European Journal of Medicinal Chemistry 47 (2012) 175–185

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Neuroprotective efficacy of quinazoline type phosphodiesterase 7 inhibitors in cellular cultures and experimental stroke model

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ARTICLE INFO

Article history:
Received 12 July 2011
Received in revised form
18 October 2011
Accepted 21 October 2011
Available online 4 November 2011

Keywords:
PDE7 inhibitors
Neuroprotection
Stroke
Quinazolines

ABSTRACT

A simple and efficient synthetic method for the preparation of quinazoline type phosphodiesterase 7 (PDE7) inhibitors, based on microwave irradiation, has been developed. The use of this methodology improved yields and reaction times, providing a scalable procedure. These compounds are pharmacologically interesting because of their *in vivo* efficacy both in spinal cord injury and Parkinson's disease models, as shown in previous studies from our group. Herein we describe for the first time that administration of one of the PDE7 inhibitors here optimized, 3-phenyl-2,4-dithioxo-1,2,3,4-tetrahydroquinazoline (compound **5**), ameliorated brain damage and improved behavioral outcome in a permanent middle cerebral artery occlusion (pMCAO) stroke model. Furthermore, we demonstrate that these PDE7 inhibitors are potent anti-inflammatory as well as neuroprotective agents in primary cultures of neural cells. These results led us to propose PDE7 inhibitors as a new class of therapeutic agents for neuroprotection.

1. Introduction

Phosphodiesterases (PDEs) selectively degrade cyclic purine nucleotides (cAMP and cGMP) that serve as second messengers in a number of cellular pathways. PDEs have proven to be druggable targets, with compounds on the market or in late-stage clinical development for a variety of diseases [1].

Theophylline was probably the first non-selective PDE inhibitor to be used clinically for the treatment of asthma, acting as bronchodilator [2]. However, the known PDEs heterogeneity led to the synthesis of highly selective inhibitors [3], which have demonstrated efficacy in a variety of disorders such as cilostazol (PDE3 inhibitor) for intermittent claudication [4], roflumilast (PDE4 inhibitor) for chronic obstructive pulmonary disease [5], and sildenafil (PDE5 inhibitor) for male erectile dysfunction and pulmonary hypertension [6].

Recently, several reports have suggested that PDEs are new targets for central nervous system (CNS) diseases [7,8]. The PDE family is comprised by 11 different subfamilies, being PDE1, PDE4, PDE7 and PDE10 highly expressed in brain. Since it has been suggested that cAMP pathways could be involved in neurodegenerative diseases, interfering with both the inflammatory and neurotransmitter cascades, selective inhibitors of these PDEs could represent a novel approach to treat several CNS diseases [9,10].

PDE7 is a cAMP-specific enzyme not modulated by rolipram (PDE4 inhibitor), expressed across a variety of brain structures apart from its expression on T-cells [11]. PDE7 emerged as a new therapeutic target, not only for a variety of immunological and immunodeficiency conditions to alleviate chronic inflammation [12], but also for several neurodegenerative disorders including multiple sclerosis (MS) in which both autoimmune system and CNS are implicated [13]. Very recently, the usefulness of this new drug target has been studied in different animal models showing preclinical positive data for the treatment of spinal cord injury [14] and Parkinson's disease (PD) [15].

Benzothiadiazine and benzothienothiadiazine derivatives, developed in our group, constituted the first described heterocyclic

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doi:10.1016/j.ejmech.2011.10.040

family of compounds with PDE7 inhibitory properties [16,17]. Afterward, several diverse heterocyclic compounds such as spiroquinazolinones, sulfonamide and thiadiazole analogs have been described as PDE7 inhibitors [13,18]. A ligand-based virtual screening using the descriptors generated by CODES program [19], pointed out the quinazoline derivatives as new PDE7 inhibitors [20]. After a preliminary synthetic optimization, new and potent quinazoline derivatives were obtained and the crystal structure of one of them within the PDE7A1 catalytic domain was solved by X-ray diffraction [21].

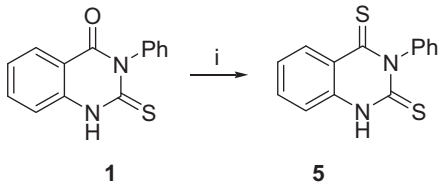
We have recently demonstrated that PDE7 inhibitors belonging to the quinazoline family enhance neuroprotection and diminish neuroinflammation in well-characterized cellular and animal models of PD and spinal cord injury [14,15]. Due to this fact, here we propose the study of a possible neuroprotective activity of quinazoline type PDE7 inhibitors, both in primary cell cultures and in a well-characterized *in vivo* model of stroke. With this aim, their synthetic pathway has been optimized and new quinazoline derivatives have been synthesized as continuation of our on-going research on new quinazoline type PDE7 inhibitors.

2. Chemistry

The synthesis of quinazoline type PDE7 inhibitors was previously described [20,21] by cyclocondensation of the corresponding functionalized anthranillic acid derivatives with isothiocyanates, subsequent thionation of the carbonyl group at 4-position and/or methylation of sulfur at 2-position when required. The major drawbacks of this procedure were the long reaction times and poor yields. Due to their promising pharmacological profile, we were interested in developing a more efficient synthetic pathway, reducing the reaction times and improving the overall yields. In this sense, we decided to prepare the PDE7 inhibitors **1**–**4** previously described [20,21] by using microwave assisted organic synthesis (MAOS) which has been successfully applied to obtain 2-thioxoquinazolinones [22]. Noteworthy is the fact that the protocol based on MAOS yielded the desired products faster than the conventional heating (Table 1).

Continuing of our research regarding quinazoline derivative synthesis, we investigated the thionation reaction of this kind of molecules by using also the MAOS technique. In this case, compound **5** (IC_{50} PDE7A = 1.04 μM [21]) was successfully obtained after reaction of **1** with Lawesson reagent in toluene, at 120 °C after 2 h, in 50% yield. The improvement of the MAOS is reflected on a significant reduction of reaction time, 2 h instead of 48 h in the conventional synthesis (Scheme 1) [21].

These results encouraged us to apply this MAOS procedure to the design of new quinazolines with major functionality with the aim of obtaining a good lead with improved drug-like properties for



1 5

Scheme 1. Reagents: (i) Lawesson reagent, toluene, 120 °C, 2 h, MW.

further development. The results are gathered in Table 2 demonstrating that this methodology is valid also for more diverse quinazolines.

The new designed quinazolines (**6**–**25**) were proposed based on the fact that a major drawback of some of the previously prepared quinazoline type PDE7 inhibitors was the lack of solubility. Thus, some modifications were done to improve the solubility and also in order to establish additional interactions in the PDE7 catalytic domain based on the X-ray data reported previously [21,23].

First, a classical isosteric replacement of the thiocarbonyl at C-2 by carbonyl group was carried out. Following with the application of isosteres the substitution of CH with N at whichever of the aromatic rings should improve also the aqueous solubility [24].

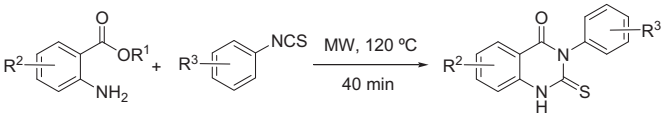
Second, in order to improve solubility by disruption of molecular planarity [25] we propose to increase the distance between the quinazoline core and the aryl group at N-3, introducing 1 or 2 methylene groups.

Third, the introduction of a variety of substituents in the fused ring was performed, to achieve an electronic enrichment of that aromatic ring in order to improve the stacking interaction with the conserved Phe416 which is a key feature in the binding mode of quinazoline type PDE7 inhibitors with the catalytic domain of PDE7A1 [21].

Table 2 summarizes the different compounds obtained with these purposes, indicating the best reaction conditions found in each case. In all cases the condensations were carried out under microwave irradiation in shorter times and easier purification procedures which confirms the convenience of this methodology for the synthesis of this kind of compounds.

The structures of all the compounds were determined by their analytical and spectroscopic data (^{1}H and ^{13}C NMR), which are described in the Experimental Section.

Table 1
MAOS of quinazoline type PDE7 inhibitors.



Comp.	R^1	R^2	R^3	Solvent	Yield	IC_{50} (PDE7A)
1	Me	H	H	Toluene	70%	5.5 μM [20]
2	Me	H	2-Br	DMSO/H_2O	60%	1.9 μM [20]
3	Me	H	2,6-diF	DMSO/H_2O	60%	5.5 μM [20]
4	H	8-Me	2-Br	Toluene	30%	1.7 μM [21]

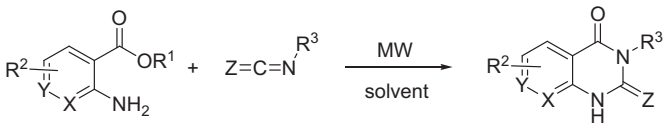
3. Biological results and discussion

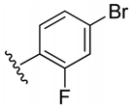
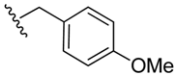
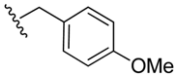
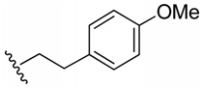
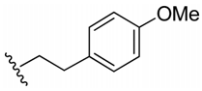
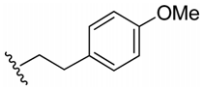
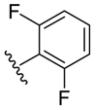
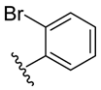
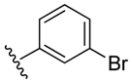
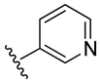
3.1. In vitro evaluation of PDE7 inhibition

The new derivatives here synthetized (**6**–**25**) were tested for their inhibitory potencies against the catalytic domain of PDE7A as described in the Experimental Section (Table 3). Among them only two of them (**12** and **13**) are potent PDE7 inhibitors in the micromolar range.

These results led us to confirm a key stacking interaction between the fused benzene ring and the enzyme. In this way we can explain the activity of derivatives **12** and **13** which have a chlorine atom attached. Electronic deficient rings have been described as the preferred over the electron rich ones when a stacking interaction is relevant in the molecular recognition in biological systems [26]. On the other hand, the modifications here proposed to increase solubility, such as the disruption of molecular planarity and bioisosteric replacements, were incompatible with optimal biological interaction. While these results are analyzed in deep by different QSAR methods, the improvement of solubility for this class of compounds is in progress, being performed by pharmaceutical technology development.

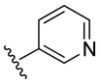
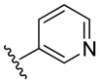
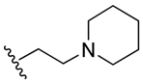
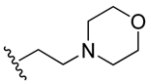
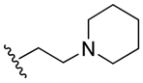
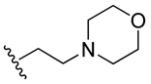
Table 2
New cyclocondensation reactions for quinazoline synthesis.



Comp.	R^1	R^2	X	Y	Z	R^3	Solvent	Temp.	Time	Yield
6	Me	–	CH	CH	O		MeCN[a]	170 °C	30 min	33%
7	Me	–	CH	CH	O		Dioxane[a]	170 °C	30 min	84%
8	Me	–	N	CH	O		MeCN[a]	170 °C	30 min	28%
9	Me	–	CH	CH	O		1,2-DME	160 °C	45 min	46%
10	H	6-Br	CH	CH	O		MeCN[a]	145 °C	45 min	17%
11	H	–	CH	N	O		MeCN[a]	170 °C	60 min	21%
12	H	8-Cl	CH	CH	S		DMSO/H_2O	120 °C	40 min	63%
13	H	8-Cl	CH	CH	S		DMSO/H_2O	120 °C	40 min	18%
14	H	6-Me,8-Br	CH	CH	S		MeCN	150 °C	45 min	55%
15	Me	6,7-diMeO	CH	CH	S		MeCN[a]	175 °C	30 min	63%
16	Me	–	N	CH	S		MeCN[a]	150 °C	30 min	5%
17	Me	–	N	CH	S		1,4-Dioxane	160 °C	30 min	18%
18	H	–	CH	N	S		MeCN[a]	150 °C	30 min	35%
19	Me	–	CH	CH	S		MeCN[a]	170 °C	45 min	87%

(*continued on next page*)

Table 2 (*continued*)

Comp.	R^1	R^2	X	Y	Z	R^3	Solvent	Temp.	Time	Yield
20	Me	6,7-diMeO	CH	CH	S		MeCN[a]	180 °C	25 min	59%
21	H	–	CH	N	S		1,2-DME	140 °C	30 min	69%
22	Me	–	CH	CH	S		1,2-DME[b]	150 °C	10 min	62%
23	Me	–	CH	CH	S		MeCN	170 °C	45 min	76%
24	Me	6,7-diMeO	CH	CH	S		MeCN[b]	175 °C	30 min	20%
25	Me	6,7-diMeO	CH	CH	S		MeCN	170 °C	45 min	59%

[a] With 2% triethylamine.
[b] With 2% DMAP.

Table 3
PDE7A inhibition of compounds **6**–**25**.

Comp.	%Inhibition PDE7A @10 μM	IC_{50} (PDE7A)
6	2.5	–
7	9.3	–
8	8.0	–
9	−7.2	–
10	−8.3	–
11	22.1	–
12	74.9	6.1 μM
13	63.4	1.8 μM
14	4.0	–
15	13.2	–
16	−3.1	–
17	12.8	–
18	9.3	–
19	10.0	–
20	19.0	–
21	2.7	–
22	21.2	–
23	13.2	–
24	12.7	–
25	17.9	–

3.2. Neuroprotective studies in vitro

Our next step was to explore whether the quinazoline type PDE7 inhibitors are neuroprotective in different cell based assays. Thus, we used primary cultures of astrocytes, microglia and neurons treated with lipopolysaccharide (LPS), a potent inflammatory agent.

The potential anti-inflammatory activity of the selected PDE7 inhibitors was first tested by evaluating the production of nitrites from primary cultured glial cells (astrocytes and microglia). Cultures were incubated with the indicated concentrations of the compounds **1**, **4**, **5**, **12** and **13** for 1 h, and then cells were cultured for another 24 h with LPS. When primary astrocytes and microglial cells were stimulated with LPS (Figs. 1 and 2) we observed a significant induction of nitrite production in the culture medium (6- and 20-fold, respectively), which was significantly diminished by PDE7 inhibitors treatment.

Continuing with the neuroprotective capacity, we investigated whether these compounds were also effective protecting neurons from the injury induced by cell-free supernatant from LPS-activated microglia. As shown in Fig. 3, treatment of neuronal cultures with compounds **1**, **4** and **5**, also resulted in a significant reduction in nitrite production.

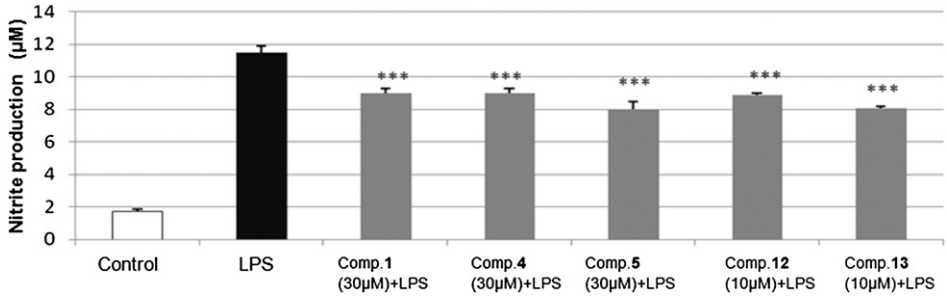


Fig. 1. Effect of PDE7 inhibitors on the inflammatory response of astrocyte cultures. Rat primary astrocyte cultures were treated for 24 h with LPS (10 μg/ml) in the absence or presence of PDE7 inhibitors and the production of nitrite was evaluated by the Griess reaction. Values represent the means ± SD from three different experiments. ***, $p \leq 0.001$.

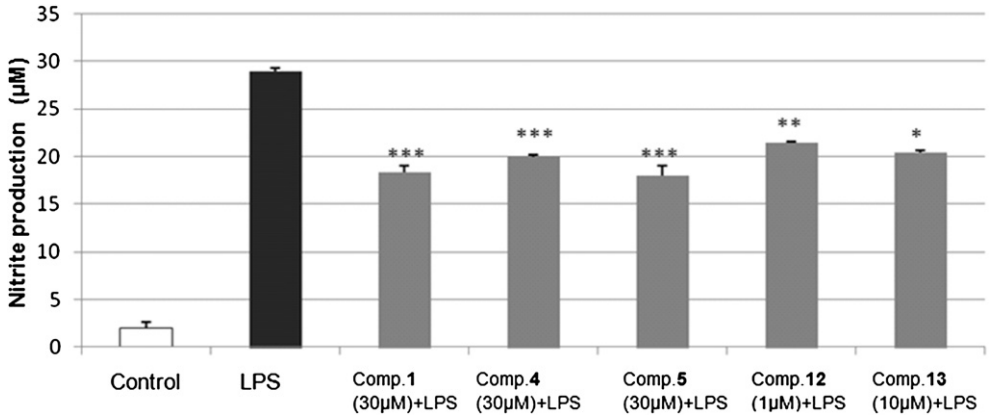


Fig. 2. Effect of PDE7 inhibitors on the inflammatory response of microglial cultures. Rat primary microglial cultures were treated for 24 h with LPS (10 μg/ml) in the absence or presence of PDE7 inhibitors and the production of nitrite was evaluated by the Griess reaction. Values represent the means ± SD from three different experiments. ***, $p \leq 0.001$, **, $p \leq 0.01$, *, $p \leq 0.05$.

Altogether these results indicate that PDE7 inhibitors, specially those of quinazoline scaffold, are potent anti-inflammatory and neuroprotective agents in primary neural cultures.

3.3. Blood brain barrier permeation prediction

Drug's penetration through the blood–brain barrier (BBB) is one of the major obstacles for the treatment of diseases in the central nervous system (CNS). The BBB is a protective barrier which is designed to keep the environment in the brain as stable as possible. It prevents many dangerous substances from entering the brain [27]. Brain penetration is influenced by several processes, such as tissue binding in the bloodstream and the brain, metabolism in the liver and the brain and active influx and efflux. Most compounds enter the brain by transcellular passive diffusion, which is driven by a concentration gradient between the blood and the brain [28]. In early drug discovery stages, evaluation of ADME (Absorption, Distribution, Metabolism, Excretion) properties is of crucial importance to reduce attrition in development process [29]. Parallel Artificial Membrane Permeability Assay (PAMPA) is a high throughput technique developed to predict passive permeability through biological membranes. In order to explore the capacity of the quinazoline derivatives to penetrate into the brain and select the best compound for *in vivo* studies, we used the PAMPA-BBB method described by Di et al. [30] which employed a brain lipid porcine membrane. The *in vitro* permeabilities (Pe) of commercial drugs through lipid membrane extract together with compounds **4** and **5** were determined. An assay validation was made comparing the reported permeability values of commercial drugs with the experimental data obtained employing this methodology. A good correlation between experimental-described values was obtained Pe (exp) = 0.9541 (bibl) − 0.536 ($R^2 = 0.987$) (see Supplementary data). From this equation and following the pattern established in the literature for BBB permeation prediction [31] we could classify compounds as CNS + when they present a permeability $> 3.26 \times 10^{-6}$ cm s^{-1}. Based on these results we can consider that compounds **4** and **5** are able to cross the BBB by passive permeation. Compound **1** was classified as CNS + in a previous experiment [15]. We selected compound **5** for *in vivo* studies due to its better IC_{50} value on the target.

3.4. Neuroprotective studies in vivo

Finally, to evaluate the neuroprotective efficacy *in vivo* we used a stroke model by permanent middle cerebral artery occlusion (pMCAO) in mice. Our data demonstrate that compound **5** (5 mg/kg; administered 15 min after pMCAO) is neuroprotective as shown by a reduction in infarct volume (15.4 ± 1.1 *vs.* 10.8 ± 1.1% of total hemisphere volume in pMCAO + vehicle *vs.* pMCAO + comp. **5**, respectively, $n = 8–10$; $p < 0.05$) and neurological deficit (6.5 ± 0.2 *vs.* 4.4 ± 0.3 points in mNSS score in MCAO *vs.* MCAO + comp. **5**, respectively, $n = 8–10$; $p < 0.05$), determined 48 h after MCAO (Fig. 4).

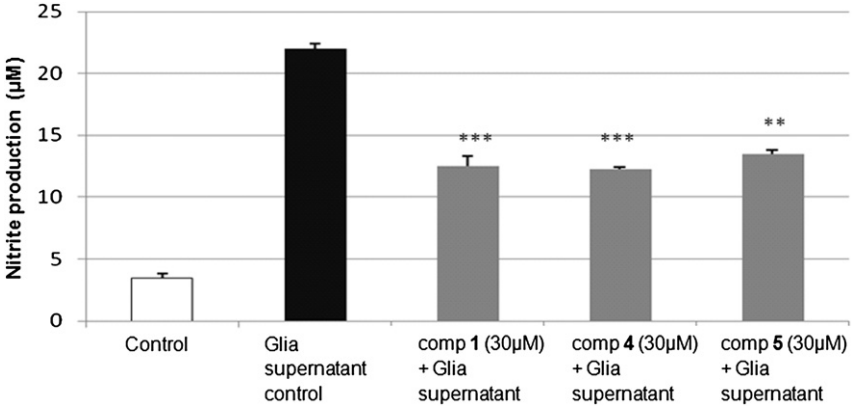


Fig. 3. Effect of PDE7 inhibitors on the inflammatory response of cultured neurons. Rat primary neuronal cultures were treated for 24 h with cell-free supernatant from LPS-stimulated microglia in the absence or presence of PDE7 inhibitors and the production of nitrite was evaluated by the Griess reaction. Values represent the means ± SD from three different experiments. ***, $p \leq 0.001$. **, $p \leq 0.01$.

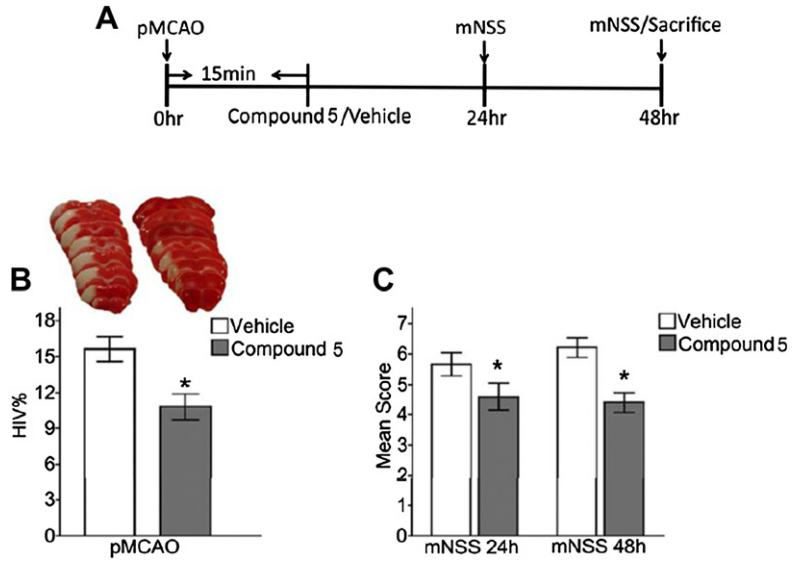


Fig. 4. (A) Pharmacological treatment and infarct outcome evaluation protocol. Effect of compound **5** on infarct volume determined 48 h after pMCAO (B) and on the modified neurological severity score (mNSS) 24 and 48 h after pMCAO (C). Data are expressed as mean value ± SEM., $n = 9–10$, $^{*}p < 0.05$ *vs*. vehicle, treated group. Unpaired t-Student's test.

4. Conclusion

Based on the results obtained here, we can conclude that PDE7 inhibitors, and specifically those of quinazoline type, present neuroprotective activity modulating nitrite production in neural cells and they also have a role in stroke by reducing the infarct volume and the associated motor deficits. These results emphasize the idea of a potential use of PDE7 inhibitors as therapeutic agents in stroke. Moreover we have provided enough data to show that MAOS is an efficient synthetic pathway for obtaining this class of potential new drugs.

5. Experimental section

5.1. Chemistry

Substrates were purchased from commercial sources and used without further purification. Melting points were determined with a Mettler Toledo MP70 apparatus. Flash column chromatography was carried out at medium pressure using silica gel (E. Merck, Grade 60, particle size 0.040–0.063 mm, 230–240 mesh ASTM) with the indicated solvent as eluent. Compounds were detected with UV light (254 nm). ^{1}H NMR spectra were obtained on the Bruker AVANCE-300 spectrometer working at 300 MHz or on a Varian INOVA 400 spectrometer working at 400 MHz. Typical spectral parameters: spectral width 10 ppm, pulse width 9 μs (57°), data size 32 K ^{13}C NMR experiments were carried out on the Bruker AVANCE-300 spectrometer operating at 75 MHz or on a Varian INOVA 400 spectrometer working at 100 MHz. The acquisition parameters: spectral width 16 kHz, acquisition time 0.99 s, pulse width 9 μs (57°), data size 32 K. Chemical shifts are reported in values (ppm) relative to internal Me_4Si and *J* values are reported in Hz. HPLC analyses were performed on Alliance Waters 2690 equipment, with a UV detector photodiode array Waters 2996 with MS detector MicromassZQ (Waters), using an XBridge C18, 3.5 μm, 200 Å (2.1 nm × 100 mm) column. The standard gradient consisted of a 10 min run from 5% to 100% of acetonitrile at a flow rate of 0.25 mL/min. Elemental analyses were performed by the analytical department at CENQUIOR (CSIC), and the results obtained were within ±0.4% of the theoretical values. The microwave assisted syntheses were carried out using a Biotage Initiator eight single-mode cavity instrument from Biotage. Experiments were performed with temperature control mode in sealed microwave process vials. The temperature was measured with an IR sensor on the outside of the reaction vessel. Stirring was provided by an *in situ* magnetic stirrer.

5.1.1. 4-Oxo-3-phenyl-2-thioxo-1,2,3,4-tetrahydroquinazoline (**1**)

A mixture of methyl anthranilate (0.25 mL, 1.98 mmol) and phenylisothiocyanate (0.20 mL, 1.98 mmol) in toluene (4 mL) was heated under microwave irradiation (40 min, 120 °C). After cooling down the reaction in an ice/salt bath, a white precipitate was formed. The solid was filtered, washed with diethylether and dried, affording **1** as a pure product (yield 70%). Mp 300–302 °C (Lit. [32] 305–306 °C); ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 13.05 (bs, 1H), 7.95 (d, $J = 7.8$ Hz, 1H, Ar-H), 7.78 (t, $J = 7.6$ Hz, 1H, H-7,), 7.51–7.27 (m, 7H, H-6, H-8, Ph); ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm): 176.0, 159.7, 139.5, 139.2, 135.5, 128.9, 128.8, 128.0, 127.3, 124.3, 116.1, 115.6; ESI MS (*m/z*): 255 $[M + H]^{+}$; purity >99% (by HPLC); Anal. $C_{14}H_{10}N_2OS$ (C, H, N, O).

5.1.2. 3-(2-Bromophenyl)-4-oxo-2-thioxo-1,2,3,4-tetrahydroquinazoline (**2**)

A mixture of methyl anthranilate (0.25 mL, 1.98 mmol) and 2-bromophenylisothiocyanate (0.25 mL, 1.98 mmol) in DMSO/H_2O (1:1) (4 mL) was heated under microwave irradiation (40 min, 120 °C). After cooling down the reaction in an ice/salt bath, a white precipitate was formed. The solid was filtered and purified by column chromatography on silica using hexane/ethyl acetate (9:1) as eluent affording **2** as a pure product (yield 60%). Mp 252–254 °C (Lit [33] 256–258 °C); ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 13.18 (bs, 1H), 7.98 (dd, $J = 7.8$, 0.9 Hz, 1H, H-5), 7.81 (ddd, $J = 8.5$, 8.4, 1.3 Hz, 1H, H-7), 7.76 (dd, $J = 8.2$, 0.6 Hz, 1H, H-Ar), 7.51–7.45 (m, 3H, H-Ar, H-8), 7.40–7.35 (m, 2H, H-6, H-Ar); ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm): 175.0, 159.0, 139.6, 138.1, 136.0, 132.7, 131.2, 130.2, 128.6, 127.5, 124.6, 122.3, 115.8, 115.7; ESI MS (*m/z*): 333/335 $[M + H]^{+}$; purity 98% (by HPLC); Anal. $C_{14}H_9BrN_2OS$ (C, H, N, O).

5.1.3. 3-(2,6-Difluorophenyl)-4-oxo-2-thioxo-1,2,3,4-tetrahydroquinazoline (**3**)

A mixture of methyl anthranilate (0.30 mL, 2.27 mmol) and 2,6-difluorophenylisothiocyanate (0.30 mL, 2.27 mmol) in DMSO/H_2O (1:1) (4 mL) was heated under microwave irradiation (40 min,

120 °C). After cooling down the reaction in an ice/salt bath, a white precipitate was formed. The solid was filtered, washed with diethylether and dried, affording **3** as a pure product (yield 60%); Mp 283–284 °C (Lit. [21] 262–263 °C); ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 13.47 (bs, 1H), 8.00 (dd, J = 7.9, 0.9 Hz, 1H, H-5), 7.85 (ddd, J = 8.4, 7.1, 0.9 Hz, 1H, H-7), 7.66–7.56 (m, 1H, H-Ar), 7.48 (d, J = 8.4 Hz, 1H, H-8), 7.41 (dd, J = 7.9, 7.1 Hz, 1H, H-6), 7.35–7.29 (m, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm): 174.7, 158.7, 157.9, 139.7, 136.7, 131.5, 127.6, 125.2, 116.2, 115.2, 114.7, 112.3; ESI MS (*m/z*): 271 [M − F]$^+$; purity >99% (by HPLC); Anal. $C_{14}H_8F_2N_2OS$ (C, H, N, O).

5.1.4. 3-(2-Bromophenyl)-8-methyl-4-oxo-2-thioxo-1,2,3,4-tetrahydroquinazoline (**4**)

A mixture of 2-amino-3-methylbenzoic acid (100 mg, 0.66 mmol) and 2-bromophenylisothiocyanate (0.089 mL, 0.66 mmol) in toluene (4 mL) was heated under microwave irradiation (40 min, 120 °C). After cooling down the reaction in an ice/salt bath, a white precipitate was formed. The solid was filtered and purified by column chromatography on silica using hexane/ethyl acetate (9:1) as eluent affording **4** as a white solid (yield 30%). Mp 209–210 °C (Lit. [21] 209–211 °C); ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 11.98 (bs, 1H); 7.86 (d, J = 7.9 Hz, 1H, H-5), 7.76 (d, J = 8.0 Hz, 1H, H-Ar), 7.65 (d, J = 7.5 Hz, 1H, H-7), 7.56–7.46 (m, 2H, H-Ar), 7.42–7.35 (m, 1H, H-Ar), 7.29 (dd, J = 7.9, 7.5 Hz, 1H, H-6), 2.53 (s, 3H, Me); ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm): 175.4, 159.0, 138.3, 138.0, 137.2, 132.7, 131.1, 130.2, 128.6, 125.3, 124.7, 124.4, 122.2, 116.0, 17.4; ESI MS (*m/z*): *267* [M − Br]; purity >99% (by HPLC); Anal. $C_{15}H_{11}BrN_2OS$ (C, H, N, O).

5.1.5. 3-Phenyl-2,4-dithioxo-1,2,3,4-tetrahydroquinazoline (**5**)

A mixture of compound **1** (50 mg, 0.196 mmol) and Lawesson's reagent (120 mg, 0.294 mmol) in toluene (4 mL) was heated under microwave irradiation (2 h, 120 °C). After cooling down the reaction in an ice/salt bath, an orange precipitate was formed. The solid was filtered and purified by column chromatography on silica using hexane/ethyl acetate (12:1) as eluent affording **5** as a pure product (yield 50%). Mp 253–256 °C (Lit. [21]. 252–254 °C); ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 13.48 (bs, 1H), 8.32 (dd, J = 8.2, 1.0 Hz, 1H, H-5), 7.78 (ddd, J = 8.3, 7.0, 1.0 Hz, 1H, H-7), 7.50–7.42 (m, 3H, H-Ar, H-6), 7.39–7.32 (m, 2H, H-Ar, H-8), 7.21 (dd, J = 8.3, 1.1 Hz, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm): 189.8, 172.8, 144.2, 135.8, 135.2, 131.7, 129.2, 128.5, 127.9, 125.2, 123.7, 115.9; ESI MS (*m/z*): 271 [M + H]$^+$; purity 98% (by HPLC); Anal. $C_{14}H_{10}N_2S_2$ (C, H, N, O).

5.1.6. 3-(4-Bromo-2-fluorophenyl)-2,4-dioxo-1,2,3,4-tetrahydroquinazoline (**6**)

A mixture of methyl anthranilate (117 mg, 0.77 mmol), 4-bromo-2-fluoro-1-isocyanatobenzene (200 mg, 0.93 mmol) and triethylamine (8 mg, 0.08 mmol) in acetonitrile (3 mL) was heated under microwave irradiation (30 min, 170 °C). After cooling down the reaction in an ice/salt bath, a pale yellow precipitate was formed. The solid was filtered, washed with diethylether and dried, affording **6** as a pure product (yield 33%). Mp 263–264 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 11.76 (s, 1H), 7.95 (dd, J = 8.1, 1.2 Hz, 1H), 7.78 (dd, J = 9.5, 2.1 Hz, 1H), 7.73 (ddd, J = 8.2, 7.3, 1.5 Hz, 1H), 7.57 (ddd, J = 8.5, 2.1, 0.8 Hz, 1H), 7.50 (t, J = 8.1 Hz, 1H), 7.26 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 161.4, 158.9, 156.4, 149.2, 139.8, 135.7, 133.0, 128.0, 127.6, 122.9, 119.6, 119.4, 115.5, 113.6; ESI MS (*m/z*): 335/337 [M + H]$^+$, purity >99% (by HPLC); Anal. $C_{14}H_8BrFN_2O_2$ (C, H, N, O).

5.1.7. 3-(4-Methoxybenzyl)-2,4-dioxo-1,2,3,4-tetrahydroquinazoline (**7**)

A mixture of methyl anthranilate (116 mg, 0.77 mmol), 1-isocyanatomethyl)-4-methoxybenzene (125 mg, 0.77 mmol) and triethylamine (8 mg, 0.08 mmol) in 1,4-dioxane (4 mL) was heated under microwave irradiation (45 min, 140 °C). After cooling down the reaction in an ice/salt bath, a pale yellow precipitate was formed. The solid was filtered, washed with diethylether and dried, affording **7** as a white solid (yield 84%). Mp 216.7 °C (Lit. [34] 213.8 °C); ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 11.48 (s, 1H, NH), 7.92 (dd, J = 7.2, 0.9 Hz, 1H, H-5), 7.65 (dt, J = 7.5, 1.6 Hz, 1H, H-7), 7.27 (d, J = 8.7 Hz, 2H, H-3′), 7.17 (t, J = 7.8 Hz, 1H, H-6), 6.85 (d, J = 7.8 Hz, 1H, H-8), 6.83 (d, J = 8.7 Hz, 2nH- H-4′), 5.00 (s, 2H, CH_2), 3.69 (s, 3H, MeO); ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm): 162.1, 158.5, 154.1, 141.2, 137.5, 132.8, 128.9, 126.0, 124.6, 117.8, 116.5, 114.7, 55,7, 43.9; ESI MS (*m/z*): 283 [M + H]$^+$, purity 97% (by HPLC); Anal. $C_{16}H_{14}N_2O_3$ (C, H, N, O).

5.1.8. 3-(4-Methoxybenzyl)-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine (**8**)

A mixture of methyl 2-aminonicotinate (150 mg, 0.98 mmol), 1-isocyanatomethyl-4-methoxybenzene (160 mg, 0.98 mmol) and triethylamine (10 mg, 0.10 mmol) in acetonitrile (3 mL) was heated under microwave irradiation (30 min, 170 °C). After cooling down the reaction in an ice/salt bath, a pale brown precipitate was formed. The solid was filtered, washed with diethylether and dried, affording **8** as a yellowish solid (yield 28%). Mp 251–253 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 12.01 (s, 1H, NH), 8.66 (d, J = 6.7 Hz, 1H), 8.31 (d, J = 7.0 Hz, 1H), 7.30 (m, 1H), 7.17 (m, 1H), 6.84 (m, 1H), 4.99 (s, 2H, CH_2); ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm): 160.4, 158.6, 152.2, 152.1, 148.4, 137.6, 130.9, 128.7, 121.1, 114.7, 108.2, 55,8, 46.1; ESI MS (*m/z*): 284 [M + H]$^+$, purity 95% (by HPLC); Anal. $C_{15}H_{13}N_3O_3$ (C, H, N, O).

5.1.9. 3-(4-Methoxyphenethyl)-2,4-dioxo-1,2,3,4-tetrahydroquinazoline (**9**)

A mixture of methyl anthranilate (292 mg, 1.93 mmol) and 1-(2-isocyanatoethyl)-4-methoxybenzene (341 mg, 1.93 mmol) in 1,2-dimethoxyethane (3 mL) was heated under microwave irradiation (45 min, 160 °C). After cooling down the reaction in an ice/salt bath, a pale brown precipitate was formed. The solid was filtered, washed with diethylether and dried, affording **9** as a white solid (yield 46%). Mp 223.8 °C (Lit. [35] 76–78 °C); ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 11.46 (s, 1H, NH), 7.94 (d, J = 7.1 Hz, 1H), 7.65 (t, J = 7.2 Hz, 1H), 7.20–7.08 (m, 4H), 6.83 (m, 2H), 4.03 (m, 2H, N-CH_2), 3.82 (s, 3H, MeO), 2.79 (m, 2H, CH_2-Ar); ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm): 161.1, 158.1, 150.3, 139.7, 135.2, 130.8, 129.9, 127.6, 122.7, 115.4, 114.1, 114.0, 55.3, 41.8, 32.8; ESI MS (*m/z*): 297 [M + H]$^+$, purity 98% (by HPLC); Anal. $C_{17}H_{16}N_2O_3$ (C, H, N, O).

5.1.10. 6-Bromo-3-(4-methoxyphenethyl)-2,4-dioxo-1,2,3,4-tetrahydroquinazoline (**10**)

A mixture of 2-amino-5-bromobenzoic acid (200 mg, 0.93 mmol), 1-(2-isocyanatoethyl)-4-methoxybenzene (213 mg, 1.20 mmol) and triethylamine (9 mg, 0.09 mmol) in acetonitrile (3 mL) was heated under microwave irradiation (45 min, 145 °C). After cooling down the reaction in an ice/salt bath, a pale yellow precipitate was formed. The solid was filtered, washed with diethylether and dried, affording **10** as a pure product (yield 17%). Mp 180–181 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.12 (m, 2H), 7.08 (m, 2H), 6.84 (m, 3H), 3.70 (s, 3H, MeO), 3.15 (m, 2H, N-CH_2-CH_2), 2.58 (m, 2H, CH_2-CH_2-Ar); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 160.7, 157.8, 157.5, 149.7, 138.6, 137.5, 131.6, 129.5 (2xCH), 129.2, 117.6, 113.8, 113.7 (2xCH), 54.9, 41.1, 35.2; ESI MS (*m/z*): 375/377 [M + H]$^+$, purity >99% (by HPLC); Anal. $C_{17}H_{15}BrN_2O_3$ (C, H, N, O).

5.1.11. 3-(4-Methoxyphenethyl)-2,4-dioxo-1,2,3,4-tetrahydropyrido[3,4-d]pyrimidine (**11**)

A mixture of 3-aminoisonicotinic acid (200 mg, 0.93 mmol), 1-(2-isocyanatoethyl)-4-methoxybenzene (213 mg, 1.20 mmol) and

triethylamine (8 mg, 0.08 mmol) in acetonitrile (3 mL) was heated under microwave irradiation (45 min, 145 °C). After cooling down the reaction in an ice/salt bath, a white precipitate was formed. The solid was filtered, washed with diethylether and dried, affording **11** as a pure product (yield 21%). Mp 238 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 8.58 (s, 1H), 8.39 (d, J = 7.1 Hz, 1H), 7.78 (d, J = 7.1 Hz, 1H), 7.13 (d, J = 8.9 Hz, 2H), 6.81 (d, J = 8.9 Hz, 2H), 4.04 (m, 2H, N-CH_2-CH_2), 3.75 (s, 3H, MeO), 2.81 (m, 2H, CH_2-Ar); ^{13}C NMR (125 MHz, DMSO-d_6) δ (ppm): 161.5, 158.3, 150.2, 143.2, 138.9, 135.1, 132.0, 130.0, 119.8, 119.5, 114.3, 55.4, 42.2, 41.6; ESI MS (*m/z*): 298 $[M + H]^+$, purity >99% (by HPLC); Anal. $C_{16}H_{15}N_3O_3$ (C, H, N, O).

5.1.12. 8-Chloro-3-(2,6-difluorophenyl)-4-oxo-2-thioxo-1,2,3,4-tetrahydroquinazoline (**12**)

A mixture of 2-amino-3-chlorobenzoic acid (140 mg, 0.818 mmol) and 2,6-difluorophenylisothiocyanate (0.105 mL, 0.818 mmol) in DMSO/H_2O (1:1) (4 mL) was heated under microwave irradiation (40 min, 120 °C). After cooling down the reaction in an ice/salt bath, a yellow precipitate was formed. The solid was filtered and purified by column chromatography on silica using hexane/ethyl acetate (9:1) as eluent affording **12** as a pure product (yield 63%). Mp 206–208 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 12.37 (bs, 1H), 7.97 (t, J = 1.2 Hz, 1H, H-7), 7.99 (t, J = 1.5 Hz, 1H, H-5), 7.66–7.56 (m, 1H, H-6), 7.40 (t, J = 7.8 Hz, 1H, H-Ar), 7.33 (tt, J = 8.2, 2.5 Hz, 2H, H-Ar); ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm): 176.0, 160.2, 158.7, 137.6, 137.4, 132.5, 127.8, 126.4, 120.0, 117.6, 113.3; ESI MS (*m/z*): 325 $[M + H]^+$; purity 98% (by HPLC); Anal. $C_{14}H_7OSN_2ClF_2$ (C, H, N, O).

5.1.13. 3-(2-Bromophenyl)-8-chloro-4-oxo-2-thioxo-1,2,3,4-tetrahydroquinazoline (**13**)

A mixture of 2-amino-3-chlorobenzoic acid (300 mg, 1.66 mmol) and 2-bromophenylisothiocyanate (0.341 mL, 2.49 mmol) in DMSO/H_2O (1:1) (4 mL) was heated under microwave irradiation (40 min, 120 °C). After cooling down the reaction in an ice/salt bath, a white precipitate was formed. The solid was filtered and purified by column chromatography on silica using hexane/ethyl acetate (9:1) as eluent affording **13** as a white solid (yield 18%). Mp 192–193 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 9.50 (s, 1H), 8.06 (dd, J = 7.9, 0.8 Hz, 1H, H-5), 7.74 (d, J = 1.4 Hz, 1H, H-7), 7.71 (d, J = 1.4 Hz, 1H, H-Ar), 7.46 (td, J = 7.6, 1.4 Hz, 1H, H-Ar), 7.35 (dd, J = 7.6, 1.7 Hz, 1H, H-Ar), 7.31 (d, J = 1.6 Hz, 1H, H-6), 7.29 (m, 1H, H-Ar); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 175.9, 158.8, 138.0, 136.1, 135.8, 134.1, 131.1, 130.6, 129.2, 128.2, 125.4, 123.0, 119.21, 117.76; ESI MS (*m/z*): 369 $[M + H]^+$; purity >99% (by HPLC). Anal. $C_{14}H_8OSN_2ClBr$ (C, H, N, O).

5.1.14. 8-Bromo-6-methyl-4-oxo-3-phenyl-2-thioxo-1,2,3,4-tetrahydroquinazoline (**14**)

A mixture of 2-amino-3-bromo-5-methylbenzoic acid (200 mg, 0.87 mmol) and isothiocyanatobenzene (152 mg, 1.13 mmol) in acetonitrile (3 mL) was heated under microwave irradiation (45 min, 150 °C). After cooling down the reaction in an ice/salt bath, a white precipitate was formed. The solid was filtered, washed with diethylether and dried, affording **14** as a pure product (yield 55%). Mp 249 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 10.89 (s, 1H), 7.94 (d, J = 1.1 Hz, 1H), 7.78 (s, 1H), 7.48 (t, J = 7.5 Hz, 2H), 7.40 (t, J = 7.3 Hz, 1H), 7.27 (d, J = 7.4 Hz, 2H), 2.37 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 176.7, 159.6, 140.1, 139.9, 136.2, 135.7, 129.7, 129.4, 128.9, 127.7, 118.5, 108.1, 20.6; ESI MS (*m/z*): 347/349 $[M + H]^+$, purity >99% (by HPLC); Anal. $C_{15}H_{11}BrN_2OS$ (C, H, N, O).

5.1.15. 6,7-Dimethoxy-4-oxo-3-phenyl-2-thioxo-1,2,3,4-tetrahydroquinazoline (**15**)

A mixture of methyl 2-amino-4,5-dimethoxybenzoate (200 mg, 0.95 mmol), isothiocyanatobenzene (179 mg, 1.32 mmol) and triethylamine (9 mg, 0.09 mmol) in acetonitrile (3 mL) was heated under microwave irradiation (30 min, 175 °C). After cooling down the reaction in an ice/salt bath, a pale orange precipitate was formed. The solid was filtered, washed with diethylether and dried, affording **15** as a white solid (yield 63%). Mp 316 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 7.45 (m, 3H), 7.26 (m, 3H), 7.02 (s, 1H), 3.90 (s, 3H), 3.82 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm): 174.8, 159.3, 155.3, 146.5, 139.4, 135.4, 129.0, 128.9, 128.1, 108.5, 106.9, 97.8, 56.0, 55.8; ESI MS (*m/z*): 315 $[M + H]^+$, purity 98% (by HPLC); Anal. $C_{16}H_{14}N_2O_3S$ (C, H, N, O).

5.1.16. 4-Oxo-3-phenyl-2-thioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine (**16**)

A mixture of methyl 2-aminonicotinate (200 mg, 1.27 mmol) and phenylisothiocyanate (0.199 mL, 1.34 mmol) with triethylamine (0.035 mL, 0.24 mmol) in acetonitrile (4 mL) was heated under microwave irradiation (30 min, 150 °C). After cooling down the reaction in an ice/salt bath, a white precipitate was formed. The solid was filtered affording **16** as a pure product (yield 5%). Mp 301–302 °C (Lit. [36] 275–276 °C); ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 13.50 (s, 1H); 8.76 (d, J = 4.6 Hz, 1H, H-7); 8.34 (d, J = 7.7 Hz, 1H, H-5); 7.50–729 (m, 6H, H-Ar, H-6); ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm): 177.8 (C-2); 160.4 (C-4); 155.6 (C-6); 154.3 (C-7); 151.0 (C-8a); 139.5 (C-Ar); 137.3 (C-Ar); 129.3 (C-Ar); 128.5 (C-Ar); 120.7 (C-4a); 112.3 (C-5); ESI MS (*m/z*): 256 $[M + H]^+$; purity > 99% (by HPLC). Anal. $C_{13}H_9N_3OS$ (C, H, N, O).

5.1.17. 3-(3-Bromophenyl)-4-oxo-2-thioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine (**17**)

A mixture of methyl 2-aminonicotinate (150 mg, 0.98 mmol) and 1-bromo-3-isothiocyanatobenzene (210 mg, 0.98 mmol) in 1,4-dioxane (3 mL) was heated under microwave irradiation (30 min, 160 °C). After cooling down the reaction in an ice/salt bath, a yellow precipitate was formed. The solid was filtered, washed with diethylether and dried, affording **17** as a pure product (yield 18%). Mp 292 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 13.51 (bs, 1H, NH), 8.74 (dd, J = 4.7, 1.8 Hz, 1H, H-7), 8.31 (dd, J = 7.8, 1.8 Hz, 1H, H-5), 7.60 (m, 1H, H-6′), 7.56 (bt, 1H, H-2′), 7.44 (t, J = 8.0 Hz, 1H, H-5′), 7.37 (dd, J = 7.8, 4.8 Hz, 1H, H-6), 7.32 (m, 1H, H-4′). ^{13}C NMR (125 MHz, DMSO-d_6) δ (ppm): 177.2, 159.9, 155.2, 150.7, 140.6, 136.8, 131.8, 131.1, 130.7, 128.4, 121.0, 120.4, 112.1; ESI MS (*m/z*): 334/336 $[M + H]^+$, purity >99% (by HPLC); Anal. $C_{13}H_8BrN_3OS$ (C, H, N, O).

5.1.18. 4-Oxo-3-phenyl-2-thioxo-1,2,3,4-tetrahydropyrido[3,4-d]pyrimidine (**18**)

A mixture of 3-aminoisonicotinic acid (150 mg, 1.03 mmol) and phenylisotiocianate (0.161 mL, 1.34 mmol) with triethylamine (0.143 mL, 0.10 mmol) in acetonitrile (4 mL) was heated under microwave irradiation (30 min, 150 °C). After cooling down the reaction in an ice/salt bath, a white precipitate was formed. The solid was filtered and purified by column chromatography on silica using hexane/ethyl acetate (6:1) as eluent affording **18** as a pure product (yield 35%). Mp 280–281 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 13.22 (s, 1H), 8.82 (s, 1H, H-6), 8.52 (d, J = 5.1 Hz, 1H, H-8), 7.81 (d, J = 5.1 Hz, 1H, H-5), 7.41 (m, 5H, H-Ar); ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm): 177.2, 159.4, 144.5, 139.3, 135.2, 129.3, 129.2, 128.6, 122.1, 119.7; ESI MS (*m/z*): 256 $[M + H]^+$; purity 98% (by HPLC). Anal. $C_{13}H_9N_3OS$ (C, H, N, O).

5.1.19. 4-Oxo-3-(pyridin-3-yl)-2-thioxo-1,2,3,4-tetrahydroquinazoline (**19**)

A mixture of methyl anthranilate (117 mg, 0.77 mmol), 3-isothiocyanatopyridine (137 mg, 1.00 mmol) and triethylamine (21 μL, 0.15 mmol) in acetonitrile (3 mL) was heated under microwave irradiation (45 min, 175 °C). After cooling down the

reaction in an ice/salt bath, an orange precipitate was formed. The solid was filtered, washed with diethylether and dried, affording **19** as a pale brown solid (yield 87%). Mp does not melt at 350 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 8.57 (m, 1H), 8.48 (bs, 1H), 7.95 (d, J = 6.9 Hz, 1H), 7.78 (m, 2H), 7.53 (dt, J = 7.8, 1.5 Hz, 1H), 7.44 (bd, 1H), 7.34 (bt, J = 8.1 Hz, 1H); ^{13}C NMR (125 MHz, DMSO-d_6) δ (ppm): 176.5, 160.4, 150.3, 149.3, 140.0, 137.4, 136.6, 136.2, 127.9, 124.9, 124.4, 116.6, 116.3; ESI MS (*m/z*): 256 $[M + H]^+$, purity > 99% (by HPLC); Anal. $C_{13}H_9N_3OS$ (C, H, N, O).

5.1.20. 6,7-Dimethoxy-4-oxo-3-(pyridin-3-yl)-2-thioxo-1,2,3,4-tetrahydroquinazoline (**20**)

A mixture of methyl 2-amino-4,5-dimethoxybenzoate (200 mg, 0.94 mmol), 3-isothiocyanatopyridine (152 mg, 1.13 mmol) and triethylamine (9 mg, 0.09 mmol) in acetonitrile (3 mL) was heated under microwave irradiation (25 min, 180 °C). After cooling down the reaction in an ice/salt bath, a yellow precipitate was formed. The solid was filtered, washed with diethylether and dried, affording **20** as a pure product (yield 59%). Mp does not melt at 350 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 12.91 (s, 1H, NH), 8.58 (dd, J = 6.8, 1.1 Hz, 1H, H-4′), 8.44 (d, J = 1.8 Hz, 1H, H-2′), 7.76 (dt, J = 11.4, 2.1 Hz, 1H, H-6′), 7.52 (dd, J = 11.4, 7.2 Hz, 1H, H-5′), 7.27 (s, 1H, H-5), 6.99 (s, 1H, H-8), 3.87 (s, 3H, MeO), 3.84; ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm): 175.2, 159.7, 155.8, 150.2, 149.1, 147.0, 137.3, 136.6, 135.9, 124.3, 108.8, 107.3, 98.3, 56.4, 56.2; ESI MS (*m/z*): 316 $[M + H]^+$, purity > 99% (by HPLC); Anal. $C_{15}H_{13}N_3O_3S$ (C, H, N, O).

5.1.21. 4-Oxo-3-(pyridin-3-yl)-2-thioxo-1,2,3,4-tetrahydropyrido[3,4-d]pyrimidine (**21**)

A mixture of 3-aminoisonicotinic acid (100 mg, 0.72 mmol) and 3-isothiocyanatopyridine (128 mg, 0.94 mmol) in 1,2-dimethoxyethane (3 mL) was heated under microwave irradiation (30 min, 150 °C). After cooling down the reaction in an ice/salt bath, a yellow precipitate was formed. The solid was filtered, washed with diethylether and dried, affording **21** as a pure product (yield 69%). Mp 341 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 13.37 (bs, 1H, NH), 8.83 (s, 1H, H-8), 8.59 (dd, J = 4.5, 1.2 Hz, 1H, H-4′), 8.52 (d, J = 5.1 Hz, 1H, H-6), 8.48 (d, J = 2.4 Hz, 1H, H-2′), 7.82 (d, J = 5.1 Hz, 1H, H-5), 7.77 (dt, J = 8.1, 1.8 Hz, 1H, H-6′), 7.54 (dd, J = 7.8, 4.8 Hz, 1H, H-5′); ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm): 176.7, 159.1, 149.5, 149.0, 144.3, 139.0, 136.7, 135.7, 134.7, 123.9, 121.6, 119.2; ESI MS (*m/z*): 257 $[M + H]^+$, purity >99% (by HPLC); Anal. $C_{12}H_8N_4OS$ (C, H, N, O).

5.1.22. 4-Oxo-3-(2-(piperidin-1-yl)ethyl)-2-thioxo-1,2,3,4-tetrahydroquinazoline (**22**)

A mixture of methyl anthranilate (50 mg, 0.33 mmol), 1-(2-isothiocyanatoethyl)piperidine (79 mg, 0.46 mmol) and a catalytic amount of DMAP in 1,2-dimethoxyethane (3 mL) was heated under microwave irradiation (50 min, 160 °C). After cooling down the reaction in an ice/salt bath, a yellow precipitate was formed. The solid was filtered, washed with diethylether and dried, affording **22** as a pure product (yield 62%). Mp 199 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 7.95 (dd, J = 7.8, 0.9 Hz, 1H), 7.74 (dt, J = 8.7, 1.5 Hz, 1H), 7.36 (d, J = 8.1 Hz, 1H), 7.30 (dt, J = 7.1, 0.9 Hz, 1H), 4.51 (t, J = 7.2 Hz, 2H, N-CH_2–CH_2-piperidine), 2.58 (t, J = 7.5 Hz, 2H, N–CH_2–CH_2-piperidine), 2.45 (bs, 4H, 2 × CH_2), 1.47 (m, 4H, 2 × CH_2), 1.35 (m, CH_2); ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm): 175.0, 170.3, 159.2, 139.0, 135.4, 127.2, 124.4, 115.5, 54.8, 42.9, 25.4, 23.8; ESI MS (*m/z*): 290 $[M + H]^+$, purity > 99% (by HPLC); Anal. $C_{15}H_{19}N_3OS$ (C, H, N, O).

5.1.23. 3-(2-Morpholinoethyl)-4-oxo-2-thioxo-1,2,3,4-tetrahydroquinazoline (**23**)

A mixture of methyl anthranilate (83 mg, 0.55 mmol) and 4-(2-isothiocyanatoethyl)morpholine (113 mg, 0.65 mmol) in acetonitrile (3 mL) was heated under microwave irradiation (45 min, 170 °C). After cooling down the reaction with an ice/salt bath, a white, precipitate was formed. The solid was filtered and washed with diethylether and dried, affording **24** as a white, silky solid (yield 76%). Mp 199 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.85 (bs, 1H, NH), 8.21 (d, J = 6.8 Hz, 1H, H-Ar), 7.64 (t, J = 6.7 Hz, 1H, H-Ar), 7.28 (m, 1H, H-Ar), 7.20 (d, J = 7.0 Hz, 1H, H-Ar), 4.79 (m, 2HN-CH_2-CH_2-morpholine), 3.91 (bs, 4H), 2.96 (m, 2H), 2.85 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 176.0, 160.2, 138.9, 135.8, 128.8, 125.4, 116.5, 67.2, 55.3, 54.1, 43.4; *m/z* (ES) 292 $(M + H)^+$; purity 98% (by HPLC); Anal. $C_{14}H_{17}N_3O_2S$ (C, H, N, O).

5.1.24. 6,7-Dimethoxy-4-oxo-3-(2-(piperidin-1-yl)ethyl)-2-thioxo-1,2,3,4-tetrahydroquinazoline (**24**)

A mixture of methyl 2-amino-4,5-dimethoxybenzoate (200 mg, 0.95 mmol), 1-(2-isothiocyanatoethyl)piperidine (224 mg, 1.32 mmol) and a catalytic amount of DMAP in acetonitrile (3 mL) was heated under microwave irradiation (30 min, 175 °C). After cooling down the reaction with an ice/salt bath, a pale yellow precipitate was formed. The solid was filtered and washed with diethylether and dried, affording **24** as a white solid (yield 20%). Mp 210 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.25 (1H, s, H-5), 6.88 (1H, s, H-8), 4.48 (2H, m, N-CH_2), 3.81 (3H, s, MeO), 3.80 (3H, s, MeO), 3.32 (1H, bs, NH), 2.53 (2H, m, CH_2-N), 2.33 (4H, bs, 2 × CH_2), 1.45 (4H, bs, 2 × CH_2), 1.34 (2H, bs, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6): δ 173.8, 158.7, 155.2, 146.7, 134.9, 107.9, 106.7, 97.7, 55.9, 55.7, 54.5, 54.2, 43.0, 25.6, 23.9; *m/z* (ES) 350 $(M + H)^+$; purity > 99% (by HPLC). Anal. ($C_{13}H_9N_3OS$) (C, H, N, O).

5.1.25. 6,7-Dimethoxy-3-(2-morpholinoethyl)-4-oxo-2-thioxo-1,2,3,4-tetrahydroquinazoline (**25**)

A mixture of methyl 2-amino-4,5-dimethoxybenzoate (115 mg, 0.55 mmol) and 4-(2-isothiocyanatoethyl)morpholine (113 mg, 065 mmol) in acetonitrile (3 mL) was heated under microwave irradiation (45 min, 170 °C). After cooling down the reaction with an ice/salt bath, a white precipitate was formed. The solid was filtered and washed with diethylether and dried, affording **25** as a white solid (yield 59%). Mp 227 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.52 (1H, s, H-Ar), 6.78 (1H, s, H-Ar), 4.83 (m, 2H, N-CH_2–CH_2-morpholine), 3.93 (s, 6H, 2 × MeO), 3.86 (m, 4H, 2 × CH_2), 2.91 (m, 4H, 2 × CH_2), 2.85 (m, 2H, N–CH_2–CH_2-morpholine); ^{13}C NMR (75 MHz, $CDCl_3$): δ 174.8, 159.9, 156.2, 147.9, 135.0, 109.3, 107.9, 97.2, 67.0, 56.9, 56.7, 55.5, 54.7, 43.7; *m/z* (ES) 352 $(M + H)^+$; purity 97% (by HPLC); Anal. ($C_{16}H_{21}N_3O_4S$) (C, H, N, O).

5.2. Biological assays

5.2.1. Phosphodiesterase inhibition assay

The methodology used for measuring PDE7A1 activity was based in a Scintillation Proximity Assay (SPA) from Perkin Elmer (TRKQ7090). The activity of PDE7A1 is measured by co-incubating the enzyme with [^{3}H]cAMP and the hydrolysis of the nucleotide is quantified by radioactivity measurement after binding of [^{3}H]AMP to scintillation binding bead.

0.02 units of PDE7A1 (Calbiochem # 524751) were incubated in a 96-well flexiplate with 5 nCi of [^{3}H]cAMP and inhibitors in 100 μl of assay buffer (contained in the kit) for 20 min at 30 °C. After the incubation time 1 mg of scintillation beads were added to each well and plate was shaken for 1 h at room temperature. Finally, beads were settled for 30 min and radioactivity was detected in a Microbeta Trilux reader.

IC_{50} values were calculated by non-linear regression fitting using GrpahPad Prism. Data (radioactivity *vs* log concentration) was fitted to a sigmoidal dose–response equation:

Y = Bottom + (Top-Bottom)/(1 + 10^((logIC$_{50}$-X)*n)), where Bottom and Top were the minimum and maximal inhibition for PDE, respectively, IC$_{50}$ was the concentration of compound that inhibited the PDE activity in a 50% and n was the slope of the concentration-response curve.

5.2.2. Primary cell cultures

Primary cortical neuronal cultures were prepared from the cerebral cortex of E18 rats, as previously reported by Luna-Medina et al [37]. Briefly, after dissecting the cerebral cortex, cells were plated on poly-lysine (20 μg/ml; Sigma) and gelatin (250 μg/ml; Sigma)-coated plates or cover-slips and the cultures were maintained in Neurobasal medium with B-27 supplements. All experiments were carried out with 7-day-old cultures.

Astrocytes were prepared from neonatal (P2) rat cerebral cortex, as previously described by Luna-Medina et al [37]. Briefly, after removal of the meninges the cerebral cortex was dissected, dissociated, and incubated with 0.25% trypsin/EDTA at 37 °C for 1 h. After centrifugation, the pellet was washed 3 times with HBSS (Gibco) and the cells were plated on non-coated flasks and maintained in HAMS/DMEM (1:1) medium containing 10% FBS. After 15 days the flasks were agitated on an orbital shaker for 12 h at 250 rpm at 37 °C, and the non-adherent oligodendrocytes and microglial cells were removed. After 7 days the flasks were agitated again to remove any remaining microglia cells and then trypsinized and expanded at a 1:5 ratio in complete medium. Primary microglial cells were prepared from 15-day-old mixed glial cultures, as described above. Fifteen-day-old mixed glial cultures were agitated on an orbital shaker for 12 h at 250 rpm at 37 °C. The supernatant was collected, centrifuged, and the cellular pellet containing the microglial cells resuspended in HAMS/DMEM (1:1) containing 10% FBS and seeded on uncoated plates. Cells were allowed to adhere for 2 h and the medium was removed to eliminate non-adherent oligodendrocytes and new fresh medium containing 10 ng/ml of GM-CSF was added. The purity of microglia obtained by this procedure was >98% as determined by immunofluorescence with the OX42 antibody.

5.2.3. CNS penetration: In vitro parallel artificial membrane permeability assay (PAMPA)-Blood brain barrier (BBB)

Prediction of the brain penetration was evaluated using a parallel artificial membrane permeability assay (PAMPA) [30]. Ten commercial drugs, phosphate buffer saline solution at pH 7.4 (PBS), Ethanol and dodecane were purchased from Sigma, Acros organics, Merck, Aldrich and Fluka. The porcine polar brain lipid (PBL) (catalog no. 141101) was from Avanti Polar Lipids. The donor plate was a 96-well filtrate plate (Multiscreen® IP Sterile Plate PDVF membrane, pore size is 0.45 μM, catalog no. MAIPS4510) and the acceptor plate was an indented 96-well plate (Multiscreen®, catalog no. MAMCS9610) both from Millipore. Filter PDVF membrane units (diameter 30 mm, pore size 0.45 μm) from Symta were used to filtered the samples. A 96-well plate UV reader (Thermoscientific, Multiskan spectrum) was used for the UV measurements. Test compounds [(3–5 mg of Caffeine, Enoxacine, Hydrocortisone, Desipramine, Ofloxacine, Piroxicam, Testosterone), (12 mg of Promazine) and 25 mg of Verapamile and Atenolol] were dissolved in EtOH (1000 μL). 100 μL of this compound stock solution was taken and 1400 μL of EtOH and 3500 μL of PBS pH 7.4 buffer were added to reach 30% of EtOH concentration in the experiment. These solutions were filtered. The acceptor 96-well microplate was filled with 180 μL of PBS/EtOH (70/30). The donor 96-well plate was coated with 4 μL of porcine brain lipid in dodecane (20 mg mL^{-1}) and after 5 min, 180 μL of each compound solution was added. 1–2 mg of every compound to be determined their ability to pass the brain barrier were dissolved in 1500 μL of EtOH and 3500 μL of PBS pH 7.4 buffer, filtered and then added to the donor 96-well plate. Then the donor plate was carefully put on the acceptor plate to form a "sandwich", which was left undisturbed for 2h and 30 min at 25 °C. During this time the compounds diffused from the donor plate through the brain lipid membrane into the acceptor plate. After incubation, the donor plate was removed. UV plate reader determined the concentration of compounds and commercial drugs in the acceptor and the donor wells. Every sample was analyzed at three to five wavelengths, in 3 wells and in two independent runs. Results are given as the mean [standard deviation (SD)] and the average of the two runs is reported. 10 quality control compounds (previously mentioned) of known BBB permeability were included in each experiment to validate the analysis set.

5.2.4. Induction of permanent focal ischemia

Adult male Swiss mice (8–10 weeks of age) were used. All animals were kept in a room with controlled temperature, a 12 h dark/light cycle and fed with standard food and water ad libitum. All experimental protocols adhered to the guidelines of the Animal Welfare Committee of the Universidad Complutense (EU directives 86/609/CEE and 2003/65/CE).

Surgery leading to focal cerebral ischemia was conducted under anesthesia with isoflurane in a mix of O_2 and N_2O (0.3/0.7 L/min). During surgery, body temperature was maintained at 37.0 ± 0.5 °C using a servo-controlled rectal probe–heating pad. The surgical procedure was a variant of that described by Chen et al. [38] and Liu et al. [39]. A small craniotomy was made over the trunk of the left middle cerebral artery and above the rhinal fissure. The permanent middle cerebral artery (MCA) occlusion (pMCAO) was done by ligature of the trunk just before its bifurcation between the frontal and parietal branches with a 9-0 suture. Complete interruption of blood flow was confirmed under an operating microscope. Additionally, the left common carotid artery was then occluded. Mice in which the MCA was exposed but not occluded served as sham-operated controls (sham). After surgery, individual animals were returned to their cages with free access to water and food.

Physiological parameters (rectal temperature, mean arterial pressure, blood glucose levels) were not significantly different between all studied groups (data not shown). No spontaneous mortality was found after MCAO with this model, and this was unaffected by the different experimental treatments.

5.2.5. Infarct outcome determination

For infarct volume determination, brains were removed 48 h after pMCAO, and cut into eight coronal brain slices of 1-mm (Brain Matrix, WPI, UK), which were stained in 2% TTC (2,3,5-triphenyltetrazolium chloride, Merck, Madrid, Spain) in 0.2 mol/L phosphate buffer. Infarct size was determined as follows: infarct volumes were measured by sampling stained sections with a digital camera (Nikon Coolpix 990, Nikon Corporation, Tokyo, Japan), and the image of each section was analyzed using ImageJ 1.44l (NIH, Bethesda, MD, USA). The digitalized image was displayed on a video monitor. With the observer masked to the experimental conditions, the ratio between the volume of the spared cortex in the damaged hemisphere (L_N) and that in the whole neocortex of the contralateral hemisphere (R) was calculated, and used to detect differences in the amount of cortex that was damaged by the infarct in each animal. To calculate the percent of hemisphere infarcted volume (HIV%) we used the formula: HIV% = [1−(L_N/R)] × 100.

To determine neurological impairment, the modified Neurological Severity Score (mNSS) was applied to every animal 24 and 48 h after pMCAO as previously described [40]. This neurological score evaluates motor symptoms (hemiparesis and gait), balance and reflexes.

Acknowledgments

The authors gratefully acknowledge the financial support of Ministry of Science and Innovation (MICINN), projects nos. SAF2007-62811, SAF2009-13015-C02-01, SAF2009-08145 and SAF2010-16365; Instituto de Salud Carlos III (ISCiii), project no. RD07/0060/0015 and RD06/0026/0005 (RETICS program) and CIBERNED; Fundación Española para la Ciencia y la Tecnología (FECYT), project no. FCT-09-INC-0367 and Consejo Superior de Investigaciones Científicas (CSIC), project no. PIE 200780I012. M. R. and D. I. P. acknowledge pre- and post-doctoral fellowship from the CSIC (JAE program) respectively. J. G. Z. was supported by the Programme Alban, scholarship No. E07D400805CO. BRAINco Biopharma is acknowledged.

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2011.10.040.

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