European Journal of Medicinal Chemistry 46 (2011) 2224–2235

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

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

Original article

# *N*-Acylaminophenothiazines: Neuroprotective agents displaying multifunctional activities for a potential treatment of Alzheimer's disease

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ARTICLE INFO

*Article history:*
Received 15 December 2010
Received in revised form
28 February 2011
Accepted 2 March 2011
Available online 10 March 2011

*Keywords:*
*N*-Acylaminophenothiazines
Butyrylcholinesterase inhibition
Neuroprotection
Oxidative stress
Okadaic acid
Beta-amyloid peptide
Calcium modulation
Alzheimer's disease

ABSTRACT

We have previously reported the multifunctional profile of *N*-(3-chloro-10*H*-phenothiazin-10-yl)-3-(dimethylamino)propanamide (**1**) as an effective neuroprotectant and selective butyrylcholinesterase inhibitor. In this paper, we have developed a series of *N*-acylaminophenothiazines obtained from our compound library or newly synthesised. At micro- and sub-micromolar concentrations, these compounds selectively inhibited butyrylcholinesterase (BuChE), protected neurons against damage caused by both exogenous and mitochondrial free radicals, showed low toxicity, and could penetrate into the CNS. In addition, *N*-(3-chloro-10*H*-phenothiazin-10-yl)-2-(pyrrolidin-1-yl)acetamide (**11**) modulated the cytosolic calcium concentration and protected human neuroblastoma cells against several toxics, such as calcium overload induced by an L-type $Ca^{2+}$-channel agonist, tau-hyperphosphorylation induced by okadaic acid and Aβ peptide.



## 1. Introduction

Neuroprotection is a valuable tool of modern medicine to potentially combat or slow down the progression of neurodegenerative conditions such as Alzheimer's disease (AD) [1]. AD is a dreadful neurological illness and the most frequent of the primary degenerative dementias. The slow but progressive impairment of the physical and neurological conditions of AD patients produces devastating effects on themselves and their caregivers and a high economic burden for the families and/or the corresponding Public Health System. At present, AD affects about 17 million people worldwide and, considering the increase and ageing of the population, this figure is estimated to increase up to about 70 million people in 2050 if the lack of an efficient treatment persists [2].

While AD symptoms are clinically well characterized, the aetiology of the illness is rather complex and many points remain unknown. Brains of AD patients show several processes which have changed their physiological function into an aberrant pathological route, including toxic polymerized forms of beta-amyloid peptide (Aβ), hyperphosphorylated tau protein, oxidative stress, synaptic failures, and a marked atrophy of the cerebral cortex with loss of cortical and subcortical neurons [3]. Increasing the complexness of AD, it has also been related with other non-neurological pathologies such as dysfunctions of lipid metabolism [4], diabetes [5] and even thyroid disorders [6].

All these processes are related among them, but the knowledge of those relationships remains still unclear in many points [7]. Even

*Abbreviations:* AChE, acetylcholinesterase; AD, Alzheimer's disease; Aβ, beta-amyloid peptide; BBB, blood-brain barrier; BuChE, butyrylcholinesterase; CNS, central nervous system; PAMPA-BBB, parallel artificial membrane permeation assay for the blood-brain barrier permeation; LDH, lactate dehydrogenase; ROS, reactive oxygen species; VDCCs, voltage-dependent calcium channels.

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doi:10.1016/j.ejmech.2011.03.003

more significant, is the fact that ageing is assumed to be the main risk factor, but the initial cause or combination of causes that triggers the cascade of related pathological processes is not yet identified. Anyway, data from human studies as well as from animal models strongly support the concept that oxidative imbalance and subsequent oxidative stress are among the earliest events in the pathogenesis of AD [8]. Oxidative stress increases with age and numerous studies point to free radicals and mitochondrial dysfunction as triggers of neurodegenerative diseases like AD [9]. Aβ and redox-active metals such as $Fe^{2+}$ and $Cu^{+}$ have been proposed to be sources of oxidative stress in AD [10]. New techniques of resonance spectroscopy have shown that the formation of hydrogen peroxide is closely related to the early stages of Aβ aggregation [11]. Furthermore, Aβ impairs the function of ion-motive ATPases, glucose and glutamate transporters, and also GTP-binding proteins [12]. This leads to disturbance of calcium homeostasis and energy metabolism, causing cell death [13].

The neuropsychiatric symptoms associated with AD are mainly related to an increasing deficit of acetylcholine which leads to a progressive collapse of cholinergic neurotransmission [14]. Consequently, most of the commercialized treatments of AD are one-target drugs, in particular inhibitors of acetylcholinesterase (AChE), which produce a temporal relief of the symptoms [15]. AChE predominates in healthy brains while butyrylcholinesterase (BuChE) is considered to play a secondary role. But in AD brains, the activity of AChE decreases while that of BuChE gradually rises [16–18]. Therefore, BuChE appears as an increasingly important therapeutic target to improve cholinergic neurotransmission, as discussed in the literature in a number of publications [19,20], including phenothiazine derivatives [21,22].

A classical approach in Medicinal Chemistry is "one molecule, one activity". However, this axiom may be inadequate in complex diseases, such as AD. For this reason, the search of candidates designed to act on multiple targets combining different properties like modulation of acetylcholine levels, neuroprotection, anti-apoptotic activity or regulation of calcium homeostasis, emerges as a new strategy for the development of new drugs for neurodegenerative disease. Probably, these compounds acting simultaneously on multiple targets will deliver greater efficacy against multifunctional diseases, such as AD, compared to single activity compounds [23]. This approach has been explored in our group and we have recently reported different multifunctional compounds with interesting neuroprotective and cholinergic properties [24–28], including a tacrine–melatonin hybrid that reduces amyloid burden and behavioural deficits in a mouse model of AD [29]. Alternatively, a fruitful strategy to discover potential multifunctional drugs is the biological evaluation of in-house libraries of compounds towards different targets [30,31].

Following this later approach, we recently described that *N*-(3-chloro-10*H*-phenothiazin-10-yl)-3-(dimethylamino)propanamide (**1**) presents an interesting multifunctional profile [32]. This compound efficiently protects the human neuroblastoma cell line SH-SY5Y from damage caused by both exogenous and mitochondrial reactive oxygen species (ROS) and also inhibits BuChE in the sub-micromolar range. In addition, it could enter into the CNS, according to an *in vitro* assay. This *N*-acylaminophenothiazine and other related molecules were synthesised and described by chemists in our Medicinal Chemistry Institute in the past [33,34], but they were never biologically tested until the beginning of our research program.

Due to its good biological profile, compound **1** was selected as a hit compound to develop a new neuroprotective family (Fig. 1). Now, we describe a series of *N*-acylaminophenothiazines that display interesting neuroprotective properties against oxidative stress, calcium overload, tau-hyperphosphorylation, and Aβ toxicity. In addition, they showed interesting cholinergic and calcium modulatory properties. Some of the compounds here studied were previously stored in our library and some others have been newly synthesised.

## 2. Results and discussion

Initially, eleven *N*-acylaminophenothiazines **2**–**12** were selected from our library of compounds. The heterocyclic system was either unsubstituted or contained a chlorine atom at different positions. In addition to molecules with a dimethylamine side chain (**1**–**4**), other compounds with related tertiary amines, such as piperidine and pyrrolidine, were evaluated (**5**–**11**). In contrast to **1** that included two methylenes between the carbonyl and the tertiary amine, compounds **2**–**11** showed only one methylene. For comparative purposes, we also chose the acetamide **12**, lacking the final tertiary amine (Fig. 1).

Purity of *N*-acylaminophenothiazines **2**–**12** was checked by HPLC analysis. Compounds with a grade of purity higher than 98% were biologically tested without further purifications. Otherwise, they were subjected to a flash column chromatography on silica gel and then evaluated. Purity and chemical structures were confirmed by combustion analysis, MS, $^{1}H$ and $^{13}C$ NMR (see Experimental Section).

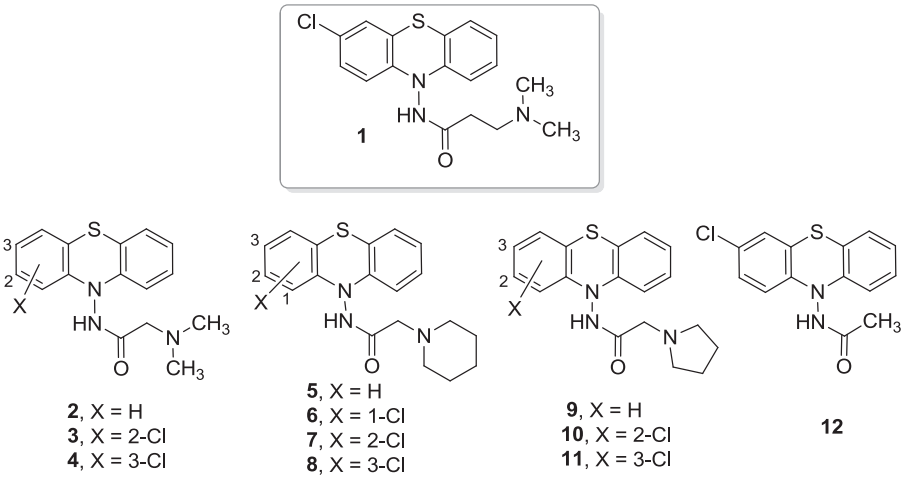



**Fig. 1.** *N*-Acylaminophenothiazines selected from our library to be biologically tested.

**Table 1**
Inhibition of equine BuChE[a] by the *N*-acylaminophenothiazines **1**–**12** from our chemical library.

| Compound | $IC_{50}$ (μM) | Compound | $IC_{50}$ (μM) |
|---|---|---|---|
| **1**[b] | 0.7 ± 0.02 | **7** | >100 |
| **2** | 1.7 ± 0.1 | **8** | 0.7 ± 0.01 |
| **3** | 7.1 ± 0.2 | **9** | 0.8 ± 0.01 |
| **4** | 5.0 ± 0.1 | **10** | 4.3 ± 0.1 |
| **5** | 0.4 ± 0.02 | **11** | 1.0 ± 0.02 |
| **6** | 2.2 ± 0.1 | **12** | >100 |

[a] BuChE (EC 3.1.1.8) from horse serum; $IC_{50}$ (tacrine) = 0.01 ± 0.0004 μM. AChE (EC 3.1.1.7) from bovine erythrocytes; $IC_{50}$ (tacrine) = 0.04 ± 0.0002 μM; compounds **1**–**12** showed no inhibition of AChE with $IC_{50}$ > 100 μM (data not shown). Results are the mean of three independent experiments ± SEM.
[b] Data from Ref. [32].

**Table 2**
Inhibition of equine BuChE[a] by new *N*-acylaminophenothiazines **13**–**19**.

| Compound | $IC_{50}$ (μM) |
|---|---|
| **13** | 1.4 ± 0.01 |
| **14** | 3.0 ± 0.1 |
| **15** | 0.8 ± 0.01 |
| **16** | 1.3 ± 0.05 |
| **17** | 0.9 ± 0.01 |
| **18** | 2.0 ± 0.01 |
| **19** | 1.5 ± 0.02 |

[a] BuChE (EC 3.1.1.8) from horse serum. $IC_{50}$ (tacrine) = 0.01 ± 0.0004 μM. AChE (EC 3.1.1.7) from bovine erythrocytes. $IC_{50}$ (tacrine) = 0.04 ± 0.0002 μM. Compounds **13**–**19** showed no inhibition of AChE with $IC_{50}$ > 100 μM (data not shown). Results are the mean of three independent experiments ± SEM.

Inhibition of AChE and BuChE was determined by following the Ellman method, using tacrine as reference [35]. Because of their lower cost and their high degree of sequence identity to the human enzymes, proteins of animal origin were used: AChE from bovine erythrocytes and BuChE from horse serum [36]. None of the compounds showed significant inhibition of AChE ($IC_{50}$ > 100 μM) (data not shown). However, they inhibited BuChE in the micro and sub-micromolar range, showing thus a marked selectivity towards this enzyme (Table 1).

Comparing compounds with the same lateral chain, the best results were obtained with unsubstituted or 3-chlorophenothiazine derivatives. Regarding the lateral chain, phenothiazine **1** ($IC_{50}$ = 0.7 μM) with two methylenes between the carbonyl group and the tertiary amine inhibited BuChE better than its analogous compound **4** which has only one methylene ($IC_{50}$ = 5.0 μM). Finally, the presence of a tertiary amine in the lateral chain seems to be necessary to inhibit BuChE, since the acetamide **12** was inactive.

On the basis of the above results, new *N*-acylaminophenothiazine derivatives bearing two methylenes in the lateral chain, fragments of pyrrolidine and 4-methylpiperazine, and different electron-donating or electron-withdrawing substituents at position 3 of the phenothiazine ring were designed and synthesised (**13**–**19**) (Scheme 1). The reaction of 2-aminobenzenethiol and 4-substituted 1-chloro-2-nitrobenzenes in the presence of potassium hydroxide in ethanol during 5 h at reflux, gave the corresponding 2-[(4-substituted-2-nitrophenyl)thio]aniline. These aromatic amines were subjected first to diazotisation with sodium nitrite and hydrochloric acid and then to reduction with stannous chloride, giving {2-[(2-nitrophenyl)thio] phenyl}hydrazines (**20**–**23**) [37]. Among them, compounds **22** and **23** have not been described before.

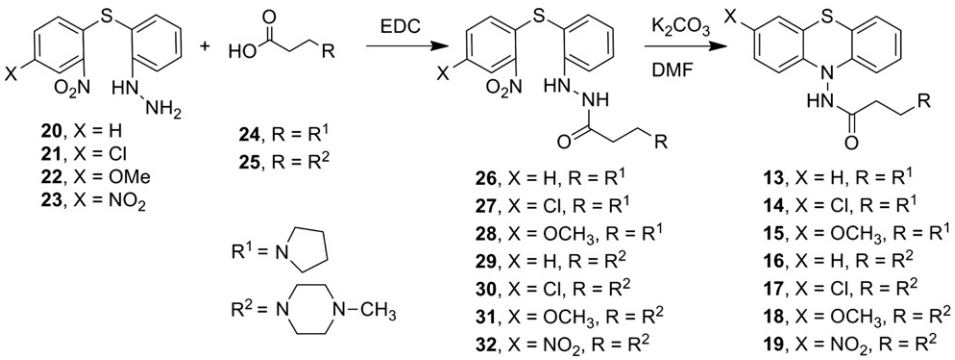
Moreover, 3-(pyrrolidin-1-yl)propanoic acid (**24**) [38] and 3-(4-methylpiperazin-1-yl)propanoic acid (**25**) [39] were obtained by reacting ethyl 3-chloropropanoate with the corresponding amine (pyrrolidine or 1-methylpiperazine), then followed by alkaline hydrolysis of the ester group. These acids were activated with EDC ([1-ethyl-3-(3-dimethylaminopropyl) carbodiimide]) and coupled with hydrazines **20**–**23** in anhydrous tetrahydrofuran solutions at room temperature to afford the corresponding *N*′-{2-[(2-nitrophenyl)thio]phenyl}-3-(pyrrolidin-1-yl)propanehydrazide **26**–**32**. Finally, solutions of these intermediates in DMF were refluxed with an equimolecular amount of $K_2CO_3$ for 15 min to obtain the desired *N*-(10*H*-phenothiazin-10-yl)-3-(pyrrolidin-1-yl)propanamides **13**–**19**, through a Smiles rearrangement [40].

All the new compounds here described (**13**–**19**, **22**, **23**, and **26**–**32**) showed analytical (HPLC and combustion analysis) and spectroscopic data (MS, $^1$H NMR, and $^{13}$C NMR) in full accordance with their structures.

The new *N*-acylaminophenothiazines **13**–**19** were evaluated as inhibitors of AChE and BuChE, showing a similar profile to derivatives **1**–**12**: a selective inhibition of BuChE with $IC_{50}$ in the micro- and sub-micromolar range (Table 2) and no inhibition of AChE with $IC_{50}$ > 100 μM (data not shown).

It is worth mentioning that the remarkable selectivity towards BuChE shown by the *N*-acylaminophenothiazines **1**–**19** could be of great importance in the development of new anti-AD therapies, since it has been described that selective BuChE inhibition increases brain acetylcholine, augments learning, and lowers beta-amyloid peptide in rodents [41]. In addition, a clinical test revealed that individuals that are deficient in BuChE show no serious physiological disadvantages, being slightly faster on simple reaction tasks, although slower on a visual perceptual matching test [42].

To explore whether these *N*-acylaminophenothiazine derivatives would be able to penetrate into the brain, we used a parallel artificial membrane permeation assay for blood-brain barrier (PAMPA-BBB).




**Scheme 1.** Synthesis of new *N*-acylaminophenothiazines **13**–**19**.

This simple and rapid model, described by Di et al. [43] and successfully applied by us to different compounds [24–27,44–48], has the advantage of predicting passive BBB permeation with high success. The *in vitro* permeabilities ($P_e$) of *N*-acylaminophenothiazines (**2**–**19**) and 15 commercial drugs through a lipid extract of porcine brain were determined using a mixture of PBS:EtOH (70:30). Assay validation was made by comparing the experimental permeability with the reported values of these commercial drugs that gave a good linear correlation, $P_e\,(\text{exptl}) = 1.24\,P_e\,(\text{bibl}) + 1.98$ ($R^2 = 0.93$). From this equation, and taking into account the limits established by Di et al. for BBB permeation [43], we found that molecules with a permeability superior to $7.0 \times 10^{-6}$ cm s$^{-1}$ would be able to cross the BBB by passive permeation. All tested *N*-acylaminophenothiazines showed permeability values over the above limit, pointing out that they would cross the BBB by passive diffusion (Table 3).

The neuroprotective capacity of *N*-acylaminophenothiazines against oxidative stress was assayed using the human neuroblastoma cell line SH-SY5Y and two toxicity models: (i) hydrogen peroxide for the generation of exogenous free radicals and (ii) the combination of rotenone plus oligomycin A for the induction of mitochondrial ROS, as a consequence of the blockade of complexes I and V of the mitochondrial electron transport chain [49]. In both models, cell viability was evaluated by measuring lactate dehydrogenase (LDH) release as a parameter of cell death.

In the first model, cells were incubated with compounds **2**–**19** at four concentrations (1, 3, 10, and 30 μM) 24 h before addition of the toxic. Thereafter, hydrogen peroxide (60 μM) was added to the cells and maintained for 24 h in the presence of the compounds. Then, cell death was evaluated by measuring the percentage of LDH released to the extracellular medium. Basal release of LDH was subtracted from the values obtained with the compounds in order to calculate the percentage of neuroprotection. Trolox, the vitamin E antioxidant moiety, was used as a positive control and the results are shown in Table 4.

Compounds **2**–**19** protected cells from the damage induced by $H_2O_2$ displaying significant percentages of neuroprotection that, in many cases, were around or exceeded the 50%. It is worth mentioning that compound **11**, derived from 3-chlorophenothiazine and (pyrrolidin-1-yl) acetamide, showed an interesting concentration–response relationship between 1 and 10 μM that could facilitate further neuroprotection studies.

The putative cytotoxic effects of **2**–**19** were studied by exposing the cells to the compounds at the highest concentration used in the above neuroprotection studies (30 μM) for 24 h [50]. With the exception of **2**, **7**, **14**, and **17** that increased cell death compared with the basal value, the rest of *N*-acylaminophenothiazines showed cell viabilities close to 100% (see Figs. S2 and S3 in Supplementary data). Thus, these compounds exhibited a wide therapeutic safety range.

**Table 3**
Permeability results from the PAMPA-BBB assay for *N*-acylaminophenothiazines **2**–**19** ($P_e$, $10^{-6}$ cm s$^{-1}$) with their predictive penetration into the CNS.

| Compound | $P_e$ ($10^{-6}$ cm s$^{-1}$)[a] | Prediction | Compound | $P_e$ ($10^{-6}$ cm s$^{-1}$)[a] | Prediction |
|---|---|---|---|---|---|
| **2** | 18.3 ± 0.6 | CNS+ | **11** | 14.9 ± 0.1 | CNS+ |
| **3** | 13.5 ± 0.2 | CNS+ | **12** | 9.8 ± 0.2 | CNS+ |
| **4** | 15.5 ± 0.1 | CNS+ | **13** | 17.3 ± 0.2 | CNS+ |
| **5** | 16.4 ± 0.5 | CNS+ | **14** | 16.4 ± 0.4 | CNS+ |
| **6** | 13.9 ± 0.2 | CNS+ | **15** | 8.7 ± 0.3 | CNS+ |
| **7** | 9.1 ± 0.3 | CNS+ | **16** | 17.0 ± 0.2 | CNS+ |
| **8** | 14.7 ± 0.2 | CNS+ | **17** | 13.8 ± 0.3 | CNS+ |
| **9** | 10.5 ± 0.3 | CNS+ | **18** | 16.8 ± 0.2 | CNS+ |
| **10** | 11.2 ± 0.3 | CNS+ | **19** | 14.6 ± 0.3 | CNS+ |

[a] PBS:EtOH (70:30) is used as solvent and data are the mean of three independent experiments ± SD.

**Table 4**
Neuroprotection (%) in the human neuroblastoma cell line SH-SY5Y against $H_2O_2$ (60 μM) at the indicated concentrations.[a]

| Compound | 1 μM | 3 μM | 10 μM | 30 μM |
|---|---|---|---|---|
| **2** | 30.5 | 26.3 | 38.6* | 22.0 |
| **3** | 15.6 | 29.5 | 42.6 | 23.2 |
| **4** | 59.8** | 62.7** | 28.2 | 9.7 |
| **5** | 27.2 | 26.1 | 9.7 | 10.1 |
| **6** | 1.1 | 9.9 | 0.0 | 32.6 |
| **7** | 66.1*** | 80.8*** | 73.8*** | 76.4*** |
| **8** | 25.4 | 55.2* | 44.9* | 17.8 |
| **9** | 13.1 | 35.6 | 18.9 | 23.3 |
| **10** | 17.4 | 48.9 | 54.8 | 55.6 |
| **11** | 54.6** | 57.7** | 79.9*** | 69.5*** |
| **12** | 40.3* | 52.2* | 37.2 | 21.2 |
| **13** | 90.1*** | 81.4*** | 88.0*** | 72.5*** |
| **14** | 34.4* | 57.2** | 29.4* | 49.0* |
| **15** | 17.8 | 11.8 | 0 | 0 |
| **16** | 82.9 | 34.5 | 22.8 | 6.3 |
| **17** | 43.9* | 56.6** | 68.6*** | 72.8*** |
| **18** | 49.5** | 42.1** | 66.5*** | 55.1*** |
| **19** | 41.3*** | 46.5*** | 46.2*** | 31.7* |
| Trolox | n.d. | n.d. | n.d. | 57.7 |

[a] Results are the mean of 4 independent experiments in triplicate. The statistical differences were calculated from the actual values of LDH release. Data on actual LDH values were normalized to calculate the % toxicity seen upon each treatment; differences between control values (in the absence of compounds) and those obtained in the presence of compounds gave the relative % protection afforded by each compound. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$.

*N*-Acylaminophenothiazines **11**, **13**, and **18**, which showed high and significant neuroprotective values towards $H_2O_2$ without putative toxic effects, were selected to be evaluated in further pharmacological assays. Firstly, they were tested against mitochondrial oxidative stress by using a combination of rotenone (30 μM) and oligomycin A (10 μM) as the toxic insult in neuroblastoma cells. Two different protocols were used: (i) *Preincubation*, wherein cells were incubated with the 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 toxics. This protocol has been used in our group in the last years to assess a possible cytoprotective effect due to activation of endogenous antioxidant pathways [51–53]. (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. Results are shown in Table 5.

All tested *N*-acylaminophenothiazines showed good levels of protection that in many cases were around or exceeded the 50%, both in pre- and in co-incubation conditions. These results suggested that **11**, **13**, and **18** probably act by a mixed mechanism: activation of endogenous antioxidant pathways and free radicals scavenging. In the case of phenothiazine **11** the main mechanism seems to be the expression of antioxidant proteins or the activation of intracellular signaling pathways involved in cell survival, elicited by the long-term preincubation of the cells with the compound [54]. However, in the case of phenothiazines **13** and **18** the free-radical scavenging activity seems to predominate since higher values of neuroprotection were achieved during short-term incubation conditions.

To assess the possible capture of mitochondrial radicals by the selected compounds, SH-SY5Y cells were loaded with the fluorescent dye 2′,7′-dichlorfluorescein-diacetate (DCFH-DA), treated with **11**, **13**, or **18** at 0.3 μM and then subjected to free-radical generation by a mixture of rotenone (30 μM) plus oligomycin A (10 μM). Trolox (0.3 μM) was also evaluated, as a positive control. All compounds tested decreased the DCFH-DA fluorescence, indicating that they could be acting, at least in part, by sequestering free radicals of mitochondrial origin.

**Table 5**
Neuroprotection (%) of **11**, **13**, **18**, and trolox 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]

| Compound | Preoccupation | | | | Co-incubation | | | |
|---|---|---|---|---|---|---|---|---|
| | 0.1 μM | 0.3 μM | 1 μM | 3 μM | 0.1 μM | 0.3 μM | 1 μM | 3 mM |
| **11** | n.d. | 55.0*** | 51.8*** | 58.9*** | n.d. | 28.6** | 44.1*** | 44.3** |
| **13** | 58.8* | 42.4 | 16.5 | n.d. | 66.1** | 63.7** | 52.6** | n.d. |
| **18** | 37.2* | 49.4* | 50.8* | n.d. | 66.3** | 57.9** | 65.4** | n.d. |
| Trolox | n.d. | n.d. | n.d. | 65.1*** | n.d. | n.d. | n.d. | 63.7*** |

[a] Results are the mean of 4 independent experiments in triplicate. The statistical differences were calculated from the actual values of LDH release. Data on actual LDH values were normalized to calculate the % toxicity seen upon each treatment; differences between control values (in the absence of compounds) and those obtained in the presence of compounds gave the relative % protection afforded by each compound. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; n.d.: not determined.

Compound **11** is a moderate free-radical scavenger, whereas *N*-acylaminophenothiazines **13** and **18** were able to capture around 24% of the generated radicals (Table 6). This value is very close to the percentage showed by trolox, a well-known antioxidant agent whose mechanism mainly involves the capture of free radicals. These results agree with the previous neuroprotection percentages against mitochondrial free radicals (see Table 5), where *N*-acylaminophenothiazine **11** exerted its maximal protection when pre-incubated before the toxics, whereas **13** and **18** were more potent when co-incubated with the stressors.

Calcium plays a fundamental role in learning and memory. Moreover, emerging findings suggest that disruptions in calcium homeostasis are implicated in neuronal degeneration that occurs in AD [55]. Thus, the ability of selected *N*-acylaminophenothiazines to modify the cytosolic calcium concentration was examined, using the human neuroblastoma line SH-SY5Y. Cells were incubated in the presence of compounds **11**, **13**, and **18** at 10 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. At 10 μM the L-type $Ca^{2+}$-channel antagonist nifedipine, which was used as positive control, caused 45% inhibition of $K^+$-evoked cytosolic [$Ca^{2+}$] increase. The blockade of calcium entry induced by compound **18**, bearing a 4-methylpiperazine moiety, was modest (19%). However, derivatives **11** and **13**, bearing a pyrrolidine ring, were able to modulate the cytosolic calcium by blocking the entry of this cation by 29 and 34%, respectively (Fig. 2).

On the basis of its previous biological results, *N*-acylaminophenothiazine **11** was selected as an illustrative compound of the series for further neuroprotective assays against calcium overload, tau-hyperphosphorylation, and Aβ peptide.

Since it is known that calcium channel blockade induces neuroprotection in several models of cell death [56,57], compound **11** was tested in SH-SY5Y neuroblastoma cells using calcium overload conditions. To increase cell damage caused by $Ca^{2+}$ entry through voltage-dependent calcium channels (VDCC), we used the L-type agonist FPL64176 under mild depolarizing conditions (20 mM $[K^+]_e$) and 5 mM $CaCl_2$ in the incubation medium for 24 h. Human neuroblastoma cells were incubated with compound **11** at concentrations of 0.3–10 μM for 24 h before incubation with FPL64176 and maintained for the 24-h period of toxic treatment. Compound **11** protected cells with a maximum of 45% at 1 μM (Table 7). Nimodipine, an L-type calcium channel blocker that was used as a positive control, produced 32% protection at 3 μM.

Okadaic acid is a toxin isolated from marine algae that induces tau-hyperphosphorylation and its subsequent aggregation into neurofibrillary tangles, both on *in vitro* and *in vivo* models [58,59]. Since pathological aggregation of tau protein is related to AD and other neurodegenerative tauopathies, it is accepted that okadaic acid-induced toxicity on human neuroblastoma cell line SH-SY5Y is a good *in vitro* model for the neuronal death linked to these diseases [60–62].

In this study, SH-SY5Y cells exposed for 24 h to okadaic acid (30 nM) in the absence of any compound, showed 61% cell death with respect to control cells (8%), measured as % of LDH release. Cells incubated with compound **11** at concentrations of 0.3, 1 and 3 μM for 24 h before addition of okadaic acid and maintained during the 24-h period of toxic exposure, significantly reduced cell death; maximum protection (32%) was achieved at 1 μM (Table 7). Galantamine, which was used as positive control [63], gave a protection of 36% at 0.3 μM.

The neuroprotective activity of compound **11** against $A\beta_{1-42}$-induced cytotoxicity in SH-SY5Y neuroblastoma cells was investigated, since this peptide is the most amyloidogenic isoform of Aβ [64]. Treatment of SH-SY5Y cells for 24 h with 30 μM $A\beta_{1-42}$ caused 42% reduction in cell viability measured as reduction of MTT. When

**Table 6**
Percentage of free-radical capture (%) by **11**, **13**, **18**, and trolox (0.3 μM) in the human neuroblastoma cell line SH-SY5Y, using the combination of rotenone (30 μM) and oligomycin A (10 μM) as stressor.[a]

| Compound | Free-radical capture (%) |
|---|---|
| **11** | 14.7 |
| **13** | 23.8* |
| **18** | 24.2* |
| Trolox | 27.8* |

[a] Results are the mean of 4 independent experiments in triplicate. *$p < 0.05$.

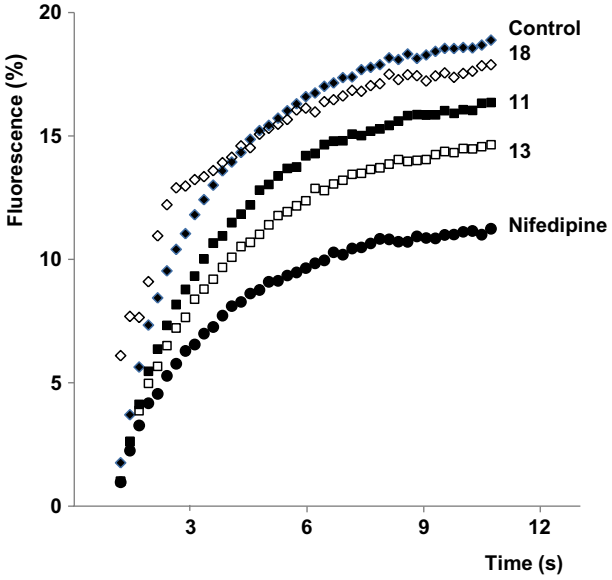



**Fig. 2.** Effects of compounds **11**, **13**, **18**, and nifedipine (10 μM) on the cytosolic [$Ca^{2+}$] increase elicited by $K^+$ (70 mM) in SH-SY5Y cells, loaded with Fluo-4/AM (4 μM). The curves are the mean of 5 independent experiments.

**Table 7**
Neuroprotection (%) in the human neuroblastoma cell line SH-SY5Y of compound **11** against the indicated toxic stressor.[a]

| Toxic stressor | 0.3 μM | 1 μM | 3 μM | 10 μM |
|---|---|---|---|---|
| $Ca^{2+}$ overload | 39.8 | 44.5* | 34.6 | 36.3 |
| Okadaic acid | 28.3** | 32.0** | 29.0* | n.d. |
| $A\beta_{1-42}$ | 91.3*** | 81.3*** | 13.6 | n.d. |

[a] Results are the mean of 4 independent experiments in triplicate. The statistical differences were calculated from the actual values of LDH release for okadaic acid or MTT reduction for $Ca^{2+}$ overload and $A\beta_{1-42}$. Data on actual LDH or MTT values were normalized to calculate the % toxicity seen upon each treatment; differences between control values (in the absence of compounds) and those obtained in the presence of compounds gave the relative % protection afforded by each compound. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$. n.d.: not determined.

cells were pre-incubated with increasing concentrations of compound **11** (0.3, 1, and 3 μM) for 24 h before and during exposure to $A\beta_{1-42}$, excellent neuroprotection values were observed that almost reached 100% viability. In fact, in the presence of *N*-acylaminophenothiazine **11** at 0.3 μM, 91% cell survival against Aβ-induced toxicity was observed (Table 7). Melatonin 10 nM used as a positive control produced 72% protection.

## 3. Conclusion

In this work, we have developed new *N*-acylaminophenothiazines that display an interesting *in vitro* multifunctional profile. They are selective inhibitors of BuChE with $IC_{50}$ values in the micro- and sub-micromolar range, and could cross the blood-brain barrier to reach their therapeutic targets in the CNS. In human neuroblastoma cells they show protective properties against damage caused by both exogenous and mitochondrial free radicals, through a mechanism that probably involves the activation of antioxidant pathways and/or the scavenging of free radicals. *N*-(3-Chloro-10*H*-phenothiazin-10-yl)-2-(pyrrolidin-1-yl)acetamide (**11**) shows an interesting $Ca^{2+}$-channel modulating activity, blocking the entry of this cation by 29% at 10 μM. This compound also protects human neuroblastoma cells against several toxic insults, such as calcium overload induced by an L-type agonist (45% at 1 μM), tau-hyperphosphorylation induced by okadaic acid (29% at 3 μM), and Aβ toxicity (91% at 0.3 μM).

Such interesting properties highlight these *N*-acylaminophenothiazines, covered by a PCT patent [65], as good candidates for further studies directed to the development of new drugs useful in the treatment of AD and other neurodegenerative diseases, like amyotrophic lateral sclerosis, Parkinson's disease or Huntington disease.

## 4. Experimental

Reagents were purchased from common commercial suppliers and were used without further purification. Solvents were purified and dried by standard procedures. Chromatographic separations were performed on silica gel (Kielgel 60 Merck of 230–400 mesh) and compounds were detected with UV light ($\lambda = 254$ nm). HPLC analyses were performed on Waters 6000 equipment, at a flow rate of 1.0 mL/min, with a UV detector ($\lambda = 214$–$274$ nm), and using a Delta Pak $C_{18}$ 5 μm, 300 Å column.

Melting points (uncorrected) were determined with a Reichert–Jung Thermovar apparatus. $^1$H NMR and $^{13}$C NMR spectra were recorded in $CD_3OD$ or $CDCl_3$ solutions using a Varian XL-400 spectrometer. Chemical shifts are reported in $\delta$ scale (ppm) relative to internal $Me_4Si$, *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 the results are within ±0.4% of the theoretical values.

### 4.1. Purification and spectroscopic data of compounds 2–12

Purity of *N*-acylaminophenothiazines **2**–**12**, which had been stored for a long time in our in-house compound library, was checked by HPLC analysis. Compounds with a purity superior to 98% were tested without further purifications. Otherwise, they were subjected to a flash column chromatography on silica gel prior to their evaluation in the biological tests. Purity was also checked by combustion analysis, and melting points were compared with those described, except for **6** and **12** that were not published before. Their chemical structures were confirmed by MS, $^1$H and $^{13}$C NMR, which are given in this work since only IR and low-resolution $^1$H NMR spectra were described in the previous papers.

#### 4.1.1. 2-(Dimethylamino)-N-(10H-phenothiazin-10-yl)acetamide (2)

Compound **2** was purified by flash column chromatography eluting with $CH_2Cl_2/CH_3OH$ (12:1). From the fractions of $R_f = 0.4$ a colourless solid of m.p. = 182–183 °C (bibl. 180–182 °C [34]) was isolated. ESI-MS *m/z* 300 $[MH]^+$, 322 $[M + Na]^+$, 621 $[2M + Na]^+$. $^1$H NMR ($CDCl_3$): $\delta = 9.17$ (s, 1H, NH), 7.08 (m, 4H, H2,4,6,8), 6.92 (dt, 2H, $J = 8.3$ Hz, $J = 1.1$ Hz, H3,7), 6.75 (dd, 2H, $J = 8.3$ Hz, $J = 1.1$ Hz, H1,9), 3.32 (s, 2H, H2′), 2.53 (s, 6H, $2CH_3$). $^{13}$C NMR ($CDCl_3$): $\delta = 169.2$ (CO), 143.0 (C9a,10a), 127.3 (C2,8), 127.1 (C4,6), 123.7 (C3,7), 120.5 (C4a,5a), 112.9 (C1,9), 62.6 (C2′), 46.7 ($2CH_3$). Purity: 100% (by HPLC). Anal. $C_{16}H_{17}N_3OS$ (C, H, N, S).

#### 4.1.2. N-(2-Chloro-10H-phenothiazin-10-yl)-2-(dimethylamino) acetamide (3)

Compound **3** was a colourless solid of m.p. 203–204 °C (bibl. 202–204 °C [34]), which was used without any purification. ESI-MS *m/z* 334 $[MH]^+$, 336 $[MH + 2]^+$, 356 $[M + Na]^+$, 689 $[2M + Na]^+$. $^1$H NMR ($CDCl_3$): $\delta = 9.23$ (s, 1H, NH), 7.01 (td, 1H, $J = 7.8$ Hz, $J = 1.3$ Hz, H8), 6.97 (dd, 1H, $J = 7.8$ Hz, $J = 1.3$ Hz, H6), 6.87 (d, 1H, $J = 8.2$ Hz, H4), 6.86 (td, 1H, $J = 7.8$ Hz, $J = 1.3$ Hz, H7), 6.81 (dd, 1H, $J = 8.2$ Hz, $J = 2.1$ Hz, H3), 6.70 (dd, 1H, $J = 7.8$ Hz, $J = 1.3$ Hz, H9), 6.67 (d, 1H, $J = 2.1$ Hz, H1), 3.25 (s, 2H, H2′), 2.44 (s, 6H, $2CH_3$). $^{13}$C NMR ($CDCl_3$): $\delta = 168.3$ (CO), 143.3 (C10a), 141.4 (C9a), 132.2 (C2), 126.7 (C4), 126.5 (C8), 126.1 (C6), 123.1 (C7), 122.4 (C3), 119.0 (C5a), 117.9 (C4a), 112.5 (C1), 112.2 (C9), 61.5 (C2′), 45.6 ($2CH_3$). Purity: 100% (by HPLC). Anal. $C_{16}H_{16}ClN_3OS$ (C, H, N, S).

#### 4.1.3. N-(3-Chloro-10H-phenothiazin-10-yl)-2-(dimethylamino) acetamide (4)

Compound **4** was a colourless solid of m.p. 182–183 °C (bibl. 180–182 °C [34]), which was used without any purification. ESI-MS *m/z* 334 $[MH]^+$, 336 $[MH + 2]^+$, 689 $[2M + Na]^+$. $^1$H NMR ($CDCl_3$): $\delta = 9.17$ (s, 1H, NH), 7.09 (td, 1H, $J = 7.7$ Hz, $J = 1.3$ Hz, H8), 7.07 (dd, 1H, $J = 7.7$ Hz, $J = 1.3$ Hz, H6), 7.03 (d, 1H, $J = 2.2$ Hz, H4), 7.00 (dd, 1H, $J = 8.9$ Hz $J = 2.2$ Hz, H2), 6.93 (td, 1H, $J = 7.7$ Hz, $J = 1.3$ Hz, H7), 6.73 (dd, 1H, $J = 7.7$ Hz, $J = 1.3$ Hz, H9), 6.63 (d, 1H, $J = 8.9$ Hz, H1), 3.29 (s, 2H, H2′), 2.49 (s, 6H, $2CH_3$). $^{13}$C NMR ($CDCl_3$): $\delta = 169.2$ (CO), 142.6 (C9a), 141.8 (C10a), 128.6 (C3), 127.6 (C8), 127.2 (C2), 126.9 (C6), 126.5 (C4), 123.9 (C7), 122.4 (C4a), 119.7 (C5a), 113.9 (C1), 113.1 (C9), 62.5 (C2′), 46.7 ($2CH_3$). Purity: 99% (by HPLC). Anal. $C_{16}H_{16}ClN_3OS$ (C, H, N, S).

#### 4.1.4. N-(10H-Phenothiazin-10-yl)-2-(piperidin-1-yl)acetamide (5)

Compound **5** was a colourless solid of m.p. 188–189 °C (bibl. 187–189 °C [34]), which was used without any purification. ESI-MS *m/z* 340 $[MH]^+$, 362 $[M + Na]^+$. 701 $[2M + Na]^+$. $^1$H NMR ($CDCl_3$): $\delta = 9.25$ (s, 1H, NH), 7.07 (m, 4H, H2,4,6,8), 6.90 (td, 2H, $J = 8.2$ Hz,

$J = 1.1$ Hz, H3,7), 6.71 (dd, 2H, $J = 8.2$ Hz, $J = 1.1$ Hz, H1,9), 3.42 (s, 2H, H2′), 2.83 (m, 4H, $2CH_2$), 1.76 (m, 4H, $2CH_2$), 1.61 (m, 2H, $CH_2$). $^{13}C$ NMR ($CDCl_3$): $\delta = 169.2$ (CO), 143.0 (C9a,10a), 127.2 (C2,8), 127.1 (C4,6), 123.7 (C3,7), 119.6 (C4a,5a), 112.9 (C1,9), 63.2 (C2′), 56.3 ($2CH_2$), 26.9 ($2CH_2$), 24.1 ($CH_2$). Purity: 98% (by HPLC). Anal. $C_{19}H_{21}N_3OS$ (C, H, N, S).

#### 4.1.5. N-(1-Chloro-10H-phenothiazin-10-yl)-2-(piperidin-1-yl) acetamide (**6**)

Compound **6** was purified by flash column chromatography eluting with hexane/ethyl acetate (1:2). From the fractions of $R_f = 0.2$ a colourless solid of m.p. = 174–175 °C was isolated [66]. ESI-MS $m/z$ 374 $[MH]^+$, 376 $[MH + 2]^+$, 396 $[M + Na]^+$, 769 $[2M + Na]^+$. $^1H$ NMR ($CDCl_3$): $\delta = 9.23$ (s, 1H, NH), 7.08 (m, 2H), 6.97 (m, 2H), 6.92 (dt, 1H, $J = 7.6$ Hz, $J = 1.1$ Hz, H7), 6.67 (d, 1H, $J = 8.3$ Hz), 6.59 (dd, 1H, $J = 6.6$ Hz, $J = 2.7$ Hz), 3.28 (s, 2H, H2′), 2.65 (m, 4H, $2CH_2$), 1.67 (m, 4H, $2CH_2$), 1.51 (m, 2H, $CH_2$). $^{13}C$ NMR ($CDCl_3$): $\delta = 169.4$ (CO), 143.9 (C10a), 142.2 (C9a), 131.2 (C1), 127.6, 127.3, 127.2, 123.9 (C7), 123.8, 120.5 (C4a), 119.3 (C5a), 112.9, 111.2, 61.8 (C2′), 55.7 (2C), 26.2 (2C), 23.5. Purity: 99% (by HPLC). Anal. $C_{19}H_{20}ClN_2OS$ (C, H, N, S).

#### 4.1.6. N-(2-Chloro-10H-phenothiazin-10-yl)-2-(piperidin-1-yl) acetamide (**7**)

Compound **7** was a colourless solid of m.p. 210–211 °C (bibl. 210–212 °C [34]), which was used without any purification. ESI-MS $m/z$ 374 $[MH]^+$, 376 $[MH + 2]^+$, 396 $[M + Na]^+$, 769 $[2M + Na]^+$. $^1H$ NMR ($CDCl_3$): $\delta = 9.22$ (s, 1H, NH), 7.09 (td, 1H, $J = 7.7$ Hz, $J = 1.2$ Hz, H8), 7.06 (dd, 1H, $J = 7.7$ Hz, $J = 1.2$ Hz, H6), 6.98 (d, 1H, $J = 8.2$ Hz, H4), 6.95 (td, 1H, $J = 7.7$ Hz, $J = 1.2$ Hz, H7), 6.90 (dd, 1H, $J = 8.2$ Hz, $J = 2.0$ Hz, H3), 6.73 (dd, 1H, $J = 7.7$ Hz, $J = 1.2$ Hz, H9), 6.71 (d, 1H, $J = 2.0$ Hz, H1), 3.31 (s, 2H, H2′), 2.68 (m, 4H, $2CH_2$), 1.69 (m, 4H, $2CH_2$), 1.53 (m, 2H, $CH_2$). $^{13}C$ NMR ($CDCl_3$): $\delta = 169.5$ (CO), 144.5 (C10a), 142.7 (C9a), 133.5 (C2), 127.9 (C4), 127.7 (C8) 127.4 (C6), 124.4 (C7), 123.7 (C3), 120.2 (C5a), 119.2 (C4a), 113.6 (C1), 113.3 (C9), 62.2 (C2′), 55.9 ($2CH_2$), 26.4 ($2CH_2$), 23.8 ($CH_2$). Purity: 99% (by HPLC). Anal. $C_{19}H_{20}ClN_3OS$ (C, H, N, S).

#### 4.1.7. N-(3-Chloro-10H-phenothiazin-10-yl)-2-(piperidin-1-yl) acetamide (**8**)

Compound **8** was a colourless solid of m.p. 196–197 °C (bibl. 194–196 °C [34]), which was used without any purification. ESI-MS $m/z$ 374 $[MH]^+$, 376 $[MH + 2]^+$, 396 $[M + Na]^+$, 769 $[2M + Na]^+$. $^1H$ NMR ($CDCl_3$): $\delta = 9.25$ (s, 1H, NH), 7.09 (td, 1H, $J = 7.9$ Hz, $J = 1.2$ Hz, H8), 7.06 (dd, 1H, $J = 7.9$ Hz, $J = 1.2$ Hz, H6), 7.04 (d, 1H, $J = 2.4$ Hz, H4), 7.01 (dd, 1H, $J = 8.6$ Hz, $J = 2.4$ Hz, H2), 6.93 (td, 1H, $J = 7.9$ Hz, $J = 1.2$ Hz, H7), 6.71 (dd, 1H, $J = 7.9$ Hz, $J = 1.2$ Hz, H9), 6.61 (d, 1H, $J = 8.6$ Hz, H1), 3.28 (s, 2H, H2′), 2.64 (m, 4H, $2CH_2$), 1.67 (m, 4H, $2CH_2$), 1.50 (m, 2H, $CH_2$). $^{13}C$ NMR ($CDCl_3$): $\delta = 169.5$ (CONH), 142.7 (C9a), 141.8 (C10a), 128.6 (C3), 127.6 (C8), 127.2 (C2), 127.0 (C6), 126.5 (C4), 123.9 (C7), 122.3 (C4a), 119.6 (C5a), 113.9 (C1), 113.0 (C9), 61.9 (C2′), 55.6 (2C), 26.1 (2C), 23.5. Purity: 100% (by HPLC). Anal. $C_{19}H_{20}ClN_3OS$ (C, H, N, S).

#### 4.1.8. N-(10H-Phenothiazin-10-yl)-2-(pyrrolidin-1-yl)acetamide (**9**)

Compound **9** was a colourless solid of m.p. 172–173 °C (bibl. 172–173 °C [34]), which was used without any purification. ESI-MS $m/z$ 326 $[MH]^+$, 348 $[M + Na]^+$, 673 $[2M + Na]^+$. $^1H$ NMR ($CDCl_3$): $\delta = 9.17$ (s, 1H, NH), 7.08 (m, 4H, H2,4,6,8), 6.92 (td, 2H, $J = 7.7$ Hz, $J = 1.1$ Hz, H3,7), 6.74 (dd, 2H, $J = 7.7$ Hz, $J = 1.1$ Hz, H1,9), 3.51 (s, 2H, H2′), 2.82 (m, 4H, $2CH_2$), 1.88 (m, 4H, $2CH_2$). $^{13}C$ NMR ($CDCl_3$): $\delta = 169.7$ (CO), 142.9 (C9a,10a), 127.3 (C2,8), 127.1 (C4,6), 123.7 (C3,7), 120.4 (C4a,5a), 113.1 (C1,9), 58.6 (C2′), 55.2 (2C), 24.2 (2C). Purity: 99% (by HPLC). Anal. $C_{18}H_{19}N_3OS$ (C, H, N, S).

#### 4.1.9. N-(2-Chloro-10H-phenothiazin-10-yl)-2-(pyrrolidin-1-yl) acetamide (**10**)

Compound **10** was a colourless solid of m.p. 204–205 °C (bibl. 204–205 °C [34]), which was used without any purification. ESI-MS $m/z$ 360 $[MH]^+$, 362 $[MH + 2]^+$, 382 $[M + Na]^+$, 741 $[2M + Na]^+$. $^1H$ NMR ($CDCl_3$): $\delta = 9.24$ (s, 1H, NH), 7.09 (td, 1H, $J = 7.8$ Hz, $J = 1.2$ Hz, H8), 7.05 (dd, 1H, $J = 7.8$, $J = 1.2$ Hz, H6), 6.95 (d, 1H, $J = 8.2$ Hz, H4), 6.93 (td, 1H, $J = 7.8$ Hz, $J = 1.2$ Hz, H7), 6.83 (dd, 1H, $J = 8.1$ Hz, $J = 2.0$ Hz, H3), 6.74 (dd, 1H, $J = 7.8$ Hz, $J = 1.2$ Hz, H9), 6.72 (d, 1H, $J = 2.0$ Hz, H1), 3.56 (s, 2H, H2′), 2.86 (m, 4H, $2CH_2$), 1,91 (m, 4H, $2CH_2$). $^{13}C$ NMR ($CDCl_3$): $\delta = 169.1$ (CO), 143.4 (C10a), 141.5 (C9a), 132.5 (C2), 127.3 (C4), 127.2 (C8) 126.9 (C6), 123.9 (C7), 123.4 (C3), 119.2 (C5a), 118.1 (C4a), 112.8 (C1), 112.5 (C9), 57.84 (C2′), 54.5 ($2CH_2$), 23.4 ($2CH_2$). Purity: 100% (by HPLC). Anal. $C_{18}H_{18}ClN_3OS$ (C, H, N, S).

#### 4.1.10. N-(3-Chloro-10H-phenothiazin-10-yl)-2-(pyrrolidin-1-yl) acetamide (**11**)

Compound **11** was purified by flash column chromatography using $CH_2Cl_2/CH_3OH$ (20:1) as eluent. From the fractions of $R_f = 0.2$ a colourless solid of m.p. = 180–181 °C (bibl. 179–180 °C [34]) was isolated. ESI-MS $m/z$ 360 $[MH]^+$, 362 $[MH + 2]^+$, 382 $[M + Na]^+$, 741 $[2M + Na]^+$. $^1H$ NMR ($CDCl_3$): $\delta = 9.23$ (s, 1H, NH), 7.09 (td, 1H, $J = 7.7$ Hz, $J = 1.3$ Hz, H8), 7.05 (dd, 1H, $J = 7.7$ Hz, $J = 1.3$ Hz, H6), 7.03 (d, 1H, $J = 2.4$ Hz $J = 2.3$ Hz, H4), 7.00 (dd, 1H, $J = 8.5$ Hz, $J = 2.4$ Hz, H4), 6.93 (td, 1H, $J = 7.7$ Hz, $J = 1.3$ Hz, H7), 6.72 (dd, 1H, $J = 7.7$ Hz, $J = 1.3$ Hz, H9), 6.62 (d, 1H, $J = 8.5$ Hz, H1), 3.51 (s, 2H, H2′), 2.80 (m, 4H), 1,88 (m, 4H). $^{13}C$ NMR ($CDCl_3$): $\delta = 169.7$ (CO), 142.5 (C9a), 141.6 (C10a), 128.5 (C3), 127.6 (C8), 127.1 (C4), 127.0 (C6), 126.4 (C2), 123.9 (C7), 122.2 (C4a), 119.5 (C5a), 114.0 (C1), 113.2 (C9), 58.5 (C2′), 55.2 (2C), 24.1 (2C). Purity: 100% (by HPLC). Anal. $C_{18}H_{18}ClN_3OS$ (C, H, N, S).

#### 4.1.11. N-(3-Chloro-10H-phenothiazin-10-yl)acetamide (**12**)

Compound **12** was a colourless solid of m.p. 222–223 °C (bibl. 223–224 °C [37]), which was used without any purification. ESI-MS $m/z$ 291 $[MH]^+$, 293 $[MH + 2]^+$. $^1H$ NMR ($CDCl_3$): $\delta = 10.5$ (s, 1H, NH), 7.13 (m, 4H), 6.93 (s, 1H), 6.80 (m, 2H), 2.11 (s, 3H, $CH_3$). $^{13}C$ NMR ($CDCl_3$): $\delta = 169.3$ (CO), 142.3, 141.8, 127.8, 127.1, 126.8, 126.5, 125.6, 123.5, 120.3, 117.2, 114.7, 113.5, 20.5. Purity: 100% (by HPLC). Anal. $C_{14}H_{11}ClN_2OS$ (C, H, N, S).

### 4.2. Synthesis of {2-[(2-nitrophenyl)thio]phenyl}hydrazines (**20**–**23**)

The corresponding 2-[(2-nitrophenyl)thio]aniline (4 mmol) was slowly added to aqueous HCl (5 mL) and the mixture was stirred at room temperature for 2 h. Then, a solution of sodium nitrite (4 mmol) in water (1.4 mL) was drop wise added, cooling with an ice bath (0–5 °C). After stirring for 1 h at this temperature, the mixture was refrigerated to −15 °C and a solution of stannous chloride dihydrate (8 mmol) in HCl (2 mL) was slowly added. The reaction mixture was stirred for 2 h at room temperature, and the solid was filtered off and treated with an excess of an aqueous solution of NaOH (20% w/w) and with water. The solid was purified on silica gel, using a flash-chromatography column and the eluents indicated in each case. Although intermediates **20** and **21** were previously described [37], their corresponding MS, $^1H$ NMR and $^{13}C$ NMR data are given here, since only IR and low-resolution $^1H$ NMR spectra were reported before.

#### 4.2.1. {2-[(2-Nitrophenyl)thio]phenyl}hydrazine (**20**)

Reagents were 2-[(2-nitrophenyl)thio]aniline (1 g, 4.06 mmol), $NaNO_2$ (0.31 g, 4.49 mmol), and $SnCl_2 \cdot 2H_2O$ (2.01 g, 8.91 mmol). Purification involved the use of hexane/ethyl acetate (8:1) as eluent. **20**: yellow solid (0.6 g, 57%) of m.p.: 139–141 °C (bibl. 138–139 °C

[37]). ESI-MS *m/z* 262 $[MH]^+$. $^1H$ NMR ($CDCl_3$): $\delta = 8.25$ (m, 1H), 7.45 (m, 3H), 7.24 (m, 2H), 6.84 (m, 2H), 6.19 (s, 1H, NH), 3.56 (s, 2H, $NH_2$). $^{13}C$ NMR ($CDCl_3$): $\delta = 152.8$, 147.1, 138.5, 138.1, 134.2, 132.9, 127.7, 126.3, 125.3, 119.5, 112.2, 111.5. Purity: 100% (by HPLC).

#### 4.2.2. {2-[(4-Chloro-2-nitrophenyl)thio]phenyl}hydrazine (**21**)

Reagents were 2-[(4-chloro-2-nitrophenyl)thio]aniline (7.0 g, 25 mmol), $NaNO_2$ (2.0 g, 29 mmol), and $SnCl_2 \cdot 2H_2O$ (12.4 g, 55 mmol). Purification involved the use of hexane/ethyl acetate (12:1) as eluent. **21**: yellow solid (2.02 g, 27%) of m.p.: 127–129 °C (bibl. 128–129 °C [37]). ESI-MS *m/z* 296 $[MH]^+$, 298 $[MH + 2]^+$. $^1H$ NMR ($CDCl_3$): $\delta = 8.24$ (d, 1H, $J = 2.4$ Hz), 7.48 (td, 1H, $J = 7.5$ Hz, $J = 1.2$ Hz), 7.43 (dd, 1H, $J = 7.5$ Hz, $J = 1.2$ Hz), 7.29 (dd, 1H, $J = 8.8$ Hz, $J = 2.4$ Hz), 7.25 (dd, 1H, $J = 7.5$ Hz, $J = 1.2$), 6.87 (td, 1H, $J = 7.5$ Hz, $J = 1.2$ Hz), 6.71 (d, 1H, $J = 8.8$ Hz). $^{13}C$ NMR ($CDCl_3$): $\delta = 152.0$, 145.4, 137.7, 135.7, 133.8, 132.8, 130.9, 128.6, 125.8, 119.4, 112.0, 110.6. Purity: 98 (by HPLC).

#### 4.2.3. {2-[(4-Methoxy-2-nitrophenyl)thio]phenyl}hydrazine (**22**)

Reagents were 2-((4-methoxy-2-nitrophenyl)thio)aniline (1.0 g, 3.6 mmol), $NaNO_2$ (0.3 g, 4.4 mmol), and $SnCl_2 \cdot 2H_2O$ (1.8 g, 8.0 mmol). Purification involved the use of hexane/ethyl acetate (8:1) as eluent. **22**: yellow solid (0.6 g, 57%) of m.p.: 115–117 °C. ESI-MS *m/z* 292 $[M + H]^+$. $^1H$ NMR ($CDCl_3$): $\delta = 7.75$ (d, 1H, $J = 2.9$), 7.46 (m, 2H), 7.23 (dd, 1H, $J = 7.6$ Hz, $J = 1.2$ Hz), 6.95 (dd, 1H, $J = 9.0$ Hz, $J = 2.9$ Hz), 6.85 (td, 1H, $J = 7.6$ Hz, $J = 1.2$ Hz) 6.68 (d, 1H, $J = 9.0$ Hz), 3.83 (s, 3H). $^{13}C$ NMR ($CDCl_3$): $\delta = 157.3$, 152.2, 145.7, 137.8, 132.3, 128.3, 128.0, 122.1, 119.2, 111.8, 111.7, 109.5, 55.9. Purity: 97% (by HPLC). Anal. $C_{13}H_{13}N_3O_3S$ (C, H, N, S).

#### 4.2.4. {2-[(2,4-Dinitrophenyl)thio]phenyl}hydrazine (**23**)

Reagents were 2-((2,4-dinitrophenyl)thio)aniline (1.0 g, 3.4 mmol), $NaNO_2$ (0.31 g, 4.5 mmol), and $SnCl_2 \cdot 2H_2O$ (2.01 g, 8.9 mmol). **23**: yellow solid (0.32 g, 30%) of m.p.: 133–137 °C. ESI-MS *m/z*307 $[MH]^+$. $^1H$ NMR ($CDCl_3$): $\delta = 9.07$ (d, 1H, $J = 2.3$), 8.12 (dd, 1H, $J = 9.0$ Hz, $J = 2.3$ Hz), 7.52 (t, 1H, $J = 7.4$ Hz), 7.44 (dd, 1H, $J = 7.4$ Hz, $J = 1.3$ Hz), 7.30–7.15 (m, 2H), 6.90–6.95 (m, 1H). $^{13}C$ NMR ($CDCl_3$): $\delta = 151.9$, 146.1, 145.1, 145.0, 137.8, 133.8, 129.0, 127.5, 125.7, 122.1, 120.6, 113.0, 110.2. Purity: 96% (by HPLC). Anal. $C_{12}H_{10}N_4O_4S$ (C, H, N, S).

### 4.3. General procedure for the synthesis of N′-{2-[(2-nitrophenyl) thio]phenyl}-3-(pyrrolidin-1-yl)propanehydrazides (**26**–**32**)

Under a nitrogen atmosphere, to a solution of 3-(pyrrolidin-1-yl) propanoic acid (**24**) or 3-(4-methylpiperazin-1-yl)propanoic acid (**25**) (10 mmol) and EDC ([1-ethyl-3-(3-dimethylaminopropyl) carbodiimide]) (10 mmol) in dry THF (10 mL), the corresponding {2-[(2-nitrophenyl)thio]phenyl}hydrazine (**20**–**23**) (10 mmol) dissolved in 10 mL of dry THF/acetonitrile (1:1) was added. The resulting mixture was stirred for 24 h at room temperature, and the solvent was removed under reduced pressure. The residue was dissolved in dichloromethane (10 mL), washed with a saturated aqueous solution of $NaHCO_3$ (3 × 10 mL), brine (3 × 10 mL), and $H_2O$ (3 × 10 mL), dried over $Na_2SO_4$, and evaporated to dryness. The resulting residue was purified on silica gel, using a flash-chromatography column and a mixture of $CH_2Cl_2$ and MeOH (9:1) as eluent.

#### 4.3.1. N′-{2-[(2-Nitrophenyl)thio]phenyl}-3-(pyrrolidin-1-yl) propanehydrazide (**26**)

Reagents were **20** (100 mg, 0.38 mmol), **24** (54.4 mg, 0.38 mmol), and EDC (59 mg, 0.38 mmol). Compound **26** was isolated as a yellow solid (66 mg, 45%) of m.p.: 145–146 °C. ESI-MS *m/z* 387 $[MH]^+$. $^1H$ NMR ($CDCl_3$): $\delta = 9.83$ (s,1H, NH), 8.20 (dd, 1H, $J = 8.2$ Hz, $J = 1.4$ Hz), 7.41 (dd, 1H, $J = 8.2$ Hz, $J = 1.4$ Hz), 7.32 (m, 2H), 7.18 (td, 1H, $J = 8.2$ Hz, $J = 1.4$ Hz), 6.85 (m, 3H), 6.81 (s,1H, NH), 2.84 (t, 2H, $J = 6.0$ Hz), 2.66 (m, 4H), 2.47 (t, 2H, $J = 6.0$ Hz), 1.81 (m, 4H). $^{13}C$ NMR ($CDCl_3$): $\delta = 171.7$ (CO), 150.2, 145.1, 137.9, 136.9, 133.8, 132.2, 127.8, 125.8, 125.2, 121.1, 113.1, 112.6, 53.6 (2C), 51.1, 32.5, 23.3 (2C). Purity: 100% (by HPLC). Anal. $C_{19}H_{22}N_4O_3S$ (C, H, N, S).

#### 4.3.2. N′-{2-[(4-Chloro-2-nitrophenyl)thio]phenyl}-3-(pyrrolidin-1-yl)propanehydrazide (**27**)

Reagents were **21** (200 mg, 0.67 mmol), **24** (96 mg, 6.76 mmol), and EDC (1.04 g, 0.67 mmol). Compound **27** was obtained as a yellow solid (220 mg, 78%) of m.p.: 147–148 °C. ESI-MS *m/z* 421 $[MH]^+$, 423 $[MH + 2]^+$. $^1H$ NMR ($CDCl_3$): $\delta = 10.18$ (s,1H, NH), 8.23 (d, 1H, $J = 2.3$ Hz), 7.46 (m, 2H), 7.35 (dd, 1H, $J = 8.6$ Hz, $J = 2.3$ Hz), 6.96 (m, 2H), 6.88 (d, 1H, $J = 8.6$ Hz), 6.75 (s,1H, NH), 2.93 (t, 2H, $J = 5.8$ Hz), 2.77 (m, 4H), 2.59 (t, 2H, $J = 5.8$ Hz), 1.88 (m, 4H). $^{13}C$ NMR ($CDCl_3$): $\delta = 171.8$ (CO), 150.6, 145.3, 137.8, 135.8, 133.9, 132.6, 131.0, 129.3, 125.7, 121.2, 112.8, 112.5, 53.6 (2C), 51.1, 32.6, 23.5 (2C). Purity: 99% (by HPLC). Anal. $C_{19}H_{21}ClN_4O_3S$ (C, H, N, S).

#### 4.3.3. N′-{2-[(4-Methoxy-2-nitrophenyl)thio]phenyl}-3-(pyrrolidin-1-yl)propanehydrazide (**28**)

Reagents were **22** (150 mg, 0.51 mmol), **24** (74 mg, 0.51 mmol), and EDC (79 mg, 0.51 mmol). Intermediate **28** was isolated as a yellow solid (150 mg, 71%) of m.p.: 148–149 °C. ESI-MS *m/z* 417 $[MH]^+$. $^1H$ NMR ($CDCl_3$): $\delta = 9.99$ (s,1H, NH), 7.74 (d, 1H, $J = 2.8$ Hz), 7.45 (dd, 1H, $J = 7.6$ Hz, $J = 1.5$ Hz), 7.39 (td, 1H, $J = 7.6$ Hz, $J = 1.5$ Hz), 7.02 (dd, 1H, $J = 9.0$ Hz, $J = 2.8$ Hz), 6.94–6.79 (m, 3H), 6.62 (s,1H, NH), 3.82 (s, 3H), 2.85 (t, 2H, $J = 6.1$ Hz), 2.63 (m, 4H), 2.43 (t, 2H, $J = 6.1$ Hz), 1.88 (m, 4H). $^{13}C$ NMR ($CDCl_3$): $\delta = 172.3$, 157.8, 150.9, 146.1, 138.9, 132.5, 129.5, 128.9, 122.4, 121.5, 114.3, 113.1, 109.9, 56.3, 53.9 (2C), 51.3, 33.3, 23.9 (2C). Purity: 100% (by HPLC). Anal. $C_{20}H_{24}N_4O_4S$ (C, H, N, S).

#### 4.3.4. 3-(4-Methylpiperazin-1-yl)-N′-{2-[(2-nitrophenyl)thio] phenyl}propanehydrazide (**29**)

Reagents were **20** (200 mg, 0.76 mmol), **25** (132 mg, 0.76 mmol), and EDC (119 mg, 0.76 mmol). Compound **29** was obtained as a yellow solid (112 mg, 35%) of m.p.: 66–67 °C. ESI-MS *m/z* 416 $[MH]^+$. $^1H$ NMR ($CDCl_3$): $\delta = 9.86$ (s,1H, NH), 8.24 (dd, 1H, $J = 8.3$, $J = 1.4$ Hz), 7.46 (dd, 1H, $J = 8.3$ Hz, $J = 1.4$ Hz), 7.40 (m, 2H), 7.22 (td, 1H, $J = 8.3$ Hz, $J = 1.4$ Hz), 7.01–6.91 (m, 3H), 6.75 (s,1H, NH), 2.67 (t, 2H, $J = 5.9$ Hz), 2.65–2.49 (m, 8H), 2.45 (t, 2H, $J = 5.9$ Hz), 2.30 (s, 3H). $^{13}C$ NMR ($CDCl_3$): $\delta = 171.9$, 150.5, 145.2, 138.0, 136.9, 133.8, 132.2, 128.0, 125.9, 125.2, 121.2, 113.3, 112.6, 54.9 (2C), 53.3, 52.4 (2C), 45.8, 31.1. Purity: 99% (by HPLC). Anal. $C_{20}H_{25}N_5O_3S$ (C, H, N, S).

#### 4.3.5. N′-{2-[(4-Chloro-2-nitrophenyl)thio]phenyl}-3-(4-methylpiperazin-1-yl)propanehydrazide (**30**)

Reagents were **21** (200 mg, 0.68 mmol), **25** (116 mg, 0.68 mmol), and EDC (105 mg, 0.68 mmol). Intermediate **30** was a yellow solid (120 mg, 39%) of m.p.: 68–69 °C. ESI-MS *m/z* 450 $[MH]^+$, 452 $[MH + 2]^+$. $^1H$ NMR ($CDCl_3$): $\delta = 9.99$ (s,1H, NH), 8.24 (d, 1H, $J = 2.3$ Hz), 7.44 (m, 2H), 7.36 (dd, 1H, $J = 8.8$ Hz, $J = 2.3$ Hz), 6.96 (m, 2H), 6.90 (d, 1H, $J = 8.8$ Hz), 6.72 (s,1H, NH), 2.69 (t, 2H, $J = 5.8$ Hz), 2.56 (m, 8H), 2.47 (t, 2H, $J = 5.8$ Hz), 2.31 (s, 3H). $^{13}C$ NMR ($CDCl_3$): $\delta = 172.2$, 150.7, 145.5, 138.3, 136.1, 134.3, 132.9, 131.5, 129.8, 126.1, 121.7, 113.2, 112.4, 55.4 (2C), 53.7, 52.9 (2C), 46.2, 31.5. Purity: 99% (by HPLC). Anal. $C_{20}H_{24}ClN_5O_3S$ (C, H, N, S).

#### 4.3.6. N′-{2-[(4-Methoxy-2-nitrophenyl)thio]phenyl}-3-(4-methylpiperazin-1-yl)propanehydrazide (**31**)

Reagents were **22** (150 mg, 0.51 mmol), **25** (89 mg, 0.51 mmol), and EDC (80 mg, 0.51 mmol). Compound **31** was isolated as a yellow solid (70 mg, 31%) of m.p.: 70–71 °C. ESI-MS *m/z* 446 $[MH]^+$, 468 $[M + Na]^+$. $^1H$ NMR ($CDCl_3$): $\delta = 9.85$ (s, 1H, NH), 7.74 (d,

1H, $J = 2.8$ Hz), 7.45 (dd, 1H, $J = 7.6$ Hz, $J = 1.5$ Hz), 7.39 (m, 1H), 7.02 (dd, 1H, $J = 9.0$ Hz, $J = 2.8$ Hz), 6.94–6.79 (m, 3H), 6.79 (s, 1H, NH), 3.82 (s, 3H), 2.68 (t, 2H, $J = 5.8$ Hz), 2.65–2.55 (m, 8H), 2.47 (t, 2H, $J = 5.8$ Hz), 2.31 (s, 3H). $^{13}$C NMR ($CDCl_3$): $\delta = 172.4$, 157.8, 150.9, 146.1, 138.9, 132.5, 129.6, 128.4, 122.4, 121.5, 114.4, 113.1, 109.9, 56.3, 55.4 (2C), 53.7, 52.9 (2C), 46.3, 31.6. Purity: 100% (by HPLC). Anal. $C_{21}H_{27}N_5O_4S$ (C, H, N, S).

#### 4.3.7. N′-{2-[(2,4-Dinitrophenyl)thio]phenyl}-3-(4-methylpiperazin-1-yl)propanehydrazide (**32**)

Reagents were **23** (125 mg, 0.41 mmol), **25** (70 mg, 0.41 mmol), and EDC (63 mg, 0.41 mmol). Intermediate **32** was obtained as a yellow solid (70 mg, 37% yield) of m.p.: 65–67 °C. ESI-MS $m/z$ 461 $[MH]^+$. $^1$H NMR ($CDCl_3$): $\delta = 9.99$ (s, 1H, NH), 9.06 (d, 1H, $J = 2.4$ Hz), 8.18 (dd, 1H, $J = 9.0$ Hz, $J = 2.4$ Hz), 7.46 (td, 1H, $J = 8.1$ Hz, $J = 1.4$ Hz), 7.43 (dd, 1H, $J = 8.1$ Hz, $J = 1.4$ Hz), 7.12 (d, 1H, $J = 9.0$ Hz), 6.95–6.99 (m, 2H), 6.69 (s, 1H, NH), 2.68 (t, 2H, $J = 5.8$ Hz), 2.61–2.51 (m, 8H), 2.45 (t, 2H, $J = 5.8$ Hz), 2.30 (s, 3H). $^{13}$C NMR ($CDCl_3$): $\delta = 172.4$, 150.8, 145.9, 144.9, 144.6, 137.8, 133.4, 129.6, 127.4, 121.8, 121.7, 113.3, 111.6, 55.2 (2C), 53.4, 52.6 (2C), 46.0, 31.3. Purity: 100% (by HPLC). Anal. $C_{20}H_{24}N_6O_5S$ (C, H, N, S).

### 4.4. Synthesis of new N-(10H-phenothiazin-10-yl)-3-(pyrrolidin-1-yl)propanamides (**13**–**19**)

A mixture of the corresponding hydrazide (0.2 mmol) (**26**–**32**) and potassium carbonate (28 mg, 0.2 mmol) in DMF (5 mL) was refluxed for 10–15 min. After cooling to room temperature, the solvent was removed under reduced pressure and the residue was purified on silica gel, using a flash-chromatography column and a mixture of $CH_2Cl_2$ and MeOH (9:1) as eluent.

#### 4.4.1. N-(10H-Phenothiazin-10-yl)-3-(pyrrolidin-1-yl)propanamide (**13**)

From **26** (54 mg, 0.14 mmol) and $K_2CO_3$ (19 mg, 0.14 mmol), the *N*-acylaminophenothiazine **13** was obtained (25 mg, 53% yield) as a yellow solid of m.p.: 195–196 °C. ESI-MS $m/z$ 340 $[MH]^+$, 362 $[M + Na]^+$, 701 $[2M + Na]^+$. $^1$H NMR ($CDCl_3$): $\delta = 10.26$ (s, 1H, NH), 7.03 (m, 4H, H2,4,6,8), 6.90 (t, 2H, $J = 7.6$ Hz, H3,7), 6.79 (d, 2H, $J = 7.6$ Hz, H1,9), 3.07 (t, 2H, $J = 5.7$ Hz, H3′), 2.76 (m, 6H, $2CH_2$ and H2′), 1.81 (m, 4H, $2CH_2$). $^{13}$C NMR ($CDCl_3$): $\delta = 169.7$ (CO), 142.2 (C9a,10a), 126.6 (C2,C8), 126.3 (C4,C6), 122.4 (C3,7), 119.6 (C4a,5a), 111.8 (C1,9), 52.9 ($2CH_2$), 50.2 (C3′), 32.0 (C2′), 22.4 ($2CH_2$). Purity: 100% (by HPLC). Anal. $C_{19}H_{21}N_3OS$ (C, H, N, S).

#### 4.4.2. N-(3-Chloro-10H-phenothiazin-10-yl)-3-(pyrrolidin-1-yl) propanamide (**14**)

From **27** (200 mg, 0.48 mmol) and $K_2CO_3$ (66 mg, 0.48 mmol), the *N*-acylaminophenothiazine **14** was obtained (90 mg, 51% yield) as a yellow solid of m.p.: 186–187 °C. ESI-MS $m/z$ 374 $[MH]^+$, 376 $[MH + 2]^+$. $^1$H NMR ($CDCl_3$): $\delta = 10.87$ (s, 1H, NH), 7.06 (m, 1H, H8), 7.03 (dd, 1H, $J = 7.9$ Hz, $J = 1.2$ Hz, H6), 7.01 (d, 1H, $J = 2.2$ Hz, H4), 6.96 (dd, 1H, $J = 2.3$, $J = 8.6$ Hz, H2), 6.90 (td, 1H, $J = 7.9$ Hz, $J = 1.2$ Hz, H7), 6.73 (dd, 1H, $J = 7.9$ Hz, $J = 1.2$ Hz, H9), 6.63 (d, 1H, $J = 8.6$ Hz, H1), 2.97 (t, 2H, $J = 5.9$ Hz, H3′), 2.73 (m, 6H, H2′ and $2CH_2$), 1.85 (m, 4H, $2CH_2$). $^{13}$C NMR ($CDCl_3$): $\delta = 171.4$ (CO), 143.0 (C9a), 142.1 (C10a), 128.5 (C3), 127.8 (C8), 127.3 (C4), 127.2 (C6), 126.6 (C2), 123.9 (C7), 121.8 (C4a), 119.2 (C5a), 113.9 (C1), 113.0 (C9), 53.7 (2C, $2CH_2$), 51.3 (C3′), 33.5 (C2′), 23.8 (2C, $2CH_2$). Purity: 98% (by HPLC). Anal. $C_{19}H_{20}ClN_3OS$ (C, H, N, S).

#### 4.4.3. N-(3-Methoxy-10H-phenothiazin-10-yl)-3-(pyrrolidin-1-yl) propanamide (**15**)

From **28** (100 mg, 0.24 mmol) and $K_2CO_3$ (33 mg, 0.24 mmol), the *N*-acylaminophenothiazine **15** was obtained (40 mg, 45% yield) as a yellow solid of m.p.: 179–181 °C. ESI-MS $m/z$ 370 $[MH]^+$. $^1$H NMR ($CDCl_3$): $\delta = 10.69$ (s, 1H, NH), 7.17 (m, 1H, H8), 7.06 (dd, 1H, $J = 7.2$ Hz, $J = 2.5$ Hz, H9), 6.89 (t, 1H, $J = 7.2$ Hz, H7), 6.82–6.72 (m, 2H, H1,6), 6.66 (d, 1H, $J = 2.6$ Hz, H4), 6.62 (dd, 1H, $J = 8.9$ Hz, $J = 2.6$ Hz, H2), 3.78 (s, 3H, $CH_3$), 3.21 (t, 2H, $J = 5.7$ Hz, H3′), 2.86 (m, 6H, H2′ and $2CH_2$), 1.63 (m, 4H, $2CH_2$). $^{13}$C NMR ($CDCl_3$): $\delta = 170.9$ (CO), 156.0 (C3), 143.6 (C9a), 136.8 (C10a), 127.3 (C8), 126.9 (C9), 123.1 (C7), 121.1 (C4a), 119.3 (C5a), 113.6 (C1), 112.8 (C6), 112.6 (C2,4), 55.6 (C, $CH_3$), 53.8 (2C, $2CH_2$), 51.1 (C3), 33.0 (C2′), 23.5 (2C, $2CH_2$). Purity: 97% (by HPLC). Anal. $C_{20}H_{23}N_3O_2S$ (C, H, N, S).

#### 4.4.4. 3-(4-Methylpiperazin-1-yl)-N-(10H-phenothiazin-10-yl) propanamide (**16**)

From **29** (60 mg, 0.14 mmol) and $K_2CO_3$ (20 mg, 0.14 mmol), compound **16** was obtained (16 mg, 30% yield) as a yellow solid of m.p.: 177–178 °C. ESI-MS $m/z$ 369 $[MH]^+$. $^1$H NMR ($CDCl_3$): $\delta = 10.48$ (s, 1H, NH), 7.06 (m, 4H, H2,4,6,8), 6.90 (td, 2H, $J = 7.9$ Hz, $J = 1.0$ Hz, H3,7), 6.75 (dd, 2H, $J = 7.9$ Hz, $J = 1.0$ Hz, H1,9), 2.86 (t, 2H, $J = 5.8$ Hz, H3′), 2.70 (t, 2H, $J = 5.8$ Hz, H2′), 2.64–2.29 (m, 8H, $4CH_2$), 2.28 (s, 3H, $CH_3$). $^{13}$C NMR ($CDCl_3$): $\delta = 170.8$ (CO), 143.1 (C9a,10a), 127.3 (C2,8), 127.1 (C4,6), 123.5 (C3,7), 119.8 (C4a,5a), 112.6 (C1,9), 54.7 ($2CH_2$), 53.4 (C3′), 52.4 ($2CH_2$), 45.7 ($CH_3$), 31.4 (C2′). Purity: 98% (by HPLC). Anal. $C_{20}H_{24}N_4OS$ (C, H, N, S).

#### 4.4.5. N-(3-Chloro-10H-phenothiazin-10-yl)-3-(4-methylpiperazin-1-yl)propanamide (**17**)

Using **30** (100 mg, 0.22 mmol) and $K_2CO_3$ (31 mg, 0.22 mmol) as reagents, the *N*-acylaminophenothiane **17** was obtained (46 mg, 51% yield), as a yellow solid of m.p.: 188–189 °C. ESI-MS $m/z$ 403 $[MH]^+$, 405 $[MH + 2]^+$, 425 $[M + Na]^+$. $^1$H NMR ($CDCl_3$): $\delta = 10.41$ (s, 1H, NH), 7.06 (m, 1H, H8), 7.03 (dd, 1H, $J = 7.7$ Hz, $J = 1.3$ Hz, H6), 7.01 (d, 1H, $J = 2.5$ Hz, H4), 6.96 (dd, 1H, $J = 8.8$ Hz, $J = 2.5$ Hz, H2), 6.91 (td, 1H, $J = 7.7$ Hz, $J = 1.3$ Hz, H7), 6.72 (dd, 1H, $J = 7.7$ Hz, $J = 1.3$ Hz, H9), 6.62 (d, 1H, $J = 8.8$ Hz, H1), 2.80 (t, 2H, $J = 5.9$ Hz, H3′), 2.64 (t, 2H, $J = 5.7$ Hz, H2′), 2.58–2.30 (m, 8H, $4CH_2$), 2.27 (s, 3H, $CH_3$). $^{13}$C NMR ($CDCl_3$): $\delta = 170.9$ (CO), 142.6 (C9a), 141.7 (C10a), 128.3 (C3), 127.5 (C8), 127.0 (C4), 126,9 (C6), 126.4 (C2), 123.5 (C7), 121.6 (C4a), 118.9 (C5a), 113.6 (C1), 112.8 (C9), 54.8 ($2CH_2$), 53.4 (C3′), 52.6 ($2CH_2$), 45.8 ($CH_3$), 31.4 (C2′). Purity: 100% (by HPLC). Anal. $C_{20}H_{23}ClN_4OS$ (C, H, N, S).

#### 4.4.6. N-(3-Methoxy-10H-phenothiazin-10-yl)-3-(4-methylpiperazin-1-yl)propanamide (**18**)

From **31** (70 mg, 0.16 mmol) and $K_2CO_3$ (22 mg, 0.16 mmol), the *N*-acylaminophenothiazine **18** was obtained (30 mg, 48% yield) as a yellow solid of m.p.: 202–203 °C. ESI-MS $m/z$ 399 $[MH]^+$. $^1$H NMR ($CDCl_3$): $\delta = 10.69$ (s, 1H, NH), 7.16 (m, 1H, H8), 7.06 (dd, 1H, $J = 7.2$ Hz, $J = 2.5$ Hz, H9). 6.91 (t, 1H, $J = 7.2$ Hz, H7), 6.78–6.70 (m, 2H, H1,6), 6.67 (d, 1H, $J = 2.5$ Hz, H4), 6.61 (dd, 1H, $J = 8.8$ Hz, $J = 2.5$ Hz, H2), 3.78 (s, 3H, $CH_3$), 2.85 (t, 2H, $J = 5.7$ Hz, H3′), 2.79–2.43 (m, 10H, H2′ and $4CH_2$), 2.37 (s, 3H, $CH_3$). $^{13}$C NMR ($CDCl_3$): $\delta = 171.3$ (CO), 156.5 (C3), 143.9 (C9a), 137.2 (C10a), 127.9 (C8), 127.4 (C9), 123.6 (C7), 120.6 (C4a), 119.9 (C5a), 113.9 (C1), 113.0 (C6), 112.9 (C2,4), 56.1 ($CH_3$), 55.2 ($2CH_2$), 53.9 (C3′), 52.4 ($2CH_2$), 46.1 ($CH_3$), 31.9 (C2′). Purity: 99% (by HPLC). Anal. $C_{21}H_{26}N_4O_2S$ (C, H, N, S).

#### 4.4.7. 3-(4-Methylpiperazin-1-yl)-N-(3-nitro-10H-phenothiazin-10-yl)propanamide (**19**)

From **32** (50 mg, 0.11 mmol) and $K_2CO_3$ (15 mg, 0.11 mmol), the *N*-acylaminophenothiazine **19** was obtained (30 mg, 67% yield) as a yellow solid of m.p.: 192–193 °C. ESI-MS $m/z$ 414 $[MH]^+$. $^1$H NMR ($CDCl_3$): $\delta = 10.87$ (s, 1H, NH), 7.85 (d, 1H, $J = 2.5$ Hz, H4), 7.82 (m, 1H, H2), 7.06 (td, 1H, $J = 7.5$ Hz, $J = 1.6$ Hz, H8), 6.97 (m, 2H, H6,7), 6.69 (m, 2H, H1, H9), 2.84 (t, 2H, $J = 5.7$ Hz, H3′), 2.68 (t, 2H, $J = 5,7$ Hz,

H2′), 2.60–2.32 (m, 8H, $4CH_2$), 2.29 (s, 3H, $CH_3$). $^{13}C$ NMR ($CDCl_3$): $\delta = 171.1$ (CO), 148.4 (C10a), 143.4 (C3), 140.9 (C9a), 128.1 (C8), 127.3 (C6), 125.2 (C7), 123.8 (C4), 122.4 (C2), 120.9 (C4a), 118.6 (C5a), 113.6 (C9), 112.3 (C1), 55.1($2CH_2$) 53.5(C3′), 52.8 ($2CH_2$), 46.0 ($CH_3$), 31.5 (C2′). Purity: 100% (by HPLC). Anal. $C_{20}H_{23}N_5O_3S$ (C, H, N, S).

### 4.5. Biochemical 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 $IC_{50}$ were calculated by UV spectroscopy, from the absorbance changes at 412 nm [35]. Experiments were performed in triplicate.

#### 4.5.2. In vitro blood-brain barrier permeation assay

Prediction of crossing the blood-brain barrier was evaluated using a parallel artificial membrane permeation assay (PAMPA), in a similar manner as previously described [43]. 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 μm) 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 μm) and the acceptor microplate was an indented 96-well plate, both from Millipore. The acceptor 96-well microplate was filled with 180 μL of PBS: ethanol (70:30) and the filter surface of the donor microplate was impregnated with 4 μL of PBL in dodecane (20 mg $mL^{-1}$). Compounds were dissolved in PBS: ethanol (70:30) at 1 mg $mL^{-1}$, filtered through a Millex filter, and then added to the donor wells (180 μL). The donor filter plate was carefully put on the acceptor plate to form a sandwich, which was left undisturbed for 4 h at 25 °C. After incubation, the donor plate is carefully removed and the concentration of compounds in the acceptor wells was determined by UV spectroscopy. Every sample is analysed at five wavelengths, in four wells and at least in three independent runs, and the results are given as the mean ± standard deviation. In each experiment, 15 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 cells

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 calf serum (FCS), 1 mM glutamine, 50 units/mL penicillin and 50 μg/mL streptomycin (reagents from GIBCO, Madrid, Spain). Cultures were seeded into flasks containing supplemented medium and maintained at 37 °C in 5% $CO_2$/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 $10^5$ cells per well and were exposed to the compounds before confluence, in DMEM free of serum.

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

Dihydrodichlorofluorescein diacetate (DCFH-DA) was used to assess intracellular ROS [67]. SH-SY5Y neuroblastoma cells were grown at confluence in 96-well black dishes. Cells were incubated with 10 μM 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) was 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.

#### 4.5.5. Measurement of cytosolic calcium concentration

SH-SY5Y 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 [$Ca^{2+}$]. 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.

#### 4.5.6. Neuroprotection against oxidative stress

To study the cytoprotective action of the compounds against cell death induced by 60 μM $H_2O_2$, or the mixture of 30 μM rotenone plus 10 μM oligomycin A, 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 stimulus that was left for an additional 24 h period. Thereafter, cell survival was assessed measuring LDH activity.

#### 4.5.7. Neuroprotection against calcium overload

To assess the neuroprotective effect of compound **11** against $Ca^{2+}$ overload, SH-SY5Y cells were incubated for 24 h to a medium with a mild depolarizing concentration of KCl (20 mM) containing 5 mM $CaCl_2$ and 0.3 μM FPL64176, an L-type $Ca^{2+}$-channel agonist, which induced $Ca^{2+}$ overload and the consequent cell death [68]. Compound **11** was administered 24 h before incubation of cells with 20 $K^+$/5$Ca^{2+}$/FPL and maintained for an additional 24-h period. At the end of the experiment, cell viability was assessed using the MTT method.

#### 4.5.8. Neuroprotection against okadaic acid-induced toxicity

To evaluate neuroprotection against okadaic acid-induced toxicity, the following protocol was used: SH-SY5Y cells were exposed for 24 h to 30 nM okadaic acid; compound **11** was added at the concentrations of 0.3, 1 or 3 μM, 24 h before cell incubation with the toxic stimuli and maintained during an additional 24-h period. At the end of the experiments, cell death was assessed by measuring the activity of lactate dehydrogenase (LDH) released.

#### 4.5.9. Neuroprotection against $A\beta_{1-42}$-induced toxicity

To assess the neuroprotective effect of compound **11** against Aβ-induced toxicity, SH-SY5Y cells were pre-incubated with the neuroprotector at the concentrations of 0.3, 1, and 3 μM for 24 h; then, they were co-incubated for another 24 h period with the compound in the presence of $A\beta_{1-42}$ 30 μM. At the end of the experiment, cell viability was assessed using the MTT method.

#### 4.5.10. Measurement of lactic dehydrogenase (LDH) activity

Extracellular and intracellular LDH activities were 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 activities and released LDH was defined as the percentage of extracellular compared to total LDH activity. Data

were expressed as the mean ± 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 determine % 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.

*4.5.11. Measurement of cell viability with MTT*

Cell viability, virtually the mitochondrial activity of living cells, was measured by quantitative colorimetric assay with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma–Aldrich, Madrid, Spain), as described previously [69]. MTT was added to all wells (final concentration 0.5 mg/mL) and allowed to incubate in the dark at 37 °C for 2 h. The tetrazolium ring of MTT can be cleaved by mitochondrial reductases in order to produce a precipitated formazan derivative. After this 2 h, the formazan produced was dissolved by adding 200 μL of DMSO, resulting in a coloured compound whose optical density was measured in an ELISA reader at 540 nm. All MTT assays were performed in triplicate. Absorbance values obtained in control cells untreated with the toxic was considered as 100% viability.

## Acknowledgments

The authors gratefully acknowledge the financial support of Spanish Ministry of Science and Innovation MICINN (projects SAF2006-01249, SAF2009-13015-C02-01, and SAF2009-12150), Community of Madrid (Programa de Actividades de I+D entre Grupos de Investigación en Biociencias, project S-SAL/0275/2006), and the Institute of Health Carlos III (Red RENEVAS, RETICS-RD06/0026). The fellowships to G.C.G.-M. and M.P.A. from CSIC and MICINN respectively, are also acknowledged. A.G.G. also would like to thank the continued support of Fundación Teófilo Hernando, Universidad Autónoma de Madrid, Spain.

## Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2011.03.003.

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