Insights into the Pharmacokinetics and In Vitro Cell-Based Studies of the Imidazoline I₂ Receptor Ligand B06

Andrea Bagán 1, José A. Morales-García 2, Christian Griñán-Ferré 3, Caridad Díaz 4, José Pérez del Palacio 4, Maria C. Ramos 4, Francisca Vicente 4, Belén Pérez 3, José Brea 6, Maria Isabel Loza 6, Mercè Pallàs 3 and Carmen Escolano 1,*

1 Laboratory of Medicinal Chemistry (Associated Unit to CSIC), Department of Pharmacology, Toxicology and Medicinal Chemistry, Faculty of Pharmacy and Food Sciences, Institute of Biomedicine (IBUB), University of Barcelona, 08028 Barcelona, Spain; abaganpolonio@ub.edu
2 The Network Center for Biomedical Research in Neurodegenerative Diseases (CIBERNED), Department of Cell Biology, School of Medicine, Complutense University (UCM), 28040 Madrid, Spain; jmoral06@ucm.es
3 Pharmacology Section, Department of Pharmacology and Toxicology, Complutense University, 28040 Madrid, Spain; christian.grinnan@ub.edu (C.G.-F.); joses.perez@ub.es (J.P.);
4 Fundación MEDINA, Centro de Excelencia en Investigación de Medicamentos Innovadores en Andalucía, Avda. del Conocimiento 34, 18016 Armilla, Spain; caridad.diaz@medinaandalucia.es (C.D.);
5 Department of Pharmacology, Therapeutic and Toxicology, Autonomous University of Barcelona, 08193 Barcelona, Spain; belen.perez@uab.cat
6 Innopharma Screening Platform, BioFarma Research Group, Centro de Investigación en Medicina Molecular y Enfermedades Crónicas (CIMUS), Universidad de Santiago de Compostela, 15706 Santiago de Compostela, Spain; pepo.brea@usc.es (J.B.); mabel.loza@usc.es (M.I.L.)
* Correspondence: cescolano@ub.edu

Abstract: The impact of neurodegenerative diseases (ND) is becoming unbearable for humankind due to their vast prevalence and the lack of efficacious treatments. In this scenario, we focused on imidazoline I₂ receptors (I₂-IR) that are widely distributed in the brain and are altered in patients with brain disorders. We took the challenge of modulating I₂-IR by developing structurally new molecules, in particular, a family of bicyclic α-iminophosphonates, endowed with high affinity and selectivity to these receptors. Treatment of two murine models, one for age-related cognitive decline and the other for Alzheimer’s disease (AD), with representative compound B06 ameliorated their cognitive impairment and improved their behavioural condition. Furthermore, B06 revealed beneficial in vitro ADME-Tox properties. The pharmacokinetics (PK) and metabolic profile are reported to de-risk B06 for progressing in the preclinical development. To further characterize the pharmacological properties of B06, we assessed its neuroprotective properties and beneficial effect in an in vitro model of Parkinson’s disease (PD). B06 rescued the human dopaminergic cell line SH-SY5Y from death after treatment with 6-hydroxydopamine (6-OHDA) and showed a crucial anti-inflammatory effect in a cellular model of neuroinflammation. This research reveals B06 as a putative candidate for advancing in the difficult path of drug discovery and supports the modulation of I₂-IR as a fresh approach for the therapy of ND.

Keywords: imidazoline I₂ receptor ligand; pharmacokinetics; bicyclic α-iminophosphonate; metabolic profile; neuroprotection; Alzheimer’s disease; Parkinson’s disease

1. Introduction

Imidazoline I₂ receptors (I₂-IR) are nonadrenergic binding sites recognized by the radioligands [³H]-idazoxan and [³H]-p-aminoclonidine, as well as and with the lowest...
affinity, by $[^{3}H]$-clonidine [1–3]. I$_2$-IR are found in the central and peripheral nervous system and many organs and tissues [4,5]. The alteration of I$_2$-IR is a hallmark in a plethora of neurodegenerative illnesses, such as brain disorders, including Alzheimer’s disease (AD), Parkinson’s disease (PD), Huntington’s disease, depression, and glial tumours [6–13]. Compounds with a high affinity for I$_2$-IR modulate many physiological and pathological processes such as analgesia and inflammation [14,15]. Two I$_2$-IR ligands, CR4056 and $[^{13}C]$BU99008, are progressing in clinical trials, unveiling the relevance of these receptors in therapeutics. The analgesic CR4056, described as the first-in-class I$_2$-IR ligand, is in phase II for osteoarthritis and postoperative dental pain, and $[^{13}C]$BU99008 is in phase I for PET diagnosis in patients suffering from AD (Figure 1) [16–18].

**Known I$_2$-IR ligands:**

![Known I$_2$-IR ligands](image)

**Our new I$_2$-IR ligands:**

![Our new I$_2$-IR ligands](image)

**Figure 1.** Structure of the known I$_2$-IR ligands (2-imidazoline nucleus in blue and imidazole nucleus in green) and our representative new I$_2$-IR ligands, MCR5 and B06.

Despite the potential of I$_2$-IR in therapeutics, their complete pharmacological characterization is still in its infancy due to their high heterogeneous nature and the absence of structural details. The disclosure of their pharmacological role relies on their modulation by I$_2$-IR ligands and the observation of the physiological responses. Known I$_2$-IR ligands are scarce and with a limited structural pattern based on the 4,5-nonsubstituted-2-heterocyclic-2-imidazoline nucleus (e.g., idazoxan, tracizoline, 2-BFI), with the only exceptions of CR4056 and LSL60101 endowing an imidazole motif (Figure 1) [19–21]. In this context, we have contributed by increasing the arsenal of ligands with structurally unprecedented new families that showed outstanding affinities to I$_2$-IR devoid of $\alpha_2$-adrenoceptors ($\alpha_2$-AR) in human brain tissues.
Therefore, in 2017, we reported the synthesis and pharmacological characterization of a family of (2-imidazolin-4-yl)phosphonates that showed excellent affinity to I$_2$-IR [22,23]. Considering that I$_2$-IR are increased in the patients that suffered from AD [24], we explored the implications of a representative member of this family, the [1-(3-chloro-4-fluorobenzyl)-5,5-dimethyl-4-phenyl-4,5-dihydro-1H-imidazol-4-yl]phosphonate, named as MCR5, in neurodegenerative diseases (ND). Our concern in this field is due to the huge worldwide impact of ND, mainly AD [25] and PD [26]. The WHO estimates that more than 50 million people are suffering from AD and about 7 million from PD. Unfortunately, these numbers are dramatically increasing every year. Aging of the population and the lack of effective treatments place AD as one of the most serious unmet medical needs that humankind must face in the upcoming years [27]. Avoiding the collapse of health systems and ameliorating the suffering of patients and families guide the scientific community efforts. As no new cognitive enhancer drug has reached the market in nearly two decades, identifying new therapeutic targets and entities is a priority for medicinal chemistry programs [28]. Accordingly, we treated with MCR5 a murine model of age-related cognitive decline, the senescence-accelerated mouse prone 8 (SAMP8), resulting in a cognitive improvement and amelioration of hallmarks. This study constituted the first experimental evidence that demonstrated the potential of the modulation of I$_2$-IR as a new therapy for AD [29]. Additionally, improvement in the behavioural and psychological symptoms of dementia, including fear–anxiety, depressive-like behaviour, and memory decline, present in most ND, were observed when treating an orally SAMP8 murine model with MCR5 [30]. There are a few studies that discuss the presence of the I$_2$-IR in post-mortem tissue from PD patients, but none describe its role in the development of PD [7].

Driven by the excellent precedented pharmacological results and the possibility of opening new structural opportunities, we reported a family of bicyclic α-iminophosphonates synthetised by a [3+2]cycloaddition reaction [31]. We selected representative diethyl (1RS,3aSR,6aSR)-5-(3-chloro-4-fluorophenyl)-4,6-dioxo-1-phenyl-1,3a,4,5,6,6a-hexahydropyrrolo[3,4-c]pyrrole-1-phosphonate, named as B06, that shares an α-phenyl-α-iminophosphonate function and a 3-chloro-4-fluorophenyl group with MCR5 (highlighted in red in Figure 1), and it is endowed with high affinity and selectivity to I$_2$-IR. Structurally, B06 does not fit into the restricted substitution pattern of I$_2$-IR ligands, with its bicyclic α-iminophosphonate structure being the first described non-imidazoline/non-imidazole-containing compound, showing outstanding affinity to I$_2$-IR. B06 displays in the membranes of the post mortem human frontal cortex, a brain area that shows an important density of I$_2$-IR and α$_2$-AR, a pK$_i$ = 8.56 ± 0.32, and a ratio of I$_2$/α$_2$ = 195 in competitive binding studies against the selective I$_2$-IR radioligand $[^3]$H2-BFI and the selective α$_2$-AR radioligand $[^3]$H[1-3H]RX821002. Note that the standard idazoxan showed a lower pK$_i$ than B06 with a value of 7.41 ± 0.63 and non I$_2$/α$_2$ selectivity. Considering the localization of I$_2$-IR in the central nervous system (CNS), the good ability of B06 to cross the blood–brain barrier (BBB) was an essential requirement with a Pe value in a parallel artificial membrane permeability assay (PAMPA) well above the threshold established for high BBB permeation. B06 was devoid of cytotoxicity in a plethora of culture cells. In silico studies offered confidence for performing in vitro assays that showed non-warnings in the physicochemical properties (solubility and chemical stability), microsomal stability, cytochromes inhibition, hERG inhibition, and plasma protein binding. A receptor characterization panel de-risked our representative compound evaluated in vivo, exhibiting hypothermic properties and neuroprotection in the murine model of age-related cognitive decline, the SAMP8, as well as in a murine model of AD, the 5xFAD [32,33]. Thus, B06 exerts a beneficial effect by improving cognition and ameliorating anxiety-like behaviour in the SAMP8 murine model. Modulation of I$_2$-IR by B06 reduces neuroinflammation, oxidative stress and calcineurin protein levels in SAMP8 [34].

To progress the promising candidate B06 in preclinical studies, here, we report parameters to be assessed in the early discovery process, such as physicochemical characteristics (chemical stability at different pH) and complete ADME parameters, such as plasma sta-
bility. To shed light on the biotransformation of B06, we undertook studies considering the stability in mouse and human liver microsomes, pharmacokinetics (PK), in vitro and in vivo metabolic profile, and the identification of metabolites of B06. To better describe the pharmacological features of B06 and considering the paucity of information on the role of I2-IR ligands in PD, we used a model of dopaminergic neurodegeneration to evaluate how B06 could protect neurons. Furthermore, since most ND such as AD and PD are characterized by the presence of an exacerbated and sustained neuroinflammatory process, which is partly responsible for the development of the disease, we have completed the study of the in vitro biological activity of B06 using a cellular model of neuroinflammation (Figure 2).

![Figure 2](image-url)

Overall, this study boosts B06 as a promising candidate for further preclinical development in AD indication or other ND diseases. The in-depth characterization of B06 will contribute to build a robust comprehensive understanding of the pharmacological implications of I2-IR and their ligands in therapeutics.

2. Results and Discussion

2.1. Solubility and Chemical Stability of B06 at Different pH

The solubility of a compound is an essential physicochemical property that drives drug absorption and optimizes its therapeutic exposure in plasma and the therapeutic target. The capabilities of the compound to be solved in different media will avoid misunderstanding in the pharmacological results. B06 showed good solubility in several media, methanol, acetonitrile, and water, and an excellent solubility of 92 µM was found in 1% DMSO and 99% PBS buffer [33]. Furthermore, the chemical stability of B06 in buffers at different pH was undertaken to measure the degradation by non-enzymatic processes (most common hydrolysis and oxidation). The pH value of the gastrointestinal tract varies from acidic in the stomach to basic in the small and large intestine, and alterations of representative compounds need to be assessed. To this end, a test solution of B06 (1 µM, 0.1% final DMSO concentration) was incubated with buffer pH 2, 5 and 7.4 at 37 ºC. Serial samples were taken at 0, 5, 15, 30, 60 and 120 min. All samples were added immediately to three volumes of methanol containing internal standard in a microtiter plate cooled to halt chemical degradation. All the samples were analysed by LC-MS/MS. The percentage of parent compound remaining at each time point relative to the 0 min sample was calculated from peak area ratios. The chemical stability assay returned the per cent parent compound remaining at each time point for B06.
The study of the graphics reveals that B06 was stable at pH 2, without significant changes in the structure up to 120 min (Figure 3, Table 1). B06 in a pH 5 media proved to be stable up to 1 h, and 25% of the compound suffered structural changes during the next hour (Figure 4, Table 2). In neutral media (pH = 7.4), B06 remained unchanged during the first minute, and at 15 min, 25% of the compound was transformed. At 30 min and pH = 7.4, 75% of B06 was detectable, with half the amount at 60 min, and only 19% at 2 h (Figure 5, Table 3). This study gave us an overview of the chemical behaviour of B06, depending on the pH of the media versus time.

![Figure 3. Chemical stability of B06 at pH = 2.](image)

**Table 1.** Percentage of B06 remaining at pH 2 versus time (0–120 min).

| Time (min) | % B06 Remaining |
|-----------|-----------------|
| 0         | 100 ± 8.84      |
| 5         | 104 ± 9.81      |
| 15        | 98 ± 11.30      |
| 30        | 106 ± 9.97      |
| 60        | 112 ± 11.2      |
| 120       | 96 ± 13.6       |

![Figure 4. Chemical stability of B06 at pH = 5.](image)

**Table 2.** Percentage of B06 remaining at pH 5 versus time (0–120 min).

| Time (min) | % B06 Remaining |
|-----------|-----------------|
| 0         | 100 ± 3.3       |
| 5         | 97 ± 6.5        |
| 15        | 100 ± 3.9       |
| 30        | 87 ± 11.3       |
| 60        | 98 ± 10.1       |
| 120       | 75 ± 8.5        |
2.2. Plasma Stability of B06 at Mouse Plasma

Stability in liver microsomes does not imply stability in plasma, where many hydrolytic enzymes are circulating. In previous studies, the mice microsomal percentage remanent of B06 at 60 min was 65.08, with a t$_{1/2}$ of 91.72 min [33]. The assessment of the plasma stability avoids misleading in vitro data and complex interpretation of the in vivo PK. To this end, 10 µM B06 in plasma mouse at 37 °C was incubated at different times (0, 60, 180 and 360 min), and acetonitrile was added for precipitating plasma protein. Then, centrifugation led to a supernatant that was analysed for sample quantification. The remaining percentage for B06 was 100% at 0 and 10 min, 69.7% at 20 min, 48.3% at 40 min and 21.6% at 60 min. Although B06 displays transformation with time, the percentage of non-modified compounds enables its in vivo activities.

2.3. Metabolic Stability of B06 in Liver Microsomes

In vitro liver microsome metabolite identification studies are essential models for assessing metabolic profiling in early drug discovery. As the compound B06 progresses through the discovery–development–clinical continuum, the goals of the metabolite analysis may alter, as will the detail with which the experiment is conducted. Early in drug discovery, the compounds being synthesized frequently have poor PK characteristics; thereby, in vitro studies are performed using liver microsomes or hepatocytes as a model to assess the metabolic profiling of the compound.

There are several reasons as to why understanding the metabolic profile of a compound is important. First, the FDA Guidance for Safety Testing of Drug Metabolites [35] recommends in vitro evaluation of interspecies differences in drug metabolism between humans and the animals expected to be used in preclinical safety assessments. If a metabolite is formed only in humans and it is absent in the animal test species, or if the metabolite is present at disproportionately higher levels in humans than in the animal species, then it may be necessary to assess the preclinical safety of the metabolite in question. Second, understanding the metabolic liability of a compound is important in directing chemistry. If a compound is rapidly cleared, then identifying which functional groups are undergoing metabolism will be valuable in understanding how the chemistry may be altered to reduce compound degradation.
2.3.1. Metabolic Stability of B06 in Human Liver Microsomes

The metabolic stability using human liver microsomes (HLM) is directly related to the elimination of the drug from the body and significantly affects efficacy and safety. In the assay, the reaction was initiated by adding HLM (1 mg/mL) to an equal volume of buffer solution containing the test compound and cofactors at the proper concentrations. Reactions without NADPH were also incubated to rule out non-NADPH metabolism or chemical instability in the incubation buffer. Verapamil was included as a positive control to monitor incubation course. All reactions were terminated using 60 µL of ice-cold acetonitrile at 0, 5, 15, 30, 45, and 60 min. The plates were centrifugated at 3500 rpm for 15 min. All experiments were conducted in triplicate. Samples were monitored for B06 disappearance by LC-MS in MRM mode according to a quantitation method for B06. The percentage of the remaining parent compound and the time course of metabolic stability for verapamil, positive control, and B06 at each incubation time are displayed in Table S2 and Figure S1 and Table 4 and Figure S2, respectively.

Table 4. Percentage of B06 remaining at each incubation time in HLM.

| Time (min) | % Compound Remaining |
|-----------|---------------------|
|           | Plus NADPH          |
| 0         | 100.00 ± 14.46      |
| 5         | 98.60 ± 9.11        |
| 15        | 77.90 ± 13.20       |
| 45        | 41.3 ± 5.83         |
| 60        | 29.40 ± 3.69        |
|           | Minus NADPH         |
| 0         | 103.2 ± 13.82       |
| 60        | 75.30 ± 1.27        |

The analysis of the percentage of B06 remaining in minus NADPH cofactor incubations did not show the metabolism of the parent from nondependent NADPH enzymes. The present study indicates that B06 displays a half-life value equal to 28.4 min (Table S3). Consequently, the predicted intrinsic clearance calculated for B06 is 23.5 mL/min/mg protein (Table S3). Attending to the classification bands typically used for categorizing compounds into low, medium or high clearance (Table S4), B06 showed hepatic clearance classified as medium clearance.

2.3.2. Metabolic Stability of B06 in Mouse Liver Microsomes

Metabolic stability of B06 in mouse liver microsomes (MLM) was carried out as in the previous section, but instead of using HLM, the study required MLM.

The percentages of the remaining parent compound and the time course of metabolic stability for verapamil, positive control, and B06 at each incubation time are displayed in Table S6 and Figure S3 and Table 5 and Figure S4, respectively. B06 showed a half-life value equal to 16.23 min (Table S7). The intrinsic clearance calculated for B06 was 153.7 mL/min/mg protein, being classified as high clearance (Table S8).

Table 5. Percentage of B06 remaining at each incubation time in MLM.

| Time (min) | % Compound Remaining |
|-----------|---------------------|
|           | Plus NADPH          |
| 0         | 100.00 ± 25.58      |
| 5         | 135.00 ± 7.06       |
| 15        | 67.50 ± 4.15        |
| 30        | 30.30 ± 5.28        |
| 45        | 15.20 ± 1.98        |
| 60        | 6.08 ± 0.01         |
|           | Minus NADPH         |
| 0         | 105.00 ± 23.97      |
| 60        | 83.20 ± 2.87        |
Note that the half-life value and the intrinsic clearance for B06 outcomes discussed above are in the same range as in liver microsomes of both species, mice and humans.

2.4. Metabolic Profiling of B06 in Liver Microsomes

The objective of the study is to evaluate the formation of the main metabolites from B06 in HLM incubations and MLM incubations preceding the in vivo metabolic study, using these same incubations to identify the possible metabolites. The assay was initiated by adding HLM solution (1 mg/mL) or MLM solution (1 mg/mL) to an equal volume of buffer solution containing B06 and cofactors. Reactions without NADPH were also incubated to rule out non-NADPH metabolism or chemical instability in the incubation buffer. Verapamil was included as positive control to monitor incubation course. All reactions were terminated using 60 µL of ice-cold acetonitrile at 0, 5, 15, 30, 45, and 60 min. The plates were centrifuged at 3500 rpm for 15 min. All experiments were conducted in triplicates. Samples were analysed by LC-HMRS in positive ionization mode. Compound incubations without NADPH and compound reference standard were used as interference control for each incubation sample.

2.4.1. Metabolic Profiling of B06 in Human Liver Microsomes

The potential metabolite 1, ethyl hydrogen [(1R,3aS,6aS)-5-(3-chloro-4-fluorophenyl)-4,6-dioxo-1-phenyl-1,3a,4,5,6,6a-hexahydropyrrolo[3,4-c]pyrrol-1-yl]phosphonate, was tentatively identified in HLM incubations from 15 to 60 min. The assigned molecular formula was C_{20}H_{17}N_2O_5FPCl, resulting in the loss of C_2H_4 (Scheme 1). The transformation can be accounted for in the hydrolysis, in a Phase I metabolic conversion, of the ethoxy group of the phosphonate function. The hydrolysis of the phosphonate ester group to the corresponding ethyl hydrogen phosphonate derivative is a plausible reaction in the physiological aqueous media (Scheme 1). In the metabolite chromatogram at 15 min, the metabolite detected was 8.4%, at 30 min was 17.8%, at 45 min was 26.2% and at 60 min was 36% of the area (page S5, Table S9).

Scheme 1. Proposed product 1 resulting from the in vitro HLM assay.

From the TOF MS/MS spectra at 15 min, several peaks were selected for assignment. The signal with the mass of the product B06 without 25 units, 451.2389, could involve the loss of a C_2H moiety. Other peaks selected for the assignment were 341.0498 that could be the fragment A without the diethylphosphonate ester of B06, and 170.0606 that could correspond to the fragment B with a 3,4-dihydro-2H-pyrole nucleus (Figure 6).
2.4.2. Metabolic Profiling of B06 in Mouse Liver Microsomes

The objective of the study is to evaluate the formation of the main metabolites from B06 in MLM incubations.

Compound 1, identified in the metabolic profile of B06 in HLM, was tentatively detected in MLM incubations from 5 to 60 min. In the metabolite chromatogram at 5 min, compound 1 was detected in 10.7%, at 15 min its area was equal to 30.9%, at 30 min its presence increased to 60.9%, at 45 min it was found to be 81.6%, and at 60 min it represented 88.9% of the area (Page S5, Table S10). Note that the half-life value and the intrinsic clearance for B06 outcomes discussed above are in the same range in HLM and MLM. These results could be a sign of a similar stay time of B06 in the organism in both animals species.

2.5. Synthesis of the Metabolite Identified in the Metabolic Profiling of B06 in Human Liver Microsomes

Metabolite 1 proposed from the biotransformation via Phase I of B06 by HLM prompted us to undertake its synthesis for unequivocal identification. To this end, the diethyl phosphonate ester group of B06 was hydrolysed by treatment with trimethylsilyl bromide in dichloromethane furnishing the desired compound 1.

The synthesized compound 1, was compared to the potential metabolite identified in both liver microsome incubations preceding the in vivo metabolic study, showing the same retention time and spectral information. Consequently, in the liver microsomes of both species, mice and humans, a metabolism of phase I occurs through the hydrolysis of the phosphonate ester furnishing 1.

2.6. In Vitro Effects of B06 in a Preclinical Model of Neurodegeneration

2.6.1. Effects of B06 on Cell Viability in a Dopaminergic Cell Line

The process of neurodegeneration results in progressive changes in the structure or function of neurons, leading to devastating neurological conditions characteristic of AD and PD [36]. Due to the lack of information about the role of I2-IR ligands in PD development, first, we investigated the neuroprotective effect of B06 on the human dopaminergic cell line SH-SY5Y. The human neuronal dopaminergic cell line SH-SY5Y has been described as an ideal system for modelling in vitro the characteristics of dopaminergic neurons because they have many features of substantia nigra neurons [37]. We first tested whether the compound per se could have a cytotoxic effect on cell cultures. For this purpose, we
treated the cells with different concentrations of the compounds from 200 nM to 20 µM (Page S18, Figure S18). None of the concentrations used were cytotoxic to the cells. Then, we conducted a dose–response experiment in order to select the lowest concentration of B06 at which the most beneficial effect was obtained (Figure 7a). From this point on, we always use that concentration (1 µM) in our analysis. Now, we tested whether B06 is able to protect the SH-SY5Y cell line from cell death induced by the toxin 6-hydroxydopamine (6-OHDA), a well-established cell model for dopaminergic neurodegeneration in which our group has extensive experience [38]. For a more in-depth analysis, the level of active caspase 3 was used to determine apoptosis (Figure 7c,d). Qualitatively, our results indicated an increase in the number of SH-SY5Y cells expressing active caspase 3 within 16 h after treatment with 6-OHDA, and this effect was reversed by the treatment with B06. A Western blot analysis was used to confirm and quantify these results (Figure 7d). For comparison, two well-known I2-IR ligands, idazoxan and 2-BFI, were included as standards (Scheme 1). The results summarized in Figure 7 clearly show that the treatment of SH-SY5Y cells with compound B06 significantly rescued human dopaminergic cells from 6-OHDA-induced apoptosis. These results are in agreement with previous observations, suggesting that I2-IR are dysregulated in patients with ND [6–8], providing new data on their role in PD in particular.

**Figure 7.** Phenotypic screening of I2-IR ligands. Neuroprotective effect of B06 in vitro. Human dopaminergic cell line SH-SY5Y was exposed to 6-OHDA (25 µM) for 16 h. Cell viability was determined by MTT assay. Values represent the mean ± SD from triplicate determinations repeated at least three times. ** p ≤ 0.01, *** p ≤ 0.001 versus 6-OHDA-treated cultures. (a) Dose–response curve of B06. In order to determine the optimal dose of use of B06 for in vitro analysis, different concentrations of B06 were used. (b) To evaluate the neuroprotective role of B06, some cultures were pre-treated with idazoxan, 2-BFI and B06 (1 µM) for 1 h before 6-OHDA treatment. Apoptotic levels were determined by active caspase 3 (green) immunodetection (c) and Western blot quantification (d). Representative images of at least three independent experiments are shown. Scale bar, 10 µm. Nuclei were counterstained with DAPI (blue). *** p < 0.001 versus 6-OHDA-treated cells. #### p < 0.001 versus control (basal) cells.
2.6.2. Anti-Inflammatory Effects In Vitro of B06

Along with the neuronal loss, neuroinflammation is a process related to the onset of the disease, and it is an important contributor to the pathogenesis and progression of these disorders [39]. Since cytotoxicity induced by the neurotoxin 6-OHDA is frequently accompanied by pronounced inflammatory activity, we next evaluated the potential anti-inflammatory effect of B06 by evaluating the production of nitrites in SH-SY5Y cultures treated with the neurotoxin. In line with the results obtained with the cell viability experiments, the treatment with B06 significantly reversed the increase in nitrite production elicited by 6-OHDA treatment (Figure 8), suggesting a convincing anti-inflammatory effect of B06. Notably, B06 demonstrated a significantly better anti-inflammatory effect in comparison with other I2-IR ligands.

In the CNS, microglia and astrocytes play a key role in regulating inflammatory responses. Moreover, I2-IR are widely distributed in the brain and primarily in glial cells [40], suggesting that this receptor plays an important role in neuroinflammation. Therefore, to further evaluate the role of B06 in inflammatory reactions, we performed experiments in different cell-based assays that mimic in part the neuroinflammatory process in PD [41]. We used primary cultures of astrocytes and microglia treated with bacterial lipopolysaccharide (LPS), a potent inflammatory agent. Then, the potential anti-inflammatory activity of B06 was tested by evaluating the production of nitrites on primary cultured glial cells. Cultures were incubated with 1 µM of the compound for 1 h, and then cells were treated with LPS for a further 24 h. When primary astrocytes and microglial cells were stimulated with LPS (Figure 8), we observed a significant induction of nitrite production (approximately seven-fold) in the culture medium, an indicator of inflammation. Those cultures previously treated with B06 showed a significant decrease in nitrite production, showing better results when compared with I2-IR ligands idazoxan and 2-BFI, thus confirming the role of B06 as a potent anti-inflammatory agent. As neuroinflammation is a hallmark of many neurodegenerative diseases, including AD and PD [42], B06 may have particular relevance for treating disorders that involve inflammation of the nervous system.

2.7. In Vivo Pharmacokinetics of B06

The physicochemical and biochemical properties of a compound determine its time course after its administration. Parameters such as concentration in bloodstream and tissues, distribution, metabolism, and elimination are fundamental for the description of the pharmacological behaviour and therapeutic characteristics of B06. To this end, after
the absence of in vitro warnings in the ADME studies and in vivo advantages of B06, we proceeded with the PK and metabolic profile studies. Note, due to the therapeutic indication addressed by B06 in the CNS, we considered the quantity of compounds that rise in brain tissues.

Previously, we reported hypothermia observed in adult rats when treated intraperitoneally with 20 and 35 mg/kg measured 1 and 2 h postinjection and in mice after repeated administration of 20 mg/kg for 5 days of B06 [33]. It is well established that hypothermia causes significant neuroprotection, which also led us to have a wide range of dose confidence for the following studies. Taking into account the absence of toxicity at high doses of B06, even at 5 days, and the dose of 5 mg/kg in drinking water during 4 weeks employed in the cognitive studies, we considered performing the PK assays at 10 mg/kg, intraperitoneally.

Thus, two-month-old mice were treated with a single dose of 10 mg/kg intraperitoneally in ten groups (n = 3), for consecutive times: 0, 5, 15, 30 min and 1, 2, 4, 6, 8, 24 h. After treatment, mice were euthanized, and the plasma and brain were collected. The experimental groups were composed of three mice, and there was no toxicity in any of the animals. Furthermore, de visu observation after euthanasia showed no toxicity in organs or tissues. These data were in accordance with the previous absence of toxicity at higher doses.

2.7.1. Pharmacokinetics of B06 in Plasma

The plasma profile and PK parameters of B06 are shown in Figure 9.

| Pharmacokinetic Parameters | Value         |
|----------------------------|---------------|
| t1/2 (min)                 | 312.81 ± 34.90|
| T_max (min)                | 15 ± 0        |
| C_max (µg/mL)              | 0.95 ± 0.62   |
| AUC0–1440 (µg/mL*min)      | 176.98 ± 37.78|
| AUC0–inf (µg/mL*min)       | 182.99 ± 38.07|
| Cl (mg/kg)/µg/mL          | 0.054 ± 0.011 |
| Vz (mg/kg)/µg/mL          | 24.56 ± 4.74  |

Figure 9. Plasma concentration of B06 at different times (5 min–24 h) after an intraperitoneal administration of 10 mg/kg. Basic PK parameters were calculated. C_max: maximum observed concentration, T_max: time of maximum observed concentration, t1/2: terminal elimination half-life, AUC0–1440: area under the curve from zero to the last sampling time, AUC0–inf: area under the curve from zero extrapolated to infinity, Cl: plasma clearance, Vz: volume of distribution.

The maximum concentration in plasma (C_max) was 0.950 ± 0.624 µg/mL and was reached at 15 min (T_max). Plasma levels, although low, remained fairly consistent for 8 h post-administration and at 24 h drug concentration, were almost undetectable. The half-life time (t1/2) was 312.8 min and the AUC0–1440/AUC0–inf,obs ratio (0.97) showed that 24 h is a good interval to define the kinetic behaviour of B06 in plasma after intraperitoneal administration at a dose of 10 mg/kg.

The maximum mean concentration found in plasma does not exceed 1% of the drug administered in a single dose intraperitoneally. Notwithstanding the low levels, the administration of B06 in drinking water at a dose of 5 mg/kg to the murine models continued at effective levels for 4 weeks. The sustained intake for the animals and the treatment time could account for the beneficial effects of B06 in the SAMP8 and 5xFAD murine models by improving cognition and ameliorating the neurodegenerative condition. In addition, a reduction in the AD hallmarks was in accordance with the new state of the animals, pointing out the variations in biochemical pathways due to the interaction of B06 with a therapeutic target.
2.7.2. Pharmacokinetics of B06 in Brain

The quantification in the brain of B06 and the calculated PK parameters are shown in Figure 10.

| Pharmacokinetic Parameters | Value (Units) |
|-----------------------------|--------------|
| t$_{1/2}$ (min)             | 256.95 ± 257.15 |
| T$_{max}$ (min)             | 15 ± 0        |
| C$_{max}$ (µg/mL)           | 0.128 ± 0.066 |
| AUC$_{0-480}$ (µg/mL*min)   | 139.49 ± 3.24 |
| AUC$_{0-\infty}$ (µg/mL*min)| 198.81 ± 6.73 |
| Cl (mg/kg)/(µg/mL)          | 0.503 ± 0.346 |

Figure 10. Brain concentration of B06 at different times (5 min–24 h) after intraperitoneal administration of 10 mg/kg. Basic PK parameters were calculated. C$_{max}$: maximum observed concentration, T$_{max}$: time of maximum observed concentration, t$_{1/2}$: terminal elimination half-life, AUC$_{0-1440}$: area under the curve from zero to the last sampling time, AUC$_{0-\infty}$: area under the curve from zero extrapolated to infinity and Cl: the brain clearance.

The B06 concentrations found in the brain show that in this organ, the drug profile is identical to that found in plasma, presenting equal T$_{max}$ and similar t$_{1/2}$, 312.81 min in plasma and 256.95 min in the brain. The ratio between brain/plasma concentrations remains constant (approximately 10%). These results indicate the consistent correlation of B06 levels between plasma and the brain when the drug is administered at a dose of 10 mg/kg.

These data are according to previous PAMPA-BBB results, confirming in vivo the high capacity of the drug to penetrate the BBB.

2.8. Metabolic Profiling In Vivo of B06

Metabolite identification studies are essential in the early stages of drug discovery and during drug and clinical development and are strongly recommended by the FDA Guidance for Industry on Safety Testing of Drug Metabolites [35]. After performing the liver microsomes studies reported above, we undertook the metabolite determination by seizing the PK study. When entering the body, B06 will undergo biotransformation via Phase I and Phase II metabolic pathways. In this framework, a study to understand the biotransformation details of B06 by identifying the main in vivo metabolites from B06 at different times (30 to 480 min) was performed. The aim was to discard toxic issues and add information on the behaviour underlying beneficial cognitive impairment in the murine model of neurodegeneration and AD after treatment with B06. To this end, an aliquot of 50.0 µL mouse plasma and 150.0 µL of cold acetonitrile was added. After vortex mixing for one minute, the samples were centrifuged for 15 min at 13,300 rpm. An aliquot of 110.0 µL of the supernatant was transferred to an auto sampler vial to analyse by LC-HRMS in positive ionization mode.

In this condition, two potential metabolites of B06, 2-hydroxyethyl ((1R,3aS,6aS)-5-(3-chloro-4-fluorophenyl)-4,6-dioxo-1-phenyl-1,3a,4,5,6,6a-hexahydropyrrolo[3,4-c]pyrrol-1-yl) phosphonate, named as 2, were tentatively identified in vivo from 30 to 480 min. The assigned molecular formula was C$_{22}$H$_{21}$N$_{2}$O$_{6}$FPCl, and taking into account the fragmentation patterns in the mass spectra, it seems that one of the oxidations occurred in one of the ethyl groups of the phosphonate ester, and the other oxidation could be predicted to be in the other ethyl group. These oxidations of Phase I accounted for the prochiral character of the phosphorous that when substituted by two ethoxy groups is lacking chirality. It is not a stereocentre, but when one of the substituents is altered, the phosphorous becomes
prochiral; therefore, both ethoxy groups are different from the stereochemical point of view (Scheme 2).

Scheme 2. Putative oxidized product of B06 resulting from the in vitro HLM assay.

The low concentration of metabolites made detection in plasma difficult due to the fact that concentrations of the parental B06 molecule were also low. The potential metabolite of B06 at 30 min was 2.5% of an oxidized B06 derivative (m/z 495.0877), at 60 min was slightly increased to 2.8%, and at 120 min was detected in 5.8%. When increasing the time to 240 min, two tentative oxidation metabolites were found with slightly different retention times, 7.82 and 7.98 min, in the chromatogram in the conditions used. The new product at 7.98 min represented 1.5%, and the former oxidized metabolite (7.82 min) was 6.3%. At 360 min, only one of the oxidized B06 proposed metabolite was detected at 6.7%, and at 480 min, the percentage was 4.5% in the order of the previous determinations.

From the TOF MS/MS spectra at 480 min, several peaks were selected for assignment. The signal with the mass of the product B06 with an additional 17 units, 495.2703, could represent the gain of an hydroxyl group. Other peaks selected for the assignment were 341.0498 that may belong to fragment A without the diethylphosphonate ester moiety of B06, 142.0646 that may correspond to fragment C, 2-phenyl-3,4-dihydro-2H-pyrrole, and 449.0453 that can be the mass of fragment D with loss of C$_2$H moiety (Figure 11).

Figure 11. MS/MS spectra at 480 min of the characteristic fragmentation pattern of B06 metabolite 2 in in vivo PK.
3. Materials and Methods

3.1. Synthesis of B06

To access enough quantities of B06 for the studies reported in this paper, we undertook its synthesis by following a previously reported procedure [33]. The [3 + 2] cycloaddition reaction between diethyl α-phenylisocyanomethylphosphonate (187 mg, 0.7 mmol) and N-(4-chloro-4-fluorophenyl)maleimide (250 mg, 1.1 mmol) in acetonitrile (6 mL) and under AgOAc (8 mg, 0.05 mmol) catalysis furnished B06 after purification by flash chromatography (189 mg, 54%). The relative stereochemistry of the three generated stereocentres was determined by X-ray crystallography. The representative data of $^1$H RMN, $^{13}$C RMN and HPLC-MS spectra are included in the Supplementary Materials (pages S19–S22) [33].

3.2. Solubility and Chemical Stability of B06 at Different pH

The detection of B06 at different pH (2, 5 and 7.4) was obtained by LC/MS/MS spectrometry with mobile phase A (0.1% formic acid in water/acetonitrile 90/10) and B (0.1% formic acid in acetonitrile/water 90/10) using an injection volume of 5 µL. The autosampler’s drawers were kept cooled at 4 °C. Diethyl (1RS,3aSR,6aSR)-5-phenyl-4,6-dioxo-1-phenyl-1,3a,4,5,6,6a-hexahydropyrrolo[3,4-c]pyrrole-1-phosphonate, an analogous molecule to B06 without halogen substitution in the N-phenyl moiety, was used as the internal standard [33]. Detection of analytes and internal standards was carried out in multiple reaction monitoring mode (MRM) with electrospray positive ionization. Mass transitions used in the method were $m/z$ 479.2/341.2 for B06 (collision energy of 22 V) and $m/z$ 427.2/289.3 for the internal standard (collision energy of 21 V). The mass spectrometric analysis can present a variability between 15–20% [43].

3.3. Plasma Stability of B06 at Mouse Plasma

Mouse plasma pooled from healthy donors extracted in citrate tubes was employed in the assay. Plates containing 10 µM B06 in plasma (total volume: 50 µL) were incubated at 37 °C at different times (0, 60, 180 and 360 min). Then, 100 µL of acetonitrile was added to precipitate the plasma protein, and the plate was centrifuged at 46,000 × g for 60 min at 5 °C. Supernatant was taken and analysed by UPLC/MS/MS for sample quantification. The stationary phase was a reverse phase Acquity UPLC® BEH C18 1.7 µm (2.1 × 50 mm) (Waters), and the mobile phase was 0.1% formic acid in water/0.1% formic acid in acetonitrile, using a gradient and flow rate of 0.6 mL/min. The chromatographic equipment employed was an UPLC QSM Waters Acquity, and the compound concentrations were calculated from the MS peak areas.

3.4. Metabolic Stability of B06 in Human Liver Microsomes

The methods and data related to this section are included in the Supplementary Materials (page S1) [44].

3.5. Metabolic Profiling of B06 in Human and Mouse Liver Microsomes

The methods and data related to this section are included in the Supplementary Materials (page S3).

3.6. Chemical Synthesis

Ethyl hydrogen [(1R,3aS,6aS)-5-(3-chloro-4-fluorophenyl)-4,6-dioxo-1-phenyl-1,3a,4,5,6,6a-hexahydropyrrolo[3,4-c]pyrrole-1-yl]phosphonate (1).

To a solution of B06 (100 mg, 0.21 mmol) in dichloromethane (5 mL), TMSBr (0.3 mL, 2.1 mmol) was added dropwise under argon. The mixture was stirred at room temperature for 1 h. The solvent was evaporated to afford 1, which was purified by a reversed-phase column chromatography (H$_2$O:ACN 95:5) [45]. $^1$H NMR (400 MHz, CD$_3$OD) δ 1.17 (t, J = 7.0 Hz, 3H, CH$_2$CH$_3$), 3.87–4.13 (m, 3H, CH$_2$CH$_3$ and H-3a), 4.21 (dd, J = 4.5, 17.5 Hz, 1H, H-6a), 6.73 (dd, J = 8.0, 18.5 Hz, 2H, ArH), 7.19 (t, J = 9.0 Hz, 1H, ArH), 7.33 (d, J = 5.5 Hz, 3H, ArH), 7.66 (s, 2H, ArH), 7.97 (s, 1H, ArH), 8.20 (d, J = 7.5 Hz, 1H,
H-3). HRMS C_{20}H_{16}ClFN_{2}O_{5}P [M + H]^- 449.0475; found, 449.0469. Purity 96.2% (t_R = 3.39 min). In addition, the compound [(1R,3aS,6aS)-5-(3-chloro-4-fluorophenyl)-4,6-dioxo-1-phenyl-1,3a,4,5,6,6a-hexahydropyrrolo[3,4-c]pyrrol-1-yl]phosphonic acid, resulting from the dihydrolysis reaction, was isolated: ^1H NMR (400 MHz, CD_3OD) δ 3.35 (s, 1H, H-3a), 4.22 (d, J = 17.0 Hz, 1H, H-6a), 6.73 (dd, J = 7.5, 18.0 Hz, 2H, ArH), 7.19 (t, J = 9.0 Hz, 1H, ArH), 7.32 (d, J = 6.5 Hz, 3H, ArH), 7.66 (s, 2H, ArH), 7.96 (s, 1H, H-3). HRMS C_{18}H_{12}ClFN_{2}O_{5}P [M + H]^- 421.0162; found, 421.0166. Purity 100% (t_R = 3.06 min).

The ^1H NMR and HPLC-MS spectra are depicted in the Supplementary Materials (Pages S20–S21 and S23–S25).

3.7. In Vitro Study of the Role of B06 in a Cellular Model of Neurodegeneration

3.7.1. SH-SY5Y Human Cell Line

Cell lines of human neuroblastoma SH-SY5Y (Sigma-Aldrich, St. Louis, MO, USA) were propagated and maintained under standard conditions (37 °C and 5% CO_2) in F12 medium/EMEM (Gibco, Waltham, MA, USA) containing 2 mM glutamine, 1% nonessential amino acids, and 15% fetal bovine serum (FBS). To test the excitotoxicity of the compounds, the cultures were treated for 24 h with the indicated compounds at 0.2, 0.5, 1, 2, 5, 10 and 20 µM. After that time, cell viability was assessed (Page S18, Figure S18) using the MTT assay. None of the concentrations used were cytotoxic to the cells. On attaining semiconfluence, cells were pretreated for 1 h with the different compounds at 1 µM. The dose was chosen based on a dose–response experiment previously performed in order to select the lowest concentration with the highest effect (Figure 7a). After that, 6-OHDA (25 µM, Sigma) was added to the cultures and incubated for 16 h. Finally, cultures were processed for cell viability assay and nitrite production.

3.7.2. Primary Glial Cultures

Mouse cerebral cortex-derived glial cells were prepared as previously described [38]. Briefly, the brain was dissected and the cerebral cortex isolated, dissociated and incubated with 0.25% trypsin/EDTA at 37 °C for 1 h. Then, the tissue was centrifugated, and after several rinses in HBSS (Gibco), cells were plated on noncoated flasks and maintained in HAMS/DMEM (1:1) medium containing 10% FBS. After 7 days on culture, flasks were agitated on an orbital shaker for 4 h at 240 rpm at 37 °C, the supernatant was collected, centrifuged, and the cellular pellet containing the microglial cells resuspended in complete medium (HAMS/DMEM (1:1) containing 10% FBS) and seeded on uncoated 96-well plates. Cells were allowed to adhere for 2 h, and the medium was removed to eliminate nonadherent oligodendrocytes. New fresh medium containing 10 ng/mL of GM-CSF was added. The remaining astroglial cells adhered to the flasks were then trypsinized, collected, centrifuged, and plated onto 96-well plates with complete medium. The purity of cultures obtained by this procedure was >98% as determined by immunofluorescence with Iba-1 (microglial marker) and GFAP (astroglial marker) antibodies. After 2 days in culture, cells were pretreated for 1 h with the compounds (1 µM), and later with bacterial lipopolysaccharide (LPS; 10 µg/mL) for 24 h. Nitrite production on cultures was then measured.

3.7.3. Cell Viability Assay and Apoptosis Measurement

Cell viability was measured using the MTT assay (Roche Diagnostic, GmbH, Basel, Switzerland), based on the ability of viable cells to reduce yellow MTT to blue formazan. Briefly, cells were cultured in 96-well plates and treated with the indicated compounds for 16 h, and then 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. To evaluate the extent of apoptotic cell death, SH-SY5Y cultures were grown on glass cover-slips in 24-well cell culture plates for immunocytochemical analysis or in P60 plates for immunoblotting. After treatment, cover-slips were washed, permeabilized and treated as previously described [41]. A rabbit anti-active caspase-3 (1:200, MAB835 R&D
Systems) primary antibody was used, followed by incubation with a 488-Alexa-labeled secondary antibody (Invitrogen, San Diego, CA, USA). Images were acquired using a Radiance 2100 confocal microscope (Bio-Rad, Hercules, CA, USA). Immunoblot analysis was performed as previously described [38]. Blots were probed with anti-human active caspase-3 (1:1000, MAB835 R&D Systems). Secondary peroxidase-conjugated donkey anti-rabbit antibody was purchased from Amersham Biosciences (GE Healthcare, Buckinghamshire, UK). Representative images of at least three independent experiments corresponding to three different samples are shown. The images of blotting were quantified using ImageJ software (Wayne Rasband, NIH, Bethesda, MD, USA). Values in the text are the mean of at least three different experiments.

3.7.4. Nitrites Measurement

Accumulation of nitrites in the media was assayed by the standard Griess reaction. After stimulation of cells with the different treatments for 16 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 read using a plate reader at 492/540 nm.

3.8. Method Validation for Quantification of \textit{B06} in Mouse Plasma and Mouse Brain

The methods and data related to this section are included in the Supplementary Materials (Pages S8–S13, Tables S12–S19 and Figures S8–S13).

3.9. In Vivo Pharmacokinetics of \textit{B06}

3.9.1. Animals

Male CD1 mice were supplied by the Laboratory Animal Service of the University of Granada (Granada, Spain) and housed in the Animal Facilities of the Center of Biomedical Investigation of the University of Granada. They were housed in standard conditions (light/dark cycle of 12 h, temperature 22 ± 0.1 °C and 50–55% moisture) with free access to food (AIN-93G diet). Sample preparation: \textit{B06} was dissolved in DMSO 10% and cyclodextrin 40% at 2 mg/mL. The calculated PK parameters for \textit{B06} were obtained from all mouse plasma and brains. Calculations were performed using noncompartmental analysis of plasma data after intraperitoneal injection by means of PK Solver 2.0. All analytical determinations of \textit{B06} were performed according to the recommendations of the FDA [46].

3.9.2. Calibration Curve for \textit{B06} in Plasma Samples

The methods and data related to this section are included in the Supplementary Materials (page S14, Figure S14).

3.9.3. Quantitation for \textit{B06} in Plasma Samples at Dose 10 mg/kg and Samples Grouping and Statistics in Plasma Samples

The methods and data related to this section are included in the Supplementary Materials (pages S14 and S15, Tables S20 and S21 and Figure S15).

3.9.4. Calibration Curve for \textit{B06} in Brain Samples

The methods and data related to this section are included in the Supplementary Materials (page S15, Figure S16).

3.9.5. Quantitation for \textit{B06} in Brain Samples at Dose 10 mg/kg and Samples Grouping and Statistics in Brain Samples

The methods and data related to this section are included in the Supplementary Materials (pages S16 and S17, Tables S22 and S23 and Figure S17).
3.10. Metabolic Profiling In Vivo of B06

The methods and data related to this section are included in the Supplementary Materials (pages S5 and S6).

3.11. Statistical Analysis

Figures 7 and 8 are expressed as the mean ± SD of triplicate determinations. Experiments were repeated at least three times, yielding similar results. Data were analysed using one-way ANOVA. Then, a significance level of \( p < 0.05 \) was applied to the post hoc statistical analyses (Tukey test). The SPSS statistical software package (version 20.0) for Windows (Chicago, IL, USA) was used.

4. Conclusions

The discovery of B06, away from the structural limits of previous I2-IR ligands, has opened the door for the comprehensive exploration of these receptors in a plethora of indications. To start with, oral treatment with B06 in murine models of neurodegeneration and AD prevented behavioural abnormalities and memory loss, reducing AD hallmarks, oxidative stress, and apoptotic and neuroinflammation markers. Preliminary ADME-Tox parameters and a safety panel proved that B06 is a suitable compound to advance in preclinical phases.

In detail, in this work, we provided information on the metabolic stability of B06 in human and mouse liver microsomes showing a medium hepatic clearance. The metabolic profile in liver microsomes lead to the proposal and unequivocal identification, after its synthesis, of a metabolite resulting from the monohydrolysis of the phosphonate ester function of the molecule. The in vivo PK study led to the calculation of relevant parameters and revealed a good correlation between the levels of B06 found in plasma and in the brain, although the low amounts of the compound quantified indicated a good penetration in the CNS.

Notably, this paper is the first study aimed to unveil the role that I2-IR ligands could have in PD. After finding the optimal dose, the neuroprotective role of B06 on the human dopaminergic cell line SH-SY5Y was determined. Furthermore, in primary cultures of astrocytes and microglia treated with LPS, B06 was shown to be an anti-inflammatory agent even better than other known ligands.

Overall, our results confirm that B06 is a promising candidate for neurodegenerative management due to its immunomodulatory and neuroprotective properties. B06 is a robust compound to be seriously considered as a modulator of I2-IR for characterizing the pharmacological profile of these receptors and for further and more advanced preclinical development in brain disorders such as AD and PD.

5. Patents

The authors declare the following competing financial interest(s): C.E., M.P., and C.G.-F. are inventors of the patent application of I2 imidazoline receptor ligands, WO2019//121853 [32].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23105408/s1.

Author Contributions: A.B.: Investigation, synthesis and purification of the intermediates and final products, spectral characterization of the compounds, writing—review and editing. J.A.M.-G.: investigation, design of the cell culture studies, in vitro studies on neuroprotective activity, analysis of the data. C.G.-F.: resources, pharmacokinetic studies. C.D.: investigation, physicochemical studies on the stability of B06 at different pH, in vitro liver microsomes studies, pharmacokinetics, metabolic profiles studies, analysis of the data, writing—review and editing. J.P.d.P., M.C.R. and F.V.: investigation, physicochemical studies on the stability of B06 at different pH, in vitro liver microsome studies, pharmacokinetics, metabolic profiles studies, analysis of the data. B.P.: analysis of pharmacokinetics data. J.B. and M.I.L.: investigation, plasma stability studies. M.P.: conceptualization, funding acquisition, supervision, analysis of the data. C.E.: conceptualization, funding acquisition,
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Abbreviations

AD Alzheimer’s disease  
α2-AR α2-adrenoceptors  
BBB blood–brain barrier  
Cl plasma clearance  
Cmax maximum observed concentration  
HLM human liver microsomes  
I2-IR imidazoline I2 receptors  
LPS bacterial lipopolysaccharide  
MLM mouse liver microsomes  
MRM reaction monitoring mode  
ND neurodegenerative diseases  
6-OHDA 6-hydroxydopamine  
PAMPA parallel artificial membrane permeability assay  
PD Parkinson’s disease  
PK pharmacokinetics  
SAMP8 senescence-accelerated mouse prone 8

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