Supporting Information

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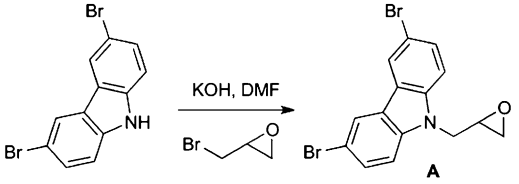
SI Materials and Methods

Accelerating Rotarod. Beginning on day 50, mice were trained on the accelerating rotarod, using Columbus Instruments Rotamex-5. Training consisted of mice being placed on a rotarod moving at 5 rpm for 300 s. Mice were trained to stay on the rotarod for the entire 300 s. If a mouse fell, it was placed back on the rotarod and the 300-s trial was started again. Training took place on two consecutive days. On day 52, mice ran their first full rotarod test, as described in *Current Protocols for Neuroscience* (1). The rotarod began at 4 rpm and accelerated to 40 rpm over 600 s, increasing by 1.25 rpm every 20 s. The time to fall was automatically recorded. Each run was separated by 20 min to allow the mice to rest, and each mouse participated in four runs. Mice were run every 7 d until the time at which they were unable to stay on the rotarod for more than 10 s for three trials.

Paw Print Analysis. Five measurements were taken: front and back stride, front and back width, and front-to-back distance, as described in *Current Protocols for Neuroscience* (2). Twenty total measures were taken for each measurement. Front stride and back stride were collected as a straight line from paw print to the following paw print. Front-to-back distance was collected as a straight line from back paw print to corresponding front paw print. Correspondence was based on closest front footprint. Width from paw print was measured by drawing a line at a 90° angle from the line connecting the previous stride and the paw being analyzed. The distance was recorded as length of line from paw to the stride line opposite the paw print. Mice attaining a score of 3 were eliminated from analysis as measurements could not be taken for a foot not being used in forward motion.

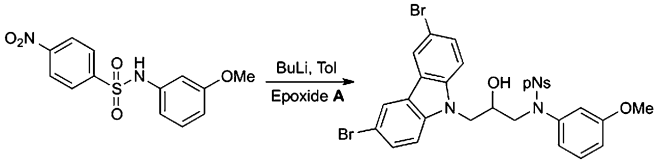
Neurological Scoring. Neurological scoring was performed every day starting with compound treatment at 80 d and was determined as follows: 0, full extension of hind legs away from lateral midline when the test mouse was suspended by its tail and could hold this for 2 s, suspended two to three times; 1, collapse or partial collapse of leg extension toward lateral midline (weakness) or trembling of hind legs during tail suspension; 2, toes curl under at least twice during walking of 12 inches or any part of foot drags along cage bottom/table; 3, rigid paralysis or minimal joint movement, foot not being used for forward motion; and 4, mouse cannot right itself within 30 s from either side. Upon reaching a score of 2, animals were given a fresh Petri dish with wet food in the dish daily. When mice achieved a score of 4 for two consecutive days, they were euthanized.

Synthesis and Preparation of P7C3A20. Scheme S1. Synthesis of P7C3-A20 3,6-dibromo-9-(oxiran-2-ylmethyl)-9H-carbazole.



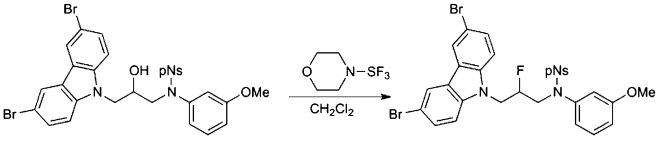
P7C3-A20 was synthesized using a modification of a procedure described previously (1). Thus, following a literature procedure (2), powdered KOH (0.103 g, 1.85 mmol) was added to a solution of 3,6-dibromocarbazole (0.500 g, 1.54 mmol) in DMF (1.5 mL) at ambient temperature and stirred for 30 min until dissolved. Epibromohydrin (0.32 mL, 3.8 mmol) was added via syringe and the reaction was stirred at room temperature overnight. Upon completion, the solution was partitioned between EtOAc and H_2O. The aqueous layer was washed three times with EtOAc, and the combined organics were washed with saturated aqueous NaCl, dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude residue was recrystallized from EtOAc/Hexane to afford epoxide **A**, 3,6-dibromo-9-(oxiran-2-ylmethyl)-9H-carbazole (80%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.10 (d, 2H, J = 2.0 Hz), 7.54 (dd, 2H, J = 2.0, 8.5 Hz), 7.31 (d, 2H, J = 8.5 Hz), 4.62 (dd, 1H, J = 2.5, 16.0 Hz), 4.25 (dd, 1H, J = 5.5, 16.0 Hz), 3.29 (m, 1H), 2.79 (dd, 1H, J = 4.0, 4.5 Hz), 2.46 (dd, 1H, J = 2.5, 5.0 Hz).

Scheme S2. *N*-(3-(3,6-dibromo-9H-carbazol-9-yl)-2-hydroxypropyl)-*N*-(3-methoxyphenyl)-4-nitrobenzenesulfonamide.



A heterogeneous mixture of *N*-(4-methoxyphenyl)-4-nitrobenzenesulfonamide (100.2 mg, 0.32 mmol) in toluene (2.5 mL, 0.13 M) under a N_2 atmosphere was cooled in a dry ice/acetone bath before dropwise addition of *n*-butyllithium (200 μL of 1.78 M in hexanes, 0.36 mmol). The reaction was stirred at –78 °C for 10 min before addition of epoxide **A**. The heterogeneous mixture was stirred at room temperature for 5 min before heating at 100 °C for 48 h. The cooled reaction was diluted with EtOAc and washed three times in 5% acetic acid solution, followed by a brine wash. The organic layer was dried over Na_2SO_4, filtered, and condensed. The crude mixture was purified by chromatography (SiO_2, 100% CH_2Cl_2) to afford the ring-opened product (88%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.23 (d, 2H, J = 8.5 Hz), 8.06 (d, 2H, J = 1.9 Hz), 7.65 (d, 2H, J = 8.5 Hz), 7.46, (dd, 2H, J = 1.9, 8.6 Hz), 7.22 (d, 2H, J = 8.8 Hz), 6.94 (d, 2H, J = 8.8 Hz), 6.83 (d, 2H, J = 9.1 Hz), 4.44 (dd, 1H, J = 3.6,14.9 Hz), 4.26–4.34 (m, 1H), 4.17–4.24 (br s, 1H), 3.81 (s, 3H), 3.62–3.75 (m, 2H). ESI m/z: 732.0 ($[M+HCOO]^-$, $C_{29}H_{24}Br_2N_3O_8S$ requires 732.0).

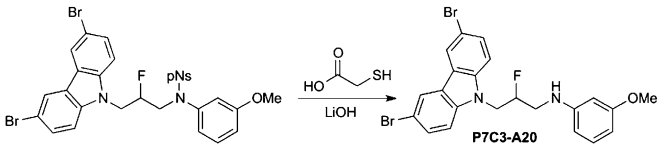
Scheme S3. *N*-(3-(3,6-dibromo-9H-carbazol-9-yl)-2-fluoropropyl)-*N*-(3-methoxyphenyl)-4 nitrobenzenesulfonamide.



The ring-opened product from the previous step was fluorinated as follows: An oven-dried 20-mL scintillation vial containing Ns-A (18.3 mg, 0.027 mmol) was purged with N_2 and charged with anhydrous CH_2Cl_2 (1.5 mL, 0.018 M). The sealed vial was cooled in a dry ice acetone bath before the dropwise addition of morpholinosulfur trifluoride (morpho-DAST, 0.053 mmol). The reaction temperature was maintained at –78 °C for 1 h and then slowly warmed to room temperature and stirred overnight. The reaction was quenched with 2.0 mL of saturated $NaHCO_3$ solution and diluted with 6 mL CH_2Cl_2 and extracted three times. The combined organics were dried over Na_2SO_4, filtered, and condensed. The crude product was carried forward without further purification. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.28 (d, 2H, J = 8.0 Hz), 8.13 (s, 2H), 7.72 (d, 2H, J = 8.7 Hz), 7.54, (d, 2H, J = 8.0 Hz), 7.21 (d, 3H, J = 8.1 Hz), 6.89 (dd, 1H, J = 2.4,

8.3 Hz), 6.67 (t, 1H, J = 2.0 Hz), 6.55 (d, 1H, J = 8.0 Hz), 4.93 (m, 1H), 4.43–4.68 (m, 2H), 4.20 (t, 1H, J = 6.2 Hz), 3.81–3.99 (m, 2H), 3.75 (s, 3H). ESI m/z: 733.9 ([M+HCOO]–, $C_{29}H_{23}Br_2FN_3O_7S$ requires 734.0).

Scheme S4. *N*-(3-(3,6-dibromo-9H-carbazol-9-yl)-2-fluoropropyl)-3-methoxyaniline (P7C3-A20).



Lithium hydroxide (3.2 mg, 0.134 mmol), DMF (0.5 mL, 0.06 M), and mercaptoacetic acid (4.2 μL, 0.060 mmol) were added to a vial containing nosyl-protected P7C3-A20 (21.0 mg, 0.030 mmol). After stirring at room temperature for 1 h, the reaction mixture was diluted with EtOAc and washed sequentially with H_2O, saturated aqueous $NaHCO_3$, H_2O (three times), and brine. The organic layer was dried over Na_2SO_4, filtered, and condensed. The crude reaction mixture was purified (SiO_2, 30% EtOAc/Hexanes + 0.2% Et_3N), to afford **P7C3-A20** (13.6 mg, 88%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.16 (d, 2H, J = 2.0 Hz), 7.56 (dd, 2H, J = 1.9, 8.7 Hz), 7.31 (d, 2H, J = 8.6 Hz), 7.11 (t, 1H, J = 8.1 Hz), 6.36 (dd, 1H, J = 2.2, 8.1 Hz), 6.23 (dd, 1H, J = 2.0, 8.0 Hz), 6.15 (t, 1H, J = 2.3 Hz), 5.11 (dddd, 1H, J = 4.6, 5.8, 10.4, 47.7 Hz), 4.60 (m, 2H), 4.39 (dm, 2H), 3.95 (t, 1H, J = 6.3 Hz), 3.75 (s, 3H). ESI m/z: 504.9 ($[M+H]^+$, $C_{22}H_{20}Br_2FN_2O$ calculated 505.0).

Pharmacokinetic Analysis of P7C3, P7C3A20, and Dimebon. Eighty-four-day-old G93A-SOD1 mice dosed daily with test compound at 20 mg/kg i.p. for 21 d were used for pharmacokinetic analysis of total P7C3, P7C3A20, and Dimebon levels in plasma, spinal cord, and brain. Six hours after the final compound dose, animals were given an inhalation overdose of CO_2 and whole blood and brain were collected. Plasma was prepared from blood and stored along with brain tissue at −80 °C until analysis. Brain and spinal cord homogenates were prepared by homogenizing the tissues in a threefold volume of PBS. Total tissue homogenate volume was estimated as volume of PBS added plus volume of brain in milliliters. For P7C3, 100 μL of plasma or brain was mixed with 400 μL of acetonitrile containing formic acid and the internal standard (IS), N-benzylamide (Sigma-Aldrich; lot 02914LH) to precipitate plasma or tissue protein and release bound drug. Conditions for P7C3A20 were similar, but used 200 μL of acetonitrile with formic acid and IS. Dimebon samples were mixed with 200 μL of methanol containing formic acid and IS. Final concentrations of formic acid and IS were 0.1% and 25 ng/mL, respectively. Extraction conditions were optimized before pharmacokinetic analysis for efficient and reproducible recovery over a 3-log range of concentrations. The samples were vortexed 15 s, incubated at room temperature for 10 min, and spun two times at 16,100 × g in a standard refrigerated microcentrifuge. The supernatant was then analyzed by liquid chromatography-tandem mass spectrometry (LC/MS/MS). Standard curves were prepared by addition of the appropriate compound to plasma or brain homogenate. A value of three times above the signal obtained in the blank plasma or brain homogenate was designated the limit of detection (LOD). The limit of quantitation (LOQ) was defined as the lowest concentration at which back calculation yielded a concentration within 20% of the theoretical value and above the LOD signal. The LOQ values for plasma and brain were as follows: P7C3, 1 ng/mL and 5 ng/mL, respectively; P7C3A20, 1 ng/mL for both; and Dimebon, 0.5 ng/mL and 1 ng/mL, respectively. Compound levels were monitored by LC/MS/MS, using an AB/Sciex 3200 Qtrap mass spectrometer coupled to a Shimadzu Prominence LC. The compounds were detected with the mass spectrometer in multiple-reaction monitoring (MRM) mode by following the precursor to fragment ion transition 474.9 → 337.8 for P7C3 [positive (pos.) mode; M+H+], 507.0 → 204.1 for P7C3A20 (pos. mode; M+H+), and 320.3 → 277.3 for Dimebon (pos. mode; $[M+H]^+$). The IS, *n*-benzylbenzamide, was monitored using a 212.1 → 91.1 transition. An Agilent XDB C18 column (50 × 4.6 mm, 5-μm packing) was used for chromatography with the following conditions: buffer A, dH_20 + 0.1% formic acid; buffer B, methanol + 0.1% formic acid, 0–1.5 min, 0% B, 1.5- to 2.5-min gradient to 100% B, 2.5–3.5 min to 100% B, 3.5- to 3.6-min gradient to 0% B, and 3.6–4.5 min to 0% B. Chromatography conditions were identical for all three compounds with the exception of Dimebon, where the initial and final concentrations of buffer B were set to 3%.

1. Jackson M, Ganel R, Rothstein JD (2002) Models of amyotrophic lateral sclerosis. *Curr Protoc Neurosci* 9.13. Available at www.currentprotocols.com.
2. Brooks SP, Trueman RC, Dunnett SB (2012) Assessment of motor coordination and balance in mice using the rotarod, elevated bridge and footprint tests. *Curr Protoc Neurosci*. Available at www.currentprotocols.com.
3. Shaw CE, et al. (1997) Familial amyotrophic lateral sclerosis. Molecular pathology of a patient with a SOD1 mutation. *Neurology* 49:1612–1616.
4. Da Cruz S, et al. (2012) Elevated PGC-1α activity sustains mitochondrial biogenesis and muscle function without extending survival in a mouse model of inherited ALS. *Cell Metab* 15:567–569.

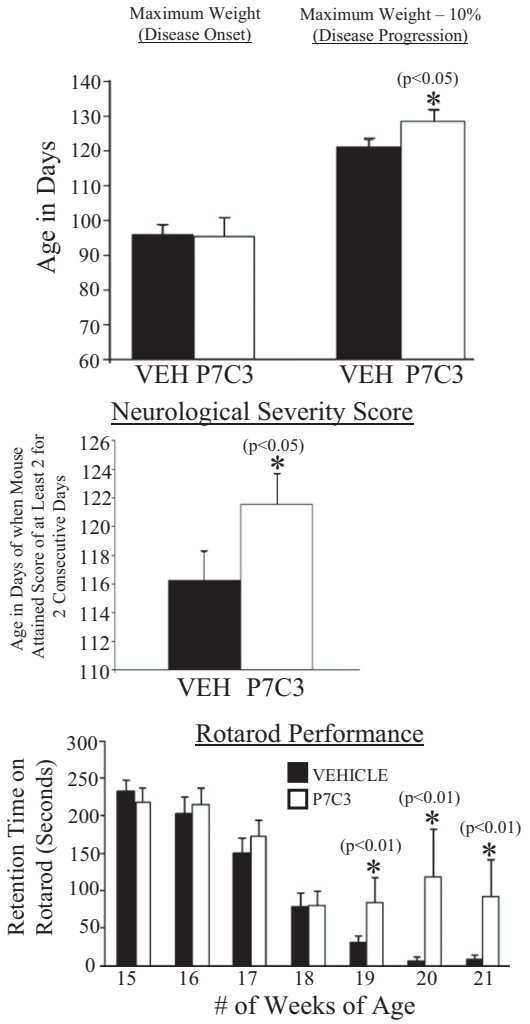


Fig. S1. Early administration (day 40) of P7C3 delays disease progression in G93A-SOD1 mutant mice. G93A SOD1 mice (beginning with $n = 30$ in each group, with all mice sibling matched across treatment groups) were treated with either vehicle or P7C3 (10 mg/kg i.p. twice daily) starting at 40 d of age. P7C3-treated mice showed a significant delay in disease progression, as evidenced by the later age by which they dropped to 10% below their maximum weight. P7C3-treated mice also attained a neurological severity score of 2 at a later age than vehicle-treated mice, again indicating that P7C3 treatment slowed disease progression. This score was determined as follows: 0, full extension of hind legs away from lateral midline when the test mouse is suspended by its tail and can hold this for 2 s, suspended two to three times; 1, collapse or partial collapse of leg extension toward lateral midline (weakness) or trembling of hind legs during tail suspension; 2, toes curl under at least twice during walking of 12 inches, or any part of paw drags along cage bottom/table; 3, rigid paralysis or minimal joint movement, paw not being used for forward motion; and 4, mouse cannot right itself within 30 s from either side. With further disease progression, vehicle-treated mice showed the expected decline in retention time on the accelerating rotarod, with retention time averaged across four trials. P7C3-treated mice, however, show a consistent trend toward improved performance on this task after onset of disease, with statistically significant improvement on days 131, 138, and 145 (*$P < 0.001$, Student's t test). All data shown are presented as mean ± SEM. All analysis was conducted blind to treatment group.

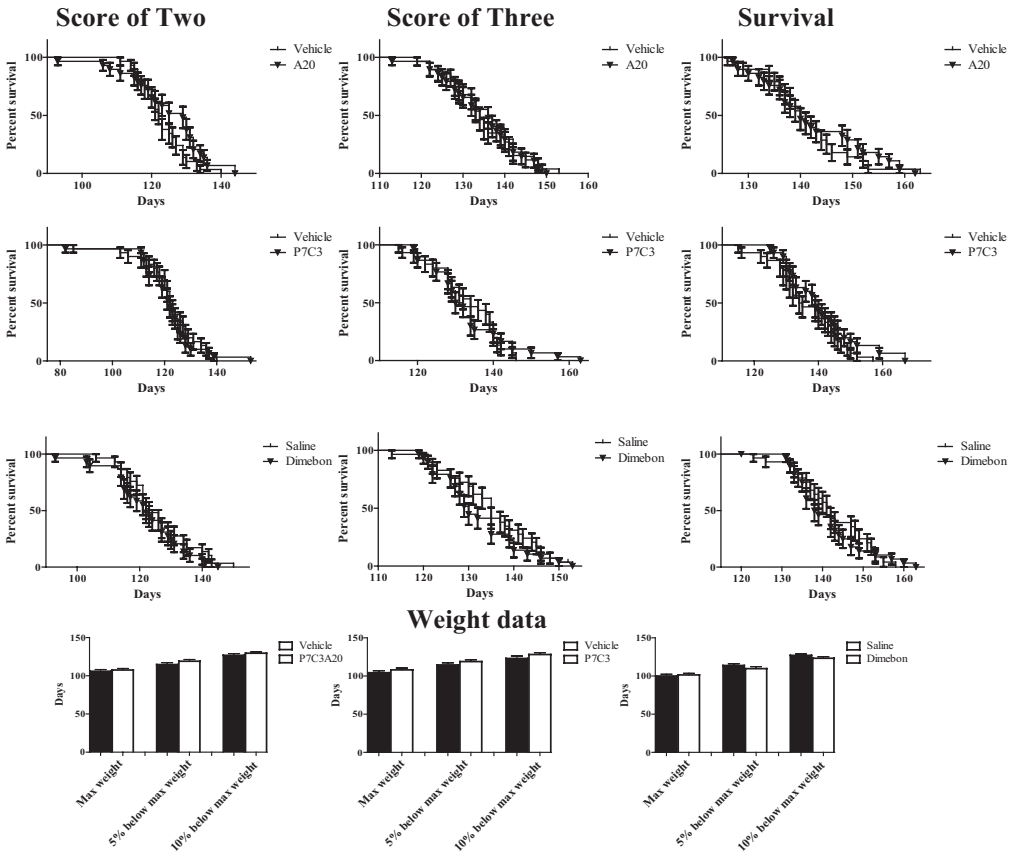


Fig. S2. Survival, neurological score, and weight loss are not improved in G93-SOD1 mutant mice when treatment with P7C3, P7C3A20, or Dimebon is initiated at the time of disease onset (day 80). G93A-SOD1 mutant mice (n = 20 per group) were tested daily for neurological score and weight change as a function of treatment with the test compounds, compared with the appropriate vehicle. There were no significant differences among treatment groups, and survival of the mice also did not vary as a function of treatment.

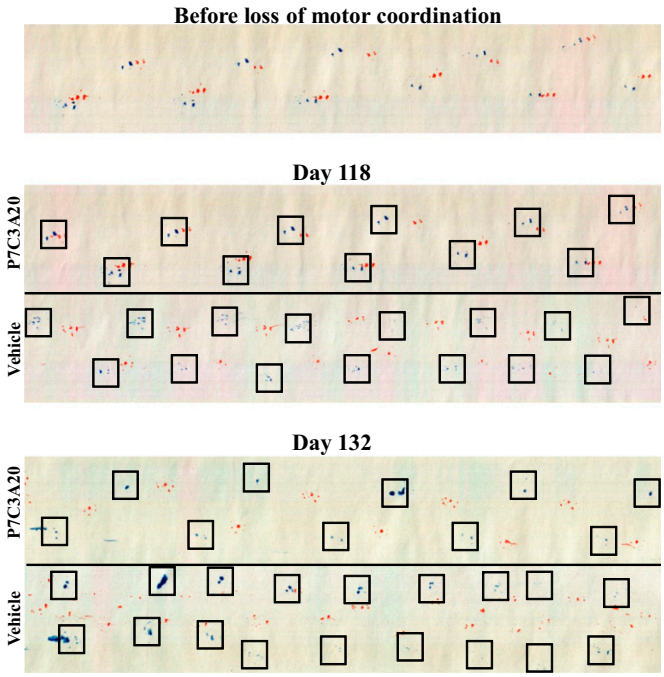


Fig. S3. Representative footprint records of G93A-SOD1 mutant mice at different ages.

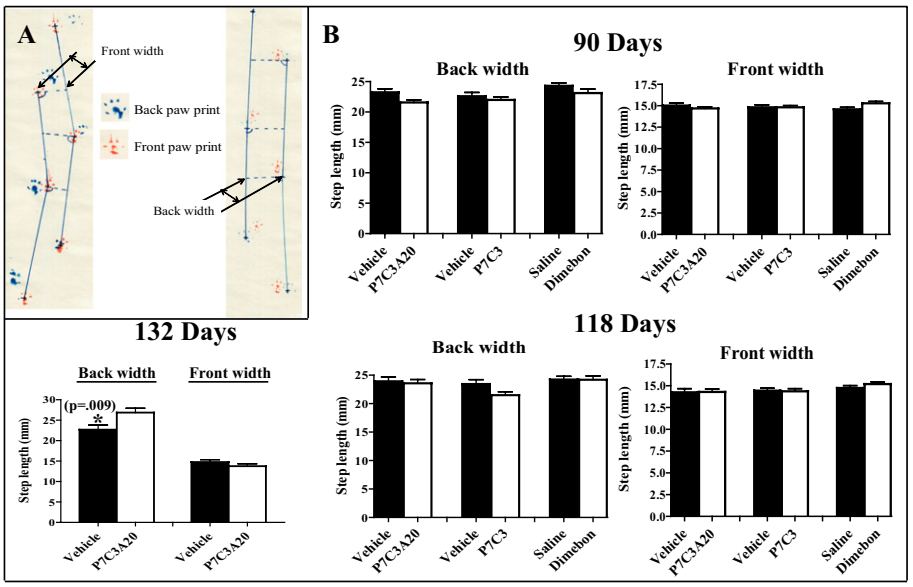


Fig. S4. Back width and front width measures of walking gait in G93A-SOD1 mutant mice. These measures show no differences as a function of disease stage or treatment group at days 90 and 118. On day 132, treatment with P7C3A20 helped preserve back width. The width from the paw print was measured by drawing a line at 90 d° from the line connected to the previous stride and the paw print being analyzed. Distance was recorded as length of the line from the paw print to the stride line opposite the paw print. All measurements were conducted blind to treatment group.

Movie S1. This movie shows a P7C3A20-treated mouse and the vehicle-treated sibling at 115 d of age. The vehicle-treated mouse falls off of the accelerating rotarod much earlier than the PC3A20-treated mouse.

Movie S1