Synthesis, biological evaluation, and molecular modeling of nitrile-containing compounds: Exploring multiple activities as anti-Alzheimer agents

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Abstract

Based on the MAO inhibition properties of aminoheterocycles with a carbonitrile group we have carried out a systematic exploration to discover new classes of carbonitriles endowed with dual MAO and AChE inhibitory activities, and Aβ anti-aggregating properties. Eighty-three nitrile-containing compounds, 13 of which are new, were synthesized and evaluated. In vitro screening revealed that 31, a new compound, presented the best lead for trifunctional inhibition against MAO A (0.34 μM), MAO B (0.26 μM) and AChE (52 μM), while 32 exhibited a lead for selective MAO A (0.12 μM) inhibition coupled to AChE (48 μM) inhibition. Computational analysis revealed that the malononitrile group can find an advantageous position with the aromatic cleft and FAD of MAO A or MAO B. However, the total binding energy can be handicapped by an internal penalty caused by twisting of the ligand molecule and subsequent disruption of the conjugation (32 in MAO B compared to the conjugated 31). Conjugation is also important for AChE as well as the hydrophilic character of malononitrile that allows this group to be in close contact with the aqueous environment as seen for 83. Although the effect of 31 and 32 against Aβ1-42, was very weak, the effect of 63 and 65, and of the new compound 75, indicated that these compounds were able to disaggregate Aβ1-42 fibrils. The most effective was 63, a (phenylhydrazinylidene)propanedinitrile derivative that also inhibited MAO A (1.65 μM), making it a potential lead for Alzheimer’s disease application.

KEYWORDS:
nitrile-containing compounds, Alzheimer’s disease, MAO inhibitors, AChE inhibitors, Aβ1-42 disaggregating agents, in silico study
1 INTRODUCTION

Alzheimer’s disease (AD), the most common neurodegenerative disease, is marked by progressive decline in a variety of higher cortical functions. AD patients share common clinical and neuropathological features, notably intracellular neurofibrillary tangles and extracellular senile plaques, the two major pathological hallmarks (Korabecny et al., 2018). Neurofibrillary tangles are formed by hyperphosphorylated tau protein, while senile plaques are aggregates of amyloid β (Aβ) peptides associated with several proteins, metal ions, and enlarged axons and synaptic terminals. Moreover, reduced levels of acetylcholine (ACh) in specific brain regions that mediate memory and learning functions, loss of neurons and synapses, high levels of metal ions like Cu$^{2+}$, Zn$^{2+}$, Fe$^{3+}$, mitochondrial dysfunction, inflammation and the associated oxidative stress (OS) represent features of this multifactorial disease (Das & Basu, 2017; Jakob-Roetne & Jacobsen, 1992). Acetylcholinesterase (AChE) hydrolyzes extracellular ACh into choline and acetic acid, thus preventing the overstimulation of nerve cells. However, in AD, loss of cholinergic neurons means that stimulation of nerve cells by ACh is reduced, and neuronal communication is hindered. AChE inhibitors improve neuronal communication by preventing the breakdown of ACh (Das & Basu, 2017).

Noncatalytic functions of AChE have been suggested in multiple physiological processes, such as cell adhesion, neurite growth, apoptosis, and Aβ aggregation (Wang & Zhang, 2019). AChE interacts with Aβ by the hydrophobic environment close to the peripheral anionic site (PAS) (Johnson & Moore, 1999; Inestrosa et al., 1996; De Ferrari et al., 2001), forming stable AChE-Aβ complexes, (Alvarez et al., 1997; Alvarez et al., 1998) and causing an increase in the neurotoxicity of the Aβ fibrils (Muñoz & Inestrosa, 1999; Reyes et al., 2004). In addition, AChE activities have also been reported to correlate with the density of Aβ deposition in the AD brain (Arendt et al., 1992). Recent experiments suggest that during the early stage of AD, Aβ enters the mitochondria increasing the generation of free radicals and might induce OS. Aβ and amyloid precursor protein (APP) were also reported in the mitochondrial membranes of post-mortem brain and found to be responsible for disruption of electron transport chain to cause irreversible neurodegeneration and cell damage (Sharma et al., 2019).

Monoamine oxidase (MAO) is the major metabolic enzyme responsible for the deactivation of the monoamine neurotransmitters generating the corresponding aldehyde, ammonia, and hydrogen peroxide as metabolic products (Ramsay, 2016). Since MAO catalytic activity produces hydrogen peroxide, the increase in MAO B activity in AD and Parkinson’s disease (PD) brain tissues is considered to contribute to the initiation of OS and the consequent deleterious effects to the cell. Thus, in addition to their ability to elevate the levels of monoamines in the brains of AD patients, both MAO A and B inhibitors may prevent OS by decreasing the formation of reactive oxygen species (Ramsay, 2016; Ramsay & Tipton, 2017). MAO B expression is generally elevated in AD (Kennedy et al., 2003), decreasing monoamine levels. Some work proposes that MAO B is also suggested to associate with β-secretase, γ-secretase and to regulate neuronal Aβ peptide levels (Hroudova et al., 2016; Schedin-Weiss et al., 2017). Elevated MAO could be also associated with the formation of neurofibrillary tangles (Hroudova et al., 2016). MAO A also influences monoamine levels, particularly serotonin in depression, (Bortolato & Shih, 2011). MAO A levels, regulated by genetic and environmental factors, including stress, hormonal deregulation and food influences, are up or down or unchanged in various brain areas (Naoi et al., 2018). Recent evidence suggests that MAO-A generated ROS is involved in quality control signaling in mitochondria (Ugun-Klusek et al., 2019; https://doi.org/10.1016/j.redox.2018.10.003).
However, the role of MAO A in the trafficking and the health (and hence energy output) of mitochondria is still poorly understood. In AD patients, MAO A level changes seem to involve multiple mechanisms influencing cell survival (Cai, 2014). Due to the multifactorial nature of AD, design strategies have been envisaged for the development of different series of multifunctional drugs. In addition to MAO inhibition, multitarget-directed ligand (MTDL) properties can include brain permeable iron chelation, ChEs inhibition, anti-Aβ aggregation, and neuroprotection among other properties (Unzeta et al., 2016).

Previous studies have indicated that the MAO inhibitory activity of several compounds is potentiated by cyanide (Davison, 1957; Ramadan et al., 2007). The potentiating effect of cyanide can be ascribed to an activation mechanism whereby cyanide assists the binding of the inhibitor to the enzyme (Davison, 1957; Juárez-Jiménez et al., 2014). A series of carbonitrile-containing amino heterocycles were examined experimentally and computationally to explore the role of nitriles in determining the inhibitory activity against MAO. Dicarbonitrile aminofurans were found to be potent, selective inhibitors against MAO A (Juárez-Jiménez et al., 2014). An earlier study examined the inhibition of MAO B by a series of C4-substituted phthalonitriles and a series of homologs lacking the nitrile units (Manley-King et al., 2012). It was found that the nitrile unit was a prerequisite for high binding affinity to both MAO A and MAO B. The high binding affinities to MAO B were ascribed to the highly polar nature of the nitrile group. Modeling studies indicated that the nitrile group interacts with the polar regions in the substrate cavity of the MAO B enzyme (Manley-King et al., 2012; Van der Walt et al., 2012; Chirkova et al., 2015). The carbonitrile function has also been introduced by other research groups in compounds either with the ChEs inhibitory activity (Samadi et al., 2010; Samadi et al., 2011; Samadi et al., 2012; Samadi et al., 2013) or dual ChEs and MAO inhibitory activities (Samadi, Chioua, Bolea et al., 2011).

Since the nitrile group may raise concerns regarding cyanide release, published information on diverse nitrile-containing drugs, research compounds, and in vivo metabolism of carbonitrile derivatives was closely examined. Structurally diverse nitrile-containing pharmaceuticals are prescribed for a variety of medical treatments, for example the selective serotonin re-uptake inhibitor and antidepressant drug citalopram, the non-purine xanthine oxidase inhibitor for the treatment of hyperuricemia febuxostat, the aromatase inhibitor and anticancer agent letrozole, and the non-nucleoside reverse transcriptase inhibitors and anti-HIV drug etravirine (Fleming et al., 2010; Jones et al., 2010). Regarding letrozole and etravirine, both exhibit two nitrile units as does the analog of etravirine, rilpivirine (Reznicek et al., 2017). In drug design, aromatic chloride is very often replaced by an aromatic nitrile since this exchange can impart desirable properties to the resulting molecule particularly when the potency is similar, or even improved. Thus, the lipophilicity of the molecule will be significantly reduced along with enhanced metabolic stability, higher aqueous solubility and reduced toxicological outcomes (Jones et al., 2010). Due to its small size, metabolic stability and ability to act as a hydrogen bond acceptor, the nitrile functional group has been incorporated into several pharmaceutical agents (Lindsay-Scott & Gallagher, 2017). A quantitative assay involving the reaction of nitriles with glutathione and cysteine has been used as a simple in vitro screen to assess potential toxicity risk of candidate compounds in drug discovery and decide whether to progress with compounds in the absence of radiolabeling studies (MacFaul et al., 2009).

Regarding metabolic stability, the nitrile group is not particularly electrophilic towards free nucleophiles, even glutathione (Fleming et al., 2010; MacFaul et al., 2009) and is metabolically quite robust (Boyd et al., 2009) unless activated by adjacent electron-
withdrawing elements (Oballa et al., 2007). Odanacatib, a selective inhibitor of cathepsin K, exhibited excellent metabolic stability in hepatocyte incubations across several species. In standard incubations (2x10⁶ cells/mL, 20 μM, 2 h), a 96% recovery of the parent compound was found in rat hepatocytes and a 98% recovery was obtained in rhesus monkey hepatocytes. High recovery (>99%) was observed in both dog and human hepatocyte incubations (Gauthier et al., 2008). In most nitrile-containing drugs, the nitrile group passes through the body metabolically unaffected. Cimetidine, an H₂ receptor antagonist, after both oral and intravenous doses in man, is oxidized to the sulfoxide (6-10%) and hydroxymethyl (4%) metabolites and conjugated to the N-glucuronide (24%), presenting unchanged drug (60-70%) (Strong & Spino, 1987). The biotransformation of entacapone, a catechol O-methyltransferase inhibitor was studied in experimental animals and man by identifying their O-glucuronides in urine and plasma; in rats and dogs, O-sulfates were also abundant (Wikberg & Vuorela, 1994). Early studies with radiolabelled cromakalim, an antihypertensive agent, showed the major urinary metabolites had unchanged nitrile groups (Kudoh, 1990; Kudoh et al., 1990). Lersivirine, a non-nucleoside reverse transcriptase inhibitor, is predominantly cleared by glucuronidation and cytochrome P450-mediated oxidation whereas hydrolysis of one of the nitrile moieties to form an amide represented less than 8% of the total in plasma (Vourvahis et al., 2010). The major urinary and plasma metabolites of verapamil, a phenethylamine vasodilator, were the N-dealkylated products which retain the α-isopropylphenylacetonitrile portion of the molecule (McIlhenny, 1971).

Release of cyanide from aromatic or fully substituted carbons is not observed (Fleming et al., 2010). Rabbits converted 2,6-dichlorobenzonitrile to an extent of approximately 2% and 23% into 2,6-dichloro-4-hydroxybenzonitrile and 2,6-dichloro-3-hydroxybenzonitrile, respectively. The hydrolysis of the nitrile group appears to occur to a very minor extent. Only insignificant amounts of the amide (<0.01%) and benzoic acid (<0.001%) derivatives were found. This was supported by the observation that rabbits did not convert 2,6-dichlorobenzamide into the corresponding acid (Wit & van Genderen, 1966). However, saturated and β,γ-unsaturated aliphatic nitriles are hydroxylated in the liver at the α-carbon to form cyanohydrins with subsequent cyanide release (Hideji & Kazuo, 1984, 1986). Mandelonitrile, an agent formerly used for the treatment of urinary tract infections, bears a H atom and OH group at the α-carbon. The cyanide plus thiocyanate excreted by Wistar rats represented 86% of the dose, hippuric acid corresponded to 71%, while 13% correlated to mandelic acid (Singh et al., 1985). Epoxidation of alkenenitriles and ring opening can potentially liberate cyanide, but epoxidation is synthetically difficult (Pryde et al., 1996; Aiiai et al., 1995; Miyashita et al., 1987) and metabolism at other sites appears more likely to occur (Fleming et al., 2010).

Based on the MAO inhibition properties of aminofurans with the carbonitrile group (Juárez-Jiménez J. et al., 2014), the present study aimed to identify classes of nitrile-containing compounds endowed with dual AChE and MAO inhibitory activities, and Aβ anti-aggregating properties. In this context, we have carried out the synthesis, biological evaluation, and molecular modeling of a number of arylidenepropanedinitriles [compounds 16-41, Scheme 1, Tables 1, 7, and Tables S1, S5, S8, and S9 (Supporting Information)], [(phenylamino)methylidene]propanedinitriles [compounds 42-52, Scheme 2, Table 2 and Table S6 (Supporting Information)], 4-amino-1-phenyl-1H-pyrrole-3-carbonitriles [compounds 53-57, Scheme 3, Table 3, and Tables S3, and S10 (Supporting Information)], and 3-amino-1-phenyl-1H-pyrrole-2,4-dicarbonitriles [compounds 58-62, Scheme 3, Tables 3, 7, and Tables S7, and S10 (Supporting Information)], (phenylhydrazinylidene)propanedinitriles [compounds 63-67, Scheme 4,
Tables 4, 7, and Table S4 (Supporting Information), 4-arylazo-3,5-diamino-1H-pyrazoles [compounds 68-72, Scheme 5, Table 5 and Table S5 (Supporting Information)], and (E,E)-4-amino-1-aryl-3-cyano-4-methoxy-2-azabutadienes (compounds 73-75, Scheme 6, Table 6).

2 MATERIALS AND METHODS

Materials and methods for Chemistry and Pharmacology are reported in Supporting Information. Caution: Storage and safety working conditions regarding malononitrile, arylidenemalononitriles and some nitrile-containing chemicals are mentioned in Supporting Information.

3 RESULTS AND DISCUSSION

3.1 Chemistry

3.1.1 Synthesis of arylidenepropanedinitriles

Arylidenepropanedinitriles 3 (compounds 16-41) (Scheme 1) were synthesized by straightforward Knoevenagel condensation of the respective aldehyde 1 with malononitrile 2 (Gazit et al, 1989). Most compounds are common starting materials or intermediates in chemical reactions (Zayed et al., 1991; Marco et al., 2004; Ding & Zhao, 2010). Interesting results were also made known regarding their capacity to activate cell resistance to oxidative stress (Turpaev et al., 2011). Here we report the MAO and AChE inhibitory properties of a few arylidenepropanedinitriles and chemical data of the new arylidenepropanedinitriles 31, 34, 35 and 36 (Scheme 1; Figure 1).

Scheme 1 here

3.1.2 Synthesis of [(phenylamino)methylidene]propanedinitriles

[(Phenylamino)methylidene]propanedinitriles type 6 (compounds 42-52) (Scheme 2) were readily synthesized by the reaction of malononitrile 2 with ethyl orthoformate 4 and the respective aniline derivative 5 (Wolfbeis, 1981). The MAO and AChE inhibitory activities are reported here as well as the data for the new [(phenylamino)methylidene]propanedinitrile derivatives 45, 48, and 52 (Scheme 2; Figure 1).

Scheme 2 here

3.1.3 Synthesis of 4-amino-1-phenyl-1H-pyrrole-3-carbonitriles and 3-amino-1-phenyl-1H-pyrrole-2,4-dicarbonitriles

The syntheses of both 4-amino-1-phenyl-1H-pyrrole-3-carbonitriles (compounds 53-57) and 3-amino-1-phenyl-1H-pyrrole-2,4-dicarbonitriles (compounds 58-62) are represented in Scheme 3 (compounds type 8). These syntheses were based on a Thorpe-Ziegler cyclization via intermediate 7 using [(phenylamino)methylidene]propanedinitrile derivatives 6 as precursors (Scheme 2), an α-halo compound (chloroacetanitrile,
chloroacetone, ethyl bromoacetate, α-bromoacetophenone) and triethylamine. (Salaheldin et al., 2008; Unverferth et al., 1998; Salaheldin et al., 2010). Pharmacological data are reported concerning MAO inhibitory activities of type 8 compounds. Additionally, chemical data are addressed to the new compounds 59 and 60 (Scheme 3; Figure 1).

Scheme 3 here

3.1.4 Synthesis of (phenylhydrazinylidene)propanedinitriles
Phenylhydrazonomalononitriles 11 (compounds 63-67) (Scheme 4) were prepared by diazotization of the appropriate arylamine 9, followed by condensation of the diazonium salt 10 with malononitrile (Krstof et al., 2006). Compounds 63 (Krstof et al., 2006), 64 (Gavlik et al., 2017), 65 (Amer et al., 2004) and 66 (Tsai & Wang, 2005) are reported in the literature while (phenylhydrazinylidene) propanedinitrile 67 (Scheme 4) is a new compound which is described here for the first time. Pharmacological data are reported concerning MAO inhibitory activities of compounds type 11. Additionally, chemical data are provided for the new compound 67 (Scheme 4; Figure 1).

Scheme 4 here

3.1.5 Synthesis of 4-arylazo-3,5-diamino-1H-pyrazoles
The 4-arylazo-3,5-diamino-1H-pyrazoles 12 (compounds 68-72) were obtained by cyclocondensation of phenylhydrazonomalononitriles 11 (compounds 63-67) with hydrazine (Krstof et al., 2006). Compounds 68 (Krstof et al., 2006; Al-Afaleq, 2000; Elgemeie et al., 2001), 69 (Al-Afaleq, 2000; Elgemeie et al., 2001), 70 (Huppatz et al., 1981), and 71 (Zhang et al., 2001) are reported in the literature while diaminopyrazole 72 (Scheme 5; Figure 1) is described here for the first time.

Scheme 5 here

3.1.6 Synthesis of (E,E)-4-amino-1-aryl-3-cyano-4-methoxy-2-azabutadienes
The (E,E)-4-amino-1-aryl-3-cyano-4-methoxy-2-azabutadienes 15 (compounds 73-75) (Scheme 6) were synthetized by reaction of benzaldehyde (13: X=H) or its derivatives with aminomalononitrile p-toluenesulphonate (14) (Scheme 6) (Gutch et al., 2001). The synthesis of compound 73 is reported (Gutch et al., 2001). Compounds 74 and 75 (Scheme 6) are new. Here we report the chemical data and the MAO inhibitory activities for the new compounds 74 and 75 (Scheme 6; Figure 1).

Scheme 6 here

Figure 1 represents the chemical structures of all the new compounds (31, 34, 35, 36, 45, 48, 52, 59, 60, 67, 72, 74, 75).
3.1.7 Diverse pyrazole derivatives (compounds 76-81) (Silva et al., 2010, Silva et al., 2011) and (phenoxyalkoxybenzylidenemalononitriles (compounds (81, 82) (Silva et al., 2013) were previously synthesized and their AChE inhibitory activities reported. Here we describe their inhibitory activities in rat mitochondria (rMAO A and rMAO B) (Table S2, Supporting Information).

3.2 Pharmacology

3.2.1 Monoamine oxidase inhibition

Compounds were screened for MAO inhibition using various assays: direct radiometric (rat mitochondrial MAO) or spectrophotometric (purified human MAO) assays or coupled fluorescent assays (any preparation). Initial screening was performed at fixed concentrations (10 or 25 μM) with 2xK_M substrates. Where inhibition greater than 70% was observed, IC_{50} values were measured. The IC_{50} values for selected inhibitors were re-evaluated in the coupled assay with horseradish peroxidase, measuring the fluorescence of resorufin produced in the presence of 2xK_M tyramine as substrate.

Table 1 shows IC_{50} values for purified human MAO A obtained using the spectrophotometric assay with kynuramine as the substrate. For the arylidene-propanedinitrile derivatives, an electron-rich substituent (F, Cl, NO_2) at the para position is detrimental to inhibition, although m-NO_2 is tolerated. Methoxy and propenyloxy substituents in the para position increase binding (19, 22) but the benzyl ring substituent in 23 prevents binding. Changing the core benzyl ring to furan gives better inhibition (30, 31 and 32).

Only one of the [(phenylamino)methylidene]propanedinitrile derivatives was active, presumably due to interactions conferred by the m-NO_2 group (49 in Table 2).

The phenylpyrrole mono- and dicarbonitriles (Table 3) were likewise poor inhibitors with only the dicarbonitrile 60 active with an IC_{50} of 6.2 μM.

The (phenylhydrazinylidene)propanedinitrile derivatives 63-67 (Table 4) were more promising with 5 active compounds. Methyl substitution in 67 was not good but the parent compound 65, the p-Cl 66 and di-methoxy 64 derivatives gave IC_{50} values around 10 μM. The best inhibitor in this series was 63, the m-NO_2 compound with an IC_{50} value of 1.65 μM.

Switching the dinitrile to a pyrazole group (Table 5) did not improve inhibition. The only active compound, the p-Cl derivative 69 was not much changed from the p-Cl derivative in the dinitrile series (66 in Table 4).

Similarly, for the aminomethoxy compounds in Table 6, only one active compound was found. The 4-CN derivative 75 gave an IC_{50} value of 1.92 μM against human MAO A, 100 times better that the parent compound 73.
Since behavioral tests are performed on rodents, some compounds were also evaluated on MAO A and MAO B present in rat liver mitochondria (RLM) as presented in the Supplementary Information. Table S1 shows the IC$_{50}$ values for arylidenepropanedinitrile derivatives on RLM, assaying MAO A with [14C]-serotonin (5-HT) and MAO B with [14C]-β-phenylethylamine (PEA). Although 16, 20, and 26 were poor inhibitors as in purified hMAO A (around 60 μM, Table 1), 20 with two methoxy substituents was a better inhibitor than the others in the RLM MAO A assay (IC$_{50}$ 15.9 μM). Compounds 24 and 25 also gave similar IC$_{50}$ values against both human and rat MAO A. The RLM study allows direct comparison of selectivity between MAO A and B (Table S1). Although both 19 and 20 inhibit RLM MAO A, the extra m-OCH$_3$ in 20 removed the inhibition of MAO B seen with the mono-OCH$_3$ compound (19), making 20 selective for MAO A. The m-NO$_2$ derivative (25) is the best non-selective inhibitor of rat MAO, but moving the NO$_2$ to the para position (24) makes the compound selective for MAO B. Thus, in rat 25 is non-selective whereas 20 is MAO A-selective and 24 is B-selective, suggesting that discrimination is based on fine steric or electronic interaction in the active sites. None of the pyrazole derivatives (compounds 76-81) and the phenoxyalkoxybenzylidenemalononitriles (compounds 81, 82) shown in Table S2 inhibited rMAO in RLM, but the arylidenepropanedinitrile series in Table S3 revealed some inhibition. rMAO A was inhibited by the rigid 40 (IC$_{50}$ = 18 μM) but more effectively by the flexible structures in 22 (3.5 μM) and 30 (0.72 μM). These compounds also inhibited rMAO B. The most effective on rMAO B were 23 (1.2 μM), and 28 (7.5 μM), neither of which inhibited MAO A in rat mitochondria.

Returning to human MAO, the inhibition of MAO A and MAO B was compared for several compound series using the coupled assay for hydrogen peroxide production. Table S4 reports the % inhibition at 25 μM and IC$_{50}$ values for (phenylhydrazinylidene)propanedinitrile compounds. Although di-methoxy substitution in 64 gives equal inhibition of MAO A (IC$_{50}$ = 22 μM) and MAO B (25 μM), the m-NO$_2$ (63) is 10 times better against MAO B (0.52 μM) than against MAO A (5.8 μM). In the same range (IC$_{50}$ values less than 10 μM), but non-selective, are 68-72 in Table S5, showing that the diaminopyrazole ring gives better inhibition than the dinitriles (63-67 in Table S4). Table S6 confirms poor inhibition of human MAO by [(phenylamino)methylidene]propanedinitriles with none inhibiting MAO A and only two
inhibiting MAO B (45 at 4.27 μM and 52 at 1.66 μM). Table S7 shows that the 4-amino-1-phenyl-1H-pyrrole-3-carbonitriles did not inhibit human MAO B, but some of the dicarbonitriles inhibited by about 50% at 25 μM. Dicarbonitrile 60 did not inhibit MAO B but was the only compound in Table S7 that inhibited MAO A (44%). Table S8 identified two arylidenepropanedinitriles that almost completely inhibited MAO A at 25 μM, namely 31 and 32 that share a furan linker.

Some compounds were also screened for inhibition of AChE (commercially cloned and purified electric eel AChE) at 10 μM and 5 μM (arylidenepropanedinitriles in Table S9 and phenylpyrrole aminocarbonitriles in Table S10). Only arylidenepropanedinitrile 60 (58% at 5 μM) and the phenylpyrrole-aminodicarbonitrile 62 (69% at 10 μM) gave inhibition. Otherwise these series were not inhibitory towards AChE.

Thus, compound 31 presents a lead for trifunctional inhibition against MAO A, MAO B and AChE (Table 7), while 32 offers a lead for selective MAO A inhibition coupled to AChE inhibition.

Table 7 here

After the primary screening in Tables 1-6 and Tables S1-S10 in Supplementary Information, a set of compounds was re-evaluated against all three human enzyme targets. Table 7 summarizes the IC₅₀ values for the human MAO and AChE enzymes with the selected set of active compounds. Adding a nitrogen to the linker between the phenyl ring and the dicarbonitrile group (25 vs 49) loses MAO A inhibition but adding two nitrogens (63) improves inhibition of both MAO A and B and introduces some weak inhibition of AChE (IC₅₀ = 44 μM). A furan linker again gives best lead compounds: 31 is a good inhibitor of both MAO A (0.34 μM) and MAO B (0.26 μM), whereas the trifluoro derivative (32) is 100-fold selective for MAO A (0.12 μM vs 15 μM for MAO B). Both 31 and 32 show some inhibition of AChE (IC₅₀ values of 52 and 48 μM, respectively), so are lead compounds to optimize for multi-target activity.

3.2.2. Thioflavin T (ThT) assay to study Aβ aggregation

For this assay four arylidenepropanedinitriles (31, 32, 33, 37), two (phenylhydrazinylidene)propanedinitriles (63, 65), and one representative of (E,E)-4-amino-1-aryl-3-cyano-4-methoxy-2-azabutadienes (75) were chosen. The capacity of these compounds to inhibit the formation of Aβ₁₋₄₂ fibrils (generated as described in Experimental section (Supporting Information) was evaluated using the thioflavin T (ThT) fluorescence assay. This assay measures changes in fluorescence intensity of ThT upon binding to amyloid fibrils (Le Vine, 1999; Sulatskaya et al., 2012).

Figure 2 here

As can be seen in Figure 2, different concentrations (20, 40 and 60 μM) of compounds 31 (p-OCH₃ electron donating group and a m-Cl electron withdrawing group) and 32 (m-CF₃ strong electron withdrawing group) slightly inhibited the formation of Aβ₁₋₄₂ fibrils, by about 25% and an insignificant 15%, respectively.

The pyrrole derivatives 33 and 37 (Figure 2) differed only in the C₅-CN electron withdrawing group in 37. Both compounds were ineffective in disaggregating Aβ₁₋₄₂ fibrils formation, being less adequate than 31 and 32 (Figure 2).
By contrast, compounds 63 and 65 (Figure 3), which differ only in the m-NO₂ electron withdrawing group present in 63, were able to disaggregate Aβ₁-42 fibrils. The most effective was 63 (inhibitor effect of 50-60%, depending on the concentration), whereas 65 (Figure 3), and 75 (Figure 3) (p<0.05) inhibited Aβ aggregation only at high concentrations (40 and 60 µM).

Figure 3 here

3.2.3 Computational chemistry studies

3.2.2.1 Docking studies on monoamine oxidases

For our study we chose the derivatives 25, 30, 31, 32, 49, 63, and 65. From these compounds only 49, 63 and 65 form tautomers with the most probable ones shown in Figure 4. The binding energies values obtained for individual structures with MAO A and MAO B are represented in Table 8.

Figure 4 here

Table 8 here

The analysis of the inhibitor locations in the MAO A model provided several common features. If present, it is the nitro group which appears to be responsible for the interaction of the active derivatives (Figure 5). The comparison of the structurally similar compounds 49, 63 and 65 showed that two inhibitors (49 and 63) directed the nitro group towards FAD, whereas for 65 (no nitro group) the dicarbonitrile group is directed towards FAD.

Figure 5 here

The main type of the interaction between the active inhibitor and FAD is a cation–π relationship, which is a kind of electrostatic interrelation (cation–quadrupol). Positive charge is located at nitrogen of nitro group and the π orbital is located at FAD; partial (but much lower) positive charge is located on the central carbon connected to the dicyano group (Figure 6). The value of the positive charge is also affected by other substituents, e.g. for 49 the atomic charge N (nitro) is diminished to 0.713 (Figure 7).

Figure 6 here
These specific features impacted the values of total binding energies (Table 8), which were higher for compound 63 due to a shorter distance from FAD (3.5Å for 63 and 3.7Å for 49) as well as two additional interactions more (cation-π interaction with Tyr407 and hydrophobic interaction with Tyr444).

Another important factor affecting the ligand interaction is the presence and location of water molecules. The original ligand harmine has water molecules near it in the binding site. The docking procedure including water molecules is rather peculiar, so water was included after docking in the procedure of complex optimization. The addition of water was important to explain the lower activity of 30 (Figure S1). Although it interacts with Gln215, binding of 30 is hindered by more effective interaction of water molecule (HOH5342) with FAD.

Inspecting the binding modes of the strongest MAO A inhibitors 31 and 32 (Figure 8), it can be seen that the benzene ring of 31 is situated near the aromatic cleft of TYR 407 and TYR 444, but the malononitrile group of 32 is more deeply immersed into this region, at a distance of 2.99 Å from C10a of flavin. Docking and optimization studies provided two preferred orientations for both compounds: one with the malononitrile group near flavin and the second one with opposite direction. It seems that it is the substitution on the benzene ring which specifies the orientation of arylidenepropanedinitriles 31 and 32 as well as that for the (phenylhydrazinylidene)propanedinitriles 63, 65 and [(phenylamino)methylidene]propanedinitrile 49. Nitro and methoxy groups in positions meta and para of the benzene ring create sufficiently strong interactions with flavin or the aromatic cleft of the catalytic center while trifluoromethyl or no substitution permits the molecule to turn malononitrile group towards flavin. The abundant numbers of attractive interactions of 31 and 32 are reflected in values of binding energy (Table 8).

The inhibition activities of compounds 31 and 32 in MAO B were different from those in MAO A. The activity of 31 stayed almost the same, while IC50 for 32 decreased in two orders from 0.12 µM to 15.2 µM. (Table 7). Docking calculations and subsequent optimization revealed the opposite orientation of the molecule 31 in MAO B compared with MAO A (Figures 8 and 9).

Both compounds interact with the gating residues Ile199 and Tyr326, which separate entrance and substrate cavities in MAO B (Milczek et al., 2011). However, in MAO B, the position of the conjugated malononitrile groups in the vicinity of the aromatic cleft was strengthened by close contact of one cyano nitrogen with hydroxyl group of Cys172 for 31 and by the interaction with internal water near FAD for 32. Due to the surrounding
residues and water, the furan ring and the malononitrile group of 32 are visibly turned against the substituted benzene ring (sum of both deviations is 44°). This fact breaks the conjugation in 32 and, finally, penalizes the position of 32 in MAO B when compared with 31, which is turned less (22°).

The water molecules can provide additional interactions for ligand and stabilize or destabilize its position. Water found in the binding of 32 in MAO B pocket is positionally similar to the water molecule participating in catalysis. So far, there has been no structural work on MAO to show the positioning of ligands with the malononitrile group in the active site.

A similar binding mode can be observed for 63. This compound is depicted in Figure S2 for the binding sites of MAO A and MAO B. The nitro group of 63 adjacent to FAD in both types of MAO is preferred, although for both enzymes reverse positions also exist (Figure S3), but with lower values of binding energy. (The differences in values of binding energy were 20.5 kJ/mol for MAO A and 4.3 kJ/mol for MAO B.). Compound 63 is bound in MAO A with Tyr407 by π-π and cation-π interaction and with FAD by cation-π interaction with distance 4.1 Å. The position is stabilized by H-bonds with Phe208 and one surrounding water molecule, as well as a hydrophobic interaction with Gln215. However, both H-bonds make the position less energetically favorable due to the twisting of malononitrile group. Interaction with Phe208 in MAO A or Ile199 in MAO B has been related to selectivity of compounds either in the role of inhibitors (Morón et al., 2000) or as substrates (Tsugeno & Ito, 1997). The stronger inhibition of MAO B with 63 was visualised as a firm position of nitro group between Tyr398 and Tyr435, H-bond with Tyr188 and π-π edge-to-edge interaction with FAD (distance 3.7 Å). The malononitrile group is held by a single H-bond with a water molecule occurring in the cavity. The resulting position keeps the whole molecule in one plane, contributing thus to the overall benefit of this mode of interaction.

For further work, the affinity of 63 towards MAO B could be enhanced by a proper substitution in 2 or 3 position from the nitro group. An interesting study would be made with a thiol substitution, as in the proximity of these two places there is Cys172, which could thus create a disulfide bond with the ligand. Another possibility would be to introduce a benzyl or phenyl group in position 1, to obtain the π-π interaction with Tyr435.

### 3.2.2.2 Docking studies on acetylcholinesterase

Docking and optimization calculations were performed to determine the binding mode of ligands to AChE for compounds 32 (Table S9), 37 (Table S9, Figure S4), 62 (Salaheldin et al., 2008) (Table S10), 82 [IC$_{50}$ (AChE = 6.0 ± 0.5 µM)] (Silva et al. 2013), (Table S2, Figures 10, 11), and 83 [IC$_{50}$ (AChE = 2.1 ± 0.5 µM)] (Silva et al. 2013) (Table S2, Figures 10, 11). In the figures, all the compounds are rendered as balls and sticks. The side chains conformations of the mobile residues are illustrated in the same color (light blue) as the ligand. Different subsites of the active site are colored as follows: catalytic triad (CT) in green, oxyanion hole (OH) in pink, anionic subsite (AS) in orange except Trp86, acyl binding pocket (ABP) in yellow, peripheral anionic site (PAS) in cyan and the non-active site residues in grey. Hydrogen bonds are represented as yellow dashed lines, π-π interactions as red lines, π-cation interactions as blue lines and hydrophobic contacts as green ones.
Table S12 summarizes the values of binding energy ($E_{bin}$) together with the inhibition activities of the chosen compounds. The binding energy values for 32 and 62 correspond to their lower activities, but 37 should be as strong inhibitor as 82 and 83 according to $E_{bin}$ value. This compound is immersed in the ligand cavity with both malononitrile and cyano groups (Figure S4) bound to Trp86 ($\pi$-$\pi$ interaction), Phe297 (hydrophobic) and Glu204 (H-bond). According to these interactions, 37 should be much stronger inhibitor than it was measured. The reason for its lower activity could be found in the value of the solvation energy (Table S11), which is very low and thus represents quite a big value of desolvation penalty in total ligand binding.

Compounds 82 and 83 contain long aliphatic chains and, for both, the malononitrile group represents the hydrophilic part in a generally hydrophobic molecule (Figure 12), which can be observed also in values of solvation energy (Table S11). The length of the aliphatic chain fits in the cavity of hAChE more comfortable for 83 than for 82 (Figure 10). Both compounds are anchored to the cavity by strong interaction between malononitrile group and Trp236 from the PAS region. Malononitrile groups, directed to the outer space, are also bound with surrounding water molecules (Figure 11). The compounds fill major parts of the ligand cavity, 83 more than 82. (Figure 11).

4 CONCLUSIONS

In this paper we have reported the synthesis and biological evaluation of 83 compounds, 13 of which are new. Based on the MAO inhibition properties of the carbonitrile group we carried out a systematic exploration to discover new classes of nitrile-containing
compounds endowed with dual AChE and MAO inhibitory activities, and Aβ anti-aggregating properties. Thus, arylidenepropanedinitriles, [(phenylamino)methylidene]propanedinitriles, 4-amino-1-phenyl-1H-pyrrole-3-carbonitriles, 3-amino-1-phenyl-1H-pyrrole-2,4-dicarbonitriles, (phenylhydrazinylidene)propanedinitriles, 4-arylazo-3,5-diamino-1H-pyrazoles and (E,E)-4-amino-1-aryl-3-cyano-4-methoxy-2-azabutadienes were synthesized and assessed.

From in vitro screening, compound 31 presents the best lead for trifunctional inhibition against MAO A, MAO B and AChE (Table 7), while 32 offers a lead for selective MAO A inhibition coupled to AChE inhibition.

Computational analysis of the binding modes of the compounds demonstrated several characteristic features. The malononitrile group, when not overcome by more attractive groups as nitro or methoxy groups, can find an advantageous position with the aromatic cleft and FAD of MAO A or MAO B. The total binding energy can be handicapped by an internal penalty caused by twisting of ligand molecule and subsequent disruption of the conjugation as happens in 32 with MAO B compared to the conjugated 31. Conjugation is also important for AChE. In addition, the hydrophilic character of malononitrile allows this group to be in close contact with the water environment in the more open binding pocket of AChE, as seen for 83 in AChE.

Although the effect of 31 and 32 against Aβ1-42 was very weak, the effect of the other compounds was quite comparable to the action observed for resveratrol (20 µM), used as a reference disaggregating compound (Silva et al. 2013). Overall, the data indicated that compounds 63, 65 and 75 (Figure 3) were able to disaggregate Aβ1-42 fibrils. The most effective was 63, a (phenylhydrazinylidene)propanedinitrile derivative that also inhibited MAO A at micromolar concentration, making it a potential lead for Alzheimer’s disease application.

CONFLICT OF INTEREST
The authors declare no conflict of interest in this research work.

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

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REFERENCES
amyloid-β-peptide change the biochemical properties of the enzyme and increase the neurotoxicity of Alzheimer’s fibrils. *Journal of Neuroscience*, 18(9), 3213-3223.


Vourvahis, M., Gleave, M., Nedderman, A.N.R., Hyland, R., Gardner, I., Howard, M., …LaBadie, R. (2010). Excretion and metabolism of Lersivirine (5-\{3,5-diethyl-1-(2-hydroxyethyl)(3,5-\textsuperscript{14}C\textsubscript{2})-1H-pyrazol-4-yl\}oxy}benzene-1,3-dicarbonitrile), a


TABLE 1  IC<sub>50</sub> values (µM) for inhibition of human MAO A by arylenepropanedinitriles 16-41

<table>
<thead>
<tr>
<th>Compound</th>
<th>X, Y, Z</th>
<th>hMAO A IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>X,Y,Z=H</td>
<td>60.7±11.4</td>
</tr>
<tr>
<td>17</td>
<td>X,Y=H; Z=CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>&gt;100</td>
</tr>
<tr>
<td>18</td>
<td>X=CH&lt;sub&gt;3&lt;/sub&gt;; Y,Z=H</td>
<td>71.3±362</td>
</tr>
<tr>
<td>19</td>
<td>X,Y=H; Z=OCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>21.4±2.70</td>
</tr>
<tr>
<td>20</td>
<td>X=H; Y,Z=OCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>58.0±12.0</td>
</tr>
<tr>
<td>21</td>
<td>X,Y,Z=OCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>288.0±33.6</td>
</tr>
<tr>
<td>22</td>
<td>X,Y=H; Z=OCH&lt;sub&gt;2&lt;/sub&gt;CH=CH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>11.3±1.08</td>
</tr>
<tr>
<td>23</td>
<td>X,Y=H; Z=OCH&lt;sub&gt;3&lt;/sub&gt;Ph</td>
<td>&gt;100</td>
</tr>
<tr>
<td>24</td>
<td>X,Y=H; Z=NO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>&gt;100</td>
</tr>
<tr>
<td>25</td>
<td>X,Z=H; Y=NO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>9.05±0.091</td>
</tr>
<tr>
<td>26</td>
<td>X,Y=H; Z=Cl</td>
<td>68.9±4.70</td>
</tr>
<tr>
<td>27</td>
<td>X,Y=H; Z=F</td>
<td>258.0±68.8</td>
</tr>
<tr>
<td>28</td>
<td>X,Y=H; Z=CN</td>
<td>27.4±7.40</td>
</tr>
<tr>
<td>29</td>
<td>X=CN; Y,Z=H</td>
<td>&gt;100</td>
</tr>
<tr>
<td>30</td>
<td>X,Y=H; Z=NO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>15.7±1.87</td>
</tr>
<tr>
<td>31</td>
<td>X=H; Y=Cl; Z=OCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>2.67±0.274</td>
</tr>
<tr>
<td>32</td>
<td>X,Z=H; Y=CF&lt;sub&gt;3&lt;/sub&gt;</td>
<td>1.43±0.265</td>
</tr>
<tr>
<td>33</td>
<td>X,Y,Z=H</td>
<td>43.4±10.6</td>
</tr>
<tr>
<td>34</td>
<td>X,Z=H; Y=OCH&lt;sub&gt;3&lt;/sub&gt;Ph</td>
<td>71.7±43.4</td>
</tr>
<tr>
<td>35</td>
<td>X=Br; Y,Z=H</td>
<td>15.9±4.07</td>
</tr>
<tr>
<td>36</td>
<td>X,Y=H; Z=CN</td>
<td>66.6±59.2</td>
</tr>
<tr>
<td>37</td>
<td>X,Z=H; Y=CN</td>
<td>166.0±27.2</td>
</tr>
<tr>
<td>38</td>
<td></td>
<td>27.2±4.47</td>
</tr>
<tr>
<td>39</td>
<td></td>
<td>172.0±28.6</td>
</tr>
<tr>
<td>40</td>
<td></td>
<td>13.7±1.06</td>
</tr>
<tr>
<td>41</td>
<td></td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

IC<sub>50</sub> values were determined spectrophotometrically with kynuramine as substrate.
**TABLE 2** IC$_{50}$ values (µM) for inhibition of human MAO A by [(phenylamino)methylidene]propanedinitriles 42-52.

![Chemical structure]

<table>
<thead>
<tr>
<th>X, Y, Z, W, Q</th>
<th>hMAO A IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>42 X,Y,Z,Q,W=H</td>
<td>&gt;100</td>
</tr>
<tr>
<td>43 X=CH$_3$; Y,Z,Q,W=H</td>
<td>&gt;100</td>
</tr>
<tr>
<td>44 X,Q=CH$_3$; Y,Z,W=H</td>
<td>&gt;100</td>
</tr>
<tr>
<td>45 X,Z,Q=CH$_3$; Y,W=H</td>
<td>78.6±70.1</td>
</tr>
<tr>
<td>46 X,Z,Q=H; Y,W=CH$_3$</td>
<td>109±72.6</td>
</tr>
<tr>
<td>47 X,W,Q=H; Y,Z=CH$_3$</td>
<td>108±24.0</td>
</tr>
<tr>
<td>48 X,W,Q=H; Y,Z=OCH$_3$</td>
<td>&gt;100</td>
</tr>
<tr>
<td>49 X,Z,W,Q=H; Y=NO$_2$</td>
<td>9.65±1.36</td>
</tr>
<tr>
<td>50 X,Y,W,Q=H; Z=Cl</td>
<td>&gt;100</td>
</tr>
<tr>
<td>51 X,Z=Cl; Y,W,Q=H</td>
<td>&gt;100</td>
</tr>
<tr>
<td>52 X,Q=CH$_3$; Y,W=H; Z=Br</td>
<td>197±90.7</td>
</tr>
</tbody>
</table>

IC$_{50}$ values were determined spectrophotometrically with kynuramine as substrate.
TABLE 3 IC$_{50}$ values (µM) for inhibition of human MAO A by 4-amino-1-phenyl-1$H$-pyrrole-3-carbonitriles 53-57 and 3-amino-1-phenyl-1$H$-pyrrole-2,4-dicarbonitriles 58-62.

<table>
<thead>
<tr>
<th>X, Y, Z, W, Q, R</th>
<th>hMAO A IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>53 X, Y, Z, W, Q=H; R=CO$_2$Et</td>
<td>36.2±6.91</td>
</tr>
<tr>
<td>54 X, Y, Z, W, Q=H; R=COCH$_3$</td>
<td>&gt;100</td>
</tr>
<tr>
<td>55 X, Y, W, Q=H; Z=Cl; R=CO$_2$Et</td>
<td>92.5±18.9</td>
</tr>
<tr>
<td>56 X, Y, W, Q=H; Z=Cl; R=COCH$_3$</td>
<td>&gt;100</td>
</tr>
<tr>
<td>57 X, Y, W, Q=H; Z=OCH$_3$; R=CO$_2$Et</td>
<td>&gt;100</td>
</tr>
<tr>
<td>58 X, Y, Z, W, Q=H; R=CN</td>
<td>1464±513</td>
</tr>
<tr>
<td>59 X, W, Q=H; Y, Z=CH$_3$; R=CN</td>
<td>113±17.4</td>
</tr>
<tr>
<td>60 X, Z, Q=H; Y, W=CH$_3$; R=CN</td>
<td>6.21±4.69</td>
</tr>
<tr>
<td>61 X, Y, W, Q=H; Z=Cl; R=CN</td>
<td>241±36.3</td>
</tr>
<tr>
<td>62 X, Y, W, Q=H; Z=OCH$_3$; R=CN</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

IC$_{50}$ values were determined spectrophotometrically with kynuramine as substrate.
<table>
<thead>
<tr>
<th>X, Y, Z</th>
<th>hMAO A IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>63 X,Z=H; Y=NO₂</td>
<td>1.65±0.17</td>
</tr>
<tr>
<td>64 X=H; Y,Z=OCH₃</td>
<td>10.7±3.79</td>
</tr>
<tr>
<td>65 X,Y,Z=H</td>
<td>9.57±3.60</td>
</tr>
<tr>
<td>66 X,Y=H; Z=Cl</td>
<td>11.4±6.79</td>
</tr>
<tr>
<td>67 X=H; Y,Z=CH₃</td>
<td>43.5±18.5</td>
</tr>
</tbody>
</table>

IC₅₀ values were determined spectrophotometrically with kynuramine as substrate.
TABLE 5  IC₅₀ values (µM) for inhibition human MAO A by 4-arylazo-3,5-diamino-1H-pyrazoles 68-72.

![Chemical structure](image)

<table>
<thead>
<tr>
<th>X, Y, Z</th>
<th>hMAO A IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>68 X,Y,Z=H</td>
<td>&gt;100</td>
</tr>
<tr>
<td>69 X,Y=H; Z=Cl</td>
<td>19.7±17.9</td>
</tr>
<tr>
<td>70 X,Z=H; Y=NO₂</td>
<td>420±250</td>
</tr>
<tr>
<td>71 X=H; Y,Z=OCH₃</td>
<td>&gt;100</td>
</tr>
<tr>
<td>72 X=H; Y,Z=CH₃</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

IC₅₀ values were determined spectrophotometrically with kynuramine as substrate.
TABLE 6 IC\textsubscript{50} values (µM) for inhibition of human MAO A by (E,E)-4-amino-1-aryl-3-cyano-4-methoxy-2-azabutadienes 73-75.

![Chemical Structure](image)

<table>
<thead>
<tr>
<th>X, Y, Z</th>
<th>hMAO A IC\textsubscript{50} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>73 X,Y,Z=H</td>
<td>251±95.1</td>
</tr>
<tr>
<td>74 X,Y=H; Z=OCH\textsubscript{2}CH\textsubscript{2}</td>
<td>113±130</td>
</tr>
<tr>
<td>75 X,Y=H; Z=CN</td>
<td>1.92±0.927</td>
</tr>
</tbody>
</table>

IC\textsubscript{50} values were determined spectrophotometrically with kynuramine as substrate.
Table 7  IC\textsubscript{50} values (µM) for inhibition of human MAO A, MAO B and AChE by selected compounds (25, 31, 32, 49, 60, 63, 65, 75).

<table>
<thead>
<tr>
<th>Comp.</th>
<th>Structure</th>
<th>IC\textsubscript{50} (µM) after 5 min preincubation</th>
<th>hMAO A</th>
<th>hMAO B</th>
<th>hAChE</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>[Structure]</td>
<td>44.7±20.6</td>
<td>24.6±9.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>[Structure]</td>
<td>0.34±0.05</td>
<td>0.26±0.11</td>
<td>52±8</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>[Structure]</td>
<td>0.12±0.03</td>
<td>15.2±5.3</td>
<td>48±2</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>[Structure]</td>
<td>&gt;100</td>
<td></td>
<td>21.5±2.5</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>[Structure]</td>
<td>(&gt;100)</td>
<td></td>
<td></td>
<td>(&gt;100)</td>
</tr>
<tr>
<td>63</td>
<td>[Structure]</td>
<td>12.2±4.1</td>
<td>3.80±0.31</td>
<td>44±5</td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>[Structure]</td>
<td>22.9±1.5</td>
<td>&lt;10%* at 25 µM</td>
<td>&lt;10%* at 100 µM</td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>[Structure]</td>
<td>3.41±0.83</td>
<td>3.45±0.52</td>
<td>71±10</td>
<td></td>
</tr>
</tbody>
</table>

\textit{ASS234}\textsuperscript{†} 0.053±0.004* 1.2±0.1\textsuperscript{‡} 0.23±0.02

\textsuperscript{†} IC\textsubscript{50} values were determined in the coupled assay with tyramine (2xK\textsubscript{M}).

\textsuperscript{‡} Bolea et al., 2011.

\textsuperscript{‡} Without preincubation (i.e., reversible binding)

Table 8  IC\textsubscript{50} values and binding energies E\textsubscript{bin} towards
<table>
<thead>
<tr>
<th>Comp.</th>
<th>Structure</th>
<th>MAO A IC50 (µM)</th>
<th>$E_{\text{bin}}$ (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td><img src="image" alt="Structure of 25" /></td>
<td>9.05 ±0.091</td>
<td>321.6</td>
</tr>
<tr>
<td>30</td>
<td><img src="image" alt="Structure of 30" /></td>
<td>15.7 ±1.87</td>
<td>338.3</td>
</tr>
<tr>
<td>31</td>
<td><img src="image" alt="Structure of 31" /></td>
<td>0.34 ±0.05</td>
<td>368.2</td>
</tr>
<tr>
<td>32</td>
<td><img src="image" alt="Structure of 32" /></td>
<td>0.12±0.03</td>
<td>377.6</td>
</tr>
<tr>
<td>49</td>
<td><img src="image" alt="Structure of 49" /></td>
<td>9.65±1.36</td>
<td>323.6</td>
</tr>
<tr>
<td>63</td>
<td><img src="image" alt="Structure of 63" /></td>
<td>1.65±0.17</td>
<td>346.7</td>
</tr>
<tr>
<td>65</td>
<td><img src="image" alt="Structure of 65" /></td>
<td>9.57±3.60</td>
<td>272.7</td>
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<tr>
<td>Harmine</td>
<td><img src="image" alt="Structure of Harmine" /></td>
<td>5-17 nM</td>
<td>395.6</td>
</tr>
</tbody>
</table>

Figure Legends
SCHEME 1 General procedure for the Knoevenagel condensation reaction regarding the syntheses of arylidene propanedinitriles of type 3 (compounds 16-41, Table 1) and the new compounds 31, 34, 35 and 36.

SCHEME 2 General procedure for the syntheses of [(phenylamino)methyldene]propanedinitriles of type 6 (compounds 42-52, Table 2) and the new compounds 45, 48 and 52.

SCHEME 3 General procedure for the syntheses of both 4-amino-1-phenyl-1H-pyrrole-3-carbonitrile and 3-amino-1-phenyl-1H-pyrrole-2,4-dicarbonitriles of type 8 (compounds 53-62, Table 3) and the new compounds 59, 60.

SCHEME 4 General procedure for the syntheses of (phenylhydrazinylidene)propanedinitrile 67, the 4-arylazo-3,5-diamino-1H-pyrazole 72, and the (E,E)-4-amino-1-aryl-3-cyano-4-methoxy-2-azabutadienes 74 and 75.

SCHEME 5 General procedure for the syntheses of 4-arylazo-3,5-diamino-1H-pyrazoles of type 12 (compounds 68-72, Table 5) and the new compound 72.

SCHEME 6 General procedure for the syntheses of (E,E)-4-amino-1-aryl-3-cyano-4-methoxy-2-azabutadienes of type 15 (compounds 73-75, Table 6) and the new compounds 74 and 75.

FIGURE 1. The structures of all the new arylidene propanedinitriles (31, 34, 35, 36), the [(phenylamino)methyldene]propanedinitriles (45, 48, 52), the 3-amino-1-phenyl-1H-pyrrole-2,4-dicarbonitriles (59, 60), the (phenylhydrazinylidene)propanedinitrile 67, the 4-arylazo-3,5-diamino-1H-pyrazole 72, and the (E,E)-4-amino-1-aryl-3-cyano-4-methoxy-2-azabutadienes 74 and 75.

FIGURE 2 Potential disaggregating effect of 31, 32, 33 and 37 on Aβ1-42 fibrils. Different concentrations (20, 40 and 60 µM) of compounds were added to Aβ1-42 samples (pre-incubated at 37°C for 24 h) and incubated at 37°C for an extra 24 h. The levels of fibrils were evaluated by the intensity of fluorescence of ThT. The fluorescence of fibrillar Aβ1-42 (fib), which was incubated at 37°C for a total time of 48 h, was also measured; and the fluorescence of other experimental conditions were indicated as arbitrary units (a.u.) related to Aβ1-42. The fluorescence of Aβ1-42 fresh solution (N-fib) was also measured as reference. Data are mean±SEM of 3-4 independent experiments.

FIGURE 3 Potential disaggregating effect of 63, 65 and 75 on Aβ1-42 fibrils. Different concentrations (20, 40 and 60 µM) of compound were added to Aβ1-42 samples (pre-incubated at 37°C for 24 h) and for another 24 h incubation period at 37°C. The levels of fibrils were evaluated by the intensity of fluorescence of ThT. The fluorescence of fibrillar Aβ1-42 (fib), which was incubated at 37°C for a total time of 48 h, was also measured; and the fluorescence of other experimental conditions were indicated as arbitrary units related to Aβ1-42. The fluorescence of Aβ1-42 fresh solution (N-fib) and of Aβ1-42 incubated with resveratrol (20 µM res), a known anti-aggregating compound, were also measured as reference. Data are mean±SEM of 3-4 independent experiments. p<0.05 statistical different from Aβ1-42 fib.

FIGURE 4 Most probable tautomers of compounds 49, 63 and 65.
FIGURE 5  Comparison of the location of 49 (magenta), 63 (element color) and 65 (green) in the proximity of FAD.

FIGURE 6  Charge distribution of compound 63.

FIGURE 7  Charge distribution of compound 49.

FIGURE 8  Interactions of 31 and 32 with MAO A.

FIGURE 9  Interactions of 31 and 32 with MAO B.

FIGURE 10  Binding mode of 82 and 83 at the active site of hAChE.

FIGURE 11  Overall view of 82 (blue, up) and 83 (down) immersed in hAChE protein (4M0F PDB model) viewed through the gorge of the protein surface. Red circles represent water molecules.

FIGURE 12  Lipophilic potential on the surface of 83. Blue – hydrophilic values, yellow – hydrophobic.
Ar \text{H} + \text{CN-CN} \rightarrow \text{Ar-CN-CN} \quad \text{Piperidine} \quad \text{EtOH, 0 °C}

31: X=Br; Y,Z=H
34: X,Z=H; Y= \text{OCH}_2\text{Ph}
35: X=Br; Y,Z=H
36: X,Y=H; Z= \text{CN}
1. TEA, 0 °C

2. Reflux

(R=CN, CO₂Et, COCH₃, COPh)

59: X=Y=CH₃, R=CN
60: X=Z=CH₃, R=CN
\[
\begin{align*}
\text{CN} + \text{HC(OEt)}_3 + \text{NH}_2 \xrightarrow{\text{MeOH, 55-60°C}} \text{CN} \\
\text{2} \quad \text{4} \quad \text{5} \quad \text{6}
\end{align*}
\]

45: X, Z, Q = CH₃; Y, W = H
48: X, W, Q = H; Y, Z = OCH₃
52: X, Q = CH₃; Y, W = H; Z = Br
9 \xrightarrow{\text{NaNO}_2, \text{HCl/H}_2\text{O}, 0^\circ\text{C}} 10 \xrightarrow{\text{CH}_2(\text{CN})_2, \text{AcONa}, 0^\circ\text{C}} 11

\text{67: } X=Y=\text{CH}_3
\[
\text{11} \xrightarrow{\text{N}_2\text{H}_4\text{H}_2\text{O} \text{ MeOH reflux}} \text{12}
\]

72: \(X, Y = \text{CH}_3\)
\[ \text{13} + \text{14} \rightarrow \text{15} \]

- Reaction conditions: MeOH, CH\(_3\)CO\(_2\)Na, rt
- 74: X = OCH\(_2\)CHCH\(_2\)
- 75: X = CN