist (17% efficacy) at 100 μM and a very weak agonist at the mu receptor, with EC50 = 13 μM. Additionally, AF710B (0.1 nM to 10 μM) did not bind to human α,β2- and α2-nicotinic receptors and to σ2R, while it showed agonistic effects on M1 mAChR, but not on M2–M5 mAChR (table 1). In addition, AF710B application altered the neurochemical profile to a state typi-
AF710B binds with high affinity to M1 mAChR showing an allosteric agonistic profile. AF710B binds with high affinity to σ1R and does not bind to σ2R (IC50 > 10 μM).

The high selectivity of AF710B for M1 mAChR and σ1R was shown in a high-throughput profiling that consists of a broad collection of 83 transmembrane (including GPCRs) and soluble receptors, channels and monoamine transporters (additional binding at 10 μM to: M2 mAChR; 5HT2A, 5HT2B, 5HT2C receptors; histamine 2 receptor; 5HT transporter. Inactive as agonist or antagonist at 10 μM on histamine 2 and 5HT2A and 5HT2C receptors and a weak agonist at μ receptor (EC50 = 13 μM); no effect on α4β2 and α7 nicotinic receptors, respectively).

<table>
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<th>Efficacy index (K_i/K_i)</th>
<th>K_i μM</th>
<th>K_h nM</th>
<th>n</th>
<th>Compound</th>
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<tr>
<td>110</td>
<td>7.7</td>
<td>70 (37%)</td>
<td>6</td>
<td>Carbachol</td>
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<tr>
<td>1.650</td>
<td>1.65</td>
<td>1 (27%)</td>
<td>6</td>
<td>Carbachol and AF710B, 0.1 nM</td>
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(For legend see next page.)
fied by downstream signaling of M1 mAChR and/or σ1R. This was detected at concentrations in the nanomolar range ∼3–4 orders of magnitude lower than effects on other screened GPCRs, ion channels and transporters mentioned above (detailed data not shown; table 1).

To characterize the interaction of AF710B with the M1 mAChR, we employed a combination of binding and functional assays. This was done first utilizing brucine, an M1-positive allosteric modulator (M1 PAM) that potentiates M1 orthosteric, but not M1 allosteric agonists [22]. Notably while brucine showed a modest cooperativity on M1 mAChR with the orthosteric agonists carbachol and AF267B, it failed to potentiate AF710B (tested in binding and calcium mobilization assays) and TBPB (another M1 allosteric agonist [23]; detailed data not shown; table 1). Thus, AF710B binds to M1 mAChR on a location different from the orthosteric site, supporting an allosteric mechanism of action of AF710B.

To further characterize the M1 allosteric profile of AF710B, we tested its effects on carbachol. AF710B (0.1 nM) potentiated carbachol binding to M1 mAChR in rat cortical membranes as evidenced by a decrease by approximately 2 orders of magnitude of the K_H for carbachol. The ratio of K_L/K_H for a given agonist directly correlates with its efficacy. In this particular case, the ratio of K_L/K_H for carbachol in the presence of 0.1 nM AF710B increased from 110 to 1,650 (fig. 3b). Thus, both the affinity and efficacy of carbachol are potentiated by AF710B.

Both M1 orthosteric and allosteric agonists increase the phosphorylation of ERK1/2 [24, 25]. The activated ERK/MAPK cascade has multiple targets, including CREB, which mediates its ability to induce memory consolidation and long-term memory formation [26, 27]. In this context we found that AF710B did not increase p-ERK1/2, yet it potentiated ERK1/2 phosphorylation induced by 10 nM carbachol (fig. 3c). In addition, AF710B increased carbachol-induced p-CREB in starved proliferated PC12M1 cells (fig. 3d).

Taken together this indicates that AF710B is functioning like an M1 PAM mAChR. However, unlike reported M1 PAMs [23], AF710B binds to M1 mAChR with a high affinity and is also a direct M1 allosteric agonist, but different in several aspects from other reported M1 allosteric agonists (see below and table 1).

AF710B Reverses the Cognitive Decline Induced by the M1 mAChR Antagonist Trihexyphenidyl via Activation of Both M1 mAChR and σ1R
To determine whether AF710B is a cognitive enhancer, we tested its effects against cognitive impairments in a PA test induced by trihexyphenidyl in rats. Trihexyphenidyl induces cognitive deficits in rodents and humans and is a CNS-penetrating and a relatively selective M1 antagonist [28].

In general, no significant difference was found in initial latency between any of the groups tested in experiments 1–3 (data not shown). In experiment 1, a significant interaction was found between trihexyphenidyl and treatments (F_2, 63 = 4.0, p < 0.023). Retention latency of trihexyphenidyl rats treated with DDW (54.2 ± 14.6 s) was significantly shorter than that of control rats treated with DDW (242.00 ± 30.60 s; p < 0.001). However, the
Table 1. AF710B: a compilation of neurochemical, behavioral and toxicological studies (see also fig. 3–5)

<table>
<thead>
<tr>
<th>Effects of AF710B</th>
<th>Results</th>
<th>Comments</th>
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<tr>
<td>Receptogram</td>
<td>See figure 3a</td>
<td>Highly potent and selective for M1 mAChR and σ1R; several effects mediated by M1 mAChR and σ1R were detected at concentrations in the nanomolar range which are lower by 3–4 orders of magnitude versus interactions with any of the other tested screens of GPCRs, ion channels and transporters (fig. 3a); the M1 PAM brucine potentiated the binding of orthosteric agonists (carbachol and AF267B) but not of AF710B (not shown); thus, AF710B is an M1 allosteric agonist</td>
</tr>
<tr>
<td>Selectivity for M1–M5 mAChR in CHO cells; [Ca2+]i readout</td>
<td>AF710B is a partial agonist at 100 μM, inactive at 1 μM; selective for M1 mAChR with no agonistic activity on M2–M5 mAChRs; enantioselectivity: AF710A is an M1 muscarinic antagonist (not shown)</td>
<td>The M1 PAM brucine potentiated the effects of the orthosteric agonists carbachol and AF267B but not the effects of AF710B and TBPB (an M1 allosteric agonist; not shown); thus, AF710B is an M1 allosteric agonist</td>
</tr>
<tr>
<td>Potentiation of effects of carbachol on M1 mAChR</td>
<td>Potentiates carbachol binding and efficacy (fig. 3b) and carbachol-mediated increase in p-ERK and p-CREB (fig. 3c, d)</td>
<td>Thus, AF710B is an M1 PAM; notably, AF710B differs from other M1 PAMs as it does not potentiate the carbachol-induced elevation of [Ca2+]i in CHO M1 cells (not shown)</td>
</tr>
<tr>
<td>Tau phosphorylation</td>
<td>Decreased tau phosphorylation in NGF-deprived and AP125-35-induced tau phosphorylation; effective at 100 μM to 1 nM</td>
<td>M1 mAChR mediated (e.g. blocked by atropine; detected in PC12M1 cells but not in PC12 cells devoid of M1 mAChR; not shown)</td>
</tr>
<tr>
<td>GSK3β activity</td>
<td>Increased inactive form of GSK3β in NGF-deprived and AP125-35-induced decrease in inactive form of GSK3β; effective at 100 μM to 1 nM</td>
<td>M1 mAChR mediated (e.g. blocked by atropine; detected in PC12M1 cells but not in PC12 cells devoid of M1 mAChR; not shown)</td>
</tr>
<tr>
<td>Neuroprotection; Bcl-2, Bax readouts</td>
<td>Neuroprotective against insults such as AP125-35-induced neurotoxicity; NGF withdrawal, effective at 1 μM to 1 nM</td>
<td>These effects of AF710B are not modulated by the M1 mAChR as can be observed both in PC12M1 and PC12 cells; AF710B-induced effects on Bax are mediated by σ1R (e.g. blocked by NE-100; not shown)</td>
</tr>
<tr>
<td>Mushroom spine stability</td>
<td>AF710B rescued mushroom spines in hippocampal neuron cultures from PS1-KI and APP-KI mice; this was prevented by the M1 mAChR antagonist pirenzepine, but not the σ1R antagonist NE-100; σ1R knockdown led to mushroom spine loss in WT hippocampal neurons; AF710B was unable to rescue this; AF267B also partially prevented mushroom spine loss in PS1-KI, but was ineffective in APP-KI cultures (fig. 4a–f)</td>
<td>This indicates that the presence of σ1R and function of M1 mAChR are required for hippocampal neuron mushroom spine stability, suggesting that AF710B may target an M1 mAChR/σ1R complex; rescue of mushroom spines by AF710B may explain its ability to improve cognitive performance in spatial memory tasks; the less effective rescue of mushroom spines by AF267B suggests that targeting M1 mAChR function alone may not be sufficient to reverse some of the AD pathologies in AD</td>
</tr>
<tr>
<td>Trihexyphenidyl-induced cognitive impairment in PA test in rats</td>
<td>Significantly effective at 1–30 μg/kg, p.o. (fig. 3e); the effects of AF710B are mediated at least in part by σ1R (e.g. blocked by NE-100; fig. 3f)</td>
<td>AF710B is the most potent cognitive enhancer (e.g. donepezil, AF267B, AF292, AF102B, AF710A; see table 2); wide safety margin (≥50,000); enantioselectivity: AF710A (10 and 1 μg/kg, p.o.) is inactive (not shown)</td>
</tr>
<tr>
<td>3xTg-AD mice</td>
<td>Figure 3</td>
<td>AF710B was highly potent in restoring cognitive decline associated with AD and with lessening BACE1, GSK3β activity, p25/CDK5, neuroinflammation, soluble and insoluble AP40, AP42, accumulation of amyloid plaques and neurofibrillary tangles; no adverse effects were observed</td>
</tr>
<tr>
<td>Acute toxicity (rats)</td>
<td>No toxic or side effects (highest tested dose, 50 mg/kg, p.o.); no gross pathological changes at necropsy</td>
<td>Maximum tolerated dose &gt;50 mg/kg, p.o. (not shown; male and female Sprague-DawleyTM rats, GLP study Harlan, Israel)</td>
</tr>
</tbody>
</table>

Receptogram See figure 3a
Potentiation of effects of carbachol on M1 mAChR
EF710B changes the effects of carbachol on M1 mAChR, demonstrating that it is M1-selective. It is also a partial agonist, as shown in the figure.

Neuroprotection; Bcl-2, Bax readouts
A new molecule, AF710B, was shown to be neuroprotective against insults such as AP125-35-induced neurotoxicity; NGF withdrawal, effective at 1 μM to 1 nM.

Mushroom spine stability
AF710B rescued mushroom spines in hippocampal neuron cultures from PS1-KI and APP-KI mice, indicating that the molecule is effective in a mouse model of AD.

Trihexyphenidyl-induced cognitive impairment in PA test in rats
AF710B was shown to be effective at 1–30 μg/kg, p.o. (fig. 3e); the effects of AF710B are mediated at least in part by σ1R (e.g. blocked by NE-100; fig. 3f).

3xTg-AD mice
Figure 3
AF710B was highly potent in restoring cognitive decline associated with AD and with lessening BACE1, GSK3β activity, p25/CDK5, neuroinflammation, soluble and insoluble AP40, AP42, accumulation of amyloid plaques and neurofibrillary tangles; no adverse effects were observed.

Discussion
Retention latency of trihexyphenidyl rats treated with AF710B at 10 and 30 μg/kg, p.o., was significantly longer than that of trihexyphenidyl rats treated with DDW (p < 0.001–0.01). The retention latency of trihexyphenidyl rats treated with AF710B at 100 μg/kg, p.o., was not different from that of trihexyphenidyl rats treated with DDW. AF710B at 10 μg/kg was very potent and significantly different from the higher dosage of 100 μg/kg, p.o. (p < 0.01). In experiment 2, a significant interaction was found between trihexyphenidyl and treatments (F2, 52 = 5.2, p < 0.009). The retention latency of trihexyphenidyl rats treated with DDW (63.9 ± 18.8 s) was significantly shorter than that of control rats treated with DDW (274.7 ± 20.8 s; p < 0.001). However, the retention latencies of trihexyphenidyl rats treated with AF710B (1 and 3 μg/kg, p.o.) were significantly longer than that of trihexyphenidyl rats treated with DDW (p < 0.001). The anti-amnesic effects of AF710B, 1 and 10 μg/kg, p.o., were replicated 2 times.
and 4 times, respectively, and the tested compound showed a long duration of action at these doses, since the retention time was significantly different versus DDW-treated trihexyphenidyl rats, both 24 and 72 h after treatment (detailed data not shown).

As AF710B binds to both M1 mAChR and σ1R, we explored whether its antiamnesic effects are blocked by NE-100, a selective σ1R antagonist [29]. In experiment 3, we found that the retention latency of trihexyphenidyl rats treated with DDW (122.0 ± 29.2 s) was significantly shorter than that of control rats treated with DDW (525.7 ± 51.0 s; p < 0.001). However, the retention latency of trihexyphenidyl rats treated with AF710B at 10 μg/kg, p.o., was significantly longer (361.7 ± 67.1 s), and NE-100 co-administered with AF710B blocked significantly the antiamnesic effect of AF710B in this model (185.4 ± 44.0 s; p < 0.02; fig. 3f). Thus, the effects of AF710B appear to be mediated at least in part by the σ1R. Notably, NE-100 was not significantly effective in inducing deficits in the PA task and also failed to affect the retention parameters of the trihexyphenidyl rats treated with AF710B (10 μg/kg, p.o., at 10 μg/kg, p.o., was significantly longer (361.7 ± 67.1 s), and NE-100 co-administered with AF710B blocked significantly the antiamnesic effect of AF710B in this model (185.4 ± 44.0 s; p < 0.02; fig. 3f). These results suggest that AF710B exerts its cognitive effects in the trihexyphenidyl model via a combined activation of both M1 mAChR and σ1R.

**AF710B Protects Synapses via Activation of M1 mAChR/σ1R**

Synaptic loss is a main feature of many neurodegenerative disorders, including AD [31]. In previous studies we determined that mushroom synaptic spines are lost in primary hippocampal neuronal cultures from PS1-KI and APP-KI mice [16, 17]. We confirmed these findings in the present experiments (fig. 4a–d). Application of AF267B (1 μM, an effective concentration on M1 mAChR-mediated readouts [2]) for 16 h starting on DIV16 partially rescued mushroom spines in PS1-KI cultures, but not in APP-KI cultures (fig. 4a, b), indicating potential beneficial effects, but only for certain forms of AD. AF267B partially reduced the prevalence of WT mushroom spines, which could be related to a slight toxic effect or depression of synaptic strength. By contrast, 30 nM AF710B did not change the proportion of mushroom spines in hippocampal cultures from nTg animals when compared with vehicle (fig. 4a, 3b). Notably, AF710B markedly rescued mushroom spine loss in both AD transgenic mouse models, nearly to the level of nTg animals (fig. 4a, b), indicating that this compound exerts a disease-modifying effect on AD neurons.

To explore the underlying molecular mechanism responsible for AF710B’s protective effect, similar studies with primary hippocampal cultures from APP-KI mice were performed in the presence of the M1 mAChR antagonist pirenzepine (1 μM) and/or the σ1R antagonist NE-100 (1 μM; fig. 4c). While the effect of AF710B on spine morphology was not affected by the blockage of σ1R, it was completely abolished in the presence of the M1 mAChR antagonist (fig. 4c, d). In addition, the combination of pirenzepine and NE-100 did not show any additive or synergistic effects in the presence of AF710B (fig. 4c, d). These results suggest that AF710B prevents mushroom spine loss in hippocampal neuron cultures from APP-KI mice by stimulating M1 mAChR activity.

We further examined the possible involvement of σ1R in mushroom spine stability in WT mouse hippocampal neuronal cultures by using lentivirus-mediated shRNA interference delivery. σ1R knockdown decreased the mushroom spine density from 38.4 ± 1.1% in hippocampal neurons transfected with scrambled siRNA to 22.1 ± 1%, which is significantly lower (p < 0.0001; fig. 4e, f). As before, treatment of cultures with 30 nM AF710B for 16 h had no effect on the mushroom spine density in control siRNA-expressing neurons (fig. 4e, f). However, AF710B treatment failed to rescue mushroom spine loss in hippocampal neurons expressing siRNA against σ1R (fig. 4e, f), indicating that the ability of AF710B to stabilize hippocampal mushroom spines requires not only M1 mAChR function, but also σ1R expression. To explain these results, we propose that AF710B may act by stimulating an M1 mAChR/σ1R complex in the synaptic spines. Further biochemical experiments will be needed to test this hypothesis.

**AF710B Mitigates Cognitive Impairments in the 3xTg-AD Mouse Model**

Given the marked effects induced by AF710B in vitro and in vivo, we sought to further investigate its effects on the cognitive decline induced by AD using the 3xTg-AD mouse model. As previously observed, 12-month-old 3xTg-AD mice performed significantly worse relative to age-matched nTg mice in the reference spatial memory version of the Morris water maze. Vehicle-treated 3xTg-AD mice exhibited longer latencies to find the platform in the training session (fig. 5a) and reduced target quadrant preference during the probe trial (fig. 5b) compared with age-matched nTg mice treated with vehicle. Notably, treatment with AF710B (10 μg/kg/day, i.p., for 2 months) improved the cognitive function of 3xTg-AD mice.
mice during the training and probe sessions of the Morris water maze (fig. 5a, b). Importantly, the improvement in the cognitive performance in the AF710B-treated 3xTg-AD mice is not directly related to changes in motor function, since no significant alterations of the swimming speed and total distance traveled in the water maze were observed when compared with vehicle-treated nTg and 3xTg-AD mice (data not shown).

**AF710B Lessens AD-Like Pathology in the 3xTg-AD Mouse Model**

Accumulation of Aβ and tau pathology coincides with the synaptic loss and cognitive decline in the 3xTg-AD mice. Therefore, we next examined for changes in Aβ pathology. We found that AF710B treatment significantly reduced levels of Aβ40 and Aβ42, in both detergent soluble and insoluble fractions (fig. 5c). Moreover, we found significantly less amyloid deposition in the AF710B-treated...
animals versus vehicle-treated animals, as indicated by the significant decrease in thioflavin S-positive plaques (fig. 5d). To elucidate the mechanism by which AF710B reduces Aβ levels, we determined whether APP-processing pathways were modified (fig. 5e). Steady-state levels of APP and α-APP-cleaving enzymes ADAM10 and ADAM17 were unaffected by AF710B treatment. Conversely, AF710B significantly diminished the expression of the putative β-secretase enzyme BACE1. Given the decline in BACE1 levels, we used the antibody 6E10 to detect full-length APP and its proteolytic fragments CTFβ [99-amino-acid-long C-terminal APP fragment produced by β-secretase cleavage (C99)] and CTFα [83-amino-acid-long C-terminal APP fragment produced by α-secretase cleavage (C83)]. As expected, the levels of C99, but not C83, were significantly diminished in the AF710B-treated 3xTg-AD mice versus vehicle-treated 3xTg-AD mice.

To examine the effect of AF710B administration on tau pathology, we first tested for changes in total p-tau levels using the anti-AT100 antibody. Notably, p-tau was found reduced in AF710B-treated 3xTg-AD mice versus vehicle-treated 3xTg-AD mice (fig. 5f). This data was further confirmed through confocal studies, which showed a reduction in AT8-positive neurons in AF710B-treated 3xTg-AD mice (fig. 5g). Biochemical analysis of p-tau revealed significant decreases in the p-tau epitopes AT100, AT270 and PHF-1 (fig. 5h). To determine if the reductions in p-tau levels were due to modulations in tau kinase function, we measured for changes in several kinases involved in tau phosphorylation. We found that AF710B administration significantly increased phosphorylation in GSK3β, at the inhibitory Ser9 residue (fig. 5h). This was also associated with an effect on p35 cleavage, since the brains of AF710B-treated 3xTg-AD mice presented diminished levels of p25 fragment. However, AF710B did not affect the steady-state levels of CDK5, GSK3β or PP2A. Overall, our data indicates that AF710B modulates tau pathology via the modulation of GSK3β and CDK5 activities.

Inflammation is another critical component of AD, and it is associated with increased numbers and/or size of microglia and astrocytes. We found significantly fewer reactive astrocytes and activated microglia in AF710B-treated animals, as indicated by the lower detection of GFAP and Iba-1, respectively. This anti-inflammatory effect was most prominent in the immediate vicinity of plaques. The immunofluorescence studies indicated lower amyloid load and glial reactivity (fig. 5i).

Discussion

We report here on AF710B, which is a novel and highly potent mixed M1 mAChR/σ1R agonist with a unique mechanism of action leading to a concomitant activation of both M1 mAChR and σ1R. Notably, AF710B showed both high potency and selectivity for the M1 mAChR and σ1R and very clean additional pharmacological profiles with regard to off-target activity (fig. 3a; table 1). AF710B exhibits an allosteric agonistic profile on M1 mAChR since very low concentrations of AF710B significantly potentiated the binding and efficacy of carbamol on M1 mAChR and some of the carbamol/M1-mediated downstream effects (e.g. ERK1/2 and CREB phosphorylation; fig. 3b–d). Furthermore, AF710B showed exceptional efficacy in vitro on downstream effects mediated by M1 mAChR and σ1R, including the ability to preserve synaptic elements (fig. 4), and in vivo in restoring cognitive deficits in animal models (trihexyphenidyl-treated rats and 3xTg-AD mice) and with lessening major hallmarks associated with AD such as BACE1, GSK3β activity, p25/CDK5, neuroinflammation, soluble and insoluble Aβ40, Aβ42, accumulation of amyloid plaques and neurofibrillary tangles (in 3xTg-AD mice; fig. 3, 5; table 1).

While pK studies have not yet been performed on AF710B, its exceptional brain penetration and target engagement can be implied from the very high potency of the compound in in vivo studies. Notably, AF710B functions as a cognitive enhancer in the PA test involving trihexyphenidyl-treated rats at unprecedented low doses of 1–30 μg/kg, p.o. (fig. 3e). The effects of AF710B on cognition are enantioselective since the enantiomer AF710A is not active at 1 and 10 μg/kg, p.o. (table 1, 2a). Strong enantioselectivity of AF710B versus AF710A was consistently observed in vitro and in vivo (table 1). How does AF710B pharmacodynamics overcome existing compounds? We found that AF710B is the most potent cognitive enhancer and with the highest safety margin versus a variety of compounds tested by us in the trihexyphenidyl model under the same experimental protocols as tested for AF710B, including, inter alia, AF710A, AF102B, AF267B, AF292 and donepezil (table 2a).

To explore the mechanism of action in vivo of AF710B in ameliorating PA impairments induced by trihexyphenidyl, we used NE-100, a selective σ1R antagonist [29]. We found that NE-100 coadministered with AF710B significantly blocked the antiamnesic effect of AF710B in this model (fig. 3f). Thus, the effects of AF710B appear to be mediated in part by the σ1R. However, NE-100 was not
significantly effective in inducing deficits in the PA task and also failed to affect the retention parameters of AF710B alone (fig. 3f). As trihexyphenidyl binds to M1 mAChR > σ1R [30] and its amnesic effects may be attributed mainly to its M1 antagonistic effects, it can be implied that AF710B exerts its cognitive effects in the trihexyphenidyl model via a unique concomitant activation of both M1 mAChR and σ1R.

Can such effects of AF710B be recapitulated with a mixture of separate agents acting on M1 mAChR and σ1R, respectively? We tested this in the trihexyphenidyl model via a combined treatment of the M1 orthosteric agonist (AF267B) with donepezil. Notably the acetylcholinesterase inhibitor donepezil, the current major treatment in AD patients, is also a potent σ1R agonist, and its effects may arise from cholinergic mechanisms, as well as
an interaction with the σ1R [32]. Importantly, we found that the effects of AF710B could not be recapitulated by the combination of AF267B + donepezil as no significant potentiation was detected and in fact the beneficial effects of the M1 agonist were unchanged by donepezil (table 2a). Thus, it can be deduced that the mechanism of action of AF710B differs from a combination of a directly acting M1 orthosteric agonist and σ1R agonist.

As AF710B potentiated the effects of carbachol on 3 tested readouts (e.g. binding, p-ERK1/2, p-CREB; fig. 3b-d), it is possible that the affinity and efficacy of the endogenous agonist acetylcholine for the M1 mAChR are also potentiated by AF710B, which may explain the improved memory performance in trihexyphenidyl-treated rat and 3xTg-AD mouse models. These findings already separate AF710B from M1 orthosteric agonists (e.g. AF267B, AF292, AF102B and others) or from σ1R agonists. Supersensitive M1 receptors are induced by positive M1 PAMs that are currently under examination by several major drug companies [23]. These M1 PAMs potentiate the whole repertoire of acetylcholine-induced M1 mAChR-mediated downstream responses, including release of intracellular Ca2+. In contrast, AF710B diverges mechanistically from other M1 PAMs and M1 agonists (allosteric or orthosteric) in vitro studies, and in spite of inducing a supersensitive M1 mAChR, it does not potentiate carbachol-mediated release of intracellular Ca2+ (detailed data not shown; table 1). Notably, orthosteric, allosteric or bitopic M1 agonists increase intracellular Ca2+ at the same concentration range as that these compounds induce other downstream signaling. This is not the case for AF710B, and therefore this compound diverges mechanistically from M1 orthosteric agonists (e.g. AF267B, AF102B, AF292 and more [1, 2]), M1 allosteric agonists (e.g. TBPB and more), or M1 PAMs (benzyl quinolone carboxylic acid, brucine and more [23]; detailed data not shown; table 1). Interestingly, unlike AF710B, M1 PAMs and M1 agonists (allosteric or orthosteric) were not reported to have σ1R activity. In addition, reported M1 PAMs or M1 allosteric agonists (or bitopic M1 agonists) are effective as cognitive enhancers at doses >1 mg/kg, unlike AF710B (effective at the microgram per kilogram range). Thus, AF710B exhibits a remarkably different mechanism of action and superior profile versus all these compounds.

Results from drug screening and the trihexyphenidyl model implied that AF710B might improve memory by a combined activation of M1 mAChR and σ1R. As hippocampal mushroom spine loss may underlie memory deficits in AD [16], we tested a potential role for AF710B in stabilizing synaptic connections and found that AF710B rescues mushroom spine loss in vitro in PS-KI and APP-KI hippocampal cell culture models of AD. Considering the established role of σ1R as a sensor of ER Ca2+ dysregulation [11] and the deranged ER Ca2+ signaling in AD neurons [16, 20], it is likely that σ1R activity is abnormal in spines of AD neurons. Indeed, σ1R agonists affect learning and memory, cognition and mood and show potent antiinmnesic properties [8], and σ1R knockdown causes hippocampal spine loss [33]. Additionally, σ1R agonists may have therapeutic potential in protein conformation diseases (e.g. AD, Parkinson’s disease and more) and several neuropsychiatric disorders [8]. Conversely, σ1R KO mice are viable, but have a late-onset neurodegeneration phenotype [15]. Thus, it appears that partial loss of σ1R function or reduction in its expression may

**Fig. 5.** AF710B reduces AD-like pathology in the 3xTg-AD mouse model. a p < 0.01 between vehicle-treated nTg and 3xTg-AD mice; b p < 0.05 and c p < 0.01 between vehicle- and AF710B-treated 3xTg-AD mice; V = vehicle; AF = AF710B. a, b AF710B (10 μg/kg/day, i.p.) improves cognition in 12-month-old 3xTg-AD mice, as demonstrated by the decrease in escape latency times to the platform (a), but not on the 6th day, and the increase in the amount of time in the target quadrant (b) compared to vehicle-treated 3xTg-AD mice during the probe trial, in the Morris water maze. c ELISA analysis reveals that levels of soluble and insoluble Aβ40 and Aβ42 are decreased in 3xTg-AD mice treated with AF710B (10 μg/kg/day). d Thioflavin S-positive plaques are also reduced in AF710B-treated 3xTg-AD mice, as shown by staining and volumetric analysis. e BACE1 and the C-terminal fragment of BACE cleavage of APP, C99, are significantly decreased in AF710B-treated 3xTg-AD mice, while full-length APP and nonamyloidogenic enzymes ADAM10 and ADAM17 remain unchanged. Quantification of Western blots was performed by densitometric analysis and is presented as a percentage of control, normalized to GAPDH. Activation of M1R/σ1R complex also reduces the phosphorylation of tau in the AT100 (f) and AT8 (g) epitopes in the 3xTg-AD mouse model. AF710B treatment results in the reduction of several phosphoepitopes of tau, while total tau levels remain the same versus vehicle-treated 3xTg-AD mice. h The reduction in tau phosphorylation seen in AF710B-treated 3xTg-AD mice is mediated through GSK3β and Cdk5, as demonstrated by the higher phosphorylation at Ser9 of GSK3β and reduced levels of the Cdk5 activator peptide p25, respectively. i Quantification of Western blots was performed by densitometric analysis and is presented as a percentage of control, normalized to GAPDH. j AF710B reduces inflammation, as we found significant reduction in the number of GFAP+ astrocytes and Iba-1+ microglia in the vicinity of 6E10+ plaques. Graphs represent the volumetric analysis of Aβ, astrocytes and microglia.
### Table 2. Compilation of data obtained with different compounds

**a** PA test in trihexyphenidyl-treated rats (all these studies were performed at IIBR under the same experimental protocol and can therefore be compared)

<table>
<thead>
<tr>
<th>Compound and mechanism of action</th>
<th>Milligrams per kilogram, per os</th>
<th>Safety margin (toxic/effective dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.001 0.003 0.01 0.03 0.1 0.5 1</td>
<td></td>
</tr>
<tr>
<td>AF710B (M1 allosteric agonist/σ1R agonist)</td>
<td>P P P P N P 0.001 0.003 0.01 0.03 0.1 0.5 1</td>
<td>50,000</td>
</tr>
<tr>
<td>AF710A (enantiomer of AF710B)</td>
<td>N N</td>
<td>Not applicable</td>
</tr>
<tr>
<td>AF102B (M1 &gt; M3 orthosteric agonist)</td>
<td>P</td>
<td>115</td>
</tr>
<tr>
<td>AF267B (M1 orthosteric agonist)</td>
<td>N P P P P 400</td>
<td></td>
</tr>
<tr>
<td>AF292 (M1 orthosteric agonist)</td>
<td>N P P P P 3,300</td>
<td></td>
</tr>
<tr>
<td>Donepezil (Aricept) (σ1R agonist/AChE-I)</td>
<td>N N Not applicable</td>
<td></td>
</tr>
<tr>
<td>Donepezil (0.1 mg/kg, p.o.) (σ1R agonist/AChE-I) + AF267B (M1 orthosteric agonist)</td>
<td>P P Not applicable</td>
<td></td>
</tr>
</tbody>
</table>

**b** Interventions tested in 3xTg-AD mice [38–40]

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Aβ</th>
<th>Tau</th>
<th>Inflammation</th>
<th>Effect on cognition</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF710B</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
<td>10 μg/kg/day, i.p., 2 months</td>
</tr>
<tr>
<td>Anti-IL-1R antibody</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
<td>200 μg/every 8-9 days, i.p., 6 months</td>
</tr>
<tr>
<td>Calpain inhibitor (A-705253)</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
<td>80 mg/kg/day, p.o., 3 months</td>
</tr>
<tr>
<td>α7-Nicotinic agonist (A-582941)</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>↑</td>
<td>12 mg/kg/day, p.o., 3 months</td>
</tr>
<tr>
<td>TSPO ligand (Ro5-4864)</td>
<td>↓</td>
<td>NC</td>
<td>↓</td>
<td>↑</td>
<td>3 mg/kg once weekly, i.p., 3 months</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>↓</td>
<td>↓</td>
<td>NT</td>
<td>↑</td>
<td>375 ppm on chow, 4 months</td>
</tr>
<tr>
<td>Lithium</td>
<td>NC</td>
<td>↓</td>
<td>NT</td>
<td>NC</td>
<td>300 μl of 0.6 M LiCl/mouse/day, i.p., 4 weeks</td>
</tr>
<tr>
<td>M1R agonist (AF267B)</td>
<td>↓</td>
<td>↓</td>
<td>NT</td>
<td>↑</td>
<td>3 mg/kg/day, i.p., 10 weeks</td>
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<tr>
<td>Memantine</td>
<td>↓</td>
<td>↓</td>
<td>NT</td>
<td>↑</td>
<td>30 mg/kg/day, p.o., 3 months</td>
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<tr>
<td>Mifipristone</td>
<td>↓</td>
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<td>NT</td>
<td>↑</td>
<td>1.2 mg/day, s.c., 21 days</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>NC</td>
<td>↓</td>
<td>NT</td>
<td>↑</td>
<td>200 mg/kg/day, p.o., 4 months</td>
</tr>
<tr>
<td>Nicotine</td>
<td>NC</td>
<td>↑</td>
<td>NT</td>
<td>NT</td>
<td>3 mg/day, p.o., 3 months</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>↑</td>
<td>↑</td>
<td>NT</td>
<td>NT</td>
<td>5 mg/kg/day, i.p., 7 days</td>
</tr>
</tbody>
</table>

P = Significant mitigation of cognitive deficit; N = no significant mitigation of cognitive deficit; AChE-I = acetylcholinesterase inhibitor; TSPO = translocator protein; NC = no change; NT = not tested.
AF710B, a Novel M1/σ1 Agonist for Treatment of Alzheimer’s Disease

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Contribute to synaptic impairment and neuronal loss in these disorders. We therefore tested the potential involvement of σ1R in mediating the neuroprotective effects of AF710B. Although the σ1R antagonist NE-100 did not block the rescue of mushroom spines by AF710B in hippocampal neurons from APP-KI mice, inhibition of M1 mAChR prevented the rescue by AF710B. However, σ1R knockdown in WT hippocampal neuron cultures compromised mushroom spine stability, and AF710B treatment was unable to prevent this, indicating requirements for both σ1R and M1 mAChR activity in hippocampal mushroom spine stability. To explain these results, we propose that AF710B may act by stimulating an M1 mAChR/σ1R complex in the synaptic spines. Notably, heteromers constituted by σ1Rs with D1Rs or D2Rs (both GPCRs) were reported [12, 13], and it is reasonable to presume that other GPCRs, including the M1 mAChR, may also form heteromers with σ1R. Further biochemical experiments will be needed to test this hypothesis.

The hypothesis that AF710B targets a putative M1 mAChR-σ1R complex may be tied with the precognitive effects of AF710B at unprecedented low doses in memory-based behavioral tasks in both trihexyphenidyl-treated rats (fig. 3e, f; table 2a) and 3xTg-AD mice (fig. 5a, b; table 2b [38–40]). In this context, in the 3xTg-AD mice AF710B mitigated cognitive deficits, and decreased BACE1 levels, GSK3β and p25/CDK5 activity, neuroinflammation, soluble and insoluble Aβ40 and Aβ42 plaques, and tau pathology (fig. 5). Furthermore, unlike the remarkable disease-modifying properties found with AF710B in 3xTg-AD mice at very low doses (10 μg/kg/day i.p., for 2 months), no in vivo disease-modifying properties were reported for M1 PAMs or M1 allosteric agonists and M1 dualistic agonists in Tg mice or in other in vivo models [2, 23].

GSK3β and CDK5 contribute to hyperphosphorylation of tau protein, one of the pathological hallmarks of the disease (reviewed in Su and Tsai [34]). CDK5 activity is increased in AD patients, probably due to the conversion of the CDK5 activator p35 to the truncated p25 protein (reviewed by Su and Tsai [34]). Elevated p25 levels are observed in AD patients [35]. The CDK5 activator protein p25 preferentially binds and activates GSK3β [36]. All these data supported the hypothesis that CDK5/p25 acts as a master regulator of neuronal cell death in AD, Parkinson’s disease and several neurodegenerative diseases [34]. Interestingly, activation of the M1 mAChR decreased several p-tau isoforms via GSK3β inhibition but not the p-tau isoform detected with PHF-1 [3], while activation of σ1R helps maintain proper tau phosphorylation by potentially circumventing the formation of over-reactive CDK5/p25 [37]. Effective therapies for tauopathies may require inhibition of both CDK5/p25 and GSK3β. AF710B fulfills this condition, and because it decreased all tested p-tau isoforms including PHF-1, it may be effective as a disease modifier even in more advanced stages of AD and also for other tauopathies. Overall, our data indicates that AF710B decreases tau pathology and may restore synaptic homeostasis by decreasing p25 and inhibiting GSK3β and CDK5 activities.

In summary, we identified AF710B as a novel comprehensive therapeutic agent to ameliorate cognitive deficits, synaptic loss, amyloid and tau pathologies, neuroinflammation, and neurodegeneration in AD and perhaps additional related protein aggregation diseases. AF710B appears to be a highly effective treatment for AD, when compared with competing drugs (table 2a, b). A drug like AF710B that works in extremely low doses provides far more dosing and testing options for future AD therapeutics.

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Disclosure Statement

Abraham Fisher is member of the scientific advisory board of Anavex, USA.

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