1-(2',5'-Dihydroxyphenyl)-3-(2-fluoro-4-hydroxyphenyl)-1-propanone (RGM079): A positive allosteric modulator of $\alpha 7$ nicotinic receptors with analgesic and neuroprotective activity

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ABSTRACT:

Acetylcholine $\alpha 7$ nicotinic receptors are widely expressed in the brain, where they are involved in the central processing of pain, as well as in neuropsychiatric, neurodegenerative and inflammatory processes. Positive allosteric modulators (PAM) show the advantage of allowing the selective regulation of different subtypes of acetylcholine receptors without directly interacting with the agonist binding site. Here we report the preparation and biological activity of a fluoro-containing compound, 1-(2',5'-dihydroxyphenyl)-3-(2-fluoro-4-hydroxyphenyl)-1-propanone (**8**, **RGM079**), that behaves as a potent PAM of the $\alpha 7$ receptors, and has a balanced pharmacokinetic profile and antioxidant properties comparable or even higher than well-known natural polyphenols. In addition, compound **RGM079** shows neuroprotective properties in AD-toxicity related models. Thus, it causes a concentration-dependent neuroprotective effect against the toxicity induced by okadaic acid (OA) in the human neuroblastoma cell line SH-SY5Y. Similarly, in primary cultures of rat cortical neurons, **RGM079** is able to restore the cellular viability after exposure to OA and amyloid peptide A β ₁₋₄₂, with cell death almost completely prevented at 10 and 30 μ M, respectively. Finally, compound **RGM079** shows *in vivo* analgesic activity in the CFA-induced paw inflammation model, after intraperitoneal administration.

INTRODUCTION

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels involved in many physiological processes. They play significant roles in cognition and neurological disorders,¹⁻³ and their activation also promotes analgesic effects in several animal models of chronic and inflammatory pain.^{4,5} They constitute an interesting family of drug targets and several years of research have generated a not negligible number of drug candidates. However, even though several nAChRs modulators have entered into clinical trials, none of them has reached the market yet. Certainly, the functional complexity of these channels, and the lack of structural information have made more difficult the design of suitable nAChR drug candidates^{6,7} It is expected that the recent disclosure of the crystal structure of the heteromeric $\alpha 4\beta 2$ nAChR⁸ would facilitate further structural studies of other receptors of the same family. This will shed deeper knowledge about the topology of the orthosteric and allosteric binding sites, which in turn will permit the design of new drugs showing higher sub-type selectivity.⁹

In particular, the $\alpha 7$ subtype ($\alpha 7$ nAChRs) are homopentameric assemblies abundantly distributed in the central nervous system,, being potential therapeutic targets for the treatment of several cognitive and memory disorders.^{1,10-12} Activation of $\alpha 7$ receptors promotes antinociceptive effects in models of neuropathic and inflammatory pain.^{13,14} Additionally, potentiation of $\alpha 7$ nAChRs has attracted considerable interest as a strategy to treat various nervous-system ailments, such as Alzheimer's disease (AD)^{15,16} or schizophrenia.¹⁷ $\alpha 7$ nAChRs are highly expressed in the hippocampus, the most affected area in AD. It is well established the presence of $\alpha 7$ nAChRs on amyloid plaques and also that they bind with high affinity to A β .¹⁸ Although the nature of this interaction is still not sufficiently clear, it has been suggested that $\alpha 7$ nAChRs are implicated in A β toxicity and in the pathogenesis of AD.¹⁹ More recently, it has been

reported that the administration of low doses of the $\alpha 7$ agonists AZD0328 and SSR180711 upregulate $\alpha 7$ nAChRs expression in hippocampus, increasing cognition in mice models.²⁰ Recent reviews evidence that the $A\beta_{1-42}$ - $\alpha 7$ /nAChR complex mediates synaptic plasticity and cognitive ability, although the exact mechanisms of regulation are still to be clarified.^{21,22} The $A\beta_{1-42}$ - $\alpha 7$ nAChR interaction is playing a crucial role in memory and cognition, and as a result several $\alpha 7$ nAChR agonists have moved into clinical trials.²¹ Similarly, in a model of neuronal death induced by okadaic acid (OA) that reproduces the typical tau (τ) hyperphosphorylation of Alzheimer's disease, it has been described that this hyperphosphorylation of tau reduces the expression of nAChRs and enhances the neurotoxicity of β -amyloid peptide.²³ In agreement with this fact, the activation of both $\alpha 7$ and $\beta 2^*$ nAChR afforded neuroprotection against OA-induced neurotoxicity.²⁴ Curiously, the promotion of Ca^{2+} entry through the $\alpha 7$ nAChR using an $\alpha 7$ -selective PAM modulator, PNU 120596, did not protect against this OA-evoked neurotoxicity.²⁴

Apart from agonists, allosteric modulation of $\alpha 7$ nAChR have been reported. Positive allosteric modulators (PAMs) are able to enhance the response evoked by acetylcholine (ACh), the endogenous ligand of AChRs. PAMs can be classified as type I and type II, according to their capacity to delay or not desensitization of the receptors. There are also compounds able to block allosteric potentiation, without affecting the orthosteric agonist responses, named silent allosteric modulators (SAMs). More recently, allosteric agonists have been described, also called ago-PAMs, which are allosteric modulators able to activate the receptor through non-orthosteric sites, without affecting the PAM action and in the absence of an orthosteric agonist. Finally, negative allosteric modulators (NAMs) are able to allosterically block the open channel.^{3,6,25} Allosteric modulation is still a valuable alternative, because it shows the advantage that could

allow the selective regulation of different subtypes of acetylcholine receptors, potentially avoiding many of the adverse effects associated with therapies that target the nAChRs endogenous agonist site.^{12,26,27} Although a more profound study of the receptor mechanism of action by allosteric activation is needed, the recent approval of BNC375, an orally available Type I PAM of nAChRs, that entered clinical phases for the treatment of cognitive impairment in Alzheimer's disease and other central nervous system dysfunctions, indicates that AChRs remain valid targets.^{28,29}

Starting from a series of polyhydroxy-substituted chalcones that behave as PAMs of $\alpha 7$ nAChRs (see compound **1**; Figure 1),³⁰ we synthesized some new polyhydroxylated 1,3-diphenylpropan-1-ones with capacity to allosterically activate the $\alpha 7$ nicotinic receptors.³¹ From this new collection of polyhydroxylated diphenylpropan-1-ones, compounds **2** and **3** were effective, although less potent, as PAMs of $\alpha 7$ nAChRs, being selected for further studies (Figure 1). In *in vivo* experiments, these compounds showed different behavior in a mechanical hyperalgesia assay. While compound **3** exhibited significant antinociceptive activity (10 mg/Kg, *iv*),³¹ no activity was found for its tetrahydroxy analogue **2** at the same dose *iv*. A brief *in vitro* study of the pharmacokinetic (PK) properties of compounds **1-3** (Figure 1) revealed a quite poor PK profile, limiting their therapeutic potential.³¹ These results prompted us to synthesize new analogues to improve the pharmacokinetic properties. In this sense, we have recently described the preparation of peptide pro-drugs of some diphenyl propanones with the aim of increasing the *in vivo* efficacy of the parent drugs, **2** and **3**, endowed with longer-lasting effects than their parent drugs.³²

Here we describe the synthesis and pharmacological characterization of a fluoro-substituted analogue of the $\alpha 7$ nAChRs PAM **3**, prepared with the aim of enhancing its permeability and its metabolic stability. Compound **RGM079**, which also behaves as a

PAM of $\alpha 7$ -nAChRs, has been evaluated for its antioxidant and neuroprotective capacity *in vitro*, and for its *in vivo* analgesic activity in a model of inflammatory pain.

Figure 1. Structure of parent compounds **1-3**.

RESULTS AND DISCUSSION

Design. Comparing the results of the brief ADME study for compounds **2** and **3**, two remarkable differences were observed.³¹ First, as could be expected, the permeability measured in CACO-2 cells of the trihydroxy substituted compound **3** is higher than that found for the more polar tetrahydroxy-substituted compound **2**. This lower permeability could be behind the lack of *in vivo* activity of compound **2**. Second, compound **3** is cleared much faster than **2**, and the half-life found for **3** is about a half relative to **2**, probably due to a quick metabolic oxidation at position 2. Therefore, to avoid this oxidative metabolic pathway, we designed the 2-fluoro derivative **7**. The incorporation of a fluorine atom at metabolically oxidizable positions is a quite common strategy in lead optimization programs, which normally improves the half-life time.³³ Moreover, this usually enhance lipophilicity of the product, which will be desirable in terms of penetration.³⁴ The benefits that fluorine substitution can entail in terms of potency, lipophilicity and PK properties are well known.³⁵

Chemistry. The parent 2-fluoro substituted chalcone **6**, was prepared by aldol condensation of 2,5-dimethoxyacetophenone (**4**) and 2-fluoro-4-methoxybenzaldehyde (**5**), both commercially available, under basic conditions. Selective reduction of the

double bond in the presence of Ph_2S , followed by deprotection of the methoxy groups with Br_3B led to compound **8 (RGM079)** in good yield (Scheme 1).

Scheme 1. Preparation of 1-(2',5'-Dihydroxyphenyl)-3-(2-fluoro-4-hydroxyphenyl)-1-propanone (**8, RGM079**).

Electrophysiology Studies. Compound **RGM079** was evaluated for its capacity to modulate the currents evoked by ACh in *xenopus* oocytes that selectively expressed $\alpha 7$ nicotinic receptors. A large potentiating effect was observed in ACh-evoked ionic currents when compound **8 (RGM079)** was applied to oocytes expressing human $\alpha 7$ nAChRs (Figure 2A). Currents decayed in a biphasic manner. At first, decay was fast, like it happens with a typical type I PAM, and then very slow, with a kinetics behavior similar to that observed with type II PAMs. Previously, we have found chalcones acting either as type I PAMs (isoliquiritigenin³⁰) or type II PAMs (2,4,2',5'-tetrahydroxychalcone, here compound **1**³⁰, and propanones **2** and **3**³¹). Interestingly, compound **RGM079** shows a mixed behavior, a quite common situation for other

modulators.²⁵ The effect of compound **RGM079** is dose-dependent with an EC₅₀ of 8.3 μ M (Table 1), and a maximal activation of about 23-fold about the current induced by 200 μ M ACh (Figure 2B), a concentration that is close to the EC₅₀ for ACh (176 μ M, see Balsera et al³⁰). The potentiating effect of **RGM079** was not observed in the absence of ACh, indicating that this compound is not acting as agonist itself. Compound **RGM079** showed similar concentration-dependent effectiveness than the most effective chalcones characterized by us (isoliquiritigenin and **1**³⁰) and clearly higher than those of other typical PAMs.³⁶ Regarding potency, compound **8** (**RGM079** (8.3 μ M)) is in-between the other two chalcones mentioned above (3.3 μ M and 25 μ M for **1** and isoliquiritigenin, respectively), and very similar to that of the reduced analogues **2** and **3** (Table 1).

Figure 2. Effect of compound **8 (RGM079)** on $\alpha 7$ nAChRs. A) Ionic currents recorded in a representative oocyte expressing human $\alpha 7$ nAChRs. Currents were evoked by 600 ms applications of ACh 200 μ M in the absence (continuous line) and in the presence (dotted line) of 10 μ M of **8 (RGM079)** and recorded at a holding potential of -80 mV. B) Concentration-response relationship for the potentiating effect of **8 (RGM079)** with ACh 200 μ M. Continuous line represents the fit to the Hill equation, with a maximal potentiating effect estimated at 22.8, and an EC₅₀ value of 8.3 μ M.

Table 1. I_{max} and EC₅₀ values for propan-1-one derivatives and comparison with chalcone analogue 1

Compound	I _{max} ^a	EC ₅₀ (μ M) ^a
1^b	34.00 $\pm$ 5.5	3.30 $\pm$ 1.6
2^b	15.46 $\pm$ 1.2	6.85 $\pm$ 0.9
3^b	15.00 $\pm$ 1.9	12.86 $\pm$ 1.4
RGM079	22.76 $\pm$ 9.8	8.34 $\pm$ 3.0

^aValues derived from the corresponding dose response curves. Responses were recorded at -80 mV and normalized with respect to that shown by only ACh (0.2 mM). Data are the mean $\pm$ SD, with n $\geq$ 6. ^bValues from reference 31.

Physicochemical Properties and Preliminary *in vitro* ADME Profile. To have an idea of the potential of the new fluoro derivative and to be able to compare it to the parent compounds, an *in vitro* physicochemical profile of compound **RGM079** was performed. The calculated physicochemical properties, like MW (< 300 Da), lipophilicity (CLogP = 2.83) and polar surface area (PSA = 77.75), are of the same order of those found for parent compounds **2** and **3**, and compatible with a good oral absorption.³¹ Concerning the *in vitro* PK data, compound **RGM079** showed aqueous solubility values quite acceptable in the three simulated fluids, comparable to those of

the parent compound **3**, and similar protein binding ability. The A-B permeability in Caco-2 cells of **RGM079** is much better than that of **2** (6-fold increase), but half that of the parent compound **3**. However, a good point is that B-A efflux is diminished for **RGM079** compared to that of **3** and specially to that of **2**. The microsomal metabolic lability values found for compound **RGM079** revealed that its metabolic stability is much higher than that found for compound **3**, showing a higher half-time value (55 versus 18 min), and still better than that of **2** (42 min, Table 2). The fact that in compounds **2** and **RGM079** the position 2 of the phenyl ring is respectively occupied by a OH- and a F- atom, while in parent compound **3** this position is free, come to reinforce our hypothesis that this position constitute an important metabolically active point in these compounds.³¹ Moreover, the intrinsic clearance value of the fluoro derivative **RGM079** is the best in this series, especially when compared to compound **3**, thus improving the possibilities of this compound for further development, and encouraging us to face further pharmacological evaluation.

Table 2. Calculated Physicochemical Properties and Short ADME Profile of Compounds 2, 3 and RGM079

Property		Compd 2 ^a	Compd 3 ^a	Compd RGM079
MW		274.27	258.27	276,26
CLogP		2.66	2.74	2.83 ^b
PSA		97.98	77.75	77.75 ^b
Log BB		-0.93 ^b	-0.59 ^b	-0.58 ^c
Solubility ^d (μM)	Intestinal fluid	184.2	188.4 (ADJ)	169.6
	PBS (pH 7.4)	176.4	159.9	186.3

	Gastric fluid	185.0	200.0 (ADJ)	186.2
% Protein bound ^e		97	97	99
Permeability Caco-2 ^f (cm/s)	A-B	3.1 x 10 ⁻⁶	33.5 x 10 ⁻⁶	17.8 x 10 ⁻⁶
	B-A	38.5 x 10 ⁻⁶	12.0 x 10 ⁻⁶	8.2 x 10 ⁻⁶
Metabolism ^g	Half-life (min)	42	18	55
	CL _{int} (μL/min/mg)	165.7	387.1	127.5

^a From reference 31. ^bCalculated using Molinspiration (<http://www.molinspiration.com>). ^cCalculated from the following equation $\text{Log BB} = -0.0148\text{PSA} + 0.152\text{CLogP} + 0.139$.³⁷ ^{d,e,f,g} Performed by CEREP (Eurofins). LCP = low chromatography purity due to compound instability at room temperature in PBS solution. ADJ = Solubility greater than 200 μM, adjusted to the maximum assay concentration (200 μM).

Antioxidant Ability. It is well known that hydroxyl substituted natural compounds have antioxidant properties, that confer them neuroprotective and anti-inflammatory effects.^{38,39} Hydroxyl groups of phenols are easily converted in phenoxy radicals by a hydrogen transfer mechanism, conferring antioxidant properties to the molecule.⁴⁰ Like for the previously prepared precursor parent compounds, the oxygen radical scavenging capacity of compound **8** was determined by an oxygen radical absorbance capacity assay (ORAC) assay. The result found, 7.66 μM trolox/μmol of pure compound, points to a not-negligible antioxidant capacity, similar to or even higher than that reported for well-known natural antioxidants.^{41,42} The value found in the ORAC assay is similar to that of resveratrol in our assay, of the same order of that described for the tetrahydroxy derivative **2**, and higher than that of the trihydroxy analog **3** (Table 3).³¹

Related to Alzheimer disease (AD), the combination of α7 nAChRs potentiation together with antioxidant properties, in a single molecule, is very interesting since it

has already led to outstanding results. This is the case of galantamine,⁴³ a marketed drug for AD treatment with antioxidant properties, and of the natural product epigallocatechin gallate that acts as a neuroprotecting agent against A β toxicity, both allosteric potentiators of the $\alpha 7$ nAChRs.⁴⁴ Therefore, we decided to explore the potential neuroprotective role of our compound in *in vitro* models of AD.

Table 3. Results of the ORAC assay

Compound	$\mu\text{M TE}/\mu\text{mol of pure compound}$
2	7.0 ± 1.8^{31}
3	2.7 ± 0.1^{31}
8	7.66 ± 0.04
Resveratrol	7.53 ± 0.39
Quercetin	6.88 ± 0.27
(+)-Catechin	2.49 ± 0.84
TE: Trolox equivalents	

Neuroprotective capacity of RGM079. Considering that $\alpha 7$ nAChR activation protects against cytotoxic stimuli related to AD,⁴⁵ like OA^{23,24} and β -amyloid,⁴⁶ compound **8** (**RGM079**) was evaluated in these neurotoxic models. OA is a phosphatase inhibitor that produces hyperphosphorylation of Tau protein, in *in vitro* and *in vivo* models, to cause neuronal degeneration, synaptic loss and memory impairments, thus resembling AD symptoms.²³

In this study, we first used the human neuroblastoma cell line SH-SY5Y that constitutes a good model for deciphering mechanisms of neuronal death and protection.^{45,47} SH-SY5Y cells were incubated with increasing concentrations of

compound **RGM079** for 24 h and then co-incubated with OA (15 nM) and the compound for an additional 24 h period. The results shown in Figure 3 reveal that **RGM079** was neuroprotective against OA-induced neurotoxicity, showing a significant concentration-dependent increase of cellular viability by 90% at 1 and 10 μ M and by 100 % at 3 μ M, respectively; these viability values were higher than that shown by PNU282987 (~ 80%), an α 7 nAChR agonist, used as positive control. When the compound was not pre-incubated and was only present during the toxic stimulus, neuroprotection values were reduced, although still significant at 1-10 μ M (Figure S1).

Figure 3. Compound 8 (RGM079) causes a concentration-dependent neuroprotective effect against the toxicity induced by okadaic acid (OA) in the human neuroblastoma cell line SH-SY5Y. (A) The protocol used: Compound **RGM079** was pre-incubated for 24 h, and then, OA was co-administered with compound **RGM079** for 24 h more. (B) Effect on cell viability, measured as MTT reduction, of increasing concentrations of compound **RGM079** against OA (15 nM) in SH-SY5Y cells. As positive control, the $\alpha 7$ agonist PNU-282987 (10 mM) was used. Data represent the mean $\pm$ SEM of 6 different experiments. *** $p < 0.001$ compared to basal; # $p < 0.05$ compared to OA; ## $p < 0.01$ compared to OA; ### $p < 0.001$ compared to OA.

The neuroprotective properties of compound **8 (RGM079)** were further confirmed in primary cortical neuronal cultures by using a 24 h pretreatment protocol, followed by 24 h incubation of the compound with the toxics (Figure 4A). We first analyzed the neuroprotective effect of **compound 8 (RGM079)** against the toxicity induced by OA used as a tau hyperphosphorylation model. As represented in Figure 4B, OA reduced viability by around 40 % and compound **RGM079** was also protective in primary neuronal cultures; at 10 μ M, cell death was almost completely prevented (Figure 4B). Then, we explored whether compound **RGM079** was able to protect against neurotoxicity evoked by $A\beta_{1-42}$, the most amyloidogenic isoform of $A\beta$. Exposure of cortical neurons to $A\beta_{1-42}$ (30 μ M) resulted in a reduced cell viability of approximately 30 %, while preincubation with increasing concentrations of **RGM079** revealed a statistically significant neuroprotection at concentrations above 10 μ M, and almost total restoration of neuronal viability was achieved at 30 μ M (Figure 4C). However, compared to the control, no relevant levels of neuroprotection were observed with $A\beta_{25-35}$ (data not shown). The crucial role of

Lys16 residue of A β_{1-42} in amyloid aggregation has been described in a study by Sato and coworkers.⁴⁸ This residue was also fundamental for the inhibitory mechanism of A β_{1-42} aggregation by catechol-type flavonoids, associated with the formation of A β_{1-42} /flavonoid adducts after flavonoid oxidation to the corresponding *o*-quinone. Considering that **RGM079** could be oxidized under aerobic conditions to a *p*-quinone, a similar mechanism of action could be suggested for the protective activity of this fluoro-compound against A β_{1-42} neurotoxicity.

Figure 4. Compound 8 (RGM079) protects rat cortical neurons in AD-toxicity related models. (A) Protocol used: Increasing concentrations of compound **8** were pre-incubated for 24 h and, thereafter, incubated with the toxic (OA or OA/A β_{1-42} ; cell viability was measured by the MTT method. (B) Effect of increasing concentrations of compound **8** against neurotoxicity induced by OA and (C) A β_{1-42} toxicity. Data represent the mean $\pm$ SEM of 6 different experiments. ***p < 0.001 compared to basal;

^{##}p< 0.01 compared to OA/ A β ₁₋₄₂; ^{###}p< 0.001 compared to the toxic alone (OA or A β ₁₋₄₂).

To know if the mechanism of neuroprotection promoted by **RGM079** was mediated by α 7 nAChR, an additional experiment was performed using α -bungarotoxin (α -Bgt). Cells were incubated 24 h with α -Bgt (100 nM), a suitable concentration to block α 7 nAChRs. Then, compound **8** together with OA were added to the culture media and incubated for an additional 24h-period. The results showed that, despite the presence of the α -Bgt, **8 (RGM079)** was able to significantly increase viability of cortical rat neuronal cells by 70% at 10 μ M. It can be concluded that the observed neuroprotective effect does not appear to be mediated by α 7 nAChRs, since it was not totally prevented by α -Bgtx (Figure 5). This result agrees with the fact that the α 7-selective PAM modulator, PNU 120596, was unable to protect against the OA-evoked neurotoxicity.²⁴ Therefore, a different mechanism should be behind the cellular protection by compound **RGM079** against OA toxicity, that probably could be related to its potent antioxidant activity.

Figure 5. The neuroprotective effect of compound 8 was not reverted by α -bungarotoxin in rat cortical neurons. (A) Protocol used: the $\alpha 7$ nAChR antagonist α -Bungarotoxin (100 nM) was incubated 2 h prior to the addition of compound 8 plus OA for an additional 24 h. (B) Cell viability, measured as MTT reduction, at the indicated experimental conditions. Data represent the mean $\pm$ SEM of 3 different experiments. ** $p < 0.01$ compared

Analgesic activity. We have previously described that compounds **1** and **3** displayed analgesic activity in an *in vivo* test of mechanical hyperalgesia after *iv* administration, which in the case of **3** was comparable to that of the PNU-120596, a known PAM of $\alpha 7$ nAChRs.^{30,31} In addition, some known polyphenols, like quercetin derivatives and resveratrol, displayed significant antinociceptive activity in different pain models.^{49,50}

As compound **RGM079** demonstrated good PAM values for $\alpha 7$ nAChRs, remarkable antioxidant capacity, and have a more balanced pharmacokinetic properties compared to its precursors **2** and **3**, here we explore its analgesic activity, after intraperitoneal (*ip*) administration, in the mechanical hyperalgesia test resulting from inflammation induced by complete Freund's adjuvant (CFA). Thus, after the intraplantar injection of CFA, the administration of **RGM079** (10 mg/Kg, *ip*) produces a significant reduction of the latency time response to mechanical stimulus (Figure 6A). The main peak of activity was observed after 1h from administration, still significant after 2 h, and then a continuous decay in the following two hours. Model PNU-120596 was also assayed in the same test after *ip* administration (Figure 6B). As observed in Figure 6, the analgesic effect exhibited by the reference compound PNU-120596 was transient and of similar duration of action than that of **RGM079**, but our compound was much more effective. The effect of **RGM079** lasted for 4h after drug inoculation, until complete disappearance, probably because of pharmacokinetic drug clearance.

Figure 6. Effect of compounds 8 (RGM079) (A) and PNU 1209596 (B) in the CFA-induced paw inflammation model. Time course of mechanical hyperalgesia in rats after injection of complete Freund's adjuvant (CFA) 0.5 mg/mL (50 μ L) into the left hind paw with and without administration of compound (10 mg/kg i.p.). The diagram shows the paw withdrawal latencies in response to mechanical stimulation ($n \geq 6$ rats/group). Data are given as mean $\pm$ SEM $n = 6$. Two-way ANOVA with Bonferroni post-hoc test. * $P < 0,05$; ** $P < 0,01$, *** $P < 0,001$.

CONCLUSIONS

Following our interest in PAM of $\alpha 7$ nAChRs, and previous promising results with 1-propanone derivatives **2** and **3**, in this work we describe the synthesis and biological characterization of a fluoro-containing analogue, 1-(2',5'-dihydroxyphenyl)-3-(2-fluoro-4-hydroxyphenyl)-1-propanone (**8**, **RGM079**), designed with the aim of improving the pharmacokinetic properties of previous model compounds. **RGM079** have a quite good, balanced pharmacokinetic profile and shows high antioxidant capacity in the ORAC assay, with TE values similar or higher than resveratrol, quercetin and catechin, all natural antioxidant polyphenols. This compound displayed significant *in vitro* neuroprotective effects against the toxicity induced by okadaic acid (OA) in a human neuroblastoma cell line (SH-SY5Y). More importantly, in AD-toxicity related models, **RGM079** restores cellular viability after exposure to OA (tau AD model) and amyloid peptide A β_{1-42} (A β AD model) using primary cultures of rat cortical neurons. The neuroprotective effect against OA is not prevented by α -Bgtx, indicating that it is not mediated by $\alpha 7$ nAChRs, in agreement with previous results for another $\alpha 7$ -selective PAM modulator (PNU 120596).²⁴ Alternatively, the potent antioxidant activity of **RGM079** could be after the observed cellular protection in this case. As for other $\alpha 7$ PAM modulators, **RGM079**, displays important *in vivo* analgesic activity in a model of inflammatory pain, showing higher efficacy than PNU 120596 after peripheral administration. Therefore, compound **8** (**RGM079**) can be considered a good lead to be used as a tool for studying $\alpha 7$ nAChR pharmacology, and its relatively simple structure could be the departure point for the future development of improved analogues.

METHODS

Chemistry. All reagents were of commercial quality. Solvents were dried and

purified by standard methods. Analytical TLC was performed on aluminum sheets coated with a 0.2 mm layer of silica gel 60 F254. Silica gel 60 (230-400 mesh) was used for flash chromatography. Analytical HPLC-MS was performed on a Waters equipment coupled to a single quadrupole ESI-MS (Waters Micromass ZQ 2000) using a reverse-phase SunFire™ C18 4.6 x 50 mm column (3.5 μ m) at a flow rate of 1 mL/min and by using a diode array UV detector. Mixtures of CH₃CN + 0.08% formic acid (solvent A) and H₂O + 0.1% formic acid (solvent B) were used as mobile phase (gradient of 15 to 95% of A in B in 5 or 10 min, as indicated in each case). HRMS (EI⁺) was carried out in an Agilent 6520 Accurate-Mass Q-TOF LC/MS equipment. NMR spectra were recorded on a Bruker-AVANCE 300 and a Varian-INOVA 400 spectrometer. Melting points were determined on a Mettler MP70 apparatus and are uncorrected.

1-(2',5'-Dimethoxyphenyl)-3-(2-fluoro-4-methoxyphenyl)-1-propen-2-one (6) To a solution of 2-fluoro-4-methoxybenzaldehyde (1g, 6.48 mmol) in EtOH (70 mL), 2,5-dimethoxyphenyl acetophenone (1.06 mL, 6.8 mmol) was added. The solution was cooled at 0°C, and an aqueous solution of NaOH 40% (2.64 mL, 25.9 mmol) was slowly added. After 15h of stirring at room temperature water was added and the organic solvent was removed at reduced pressure. The organic phase was extracted with EtOAc, and the organic extracts were washed with H₂O and brine. After drying over MgSO₄, the solvent was removed to dryness and the resulting residue was purified in a column chromatography, using a gradient of EtOAc in hexane from 15 to 35%, to afford 1.31 g (64%) of the title compound. Yellow solid, Mp 85-87 °C. HPLC, t_R = 9.47 min (10 min gradient: 15 to 95% of A in B). ¹H NMR (400 MHz, CDCl₃) δ : 3.81 (s, 3H, OCH₃), 3.83 (s, 3H, OCH₃), 3.85 (s, 3H, OCH₃), 6.64 (dd, 1H, J = 12.5, 2.5 Hz, 3-H), 6.72 (dd, 1H, J = 8.7, 2.5 Hz, 5-H), 6.93 (d, 1H, J = 9.0 Hz, 3'-H), 7.02 (dd, 1H, J = 9.0, 3.2 Hz, 4'-H), 7.18 (d, 1H, J = 3.2 Hz, 6'-H) 7.39 (d, 1H, J = 16.0 Hz, =CH_a), 7.53 (t, 1H, J =

8.6, 6-H), 7.71 (d, 1H, $J = 16.1$ Hz, =CH β). ^{13}C NMR (75 MHz, CDCl_3) δ : 55.8 (OCH_3), 56.0 (OCH_3), 56.6 (OCH_3), 102.01 (d, C-3, $J = 25.5$ Hz), 110.9 (d, C-5, $J = 3$ Hz), 113.5 (C-6'), 114.5 (C-3'), 116.1 (d, C-1, $J = 12$ Hz), 119.2 (C-4'), 126.9 (d, C $_{\alpha}$, $J = 6.7$ Hz), 129.9 (C-1'), 130.5 (d, C-6, $J = 2$ Hz), 136.1 (d, C $_{\beta}$, $J = 2.2$ Hz), 152.7 (C-2'), 153.7 (C-5'), 162.6 (d, C-4, $J = 11.2$ Hz), 162.8 (d, C-2, $J = 252$ Hz), 192.6 (CO) ppm. EM (ESI $^+$): m/z 317.2 ($\text{M} + \text{H}$) $^+$.

1-(2',5'-Dimethoxyphenyl)-3-(2-fluoro-4-methoxyphenyl)-1-propanone (7)

To a solution of compound **6** (0.73 g, 2.3 mmol) in MeOH (70 mL) and diphenylsulfide (28 μL , 0.023 mmol), Pd/C 10% (73 mg) was added. After hydrogenation at room temperature and 20 psi during 20 h, the catalyst was filtered off, the solvent evaporated under vacuum, and the residue purified by flash chromatography (EtOAc/hexane 1:4). White amorphous solid, 626 mg (85 %). Mp 60-63 $^{\circ}\text{C}$. HPLC-MS (Sunfire): $t_R = 5.53$ min (5 min gradient: 15 to 95% of A in B). ^1H NMR (400 MHz, CDCl_3) δ : 2.89 (t, 2H, CH $_2$, 3 Pr), 3.19 (t, 2H, CH $_2$, 2 Pr), 3.69 (s, 3H, OMe), 3.70 (s, 3H, OMe), 3.75 (s, 3H, OMe), 3.79 (s, 3H, OMe), 6.52 (m, 2H, 3-H, 5-H), 6.81 (d, $J = 9.0$ Hz, 1H, 3'-H), 6.93 (dd, $J = 9.0, 3.2$ Hz, 1H, 4'-H), 7.05 (t, $J = 8.8$ Hz 1H, 6-H), 7.27 (d, 2H, $J = 3.1$ Hz, 6'-H) ppm. ^{13}C NMR (75 MHz, CDCl_3) δ : 23.6 (CH $_2$), 44.2 (CH $_2$), 55.6 (OMe), 55.9 (OMe), 56.1 (OMe), 101.7 (d, C-3, $J = 25.5$ Hz), 109.6 (d, C-5, $J = 3.0$ Hz), 113.2 (C-6'), 114.0 (C-3'), 120.1 (C-4'), 120.4 (d, C-1, $J = 21.7$ Hz), 128.4 (C-1'), 131.5 (d, C-6, $J = 6.7$ Hz), 153.3 (C-2'), 153.5 (C-3'), 159.3 (d, C-4, $J = 10.5$ Hz), 161.3 (d, C-2, $J = 243$ Hz), 201.2 (CO) ppm. MS (ESI $^+$): m/z 319.4 ($\text{M} + \text{H}$) $^+$.

1-(2',5'-Dihydroxyphenyl)-3-(2-fluoro-4-hydroxyphenyl)-1-propanone (8)

To a previously cooled solution (0 $^{\circ}\text{C}$) of compound **7** (0.31 g, 0.97 mmol) in dry DCM (24 mL), a 1M solution of BBr $_3$ in DCM (5.84 mL, 5.84 mmol) was slowly added, under Ar atmosphere. After stirring 7 h at room temperature under Ar, H $_2$ O was added

to the reaction mixture. The solid precipitate formed was filtered off, and washed with H₂O and DCM. Then, the liquid phase was extracted with EtOAc. The organic extracts were washed with H₂O and brine, dried over Na₂SO₄ and evaporated. The crude product was purified by column chromatography (Eluent: gradient from 20 to 50% of EtOAc in hexane) to led to 250 mg (93 %) of an amorphous, white solid. Mp = 136-137 °C. HPLC (Sunfire): t_R = 8.01 min (10 min gradient: 15 to 95% of A in B). ¹H NMR (400 MHz, DMSO-d₆) δ: 2.84 (t, J = 7.3 Hz, 2H, H-3 Pr), 3.26 (t, J = 7.4 Hz, 2H, H-2 Pr), 6.53 (dm, 2H, 3-H, 5-H), 6.81 (d, J = 8.8 Hz, 1H, 3'-H), 6.98 (dd, J = 8.8, 2.9 Hz, 1H, 4'-H), 7.11 (t, J = 9.3 Hz, 1H, 6-H), 7.19 (d, 1H, J = 2.8 Hz, 1H, 6'-H), 9.15 (s, 1H, OH), 9.66 (s, 2H, OH), 11.19 (s, 1H, OH) ppm. ¹³C NMR (125 MHz, DMSO-d₆) δ: 22.3 (C3 Pr), 39.5 (C₂, Pr, together with the DMSO signal), 102.4 (d, C-3, J = 24.7 Hz), 111.3 (C-5), 114.7 (C-6'), 117.3 (d, C-1, J = 15.7 Hz), 118.4 (C-1'), 120.2 (C-3'), 124.3 (C-4'), 130.9 (d, C-6, J = 6.7 Hz), 149.4 (C-2'), 153.5 (C-5'), 157.3 (d, C-4, J = 11.6 Hz), 160.9 (d, C-2, J = 132 Hz), 204.3 (CO) ppm. MS (ES⁺): m/z 277.3 (M+H)⁺.

Oocyte Expression and Electrophysiological Studies. The α7 nAChR cDNA was cloned in a derivative of the pSP64T vector containing part of the pBluescript polylinker. Capped mRNA was synthesized *in vitro* using SP6 RNA polymerase, the mMESSAGE-mMACHINE kit from Ambion (Thermo Fisher Scientific, Madrid, Spain) and the pSP64T derivative mentioned above. Defolliculated *Xenopus laevis* oocytes were injected with 5 ng of α7 cRNA in 50 nL of sterile water. All experiments were performed within 2-3 days after cRNA injection. Unless otherwise specified, compound **RGM079** was pre-applied in the bath for 2 min and then co-applied with ACh through a pipette held very close to the oocyte for fast application. Functional expression of α7 nAChR was estimated as the peak ionic current evoked by 0.6 s application of 0.2 mM ACh at -80 mV. All experiments were performed at 22°C. Current records were

measured with Clampfit 10.0 (MDS Analytical Technologies, Sunnyvale, CA, USA). Normalized peak currents were obtained by dividing the maximum value of the current obtained in the presence of compound by the maximum value of the current obtained in control conditions. Dose-response curves for the peak current obtained with ACh were fitted to the Hill equation: Normalized current = $I_{\max}/(1 + (EC_{50}/[ACh])^{nH})$. Data are expressed as mean $\pm$ SEM.

Oxygen Radical Absorbance Capacity (ORAC) Experiment. The ORAC assay was performed following Ou et al.⁵¹ as modified by Davalos et al.⁵² All samples and reagents were dissolved in phosphate buffer (75 mM; pH 7.4). The reaction was performed in a final volume of 200 μ l: 20 μ l test samples or 20 μ l Trolox solutions (0.2 – 2 nM), 120 μ l fluorescein solution (1.17 mM) and 60 μ l 2,2'-azo-bis-(2-methylpropionamide) dihydrochloride (AAPH) 1.3% solution (all from Sigma Aldrich) were added to a black 96-well plate. The fluorescence was recorded at 37 °C every 55 s for 95 min using a fluorimeter (SpectraMax M2; Molecular Devices, California, USA), with excitation and emission wavelengths of 480 and 520 nm, respectively. All samples were tested in triplicate. ORAC values were expressed as μ mol Trolox equivalents/ μ mol of pure compound.

Culture of SH-SY5Y cells. SH-SY5Y cells were maintained in culture medium containing 10 % inactivated fetal bovine serum, 15 nonessential aminoacids, 1 mM sodium pyruvate (Invitrogen, Madrid, Spain), F12 nutrient medium (Ham12), MEM medium (Eagle's minimum essential medium) (Sigma Aldrich, Madrid, Spain), NaHCO₃, 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen, Madrid, Spain) in H₂O miliQ. Cells were grown initially in a flask and sub-cultured in 48-well plates at a density of 1×10^5 cells/well. Cells were maintained in an incubator in a humid atmosphere at 37°C with 5 % CO₂; they were used between 4-12 passages.

Culture of rat primary cortical neurons. Rat primary cortical neurons were isolated from 18-days rat embryos. For this purpose, pregnant Sprague-Dawley (SD) rats were decapitated and the embryos were removed by caesaren section. Afterwards, the brains were removed in cool HBBS solution and the cortex were disgreagated in Neurobasal medium (Gibco – Invitrogen) supplemented with 10% foetal bovine serum (FBS, Gibco – Invitrogen). Cells were seeded in Neurobasal + 10% FBS at a density of 100.000 cells/well in poly-D-lysine treated 96 well plates (Sigma-Adrich, Spain). Once cells were adhered, medium was change to Neurobasal and B27 plus OA (antioxidants) (Gibco – Invitrogen). After 10 days of culture cortical neurons were treated as explain afterwards with Neurobasal medium plus B27 but without OA.

Induction of neurotoxicityThe toxics used were OA at 15 nM (Sigma-Adrich, Spain) or beta-amyloid₁₋₄₂ (Bachem, Germany) at 30 μ M and they were incubated for 24 h. For neuroprotection studies, compound **8**, at increasing concentrations, was incubated 24 before and during the 24 h period exposure to the toxic. Beta-amyloid₁₋₄₂ was not aggregated before adding it to the cells. When α -bungarotoxin (100 μ M) was given, it was incubated at the same time as compound **8**.

Evaluation of cell viability Cell viability was assessed by the detection of mitochondrial activity in living cells using the colorimetric analysis of Blue Tetrazolium Bromide Thiazolyl (MTT) (Sigma-Aldrich, Spain), previously described by Denizot.⁵³ Upon completion of the experiments, 50 μ l of reagent MTT was added to each well to achieve a final concentration of 0.5 mg/ml; then, the cells were kept for 2 h in an incubator at 37 °C with 5% CO₂ and 95 % air. Finally, 200 μ l of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan salt and absorbance was measured in an ELISA reader at 540 nm. The absorbance obtained in basal conditions was taken as 100 % cell viability.

Analgesic assay. Male Wistar rats (250-300 g) were obtained from Janvier France. All experiments were approved by the Institutional Animal and Ethical Committee of the Universidad Miguel Hernandez where experiments were conducted and they were in accordance with the guidelines of the Economic European Community and the Committee for Research and Ethical Issues of the International Association for the Study of Pain. All parts of the study concerning animal care were performed under the control of veterinarians.

CFA emulsion (1:1 oil/saline, 0.5 mg/mL) was injected into the plantar surface (50 μ L) of the left hind paw of rats.⁵⁴ Compounds were administered at 10 mg/kg i.v. 24 h after CFA injection.

The mechanical allodynia was monitored 24 h after CFA injection and up to 4 h after administering the compounds. Paw withdrawal latency to mechanical stimulation was assessed with an automated testing device consisting of a steel rod that is pushed against the plantar surface of the paw with increasing force until the paw is withdrawn (Dynamic Plantar Aesthesiometer; Ugo Basile). The maximum force was set at 50 g to prevent tissue damage and the ramp speed was 2.5 g/s. Rats were placed in test cages with a metal grid bottom. They were kept in the test cages for 30-40 min to allow accommodation. The paw withdrawal latency was obtained as the mean of 3 consecutive assessments at each time point (at least 10s between repeated measurements of the same paw).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the

Methods ADME-Tox study and co-incubation experiment with OA in SH-Y5S5 cells (pdf).

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Author Contributions

RGM and MJPdV have the idea, were in charge of the research and synthesized the compounds. CFM and MGL carried out the neuroprotection studies. RTM, SGR, AFC and AFM carried out the in vivo nociception studies. JC, FS, SS and MC performed the electrophysiology. SMF and MM measured the antioxidant activity. RGM and MJPdV wrote the manuscript. All authors have participated in the writing refinement and have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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TOC

Neuroprotective

PAM α 7 nACh
 $I_{max} = 22.7$; $EC_{50} = 8.3 \mu M$
 Antioxidant, 7.7 TE (ORAC)
 $t_{1/2} = 55$ min.

Analgesic