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Original article

Dibenzo[1,4,5]thiadiazepine: A hardly-known heterocyclic system with neuroprotective properties of potential usefulness in the treatment of neurodegenerative diseases



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ABSTRACT

In this work we describe a new family of dibenzo[1,4,5]thiadiazepines (**2–12**) that showed an interesting *in vitro* biological profile, namely neuroprotective and antioxidant properties, as well as blockade of cytosolic calcium entry. They showed no cytotoxic effects and the majority were predicted as CNS-permeable compounds. In human neuroblastoma cells they displayed good neuroprotective properties against mitochondrial oxidative stress which, in many cases, almost reached the full protection (>90%) when compounds were incubated with cells 24 h before the addition of toxic stressors. In co-incubation conditions these figures were smaller, although some compounds maintained an interesting level of neuroprotection, higher than 50%. Four selected compounds (**2**, **5**, **8**, and **11**) were found to be effective antioxidant agents by sequestering mitochondrial radical oxygen species (ROS). Moreover, compound **2** showed a remarkable calcium-channel modulating activity. The interest of these compounds is increased by the fact that dibenzo[1,4,5]thiadiazepine is a barely known structure that is not difficult to synthesize and presents very few described derivatives, opening a new and broad line of research in Medicinal Chemistry.

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1. Introduction

The known figures of the prevalence of Alzheimer's disease (AD), the most extended senile dementia, are really impressive and worrying. Although age is the highest risk factor for AD, about 5.2 million USA citizens of all ages are thought to suffer AD in 2013; among them, approximately 4% are under 65 years old, 13% are 65–74, 44% are 75–84 and 38% are 85 years and older. Namely, one out

of nine (11%) USA citizens age 65 and older [1]. The terrible symptoms and long process of AD, the subsequent impact on the caregivers and burden for the Public Health Systems and families are also well-known.

AD is a multifunctional disease in which a number of physiological processes change progressively into closely connected pathological ones. The most striking hallmarks of these changes are the occurrence of amyloid senile plaques, neurofibrillary tangles and dysfunction of the cholinergic neurotransmission in the framework of a massive neuronal loss, as well as other not so visible events such as a progressive failure of the endogenous antioxidant systems [2] and an imbalance in the cytosolic calcium concentrations [3]. Thus, several neuroprotective strategies involving the capture of mitochondrial free radicals [4,5] or the cytosolic calcium modulation [6] have been proposed for the treatment of AD.

Continuously, new structures designed to act on one (standard drugs) or more than one (multifunctional drugs) of the pathological processes of AD appear published [7]. Of course, the ultimate goal

Abbreviations: AD, Alzheimer's disease; AChE, acetylcholinesterase; BBB, blood–brain barrier; BuChE, butyrylcholinesterase; CNS, central nervous system; DCFH-DA, 2',7'-dichlorofluorescein-diacetate; HPLC-MS, high performance liquid chromatography–mass spectrometry; LDH, lactate dehydrogenase; PAMPA, parallel artificial membrane permeation assay; ROS, reactive oxygen species.

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of all these works is to find out a new treatment to cure, or at least alleviate, the disease. However, multi-target drugs may provide more therapeutic advantages in the treatment of such a complex pathology than single-target drugs [8–10]. Indeed, by applying the multifunctional approach, in recent years we reported several families of hybrid compounds that combine neuroprotective, cholinergic, antioxidant and beta-amyloid reducing properties [11–16]. Some of these hybrids have proved their usefulness in murine models of AD and stroke [17–19].

Another rewarding strategy to discover new multifunctional drugs is the biological evaluation of compounds proceeding from in-house libraries towards different targets. Many groups of medicinal chemistry synthesized lots of products that, in many cases in the past, were evaluated in one activity and then, stored and forgotten. Nowadays, the *in vitro* biological tests are widely generalized and those in-house libraries are treasures of structures frequently related to active compounds. Our group possesses a broad in-house library of compounds which has already produced interesting results in our line of research aimed to the study of new drugs for neurodegenerative diseases [20–22].

Following this later approach, in a first exploratory work we studied some biological properties of a group of compounds selected from our library, five phenothiazines and a unique derivative of the very little-known heterocyclic system 1,4,5-dibenzothiadiazepine [23]. The promising results obtained encouraged us to develop two new families, taking a representative product of each series as hit compounds. The development of the first selected structure yielded a series of *N*-acylaminophenothiazine derivatives that displayed interesting properties as multifunctional neuroprotective agents [24]. Phenothiazine is a well-known heterocyclic system present in many commercial drugs such as fluphenazine, chlorpromazine, promethazine (Phenergan) and many others [25]. Phenothiazines are also useful as neurogenic and neuroprotective agents [26] and as potential drugs for the treatment of Creutzfeldt-Jakob and other prion diseases [27].

The second selected hit from our initial work was 1,4,5-(4-chloro-5,6-dihydro-5,6-diacetyl)dibenzo[*b,f*]thiadiazepine **1** as a possible bioisostere of the phenothiazine system, which showed an interesting multifunctional profile: neuroprotection in the human neuroblastoma cell line SH-SY5Y against oxidative stress produced by free ROS, both of exogenous and mitochondrial origin; modulation of the voltage-dependent calcium channels; not cytotoxic effects *per se*; and ability to cross the blood–brain barrier (BBB) [23]. Compound **1** and other 1,4,5-dibenzothiadiazepine derivatives were described in 1982 by Corral et al., from our Medicinal Chemistry Institute [28], but they were never biologically tested until we started our screening program.

In addition and as far as we know, biological properties of 1,4,5-dibenzothiadiazepine derivatives were absolutely unexplored and even from a chemical point of view, it is a hardly-known heterocyclic system.

Thus, the structural novelty and the promising results founded in the 1,4,5-dibenzothiadiazepine **1** prompted us to carry out a more extensive study of the pharmacological properties of this family of compounds. In this work, we describe a series of CNS-permeable 1,4,5-dibenzothiadiazepines that showed neuroprotective properties against mitochondrial oxidative stress, as well as calcium modulatory and antioxidant properties. Some compounds were taken from our library and others have been newly synthesized.

2. Results and discussion

In addition to the original 1,4,5-(4-chloro-5,6-dihydro-5,6-diacetyl)dibenzo[*b,f*]thiadiazepine **1**, there were three more

derivatives: **2**, **3** (2-Cl and 3-Cl respectively) and **4** (2-Cl basic heterocycle) in our library of compounds, all of them synthesized by the Corral's group [28] (Fig. 1). Their purity was checked by HPLC-MS and their structures confirmed by ¹H, ¹³C NMR and MS. Compounds **2** and **3** were purified by flash column chromatography (see Experimental Section), yielding two crystalline solids which showed purities higher than 95% (HPLC-MS). The stored product **4** was pure enough (97%) to be used without any new purification.

With the aim to extend this initial set of compounds, new dibenzo[1,4,5]thiadiazepines were obtained (**5–12**), following the synthetic method formerly described [28]. Hydrazines were diacylated to the hydrazides **13–15**, which then yielded the dibenzothiadiazepines **5** (60%) and **6** (40%) by cyclization in a base-catalyzed Smiles rearrangement. All the hydrazines used in this work, except the trifluoromethylated derivative (see Experimental Part) had previously been obtained as common starting products for the synthesis of *N*-acylaminophenothiazines [24]. The mono-acylated product **7** (50%) was obtained instead of the expected diacylated analog when the substituent was a nitro group. Yields were not optimized because our chemical objective was simply to obtain enough amounts of pure products to carry out their biological evaluation.

The same procedure, although a little more complicated, was employed to obtain the derivatives **8–12** (Scheme 1). 2-Nitro-5- and 2-nitro-6-chloro-2'-hydrazinodiphenyl sulfides were acylated with bromoacetyl and 2-bromopropionyl chloride to generate the hydrazides **16–18** which were treated with selected secondary amines to yield the new hydrazides **21–23**. There was no need of obtaining **16** and **17** because hydrazides **19** and **20** were also stored in our in-house library, in enough purity and amount to be used as synthetic intermediates. Finally, these compounds were subjected to cyclization in the same conditions that the previous derivatives, affording the expected dibenzothiadiazepines **8–12**.

Compounds **2–12** were initially tested as acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitors, following the well-known Ellman's method and using tacrine as a reference [29]. Enzymes of mammalian origin were used, namely AChE from bovine erythrocytes and BuChE from horse serum, due to their high degree of sequence identity to the human proteins [30] and their lower cost. As it could be foreseen from the results previously obtained from **1** [23], none of the compounds inhibited AChE (data not shown) and only **9** and **10** showed a sub-micromolar inhibition of BuChE (IC₅₀ = 0.9 and 0.7 μM respectively) (Table 1).

A basic condition of any compound to act on neurodegenerative processes is to penetrate into the brain, that is, to be able of crossing the blood–brain barrier (BBB). To examine the capability of our compounds to pass this barrier, we used a parallel artificial membrane model [31]. This is a fairly easy and successful method to predict the passive CNS permeation that our group had previously optimized in order to be applied to investigational compounds with limited aqueous solubility and that was employed to study many structurally different families [11,13–16,24]. The permeabilities of compounds **2–12** through a lipid extract of porcine brain were determined using a mixture 70:30 of phosphate buffered saline solution and ethanol (PBS:EtOH) (Table 1). In each experiment 10 commercial drugs were also evaluated for assay validation. The graphic representation of experimental permeabilities vs. reported values of such well-known drugs gave a lineal correlation, $P_e(\text{exptl}) = 0.57 P_e(\text{bibl}) + 2.36$ ($R^2 = 0.919$). From this equation and taking into account the described limits for BBB permeation, we established that compounds with permeability values above $4.6 \cdot 10^{-6} \text{ cm s}^{-1}$ could penetrate into the CNS by passive diffusion (CNS+), whereas products with P_e below $3.5 \cdot 10^{-6} \text{ cm s}^{-1}$ could not enter (CNS–). Between these values, the CNS permeation was considered as uncertain (CNS+/-). As shown in Table 1, with the

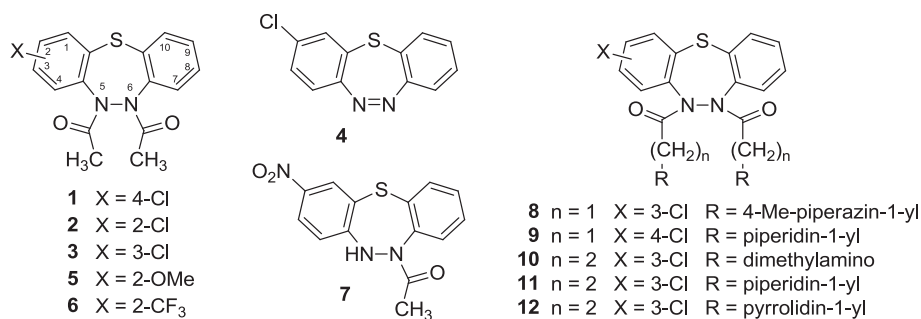
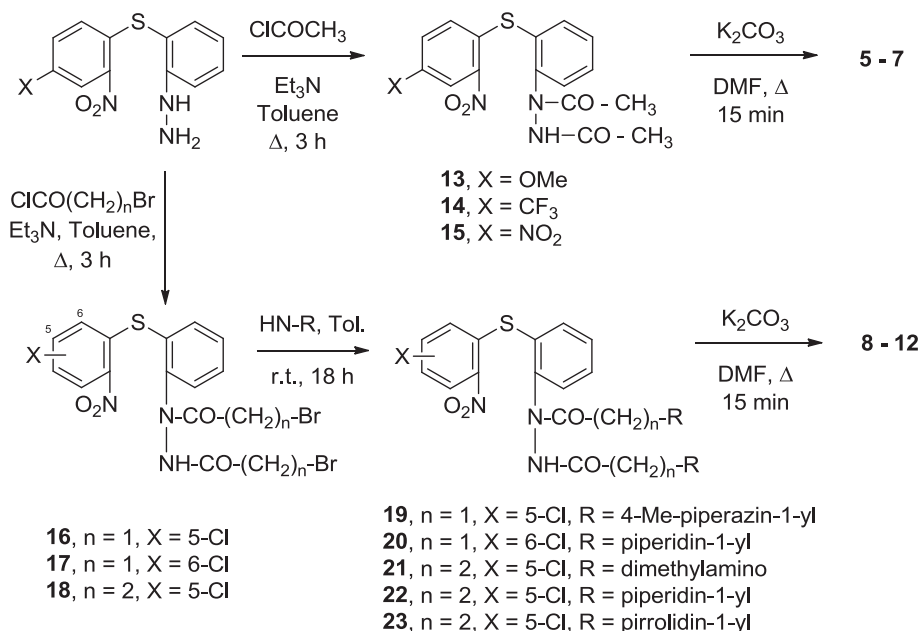


Fig. 1. Dibenzo[1,4,5]thiadiazepines studied in this work. Compounds **1–4** were taken from our library and **5–12** have been newly synthesized.



Scheme 1. Synthesis of the new dibenzo[1,4,5]thiadiazepines **5–12**.

exception of compounds **6**, **7** and **12** that could have some difficulties to penetrate into the brain, the rest of dibenzo[1,4,5]thiadiazepines would be able to cross BBB and reach their therapeutic targets.

To explore the therapeutic potential of dibenzo[1,4,5]thiadiazepines **2–12** their cell viability and neuroprotective capacity against mitochondrial oxidative stress were evaluated using the human neuroblastoma cell line SH-SY5Y and the mixture of rotenone and oligomycin A as the toxic insult. The combination of such toxics induces mitochondrial ROS as a consequence of the blockade of complex I and V of the mitochondrial electronic chain, being a good model of endogenous oxidative stress [32]. Two different protocols were used: (i) Pre-incubation, wherein cells were incubated with each compound for 24 h before the addition of rotenone plus oligomycin A, and then maintained for an additional 24 h period in the presence of the mixture of toxics. This protocol is useful to explore the possible cytoprotective effect due to the activation of endogenous antioxidant pathways. (ii) Co-incubation, wherein the compound and the combination rotenone plus oligomycin A were added at the same time and incubated for 24 h. In this case, a cytoprotective effect would indicate that the compound could be acting as a free radical scavenger [33]. In both protocols, compounds were tested at three concentrations (0.3, 1, and 3 μ M) and the percentage of cell death was determined by measuring the

amount of lactate dehydrogenase (LDH), an enzyme that is released to the extracellular medium when neurons die. To calculate the percentage of neuroprotection the basal liberation of LDH was subtracted from the value obtained in the presence of each compound. Trolox, the vitamin E antioxidant substructure, was used as a positive control and the results are shown in Table 2.

Firstly, possible cytotoxic effects of **2–12** were studied by exposing cells to compounds at the highest concentration used in the neuroprotection studies (3 μ M) for 24 h. In all cases, LDH percentage was roughly the same as the basal value (data not shown), suggesting that cell viability reached 100% and that dibenzo[1,4,5]thiadiazepines **2–12** are non-toxic products at the highest tested concentration.

All dibenzo[1,4,5]thiadiazepines **2–12** protected cells from the damage induced by the mixture of rotenone and oligomycin A, displaying very high percentages of mitochondrial protection that, in many cases, exceeded the 90% and almost reached the 100% in pre-incubation conditions. However, in the co-incubation experiments these figures were smaller, between 10 and 60%. These results suggested that the neuroprotective properties of dibenzo[1,4,5]thiadiazepines **2–12** are due to a mixed mechanism of action, in which clearly predominates the activation of endogenous antioxidant pathways vs. the direct capture of radical oxygen species (ROS). However, in the case of trolox its principal mechanism seems

Table 1Inhibition of BuChE^a and prediction of the blood–brain penetration of dibenzo[1,4,5]thiadiazepines **2**–**12**.

| Comp. | BuChE | PAMPA-BBB assay | |
|----------------|------------------------------------|--|------------|
| | IC ₅₀ (μM) ^b | P _e (10 ^{−6} cm s ^{−1}) ^b | Prediction |
| 2 | >100 | 10.1 ± 0.1 | CNS+ |
| 3 | >100 | 12.3 ± 0.5 | CNS+ |
| 4 | >100 | 12.3 ± 0.2 | CNS+ |
| 5 | >100 | 12.6 ± 0.3 | CNS+ |
| 6 | >100 | 4.0 ± 0.1 | CNS+/- |
| 7 | >100 | 3.9 ± 0.1 | CNS+/- |
| 8 | >100 | 5.9 ± 0.2 | CNS+ |
| 9 | 0.9 ± 0.01 | 7.3 ± 0.2 | CNS+ |
| 10 | 0.7 ± 0.02 | 14.2 ± 0.1 | CNS+ |
| 11 | >100 | 5.3 ± 0.2 | CNS+ |
| 12 | >100 | 3.7 ± 0.1 | CNS+/- |
| Tacrine | 0.01 ± 0.001 | nd | |

^a BuChE (EC 3.1.1.8) from horse serum.^b Results are the mean of three independent experiments ± SEM.**Table 2**Neuroprotection (%) of dibenzo[1,4,5]thiadiazepines **2**–**12** in the human neuroblastoma cell line SH-SY5Y against the combination of rotenone (30 μM) and oligomycin A (10 μM) at the indicated concentrations, using pre- and co-incubation conditions^a.

| Comp. | Pre-incubation | | | Co-incubation | | |
|---------------|----------------|---------|---------|---------------|---------|---------|
| | 0.3 μM | 1 μM | 3 μM | 0.3 μM | 1 μM | 3 μM |
| 2 | 64.8*** | 76.6*** | 81.5*** | 49.0*** | 59.2*** | 63.2*** |
| 3 | 79.8*** | 88.9*** | 91.1*** | 9.3* | 38.3* | 38.3* |
| 4 | 90.3*** | 90.2*** | 84.4*** | 40.5*** | 31.4*** | 38.8*** |
| 5 | 89.7*** | 97.0*** | 95.7*** | 49.9* | 50.1* | 53.8* |
| 6 | 76.4*** | 91.1*** | 92.2*** | 20.7** | 35.0*** | 35.8*** |
| 7 | 83.8** | 75.8* | 72.7* | 26.2* | 27.8* | 21.9* |
| 8 | 86.0*** | 96.4*** | 97.5*** | 50.6*** | 42.6*** | 35.7*** |
| 9 | 90.5*** | 83.9*** | 83.4*** | 43.3*** | 38.2*** | 35.6*** |
| 10 | 75.9*** | 92.1*** | 96.1*** | 8.1* | 20.4* | 15.9* |
| 11 | 84.6*** | 90.4*** | 96.4*** | 53.0*** | 55.4*** | 63.0*** |
| 12 | 99.3*** | 94.6*** | 92.4*** | 40.7*** | 45.9*** | 32.7*** |
| Trolox | n.d. | n.d. | 56.1*** | n.d. | n.d. | 55.7*** |

^a *p* < 0.05, ***p* < 0.01, ****p* < 0.001 respect to control; n.d.: not determined.^a Results are the mean of 4 independent experiments in triplicate.

to be the radical trapping, because it showed the same percentage of protection in both conditions (around 56%).

On the basis of the above results, the CNS-permeable dibenzo[1,4,5]thiadiazepines **2**, **5**, **8**, and **11**, which in co-incubation conditions displayed a percentage of neuroprotection higher than 50%, were selected to corroborate their capacity to capture mitochondrial radicals in the human neuroblastoma cell line. Thus, SH-SY5Y cells were loaded with the fluorescent dye 2',7'-dichlorofluorescein diacetate (DCFH-DA), treated with selected compounds at 0.3 μM and then subjected to free-radical generation by the mixture of rotenone (30 μM) plus oligomycin A (10 μM). Trolox (0.3 μM), a well-known potent ROS-scavenger, was also evaluated as a positive control. All tested compounds decreased the DCFH-DA fluorescence with the same strength than trolox (Table 3), showing that they are effective agents sequestering mitochondrial free radicals.

On the other hand, calcium is involved in many intracellular signaling pathways, including learning and memory processes. It has been described that disruptions in calcium homeostasis have been implicated in neuronal degeneration [34], and that preventing intracellular calcium overload could be a useful strategy to combat AD pathology [35]. Thus, the ability of above-selected dibenzo[1,4,5]thiadiazepines to reduce the cytosolic calcium concentration was examined, using the human neuroblastoma cell line SH-SY5Y. Cells were loaded with the fluorescent calcium probe Fluo-4/AM

(4 μM) for 1 h, incubated in the presence of compounds **2**, **5**, **8**, and **11** (3 μM) for 10 min and then stimulated with a concentrated solution of potassium chloride, so that the final K⁺ concentration in the medium was 70 mM. The L-type Ca²⁺-channel antagonist nifedipine was also assayed at the same concentration as a positive control, giving 48% inhibition of K⁺-evoked cytosolic calcium increase (Table 3). Whereas the blockade of calcium entry induced by compound **8** was under 10%, compounds **5** and **11** showed slightly better results, around 15%. In this assay, our best compound was the diacetylated dibenzo[1,4,5]thiadiazepine **2**, which modulated the cytosolic calcium by blocking the entry of this cation by 22%, about a half of the nifedipine value.

3. Conclusions

New promising neuroprotective agents derived from the hardly-known dibenzo[1,4,5]thiadiazepine system are here described. At micro- and sub-micromolar concentrations, these compounds provide almost a full-protection against mitochondrial oxidative stress when compounds were incubated with cells 24 h before the addition of stressors. When compounds were added at the same time than the toxic event the neuroprotection percentage was smaller, although in some compounds was superior to 50%. These results suggested that the neuroprotective properties of dibenzo[1,4,5]thiadiazepines **2**–**12** are due to a mixed mechanism of action, in which predominates the activation of endogenous antioxidant pathways vs. the direct capture of ROS. Four CNS-permeable compounds (**2**, **5**, **8**, and **11**) were found as effective antioxidant agents as trolox sequestering mitochondrial ROS. Moreover, compound **2** showed an interesting calcium-channel modulating activity, blocking the entry of this cation by 22%, about a half of the nifedipine value.

Such interesting profile, in addition to the almost novelty of this heterocyclic system, highlight these dibenzo[1,4,5]thiadiazepines as valuable candidates in the search for new treatments of neurodegenerative diseases.

4. Experimental

Solvents were purified and dried using standard methods, distillation under nitrogen atmosphere and sodium wire (THF). Reagents and anhydrous DMF were acquired from the usual commercial suppliers and used without further purification. Chromatographic separations were performed on silica gel (Kieselgel 60 Merck of 230–400 mesh) and compounds were detected with UV light (λ = 254 nm). HPLC-MS analyses were performed on an equipment composed by the separation module Alliance 2695, the

Table 3Percentage of free-radical capture (%) and blockade of cytosolic calcium entry (%) by compounds **2**, **5**, **8**, and **11** in the human neuroblastoma cell line SH-SY5Y.^a

| Comp. | Free-radical capture (%) ^b | Blockade of cytosolic calcium entry (%) ^c |
|------------|---------------------------------------|--|
| 2 | 24.3*** | 21.5* |
| 5 | 21.6** | 13.1* |
| 8 | 24.3*** | 9.3* |
| 11 | 18.8*** | 16.2* |
| Trolox | 22.3*** | n.d. |
| Nifedipine | n.d. | 47.9*** |

^a Results are the mean of 3 independent experiments; **p* < 0.05, ***p* < 0.01, ****p* < 0.001 respect to control; n.d.: not determined.^b Compounds at 0.3 μM; fluorescent dye: DCFH-DA; stressor: rotenone (30 μM) and oligomycin A (10 μM).^c Compounds at 3 μM; fluorescent dye: Fluo-4/AM (4 μM); cytosolic calcium overload elicited by K⁺ (70 mM).

photodiode array Waters 2996, and the quadrupole mass analyser Micromass ZQ. A SunFire C18 column, 91 Å, 3.5 µm, 4.6 mm × 50 mm was used at a flow rate of 1 mL/min and a gradient of (10–100)% B over 5 min. The mobile phase conditions were as follow: eluant A: water/0.1% HCO₂H; eluant B: MeCN/0.1% HCO₂H. Retention times (*t_R*) are reported in minutes.

Melting points (uncorrected) were determined with a Reichertert-Jung Thermovar apparatus. ¹H NMR and ¹³C NMR spectra were recorded in CD₃OD or CDCl₃ solutions using a Varian XL-400 spectrometer. Chemical shifts are reported in δ scale (ppm) relative to internal Me₄Si, *J* values are given in Hertz, and spin multiplicities are expressed as s (singlet), d (doublet), t (triplet), or m (multiplet). Mass spectra (MS) were obtained by electron spray ionization (ESI) in positive mode using a Hewlett–Packard MSD 1100 spectrometer. Elemental analyses were carried out in a Perkin-Elmer 240C equipment in the Centro de Química Orgánica Manuel Lora-Tamayo (CSIC) and all results are within ±0.4% of the theoretical values.

4.1. Compounds from our in-house library: **2–4** and **19, 20**. Purification and spectroscopic data

These four compounds had been stored a long time in our in-house library and they were initially examined by HPLC. Two of them (**2** and **3**) were purified by a flash column chromatography on silica gel, while **19** and **20** kept purities higher than 98% and were used directly as intermediates in the following synthetic step. Their chemical structures were confirmed by MS, ¹H and ¹³C NMR and the data obtained are given in this work.

4.1.1. 2-Chloro-5,6-dihydro-5,6-diacetyldibenzo[b,f][1,4,5]thiadiazepine (**2**)

This compound was purified by a flash column chromatography eluted with hexane:EtOAc (5:1) and isolated from the fractions *R_f* = 0.4 as a colorless solid of m.p. 148–149 °C (bibl. 150–151 °C [28]). ESI-MS *m/z* = 333 [MH]⁺, 335 [MH + 2]⁺. ¹H NMR (CDCl₃): δ (ppm) = 7.67–7.03 (m, 7H), 2.12 (s, 3H), 2.02 (s, 3H). ¹³C NMR (CDCl₃): δ (ppm) = 170.1, 168.2, 140.4, 139.6, 130.4, 129.7, 129.4, 128.7, 128.3, 127.6, 127.3, 126.1, 122.5, 117.8, 22.0, 21.9. HPLC-MS purity: 100%, *t_R* = 4.95 min. Anal. C₁₆H₁₃ClN₂O₂S (C, H, N, S).

4.1.2. 3-Chloro-5,6-dihydro-5,6-diacetyldibenzo[b,f][1,4,5]thiadiazepine (**3**)

This compound was purified by a flash column chromatography eluted with hexane:EtOAc (4:1). It was isolated from the fractions *R_f* = 0.5 as a colorless solid of m.p. 196–197 °C (bibl. 200–202 °C [28]). ESI-MS *m/z* = 333 [MH]⁺, 335 [MH + 2]⁺. ¹H NMR (CDCl₃): δ (ppm) = 7.63–7.09 (m, 7H), 2.12 (s, 3H), 2.03 (s, 3H). ¹³C NMR (CDCl₃): δ (ppm) = 167.2, 166.8, 140.1, 139.4, 131.0, 129.6, 129.4, 129.1, 128.6, 128.0, 127.7, 127.0, 125.9, 125.6, 21.8, 21.7. HPLC-MS purity: 97%, *t_R* = 4.98 min. Anal. C₁₆H₁₃ClN₂O₂S (C, H, N, S).

4.1.3. 3-Chlorodibenzo[b,f][1,4,5]thiadiazepine (**4**)

This compound remained in our chemical library as a colorless solid of m.p. 121–123 °C (bibl. 127–128 °C [28]). Its structure was confirmed and it did not need any purification. EM: *m/z* = 247 [MH]⁺, 249 [MH + 2]⁺. ¹H NMR (400 MHz, CDCl₃): δ (ppm) = 7.60 (d, 1H, *J* = 7.4 Hz), 7.52 (d, 1H, *J* = 7.4 Hz), 7.46 (dd, 1H, *J* = 7.4, *J* = 2.0 Hz), 7.39 (dd, 1H, *J* = 7.4, *J* = 2.0 Hz), 7.34–7.27 (m, 3H). ¹³C NMR (CDCl₃): δ (ppm) = 151.6, 150.0, 135.5, 132.4, 131.8, 131.2, 130.2, 129.9, 129.5, 129.4, 128.7, 127.6. HPLC-MS purity: 97%, *t_R* = 5.06 min. Anal. C₁₂H₇ClN₂S (C, H, N, S).

4.1.4. *N*-[2-(5-Chloro-2-nitrothiophenyl)phenyl]-*N,N'*-bis(4-methylpiperazin-1-yl)diacetylhydrazine (**19**)

This intermediate remained in our chemical library as a yellowish solid of m.p. 118–120 °C. Its structure was confirmed by MS, ¹H and ¹³C NMR and its purity by HPLC, and was used without a further purification. ESI-MS: *m/z* = 576 [MH]⁺, 578 [MH + 2]⁺. ¹H NMR (400 MHz, CDCl₃): δ (ppm) = 8.92 (s, 1H), 8.23 (d, 1H, *J* = 8.9 Hz), 7.70 (dd, 1H, *J* = 8.9 Hz, *J* = 1.5 Hz), 7.54 (m, 2H), 7.19 (m, 2H), 6.72 (d, 1H, *J* = 1.5 Hz), 3.15 (s, 2H), 3.05 (s, 2H), 2.22–2.59 (m, 16H), 2.18 (s, 6H). ¹³C NMR (CDCl₃): δ (ppm) = 170.4, 168.4, 144.3, 141.2, 139.3, 138.4, 132.6, 132.1, 131.9, 130.2, 128.1, 127.2, 126.8, 125.8, 60.7, 59.9, 54.7 (4C), 54.5 (4C), 45.6 (2C). HPLC-MS purity: 98%, *t_R* = 2.40 min.

4.1.5. *N*-[2-(6-Chloro-2-nitrothiophenyl)phenyl]-*N,N'*-bis(piperidin-1-yl)diacetylhydrazine (**20**)

This compound remained in our chemical library as a yellowish solid of m.p. 119–122 °C, pure enough to be used as intermediate without a further purification. ESI-MS: *m/z* = 546 [MH]⁺, 548 [MH + 2]⁺. ¹H NMR (400 MHz, CDCl₃): δ (ppm) = 9.33 (s, 1H), 7.72 (dd, 1H, *J* = 7.5 Hz, *J* = 1.0 Hz), 7.60 (m, 3H), 7.50 (td, 1H, *J* = 8.06 Hz, *J* = 1.2 Hz), 7.15 (m, 2H), 3.23 (s, 2H), 3.06 (s, 2H), 2.65 (m, 8H), 1.67 (m, 8H), 1.56 (m, 4H). ¹³C NMR (CDCl₃): δ (ppm) = 169.0, 168.4, 141.1, 140.3, 132.6, 132.5, 130.3, 129.9, 129.3, 128.8, 122.1, 126.1, 121.4, 121.3, 60.9, 59.0, 55.7 (4C), 26.2 (4C), 23.5 (2C). HPLC-MS purity: 98%, *t_R* = 2.50 min.

4.2. Synthesis of hydrazines

4.2.1. 2-[(4-Trifluoromethyl-2-nitrophenyl)thio]phenylhydrazine

It was synthesized following the general procedure described in Ref. [36], using 2-[(4-trifluoromethyl-2-nitrophenyl)thio]aniline (0.9 g, 2.86 mmol), NaNO₂ (0.21 g, 3.15 mmol) and SnCl₂·2H₂O (1.41 g, 6.30 mmol). Purification involved the use of hexane/ethyl acetate (8:1) as eluant. Yellow solid (0.40 g, 43%) of m.p. 141–143 °C. ESI-MS: *m/z* = 330 [MH]⁺, 353 [MH + Na]⁺. ¹H NMR (CDCl₃): δ (ppm) = 8.52 (d, 1H, *J* = 1.0), 7.52 (m, 2H), 7.44 (dd, 1H, *J* = 7.9 Hz, *J* = 1.2 Hz), 7.29 (dd, 1H, *J* = 7.9 Hz, *J* = 1.2 Hz), 6.89 (m, 2H), 6.12 (s, 1H), 3.38 (s, 2H). ¹³C NMR (75 MHz, CDCl₃): δ (ppm) = 152.5, 145.3, 142.4, 138.0, 133.5, 130.2, 128.9, 127.9, 125.1, 123.9, 120.5, 112.6, 110.0. HPLC-MS purity: 95%, *t_R* = 2.10 min.

4.3. Hydrazides, *N,N'*-diacylhydrazines

N,N'-Diacylhydrazines **13–15** were synthesized following a very general method. Derivatives **21–23** required a two-steps approach, the acylation of the corresponding hydrazine with 2-bromopropionyl chloride to yield **18**, which was treated with the chosen amine in order to obtain **21–23**. As explained above, hydrazides **19** and **20** were present in our in-house library of compounds and were not synthesized again.

General procedure: Acetyl chloride (1.30 mmol) was added on an ice-cooled solution of the selected hydrazine (0.65 mmol) and triethylamine (1.30 mmol) in anhydrous toluene (3 mL). Then, the resulting solution was refluxed (3 h), the solvent removed under reduced pressure and the residue purified on silica gel, using a chromatography column and a mixture of hexane/ethyl acetate (1:1) as eluant.

4.3.1. *N*-[2-(4-Methoxy-2-nitrothiophenyl)phenyl]-*N,N'*-diacetylhydrazine (**13**)

Following the general procedure, this product was obtained (48%) as a yellow solid of m.p. 96–98 °C. ESI-MS: *m/z* = 376 [MH]⁺, 399 [MH + Na]⁺, 775 [2 MH + Na]⁺. ¹H NMR (DMSO): δ (ppm) = 10.85 (s, 1H), 7.65 (d, 1H, *J* = 2.8 Hz), 7.51–7.25 (m, 4H),

7.22 (dd, 1H, $J = 9.0$ Hz, $J = 2.8$ Hz), 6.99 (d, 1H, $J = 9.0$ Hz), 3.81 (s, 3H), 2.04 (s, 3H), 1.72 (s, 3H). ^{13}C NMR (DMSO): δ (ppm) = 171.9, 169.1, 159.1, 149.7, 143.9, 135.2, 133.2, 131.1, 130.3, 129.6, 126.2, 121.4, 110.1, 109.2, 56.5, 21.9, 20.7. HPLC-MS purity: 98%, $t_R = 4.58$ min.

4.3.2. *N*-[2-(4-Trifluoromethyl-2-nitrothiophenyl)phenyl]-*N,N'*-diacetylhydrazine (**14**)

Following the general procedure, this product was obtained (53%) as a yellow solid of m.p. 188–190 °C. ESI-MS: $m/z = 414$ $[\text{MH}]^+$. ^1H NMR (DMSO): δ (ppm) = 10.79 (s, 1H), 8.47 (s, 1H), 7.70 (m, 1H), 7.61 (m, 3H), 6.46 (m, 1H), 7.05 (m, 1H), 2.02 (s, 3H), 1.82 (s, 3H). ^{13}C NMR (DMSO): δ (ppm) = 171.8, 168.5, 145.1, 144.9, 143.2, 138.1, 132.2, 130.6, 129.6, 129.1, 127.8, 126.1, 125.6, 125.1, 122.5, 21.7, 20.3. HPLC-MS purity: 100%, $t_R = 4.78$ min.

4.3.3. *N*-[2-(2,4-Dinitrothiophenyl)phenyl]-*N,N'*-diacetylhydrazine (**15**)

Following the general procedure, this product was obtained (57%) as a yellow solid of m.p. 156–158 °C. ESI-MS: $m/z = 391$ $[\text{MH}]^+$. ^1H NMR (DMSO): δ (ppm) = 10.83 (s, 1H), 8.87 (d, 1H, $J = 2.5$ Hz), 8.33 (dd, 1H, $J = 2.5$ Hz, $J = 9.1$ Hz), 7.62 (m, 2H), 7.48 (m, 2H), 7.06 (d, 1H, $J = 9.1$ Hz), 2.04 (s, 3H), 1.83 (s, 3H). ^{13}C NMR (DMSO): δ (ppm) = 172.4, 169.5, 147.1, 145.6, 145.2, 144.7, 138.9, 137.4, 132.7, 131.2, 130.4, 129.4, 127.9, 121.7, 22.5, 21.1. HPLC-MS purity: 100%, $t_R = 4.32$ min.

4.3.4. *N*-[2-(5-Chlor-2-nitrothiophenyl)phenyl]-*N,N'*-di(2-bromopropionyl)hydrazine (**18**)

Following the general procedure but using 2-bromopropionyl chloride as acylating agent and dichloromethane/methanol (9:1) as eluant, this product was obtained (30%) as a yellow solid. ESI-MS: $m/z = 566$ $[\text{MH}]^+$, 568 $[\text{MH} + 2]^+$. ^1H NMR (DMSO): δ (ppm) = 11.05 (s, 1H), 8.22 (d, 1H, $J = 9.0$ Hz), 7.66 (m, 3H), 7.50 (m, 2H), 6.76 (d, 1H, $J = 2.0$ Hz), 3.66 (t, 2H, $J = 6.0$ Hz), 3.54 (t, 2H, $J = 6.6$ Hz), 3.0 (m, 2H), 2.70 (m, 2H). ^{13}C NMR (DMSO): δ (ppm) = 171.1, 169.3, 144.6, 143.8, 140.1, 139.0, 137.1, 131.9, 130.0, 129.4, 127.9, 128.3, 127.3, 125.8, 36.2, 35.4, 28.8, 27.4. HPLC-MS purity: 90%, $t_R = 5.33$ min.

4.3.5. *N*-[2-(5-Chlor-2-nitrothiophenyl)phenyl]-*N,N'*-di(2-dimethylaminopropionyl)hydrazine (**21**)

This derivative was synthesized following a general procedure of amination: hydrazide **18** (0.88 mmol) reacted with dimethylamine (3.52 mmol) in anhydrous toluene (6 mL) at room temperature. After remaining stirred overnight, solvent was evaporated, the residue washed (NaHCO₃ 5%), dried and purified on silica gel, using a chromatography column and a mixture of dichloromethane/methanol (9:1) and ammonium hydroxide 2% as eluant. The product was obtained (61%) as a yellow solid. ^1H NMR (DMSO): δ (ppm) = 10.84 (s, 1H), 8.22 (d, 1H, $J = 9.0$ Hz), 7.56 (d, 1H, $J = 9.0$ Hz), 7.85 (m, 1H), 7.22 (m, 4H), 3.80 (t, 2H), 3.75 (t, 2H), 2.95 (s, 12H), 2.72 (t, 2H), 2.65 (t, 2H). ^{13}C NMR (DMSO): δ (ppm) = 173.4, 170.9, 145.1, 139.8, 139.0, 138.0, 136.8, 136.3, 131.9, 130.8, 129.2, 128.6, 127.2, 125.8, 60.1, 58.7, 47.3, 42.0, 30.7 (2C), 28.6 (2C). HPLC-MS purity: 90%, $t_R = 2.01$ min.

4.3.6. *N*-[2-(5-Chlor-2-nitrothiophenyl)phenyl]-*N,N'*-di[(2-dipiperidin-1-yl)propionyl]hydrazine (**22**)

Applying the same method, this hydrazide was obtained (75%) as a yellow solid. ESI-MS: $m/z = 574$ $[\text{MH}]^+$, 576 $[\text{MH} + 2]^+$. ^1H NMR (DMSO): δ (ppm) = 10.84 (s, 1H), 8.22 (d, 1H, $J = 9.0$ Hz), 7.54 (d, 1H, $J = 9.0$ Hz), 7.85–7.70 (m, 1H), 7.20–6.96 (m, 4H), 3.78 (t, 2H), 3.71 (t, 2H), 2.30–2.10 (m, 12H), 1.50–1.20 (m, 12H). ^{13}C NMR (DMSO): δ (ppm) = 173.4, 170.9, 145.1, 143.8, 140.1, 139.2, 138.0, 136.3, 131.9, 129.2, 128.6, 127.7, 126.6, 125.8, 54.1, 53.7, 53.6, 53.3, 30.1, 30.9, 30.7,

29.8, 25.5 (2C), 24.0 (2C), 23.8 (2C). HPLC-MS purity: 90%, $t_R = 2.60$ min.

4.3.7. *N*-[2-(5-Chlor-2-nitrothiophenyl)phenyl]-*N,N'*-di[(2-dipirrolidin-1-yl)propionyl]hydrazine (**23**)

Applying the same method, this hydrazide was obtained (65%) as a yellow solid. ESI-MS: $m/z = 546$ $[\text{MH}]^+$, 548 $[\text{MH} + 2]^+$. ^1H NMR (DMSO): δ (ppm) = 10.91 (s, 1H), 8.22 (d, 1H, $J = 9.0$ Hz), 7.60 (d, 1H, $J = 9.0$ Hz), 7.83–7.72 (m, 1H), 7.44–6.40 (m, 4H), 3.77 (t, 2H), 3.70 (t, 2H), 2.45–2.15 (m, 12H), 1.70–1.50 (m, 8H). ^{13}C NMR (DMSO): δ (ppm) = 173.1, 170.6, 145.1, 143.8, 140.1, 139.2, 138.2, 136.9, 132.0, 130.7, 129.6, 128.6, 127.8, 125.8, 53.4, 53.1, 51.2, 50.4, 32.8 (4C), 23.1 (4C). HPLC-MS purity: 90%, $t_R = 2.49$ min.

4.4. General method for the synthesis of dibenzothiadiazepines

Hydrazides were dissolved in DMF and refluxed for 15 min with an equimolecular amount of K₂CO₃. Then, solvent was evaporated and the residue purified through reverse-phase chromatography using H₂O/acetonitrile (95:5) as eluant.

4.4.1. 2-Methoxy-5,6-dihydro-5,6-diacetyldibenzo[b,f][1,4,5]thiadiazepine (**5**)

Following the general procedure, this product was obtained (60%) as a colorless solid, m.p.: 99–101 °C. ESI-MS: $m/z = 329$ $[\text{MH}]^+$, 351 $[\text{M} + \text{Na}]^+$, 679 $[2\text{M} + \text{Na}]^+$. ^1H NMR (CDCl₃): δ (ppm) = 7.79–6.73 (m, 7H), 3.81 (s, 3H), 2.19 (s, 3H), 2.05 (s, 3H). ^{13}C NMR (CDCl₃): δ (ppm) = 167.8, 167.6, 159.2, 139.6, 133.2, 132.2, 130.9, 129.9, 129.2, 128.1, 127.3, 125.9, 112.4, 111.8, 55.9, 22.4, 22.1. HPLC-MS purity: 97%, $t_R = 4.53$ min. Anal. C₁₇H₁₆N₂O₃S (C, H, N, S).

4.4.2. 2-Trifluoromethyl-5,6-dihydro-5,6-diacetyldibenzo[b,f][1,4,5]thiadiazepine (**6**)

Following the general procedure, this product was obtained (40%) as a colorless solid, m.p.: 125–127 °C. ESI-MS: $m/z = 367$ $[\text{MH}]^+$. ^1H NMR (CDCl₃): δ (ppm) = 7.82–7.27 (m, 7H), 2.21 (s, 3H), 2.09 (s, 3H). ^{13}C NMR (CDCl₃): δ (ppm) = 169.8, 168.4, 142.2, 135.2, 129.8, 129.0, 128.6, 128.2, 127.9, 127.5, 127.1, 126.5, 124.6, 123.2, 122.6, 22.0, 21.4. HPLC-MS purity: 100%, $t_R = 5.17$ min. Anal. C₁₇H₁₃F₃N₂O₂S (C, H, N, S).

4.4.3. 2-Nitro-5,6-dihydro-6-acetyldibenzo[b,f][1,4,5]thiadiazepine (**7**)

Following the general procedure, this product was obtained (50%) as a yellow solid, m.p.: 111–113 °C. ESI-MS: $m/z = 302$ $[\text{MH}]^+$. ^1H NMR (CDCl₃): δ (ppm) = 8.15 (d, 1H, $J = 2.4$ Hz), 7.88 (dd, 1H, $J = 8.9$ Hz, $J = 2.5$ Hz), 7.62 (dd, 1H, $J = 5.7$ Hz, $J = 3.2$ Hz), 7.44 (m, 2H), 7.37 (m, 1H), 7.32 (s, 1H), 6.80 (d, 1H, $J = 8.8$ Hz), 2.02 (s, 3H). ^{13}C NMR (CDCl₃): δ (ppm) = 170.5, 151.2, 144.2, 141.6, 132.8, 131.4, 130.6, 129.9, 127.7, 126.4, 123.3, 117.1, 116.4, 20.6. HPLC-MS purity: 100%, $t_R = 4.56$ min. Anal. C₁₄H₁₁N₃O₃S (C, H, N, S).

4.4.4. 3-Chloro-5,6-dihydro-5,6-di(4-methylpiperazin-1-yl)acetyldibenzo[b,f][1,4,5]thiadiazepine (**8**)

Following the general procedure, this product was obtained (31%) as a colorless solid, m.p.: 174–176 °C. ESI-MS: $m/z = 529$ $[\text{MH}]^+$, 531 $[\text{MH} + 2]^+$. ^1H NMR (CDCl₃): δ (ppm) = 7.70–7.12 (m, 7H), 3.25–3.20 (m, 4H), 2.62–2.0 (m, 22H). ^{13}C NMR (CDCl₃): δ (ppm) = 167.5, 167.2, 140.5, 139.8, 130.8, 130.2, 129.2, 128.6, 128.3, 127.8, 127.6, 127.4, 127.2, 125.9, 59.8, 59.6, 54.8 (2C), 54.7 (2C), 52.7 (2C), 52.5 (2C), 45.9 (2C). HPLC-MS purity: 100%, $t_R = 3.03$ min. Anal. C₂₆H₃₃ClN₆O₂S (C, H, N, S).

4.4.5. 4-Chloro-5,6-dihydro-5,6-di(piperidin-1-yl)acetyldibenzo[b,f][1,4,5]thiadiazepine (**9**)

Following the general procedure, this product was obtained (39%) as a colorless solid, m.p.: 131–133 °C. ESI-MS: m/z = 499 [MH]⁺, 501 [MH + 2]⁺. ¹H NMR (CDCl₃): δ (ppm) = 7.62–7.10 (m, 7H), 3.44–3.01 (m, 4H), 2.98–2.17 (m, 20H). ¹³C NMR (CDCl₃): δ (ppm) = 169.4, 166.5, 139.4, 134.5, 133.4, 130.6, 130.1, 128.6, 128.3, 127.9, 127.1, 126.7, 125.8, 124.9, 61.3, 60.6, 54.2 (2C), 54.0 (2C), 25.7 (2C), 25.6 (2C), 23.8, 23.5. HPLC-MS purity: 97%, t_R = 4.91 min. Anal. C₂₆H₃₁ClN₄O₂S (C, H, N, S).

4.4.6. 3-Chloro-5,6-dihydro-5,6-di(2-dimethylamino)propionylidibenzo[b,f][1,4,5]thiadiazepine (**10**)

Following the general procedure, this product was obtained (25%) as a colorless solid, m.p.: 138–140 °C. ESI-MS: m/z = 447 [MH]⁺, 449 [MH + 2]⁺. ¹H NMR (CDCl₃): δ (ppm) = 8.07–7.24 (m, 7H), 2.82–2.69 (m, 8H), 2.48–2.40 (m, 12H). ¹³C NMR (CDCl₃): δ (ppm) = 173.1, 170.5, 141.2, 138.9, 132.2, 131.3, 131.0, 130.8, 130.4, 130.0, 129.8, 129.4, 129.0, 128.2, 55.8, 55.4, 44.7 (2C), 44.1 (2C), 30.8, 30.9. HPLC-MS purity: 96%, t_R = 4.20 min. Anal. C₂₂H₂₇ClN₄O₂S (C, H, N, S).

4.4.7. 3-Chloro-5,6-dihydro-5,6-di[2-(piperidin-1-yl)]propionylidibenzo[b,f][1,4,5]thiadiazepine (**11**)

Following the general procedure, this product was obtained (22%) as a colorless solid, m.p.: 151–153 °C. ESI-MS: m/z = 527 [MH]⁺, 529 [MH + 2]⁺. ¹H NMR (CDCl₃): δ (ppm) = 8.27–7.22 (m, 7H), 3.73–1.40 (m, 28H). ¹³C NMR (MeOH): δ (ppm) = 173.1, 170.5, 141.1, 138.9, 132.1, 131.8, 130.9, 130.8, 130.6, 130.4, 129.8, 129.3, 128.6, 128.2, 55.2, 54.8 (2C), 54.6 (2C), 53.7, 30.6, 29.7, 24.2 (2C), 24.1 (2C), 22.5, 22.4. HPLC-MS purity: 100%, t_R = 4.27 min. Anal. C₂₈H₃₅ClN₄O₂S (C, H, N, S).

4.4.8. 3-Chloro-5,6-dihydro-5,6-di[2-(pyrrolidin-1-yl)]propionylidibenzo[b,f][1,4,5]thiadiazepine (**12**)

Following the general procedure, this product was obtained (20%) as a colorless solid, m.p.: 180–182 °C. ESI-MS: m/z = 499 [MH]⁺, 501 [MH + 2]⁺. ¹H NMR (CDCl₃): δ (ppm) = 8.05–7.18 (m, 7H), 3.64–1.92 (m, 24H). ¹³C NMR (MeOH): δ (ppm) = 173.1, 170.5, 141.2, 138.9, 132.2, 131.3, 131.0, 130.8, 130.4, 130.0, 129.8, 129.4, 129.0, 128.2, 55.6 (2C), 55.4 (2C), 51.6, 51.4, 31.7, 31.1, 24.0 (2C), 23.9 (2C). HPLC-MS purity: 97%, t_R = 4.19 min. Anal. C₂₆H₃₁ClN₄O₂S (C, H, N, S).

4.5. Biological studies

4.5.1. Cholinesterase inhibitory activities

Acetylcholinesterase (AChE, EC 3.1.1.7) from bovine erythrocytes (0.25–1.0 unit/mg, lyophilized powder) and butyrylcholinesterase (BuChE, EC 3.1.1.8) from equine serum (10 units/mg protein, lyophilized powder) were purchased from Sigma. Compounds were measured in 100 mM phosphate buffer pH 8.0 at 30 °C, using acetylthiocholine and butyrylthiocholine (0.4 mM) as substrates, respectively. In both cases, 5,5'-dithio-bis(2-nitrobenzoic)acid (DTNB, Ellman's reagent, 0.2 mM) was used and the values of IC₅₀ were calculated by UV spectroscopy, from the absorbance changes at 412 nm [29]. Experiments were performed in triplicate.

4.5.2. In vitro blood–brain barrier permeation assay

Prediction of the brain penetration was evaluated using a parallel artificial membrane permeation assay (PAMPA-BBB), in a similar manner as previously described [11,13,14,16,31]. Pipetting was performed with a semi-automatic robot (CyBi®-SELMA) and UV reading with a microplate spectrophotometer (Multiskan Spectrum, Thermo Electron Co.). Commercial drugs, phosphate

buffered saline solution at pH 7.4 (PBS), and dodecane were purchased from Sigma, Aldrich, Acros, and Fluka. Millex filter units (PVDF membrane, diameter 25 mm, pore size 0.45 μ m) were acquired from Millipore. The porcine brain lipid (PBL) was obtained from Avanti Polar Lipids. The donor microplate was a 96-well filter plate (PVDF membrane, pore size 0.45 μ m) and the acceptor microplate was an indented 96-well plate, both from Millipore. The acceptor 96-well microplate was filled with 200 μ L of PBS:ethanol (70:30) and the filter surface of the donor microplate was impregnated with 4 μ L of PBL in dodecane (20 mg mL⁻¹). Compounds were dissolved in PBS: ethanol (70:30) at 100 μ g mL⁻¹, filtered through a Millex filter, and then added to the donor wells (200 μ L). The donor filter plate was carefully put on the acceptor plate to form a sandwich, which was left undisturbed for 240 min at 25 °C. After incubation, the donor plate is carefully removed and the concentration of compounds in the acceptor wells was determined by UV–Vis spectroscopy. Every sample is analyzed at five wavelengths, in four wells and at least in three independent runs, and the results are given as the mean \pm standard deviation. In each experiment, 10 quality control standards of known BBB permeability were included to validate the analysis set.

4.5.3. Culture of the human neuroblastoma cell line SH-SY5Y

SH-SY5Y cells, at passages between 3 and 16 after de-freezing, were maintained in a Dulbecco's modified Eagle's medium (DMEM) containing 15 non-essential amino-acids (NEAAs) and supplemented with 10% fetal bovine serum (FBS), 1 mM glutamine, 50 units/mL penicillin and 50 μ g/mL streptomycin (reagents from GIBCO, Madrid, Spain). Cultures were seeded into flasks containing supplemented medium and maintained at 37 °C in 5% CO₂/humidified air. Stock cultures were passaged 1:4 twice weekly. For cell viability/cell death experiments, SH-SY5Y cells were sub-cultured in 48 well plates at a seeding density of 10⁵ cells per well and were exposed to the compounds before confluence, in DMEM free of serum.

4.5.4. Cell viability experiments

To study the cytotoxic effects of compounds alone, cells were plated at a density of 10⁵ cells per well at least 48 h before the toxicity measurements. Cells were exposed for 24 h to the compound at 3 μ M, and the quantitative assessment of cell death was made by measurement of the percent of the intracellular enzyme lactate dehydrogenase (LDH) released to the extracellular medium (cytotoxicity detection kit, Roche). The quantity of LDH was evaluated in a microplate reader (Anthos 2010 or Labsystems iMES Reader MS) at 492 nm (λ excitation) and 620 nm (λ emission).

4.5.5. Neuroprotection studies

To study the cytoprotective action of the compounds against cell death induced by the mixture of rotenone (30 μ M) and oligomycin A (10 μ M) in the experiments of pre-incubation, drugs were given at time zero and maintained for 24 h. Then, the media were replaced by fresh media still containing the drug plus the cytotoxic insult, which was left for an additional 24 h period. Thereafter, cell survival was assessed measuring LDH activity. For the experiments of co-incubation the compounds and the combination rotenone plus oligomycin A were added at the same time and incubated for 24 h.

4.5.6. Evaluation of selected compounds as free-radical scavengers in SH-SY5Y cells

Dihydrodichlorofluorescein diacetate (DCFH-DA) was used to assess intracellular ROS [37]. SH-SY5Y neuroblastoma cells were grown at confluence in 96-well black dishes. Cells were incubated with 10 μ M DCFH-DA for 45 min and basal fluorescence was

measured in a fluorescence microplate reader (FLUOstar Optima, BMG, Germany). Then, a mixture of rotenone (30 μM), oligomycin A (10 μM), and the tested compound (0.3 μM) were added at time zero ($t = 0$). After 120 min, changes in fluorescence were measured, using 485 nm and 520 nm as wavelengths of excitation and emission, respectively.

4.5.7. Measurement of cytosolic calcium concentration

SHSY5Y neuroblastoma cells were grown at confluence in 96-well black dishes. Cells were loaded with Fluo-4/AM (4 μM) for 1 h at 37 °C in Eagle's minimal essential medium. Then, cells were washed twice with Krebs Hepes solution and kept at room temperature for 15 min before the beginning of the experiment. Compounds were incubated 10 min before K^+ (70 mM) was applied to evoke the increment of cytosolic $[\text{Ca}^{2+}]$. At the end of the experiment, Triton X-100 (5%) and MnCl_2 (1 mM) were applied to record maximal and basal fluorescence, respectively. Fluorescence was measured in a fluorescence microplate reader (FLUOstar Optima, BMG, Germany). Wavelengths of excitation and emission were 485 and 520 nm, respectively.

4.5.8. Measurement of LDH activity

Extracellular and intracellular LDH activity was measured by UV–vis using a cytotoxicity cell death kit (Roche-Boehringer, Mannheim, Germany) according to the manufacturer's indications. Total LDH activity was defined as the sum of intracellular and extracellular LDH activity and released LDH was defined as the percentage of extracellular compared with total LDH activity. Data were expressed as the mean ($\pm\text{SEM}$) of at least three different cultures in quadruplicate. LDH released was calculated for each individual experiment considering 100% the extracellular LDH released by the vehicle with respect to the total. To determinate percent protection, LDH release was normalized as follows: in each individual triplicate experiment, LDH release obtained in non-treated cells (basal) was subtracted from the LDH released upon the toxic treatment and normalized to 100% and that value was subtracted from 100.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at <http://dx.doi.org/10.1016/j.ejmech.2014.04.075>. These data include MOL files and InChIKeys of the most important compounds described in this article.

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