Selective Androgen Receptor Modulators Antagonize Apolipoprotein E4-Induced Cognitive Impairments

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Abstract: While tissue specific effects of selective androgen receptor modulators (SARMs) outside the brain have been reported, little is known about brain specific SARMs. Here we show that SARMs upregulate androgen receptor levels in brain following castration and antagonize impairments in hippocampus-dependent novel location recognition and spatial memory retention in apoE4 female mice. Together with the reduced androgen levels in aged men and women and the beneficial effects of androgens on brain function and pathology in Alzheimer's disease-related models, these data support the therapeutic potential of SARMs for age-related cognitive decline and Alzheimer's disease.

Keywords: Open field, Object recognition, Water maze, Mouse, Androgen receptor, Castration.

INTRODUCTION

Reduced androgen levels in aged men and women might be risk factors for age-related cognitive decline and Alzheimer's disease (AD). Ongoing clinical trials are designed to evaluate the potential benefit of estrogen in women and of testosterone in men. Apolipoprotein E (apoE) is important for lipoprotein and cholesterol metabolism and implicated in nerve development and regeneration, neurite outgrowth and neuroprotection [1]. Expression of apoE4, a risk factor for age-related cognitive decline and AD compared to apoE2 and apoE3, in *Apoe*$^{-/-}$ mice leads to age-related impairments in spatial learning and memory in female, but not male, mice [2, 3]. These impairments are observed in mice that express apoE4 in neurons or astrocytes [4] and are therefore independent of the cellular source of apoE. They require apoE4 and are not seen in *Apoe*$^{-/-}$ female mice, consistent with a pathogenic gain of function of apoE4. Effects of apoE4 on androgen receptor (AR) function might contribute to these cognitive impairments. Compared to age-matched wild-type and *ApoE*$^{-/-}$ mice, expression of apoE4, but not apoE3, reduced cytosolic AR binding to androgens in the cortex of female and male mice and treatment of apoE4 female mice with testosterone or dihydrotestosterone antagonized their cognitive impairments [5].

ApoE4 also has effects on AR function in the cholinergic basal forebrain, participating in behavioral processes such as attention and memory associated with aging and AD [6]. In the vertical limb of the diagonal band of Broca, a major cholinergic nucleus in the basal forebrain affected in AD, the presence of AD pathology or apoE4 negatively correlates with the percentage of AR-positive neurons in women, but not in men.

ApoE4 might also have detrimental effects of on AR function outside the brain. Peritoneal macrophages, isolated from transgenic mice that expressed apoE4 and stimulated with interferon-γ either alone or in combination with synthetic double-stranded RNA (Poly I:C) or lipolysaccharide showed a greater release of the inflammatory mediators nitric oxide and tumor necrosis factor-α (TNF-α) than those isolated from male transgenic mice that expressed apoE3 [7, 8]. This effect was sex-dependent, as it was not seen in peritoneal macrophages from female apoE3 and apoE4 homozygous mice, and involved androgens, consistent with the increase in nitric oxide synthase (NOS) activity in the brains of rats following castration and decrease in NOS activity following administration of the dihydrotestosterone [9].

Selective androgen receptor modulators (SARMs) are tissue specific and do not have side effects associated with the use of conventional androgens. Effects of SARMs outside the brains have been reported. For example, in rats SARMs restored levator ani muscle mass to levels expressed in intact controls [10], muscle strength and body composition and prevented bone loss in orchidectomized animals [11], prostate tumor growth, and orchidectomy-induced bone loss [12]. SARMs are also promising for effects in the brain. In this study, we tested whether SARMs can antagonize apoE4-induced cognitive impairments.

METHODS

Animals

Male Sprague-Dawley rats (200-225 g) from Charles River Laboratories were used for the immunohistochemical studies. Six-month-old human neuron-specific enolase (NSE) apoE4 transgenic mice bred in our mouse colony and genotyped as described [2] were used for the behavioral studies. The rat and mouse studies were conducted in accordance with the policies and recommendations of the NIH guidelines for the handling and use of laboratory animals and were approved by the Animal Care and Use Committee of ACADIA Pharmaceuticals and OHSU, respectively.

Castration of Rats and Pharmacological Treatments

To determine the ability to affect AR receptor level and function in brain, rats were castrated or sham-operated and

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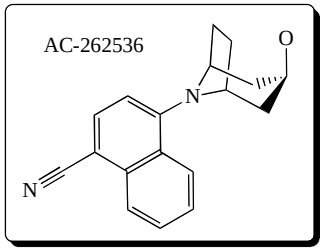
allowed to recover for 5 days. Thereafter, they were treated (s.c.) for 14 consecutive days with vehicle, testosterone propionate (TP) at 0.1, 1, or 10 mg/kg or with SARM AC-262536. Twenty four hours following the last injection, the animals were killed and their brains processed for immunohistochemistry.



R-SAT™ Assays

R-SAT™ (Receptor Selection and Amplification Technology) is a proprietary cell-based functional assay that allows one to monitor receptor-dependent proliferative responses of various receptor classes including nuclear receptors [13-15]. Its principle resides in the genetic selection and amplification of nuclear receptors in a ligand-dependent manner. This process is achieved by partial cellular transformation *via* loss of contact inhibition and growth factor dependency. Monitoring is achieved by transfecting the cells with a β-galactosidase reporter gene vector whose expression is under a constitutively active promoter. Briefly, mouse NIH3T3 fibroblasts were plated overnight in 96wells plates in DMEM 10 % calf serum (Hyclone) and grown to 60-70 % confluency prior to transfection. Transient transfections were performed using Polyfect (Qiagen) according to manufacturer's instructions. Typically a transfection mix would consist of expression vectors encoding the androgen receptor (200ng), β-galactosidase (500ng) and the coactivators SRC1, DRIP205 and GRIP1 (10ng each). Such a transfection mix would be sufficient to transfect 30 96-wells. Sixteen hours post-transfection, cells were incubated with different doses of ligand in DMEM containing 30% Ultraculture (Hyclone) and 0.4% calf serum (Hyclone) to generate a dose response curve. After 5 days, plates were developed by adding onto the washed cells a solution containing the β-galactosidase substrate o-nitrophenyl-d-galacto pyranoside ONPG (in phosphate buffered saline with 5% Nonidet P40 detergent). Plates were read using a microplate reader at 420nm. Data from RSAT™ assays were fit to the equation: $r = A + B(x/(x + c))$, where A = minimum response, B = maximum response minus minimum response, c = EC50, r = response, and × = concentration of ligand. Curves were generated using the curve fitting softwares Excel Fit and GraphPad Prism (San Diego, CA).

Luciferase Reporter Gene Assays

Human breast carcinoma MDAkb2 cells (which are stably transfected with the mouse mammary tumor virus (MMTV) promoter linked to luciferase, and express endogeneous AR) were grown in DMEM 10% charcoal-stripped FBS (Gibco BRL), plated at 10,000 cells per 96-well (100 µl/well) onto luciferase assay plates. The day after plating, the medium was replaced with fresh one and varying concentrations of ligand. Twentyfour hours later, the medium was removed from plates and cells extracts were then lyzed and Luciferase activity measured using a commercially available kit (Promega).

Binding Assays

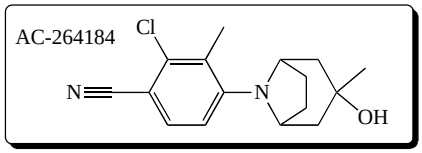
Hamster DDT cells were grown in DMEM 5% charcoal-stripped FBS in presence of 10 nM Testosterone. Cells were plated at 25,000 cells / well in a 24-well plate (500 µl / well) and grown to 80% confluency (typically 3 days). At that stage, the media was replaced and ^{3}H-DHT added to a final concentration of 2 nM, along with varying concentrations of the test ligands. Cells were incubated for an additional 24 hour period. Cells were then washed multiple times with ice cold HBSS (Hank's Balanced Salt Solution) then resuspended in 100 % ethanol (100 µl /well). Plates were then sealed and shaken for 6 hours. Extracts were then quantified in a Beckman scintillation counter.

Immunohistochemistry

Animals were overdosed with isofluorane and perfused transcardially with PBS and 4% paraformaldehyde. Brains were stored overnight in 30% sucrose before sectioning. Tissue was sectioned coronally through the hippocampus and collected in cryoprotectant media and then rinsed in PBS prior to staining. Thereafter, endogenous peroxidase activity was eliminated by placing sections into 3% H_2O_2 in PBS for 10 min. Sections were incubated for at least 45 min in PBS/avidin and 0.1% Triton X-100 with 8% Normal Serum to block nonspecific binding sites before being incubated with either a-NeuN monoclonal mouse antibody (1:500, Chemicon) or a polyclonal rabbit AR antibody (PG-21, 1:50, lot #30019, Upstate) over night at 4ºC. After the primary antibody, control sections were incubated with a biotinylated anti-mouse antibody and all others were incubated with a biotinylated anti-rabbit antibody for 1 hour at room temperature (1:250, Vector, USA). Then, sections were incubated with the ABC kit (Vector, USA) for 30 min, and specifically bound antibody was revealed by using metal enhanced 3.3'-diaminobencidine tetrahydrochloride as chromogen, and a stable peroxide buffer (DAB kit, Pierce USA). Sections were rinsed in PBS and mounted on glass slides and coverslipped.

Behavioral Testing and Pharmacological Treatments of Mice

To determine the effects of SARMs on behavioral performance, 6-month-old NSE-apoE4 female (*n* = 6 mice/treatment) mice were treated with vehicle, SARM AC-262536 (30 mg/kg) or SARM AC-264184 (3 mg/kg). These doses were selected based on preliminary data assessing effects of the SARMs on AR levels in castrated rats. The treatments were administered subcutaneously daily, starting one week prior to cognitive testing. The mice were tested for measures of activity and anxiety in the open field in week 1, for novel location and novel object recognition in week 2, and for spatial learning and memory in the water maze in week 3. The open field was performed as described [2].



Novel Location and Novel Object Recognition

After habituation to an open field, the mice were trained in three consecutive trials and than tested in two consecutive trials with a 5–min inter-trial interval. For both the training and testing sessions, three objects were placed in the open field, and the animal was allowed to explore for 10 min. All objects were only used once and replicas were used in subsequent trials. Five min after the training trials, the animals were tested for recognition of the novel location of one of the familiar objects. Five min after the novel location test, the animals were tested for novel object recognition. The time spent exploring each object during the training and testing sessions was recorded by an observer.

Water Maze

In the water maze test, the mice were first trained to locate a visible platform in a water maze surrounded by a curtain in 5 sessions, and subsequently trained to locate a platform hidden beneath opaque water in 6 sessions. There were two sessions per day 3.5 hr apart and each session consisted of 3 60-sec trials each (at 10-min intervals). During visible

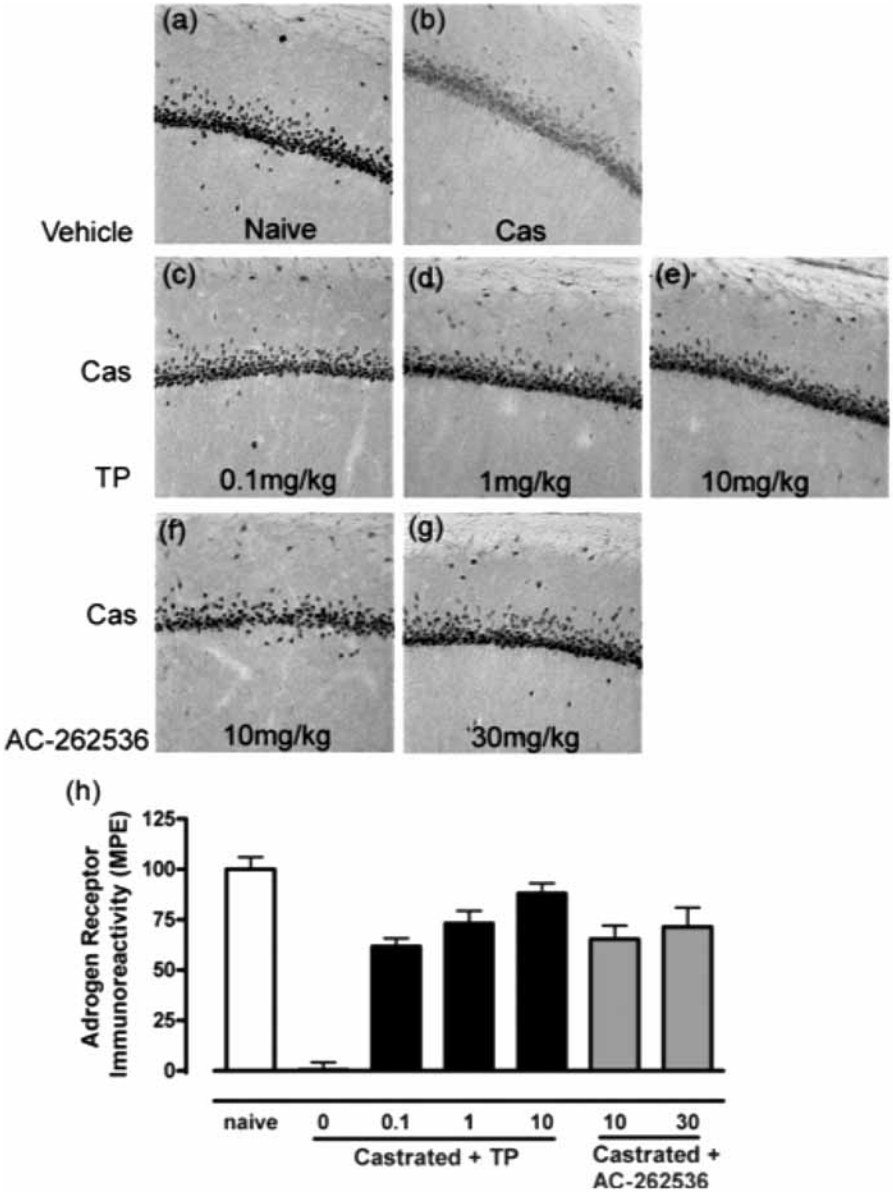


Fig. (1). AR immunoreactivity in naïve rats treated with vehicle (**a**) and castrated rats treated with vehicle (**b**), TP at 0.1 (**c**), 1, (**d**), or 10 (**e**) mg/kg, or AC-262536 at 10 (**f**) or 30 (**g**) mg/kg. h. Quantification of AR immunoreactivity. *n* = 8 rats/treatment.

platform training, the platform location was changed in each session. The pool had a diameter of 140 cm and was filled with opaque water (21-23˚C). Mice that failed to find the hidden platform within 60 sec were put **on** it for 15 sec. During the visible platform training, the platform was moved to a different quadrant for each session. During the hidden platform training, the platform location was kept constant for each mouse (in the center of the target quadrant). The starting point at which the mouse was placed into the water was changed for each trial. A 60 sec probe trial (platform removed) was performed 1 hr after sessions 8 and 11. For probe trial data analysis, cumulative distance to the platform location during the hidden sessions was calculated or the pool was divided into four quadrants and the % time spent in each quadrant was calculated. The data were recorded with a Noldus Instruments Ethovision video tracking system (Noldus Information Technology, Sterling, VA) set to analyze six samples per second.

Statistical Analysis

Data are expressed as mean ± SEM. The data were analyzed by ANOVA, followed by Tukey-Kramer posthoc tests when appropriate, or repeated measures ANOVA (session x treatment) for learning curves, using SPSS software (SAS Institute Inc., Cary, NC). For probe trial analysis, a two-way trial x treatment ANOVA was performed. A probability value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Castration dramatically reduced AR levels (Fig. **1b**), compared to those in intact animals (Fig. **1a**). This reduction in AR levels is unlikely the result of potencial cell loss in the castrated animals as treatment with either testosterone or the SARM resulted in AR immunoreactivity levels comparable to wild-type mice. Like testosterone propionate (TP) (Fig. **1c-e, h**), SARM AC-262536 (Fig. **1f-h**) dose-dependently restored AR levels in castrated rats [20]. Several biochemical assays were used to characterize the activities of AC-262536 and AC-264184 at AR. In a functional cell based assay, the % efficacy and pEC50 (–log[EC50]) were for testosterone (102 ± 11; 8.7 ± 0.2, n = 30), AC-262536 (67 ± 9; 7.9 ± 0.3, n = 11), and AC-264184 (81 ± 7; 8.9 ± 0.2, n = 10). In a AR transcriptional activity luciferase assay, the % efficacy and (pEC50 were for testosterone (104 ± 24; 8.5 ± 0.6, n = 4), AC-262536 (72 ± 9; 8.8 ± 0.1, n = 5), and AC-264184 (86 ± 11; 9.6 ± 0.1, n = 4). Finally, in a AR binding assay the pKi (–log[KI]) were for testosterone 8.9 ± 0.3, n = 5 and for AC-262536 8.3 ± 0.2, n = 4 (n is the number of experiments done in triplicates). These data demonstrate the activities of AC-262536 and AC-264184 at AR, with the latter one being about 10 times more potent.

Testosterone and DHT antagonize cognitive deficits in 6-month-old apoE4 female mice in spatial memory retention in the water maze probe trials [5]. To determine whether SARMs can antagonize cognitive impairments in apoE4 female mice, 6-month-old apoE4 female mice were treated with vehicle, AC-262536 (30 mg/kg) or AC-264184 (3 mg/kg) (n = 6 mice/treatment). To assess potential effects on measures of activity or anxiety, the mice were first tested in the open field. No effects of the SARMs were detected on measures of activity or anxiety in the open field (distance moved: F = 0.56, p = 0.58; % time spent in center of the open field: F = 0.38, p = 0.68). Next the mice were tested for novel location and novel object recognition. Vehicle-treated apoE4 mice showed impaired hippocampus-dependent novel location recognition (Fig. **2a**). In contrast, apoE4 mice treated with either SARM showed novel location recognition and explored the object in the novel location more than in the old location (Fig. **2a**). Most mice showed hippocampus-independent novel object recognition (Fig. **2b**). In the vehicle group, 2 out of 6 mice did not show preference (p = 0.08) and in the AC-262536 treated mice 1 out of 6 mice did not show preference ($p < 0.05$). In the AC-264184 treated group, all mice showed preference ($p < 0.0001$). Finally the mice were tested in the water maze. There were no effects of treatment on swim speeds during the visible sessions (F = 1.646, p = 0.224, vehicle: 16.70 ± 0.54 cm/sec; AC-262536: 15.54 ± 0.54 cm/sec; AC-264184: 15.49 ± 0.50 cm/sec). All groups showed learning and improved their performance with training, analyzed as distance swam from the platform location (cumulative distance to the target location), during the visible (F = 26.012, $p < 0.0001$) and hidden (F = 6.199, $p < 0.001$) sessions. There was a treatment x session interaction for the visible (F = 12.38, $p < 0.0001$) and hidden (F = 12.38, $p < 0.0001$) sessions. There was also an effect of treatment during the hidden sessions (F = 4.13, $p < 0.05$). Similar results were obtained for time to reach the platform (platform) and total distance moved. One hour following the fourth and seventh hidden session the mice were tested in a probe trial (platform removed) and the cumulative distance to the platform location used during the hidden sessions was calculated. There was a an effect of probe trial (repeated measures ANOVA, F = 25.87, $p < 0.0001$) and probe trial x treatment interaction (repeated measures trial x treatment ANOVA, F = 7.274, $p < 0.006$). While vehicle-treated apoE4 female mice did not improve from the first to the second probe trial, SARM-treated apoE4 mice did (Fig. **2d**). When the probe trial data were analyzed as % time spent searching in the target quadrant where the platform was located during the hidden sessions versus the other three quadrants, AC-264184 showed already spatial memory retention in the first probe trial (Fig. **2e**), while the other two groups did not. In the second probe trial, vehicle-treated apoE4 mice still did not show spatial memory retention while apoE4 mice treated with either SARM did (Fig. **2f**). These data demonstrate that, like testosterone and DHT, SARMs, antagonize cognitive deficits in 6-month-old apoE4 female mice.

In nondemented healthy elderly women salivary testosterone levels were lower in apoE4- than non-apoE4 carrying women [16]. Therefore, stimulation of AR-mediated signaling by SARMs might be particularly valuable in apoE4-carrying women. As in men age-related testosterone depletion is a risk factor for AD and testosterone and apoE4 interact in the risk of AD [17, 18] and DHT protects against acceleration of AD-like neuropathology in male mice containing three other AD-related genes (APP_{SWE}, $PS1_{M146V}$, tau_{P301}) [19], SARMs without effects in the prostate would also be valuable. Therefore, increased efforts are warranted to assess

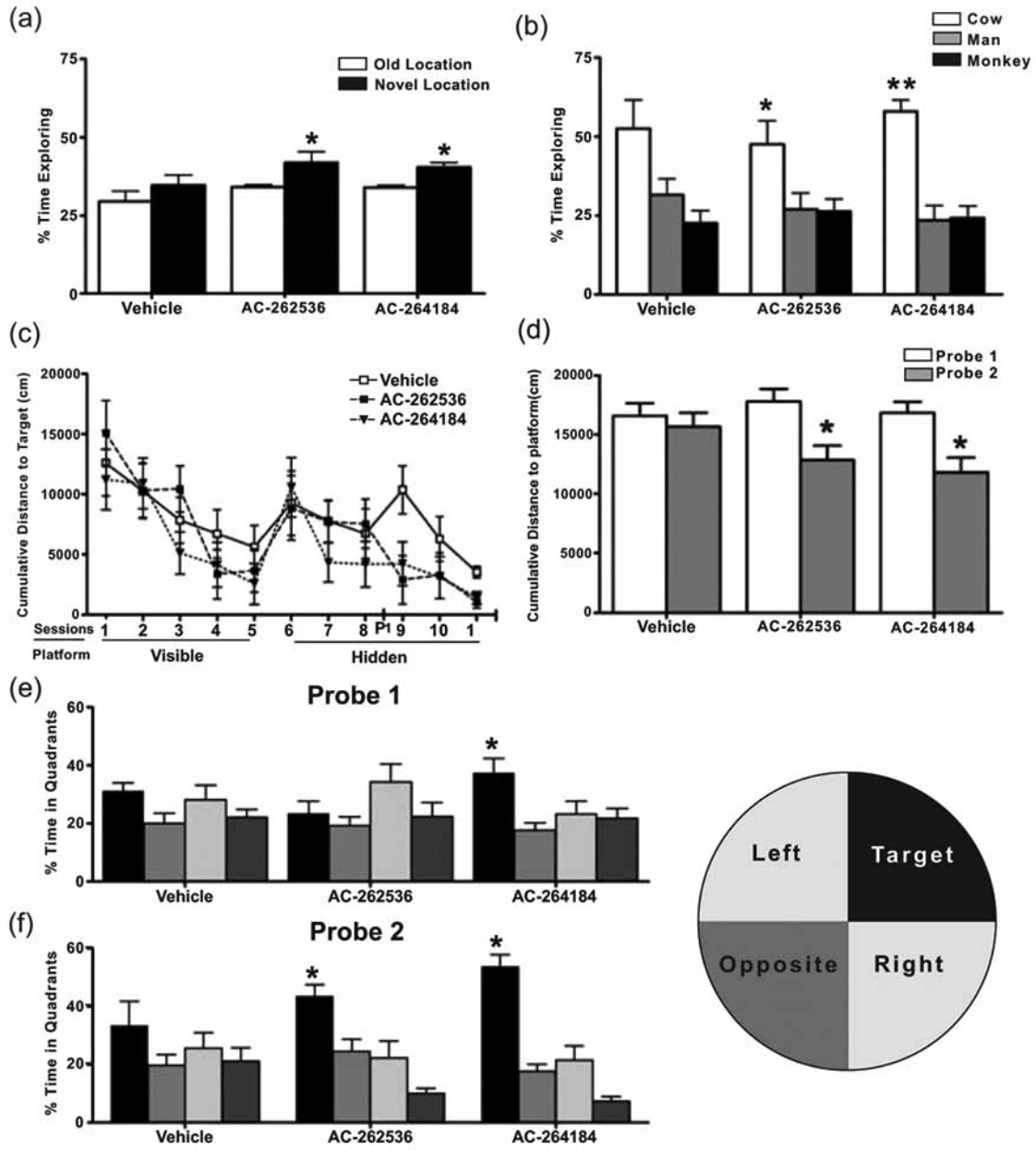


Fig. (2). Effects of AC-262536 and AC-264184 on novel location recognition (**a**), novel object recognition (**b**), water maze learning (**c**) and spatial memory retention in the water maze probe trials, as analyzed by cumulative distance to the platform location during the hidden sessions (**d**) or % time spent in the different quadrants of the pool following the 8th (**e**) and 11th session (**f**). (a) *p < 0.04 versus old location; (b) *p < 0.05 versus man or monkey, **p < 0.0001 versus man or monkey; (d) *p < 0.05 versus Probe 1; (e) and (f) *p < 0.05 versus any other quadrant. *n* = 6 mice/treatment.

the therapeutic effects of SARMs in age-related cognitive decline and AD in women and men.

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STATEMENT OF INTEREST

Drs Raber and Acevedo have no conflict of interest to reports. Drs Piu, Gardell and Risso Bradley are present or past employees of ACADIA Pharmaceuticals.

REFERENCES

[1] Mahley, R. W. *Science*, **1988**, *240*, 622.
[2] Raber, J.; Wong, D.; Buttini, M.; Orth, M.; Bellosta, S.; Pitas, R. E.; Mahley, R. W.; Mucke, L. *Proc. Natl. Acad. Sci. USA*, **1998**, *95*, 10914.
[3] Raber, J.; Wong, D.; Yu, G.-Q.; Buttini, M.; Mahley, R. W.; Pitas, R. E.; Mucke, L. *Nature*, **2000**, *404*, 352.
[4] Van Meer, P.; Acevedo, S.; Raber, J. *Beh. Brain Res.*, **2007**, *176*, 372.
[5] Raber, J.; Bongers, G.; LeFevour, A.; Buttini, M.; Mucke, L. *J. Neurosci.*, **2002**, *22*, 5204.
[6] Ishunina, T.; Fisser, B.; Swaab, D. *Exp. Neurol.*, **2002**, *176*, 122.
[7] Brown, C.; Wright, E.; Colton, C.; Sullivan, P. M.; Laskowitz, D. T.; Vitek, M. *Free Radic. Biol. Med.*, **2002**, *32*, 1071.

[8] Brown, C.; Xu, Q.; Okhubo, N.; Vitek, M.; Colton, C. *Endocrinology*, **2003**, *148*, 3383.
[9] Sing, R.; Pervin, S.; Shryne, J.; Gorski, R.; Chaudhuri, G. *Proc. Natl. Acad. Sci. USA*, **2000**, *97*, 3672.
[10] Sun, C.; Robl, J.; Wang, T.; Huang, Y.; Kuhns, J.; Lupisella, J.; Beehler, B.; Golla, R.; Sleph, P.; Seethala, R.; Fura, A.; Krystek, S. J.; An, Y.; Malley, M.; Sack, J.; Salvati, M.; Grover, G.; Ostrowski, J.; Hamann, L. *J. Med. Chem.*, **2006**, *49*, 7596.
[11] Gao, W.; Reiser, P.; Coss, C.; Phelps, M.; Kearbey, J.; Miller, D.; Dalton, J. *Endocrinology*, **2005**, *146*, 4887.
[12] Allan, G.; Lai, M.; Sbriscia, T.; Linton, O.; Haynes-Johnson, D.; Bhattacharjee, S.; Dodds, R.; Fiordeliso, J.; Lanter, J.; Sui, Z.; Lundeen, S. *J. Steroid Biochem. Mol. Biol.*, **2007**, *103*, 76.
[13] Burstein, E.; Piu, F.; Ma, J.; Weismann, J.; Currier, E.; Nash, N.; Weiner, D.; Spalding, T.; Schiffer, H.; Del Tredici, A.; Brann, M. *Curr. Pharm. Des.*, **2006**, *12*, 1717.
[14] Piu, F.; Magnani, M.; Ader, M. *Oncogene*, **2002**, *21*, 3579.
[15] Piu, F.; Gauthier, N.; Olsson, R.; Currier, E.; Lund, B.; Croston, G.; Hacksell, U.; Brann, M. R. *Biochem. Pharmacol.*, **2005**, *71*, 156.
[16] Berteau-Pavy, F.; Park, B.; Raber, J. *Neuroscience*, **2007**, *147*, 6.
[17] Hogervorst, E.; Lehmann, D.; Warden, D.; McBroom, J.; Smith, A. D. J. Geriatr. *Psychiatry*, **2002**, *17*, 938.
[18] Hogervorst, E.; Williams, J.; Budge, M.; Barnetson, L.; Combrinck, M.; Smith, A. D. *Neuroendocrinol. Lett.*, **2001**, *22*, 163.
[19] Rosario, E.; Carroll, J.; Oddo, S.; LaFerla, F.; Pike, C. J. J. *Neuroscience*, **2006**, *26*, 13384.
[20] Piu, F.; Gardell, L.; Son, T.; Schlienger, N.; Lund, B.; Schiffer, H.; Vanover, K.; Davis, R.; Olsson, R.; Bradley, S. *J. Steroid. Biochem. Mol. Biol.*, **2008**, *109*, 129.