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<th>Journal:</th>
<th>ACS Chemical Neuroscience</th>
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<td>Manuscript ID</td>
<td>cn-2017-00424a</td>
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<tr>
<td>Manuscript Type:</td>
<td>Article</td>
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<td>Date Submitted by the Author:</td>
<td>05-Nov-2017</td>
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A focused library of psychotropic analogs with neuroprotective and neuroregenerative potential

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ABSTRACT

Overcoming the lack of effective treatments and the continuous clinical trial failures in neurodegenerative drug discovery might require a shift from the prevailing paradigm targeting pathogenesis to the one targeting simultaneously neuroprotection and neuroregeneration. In the studies reported herein, we sought to identify small molecules that might exert neuroprotective and neuroregenerative potential as tools against neurodegenerative diseases. In doing so, we started from the reported neuroprotective/neuroregenerative mechanisms of psychotropic drugs featuring a tricyclic alkylamine scaffold. Thus, we designed a focused-chemical library of 36 entries aimed at exploring the structural requirements for efficient neuroprotective/neuroregenerative cellular activity, without the manifestation of toxicity. To this aim, we developed a synthetic protocol which overcame the limited applicability of previously reported procedures. Next, we evaluated the synthesized compounds through a purposely developed phenotypic screening pipeline, based on primary neuronal systems. Phenothiazine 2Bc showed improved neuroregenerative and neuroprotective properties with respect to reference drug desipramine (2Aa). Importantly, we have also shown that 2Bc outperformed currently available drugs in cell models of Alzheimer's and Parkinson's diseases.

Keywords: neurodegenerative diseases, psychotropic agents, phenotypic screening, phenothiazines, neuroprotection, neural regeneration
**Introduction**

The progress in understanding processes and mechanisms involved in neurodegenerative diseases, such as Alzheimer’s disease (AD), has been impressive in the last decade. Despite that, no effective drug or drug candidate has been identified in the wake of that understanding.\(^1\) The current discouraging record of translation has been attributed to various factors, spanning from the lack of sufficient target engagement to the presence of serious toxic effects.\(^1\) Furthermore, the multifactorial etiology and complex pathophysiology of AD,\(^1\) together with the absence of diagnostic methods that allow detecting the disease earlier,\(^2\) has likely contributed to AD drug failures. Closely related to this is the paucity of validated drug targets, which has been exacerbated by the recent Phase III failures of investigational drugs designed to reduce amyloid-β (Aβ) plaque formation.\(^3\) On the other hand, the marketed drugs (three acetylcholinesterase inhibitors and one N-methyl-D-aspartate (NMDA) antagonist) were not generated from recent molecular insights, but from previous neurotransmitter system research. Unfortunately, they show limited ability to modify disease course, offering only transient benefit to patients.\(^4\) This situation is not different from that of Parkinson’s disease (PD), where similar symptomatic treatments are available.\(^5\)

These frustrating aspects fueled many researchers to question whether the viability of neurodegenerative targets for treating AD and related dementia might yield disease-modifying drugs.\(^6\) Indeed, in addition to the classical “positive” neurodegeneration lesions, such as Aβ plaques and neurofibrillary tangles, characteristic losses of neurons and synaptic elements are core “negative” features.\(^7\) Particularly, a significant reduction of neuronal density caused by cell death in specific hippocampal areas is one of the major hallmarks of AD progression.\(^7\) This suggests the need for restorative intervention and prompts the pharmaceutical community to
explore the emerging possibility of targeting neuroprotection and neuroregeneration. In principle, activating endogenous neuroprotective pathways and sustaining brain neurogenesis could be more effective than blocking Aβ- and tau-mediated toxic cascades leading to neurodegeneration. On this basis, small molecules might prevent death and restore function to damaged neurons by directly targeting neuroprotective mechanisms. Thus, the prospect of developing new chemical entities capable of modulating regenerative processes in vivo, has attracted much interest from the neurodegenerative diseases drug discovery community. However, due to the complexity of the involved processes, a distinct molecular target for drug discovery endeavors has not been prioritized yet. Conversely, phenotypic approaches, i.e., to find compounds that modulate key phenotypes of dementia, without requirement for the detailed mechanistic knowledge that is often lacking, has gained a new momentum.

With these concepts in mind, we were interested in small molecules that, by crossing the blood–brain barrier (BBB) and gaining access to the central nervous system (CNS), might promote a neuroprotective and neuroregenerative phenotype in diseased neuronal cells, while showing no toxic effect. Indeed, in addition to lack of efficacy, low BBB penetration and liver toxicity are still frequent reasons for AD clinical trial discontinuation. Thus, following a chemocentric approach, we looked for chemotypes possessing these distinct features.

We were illuminated by the reported neuroprotective/neuroregenerative mechanisms of psychotropic drugs. Particularly, the effect on neurogenesis of tricyclic antidepressants (TCA) has been widely recognized. It has been demonstrated that chronic TCA treatment increases the number of hippocampal granule cells and protects neurons from further damage, or possibly even reverse the damage that has already occurred. Thus, imipramine (1Aa in Figure 1) has been proposed as a hippocampal neurogenic agent, although it is hard to relate this effect to its
complex pharmacodynamic mechanisms.\textsuperscript{19} Similarly, phenothiazine (PTZ) antipsychotic drugs, such as chlorpromazine (1Ab in Figure 1), have shown neuroprotective effects by protecting mitochondria and preventing apoptosis,\textsuperscript{20} and by promoting neuronal regeneration and survival.\textsuperscript{21} Knowingly, TCA and PTZ share similar chemical motifs: \textit{i.e.}, a tricyclic moiety connected to an amino functionality through an alkyl linker. More generally, they all belong to the arylalkylamine class, which represents one of the oldest and most frequently encountered structural motifs among CNS-active, naturally occurring or synthetic small molecules.\textsuperscript{22} Thus, they might be considered “privileged” for the synthesis of a hypothesis-driven library of potential neuroprotective/neuroregenerative small molecules.\textsuperscript{23}

\textbf{Figure 1.} Design strategy to subsets 1-3.
On this basis, we report the results of an in vitro evaluation of a focused chemical library (subsets 1-3 in Figure 1) aimed at exploring the structural requirements for efficient neuroprotective/neuroregenerative cellular activity, without the manifestation of toxicity. The library has been built around the selected tricyclic alkylamine scaffold (Figure 1) (i) by diversifying the tricyclic portion (a-f), (ii) by changing the methylene units of the linker (A and B), (iii) by varying the amino group from a tertiary (subset 1) to a secondary (2), or primary amine (3). As illustrated in Figure 1, we selected three tricyclic scaffolds. They were the dibenzoazepine a of 1Aa, as well as the 10,11-dihydro-dibenazepine e of the mood stabilizer carbamazepine (1 in Figure S1), which has been similarly shown to induce neuronal differentiation and neuroprotection. We also included differently substituted PTZs (b-d). Interestingly, several PTZ derivatives have been shown to exert neuroprotective effects through different mechanisms. The choice of the carbazole moiety f has been motivated by the fact that it belongs to aminopropyl carbazole P7C3 (2 in Figure S1), with outstanding neuroregenerative and neuroprotective profile in various neurodegenerative conditions. We came up with the design of a hypothesis-driven phenotypic library of 36 entries.

Once the library has been synthesized, a cellular pipeline has been set up to screen subsets 1A(B)a-f, 2A(B)a-f, and 3A(B)a-f in a target-agnostic fashion.

Chemistry

The reported synthetic route for TCA and PTZ are obsolete and hazardous procedures, optimized as industrial processes more than half a century ago. In particular, a challenging step is the functionalization of the aromatic nitrogen, which involved N-alkylation by using strong
alkali metals or tedious multistep routes exploiting toxic reagents.\textsuperscript{28} These undesirable characteristics result in limited applicability to expedite broad SAR studies. To overcome this challenge, we turned our attention to a one-pot reductive $N$-alkylation procedure previously reported by Righi.\textsuperscript{29} It was extended to the direct $N$-alkylation of a-f with acetals 7 and 8, in the presence of triethylsilane (TES), as nontoxic and stable reducing agent (Scheme 2).

\begin{scheme}
\centering
\includegraphics[width=\textwidth]{Scheme1.png}
\caption{Synthesis of acetal intermediates 7 and 8.}\label{Scheme1}
\end{scheme}

\begin{quote}
\textsuperscript{a}Reagents and conditions: a. NaBH$_4$, NiSO$_4$, Ac$_2$O, MeOH, rt (56%); b. TFAA, TEA, THF, N$_2$, rt (75%).
\end{quote}

To obtain acetamide 7 from nitrile 5, we slightly modified a procedure involving the use of a large excess of NaBH$_4$ (7 eq.), NiSO$_4$ as catalyst, and acetic anhydride as trapping agent, to prevent dimerization side reaction, often occurring during the reduction of nitriles to primary amine (Scheme 1).\textsuperscript{30} The synthesis of 8 was accomplished by reacting commercially available amine 6 with trifluoroacetic anhydride (TFAA).

With the acetals 7 and 8 in hands, the reductive $N$-alkylation reaction of tricyclic aromatic amines a-f was performed (Scheme 2).\textsuperscript{29} This provided alkylamide derivatives 4 in very good yields (62\%-95\%). Probably, the lower yields for 4A(a-f) (62\%-82\%), compared to those for 4B(a-f) (70\%-95\%), reflects the lower reactivity of acetamide 7 with respect to trifluoroacetamide 8. The subsequent hydrolysis of 4 to afford primary amines of series 3 involved two different procedures. In the first one, treatment of 4A(a-f) under microwave (MW)
irradiation with a strong base (KOH) gave the primary amines 3A(a-f) in good to excellent yield (72%-98%). It should be noted that under conventional reflux conditions, dramatic differences have been observed in terms of both yields (45%-67%) and decreased reaction time (2 days vs. 90 min). Conversely, hydrolysis of 4B(a-f) under milder reaction conditions (K₂CO₃) led to primary amines 3B(a-f) in quantitative yield.

Scheme 2. Synthesis of target subsets 1-3.a

Reagents and conditions: a. 7 or 8, TES, TFA, DCM, rt (62%-95%); b. KOH, MeOH/H₂O, MW, 150 W, 160 °C (72%-98%); c. K₂CO₃, MeOH/H₂O, rt (quantitative); d. HCOOH, HCOH, H₂O, MW, 50 W, 100 °C (63%-74%); e. HCOOH, HCOH, H₂O, 80 °C (45%-84%); f. HCOH, K10-clay, NaBH₄, MeOH, MW, 50 W, 80 °C (24%-44%).

To synthesize tertiary amines of subset 1, we developed a MW-assisted Eschweiler–Clarke procedure, which led to consistent increases in yields (63%-74%) and shortening of reaction time (from 10 h to 30 min), compared to conventional heating protocol. However, MW irradiation...
failed in the case of PTZ 3A(b-d) and 3B(b-d), which displayed an excessive reactivity under these conditions. Thus, conventional heating (80°C) for 8-10 h provided 1A(b-d) and 1B(b-d) in satisfactory to good yields (45%-84%). Finally, primary amines of series 3 were converted to the corresponding secondary amines 2 by exploiting a modified version of a MW-assisted reductive amination. This was based on the use of paraformaldehyde and acidic clay montmorillonite K-10 as green solid catalyst. Further optimization of reaction conditions led to subset 2 in acceptable yields (24%-44%). All the final compounds were characterized by 1H- and 13C-NMR, MS analyses and their purity confirmed to be ≥ 95% by analytical HPLC.

**Results and Discussion**

With the 36 library compounds in hand, we were interested to implement a phenotypic screening pipeline aimed to identify those more effective in counteracting the regenerative failure typical of injured neuron and, at the same time, enhancing neurogenesis. However, generally speaking, hit selection primarily based on potency might lead to molecules from which it proves difficult to eliminate toxicity. Accordingly, the use of in vitro toxicity screens to facilitate prioritization of compounds for further testing was recently emphasized as a critical need. On this basis and considering our academic environment, we thought that cytotoxicity determination as primary screening might improve effectiveness in a timely and cost-effective manner. In particular, an unbiased phenotypic screening pipeline has been defined, whose main goals are: (i) to make a preliminary screening of molecules for neuronal and hepatic viabilities; (ii) to test the neuroprotective properties of the less toxic compounds against neurodegeneration caused by low serum and K+ deprivation in rat primary neurons; iii) to assess the most promising
compounds for their neurogenic activity in adult mouse derived neurospheres (NS); iv) to assess BBB permeability.

(i) First, we tested all subsets 1-3 on primary rat cerebellar granule neurons (CGNs), in order to proceed only with those molecules showing no neurotoxicity. Primary cells provide higher-quality models than immortalized cell lines as they form synapses and incorporate significant neuromodulatory and trophic inputs. Particularly, CGNs are considered a reliable model for studying cellular and molecular mechanisms of survival/apoptosis and neurodegeneration/neuroprotection. Thus, CGNs were exposed to 1-3 for 24 h at concentrations ranging from 0 to 50 µM, and cell viability measured by MTT assay (Figure 2). Anti-AD drug galantamine (3 in Figure S1) was used as positive control (Figure S2A). As expected, being these compounds mainly derived by FDA-approved or investigational drugs, they showed a general low toxicity at 5 µM concentration. Most of them showed toxicity only at higher concentrations (25 and 50 µM). In particular, the most toxic subset was 3A, featuring a primary amino group and a linker of three methylenes. As a general trend, we experienced a lower neurotoxicity for tricyclic systems c (unsubstituted PTZ) and f (carbazole). In addition, we noted that CGNs treated with 1Af, 2Aa, and 2Bc showed slight increased MTT values compared to untreated cells. This might be indicative of increased cellular metabolic activity, hence greater viability of the neurons under the assay condition. In addition to neurotoxicity, the assessment of hepatotoxicity would be of critical importance for the drug-likeness of the library. Indeed, both TCA and PTZ drugs can induce liver toxicity. Furthermore, aging, comorbidity and subsequent polytherapy significantly contribute to increase the risk of pharmacological side effects (i.e., liver injury) and drug interactions in many AD patients. Experiments were performed in human hepatoma cell line (HepG2) using those compounds that show no significant neurotoxicity (20
out of the starting 36). Anti-AD drug tacrine (4 in Figure S1) was used as positive control (Figure S2B). Thus, 1Aa, 1Ac, 1Af, 1Bb, 1Bd, 1Be, 2Aa, 2Ab, 2Ad, 2Ae, 2Af, 2Bb, 2Bc, 2Bd, 2Bf, 3Aa, 3Ac, 3Af, 3Bc, 3Af were incubated at 0-50 µM for 24 h. 1Aa, 1Bd, 2Ab, 2Ad, 2Bb, 2Bd, 2Bf, 3Bc, 3Af, 3Ac and 3Aa negatively affected hepatic viability, whereas no dramatic variation was found for 1Ac, 1Af, 1Bb, 2Aa, 2Ae, 2Af, 2Bc, 3Ac, and 3Ae (Figure S3).
Figure 2. Toxicity of 1-3 to differentiated CGNs after 24 h treatment. The red line marks cytotoxicity of 50%. Results are expressed as percentage of controls and are the mean ± SE of at least 3 different experiments, each run in triplicate.

Taken these data together, 1Af, 1Bb, 2Aa, 2Ae-f and 2Bc, as the less neurotoxic and hepatotoxic derivatives, were progressed further into the screening cascade to assess their neuroprotection profile.

(ii) To this end, we tested the ability of 1Af, 1Bb, 2Aa, 2Ae-f and 2Bc to support survival of primary CGN cultures, in which oxidative stress and neuronal death had been induced by serum and K+ withdrawal. Since this insult leads to partial ATP depletion, K+ loss, and ultimately apoptosis, it represents a useful model of neuronal injury, as well as neuronal senescence. As shown in Figure 3, deprived CGNs showed significantly decreased viability, whereas the addition of PTZ 1Bb and 2Bc at 1 and 5 µM was able to rescue neurons. Particularly, 2Bc increased CGNs viability up to 120%. Conversely, 1Af, 2Aa, and 2Ae-f reduced CGN viability. Thus, a linker length of two methylenes and the presence of a PTZ seem important features for neuroprotection. Interestingly, the collected data are in agreement with the literature, and strongly corroborate the idea that the PTZ scaffold is associated with promising neuroprotective effects.
Figure 3. Neuroprotection of 1Af, 1Bb, 2Aa, 2Ae, 2Af and 2Bc at different concentrations (0-10 µM) on serum and K⁺ deprivation-induced neuronal death in CGNs. White bars represent serum deprivation (control condition), whereas black bars serum and K⁺ deprivation. Results are the mean ± S.E. of at least 3 different experiments, each run in triplicate.

(iii) In addition to identifying molecules capable of rescuing neuronal death associated with low serum and K⁺ deprivation, we looked for compounds capable of enhancing neuronal regeneration. The ability to simultaneously stimulate neural progenitor proliferation and provide neuroprotection has been proposed as a useful trait for AD drug candidates.³⁸ Thus, we used the
NS model system to evaluate the NS formation capacity of the tested compounds by quantifying NS size and number. In addition to the most neuroprotective 2Bc, we also tested 2Aa and 2Af because of the known neurogenic activity of TCA and carbazole derivatives (see above). Thus, NS were derived from the subventricular zone of adult mice and NS proliferation assays were set up after 15 days (Figure S4). In particular, NS cultures were plated and after 30 min incubated for 4 days with 2Aa, 2Af, and 2Bc at 1, 2.5, and 5 µM concentrations. After 4 days, compounds 2Bc and 2Af (at 1 µM) increased the average NS size by 34% and 40%, compared to control (Figure 4A). Conversely, no significant effect was observed for 2Aa. However, at 5 µM concentration, all compounds decreased average NS size. Thus, it appears that higher concentration of 2Aa, 2Af, and 2Bc negatively affects NS formation because of toxic effects. Particularly, at 5 µM, 2Bc and 2Af induced a marked reduction of average size down to 22% and 19%, respectively.

Regarding the average NS number (Figure 4B), no differences were observed at 1 µM concentration in comparison to control. In contrast, at 5 µM concentration, a higher average number of NS with 2Bc (149%) and 2Af (157%) was determined.

The collected results suggest that, at 1 µM, 2Bc and 2Af have higher NS formation capacity than 2Aa, for which a regenerative profile has been claimed.39
Figure 4. Effect of 2Aa, 2Af, and 2Bc on the proliferation and of mice primary NS by analyzing size (A) and number (B). Each bar represents the percentage of control and is the mean ± SE of at least 3 different experiments, each run in six replicates.

(iv) Despite the high potential as BBB permeable compounds (see also their compliance with FAF-drugs3 prediction in SI), we confirmed the CNS penetration ability of 2Aa, 2Af and 2Bc in a PAMPA-BBB test. Starting from a validation study on known CNS permeable drugs and comparing the obtained permeability values with the corresponding literature data, we verified their PAMPA-BBB permeability (Table S1).
Encouraged by the promising profile of 2Bc in terms of both neuroprotective and neuroregenerative properties, we sought to evaluate its therapeutic potential. We tested 2Bc in established cellular models of neurodegenerative diseases, mimicking distinct aspects of neurodegeneration (i.e., Aβ-, 6-hydroxydopamine (6-OHDA)-, and glutamate-induced neurotoxicity and neuronal death), in comparison with AD and PD approved drugs. This would allow verifying whether the identified compound, with the predefined neuroprotective and neuroregenerative profile, could outperform currently available drugs. To this end, primary CGNs were pretreated (2 h) with serial concentrations of 2Bc or AD drug galantamine (3 in Figure S1) (5–25 µM) in the presence of toxic Aβ1–42 (25 µM) for 24 h (Figure 5A). As expected, viability of GCNs exposed to Aβ was reduced to 80% compared to the control. Notably, a pretreatment of CGNs with 2Bc (5–25 µM) reversed Aβ-induced neuronal death with a maximal effect obtained at 5 µM (viability increased to 129%). Conversely, such effect was not observed for 3 at the same concentration. Interestingly, 2Bc was not able to block Aβ-induced toxicity upon co-treatment with Aβ (data not shown).

The protective effect of 2Bc was further assessed in the 6-OHDA cellular model of PD. CGNs were found to be sensitive to 20 µM of 6-OHDA, which significantly decreased cell viability by 20% (Figure 5B). Again, pre-treatment (2 h) with 2Bc prevented neuronal death in a concentration-dependent inverse manner, with a maximal effect observed at 5 µM. Gratifyingly, 2Bc showed neuroprotective effects comparable to those elicited by the anti-PD dopaminergic drug apomorphine (5 in Figure S1) at 5 µM, whereas, 2Bc turned out to be more effective at 10 and 25 µM. Also in this case, co-treatment experiments failed to rescue 6-OHDA-induced neuronal death (data not shown).
To have a wider picture of the neuroprotective capability of 2Bc, we considered excitotoxicity as another valuable model to mimic neurodegeneration.\textsuperscript{33} We investigated and compared the neuroprotection of 2Bc and memantine (6 in Figure S1), as the marketed NMDA receptor antagonist, against glutamate-induced excitotoxicity. CGNs were pretreated with 2Bc for 2 h before adding 100 µM glutamate/10 µM glycine insult. As shown in Figure S5, 2 h pretreatment with 2Bc at all tested concentrations resulted ineffective in rescuing CGNs against glutamate-induced excitotoxicity, whereas 5 was protective in a dose-dependent manner.

**Figure 5.** Neuroprotective effects of 2Bc on Aβ- and 6-OHDA-induced neurotoxicity in CGNs. Cells were pretreated (2h) at various concentrations in the presence of Aβ\textsubscript{1-42} (25 µM) (A) or 6-
OHDA (20 µM) (B), for 24 h. Results are the mean ± S.E. of 2 different experiments, each run in quadruplicate.

Interestingly, in this case, both co- and pre- treatments with 2Bc were ineffective in reversing glutamate-induced neurotoxicity. This might indicate that the underlying mechanisms to the observed neuroprotective effects of 2Bc could not involve the glutamatergic system.

Microglia is among the most potent modulators of CNS repair/regeneration. Particularly, microglial polarization, i.e. the shift from a neurotoxic (M1) to a neuroprotective phenotype (M2), is crucial to make the brain microenvironment permissive to neuroregeneration. To this end, we tested whether 2Bc could trigger microglia M1/M2 switch by evaluating the inducible nitric oxide synthase (iNOS), and triggering receptor expressed on myeloid cells 2 (TREM2) profiles expression and nitrite production upon inflammatory stimuli with lipopolysaccharide (LPS) (100 ng/mL) (Figure 6). In LPS-treated primary cultured glial cells, we observed a substantial induction of the M1 marker iNOS, which was dose-dependently reduced by treatment with 2Bc. However, 2Bc did not counteract nitrite production. Encouragingly, in the case of classical M2 marker, 2Bc did not affect TREM2 expression at all the tested concentrations. Given such a profile, 2Bc (at 5 µM) resulted as a peculiar modulator of glial cell-derived inflammation able, in principle, to revert the pro-inflammatory phenotype and sustain and favor the endogenous neuroregenerative processes. Furthermore, this result is of great therapeutic relevance as abnormal or prolonged glial cell activation is one of the main contributors to the pathogenesis and progression of neurodegenerative disorders.
Figure 6. Immunomodulatory effects of 2Bc in glial cells were evaluated through Western blot analysis of iNOS and TREM2 expression as microglial polarization markers (A), and relative densitometries of iNOS (B) and TREM2 (D) expressions, as well as the indirect extent of released NO trough nitrite measurement in the medium (C). GAPDH was used as loading control. Densitometric results are expressed as percentage of LPS only and are the mean ± SE of three different experiments.
Conclusions

Based on the reported neuroprotective/neuroregenerative properties of psychotropic drugs, a new hypothesis-driven chemical library of 36 tricyclic arylalkylamines has been designed. Thanks to the development of a new versatile procedure, the library has been synthesized and investigated in a phenotypic screening pipeline aimed at selecting entries displaying simultaneously neuroprotective and neuroregenerative effects, while devoid of neurotoxicity and hepatotoxicity. By using primary neurons and NS cultures, we have identified PTZ 2Bc, which functions as a neuroprotective and neuroregenerative phenotypic hit. Remarkably, it outperforms drug 2Aa, a well-known neurogenic agent, and displays immunomodulatory effects on microglia. Importantly, its higher efficacy with respect to currently available drugs (galantamine and apomorphine) in two cell models of AD and PD seems to corroborate the starting idea that targeting neuroprotection/neuroregeneration rather than neurodegeneration might be a reasonable strategy for neurodegenerative drug discovery. In addition, these preliminary findings appear to validate the tricyclic arylalkylamine structure, not only for the well-known psychotropic effects, but as a privileged chemotype against neurodegeneration.

Methods

Chemistry

General chemical methods

All the commercial available reagents and solvents were used purchased from Sigma-Aldrich (Italy), TCI chemicals (Europe) and Alfa Aesar (Germany) without purification. Column
chromatography purifications were performed under "flash conditions" using Sigma-Aldrich silica gel grade 9385, 60 Å, 230-400 mesh. CEM Discover SP focused microwave reactor was used for microwave-mediated reactions. Thin layer chromatography (TLC) separations were performed on 0.20 mm silica gel 60 F254 plates (Merck, Germany), which were visualized by exposure to ultraviolet light (254 and 366 nm), iodine and potassium permanganate stains. Reactions involving generation or consumption of amine were visualized by using bromocresol green (0.04% in EtOH made blue by NaOH) following heating of the plate. Compounds were named following IUPAC rules as applied by ChemDraw Professional 15.0. NMR experiments were run on Varian VXR 400 (400 MHz for $^1$H; 100 MHz for $^{13}$C). Spectra were acquired at 300 K, using CDCl$_3$ and CD$_3$OD as solvents. Chemical shifts for $^1$H and $^{13}$C spectra were recorded parts per million (ppm) using the residual non-deuterated solvent as the internal standard. Data are reported as follows: chemical shift (ppm), multiplicity (indicated as: s, singlet; br s, broad singlet; exch, exchangeable proton with D$_2$O; d, doublet; t, triplet; q, quartet; p, pentet; m, multiplet and combinations thereof), coupling constants ($J$) in Hertz (Hz) and integrated intensity. The molecular mass was determined by low-resolution mass spectra ESI-MS, recorded on a Waters ZQ 4000 apparatus. Target compounds were converted into the corresponding hydrochloride salts and were assessed for purity ($\geq$95%) by analytical RP-HPLC (see Supporting Information and Table S2 for experimental details). All compounds, with the exception of 2Bd and 3Bd, were previously synthesized using different methodologies for other applications.$^{44-48}$

**General procedure for the preparation of tertiary amine (subset 1). Method A:** To a suspension of primary amines 3 (1 mmol) in H$_2$O (4 mL), 37% aq. formaldehyde (10 mmol) and 90% aq. formic acid (5 mmol) solutions were sequentially added in a pressure-tight tube cooled
to 0 °C. After warming up at rt, the mixture was submitted to MW irradiation for 1 h at 100°C, with an irradiation power of 50 W. The reaction mixture was neutralized with NaHCO₃, followed by extraction with DCM (3 x 15 mL). The collected organic extracts were dried over Na₂SO₄, and evaporated in vacuum to give crude products, which were purified by column chromatography on silica gel with an eluent of DCM/MeOH/33% aq. NH₃ solution (9.5: 0.5: 0.05) to give 1Aa, 1Ae-f and 1Ba, 1Be-f. **Method B:** To a suspension of primary amines 3 (1 mmol) in H₂O (4 mL), 37% aq. formaldehyde (10 mmol) and 90% aq. formic acid (5 mmol) solutions were sequentially added in a pressure tube cooled to 0 °C. After 5 min, the reaction was allowed to reach rt, and then was warmed at 80°C for 8 h. The reaction mixture was neutralized with NaHCO₃, followed by extraction with DCM (3 x 15 mL). The collected organic extracts were dried over Na₂SO₄, and evaporated in vacuum to give crude products, which were purified by column chromatography on silica gel with an eluent of DCM/MeOH/33% aq. NH₃ solution (9.5: 0.5: 0.05) to give 1Ab-d and 1Bb-d.

**3-(10,11-Dihydro-5H-dibenzo[b,f]azepin-5-yl)-N,N-dimethylpropan-1-amine (1Aa).**

The title compound 1Aa was obtained as pale yellow oil by using Method A. Yield 61%.

¹H NMR (400 MHz, CDCl₃) δ 7.16 – 6.93 (m, 6H), 6.97 – 6.87 (m, 2H), 3.76 (t, J = 6.9 Hz, 3H), 3.15 (s, 4H), 2.31 (t, J = 7.8 Hz, 2H), 2.14 (s, 6H), 1.72 (p, J = 7.1 Hz, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 148.28, 134.19, 129.74, 126.32, 122.36, 119.97, 57.63, 48.82, 45.45, 32.20, 26.11.


**3-(2-Chloro-10H-phenothiazin-10-yl)-N,N-dimethylpropan-1-amine (1Ab).**

The title compound 1Ab was obtained as yellow oil by using Method B. Yield 66%.
\( ^1 \)H NMR (400 MHz, CDCl\(_3\)) δ 7.20 – 7.11 (m, 2H), 7.03 – 7.01 (m, 1H), 6.95 – 6.87 (m, 4H), 3.88 (t, \( J = 7.7 \) Hz, 2H), 2.40 (t, \( J = 7.0 \) Hz, 2H), 2.22 (s, 6H), 1.97 – 1.90 (m, 2H). \( ^{13} \)C NMR (100 MHz, CDCl\(_3\)) δ 146.52, 144.51, 133.21, 127.84, 127.47, 124.82, 123.53, 122.83, 122.20, 115.85, 115.78, 57.00, 45.56, 45.45, 25.06. MS(ESI): \( m/z \) C\(_{17}\)H\(_{19}\)ClN\(_2\)S; calcd. [M]: 318.86, found [M + H]\(^+\): 319, [M + Na]\(^+\): 341.

**\( N,N \)-Dimethyl-3-(10H-phenothiazin-10-yl)propan-1-amine (1Ac).**

The title compound 1Ac was obtained as yellow oil by using Method B. Yield 74%.

\( ^1 \)H NMR (400 MHz, CDCl\(_3\)) δ 7.15 – 7.11 (m, 4H), 6.91 – 6.86 (m, 4H), 3.92 – 3.88 (m, 2H), 2.40 (t, \( J = 7.1 \) Hz, 2H), 2.19 (s, 6H), 1.92 (p, \( J = 7.0 \) Hz, 2H). \( ^{13} \)C NMR (100 MHz, CDCl\(_3\)) δ 150.35, 145.24, 127.42, 127.17, 125.13, 122.38, 115.49, 57.14, 45.54, 45.36, 25.21. MS(ESI): \( m/z \) C\(_{17}\)H\(_{20}\)N\(_2\)S; calcd. [M]: 284.42, found [M + H]\(^+\): 285, [M + Na]\(^+\): 307.

**\( N,N \)-Dimethyl-3-(2-(trifluoromethyl)-10H-phenothiazin-10-yl)propan-1-amine (1Ad).**

The title compound 1Ad was obtained as yellow oil by using Method B. Yield 48%.

\( ^1 \)H NMR (400 MHz, CDCl\(_3\)) δ 7.18 – 7.05 (m, 5H), 6.93 – 6.89 (m, 2H), 3.92 (t, \( J = 7.0 \) Hz, 2H), 2.39 (t, \( J = 7.0 \) Hz, 2H), 2.19 (s, 6H), 1.96 – 1.88 (m, 2H). \( ^{13} \)C NMR (100 MHz, CDCl\(_3\)) δ 145.64, 144.39, 127.51 (q, \( J = 272 \) Hz), 123.03, 118.99, 115.84, 111.90, 56.86, 45.21, 24.73. MS(ESI): \( m/z \) C\(_{18}\)H\(_{19}\)F\(_3\)N\(_2\)S; calcd. [M]: 352.42, found [M + H]\(^+\): 353, [M + Na]\(^+\): 375.

**3-(5H-Dibenzo[b,f]azepin-5-yl)-N,N-dimethylpropan-1-amine (1Ae).**

The title compound 1Ae was obtained as yellow oil by using Method A. Yield 64%.
1H NMR (400 MHz, CDCl₃) δ 7.19 (t, J = 8.0 Hz, 2H), 7.03 – 6.87 (m, 6H), 6.67 (s, 2H), 3.70 (t, J = 6.9 Hz, 2H), 2.31 (t, J = 7.3 Hz, 2H), 2.09 (s, 6H), 1.71 – 1.62 (m, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 150.89, 133.91, 132.11, 129.11, 128.79, 123.22, 120.40, 57.35, 48.65, 45.51, 25.71.


3-(9H-Carbazol-9-yl)-N,N-dimethylpropan-1-amine (1Af).

The title compound 1Af was obtained as yellow oil by using Method A. Yield 56%.

1H NMR (400 MHz, CDCl₃) δ 8.10 – 8.07 (m, 2H), 7.47 O 7.42 (m, 4H), 7.21 (t, J = 4.0 Hz, 2H), 4.43 – 4.34 (t, J = 7.0 Hz, 2H), 2.29 (t, J = 6.8 Hz, 2H), 2.22 (s, 6H), 2.06 – 1.97 (m, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 140.44, 125.56, 122.78, 120.25, 118.71, 108.71, 56.61, 45.38, 40.65, 26.90. MS(ESI): m/z C₁₇H₂₀N₂; calcd. [M]: 252.36, found [M + H]⁺: 253, [M + Na]⁺: 275.

2-(10,11-Dihydro-5H-dibenzo[b,f]azepin-5-yl)-N,N-dimethylethan-1-amine (1Ba).

The title compound 1Ba was obtained as colorless oil by using Method A. Yield 56%.

1H NMR (400 MHz, CDCl₃) δ 7.14 – 7.02 (m, 6H), 6.94 – 6.85 (m, 2H), 3.91 – 3.83 (m, 2H), 3.13 (s, 4H), 2.50 – 2.43 (m, 2H) 2.25 (s, 6H). ¹³C NMR (100 MHz, CDCl₃) δ 146.93, 145.31, 131.47, 129.20, 127.03, 124.85, 53.40, 39.99, 34.63, 31.85. MS(ESI): m/z C₁₈H₂₀N₂; calcd. [M]: 266.39, found [M + H]⁺: 267, [M + Na]⁺: 289.

2-(2-Chloro-10H-phenothiazin-10-yl)-N,N-dimethylethan-1-amine (1Bb).

The title compound 1Bb was obtained as colorless oil by using Method B. Yield 72%.

1H NMR (400 MHz, CDCl₃) δ 7.21 – 7.08 (m, 2H), 7.01 (d, J = 8.1 Hz, 1H), 6.95 – 6.87 (m, 4H), 3.98 – 3.95 (m, 2H), 2.75 – 2.62 (m, 2H), 2.33 (s, 6H). ¹³C NMR (100 MHz, CDCl₃) δ
N,N-Dimethyl-2-(10H-phenothiazin-10-yl)ethan-1-amine (1Bc).

The title compound 1Bc was obtained as colorless oil by using Method B. Yield 81%.

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.17 - 7.12 (m, 4H), 6.93 - 6.90 (m, 4H), 4.01 (t, $J = 7.0$ Hz, 2H), 2.73 (t, $J = 7.0$ Hz, 2H), 2.34 (s, 6H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 145.01, 127.45, 127.33, 124.70, 122.55, 115.27, 105.58, 56.55, 46.29, 45.82. MS(ESI): m/z C$_{16}$H$_{17}$ClN$_2$S; calcd. [M]: 304.84, found [M + H]$^+$: 305, [M + Na]$^+$: 327.

N,N-Dimethyl-2-(2-(trifluoromethyl)-10H-phenothiazin-10-yl)ethan-1-amine (1Bd).

The title compound 1Bd was obtained as yellowish oil by using Method B. Yield 45%.

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.18 - 7.09 (m, 4H), 6.94 - 6.91 (m, 3H), 4.03 - 3.95 (m, 2H), 2.71 - 2.67 (m, 2H) 2.32 (s, 6H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 145.39, 144.18, 127.61 (q, $J = 273$ Hz), 123.54 (q, $J = 33$ Hz), 119.10, 115.55, 111.85, 111.81, 109.99, 56.46, 46.75, 45.83. MS(ESI): m/z C$_{16}$H$_{17}$F$_3$N$_2$S; calcd. [M]: 338.39, found [M + H]$^+$: 339, [M + Na]$^+$: 361.

2-(5H-Dibenzo[b,f]azepin-5-yl)-N,N-dimethylethan-1-amine (1Be).

The title compound 1Be was obtained as yellow oil by using Method A. Yield 58%.

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.22 (t, $J = 7.8$ Hz, 2H), 7.04 (m, $J = 7.1$ Hz, 4H), 6.98 (t, $J = 7.3$ Hz, 2H), 6.71 (s, 2H), 3.89 (t, $J = 8.1$ Hz, 2H), 2.55 (t, $J = 8.1$ Hz, 2H), 2.29 (s, 6H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 150.74, 133.81, 132.08, 129.11, 128.90, 123.37, 120.18, 57.22, 49.29, 45.79. MS(ESI): m/z C$_{18}$H$_{20}$N$_2$; calcd. [M]: 264.37, found [M + H]$^+$: 265, [M + Na]$^+$: 287.
2-(9H-Carbazol-9-yl)-N,N-dimethylethan-1-amine (1Bf).

The title compound 1Bf was obtained as colorless oil by using Method A. Yield 56%.

$^1$H NMR (400 MHz, CDCl$_3$) δ 8.09 (t, $J = 7.8$ Hz, 1H), 7.52 – 7.35 (m, 4H), 7.30 – 7.13 (m, 2H), 4.43 (t, $J = 7.8$ Hz, 2H), 2.76 – 2.67 (m, 2H), 2.37 (s, 6H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 120.38, 120.13, 119.48, 118.92, 118.58, 108.43, 57.15, 45.44, 43.44. MS(ESI): m/z C$_{16}$H$_{18}$N$_2$; calcd. [M]: 238.33, found [M + H]$^+$: 239, [M + Na]$^+$: 261.

General procedure for the preparation of secondary amine (subset 2). To a solution of paraformaldehyde (1 mmol) in MeOH (5 mL), 20 mg of clay K10 was added and left stirred at rt for 15 min in a pressured-microwave tube. Then, the appropriate primary amine 3 (1.5 mmol) was added to the reaction mixture and was submitted to MW irradiation for 30 min at 80 °C, with an irradiation power of 50 W. A mixture of 10 mg of clay K10 with NaBH$_4$ (1.5 mmol) was then added to the reaction after cooling down to 0 °C. Subsequently, the reaction mixture was irradiated again for 10 min at 80 °C. It was then diluted with MeOH, filtered, concentrated to dryness and purified by flash chromatography on silica gel using a gradient of DCM/MeOH/33% aq. NH$_3$ solution (from 9.5: 0.5: 0.05 to 9: 1: 0.1) to give secondary amine subset 2.

3-(10,11-Dihydro-5H-dibenzo[b,f]azepin-5-yl)-N-methylpropan-1-amine (2Aa).

The title compound 2Aa was obtained as pale yellow oil. Yield 44%. $^1$H NMR (400 MHz, CDCl$_3$) δ 7.15 – 7.09 (m, 4H), 6.90 (t, $J = 8.0$ Hz, 2H), 3.81 (t, $J = 6.7$ Hz, 2H), 3.18 (s, 4H), 2.62 (t, $J = 7.1$ Hz, 1H), 2.38 (s, 3H), 1.80 – 1.77 (m, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ
3-(2-Chloro-10H-phenothiazin-10-yl)-N-methylpropan-1-amine (2Ab).

The title compound 2Ab was obtained as yellow oil. Yield 30%.

$^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 7.13 – 7.07 (m, 2H), 7.03 (d, $J = 8.7$ Hz, 1H), 6.98 – 6.95 (m, 4H), 4.04 (t, $J = 7.7$ Hz, 2H), 3.33-3.32 (m, 2H), 2.73 (t, $J = 7.0$ Hz, 2H), 2.34 (s, 3H). $^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 146.50, 144.30, 133.08, 127.62, 127.33, 127.02, 124.85, 123.93, 122.88, 122.13, 115.67, 115.48, 55.61, 48.20, 47.98, 47.77, 47.56, 47.34, 46.92, 45.14, 44.30.

MS(ESI): $m/z$ C$_{18}$H$_{22}$N$_2$; calcd. [M]: 266.39, found [M + H]$^+$: 267, [M + Na]$^+$: 289.

N-Methyl-3-(10H-phenothiazin-10-yl)propan-1-amine (2Ac).

The title compound 2Ac was obtained as yellow oil. Yield 28%.

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.14 – 7.10 (m, 3H), 6.91 - 6.85 (m, 5H), 3.91 (t, $J = 6.7$ Hz, 2H), 2.67 (t, $J = 7.1$ Hz, 2H), 2.35 (s, 3H), 1.96 (p, $J = 6.7$ Hz, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 145.20, 127.46, 127.24, 125.31, 122.50, 115.57, 77.44, 77.13, 76.81, 49.36, 45.27, 36.10, 26.68.

MS(ESI): $m/z$ C$_{16}$H$_{17}$ClN$_2$S; calcd. [M]: 304.84, found [M + H]$^+$: 305, [M + Na]$^+$: 327.

N-Methyl-3-(2-(trifluoromethyl)-10H-phenothiazin-10-yl)propan-1-amine (2Ad).

The title compound 2Ad was obtained as yellow oil. Yield 24%.

$^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 7.24 - 7.12 (m, 5H), 7.04 (s, 1H), 6.97 (s, 1H), 4.04 – 3.97 (m, 2H), 2.68 (m, 2H), 2.31 (s, 3H), 1.95 (m, 2H). $^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 145.64, 144.39, 127.51 (q, $J = 271$ Hz), 123.04
(q, \( J = 34 \) Hz), 119.03, 118.99, 115.84, 111.87, 56.99, 45.54, 45.52, 24.99. MS(ESI): m/z C\textsubscript{17}H\textsubscript{17}F\textsubscript{3}N\textsubscript{2}S; calcd. [M]: 338.39, found [M + H]\(^+\): 339, [M + Na]\(^+\): 361.

3-(5H-Dibenzo[b,f]azepin-5-yl)-N-methylpropan-1-amine (2Ae).

The title compound 2Ae was obtained as yellow oil. Yield 36%.

\(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 7.24 (t, \( J = 7.8 \) Hz, 2H), 7.07 - 6.96 (m, 4H), 6.74 (s, 2H), 3.80 (t, \( J = 6.4 \) Hz, 2H), 2.70 (t, \( J = 6.7 \) Hz, 2H), 2.32 (s, 3H), 1.78 (t, \( J = 6.6 \) Hz, 2H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \( \delta \) 150.40, 133.72, 132.09, 129.17, 128.93, 123.51, 120.17, 49.61, 48.66, 35.54, 26.03. MS(ESI): m/z C\textsubscript{18}H\textsubscript{20}N\textsubscript{2}; calcd. [M]: 264.37, found [M + H]\(^+\): 265, [M + Na]\(^+\): 287.

3-(9H-Carbazol-9-yl)-N-methylpropan-1-amine (2Af).

The title compound 2Af was obtained as yellowish oil. Yield 35%.

\(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 8.10 (d, \( J = 7.8 \) Hz, 2H), 7.45 (m, 4H), 7.23 (t, \( J = 7.9 \), Hz, 2H), 4.39 (t, \( J = 6.8 \) Hz, 2H), 2.61 (t, \( J = 6.9 \) Hz, 2H), 2.40 (s, 3H), 2.05 (p, \( J = 6.9 \) Hz, 2H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \( \delta \) 140.48, 125.73, 122.89, 120.40, 118.89, 108.72, 49.16, 40.76, 36.27, 29.01. MS(ESI): m/z C\textsubscript{16}H\textsubscript{18}N\textsubscript{2}; calcd. [M]: 238.33, found [M + H]\(^+\): 239, [M + Na]\(^+\): 261.

2-(10,11-Dihydro-5H-dibenzo[b,f]azepin-5-yl)-N-methylethan-1-amine (2Ba).

The title compound 2Ba was obtained as yellow oil. Yield 37%.

\(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 7.14 - 7.08 (m, 6H), 6.91 (m, 2H), 3.87 (t, \( J = 6.3 \) Hz, 2H), 3.15 (s, 4H), 2.75 (t, \( J = 6.3 \) Hz, 2H), 2.35 (s, 3H). \(^{13}\)C NMR (100 MHz, CD\(_3\)OD) \( \delta \) 147.44, 134.50, 129.82, 126.42, 123.32, 119.00, 46.72, 46.42, 32.69, 31.52. MS(ESI): m/z C\textsubscript{17}H\textsubscript{20}N\textsubscript{2}; calcd. [M]: 252.36, found [M + H]\(^+\): 253, [M + Na]\(^+\): 275.
2-(2-Chloro-10H-phenothiazin-10-yl)-N-methylethan-1-amine (2Bb).

The title compound 2Bb was obtained as yellow oil. Yield 29%.

$^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 7.21 – 7.10 (m, 4H), 6.97 – 6.91 (m, 3H), 4.03 (t, $J = 6.3$ Hz, 2H), 3.29 (s, 3H), 2.87 (t, $J = 6.3$ Hz, 2H). $^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 146.59, 144.32, 133.14, 127.68, 127.37, 127.07, 125.22, 124.28, 123.00, 122.27, 115.92, 115.70, 47.56, 45.96, 34.42. MS(ESI): $m/z$ C$_{16}$$H_{15}$$Cl$$N_2$$S$; calcd. [M]: 290.81, found [M + H]$^+$: 291, [M + Na]$^+$: 313.

N-Methyl-2-(10H-phenothiazin-10-yl)ethan-1-amine (2Bc).

The title compound 2Bc was obtained as yellow oil. Yield 38%.

$^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 7.31 – 7.25 (m, 4H), 7.17 – 6.98 (m, 4H), 4.35 (t, $J = 5.8$ Hz, 2H), 3.43 (t, $J = 5.8$ Hz, 2H), 2.71 (s, 3H). $^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 144.51, 127.45, 127.42, 126.33, 123.21, 115.40, 48.19, 47.98, 47.77, 47.56, 47.34, 47.13, 46.92, 45.60, 42.84, 32.73. MS(ESI): $m/z$ C$_{16}$$H_{16}$$N_2$$S$; calcd. [M]: 256.37, found [M + H]$^+$: 257, [M + Na]$^+$: 279.

N-Methyl-2-(2-(trifluoromethyl)-10H-phenothiazin-10-yl)ethan-1-amine (2Bd).

The title compound 2Bd was obtained as yellow oil. Yield 26%.

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.21 – 7.11 (m, 5H), 6.98 – 6.93 (m, 2H), 4.02 (t, $J = 6.8$ Hz, 2H), 2.73 – 2.70 (m, 2H), 2.34 (s, 3H). $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 145.96, 144.75, 128.29 (q, $J = 271$ Hz), 123.70 (q, $J = 35$ Hz), 119.67, 116.12, 112.42, 57.03, 47.32, 46.40. MS(ESI): $m/z$ C$_{16}$$H_{13}$$F_3$$N_2$$S$; calcd. [M]: 324.37, found [M + H]$^+$: 325, [M + Na]$^+$: 347.

2-(5H-Dibenzo[b,f]azepin-5-yl)-N-methylethan-1-amine (2Be).
The title compound 2Be was obtained as yellow oil. Yield 42%.

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.26 – 7.21 (m, 2H), 7.06 – 6.98 (m, 6H), 6.73 (s, 2H), 3.87 (t, $J$ = 5.9 Hz, 2H), 2.70 (s, 3H). $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 150.17, 134.02, 132.05, 129.18, 128.92, 123.63, 120.67, 49.78, 48.64, 35.82. MS(ESI): $m/z$ C$_{17}$H$_{18}$N$_2$; calcd. [M]: 250.35, found [M + H]$^+$: 251, [M + Na]$^+$: 272.

2-(9H-Carbazol-9-yl)-N-methylethan-1-amine (2Bf).

The title compound 2Bf was obtained as yellow oil. Yield 31%.

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.10 (d, $J$ = 7.8 Hz, 2H), 7.47 (d, $J$ = 4.0 Hz, 4H), 7.25 (q, $J$ = 3.8 Hz, 2H), 4.45 (t, $J$ = 6.1 Hz, 2H), 3.05 (m, 2H), 2.40 (s, 3H). $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 140.52, 125.76, 122.91, 120.36, 119.05, 108.70, 43.15, 41.33, 29.71. MS(ESI): $m/z$ C$_{17}$H$_{18}$N$_2$; calcd. [M]: 224.31, found [M + H]$^+$: 225, [M + Na]$^+$: 247.

**General procedure for the preparation of primary amine (subset 3) Method C**: To a solution of acetamides 4A(a-f) (1 mmol) in a mixture of MeOH: H$_2$O (2:1, 5 mL), KOH (7.5 mmol) was added in a pressure-tight microwave tube. The resulting mixture was submitted to MW irradiation for 90 min at 160 °C, with an irradiation power of 150 W. After evaporation of the solvents under vacuum, the resulting mixture was purified by flash chromatography using as eluent a mixture of DCM: MeOH: 33% aq. NH$_3$ solution (9:1:0.1) to give the title compounds 3A(a-f).

**Method D**: To a solution of trifluoroacetamides 4B(a-f) (1 mmol) in a mixture of MeOH: H$_2$O (2:1, 3.5 mL), K$_2$CO$_3$ (7.88 mmol) was added and the resulting mixture is stirred at rt for 4-6 h. After evaporation of MeOH under vacuum, the resulting aqueous residues was diluted with H$_2$O (10 mL) and extracted with DCM (3 x 10 mL). The organic phases were
reunited, washed with brine, dried over Na$_2$SO$_4$, filtered and evaporated in vacuum to give the title compounds 3B(a-f), without any further purification.

3-(10,11-Dihydro-5H-dibenzo[b,f]azepin-5-yl)propan-1-amine (3Aa).

The title compound 3Aa was obtained as colorless oil by using Method C. Yield 78%.

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.15 - 7.07 (m, 6H), 6.98 – 6.87 (m, 2H), 3.79 (t, $J = 6.7$ Hz, 2H), 3.16 (s, 4H), 2.71 (t, $J = 7.0$ Hz, 2H), 1.71 (p, $J = 6.8$ Hz, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 148.27, 134.23, 129.82, 126.34, 122.47, 119.91, 47.94, 39.90, 32.18, 31.69. MS(ESI): m/z C$_{17}$H$_{20}$N$_2$; calcd. [M]: 252.36, found [M + H]$^+$: 253, [M + Na]$^+$: 275.

3-(2-Chloro-10H-phenothiazin-10-yl)propan-1-amine (3Ab).

The title compound 3Ab was obtained as yellow oil by using Method C. Yield 87%.

$^1$H NMR (400 MHz, CD$_3$OD) δ 7.13 (t, $J = 8.0$ Hz, 1H), 7.04 (d, $J = 8.1$ Hz, 1H), 6.97 – 6.84 (m, 4H), 6.82 (d, $J = 8.3$ Hz, 1H), 3.82 (t, $J = 6.7$ Hz, 2H), 2.67 (t, $J = 7.1$ Hz, 2H), 1.82 (m, 2H). $^{13}$C NMR (100 MHz, CD$_3$OD) δ 146.64, 144.45, 133.00, 127.58, 127.31, 127.02, 124.86, 123.89, 122.76, 121.98, 115.94, 115.63, 44.64, 38.80, 29.27. MS(ESI): m/z C$_{15}$H$_{15}$CIN$_2$S; calcd. [M]: 290.81, found [M + H]$^+$: 291, [M + Na]$^+$: 323.

3-(10H-Phenothiazin-10-yl)propan-1-amine (3Ac).

The title compound 3Ac was obtained as yellowish oil by using Method C. Yield 98%.

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.14 - 7.18 (m, 4H), 6.96 – 6.86 (m, 4H), 3.95 (t, $J = 6.7$ Hz, 2H), 2.82 (t, $J = 6.8$ Hz, 2H), 1.95 (q, $J = 6.7$ Hz, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 145.26, 127.53,
127.25, 125.37, 122.53, 115.58, 53.45, 44.79, 39.63, 30.09. MS(ESI): *m/z* C$_{15}$H$_{16}$N$_2$S; calcd. [M]: 256.37, found [M + H]$^+$: 257, [M + Na]$^+$: 279.

3-(2-(Trifluoromethyl)-10H-phenothiazin-10-yl)propan-1-amine (3Ad).

The title compound 3Ad was obtained as yellowish oil by using Method C. Yield 48%.

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.20 – 7.03 (m, 4H), 6.93 - 6.87 (m, 3H), 3.94 (t, $J$ = 6.7 Hz, 2H), 2.80 (t, $J$ = 6.7 Hz, 2H), 1.89 (t, $J$ = 6.7 Hz, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 145.69, 144.38, 130.02 (q, $J$ = 34 Hz), 127.51 (q, $J$ = 272 Hz), 124.25, 123.13, 122.81, 120.11, 119.16, 115.93, 111.94, 44.93, 39.51, 30.11. MS(ESI): *m/z* C$_{16}$H$_{15}$F$_3$N$_2$S; calcd. [M]: 324.37, found [M + H]$^+$: 325, [M + Na]$^+$: 347.

3-(5H-Dibenzo[b,f]azepin-5-yl)propan-1-amine (3Ae).

The title compound 3Ae was obtained as yellow solid by using Method C. Yield 79%.

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.24 (t, $J$ = 7.7 Hz, 2H), 7.07 – 6.92 (m, 6H), 6.71 (s, 2H), 3.78 (t, $J$ = 6.5, 2H), 2.74 (t, $J$ = 6.7 Hz, 2H), 1.69 (t, $J$ = 6.6 Hz, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 188.54, 150.76, 133.85, 132.14, 129.14, 128.80, 123.31, 120.26, 48.20, 40.05, 30.68. MS(ESI): *m/z* C$_{17}$H$_{18}$N$_2$; calcd. [M]: 250.35, found [M + H]$^+$: 251, [M + Na]$^+$: 273.

3-(9H-Carbazol-9-yl)propan-1-amine (3Af).

The title compound 3Af was obtained as yellow oil by using Method C. Yield 77%.

$^1$H NMR (400 MHz, CDCl$_3$) δ 8.08 (d, $J$ = 7.8 Hz, 2H), 7.49 – 7.38 (m, 4H), 7.27 – 7.17 (m, 2H), 4.40 (t, $J$ = 6.8 Hz, 2H), 2.74 (t, $J$ = 6.9 Hz, 2H), 2.01 (p, $J$ = 6.9 Hz, 2H), 1.67 (s, 1H). $^{13}$C
NMR (100 MHz, CDCl₃) δ 140.37, 125.60, 120.32, 118.78, 108.59, 40.44, 39.58, 32.44.


2-(10,11-Dihydro-5H-dibenzo[b,f]azepin-5-yl)ethan-1-amine (3Ba).

The title compound 3Ba was obtained as colorless oil by using Method D. Yield 98%.

¹H NMR (400 MHz, CDCl₃) δ 7.11 (m, 6H), 6.93 (t, J = 7.3 Hz, 2H), 3.81 (t, J = 6.0 Hz, 2H), 3.18 (s, 4H), 2.86 (s, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 148.04, 134.23, 129.91, 126.43, 122.70, 120.02, 53.98, 39.82, 32.27. MS(ESI): m/z C₁₆H₁₈N₂; calcd. [M]: 238.33, found [M + H]⁺: 239, [M + Na]⁺: 261.

2-(2-Chloro-10H-phenothiazin-10-yl)ethan-1-amine (3Bb).

The title compound 3Bb was obtained as yellow oil by using Method D. Yield 96%.

¹H NMR (400 MHz, CDCl₃) δ 7.17 – 7.12 (m, 2H), 7.02 (d, J = 8.2 Hz, 1H), 6.96 – 6.82 (m, 4H), 3.92 (t, J = 5.9 Hz, 2H), 3.03 (t, J = 5.9 Hz, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 145.22, 127.61, 127.27, 125.88, 122.73, 115.80, 50.46, 38.81. MS(ESI): m/z C₁₄H₁₃ClN₂S; calcd. [M]: 276.78, found [M + H]⁺: 277, [M + Na]⁺: 299.

2-(10H-Phenothiazin-10-yl)ethan-1-amine (3Bc).

The title compound 3Bc was obtained as colorless oil by using Method D. Yield 98%.

¹H NMR (400 MHz, CDCl₃) δ 7.17 – 7.10 (m, 4H), 6.96 – 6.84 (m, 4H), 3.96 (t, J = 5.9 Hz, 2H), 3.03 (t, J = 5.9 Hz, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 145.21, 127.59, 127.24, 125.87, 122.70, 115.78, 109.98, 50.51, 38.82. MS(ESI): m/z C₁₄H₁₄N₂S; calcd. [M]: 242.34, found [M + H]⁺: 243, [M + Na]⁺: 265.
2-(2-(Trifluoromethyl)-10H-phenothiazin-10-yl)ethan-1-amine (3Bd).

The title compound 6 was obtained as yellow oil by using Method D. Yield 97%.

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.29 – 7.13 (m, 4H), 7.10 – 7.05 (m, 1H), 7.02 – 6.88 (m, 2H), 4.02 (t, $J$ = 5.9 Hz, 2H), 3.08 (t, $J$ = 5.9 Hz, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 145.76, 144.26, 127.67 (q, $J$ = 273 Hz), 124.87, 123.38, 119.36, 116.15, 112.27, 112.24, 50.66, 38.65. MS(ESI): $m/z$ C$_{15}$H$_{13}$F$_3$N$_2$S; calcd. [M]: 310.34, found [M + H]$^+$: 311, [M + Na]$^+$: 333.

2-(5H-Dibenzo[b,f]azepin-5-yl)ethan-1-amine (3Be).

The title compound 3Be was obtained as yellow solid by using Method D. Yield 98%.

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.28 – 7.19 (m, 2H), 7.11 – 6.92 (m, 6H), 6.73 (s, 2H), 3.81 (t, $J$ = 1.5 Hz, 2H), 2.81 (m, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 150.42, 134.13, 132.12, 129.19, 128.84, 123.52, 120.70, 53.84, 39.31. MS(ESI): $m/z$ C$_{16}$H$_{16}$N$_2$; calcd. [M]: 236.32, found [M + H]$^+$: 237, [M + Na]$^+$: 259.

2-(9H-Carbazol-9-yl)ethan-1-amine (3Bf).

The title compound 3Bf was obtained as pale yellow oil by using Method D. Yield 97%.

$^1$H NMR (400 MHz, CDCl$_3$) δ 8.13 (dt, $J$ = 7.8, 1.1 Hz, 2H), 7.53 – 7.41 (m, 4H), 7.27 (m, 2H), 4.35 (t, $J$ = 6.2 Hz, 2H), 3.18 (t, $J$ = 6.1 Hz, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 140.65, 125.77, 122.92, 120.41, 119.07, 108.76, 46.21, 41.33. MS(ESI): $m/z$ C$_{14}$H$_{14}$N$_2$; calcd. [M]: 210.28, found [M + H]$^+$: 211, [M + Na]$^+$: 243.
Biology

Cell Cultures

Primary mixed glial cell cultures were prepared from cerebral cortex of newborn Wistar rats (*Rattus norvegicus*), whereas primary cultures of cerebellar granule neurons (CGNs) were prepared from 7 day-old pups of the same rat strain, as previously described. All animal experiments were authorized by the University of Bologna bioethical committee (Protocol n° 17-72-1212) and performed according to Italian and European Community laws on the use of animals for experimental purposes. For cerebellar granule cultures, cells were dissociated from cerebella and plated on 96 well plates, previously coated with 10 µg/mL poly-L-lysine, at a density of $3 \times 10^5$ cells/0.2 mL medium/well in BME supplemented with 100 mL/L heat-inactivated FBS (Life technologies), 2 mmol/L glutamine, 100 µmol/L gentamicin sulphate and 25 mmol/L KCl (all from Sigma-Aldrich). 16 h later, 10 µM cytosine arabinofuranoside (Sigma-Aldrich) was added to avoid glial proliferation. After 7 days in vitro, differentiated neurons were shifted to serum free BME medium containing 25 mmol/L KCl without serum and different treatments were performed. Microglial cells were obtained from mixed glial cell cultures after mechanical shaking, and then resuspended in fresh BME medium with 2 mmol/L glutamine and 100 µmol/L gentamicin sulphate, no serum and plated on uncoated 35 mm Ø dishes at a density of $1.5 \times 10^6$ cells/1.5 mL medium/well for Western blot analysis.

HepG2 cells (human hepatocellular liver carcinoma cell line from American Type Culture Collection, ATCC), were grown in DMEM supplemented with 10% FBS and 50 units/mL of penicillin/ streptomycin (Life Technologies i) at 37 °C in a humidified atmosphere containing 5% CO2. For the experiments, cells (0.5 × 105 cells/well) were seeded in 96-well plate in
complete medium; after 24 h, the medium was removed, and cells were exposed to the increasing concentrations of previously selected non-neurotoxic compounds (0, 10, 25, and 50 µM) in DMEM with no serum for further 24 h and survival was measured through MTT assay.

**MTT assay**

The viability of the different cell types (CGNs and HepG2) exposed to increasing concentrations of the studied compounds (0, 10, 20 and 50 µM) for 24 h was evaluated through the MTT assay. Briefly, thiazolyl blue was added to the culture medium at a final concentration of 0.1 mg/mL. Following a 20 min incubation for CGNs and 10 min for HepG2 cells at 37 °C in the dark, the MTT precipitate was dissolved in 0.1 M Tris-HCl pH 7.5 buffer containing 5% Triton X-100 (all from Sigma-Aldrich) and absorbance was read at 570 nm in a multiplate spectrophotometric reader (Bio-Rad). Drugs galantamine and tacrine were used as positive controls for CGNs and HepG2, respectively (Figure S2).

**Nitrites Measurement**

Primary cultures of microglia were incubated with LPS (100 ng/mL) in presence or absence of the selected compound 2Bc at increasing concentrations (5, 10, 25 µM) for 24 h. Nitrite accumulation in cell media was assayed by the standard Griess reaction. Culture media were then collected, centrifuged to remove residual cells and mixed with an equal volume of Griess reagent (Sigma). Lastly, samples were incubated at RT for 15 min and absorbance was read using a plate reader at 492/540 nm.
Western Blot Analysis

Microglial and astrocytic cells exposed to LPS (100 ng/mL) in presence or absence of different concentrations of $2\text{Be}$ (0, 5, 10 and 20 µM) for 24 h were directly lysed in ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 1% SDS, 0.05% protease inhibitor cocktail) and protein content was determined by using the Lowry method. 20 µg of protein extract were resuspended in 20 µL of loading buffer (0.05 M Tris-HCl pH 6.8; 40 g/L sodium dodecyl sulfate; 20 mL/L glycerol; 2 g/L bromophenol blue and 0.02 M dithiothreitol; all from Sigma-Aldrich) and loaded onto 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE; Bio-Rad Laboratories SrL, Segrate, MI, IT). After electrophoresis and transfer onto nitrocellulose membranes (GE Healthcare Europe GmbH, Milano, MI, IT), membranes were blocked for 1 h in 5% non-fat milk (Bio-Rad)/0.1% Tween-20 in PBS (Sigma-Aldrich), pH 7.4, and incubated overnight at 4°C with primary antibodies (rabbit polyclonal anti-iNOS or anti-TREM2 1:1000, both from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, or mouse monoclonal anti-β-actin, 1:2000, Sigma-Aldrich) in 0.1% Tween-20/PBS. Membranes were then incubated with an anti-rabbit or anti-mouse secondary antibody conjugated to horseradish peroxidase (1:2000; Santa Cruz), for 90 min at RT in 0.1% Tween-20/PBS. Labeled proteins were detected by using the enhanced chemiluminescence method (ECL; BioRAD) with a Chemidoc (BioRad) chemiluminescence detector. Densitometric analysis was performed by using Image J software from NIH.

Neuroprotection assays
A preliminary screening for neuroprotection has been carried to test the compounds 1Af, 1Bb, 2Aa, 2Ae, 2Af and 2Bc. To this aim, differentiated primary CGNs cultures has been shifted in a serum free medium with 25 mM KCl (control condition) or 5 mM KCl (serum/potassium deprivation) in presence increasing concentrations of the compounds (0, 1.5 and 10 µM).

For the neuroprotective effect against AD/PD stimuli, increasing concentrations (0.5, 10 and 25 µM) of the selected compound 2Bc have been tested on differentiated GCNs. As a model of AD neurodegeneration, Aβ toxicity was evaluated; briefly, aggregated synthetic Aβ1−42 from Biopeptide Co., Inc. (San Diego, CA) was prepared by incubating the synthetic peptide in 95% PBS/5% DMSO (5 mM) at 37 °C for 72 h, sonicating, and further centrifuging at 15000g at RT for 10 min. Aggregated Aβ1−42 (10 µM) was used to treat differentiated CGNs. Increasing concentrations (0, 5, 10 or 25 µM) of selected 2Bc compound or of galantamine, as reference benchmark drug, have been tested in co-treatment with Aβ or with both a 2h pre-treatment and a 24 h co-treatment in serum-free medium.

As an in vitro model of PD neurodegeneration, 6-hydroxydopamine (6-OHDA) toxicity was evaluated on differentiated CGNs. CGNs cultures were switched to serum-free medium in the presence or absence of 20 µM 6-OHDA (Sigma-Aldrich). Increasing concentrations (0, 5, 10 or 25 µM) of selected compound 2Bc or of apomorphine, as reference benchmark drug, have been tested in co-treatment with 6-OHDA or with both a 2h pre-treatment and a 24 h co-treatment in serum-free medium.

As a model of excitotoxicity, glutamate toxicity was evaluated on differentiated CGNs. CGNs cultures were switched to serum-free medium in the presence or absence of 100 µM glutamate/10 µM glycine (Sigma-Aldrich). Increasing concentrations (0, 5, 10 or 25 µM) of
selected compound 2Bc or of mementine, as reference benchmark drug, have been tested in co-
treatment with glutamate/glycine or with both a 2h pre-treatment and a 24 h co-treatment with
the excitotoxic stimulus in serum-free medium.

Following all above-described stimuli, neuronal survival has been evaluated through MTT assay.

**Neurosphere Cultures**

All animal care and handling was carried out in accordance with European Union guidelines
(directives 86/609/EEC and 2010/63/EU) and Italian legislation and all protocols were approved
by the University of Bologna Ethical committee. All efforts were made to ameliorate animal
suffering and reduce the number of animals used.

Neurosphere cultures were derived from the subventricular zone (SVZ) of 3 C57BL6/N male
adult mice (8-week old) and induced to proliferate using established passaging methods to
achieve optimal cellular expansion according to published protocols. Briefly, mice were
anesthetized with 10 mg/kg xylazine after intraperitoneal injection and then subject to cervical
dislocation. Brains were promptly collected, rinsed with cold PBS and transferred into a 10cm
Petri dish containing cold PBS and placed under a dissecting microscope. The SVZ was then
dissected, minced and digested using 0.7 mg/ml papain, 0.2 mg/ml cysteine and 0.2 mg/ml
EDTA (all from Sigma Aldrich) before being gently disaggregated. The resulting cell suspension
was plated into 2 wells from 12-well plates/animal in complete DMEM-F12 medium [DMEM-
F12 medium (Gibco) supplemented with 10 µg/ml insulin from bovine pancreas (Sigma
Aldrich), 1% N2 (Gibco), 1% B27 (Gibco), 20 ng/ml fibroblast growth factor-2 (FGF-2,
Peprotech Cat No. 100-18B) and 20 ng/ml of epidermal growth factor (EGF, Peprotech Cat No.
Cells were passaged every 5 days by mechanical procedures and maintained with alternative daily addition of FGF-2 (20 ng/ml) and EGF (20 ng/ml). Neurosphere proliferation experiments were set up after passage 3 (15 days after SVZ dissection). For this purpose, after neurosphere cell culture passaging, 5,000 cells/well were plated in 96-well plates in complete DMEM-F12 medium and after 30 minutes, tested compounds (diluted in DMSO) were added, whereas 0.5% DMSO was used as a control condition. Each condition was repeated in six replicates and cells were allowed to grow at 37°C and 5% CO2 in an incubator for 4 days. After 4 days in culture, 5 different image fields per well (6 wells per condition) were acquired by using an Eclipse TE 2000-S microscope (Nikon) in bright field mode using the 10X objective. Acquired images were then analyzed by using Fiji ImageJ by using the publicly available cell colony edge macro and only neurospheres with an area bigger than 400 µm² were considered after which results were expressed as average neurosphere number and average neurosphere size. Quantitative analyses were carried out in three separate experiments, calculating the mean ± SEM and using a two-tailed Student’s t-test to determine statistically relevant changes.

ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website. Figures S1-5; Table S1-2; Chemistry: synthesis and characterization of intermediates, compounds purity and copies of NMR spectra; CNS penetration: in vitro PAMPA-BBB test; PAINS behavior assessment (PDF). Molecular formula strings, calculated physicochemical-properties and PAINS behavior assessment of subsets 1-3 (CSV)
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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally.

Funding Sources

This work was supported by the University of Bologna (GrantRFO 2016). Financial support from MINECO (grant no. BFU2014-57494-R to AVM).

Notes

Any additional relevant notes should be placed here.

ACKNOWLEDGMENT

EU acknowledges Novamolstam, Spinner Emilia Romagna regional Ph.D. program (Ph.D. fellow 2013–2015) and University of Bologna for a postdoctoral grant. LEPA thanks COST Action CA15135 “Multi-target paradigm for innovative ligand identification in the drug discovery process (MuTaLig)” for a STSM grant that enabled him to work for one month in the laboratory of AVM at CSIC (Madrid). We also acknowledge Drs M.P. Costi, M. Mor, and D. Huryn for useful discussions. Technical assistance of M. Ciorraga is also acknowledged.

ABBREVIATIONS

6-OHDA, 6-hydroxydopamine; Aβ, amyloid-β; AD, Alzheimer’s disease; BBB, blood–brain barrier; CGNs, cerebellar granule neurons; CNS, central nervous system; HepG2, human
hepatoma cell line; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NMDA, N-methyl-D-aspartate; NS, neurosphere; PD, Parkinson’s disease; PTZ, phenothiazines; SAR, structure-activity relationships; TCA, tricyclic antidepressants; TREM2, Triggering Receptor Expressed on Myeloid cells 2.

REFERENCES


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