Supporting Information: AC-186, a Selective Nonsteroidal Estrogen Receptor ß Agonist, Shows Gender specific Neuroprotection in a Parkinson's Disease Rat Model

*Krista McFarland, Diana Price, Christopher N. Davis, Jian-Nong Ma, Douglas W. Bonhaus, Ethan S. Burstein, Roger Olsson**

Brain: plasma exposure

The brain:plasma (B:P) ratio of **AC-186** was evaluated after intravenous and subcutaneous administration in Sprague-Dawley rats. After subcutaneous dosing at 10 mg/kg, the average brain-to-plasma ratio (B:P) of **AC-186** increased linearly ($r^2 = 0.91$) as a function of time. At 4 (B:P= 3.5) and 12 (B:P= 4) hours the ratio was significantly higher than at 1 (B:P= 1) or 2 (B:P= 2) hours. Over the time period from 0-12h there were brain concentrations >100nM, which is around 10X the pEC_{50} at ERβ and at 2h (C_{max}) the brain concentration was around 3uM.

The pharmacokinetics of AC-186 in male and female rats was compared after SC dosing. No large gender difference was observed in the pharmacokinetics of AC-186. The clearance and volume of distribution of AC-186 was somewhat higher in males than in females, whereas the T1/2 was very similar, the exposure was somewhat higher in females than in males. The brain exposure and brain:plasma ratios where similar in both males and females.

In vitro safety

Ames assays with and without Arochlor-induced rat liver S9 fractions. There was no effect of AC-186 (top concentration 100 uM) in the Ames assay at any concentration tested.

Micronucleus/chromosomal aberration assays with and without S9 fractions. There was no effect of AC-186 (top concentration 100 uM) in any concentration tested.

AC-186 was tested at hERG, and showed no activity up to the max dose tested 10uM.

Cyp inhibition was tested at 6 isoenzymes, CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4. The highest inhibition was found at CYP2C19 (1.5-5 uM) and CYP2C9 (>5uM).

In vivo safety

The Maximum Tolerated Dose (MTD) of the AC-186 was evaluated in male and female Sprague-Dawley rats after single dose. AC-186 was administered subcutaneously at 100, 300, and 1000 mg/kg. The administered doses were well tolerated by both male and female rats. Based on clinical observations MTD for AC-186 was determined as 1000 mg/kg or higher.

Irwin screen

No CNS liabilities where seen at 100mg/kg s.c. in a modified Irwin screen: Body posture, Muscle tone, Respiration, Skin color, Amount of activity, Type of activity, Horizontal Wire test, Tremors/Proconvulsive behaviors (twitches, popping, seizures, straub tail, piloerection etc.), Salivation, Feces, Vocalization, Toxicity

In vivo selectivity

ERß/ERα selectivity in vivo was assessed by uterine hypertrophy, which is driven by ERα receptor activation (Piu et al., 2008), and thus is a good way to assess ERß/ERα selectivity in vivo. Immature female rats treated for 4 days with either vehicle, the ERα selective agonist PPT

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(400-fold selective for ERα over ERß), or various doses of **AC-186** were sacrificed and their uterine mass measured. In accordance with previous reports, we confirmed that PPT induced robust uterine hypertrophy, causing a roughly 100% increase in uterine mass compared with vehicle. In contrast, **AC-186** did not have significant effects on uterine hypertrophy up to 100 mg/kg/day.

In vivo experiment of AC-186 in ovariectomized female rats

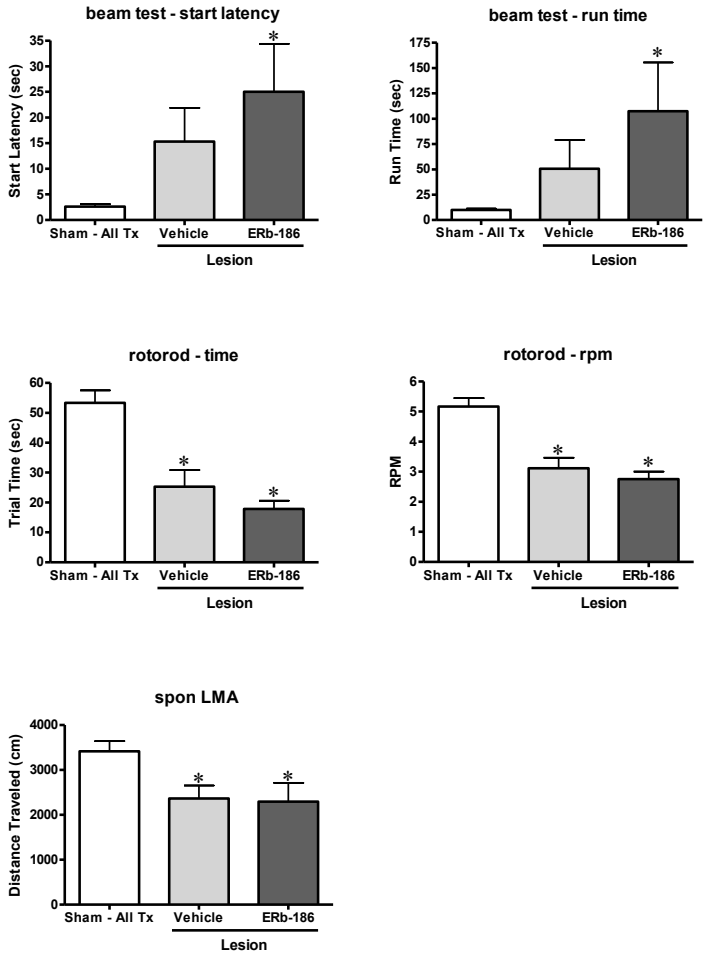

Figure 1. Motor performance evaluations of sham (all treatments combined) and 6-hydroxydopamine-lesioned animals treated with sesame oil vehicle (Veh), **AC-186**. Start latency and time required to traverse the challenging beam, respectively; Time animals remained on the rotorod on the test trials; Distance traveled during a 15 min spontaneous locomotor session. For each of these measures of motoric ability, 6-OHDA-lesions impaired performance (*$p<0.05$ *vs. sham-lesioned animals*), and treatment with an ERβ agonist (**AC-186**) did not prevent the impairments in female rats. Data were analyzed using one-way ANOVAs, followed by Bonferroni's multiple comparison post hoc analyses.

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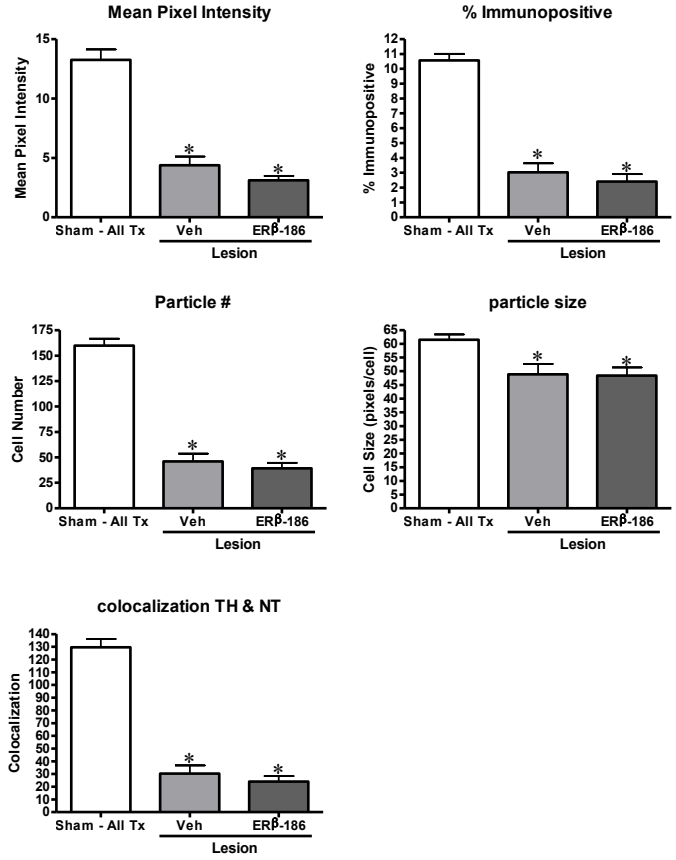

Figure 2. Tyrosine hydroxylase immunofluorescence (TH+) in the SN following sham- or 6-OHDA-lesion. 6-OHDA lesions produced (A) reduced TH+ cell counts in the SN, (B) reduced percentage of the image that was TH+, (C) reduced mean cell size, and (D) reduced mean pixel intensity of immunofluorescent pixels (*all *p<0.05 vs. sham control subjects*). Treatment with AC-186 did not ameliorated 6-OHDA lesion-induced reductions in cell number in female rats (**p<0.05 vs. Vehicle/6-OHDA group*). Data were analyzed with one-way ANOVAs followed by Bonferroni's post hoc comparisons.

In vitro receptor pharmacology

R-SAT® assays(Burstein et al., 2006)

R-SAT® (Receptor Selection and Amplification Technology) assays were performed essentially as described previously with the following modifications. Briefly, NIH-3T3 fibroblasts were plated overnight in 96-wells plates in DMEM 1% PSG (Penicillin–streptomycin–L-glutamine), 10% calf serum (Hyclone) and grown to 60–70% confluency prior to transfection. Transient transfections were performed with 1 to 10 ng/well of receptor DNA, and 30 ng/well pSI-ß-galactosidase (Promega, Madison, WI) per well of a 96-well plate using Polyfect (Qiagen, Valencia, CA) according to the manufacturer's instructions. One day after transfection media was changed and cells were combined with ligands in DMEM supplemented with 30%

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Ultraculture synthetic supplement (Cambrex, Walkersville, MD), 1% PGS and 0.4% calf serum (Hyclone) to a final volume of 200 ul/well. All data were analyzed using the computer programs Excel Fit and GraphPad Prism software (San Diego). After 5 days, plates were developed by removing the media and 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 P-40 detergent). β-galactosidase activity was quantified as absorbance at 420 nm using a microplate reader (Bio-Tek EL 310 or Molecular Devices). All data were analyzed using the computer programs Excel Fit and GraphPad Prism software (San Diego).

Burstein, E. S., Piu, F., Ma, J.-N., Weissman, J. T., Currier, E. A., Nash, N. R., et al. (2006). Integrative functional assays, chemical genomics and high throughput screening: harnessing signal transduction pathways to a common HTS readout. *Current pharmaceutical design*, *12*(14), 1717–1729.

Piu, F., Cheevers, C., Hyldtoft, L., Gardell, L. R., Del Tredici, A. L., Andersen, C. B., et al. (2008). Broad modulation of neuropathic pain states by a selective estrogen receptor beta agonist. *European journal of pharmacology*, *590*(1-3), 423–429. doi:10.1016/j.ejphar.2008.05.015