Glycogen Synthase Kinase-3 Inhibitors as Potent Therapeutic Agents for the Treatment of Parkinson Disease.

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S Supporting Information

ABSTRACT: Parkinson's disease (PD) is a devastating neurodegenerative disorder characterized by degeneration of the nigrostriatal dopaminergic pathway. Because the current therapies only lead to temporary, limited improvement and have severe side effects, new approaches to treat PD need to be developed. To discover new targets for potential therapeutic intervention, a chemical genetic approach involving the use of small molecules as pharmacological tools has been implemented. First, a screening of an in-house chemical library on a well-established cellular model of PD was done followed by a detailed pharmacological analysis of the hits. Here, we report the results found for the small heterocyclic derivative called SC001, which after different enzymatic assays was revealed to be a new glycogen synthase kinase-3 (GSK-3) inhibitor with $IC_{50} = 3.38 \pm 0.08 \mu M$.

To confirm that GSK-3 could be a good target for PD, the evaluation of a set of structurally diverse GSK-3 inhibitors as neuroprotective agents for PD was performed. Results show that inhibitors of GSK-3 have neuroprotective effects *in vitro* representing a new pharmacological option for the disease-modifying treatment of PD. Furthermore, we show that SC001 is able to cross the blood–brain barrier, protects dopaminergic neurons, and reduces microglia activation in *in vivo* models of Parkinson disease, being a good candidate for further drug development.

KEYWORDS: GSK-3 inhibitors, Parkinson's disease, drug discovery, neuroprotection

Parkinson's disease (PD) is a devastating neurodegenerative disorder and the second most common neurodegenerative disease after Alzheimer's disease, characterized by the loss of dopamine-producing neurons (dopaminergic neurons) in a specific brain region, the ventral midbrain. Although the selective loss of dopaminergic neurons from the substantia nigra pars compacta (SNpc) is the pathological hallmark of PD, cell loss also occurs in the locus coeruleus, dorsal nuclei of the vagus, nucleus basalis of Meynert, and some other catecholaminergic brain stem structures.¹ The cells loss leads to the deficit of dopamine in areas where these neurons project and to an inadequate control of motor function.

Even though PD is regarded as a sporadic disorder, remarkably few environmental triggers have so far been identified.^{2–5} Similar to other neurodegenerative diseases, aging is the major risk factor, although around 10% of people with the disease are younger than 45 years of age. The median age of onset is 60 years, and the mean duration of the disease from diagnosis to death is 15 years.³ An understanding of the mechanisms underlying the development and progression of PD pathology is critical for the development of neuroprotective therapies. Several mechanisms have been implicated as crucial

to PD pathogenesis: oxidative stress, mitochondrial dysfunction, protein aggregation and misfolding, inflammation, excitotoxicity, and apoptosis. No one mechanism appears to be primary in all cases of PD, and these pathogenic mechanisms likely act synergistically through complex interactions to promote neurodegeneration.^{6–8}

Current therapies are based on dopamine replacement, which reduces motor handicap and alleviates associated depression and pain. However, this pharmacological approach only leads to temporary, limited improvement of patient's quality of life, because chronic treatment with L-DOPA has severe side effects. Thus several new approaches to treat PD are under development with the aim to stop or delay the neurodegeneration that takes place in this disease.

Because our research group has great experience in the discovery and development of innovative drugs for neurodegenerative diseases,^{9–11} we follow a forward chemical genetic approach,¹² exploiting the use of small molecules as

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Figure 1. Phenotypic screening of small molecule chemical library. Typical plot obtained in our chemical genetic approach to discover new pharmacological targets for PD. Results for 27 different compounds including SC001 are depicted. SH-SY5Y cells were exposed to 35 μ M 6-OHDA during 24 h in the presence or absence of the small heterocyclic compounds (10 μ M). The number of viable cells was measured by MTT assay. Each data point represents the mean $\pm$ SD of four replications in three different experiments. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ versus 6-OHDA-treated cultures.

pharmacological tools to discover new targets for further pharmacological intervention. Moreover, the chemogenomics approach has the great advantage of revealing simultaneously some new leads for further development. First, through a blind screening of diverse heterocyclic small molecules from our in-house chemical library on a well-established cellular model of PD,¹³ we selected those compounds able to prevent dopaminergic neural cell death from the neurotoxicity caused by 6-hydroxydopamine (6-OHDA). In a second step, the selected compounds were studied in depth to discover their pharmacological target, this task being the major challenge in the chemical genetic approach used. After the phenotypic screening of 416 different compounds, we selected 39 chemically diverse molecules that were able to protect the cells for deeper *in vitro* studies.

Different enzymatic assays were performed that allow us to classify our 39 hits in different categories: (i) oxygen free radical scavengers, which are known to have positive effects in preventing or attenuating PD¹⁴ (the finding of these compounds validates the phenotypic discovery assay here used); (ii) phosphodiesterase 7 (PDE7) inhibitors, which allow us to propose PDE7 as a new and good target for PD;¹⁵ (iii) GSK-3 inhibitors. Among all these different compounds, we have selected the small heterocyclic derivative called SC001 (which we found to be a GSK-3 inhibitor) for further analysis.

The findings presented here demonstrate that GSK-3 inhibition can protect dopaminergic neurons against different

insults, and they provide support for the therapeutic potential of GSK-3 inhibitors, specifically SC001, in the treatment of PD.

RESULTS AND DISCUSSION

Screening of Chemical Library for Effects on Cell Viability in SH-SY5Y Cells. We analyzed the ability of several small molecules with different chemical scaffolds to protect the human neuroblastoma cell line SH-SY5Y from cell death induced by the toxin 6-OHDA. To this end, cells were pretreated for 1 h with 10 μ M of each compound before 6-OHDA exposure, and cell viability was analyzed 24 h later. As expected, treatment of SH-SY5Y cells with 6-OHDA resulted in a significant cell death (40%). Compounds that induced neuroprotection against 6-OHDA-induced cell death (cell death <35%) were selected for further studies. After the screening of 436 molecules from our in-house chemical library, 39 small heterocyclic derivatives (9% success) were selected as primary hits. Figure 1 shows results for 27 of these compounds including the molecule named SC001.

Target Identification for the New Hits. These 39 selected compounds, at a concentration of 10 μ M, were tested against different kinases such as GSK-3 β and CK-1 δ , phosphodiesterases such as PDE7B and PDE10A, and also as antioxidant agents following the ORAC methodology.¹⁶ In this way, we have been able to identify from the previous hits, 10 chemically diverse molecules with antioxidant properties (26% of positive hits). Because this is a well-known mechanism of action to interfere with PD, the identification of these

Figure 2. GSK-3 inhibitor focused library tested in a Parkinson's disease *in vitro* cellular model. Chemical structures and GSK-3 biological inhibition (IC₅₀ values) of the small molecules used for the target confirmation step of the chemogenomics drug/target discovery approach. SH-SY5Y cells were exposed to 35 μM 6-OHDA, 24 h in the presence or absence of the GSK-3 inhibitors at different concentrations (0.5–10 μM). The number of viable cells was measured by MTT assay. Each data point represents the mean ± SD of four replications in three different experiments. **p* ≤ 0.05, ***p* ≤ 0.01, ****p* ≤ 0.001 versus 6-OHDA-treated cultures.

Figure 3. GSK-3 inhibitors focused library tested in a Parkinson's disease *in vitro* cellular model. Chemical structures and GSK-3 biological inhibition (IC₅₀ values) of the small molecules used for the target confirmation step of the chemogenomics drug/target discovery approach. SH-SY5Y cells were exposed to 35 μM 6-OHDA, 16 h in the presence or absence of the GSK-3 inhibitors at different concentrations (0.5–10 μM). The amount of lactate dehydrogenase (LDH) released was measured. Each data point represents the mean ± SD of four replications in three different experiments. **p* ≤ 0.05, ***p* ≤ 0.01, ****p* ≤ 0.001 versus 6-OHDA-treated cultures.

compounds validates our phenotypic assay. Moreover, three different derivatives showed inhibition of PDE7B (8% of positive hits), which led us to identify PDE7 as a new target for PD.¹⁵ Thanks to this chemogenetic screening, the PDE7 inhibitor called S14 is in regulatory toxicological studies to enter into clinical trials for PD (Martinez et al. WO2010133742) and other new potential targets such as PDE10A are under evaluation. In the present work, results obtained for the evaluation candidate called SC001 are reported. Regarding GSK-3 evaluation, we have identified 8% of positive hits, and the above-mentioned target evaluation led to the discovery of a structurally diverse GSK-3 inhibitor with an IC_{50} value of $3.38 \pm 0.08 \mu\text{M}$. SC001 did not show any antioxidant activity on ORAC assay, and it showed 12%, 14%, and 2% inhibition of CK-1 δ , PDE7A, and PDE10A, respectively.

Effects of Different GSK-3 Inhibitors on Cell Viability in SH-SY5Y Cells. To span the biological relevance of GSK-3 as a valid target for PD, we selected a focused small chemical library comprising different chemical and biological GSK-3 inhibitors for this study. Among these, we have tested paullones (alsterpaullone) and maleimides (VP 3.36, VP 3.16, and VP 3.40)¹⁷ as ATP competitive GSK-3 inhibitors, two different iminotriazoles (VP 1.14 and VP 1.16) as substrate competitive GSK-3 inhibitors,¹⁸ the bromomethylmaleimide VP 3.35 as an irreversible GSK-3 inhibitor,¹⁷ and the quinoline VP0.7 as an allosteric GSK-3 modulator.¹⁹ The chemical structures of all these compounds together with the values of their IC_{50} are depicted in Figure 2. The ability to protect the SH-SY5Y cell line from cell death induced by the toxin 6-OHDA was evaluated. To this end, cells were pretreated for 1 h with different doses of the GSK-3 inhibitors, including SC001, before 6-OHDA exposure. Cell viability was analyzed 24 h later. While treatment of SH-SY5Y cells with 6-OHDA resulted in a significant cell death (around 40%), addition of the compounds induced neuroprotection against 6-OHDA albeit at different concentrations due to different IC_{50} values and cell membrane permeability for the different inhibitors. Similar data were obtained when liberation of lactate dehydrogenase (LDH) was measured (Figure 3). We observed that incubation with these compounds afforded significant protection against 6-OHDA-induced cell death, lowering elevated LDH levels. These data confirm that GSK-3 is a target pharmacologically modulable to protect against neuronal cell death from 6-OHDA toxicity.

Neuroprotective and Anti-inflammatory Effects of SC001. To further evaluate the possible role of SC001 in other models of neuronal cell death, we performed experiments using murine hippocampal HT22 cells. We tested the capacity of SC001 to prevent HT22 cell death induced by glutamate. As shown in Figure 4A, glutamate elicited an induction in the number of cells dead. The addition of SC001 protected neuronal cultures of glutamate-induced excitotoxicity. These observations indicate that the protective role of SC001 is not restricted to a specific insult.

Because GSK-3 is involved in microglial activation,²⁰ the potential anti-inflammatory activity of SC001 was next tested by evaluating the production of nitrites from cultured microglial cells. Microglia were incubated with SC001 for 1 h and then cells were cultured for another 24 h with LPS. As shown in Figure 4B, SC001 significantly inhibited the production of nitrites. The effects of the compound were not caused by a loss of cell viability, because 24-h exposure of microglial cells to SC001 did not alter cell viability (data not shown). These data

Figure 4. Neuroprotective and anti-inflammatory effect of SC001 *in vitro*. (A) Neuronal HT22 cells were exposed to glutamate (3 mM) for 24 h in the presence or absence of SC001 (10 μM), and the number of viable cells was measured by MTT assay. (B) Glial cell cultures were treated with LPS (10 $\mu\text{g}/\text{mL}$) in the presence or absence of SC001 (10 μM), and the production of nitrite was measured by the Griess reaction. Each data point represents the mean $\pm$ SD of four replications in three different experiments. *** $p \leq 0.001$, versus glutamate- or LPS-treated cultures.

suggest that SC001 helps to modulate the production of proinflammatory mediators in microglial cells.

SC001 Penetrates into the Brain in an *in Vitro* Experimental Model. One of the main obstacles for the treatment of diseases of the central nervous system (CNS) is the capacity of the drugs to penetrate into the brain through the blood–brain barrier (BBB) at therapeutic concentrations. Therefore the ability of the candidate SC001 to enter in the brain by passive diffusion was experimentally evaluated. Parallel artificial membrane permeation assay (PAMPA methodology) is a high-throughput technique developed to predict passive permeability through biological membranes (see Supporting Information). Here, we used the PAMPA-BBB method described by Di et al.,²¹ which employs a brain lipid porcine membrane. The *in vitro* permeabilities (P_e) of commercial drugs through lipid membrane extract together with compound SC001 were determined and are described in Supplementary Table 1, Supporting Information. An assay validation was made comparing the reported permeability values of commercial drugs with the experimental data obtained employing this methodology. A good correlation between experimentally described values was obtained $P_e(\text{exptl}) = 1.5631(\text{bibl}) - 1.6741$ ($R^2 = 0.9842$) (Supplementary Figure 1, Supporting Information). From this equation and following the pattern established in the literature for BBB permeation prediction,²² we could classify compounds as CNS+ when they present a permeability $>4.47 \times 10^{-6} \text{ cm s}^{-1}$. Based on these results, we can consider that compound SC001 is able to cross the BBB by passive permeation (Supplementary Table 1, Supporting Information).

Effect of SC001 in *in vivo* Models of Parkinson's Disease. To further substantiate the neuroprotective role of GSK-3 inhibitors at the *in vivo* level and in view of the BBB results obtained, we next examined the effect of SC001 in two different models of Parkinson's disease: unilateral stereotaxic injection of lipopolysaccharide (LPS, inflammatory model) or 6-OHDA (oxidative stress model) injection into the *substantia*

A Neuroinflammation animal model

B Neurodegeneration animal model. Intracerebral administration.

C Neurodegeneration animal model. Intraperitoneal administration.

Figure 5. Experimental approach. Study of the anti-inflammatory and neuroprotective effect of SC001 *in vivo*. (A) Neuroinflammation model. LPS (10 μ g in 2.5 μ L PBS) alone or in combination with SC001 (15 nmol) was injected into the right side of the SNpc as indicated in Methods. Animals were sacrificed 72 h later, and brains were isolated. (B) 6-OHDA model with SC001 intracerebral administration. 6-OHDA (9 μ g in 2.5 μ L PBS) alone or in combination with SC001 (15 nmol) was injected into the right side of the SNpc as indicated in Methods. After 20 days, animals were sacrificed, and brains were obtained. (C) 6-OHDA model with SC001 intraperitoneal administration. Rats were injected with 6-OHDA into the right side of the SNpc, and 10 days later, the animals were treated with SC001 (150 μ M) intraperitoneally every 2 days. Animals were sacrificed 10 days after SC001 treatment. ic, intracerebral; ip, intraperitoneal.

nigra pars compacta (SNpc) of adult rats. 6-OHDA injection into the SNpc of rodents causes the destruction of the nigrostriatal pathway, mediated by an oxidative stress, and so loss of dopaminergic input to the striatum.^{23–25} The treatment with LPS leads to a loss of dopaminergic cells and activation of microglial cells with the subsequent release of neurotoxic factors.^{26–29} A diagram showing the experiments performed is presented in Figure 5. Because compound SC001 had been predicted as a BBB permeable drug, an intraperitoneally (ip) administration of this compound was also designed.

Histological analysis by Nissl staining of LPS-lesioned animals revealed an extensive cell loss in the SNpc of these animals (Figure 6). Injection of SC001 together with LPS resulted in a considerable protection against LPS-induced neuronal loss. By using specific anti-tyrosine hydroxylase-(TH) antibody, we also observed a significant preservation of dopaminergic neurons in SC001-injected rats compared with abundant dopaminergic neuron damage after injection of LPS. Quantitative analysis showed a decrease of 80%, compared with control rats, in the number of dopaminergic neurons in the SNpc after LPS injection. On the other hand, in the SC001-injected group only a moderate decrease (8%) in dopaminergic cells was detected 72 h after LPS injection.

To assess whether treatment with SC001 also had an effect on the integrity of dopaminergic neurons in a 6-OHDA model, animals were treated with the compound at the same time of 6-OHDA injury or 10 days later (Figure 6). The loss of TH⁺ cells in the SNpc in the 6-OHDA-lesioned animals was 82% three

weeks after lesion, compared with vehicle-treated animals. In contrast, treatment with SC001 at the same time of 6-OHDA injury resulted in a significant neuroprotective effect of TH⁺ cells in the SNpc. Dopaminergic cell loss in this group of animals was only 10%. Concerning the effects of SC001 administered 10 days post 6-OHDA lesioning, we also observed a significant neuroprotective effect of SC001 of dopaminergic neurons. In animals treated ip with SC001 10 days after lesion induction, the loss of TH⁺ cells was reduced to 14% compared with the massive dopaminergic cell loss detected in 6-OHDA-injured animals. Overall, these results indicate that GSK-3 inhibition by the SC001 compound has significant neuroprotective effects of dopaminergic cells in the SNpc both when administered at the same time and when administered 10 days post-6-OHDA-lesioning, which is in agreement with the ability of this compound to enter into the brain, shown by the *in vitro* PAMPA analysis.

Finally, since one of the events that takes place in the SNpc after LPS injury is the activation of microglial cells, which is in great part responsible for the dopaminergic cell loss, we analyzed CD11b expression as a marker of this microglial activation (Figure 7). CD11b⁺ cells were not observed in the ipsilateral part of vehicle-injected animals. Three days after LPS injection, a strong CD11b immunoreactive signal was clearly observed in the SNpc. This increase in microgliosis was almost completely abolished by the GSK-3 inhibitor SC001. Altogether these results suggest that treatment with the GSK-3 inhibitor called SC001 protects against 6-OHDA- and LPS-induced loss

Figure 6. Effect of SC001 on dopaminergic cell death *in vivo*. Rats were treated with LPS or 6-OHDA alone or in combination with the compound SC001 as indicated in Methods and shown schematically in Figure 5. (A) Brains were removed, and tissue sections were processed for Nissl stain to label neurons or tyrosine hydroxylase (TH) immunoreactivity to label dopaminergic neurons. Scale bars, 500 μ m. Inset scale bars, 100 μ m. (B) Quantification of the numbers of neurons stained with cresyl violet (Nissl stain) or TH-immunoreactive cells. Values represent the mean $\pm$ SD, expressed as a percentage of vehicle-treated animals, from three different experiments, four animals per experiment per experimental group, and five independent sections per animal. *** $p \leq 0.001$ versus LPS-treated animals. ### $p \leq 0.001$ versus 6-OHDA-treated animals. ic, intracerebral; ip, intraperitoneal.

Figure 7. Effect of SC001 on *in vivo* inflammation. Lipopolysaccharide (LPS, 10 μ g) or vehicle was injected unilaterally into the adult substantia nigra pars compacta of adult rats. A group of animals also received SC001 (15 nmol) together with LPS. After 72 h, the brains were removed, and tissue sections were processed for CD11b (OX-42) immunoreactivity to label activated microglia. Scale bars, 100 μ m. Quantification of the reactive cells is expressed as the mean $\pm$ SD from three different experiments, four animals per experiment per experimental group, and five independent sections per animal. *** $p \leq 0.001$ versus LPS-treated animals.

of dopaminergic cells and that this neuroprotective effect is associated with the inhibition of microglial activation.

CONCLUSIONS

GSK-3, a highly conserved and widely expressed serine/threonine protein kinase, plays an important role in a number of physiological processes ranging from glycogen metabolism to cell proliferation and differentiation and microtubule stability.^{30–34} Although misregulation of GSK-3 has been well established in human disorders such as bipolar disorder, schizophrenia, Alzheimer's disease, and cancer,^{35–37} the role of GSK-3 in PD is controversial and much less known. Our results, obtained by a chemogenetics approach, strongly support the idea that GSK-3 inhibition may represent a valid therapeutic strategy for the treatment of Parkinson's disease.

We have here shown the neuroprotective effects of different biological and chemical GSK-3 inhibitors in an *in vitro* model of dopaminergic cell death using the human neuroblastoma cell line SH-SY5Y. We show that all the inhibitors tested attenuated 6-OHDA-induced neuronal cell death as measured by cell viability (MTT and LDH) assays. These results are consistent with a previous study indicating that TDZD-8, an inhibitor of GSK-3, eliminated 6-OHDA-induced apoptosis of these cells.³⁸ Also, other authors have shown that inhibition of GSK-3 can result in neuroprotection in two different models of Parkinson's disease (rotenone and MPTP injuries).^{39–41}

Using the new GSK-3 inhibitor SC001, here discovered, we have also demonstrated that inhibition of this enzyme results in a significant neuroprotection of the hippocampal cell line HT22 and in an anti-inflammatory action in primary cultures of astrocytes treated with lipopolysaccharide. Because inflammation plays a central role in the pathogenesis of several brain disorders and neuronal injury⁴² as a consequence of activation of astrocytes and microglial cells,⁴³ our results suggest that

GSK-3 inhibitors could be of therapeutic value not only in PD but also in those brain diseases where inflammation processes are involved. Other authors have also shown that GSK-3 inhibition could be of therapeutic value in inflammatory conditions.⁴⁴ We extended these *in vitro* studies by evaluating the anti-inflammatory and neuroprotective actions of direct administration of SC001 into the SNpc of adult rats using two classical models of PD, 6-OHDA- and LPS-lesioning. Administration of this compound causes a significant preservation of dopaminergic cells in the SNpc in these two paradigms of PD. Importantly, the effects of SC001 were observed not only when administered the same day as the toxin 6-OHDA but also when the administration occurred 10 days later. This ability to preserve dopaminergic cells in animals with already established lesions is particularly significant for further clinical intervention since patients present motor symptoms once the nigrostriatal pathway has degenerated substantially.⁴⁵ Finally, and in agreement with the *in vitro* data, our results indicate that SC001 significantly reduced the accumulation of reactive microglia in the striatum of LPS-lesioned rats. Since administration of the endotoxin LPS in rats induces a robust glial activation and a subsequent dopaminergic cell loss that parallels many aspects of PD,^{26,27} the data presented here suggest that, at least in part, the SC001 compound induces a protection of dopaminergic cells through anti-inflammatory mechanisms.

Collectively, our results provide conclusive evidence for a role of GSK-3 on dopaminergic neuroprotection and suggest that GSK-3 inhibitors, and specially SC001, might be particularly well suited for potential novel therapies in the treatment of PD.

METHODS

Neuronal Cell Culture. The human neuroblastoma SH-SY5Y cell line was obtained from Sigma-Aldrich and propagated in F12 medium/EMEM containing glutamine (2 mM), 1% nonessential amino acids, and 15% fetal bovine serum (FBS) under humidified 5% CO₂ and 95% air. On attaining semiconfluence, cells were treated with 6-OHDA (35 μ M, Sigma) for 24 h. Some cultures were pretreated for 1 h with the different compounds at several concentrations ranging from 0.5 to 10 μ M. After treatment, cultures were processed for cell viability and LDH measurement.

The mouse hippocampal HT22 cell line, seeded at a density of 5000 cells/well in 96-multiwell plates, were grown in DMEM supplemented with 10% FBS containing penicillin (100 units/mL) and streptomycin (100 μ g/mL) and incubated at 37 °C under 5% CO₂. Some cultures were treated with SC001 (10 μ M) added to the culture medium 2 h before exposure to glutamate (3 mM). After 24 h in culture a viability assay was performed.

Cell Viability Assay. Cell viability was measured using the MTT assay (Roche Diagnostic, GmbH), based on the ability of viable cells to reduce yellow MTT to blue formazan. After treatment, cells were incubated with MTT (0.5 mg/mL, 4 h) and subsequently solubilized in 10% SDS/0.01 M HCl for 12 h in the dark. The extent of reduction of MTT was quantified by absorbance measurement at 595 nm according to the manufacturer's protocol.

LDH Release Assay. Cytotoxicity was assessed by measuring the levels of lactate dehydrogenase (LDH) released into the culture medium 16 h after the different treatments. LDH activity was measured using a Cytotoxicity Detection kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) and quantified by measuring absorbance at 490 nm.

GSK-3 Inhibition. The method of Baki et al.⁴⁶ was followed for measuring the inhibition of GSK-3 β . Kinase-Glo luminescent assays (Promega Biotech Ibérica, SL) were performed in assay buffer using black 96-well plates. In a typical assay, 10 μ L (10 μ M) of test

compound (dissolved in dimethyl sulfoxide [DMSO] at 1 mM concentration and diluted in advance in assay buffer to the desired concentration) and 10 μ L (20 ng) of GSK-3 β (Millipore Iberica S.A.U.) were added to each well followed by 20 μ L of assay buffer containing 25 μ M substrate, the prephosphorylated polypeptide GS-2 (Millipore Iberica S.A.U.), and 1 μ M ATP (Sigma-Aldrich). Assay buffer contained 50 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM EGTA, and 15 mM magnesium acetate. The final DMSO concentration in the reaction mixture did not exceed 1%. After 30 min incubation at 30 $^{\circ}$ C, the enzymatic reaction was stopped with 40 μ L of Kinase-Glo reagent. Glow-type luminescence was recorded after 10 min using a FLUOstar Optima (BMG Labtechnologies GmbH, Offenburg, Germany) multimode reader. The activity is proportional to the difference of the total and consumed ATP. The inhibitory activities were calculated on the basis of maximal activities measured in the absence of inhibitor. The IC₅₀ was defined as the concentration of each compound that reduces by 50% the enzymatic activity with respect to that without inhibitors.

CK1 δ Assay Protocol. The "Kinase-Glo" kit from Promega (Promega Biotech Ibérica, SL) was used to screen compounds for activity against CK1 δ (Millipore Iberica S.A.U.). Kinase-Glo assays were performed in assay buffer using black 96-well plates. In a typical assay, 10 μ L of test compound (dissolved in dimethyl sulfoxide [DMSO] at 1 mM concentration and diluted in advance in assay buffer to the desired concentration) and 10 μ L (16 ng of CK1 δ) of enzyme were added to each well followed by 20 μ L of assay buffer containing 0.1% casein as substrate and 4 μ M ATP. Assay buffer contained 50 mM HEPES, pH 7.5; 0.01% Brij-35; 10 mM MgCl₂; 1 mM EGTA; and 0.01% NaN₃. The final DMSO concentration in the reaction mixture did not exceed 1%. After 60-min incubation at 30 $^{\circ}$ C, the enzymatic reaction was stopped with 40 μ L of Kinase-Glo reagent. Glow-type luminescence was recorded after 10 min using a FLUOstar Optima (BMG Labtechnologies GmbH, Offenburg, Germany) multimode reader. The activity is proportional to the difference of the total and consumed ATP. The inhibitory activities were calculated on the basis of maximal activities measured in the absence of inhibitor. The IC₅₀ was defined as the concentration of each compound that reduces by 50% the enzymatic activity with respect to that without inhibitors.

Oxygen Radical Absorbance Capacity Assay. The ORAC-FL method of Ou et al.⁴⁷ partially modified by BMG LABTECH (BMG LABTECH Application note 148 (2006) ORAC Assay on the FLUOstar OPTIMA to Determine Antioxidant Capacity. <http://www.bmglabtech.com/application-notes/fluorescence-intensity/orac-148.cfm>) was followed using a FLUOstar Optima (BMG Labtechnologies GmbH, Offenburg, Germany) with 485 excitation and 520 emission filters. 2,2'-Azobis-(amidinopropane) dihydrochloride (AAPH), ($\pm$)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox), and fluorescein (FL) were purchased from Sigma-Aldrich. The reaction was carried out in 75 mM phosphate buffer (pH 7.4), and the final reaction mixture was 200 μ L. Antioxidant (25 μ L) and FL (150 μ L; 10 nM) solutions were placed in a black 96-well microplate (96F untreated, Nunc™). The mixture was preincubated for 30 min at 37 $^{\circ}$ C, and then, AAPH solution (25 μ L, 240 mM) was added rapidly using a multichannel pipet. The microplate was immediately placed in the reader, and the fluorescence was recorded every 90 s for 90 min. The microplate was automatically shaken prior each reading. Samples were measured at four different concentrations (10–1 μ M). A blank (FL + AAPH in phosphate buffer) instead of the sample solution and four calibration solutions using trolox (10–1 μ M) were also carried out in each assay. All the reaction mixtures were prepared in duplicate, and at least three independent assays were performed for each sample. Raw data were exported from the Fluostar Optima Software to an Excel sheet for further calculations. Antioxidant curves (fluorescence vs time) were represented, and the AUC is calculated as $AUC = f_1/f_0 + f_i/f_0 + \dots + f_{34}/f_0 + f_{35}/f_0$, where f_0 = initial fluorescence reading at 0 min and f_i = fluorescence reading at time i . The net AUC is obtained by subtracting the AUC of the blank from that of a sample. The relative trolox-equivalent ORAC value is calculated as: relative ORAC value = $[(AUC_{\text{sample}} - AUC_{\text{blank}})/(AUC_{\text{Trolox}} - AUC_{\text{blank}})](\text{molarity of Trolox/molarity of sample})$.

GSK-3 Inhibitors. Alsterpaullone was purchased from Calbiochem. 5-Iminothiazoles (VP 1.14 and VP 1.16), maleimides (VP 3.16, VP 3.35, and VP 3.36), and the quinoline derivative VP 0.7 were synthesized in the Instituto de Química Médica-CSIC following described procedures.^{17–19}

Primary Glial Cell Cultures. Microglial cultures were prepared from the cerebral cortex of 2-day old rats as previously described.⁴⁸ Briefly, after removal of the meninges, the cerebral cortex was dissected and mechanically dissociated by gently pipetting through tips of small diameter pipets. After centrifugation, the pellet was washed with cold HBSS (Gibco), and the cells were plated on 75 cm² flasks, previously incubated with poly(D-lysine) (20 μ g/mL), and allowed to grow in DMEM medium containing 10% FBS, 10% horse serum, and 1% penicillin/streptomycin at 37 $^{\circ}$ C in a humidified incubator (5% CO₂). After 7 days, flasks were agitated on an orbital shaker for 4 h at 230 rpm at 37 $^{\circ}$ C, and microglial cells were collected from supernatant. After centrifugation at 1050 rpm for 10 min, isolated microglial cells were seeded in 96-well dishes. Cultures were pretreated with SC001 (10 μ M) 1 h before exposure to LPS (10 μ g/mL), and after 24 h in culture, nitrite production was measured.

Nitrite Measurement. Accumulation of nitrites was assayed by the standard Griess reaction. After stimulation of cells with the different treatments for 24 h, supernatants were collected and mixed with an equal volume of Griess reagent (Sigma-Aldrich). Samples were then incubated at room temperature for 15 min and absorbance was read using a plate reader at 492/540 nm.

Animal Experiments. Adult male Wistar rats (8–12 weeks old) were used in this study. All procedures with animals were specifically approved by the "Ethics Committee for Animal Experimentation" of the Instituto de Investigaciones Biomédicas (CSIC-UAM) and carried out in accordance with the protocols issued, which followed National (normative 1201/2005) and International recommendations (normative 86/609 from the European Communities Council). Adequate measures were taken to minimize pain or discomfort of animals.

LPS and 6-OHDA Injection *in Vivo*. The animals divided into six groups, with at least four rats in each group, were properly anesthetized and placed in a stereotaxic apparatus (Kopf Instruments, CA). LPS (10 μ g in 2.5 μ L PBS) or 6-OHDA (9 μ g in 2.5 μ L PBS containing 0.02% ascorbic acid) alone or in combination with SC001 (15 nmol) was injected into the right side of the SNpc (coordinates from Bregma: posterior –4.8 mm; lateral +2.0 mm; ventral +8.2 mm, according to the atlas of Paxinos and Watson). The dose of LPS and 6-OHDA was chosen based on previous published data.^{15,27,29,49,50} Control animals of the same age were injected with PBS. The amount of SC001 injected was calculated taking into account the distribution volume of this cerebral area and the effective dose observed in the *in vitro* experiments. Rats were then housed individually to recover and sacrificed 72 h after lesioning with LPS or 3 weeks after lesioning when 6-OHDA was used. We also used a more clinically relevant treatment regime, that is, injection with SC001 after lesion induction to see whether the compound could rescue degenerating neurons. To this end, one group of rats lesioned with 6-OHDA were injected intraperitoneally on alternate days with SC001 (150 μ M) 10 days after lesioning and sacrificed 10 days later (Figure 5).

Histology and Immunohistochemistry. Animals previously anaesthetized were perfused transcardially with 4% paraformaldehyde, and brains were obtained as previously described.¹⁵ Briefly, brains were removed, postfixed in the same solution at 4 $^{\circ}$ C overnight, cryoprotected, and frozen, and 30 μ m coronal sections were obtained in a cryostat. Free-floating sections were then processed for cresyl violet (Nissl stain) or immunohistochemistry. Dopaminergic neurons were stained with a rabbit anti-tyrosine hydroxylase antibody (Chemicon/Millipore, USA), and activated glia were immunodetected with a mouse anti-CD11b antibody (Serotec, Germany). After being dehydrated, cleared, and mounted with DePeX (Serva, Heidelberg, Germany), samples were examined with a Nikon eclipse 90i microscope, equipped with a DS-Fi1 digital camera (Amsterdam, The Netherlands). Four animals from each experimental group were analyzed. Neuronal integrity and specifically dopaminergic cell death was assessed by counting the percentage of Nissl-stained and TH⁺

cells, respectively, in the SNpc in four well-defined high magnification ($\times 400$) fields per animal, using a computer-assisted image analysis software (Soft Imaging System Corp). Microgliosis was quantified similarly.

Statistics Analysis. Statistical comparisons for significance among different groups of animals were performed by ANOVA followed by Newman–Keuls' test for multiple comparisons. Student's *t*-test was used to analyze statistical differences between cells. Differences were considered statistically significant at $p < 0.05$.

■ ASSOCIATED CONTENT

● Supporting Information

In vitro parallel artificial membrane permeability assays (PAMPA), data on the permeability in the PAMPA-BBB assay of 10 commercial drugs and the SC001 compound, and linear correlation between experimental and reported permeability of commercial drugs using the PAMPA-BBB assay. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

A.P.-C. and A.M. conceived and designed the experiments, analyzed data, and wrote the manuscript. C.G. and A.S. designed experiments, analyzed data, and wrote the manuscript. J.A.M.-G. and C.S. helped design the experiments, analyzed data, and performed the experiments. S.A.-G. performed the experiments. C.G. and A.M. coordinated the chemogenomic approach and analyzed data. S.C. controlled the compound library. V.P. synthesized and optimized the compounds. C.P. evaluated enzymatic activities. D.I.P. performed PAMPA studies and analyzed data.

Author Contributions

‡These authors contributed equally to this work.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

6-OHDA, 6-hydroxydopamine; BBB, blood–brain barrier; CNS, central nervous system; GSK-3, glycogen synthase kinase-3; LPS, lipopolysaccharide; PAMPA, parallel artificial membrane permeation assay; PD, Parkinson's disease; PDE, phosphodiesterase; SNpc, substantia nigra pars compacta

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