Small molecule modulator of sigma 2 receptor is neuroprotective and reduces cognitive deficits and neuro-inflammation in experimental models of Alzheimer’s disease

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Abstract

Accumulating evidence suggests that modulating the sigma 2 receptor (Sig2R) can provide beneficial effects for neurodegenerative diseases. Herein, we report the identification of a novel class of Sig2R binding ligands and their cellular and in vivo activity in experimental models of Alzheimer’s disease (AD). We report that SAS-0132 and DKR-1051, selective ligands of Sig2R, modulate intracellular Ca\textsuperscript{2+} levels in human SK-N-SH neuroblastoma cells. The Sig2R antagonists SAS-0132 and JVW-1009 are neuroprotective in a C. elegans model of amyloid precursor protein-mediated neurodegeneration. Since this neuroprotective effect is replicated by genetic knockdown and knockout of vem-1, the ortholog of progesterone receptor membrane component-1 (PGRMC1), it indicates that Sig2R ligands modulate a PGRMC1-related pathway. Last, we demonstrate that SAS-0132 improves cognitive performance both in the Thy-1 hAPP\textsuperscript{Lond/Swe+} transgenic mouse model of AD and in healthy wild-type mice. These results demonstrate that Sig2R is a promising therapeutic target for neurocognitive disorders including AD.

Keywords

Alzheimer’s disease; AD; sigma 1 receptor; Sig1R; sigma 2 receptor; Sig2R; progesterone receptor membrane component-1; PGRMC1

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Disclosures

The authors have no conflicts of interest to declare.
INTRODUCTION

Alzheimer’s disease (AD) is currently the sixth leading cause of death in the US (Reitz & Mayeux 2014, James et al. 2014). The hallmarks of AD include neuronal loss, neuroinflammation, and the accumulation of amyloid plaques and neurofibrillary tangles (Mattson 2004, Citron 2010, Huang & Mucke 2012). The multifaceted pathology of AD makes it difficult to develop effective treatments. Current therapies for AD rely upon transiently mitigating some of its symptoms. However, these medications do not alter pathology underlying the disease (Cummings et al. 2014), and there remains an unmet need for therapeutic approaches to treat AD.

It is notable that modulating sigma receptors, for which the two known subtypes are sigma 1 (Sig1R) and sigma 2 (Sig2R) (Nguyen et al. 2014a, Matsumoto et al. 2007), has only recently been explored as a possible strategy for treating neurological disorders. Both sigma receptor subtypes are expressed in the central nervous system (CNS) and distinguished from one another based upon their affinity for different ligands and biological profiles. Sig1Rs, which have been cloned, are chaperone proteins that reside in the endoplasmic reticulum-mitochondrion interface as well as in nuclear and plasma membranes (Su et al. 2010, Hashimoto 2013, Su et al. 2016); the structure of a trimer of Sig1R has been characterized by X-ray crystallography (Schmidt et al. 2016). Although Sig2Rs have not been cloned, they are widely distributed in the CNS and are implicated in cellular processes relevant to cancer and CNS disorders (Bowen 2000, Mach et al. 2013, Huang et al. 2014, Guo & Zhen 2015). Recent photo affinity and fluorescent-probe experiments suggest that the putative Sig2R binding site is the progesterone receptor membrane component-1 (PGRMC1) protein complex (Xu et al. 2011). PGRMC1 is a cytochrome-like single-transmembrane protein that has been structurally characterized as a heme-bound dimer (Kabe et al. 2016) and is involved in neuroprotection and axonal migration (Rohe et al. 2009, Runko & Kaprielian 2004, Cahill 2007). However, other reports conclude PGRMC1 and Sig2R are distinct molecular entities (Abate et al. 2015, Chu et al. 2015). These two views are not necessarily mutually exclusive, and further work is needed to resolve this issue. Notwithstanding this controversy, we originally identified the compounds described herein as Sig2R binding ligands and found they modulate a PGRMC1-related pathway, thus we will refer to the biological target by the descriptor Sig2R/PGRMC1.

We became interested in Sig2R/PGRMC1 several years ago as a possible target for drug discovery in neurodegenerative diseases because of its widespread occurrence in the CNS and its putative involvement in calcium homeostasis. Within the CNS, Sig2R/PGRMC1 is highly expressed in the cortex and hippocampus, two regions known to be important for cognitive function (Izzo et al. 2014b, French & Pavlidis 2011). Moreover, Sig2R/PGRMC1 signaling has been implicated in cellular processes relevant to CNS diseases, including neuroprotection, axonal migration, and mitochondrial protection (Cahill 2007, Qin et al. 2015). For example, progesterone, which binds to both Sig2R and PGRMC1 (Chu et al. 2015), exerts neurogenic and neuroprotective effects in neural progenitor cells derived from adult rat brain (Liu et al. 2009). In the context of AD-related pathology, afobazole, which is a pan-selective Sig1R-Sig2R ligand, reduces neurotoxic microglia stimulation and apoptosis induced by fragments of amyloid beta (Aβ) (Behensky et al. 2013). Recent work conducted
by Cognition Therapeutics also show that Aβ oligomers are Sig2R/PGRMC1 ligands and that blocking the binding of these oligomers to Sig2R/PGRMC1 mitigates the synaptotoxic effects of Aβ oligomers (Izzo et al. 2014a, Izzo et al. 2014b). Moreover, exposure of cultured neurons to Aβ oligomers produces progressive upregulation of Sig2R/PGRMC1 (Izzo et al. 2014b). This study also revealed that density of Sig2R/PGRMC1 remains unchanged in severely demented AD patients compared to the age-matched normal individuals despite a high degree of cell loss, suggesting that Sig2R/PGRMC1 may be upregulated in AD patients. These findings suggest Sig2R/PGRMC1 is involved in AD pathology. Thus, we hypothesized that ligands that modulate Sig2R/PGRMC1 may hold significant promise as medicinal agents to treat AD.

As part of a broad effort to identify novel compounds with possible applications in neuroscience (Sahn et al. 2014, Martin 2013), we screened collections of substituted heterocyclic compounds against a variety of CNS proteins in the Psychoactive Drug Screening Program (PDSP) (Besnard et al. 2012). We thus identified a subclass of substituted norbenzomorphans that bind to Sig2R with high affinity (Sahn & Martin 2012, Sahn et al. 2016). This is an important discovery because norbenzomorphans have compact molecular scaffolds and may be easily modified for structure-activity-relationship studies. Moreover, they are structurally distinct from all other known Sig2R binding ligands (Mach, Zeng, and Hawkins 2013; Huang et al. 2014; Guo and Zhen 2015). Of the initial set of compounds, SAS-0132 emerged as an attractive candidate for further study because it exhibits 9-fold selectivity for Sig2R over Sig1R, and has low off-target affinity for most other CNS-relevant targets. With this newly identified Sig2R/PGRMC1 ligand in our hands as a pharmacological tool, we tested our hypothesis that modulation of Sig2R/PGRMC1 might lead to beneficial effects in the treatment of AD.

Here, we report that the Sig2R/PGRMC1 antagonist SAS-132 produces neuroprotective, cognitive enhancing, and anti-inflammatory effects in animal models of neurodegeneration. These results suggest that Sig2R/PGRMC1 represents a promising target to treat neurodegenerative diseases.

MATERIALS AND METHODS

Intracellular calcium assay

Calcium responses were measured using a Calcium 6-QF assay kit (Molecular Devices, Sunnyvale, CA) in human SK-N-SH neuroblastoma cells as described in the Appendix supplemental information. Effects of DKR-1051 or SAS-0132 were tested at final concentrations of 0.3, 3, 10, 30, or 100 µM. Effects of SAS-0132 on the calcium response induced by DKR-1051 (100 µM) were tested at final concentrations of 0.1, 0.3, 3, 10, or 30 µM.

C. elegans strains, maintenance, and transgenesis

C. elegans were grown and maintained according to the standard method (Brenner 1974). Transgenic animals were generated through the MOSSCI technique as previously described.
C. elegans pharmacological and RNAi treatments

For pharmacological treatment alone, plates contained either vehicle or compound dissolved in DMSO (final concentration of 200 µM and 0.2% DMSO). For pharmacological and RNAi co-treatment, the vem-1 (C. elegans homolog of PGRMC1) RNAi colony was selected from the Ahringer library (Genesequence) and grown overnight in LB with 50 µg/mL of carbenicillin. After 24 h, this culture was seeded onto carbenicillin-containing NGM-agar plates with or without 1 mM isopropylthiogalactoside (IPTG) for RNAi treatment. These plates also contained either vehicle or the Sig2R/PGRMC1 ligands dissolved in DMSO (final concentration of 200 µM and 0.2% DMSO). Plates that contained vehicle but did not contain IPTG were considered as sham control plates. The RNAi bacteria were induced overnight at room temperature for dsRNA expression.

C. elegans neurotoxicity assay

Well-fed, age-synchronized larvae 4 (L4) were picked onto seeded plates with the sterility compound FUdR (0.12 mM) to prevent progeny from hatching. Larvae were then allowed to age at 20°C on seeded plates for ~24 h (D1 adult), ~72 h (D3 adult) or ~116 h (D5 adult) to assess age-related neurodegeneration. Animals were immobilized on 2% agar pads and scored for neuronal health at 40× within 1 h of 0.7 mM sodium azide treatment. GFP-filled neurons were scored for neuronal health by an observer blind to condition, and were considered degenerated if they displayed shrinking and/or dimming of the soma (see Figure 3B: the VC5 neuron in the D3 animal is considered degenerated). The same observer scored neurodegeneration in each dataset. The percentage degeneration of VC4 and VC5 neurons was measured out of the total number of neurons scored (>60 neurons). The percentage that succumbed to APP-induced degeneration was compared for different groups (strain, drug condition) using planned Fisher’s exact tests (Zar 1999).

Experimental designs for in vivo studies

All animals were single-housed at the start of drug administration and kept under a reverse light-dark cycle with lights off at 8:30 AM and on at 8:30 PM in a temperature- and humidity-controlled environment and given food and water ad libitum. All experiments were in accordance with protocols approved by the Stanford University Administrative Panel for Laboratory Animal Care and conformed to the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. For pharmacokinetic (PK) study, male C57Bl/6J mice at 2–3 month age were used (Jackson Laboratory). For efficacy studies, male Thy1-hAPP\textsuperscript{Lond/Swe+} (APP\textsuperscript{Lond/Swe+}) and their age-matched (4.5 to 6.7 month-old) wild-type (WT) littermates were used as a mouse model of AD (Rockenstein et al. 2001). This transgenic line was maintained by crossing heterozygous Thy1-hAPP\textsuperscript{Lond/Swe+} mice with C57BL/6J breeders. The genotype of all mice was determined by PCR using tail-tip DNA.

For the PK study, a total of 9 C57Bl/6J mice (n=3 per route) were used. For the efficacy studies, two separate cohorts of APP\textsuperscript{Lond/Swe+} mice were used in two independent studies. In study I, a total of 43 mice were used in 4 experimental groups (n=9–12 mice per group) to...
assess the chronic daily dosing effect of SAS-0132 at 10mg/kg. In study II, a total of 38 mice were used in 8 experimental groups (n=4–5 mice per group) to assess the dose-response effects of chronic daily dosing of SAS-0132 at 3, 10, and 30mg/kg. Since the maximum feasible number of mice to be tested during a testing day is 35–45, we had to reduce the group size to 4–5 animals per group in the dose response study. For details, see Supplemental table 1.

**PK study**

Mice were dosed with 10 mg/kg of SAS-0132 intravenously, subcutaneously, or via oral gavage. Retro-orbital blood samples were collected before drug administration, and 5 min, 30 min, 1 h after drug administration. Three hours post-dose, mice were sacrificed, and blood samples were collected by cardiac puncture. Subsequently, brains were collected after perfusion with PBS. Plasma was immediately separated by centrifugation and stored at −80°C until analysis. The brain tissue samples were homogenized using distilled water and the homogenates were stored at −80°C until analysis. The concentration of SAS-0132 was then determined using LC/MS. The bioavailability was calculated as the ratio of subcutaneous or oral to intravenous area under the curve calculated up to the last measured time point ($AUC_{(0-3h)}$).

**Drug Treatment and Behavioral Testing**

SAS-0132 (purity > 95%; HPLC) was synthesized as previously described (Sahn et al. 2016). For the *in vivo* studies, solutions of SAS-0132 were freshly prepared daily before administration as described in the Appendix supplemental information. In the *in vivo* efficacy studies, APP$^{Lond/Swe+}$ and WT littermates were pseudo-randomly assigned to vehicle or drug groups based on the body weight and the locomotor activity determined in the pre-drug administration activity chamber test so that experimental groups were balanced for the body weight and locomotor activity. In the chronic daily dosing study, APP$^{Lond/Swe+}$ and WT mice were administered vehicle or SAS-0132 at a dose of 10 mg/kg once a day for 64 days. In the chronic daily dosing dose-response study, APP$^{Lond/Swe+}$ and WT mice were administered vehicle or SAS-0132 at doses of 3, 10, or 30 mg/kg daily for 60 days. Our previous studies have shown 4 weeks of treatment is sufficient to detect chronic effects of drugs with a diverse range of mechanisms in this mouse model (Nguyen et al. 2014b). Thus, all animals from the *in vivo* study cohorts were evaluated in a battery of cognitive and behavioral tests in the following order during the animal’s active cycle: Activity chamber, Social discrimination, Morris Water Maze (MWM, only in the dose-response study), and Y-maze tests as previously described during the last 4–9 weeks of drug administration (Coutellier et al. 2014, Faizi et al. 2012). See the Appendix supplemental information for behavioral testing procedure. All behavior tests were performed blindly by investigators being blind to the treatment groups and genotypes. More specifically, the animals were housed singly and were coded with animal IDs corresponding to the earmarks. All data collections were done using these codes and the data were processed and analyzed blindly based on group codes independent of treatment and genotype. The dosing was performed by dosing personnel who were not collecting experimental data from the animals. All results were re-analyzed and confirmed by a second investigator who was unaware of the experimental groups. In both efficacy studies, mice were tested in two cohorts divided
between morning and afternoon sessions with treatment groups balanced between sessions. SAS-0132 was subcutaneously administered in the late afternoon except on those days behavioral testing occurred in which mice were dosed after testing to minimize acute effects of the drug administration on behavior. No exclusion was made for the behavior test analyses with the exception of MWM. In MWM, a total of 6 mice from WT-vehicle, APP\textsuperscript{Lond/Swe+}.3 mg/kg, APP\textsuperscript{Lond/Swe+}.10 mg/kg, and APP\textsuperscript{Lond/Swe+}.30 mg/kg groups were excluded based on extensive floating and swimming inability.

**Tissue collection and biochemical analysis**

After the behavioral testing was completed, mice were sacrificed and perfused with PBS, and the brains were collected. The brains were sagittally bisected; half of the brain was frozen and stored at −80°C until use; the other half was placed overnight in 4% formalin and kept at 4°C in 30% sucrose until processed. To determine if chronic administration of SAS-0132 leads to any toxic effects, blood and organs were collected and used for blood chemistry and histopathology studies. To determine the effects of SAS-0132 on AD-related pathology, quantitative RT-PCR (qRT-PCR), ELISA and immunohistochemical analyses were performed following the previously reported methods (Schmittgen & Livak 2008, Maier et al. 2008, Johnson-Wood et al. 1997). Details are described in Appendix supplemental information.

**Statistics**

In order to obtain 80% power in our in vivo efficacy studies, we conducted a power-analysis using our historical data to detect a statistically significant difference of 20% in one single behavioral task and determined to use 9–12 mice per group when feasible.

Statistical analyses were performed with the IBM SPSS Statistical Package 22.0 and Prism 5.0 (GraphPad Software). The calcium assay and qRT-PCR data were analyzed by one-way analyses of variance (ANOVA), followed by Dunnett’s test for post-hoc analysis. *C. elegans* neurodegeneration data analyses were performed with Fisher’s exact tests. Behavioral and biochemical data analyses were performed with either t-test (Y-maze and social discrimination tests, immunohistochemistry data), repeated measures of ANOVA, or ANOVA followed by Fisher’s LSD or Dunnett’s post-hoc analyses (MWM). In all cases, outliers were excluded according to Grubbs’ test and p < 0.05 was considered to be significant.

**RESULTS**

**Biological properties of SAS-0132 and related Sig2R/PGRMC1 binding ligands in vitro**

**Sigma receptor binding affinities of some norbenzomorphans—**SAS-0132 (Figure 1) is a member of a novel family of Sig2R binding ligands with a 9-fold preference for Sig2R (K\textsubscript{i} 90 nM) over Sig1R (K\textsubscript{i} 841 nM) (Sahn et al. 2016). It also exhibits low off-target affinity (K\textsubscript{i} > ~0.5 to 10 µM) for most CNS-relevant targets, including opioid, adrenergic, nACR, mAChR, and NMDA receptors, as well as the neurotransmitter transporters and ion channels (Supplemental table 2). Two structurally related compounds, DKR-1005 and DKR-1051 also show similar degree of selectivity for Sig2R over Sig1R,
whereas another structural analog JVW-1009 is approximately equipotent for both SigR subtypes (Figure 1).

**Effects of Sig2R binding ligands on intracellular calcium**—Sig2R agonists have been reported to modulate intracellular Ca\(^{2+}\) levels (Vilner & Bowen 2000, Cassano et al. 2009). Thus, we tested the effects of DKR-1051 and SAS-0132 on intracellular Ca\(^{2+}\) levels in human SK-N-SH neuroblastoma cells. DKR-1051 induces concentration-dependent increases in intracellular Ca\(^{2+}\) concentration, suggesting that it has agonist properties (Figure 2A). SAS-0132 fails to produce a significant calcium response at concentrations up to 30 µM, but induces significant increases in intracellular Ca\(^{2+}\) at 100 µM (Figure 2B). At 100 µM, the effects of SAS-0132 on intracellular Ca\(^{2+}\) are weaker than the effects of DKR-1051. As SAS-0132 has no effect on intracellular Ca\(^{2+}\) at concentrations up to 30 µM, we examined whether these lower concentrations of SAS-0132 can antagonize the calcium response induced by DKR-1051. To evaluate the antagonistic effects of SAS-0132, SAS-0132 was applied prior to adding the putative Sig2R/PGRMC1 agonist DKR-1051 in order to allow SAS-0132 to first interact with the receptor in the absence of DKR-1051. To maximize the signal window to detect antagonistic effects, we also used the high concentration of DKR-1051 (i.e., 100 µM), which produces a robust calcium response. Notably, SAS-0132 significantly attenuated the DKR-0151-induced calcium response at the lower concentrations which are not associated with significant calcium response (Figure 2C).

**Sig2R/PGRMC1 antagonists are neuroprotective**—Given that Sig2R/PGRMC1 appears to be involved in neuroprotection (Izzo et al. 2014b, Cahill 2007, Rohe et al. 2009), we queried whether any of our compounds might improve the survival of neurons in a *C. elegans* model of neurodegeneration. This novel transgenic model has a single copy insertion of human amyloid precursor protein 695 (SC_APP) under a pan-neuronal promoter in addition to the endogenous worm APP-like protein, *apl-1*. VC class neurons along the ventral cord are genetically manipulated to express a fluorescent label, so neurodegeneration in the transparent bodies of live worms can be easily monitored (Figures 3A,B). Degeneration in two cholinergic neurons, VC4 and VC5, is characterized by shrinking somas visible in both fluorescence and phase-contrast images, and dimming GFP-labeling (Figure 3B). The presence of SC_APP in this worm model increases the rate of degeneration (% degeneration out of the total number of neurons scored) of these cholinergic neurons in an age-dependent manner (Figure 3C). Notably, neuronal degradation in the SC_APP strain was significantly diminished by two different loss-of-function alleles of *vem-1*, a *C. elegans* ortholog of PGRMC1 (Figure 3D; Fisher’s exact test (n = 194–230), SC_APP strain vs. each SC_APP; *vem-1(null)* strain, p < 0.01 and 0.001, respectively). Neurodegeneration was similarly diminished by pharmacological modulation of VEM-1 with Sig2R/PGRMC1 binding ligands (Figure 3E). A 5-day treatment of SC_APP worms with SAS-0132 and the structural analog JVW-1009 (200 µM) decreased neurodegeneration on day 5 of adulthood when compared to the vehicle treated group (Figure 3E; Fisher’s exact test (n = 116–378), SAS-0132 or JVW-1009 vs. vehicle, p < 0.01). In contrast, neurodegeneration was exacerbated in SC_APP worms treated with DKR-1005 relative to the control group (Figure 3E; Fisher’s exact test (n = 110–378), DKR-1005 vs. vehicle, p < 0.01). Importantly,
JVW-1009 and SAS-0132 both reduced neurodegeneration in SC_APP C. elegans but provided no further neuroprotection in animals with vem-1 (PGRMC1) expression knocked-down by RNAi (Figure 3F; Fisher’s exact test (n = 110–142), sham vs. RNAi, p < 0.001; RNAi vs. JVW-1009 or double treatment, n.s., Figure 3G; Fisher’s exact test (n = 130–138), sham vs. RNAi, p<0.01; RNAi vs. SAS-0132 or double treatment, n.s.). This suggests that neuroprotection occurs via inhibition of a PGRMC1-mediated pathway. As the effects of the Sig2R/PGRMC1 null mutations are redundant to the effects of JVW-1009 and SAS-0132 and contrary to the effects of DKR-1051, we have classified JVW-1009 and SAS-0132 as Sig2R/PGRMC1 antagonists and DKR-1005 as a Sig2R/PGRMC1 agonist.

**SAS-0132 is bioavailable and crosses the blood-brain barrier**

The finding that the Sig2R antagonists SAS-0132 and JVW-1009 have neuroprotective effects suggested that they might mitigate neuronal damage in neurodegenerative diseases such as AD. Toward evaluating this intriguing possibility, we determined the PK properties of SAS-0132. Detectable levels of SAS-0132 were measured in plasma up to 3 h post-administration of 10 mg/kg through intravenous, subcutaneous, and oral administration (Figure 4A). The bioavailability of SAS-0132 was 45% and 7% after subcutaneous and oral administration, respectively (Figure 4B). Consistent with its favorable ClogD (3.5; pH=7.4), SAS-0132 readily crosses the blood-brain-barrier and achieves a brain concentration of 3.8 µM (subcutaneous administration) and brain/plasma ratio above 7 through all routes of administration at 3 h post-administration (Figures 4C,D). The finding that only a trace (1.6 ng/g) of SAS-0132 could be detected in brain homogenates 24 h after a single injection of SAS-0132 (10 mg/kg, subcutaneous administration) suggests that it does not accumulate in the brain.

**SAS-0132 improves cognitive performance in APP<sup>Lond/Swe+</sup> mice**

Having confirmed the bioavailability and brain permeability of SAS-0132, we assessed whether chronic treatment with SAS-0132 might alleviate the cognitive deficits associated with AD in an animal model of the disease. We selected the APP<sup>Lond/Swe+</sup> transgenic mouse model, which expresses human APP751 cDNA containing the London (V717I) and Swedish (K670M/N671L) mutations under the regulatory control of the murine Thy1 gene (Rockenstein et al. 2001, Rockenstein et al. 2007); these animals exhibit cognitive deficits in several behavioral tests (Nguyen et al. 2014b, Faizi et al. 2012, Coutellier et al. 2014). Two separate cohorts of mice were used to assess chronic daily dosing and chronic daily dosing dose-response effects of SAS-0132. Importantly, all behavioral tests were conducted prior to administration of the daily dose of SAS-0132 when SAS-0132 from the previous day’s dosing is expected to be cleared from the brain.

In the chronic daily dosing study, daily dosing of APP<sup>Lond/Swe+</sup> mice and their WT littermates with SAS-0132 (10 mg/kg) was initiated at 4.5 months of age and continued for 64 days. In the sociability test performed after 35 days of dosing, vehicle-treated WT animals showed a normal sociability by showing preference for the cup containing a mouse over the empty cup; this preference was not significantly altered by SAS-0132 treatment (Figure 5A, t-test, mouse versus empty cup, * p < 0.05). In contrast, vehicle-treated APP<sup>Lond/Swe+</sup> mice exhibited impaired sociability and showed no preference between the
mouse and empty cup (Figure 5A, t-test, mouse vs. empty cup, n.s.). The SAS-0132 treated APP\textsuperscript{Lond/Swe+} mice showed a distinct preference for the cup containing a mouse over the empty cup (Figure 5A, t-test, mouse vs. empty cup, * \( p < 0.05 \)). In the Y-maze test performed after 50 days of dosing, vehicle-treated APP\textsuperscript{Lond/Swe+} mice were impaired as assessed by low spontaneous alternation behavior in contrast to the WT mice (Figure 5B, one-sample t-test vs. 50% theoretical mean, ** \( p < 0.01 \), *** \( p < 0.001 \)). APP\textsuperscript{Lond/Swe+} mice treated with SAS-0132 showed an alternation level comparable to vehicle-treated WT mice (Figure 5B, one-sample t-test vs. 50% theoretical mean, * \( p < 0.05 \)). Total arm entries were not different among the four groups (Figure 5B).

In the chronic daily dosing dose-response study, APP\textsuperscript{Lond/Swe+} mice and their WT littermates were aged to 6.7 months prior to initiating the administration of SAS-0132 at doses of 3, 10, and 30 mg/kg for a total of 60 days. In a social discrimination task performed after 30 days of dosing, all groups show normal sociability except for WT and APP\textsuperscript{Lond/Swe+} mice treated with 10 mg/kg of SAS-0132 (Data not shown). We attribute this observation to animal variations because the higher dose of 30 mg/kg did not impair sociability. The social discrimination test was performed 1 day after the sociability test, and only APP\textsuperscript{Lond/Swe+} mice treated with 3 mg/kg of SAS-0132 showed a significant preference for a novel mouse over a familiar one (Figure 6A, t-test, familiar vs. novel). In the Y-maze test, which was performed after 45 days of dosing, vehicle-treated APP\textsuperscript{Lond/Swe+} mice showed a deficit in spontaneous alternation behavior (Figure 6B, one-sample t-test vs. 50% theoretical mean, n.s.), whereas those treated with 3 and 10 mg/kg of SAS-0132 showed normal spontaneous alternation behavior (Figure 6B, one-sample t-test vs. 50% theoretical mean, n.s.).

Administration of 3 and 10 mg/kg of SAS-0132 to WT mice did not affect their performance, but those dosed with 30 mg/kg showed impaired alternation behavior, suggesting that high doses of SAS-0132 might have adverse effects on memory (Figure 6B, one-sample t-test vs. 50% theoretical mean, n.s.). The number of total arm entries was not different among groups (Figure 6B). The MWM test was initiated after 35 days of dosing. During the hidden platform learning period, WT mice treated with 3 and 30 mg/kg of SAS-0132 found the escape platform faster than the vehicle-treated WT mice (Figure 6C, repeated measures of ANOVA, * \( p < 0.05 \), Dunnett’s post-hoc analysis, * \( p < 0.05 \)). When the platform was removed, vehicle-treated WT mice did not show target quadrant preference, whereas WT mice treated with 3 mg/kg of SAS-0132 showed a clear preference for the target quadrant (Figure 6C, t-test, target vs. non-target quadrant, * \( p < 0.01 \)). Administration of SAS-0132 had no effect on the cognitive functions of APP\textsuperscript{Lond/Swe+} animals during the hidden platform learning phase (Figure 6D). After the spatial learning phase, the reversal learning hidden platform trial was performed. On day 2 of this training, WT mice treated with 10 and 30 mg/kg of SAS-0132 found the escape platform faster than WT mice treated with vehicle (Figure 6E, one-way ANOVA, * \( p < 0.05 \), Fisher’s LSD, * \( p \leq 0.05 \)). With the exception of the group treated with 3 mg/kg of SAS-0132, all WT groups demonstrated recall and showed target quadrant preference during the reversal learning probe trial (Figure 6E, paired t-test, target versus non-target quadrant, * \( p < 0.05 \), *** \( p < 0.001 \)). There were no significant effects of SAS-0132 treatment in APP\textsuperscript{Lond/Swe+} mice during the reversal learning trials (Figure 6F). During the reversal learning probe trial, vehicle-treated APP\textsuperscript{Lond/Swe+} mice did not show a preference for the target quadrant over the
non-target quadrants, whereas APP\textsuperscript{Lond/Swe+} mice treated with 10 mg/kg of SAS-0132 showed target quadrant preference, indicating that SAS-0132 treatment reversed the reversal training probe deficits in the APP\textsuperscript{Lond/Swe+} group (Figure 6F, paired t-test, target vs. non-target quadrant, * \( p < 0.05 \)).

In both chronic daily dosing and chronic daily dosing dose-response studies, animals treated with SAS-0132 remained healthy and exhibited no detectable abnormalities. Locomotor activity measured in the activity chamber test after 24 days of treatment was not significantly different between vehicle- and SAS-0132-treated mice, indicating that general locomotor activity were not affected by SAS-0132 (data not shown). Comprehensive blood chemistry and histopathology studies conducted using plasma and organs obtained from WT mice treated with vehicle and SAS-0132 (10 mg/kg/d for 64 days) revealed no signs of SAS-0132 induced toxicity (Supplemental tables 3–4).

**Effects of SAS-0132 treatment upon Aβ pathology, synaptic loss, and neuroinflammation**

Upon completion of the behavioral tests, we queried whether the cognitive-enhancing effects of SAS-0132 might be attributable to its effects on AD-related pathological features. In vehicle treated APP\textsuperscript{Lond/Swe+} mice from the chronic daily dosing study (6.5 months old at tissue collection), increased intracellular Aβ immunoreactivity was observed in the cortex, hippocampus, and amygdala, relative to vehicle-treated WT group (Supplemental figure 1A, *** \( p < 0.001 \) vs. WT groups, independent samples t-test). APP\textsuperscript{Lond/Swe+} animals treated with SAS-0132 displayed Aβ immunoreactivity similar to that seen in the vehicle-treated APP\textsuperscript{Lond/Swe+} groups, indicating that chronic treatment with SAS-0132 does not affect levels of intracellular Aβ (Supplemental figure 1A). Similarly, ELISA analysis using brains of these animals revealed no significant difference in soluble and insoluble Aβ40 and Aβ42 between vehicle- and SAS-0132-treated APP\textsuperscript{Lond/Swe+} mice (Supplemental figure 1B, independent samples t-test). Collectively, these data indicate that SAS-0132 does not affect Aβ burden in the APP\textsuperscript{Lond/Swe+} mouse model of AD.

Because Aβ is known to be toxic to synapses and to induce a neuroinflammatory response (Shankar et al. 2007, Koffie et al. 2009, Meyer-Luehmann et al. 2008, Halle et al. 2008), we determined whether SAS-0132 affects synaptic pathology and the neuroinflammatory response. Quantifying the immunoreactivity of the pre-synaptic marker synaptophysin revealed a significant reduction in synaptophysin levels in the CA3 region of APP\textsuperscript{Lond/Swe+} mice compared to WT mice (chronic daily dosing study cohort, 6.5 months old at tissue collection), but chronic treatment with SAS-0132 (10 mg/kg/d) did not alter the extent of synapse loss (Supplemental figure 2). With respect to neuroinflammation, quantitative immunohistochemistry with iba1 did not reveal any differences in microglia/monocyte-derived macrophages in vehicle-treated APP\textsuperscript{Lond/Swe+} mice relative to vehicle-treated WT mice (chronic daily dosing study cohort, 6.5 months old at tissue collection). There were no drug-related effects on immunoreactivity for iba1 in either WT or APP\textsuperscript{Lond/Swe+} mice (Supplemental figure 2). On the other hand, qRT-PCR analysis revealed that APP\textsuperscript{Lond/Swe+} animals had elevated inflammatory markers TNFa, CD14, and IL1β at 8.75 months (dose-response cohort), although elevation of the IL1β was not significant at 8.75 months (dose-
response cohort) (Figure 7). Notably, treatment with SAS-0132 decreased the expression of the inflammatory cytokine IL1β (Figure 7).

**DISCUSSION**

In this study, we report the identification of a novel norbenzomorphan class of Sig2R/PGRMC1 ligands and their cellular and in vivo activity. The Sig2R/PGRMC1 ligands reported here have K_i values in the low nanomolar range, which are comparable to the K_i values of other known Sig2R/PGRMC1 ligands (Guo & Zhen 2015, Huang et al. 2014, Vilner & Bowen 2000). The three ligands (i.e. SAS-0132, DKR-1005, and DKR-1051) display selectivity for Sig2R/PGRMC1 over the Sig1R, with selectivity ratios greater than 9. The discovery of this novel class of Sig2R/PGRMC1 ligands is of great importance, as these norbenzomorphans have favorable pharmacokinetic attributes required for CNS indications. In addition, they have compact molecular scaffolds and are amenable to structure-activity relationship studies. Thus, norbenzomorphan analogs may be promising candidates for further development into drug leads.

Regulation of intracellular Ca^{2+} is one important cellular process mediated by Sig2R/PGRMC1, and structurally diverse Sig2R agonists are known to promote the release of calcium from the endoplasmic reticulum and mitochondria (Cassano et al. 2009, Vilner & Bowen 2000, Brent et al. 1996). We have shown that DKR-1051 produces concentration-dependent increases in intracellular Ca^{2+} levels, suggesting that DKR-1051 has agonist properties (Figure 2A). The other Sig2 binding ligand SAS-0132 failed to produce a significant calcium response at up to 30 µM. At 100 µM, however, SAS-0132 increased intracellular Ca^{2+} levels, but its effects on intracellular Ca^{2+} levels were weaker than the effects of DKR-1051 (Figures 2A–2B). Interestingly, SAS-0132 attenuated the calcium response induced by DKR-1051 at the lower concentrations that have no effect on intracellular Ca^{2+} levels alone (Figure 2C). Collectively, this observation suggests that SAS-0132 is an antagonist of Sig2R/PGRMC1 that has partial agonist activity. At the molecular level, Sig2R/PGRMC1 could potentially modulate intracellular Ca^{2+} by indirectly influencing the activity of ion-channels expressed in the endoplasmic reticulum, mitochondria, and cell membrane (Vilner & Bowen 2000). As Sig2R/PGRMC1 is localized in high density to subcellular fractions known to store calcium (Basile et al. 1992), another possibility is that Sig2R/PGRMC1 directly gate the release of calcium. Further studies will be needed to determine the exact mechanisms by which Sig2R agonists increase intracellular calcium concentrations. Sig2R/PGRMC1-mediated calcium mobilization has important clinical implications because intracellular Ca^{2+} plays a critical role in learning and memory, and is strongly implicated in neuronal health (Bezprozvanny 2009). Perturbed Ca^{2+} homeostasis also appears to be involved in the dysfunction and death of neurons (Demuro et al. 2010, Mattson 1994), and its probable role in AD pathogenesis is supported by studies of animal models of AD as well as of patients (Bezprozvanny & Mattson 2008, Zhang et al. 2015, Chakroborty et al. 2012). Because Ca^{2+} dyshomeostasis is a pathological feature of AD and other neurodegenerative diseases (Berridge 2010), restoring Ca^{2+} homeostasis via modulation of Sig2R/PGRMC1 may be a promising new approach to treat neurodegenerative disorders.
**C. elegans** is well-suited as a model organism for human diseases because it has clear orthologs for two-thirds of all human genes (Hulme & Whitesides 2011, Consortium 1998, Sonnhammer & Durbin 1997, Lai et al. 2000, Kuwabara & O’Neil 2001). Relevant to our study, *C. elegans* expresses *vem-1*, an ortholog of PGRMC1, as well as other genes involved in AD-related pathways (Link 2006, Simonsen et al. 2012, Safra et al. 2013, Haynes & Ron 2010, Runko & Kaprielian 2004). Our results demonstrate that single copy insertion of the human APP gene into the *C. elegans* genome leads to age-dependent degeneration of cholinergic neurons. While previous studies have not examined age-dependent neurodegeneration, multi-copy overexpression of the *C. elegans* ortholog of APP (*apl-1*) or human APP has been shown to cause behavioral dysfunction and partial lethality in *C. elegans* (Hornsten et al. 2007, Ewald et al. 2012). Importantly, the neurodegeneration we observed in transgenic APP worms was diminished by genetic manipulation of PGRMC1 or by pharmacological inhibition with SAS-0132 and JVW-1009. Conversely, neurodegeneration was exacerbated by treatment with the agonist DKR-1005 (Figure 3). These findings provide *in vivo* genetic evidence that PGRMC1 is critically involved in APP-mediated neurodegeneration, and the Sig2R antagonists SAS-0132 and JVW-1009 exert neuroprotection via a PGRMC1-related pathway.

In chronic daily dosing and chronic daily dosing dose-response studies, significant cognitive enhancing effects of SAS-0132 were observed in both transgenic and WT animals. In the chronic daily dosing study, SAS-0132 rescued AD-related sociability deficits and spatial memory deficits (Y-maze-test). In the chronic daily dosing dose-response study, SAS-0132 rescued deficits in the social discrimination test (3 mg/kg) as well as deficits in spatial memory in the Y-maze test (3 mg/kg and 10 mg/kg). SAS-0132 also led to improvements in spatial and long-term memory in both WT (3, 10 and 30 mg/kg) and APP<sub>Lond/Swe+</sub> mice (10 mg/kg) in the MWM test. Although the cognitive enhancing effects of SAS-0132 shown in the chronic daily dosing dose-response study should be interpreted cautiously due to the small sample size, it is noteworthy that the effects of SAS-0132 were sufficiently large to be detected despite the low statistical power. Importantly, cognitive performance of the animals was evaluated prior to administration of the daily dose of SAS-0132. Because SAS-0132 is largely cleared from the brain within 24 hours, the observed improvements in cognitive performance likely arose from sustained modulation of Sig2R/PGRMC1 rather than from an acute effect from treatment with SAS-0132.

There are several mechanisms that might explain the cognition-enhancing effects of SAS-0132. First, SAS-0132 could produce pro-cognitive effects by being neuroprotective against APP-mediated neuronal toxicity. For example, the Sig2R binding ligands CT01344 and CT01346 were found to block binding of Aβ oligomers to Sig2R/PGRMC1 and restore AD-related behavioral deficits in the APP<sub>Lond/Swe+</sub> mouse model of AD (Izzo et al. 2014a). Second, by regulating intracellular calcium levels, SAS-0132 treatment may counteract Aβ related dysregulation of calcium homeostasis. For example, application of Aβ oligomers to cultured neurons induces an unregulated flux of Ca<sup>2+</sup> through the plasma membrane via various mechanisms that include activating endogenous receptors coupled to Ca<sup>2+</sup> influx, disrupting membrane lipid integrity, and forming Ca<sup>2+</sup>-permeable channels (Arispe et al. 1993, Rovira et al. 2002, Kayed et al. 2004, Demuro et al. 2010). Dysregulated Ca<sup>2+</sup> homeostasis can trigger detrimental processes such as apoptosis and free radical formation,
and these adverse events can eventually lead to cognitive deficits. Thus, with its ability to modulate intracellular Ca\(^{2+}\) concentration, SAS-0132 may lead to cognitive enhancing effects. Last, the benefits of SAS-0132 might not be exclusively related to its effects on APP related pathways, but result from more generalized mechanisms that are not specific to APP. For example, in our dose-response study, we discovered that chronic treatment of WT mice with SAS-0132 improved spatial and long-term memory in the MWM test. Although further studies are required to confirm this finding, these cognitive enhancing effects of SAS-0132 in healthy animals certainly warrant further study.

In contrast to its significant effect on cognition, SAS-0132 had no discernible effects upon several markers of AD pathology, including A\(\beta\) burden and synaptic loss. As neuroinflammation is one of the main pathological features of AD, we also sought to determine the effects of SAS-0132 on neuroinflammation. In the chronic daily dosing study, we were unable to evaluate the effects of SAS-0132 on AD-related neuroinflammation, as APP\(^{Lond/Swe+}\) mice showed little evidence of neuroinflammation. Although this finding is interesting as high levels of A\(\beta\) and behavioral deficits associated with AD are present in this APP\(^{Lond/Swe+}\) mice at this age, it is in line with a previous study that reported the lack of signs of neuroinflammation in this mouse model at 8 months of age (Lull et al. 2011). Multiple lines of transgenic models of AD have been shown to develop AD-related neuropathology in an age-dependent manner (Abbas et al. 2002, Sly et al. 2001, Oakley et al. 2006, Rockenstein et al. 2001). Thus, it is possible that the APP\(^{Lond/Swe+}\) mice model may show evidence of neuroinflammation at older age. Indeed, we observed significant increases in expression of neuro-inflammatory genes in APP\(^{Lond/Swe+}\) mice compared to WT mice in the chronic daily dosing dose-response study (8.7 months of age at tissue collection). Chronic treatment with SAS-0132 was shown to decrease AD-related neuroinflammatory responses as measured by reduced brain levels of the inflammatory cytokine IL1\(\beta\). Because chronic inflammation in response to neuronal damage is thought to accelerate and potentially underlie disease progression in AD (Heneka et al. 2015), the anti-inflammatory effects of SAS-0132 might be partly responsible for its cognitive enhancing effects.

In this study, we report that pharmacological and genetic inhibition of Sig2R is neuroprotective, cognitive enhancing, and anti-inflammatory in experimental models of AD and excessive amyloidosis. Regulation of intracellular calcium concentration by Sig2R modulation could be responsible for these beneficial effects. Further mechanistic studies will be needed to investigate the role of the Sig2R/PGRMC1 in modulation of neuroinflammation and cellular signaling, leading to neuroprotection and modulation of intracellular calcium homeostasis. The fact that the Sig2R/PGRMC1 antagonist SAS-0132 has significant benefits when it is chronically administered to symptomatic animals inspires the intriguing question of whether treatment of presymptomatic animals could prevent the development of AD and/or cognitive deficits. Our findings clearly demonstrate the significant potential of modulating pathways mediated by Sig2R/PGRMC1 as a novel mechanism to treat AD. This hypothesis is supported by recent work conducted by Izzo and coworkers, who have shown that CT01344 and CT01346: (1) block binding of A\(\beta\) oligomers to Sig2R/PGRMC1 on neurons (Izzo et al. 2014a, Izzo et al. 2014b); (2) prevent and reverse the effects of A\(\beta\) oligomers on membrane trafficking and synapse loss in neuronal culture.
(Izzo et al. 2014a); and (3) reverse cognitive deficits in a mouse model of AD (Izzo et al. 2014a). These collective discoveries suggest that modulating pathways mediated by Sig2R/PGRMC1 is meritorious as a new approach to study neurodegenerative conditions and, if shown to be safe and well tolerated, should be further evaluated.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

**Acknowledgments and dedication**

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**Abbreviations used**

- **AD** Alzheimer’s disease
- **Sig1R** sigma 1 receptor
- **Sig2R** sigma 2 receptor
- **PGRMC1** progesterone receptor membrane component-1

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http://www.proteinatlas.org/ENSG00000101856-PGRMC1/tissue


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Figure 1.
Sig2R binding ligands with neuromodulatory (JVW-1009, SAS-0132, DKR-1005, and DKR-1051) and cognition enhancing activity (SAS-0132).
Figure 2. Effects of the Sig2R ligands on intracellular calcium in SK-N-SH neuroblastoma cells
(A) DKR-1051 induces concentration-dependent increases in intracellular calcium (### \( p < 0.001 \) versus DKR-1051 (0 µM), one-way ANOVA followed by Dunnett’s multiple comparison test). (B) SAS-0132 does not induce significant increases in intracellular calcium up to 30 µM, but produces a significant calcium response at 100 µM (### \( p < 0.001 \) versus SAS-0132 (0 µM), one-way ANOVA followed by Dunnett’s multiple comparison test). (C) SAS-0132 attenuated the calcium response induced by DRK-1051 (### \( p < 0.001 \) versus DKR-1051 (0 µM); * \( p < 0.05 \) and ** \( p < 0.01 \) versus DKR-1051 (100 µM), one-way ANOVA followed by Dunnett’s multiple comparison test). Data are presented as mean ± SEM from 3–4 independent experiments performed in duplicate or triplicate.
Figure 3. Neuroprotective effects of SAS-0132

(A) In our *C. elegans* model of neurodegeneration, transgenic worms carry a single copy insertion of human amyloid precursor protein (SC_APP). VC class cholinergic neurons express GFP. (B) Representative fluorescence and phase contrast images of VC4 and 5 neurons in transgenic SC_APP *C. elegans* (on day 1 and 3 of adulthood). (C) Transgenic *C. elegans* expressing SC_APP display age-dependent degeneration of VC4 and 5 compared to wild-type (WT) controls (** p < 0.01 versus WT control, planned comparisons with Fisher’s exact test, n=60–128). (D) Neurodegeneration in these transgenic *C. elegans* is significantly diminished with two different null alleles of *vem-1*, an ortholog of PGRMC1 (** p < 0.01 and *** p < 0.001 versus transgenic hAPP animals with WT PGRMC1, planned comparisons with Fisher’s exact test, n=194–230) (E) DKR-1005 exacerbates the neurodegeneration shown in the transgenic SC_APP worm, while JVW-1009 and SAS-0132 decrease the neurodegeneration in the transgenic SC_APP worm (** p < 0.01 versus vehicle group, planned comparisons with Fisher’s exact test, n=110–378) (F) Knockdown of PGRMC1 or pharmacological modulation of Sig2R/PGRMC1 with JVW-1009 ameliorates the neurodegeneration shown in the transgenic *C. elegans* (** p < 0.01 versus vehicle group, planned comparisons with Fisher’s exact test, n=110–378).
JWW-1009(−)/PGRMC1 RNAi (−) group, planned comparisons with Fisher’s exact test, n=110–142). JWW-1009 provides no further effects in animals with reduced PGRMC1 expression. (G) Knockdown of PGRMC1 or pharmacological modulation of Sig2R/PGRMC1 with SAS-0132 ameliorates the neurodegeneration shown in the transgenic C. elegans (** p < 0.01 versus SAS-0132(−)/PGRMC1 RNAi (−) group, planned comparisons with Fisher’s exact test, n=130–138). SAS-0132 provides no further effects in animals with reduced PGRMC1 expression.
Figure 4. Pharmacokinetics of SAS-0132

(A) Plasma concentration of SAS-0132 in mice plasma collected over 3 h post-dose after a single injection of SAS-0132 (10 mg/kg) via intravenous (IV), subcutaneous (SC), and oral (PO) administration (n = 3 per route). (B) Bioavailability of SAS-0132 by SC and PO dosing (n = 3 per route). (C,D) Brain concentration and brain/plasma ratio of SAS-0132 3 h after the administration of SAS-0132 (n = 3 per route). Data are represented as mean ±SEM.
Figure 5. Effects of SAS-0132 on cognitive functions in the chronic daily dosing study
(A) In the sociability test, vehicle-treated wild-type (WT) mice explored a cup containing a C57Bl/6 mouse more than an empty cup, showing normal sociability. APP<sub>Lond/Swe+</sub> mice treated with vehicle showed lack of sociability, while APP<sub>Lond/Swe+</sub> mice treated with SAS-0132 (10 mg/kg) showed normal sociability (t-test, mouse versus empty cup, *p < 0.05, ** p < 0.01, n =9–12 per group). (B) In the Y-maze, WT mice showed spontaneous alternation behavior, indicating a normal spatial memory. The vehicle-treated APP<sub>Lond/Swe+</sub> mice showed impaired spontaneous alternation performance. The APP<sub>Lond/Swe+</sub> mice treated with SAS-0132 (10 mg/kg) showed spontaneous alternation behavior to a level comparable to the WT mice (One-sample t-test vs 50% theoretical mean, *p < 0.05, ** p < 0.01, *** p < 0.001, n=9–12 per group). The number of total entries were not significantly different among groups (one-way ANOVA). Data represent mean ± SEM. Wild-type, WT; APP<sub>Lond/Swe+</sub>, APP.
Figure 6. Effects of SAS-0132 on cognitive function in the chronic daily dosing dose-response study

(A) In the social recognition test, only the APP$^{Lond/Swe+}$ mice group treated with 3 mg/kg of SAS-0132 distinguished between a novel and familiar mouse, preferring a novel mouse (t-test, familiar versus novel, ***p < 0.001, n = 3 – 5 per group). (B) In the Y-maze test, the APP$^{Lond/Swe+}$ mice showed impaired spontaneous alternation behavior, which was restored by chronic administration of SAS-0132. Both wild-type (WT) and APP$^{Lond/Swe+}$ mice dosed with 30 mg/kg showed impaired spontaneous alternation behavior (One-sample t-test vs 50% theoretical mean, *p < 0.05, **p < 0.01, n = 3 – 5 per group). The number of total...
entries was not significantly different among groups (One-way ANOVA). (C) WT mice groups treated with 3 and 30 mg/kg SAS-0132 showed faster escape latency compared to vehicle-treated WT mice during the hidden platform training (Repeated measures of ANOVA, * $p < 0.01$ versus vehicle-treated WT, $n = 3 – 5$ per group). During the probe trial, WT mice treated with 3 mg/kg of SAS-0132 showed target quadrant preference (t-test, target versus non-target quadrant, * $p < 0.01$), while the other three groups did not show this preference ($n = 3 – 5$ per group). (D) APP$^{Lond/Swe+}$ mice showed signs of learning as indicated by preference for the target over the non-target quadrant during the probe trial, although not significant ($n = 3 – 5$ per group). Three of the five mice in the 30 mg/kg APP$^{Lond/Swe+}$ group did not perform the task. Thus, this group was excluded from the analyses. (E) On the second day of the reversal learning training, WT mice treated with 10 and 30 mg/kg SAS-0132 found the escape platform significantly faster relative to vehicle-treated WT mice (one-way ANOVA, * $p < 0.05$ post-hoc, $n = 3 – 5$ per group). During the reversal probe trial, all WT groups showed target quadrant preference with the exception of the 3 mg/kg dose group (paired t-test, * $p < 0.05$, *** $p < 0.001$, $n = 3 – 5$ per group). (F) The reversal learning training revealed no main effect of drug on learning in APP$^{Lond/Swe+}$ mice (repeated measures of ANOVA), although a non-significant decreased escape latency was observed for APP$^{Lond/Swe+}$ mice treated with 3 and 10 mg/kg SAS-0132 compared to the vehicle-treated APP$^{Lond/Swe+}$ mice ($n = 3 – 5$ per group). During the reversal probe trial, APP$^{Lond/Swe+}$ vehicle-treated mice did not show target quadrant preference. APP$^{Lond/Swe+}$ mice treated with 10 mg/kg SAS-0132 showed preference for the target over the non-target quadrant (paired t-test, ** $p < 0.01$, $n = 3 – 5$ per group). Three of five mice in the 30 mg/kg APP$^{Lond/Swe+}$ group did not pass the swimming performance criteria. Thus, this group was excluded from the analyses. Three additional mice in 3 other groups (WT-vehicle, APP$^{Lond/Swe+}$, 3 and 10 mg/kg), were also excluded for the same reason. Data represent mean ± SEM. Wild-type, WT; APP$^{Lond/Swe+}$, APP.
Figure 7. Effects of SAS-0132 on neuroinflammation in the chronic daily dosing dose-response study

TNFα, CD14, and IL1β expression was elevated in the vehicle-treated APP\textsuperscript{Lond/Swe+} mice compared to wild-type (WT) counterparts, although not significantly. SAS-0132 treatment attenuated increases in IL1β expression. Bar graphs depict mRNA expression in cortical tissue from WT and APP\textsuperscript{Lond/Swe+} mice. mRNA expression was normalized relative to vehicle-treated WT mice for each gene. Data represent mean ± SEM. n= 3–5 per group. Wild-type, WT; APP\textsuperscript{Lond/Swe+}, APP.