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Short communication

Synthesis of 6-amino-1,4-dihydropyridines that prevent calcium overload and neuronal death

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Abstract

The synthesis and pharmacology of 6-amino-1,4-dihydropyridines, such as ethyl 6-amino-4-aryl-5-cyano-1,4-dihydro-2-methyl-3-pyridinecarboxylic acids (**3**–**16**) and 2-amino-4-aryl-7,7-dimethyl-5-oxo-1,4,5,6,7,8-hexahydro-3-quinolinenitriles (**17**–**21**) are described. Compounds **18** and **21**, at the concentration of 0.3 μM, proved to be the best blockers of the [Ca^{2+}] overload induced by depolarization with high [K^+] of SH-SY5Y neuroblastoma cells, with values of 63.8% and 50.4%, respectively. Most of the compounds induced a remarkable neuroprotective effect against toxicity caused by high [K^+]-elicited [Ca^{2+}] overload, and against H_2O_2-generated free radicals, in SH-SY5Y cells.

Keywords: 6-Amino-1,4-dihydropyridines; Ethyl 6-amino-4-aryl-5-cyano-1,4-dihydro-2-methyl-3-pyridinecarboxylic acids; 2-Amino-4-aryl-7,7-dimethyl-5-oxo-1,4,5,6,7,8-hexahydro-3-quinolinenitriles; Voltage-dependent calcium channels; Neuroprotection

1. Introduction

1,4-Dihydropyridine (1,4-DHP) [1] calcium antagonists that produce vascular smooth muscle relaxation, specially in arterial beds, such as nifedipine (**1**) or nimodipine (**2**) (Fig. 1), are relevant drugs for the treatment of peripheral vascular diseases such as angina pectoris and hypertension [2]. The design of DHP–calcium channel modulators has prompted studies to investigate the functional and geometrical requirements at the DHP binding site [3]. Structure–activity relationships (SAR) show that the combination of the substituents at the C3, C4 and C5 positions of nifedipine (**1**) modulates the activity [4] and tissue selectivity [5], while the nature and position of C4-aryl ring substituent were determinant of voltage-dependent calcium channel (VDCC) antagonist activity. In general, the presence of an aryl group at C4, and esters, acyl, sulphonyl or nitrile groups at C3 and C5 of the 1,4-DHP ring has proved to be a fundamental requirement for the pharmacological activity [6–8]. In spite of the widely developed chemistry of the 1,4-DHPs, little is known about 1,4-DHPs bearing substituents other than hydrogen atoms or alkyl groups at C2 and C6 [9,10]. In this context, it is surprising to recognize the scarce number of studies that report the pharmacology of 6-amino substituted 1,4-DHPs (**I**) (Fig. 1). A survey of the current literature has shown that compounds of type **I** have been described in the literature [11,12], and claimed to be active in the central nervous system, but without reporting the biological relevant data [13]. Similarly, related 2-amino-4-aryl-7,7-dimethyl-5-oxo-1,4,5,6,7,8-hexahydro-3-quinolinenitriles (**II**) (Fig. 1) are also well known [14], but to the best of our knowledge, their pharmacology has never been addressed.

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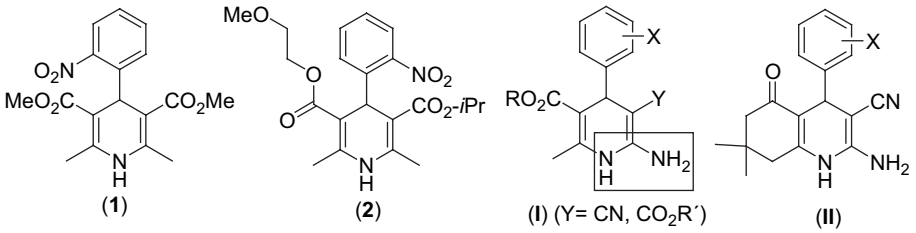


Fig. 1. Nifedipine (**1**), nimodipine (**2**), and general structure for the target 1,4-DHP (**I**, **II**).

On the other hand, amlodipine, a 1,4-DHP in clinical use, and carvedilol prevent cytotoxicity in cortical neurons isolated from stroke-prone spontaneously hypertensive rats [15]. Protective effects of a selective L-type voltage-sensitive calcium channel blocker, S-312-d, have been reported [16]. Similarly, a 1,4-DHP blocker such as nimodipine (**2**) (Fig. 1) behaves as neuroprotectant drug [17]. Thus, it seems reasonable to study the effects of new synthetic 1,4-DHPs on the increase of $[Ca^{2+}]$ elicited by depolarization with high $[K^+]$ (70 mM) in SH-SY5Y human neuroblastoma cells, as well as their possible effect on cell viability.

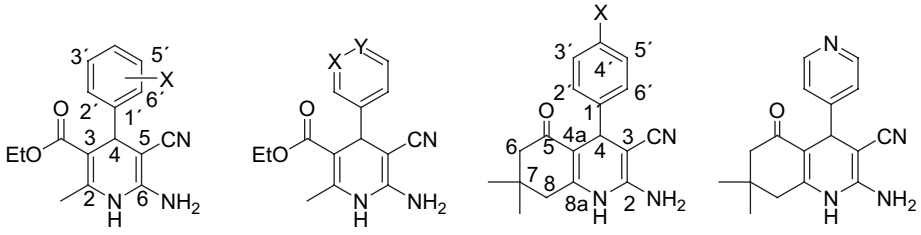
These facts prompted us to synthesize the readily available ethyl 6-amino-4-aryl-5-cyano-1,4-dihydro-2-methyl-3-pyridinecarboxylic acids (**3**–**16**)/2-amino-4-aryl-7,7-dimethyl-5-oxo-1,4,5,6,7,8-hexahydro-3-quinolinecarbonitriles (**17**–**21**) (Fig. 2), and test them to a pharmacological screening including VDCC blockade, and neuroprotection.

2. Results and discussion

In the family of ethyl 6-amino-4-aryl-5-cyano-1,4-dihydro-2-methyl-3-pyridinecarboxylic acids (**3**–**16**), compounds **3** [12], **4** [18], and **6**–**12** [18] (Fig. 2) have been reported. The other analogues (**5**, **13**–**16**) have been easily synthesized [12] starting from the corresponding substituted benzaldehydes *via* the 2-arylmethylene-3-oxo-butanoic acid ethyl esters **22** [19], **23** [20], **25** [21] and **26** [21] (Scheme 1). 2-[(4′-Biphenyl)methylene]-3-oxo-butanoic acid ethyl ester (**24**) has been synthesized here for the first time from the biphenyl-4-carboxaldehyde. Treatment of compounds **22**–**26** with 3,3-diaminoacrylonitrile [12], obtained *in situ* from ethyl 2-cyanoacetimidate hydrochloride (**27**) in the presence of ammonium acetate, gave the 4-amino-1,4-DHPs (**5**, **13**–**16**) in good yields (Scheme 1).

In the group of 2-amino-4-aryl-7,7-dimethyl-5-oxo-1,4,5,6,7,8-hexahydro-3-quinolinenitriles (**17** [14], **18** [22], **19** [14], **20** [14]), compound **21** has been prepared here for the first time by reacting [2-(4-pyridyl)methylene]malononitrile (**28**) [23] with 3-amino-5,5-dimethyl-2-cyclohexenone (**29**) [24] in methanol at reflux, in the presence of ammonium acetate (Scheme 2).

With these compounds in hands, we first carried on the evaluation of cytosolic $[Ca^{2+}]$ concentrations in the presence of the compounds **3**–**21**. A control with nimodipine (**2**) (Fig. 1), a blocker of L-type VDCC, was included in every individual experiment. All compounds were assayed at the concentration of 0.3 μM. The results are shown in Table 1. As can be noticed, most of the compounds blocked the increase in $[Ca^{2+}]$ induced



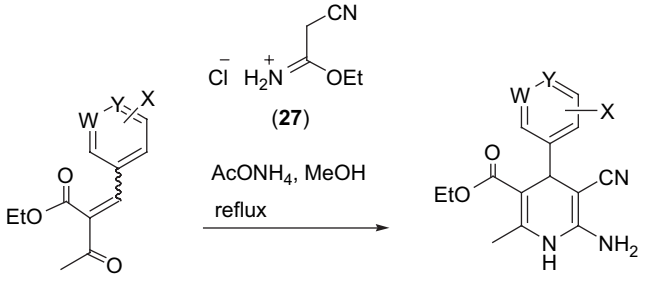
(**3**) X = H
(**4**) X = 4'-F
(**5**) X = 2'-CF_3
(**6**) X = 2'-NO_2
(**7**) X = 3'-NO_2
(**8**) X = 4'-NO_2
(**9**) X = 4'-Me
(**10**) X = 2'-OMe
(**11**) X = 3'-OMe
(**12**) X = 4'-OMe
(**13**) X = 3',4'-di-OMe
(**14**) X = 4'-C_6H_5

(**15**) X = N, Y= CH
(**16**) X = CH, Y= N

(**17**) X = H
(**18**) X = F
(**19**) X = Me
(**20**) X = OMe

(**21**)

Fig. 2. Target 6-amino-1,4-DHP.



(**22**) W,Y = CH,X = 2'-CF_3
(**23**) W,Y = CH,X = 3',4'-di-OMe
(**24**) W,Y = CH,X = 4'-Ph (76%)
(**25**) W = N,Y = CH,X = H
(**26**) W = CH,Y = N,X = H

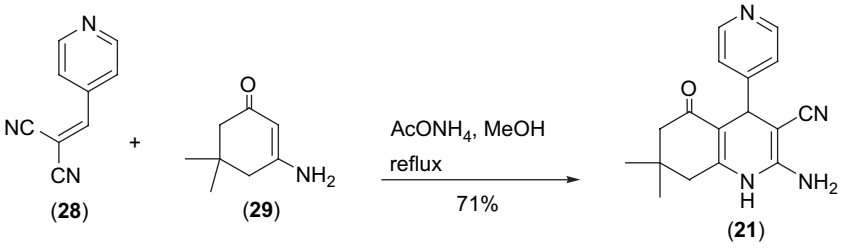
(**5**) (43%)
(**13**) (77%)
(**14**) (60%)
(**15**) (66%)
(**16**) (85%)

Scheme 1. Synthesis of 1,4-DHP (**5**, **13**–**16**).

by high [K^+] in a statistically significant manner, the values ranging between 7.2% and 63.9%. From the SAR analysis, some conclusions can be drawn. In general, 2-amino-4-aryl-3-cyano-7,7-dimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinolines (**17**–**21**) induced higher blockade of [Ca^{2+}] elevation than 6-amino-4-aryl-5-cyano-1,4-dihydropyridines (**3**–**16**); for instance, the most potent derivatives found in this study, the hexahydroquinolines **18** and **21**, with a 4′-F and a 4′-pyridyl substituent, respectively, showed blockade values of 63.9% and 50.4%, respectively (compared with the 1,4-DHP analogues **4** and **16**, showing a 7.2% and 32.6% blockade). It is also very clear that in the 2-amino-4-aryl-3-cyano-7,7-dimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline family of compounds (**17**–**21**), the most potent bears electron-withdrawing groups such as 4′-F or 4′-pyridyl. On the other hand, compounds that possess electron-donating groups [**19** (4′-Me) and **20** (4′-MeO)] only show modest blockade values. Very interestingly, compounds **18** and **21** were more active than nimodipine (**2**). In the 6-amino-4-aryl-5-cyano-1,4-dihydropyridine family of compounds (**3**–**16**), the most potent molecule corresponded to the unsubstituted member (**3**) with a 34.0% blockade, followed by substituted analogues with electron-withdrawing groups such as 4′-pyridyl (32.6%) (**16**), 2′-CF_3 (30.4%) (**5**), and 3′-NO_2 (27.8%) (**7**). Considering the same substituent, for instance a nitro or methoxy group, the most potent compounds were **7** and **11**, bearing these groups at C3′. Note also that compound **13**, with two methoxy groups at C3′ and C4′, is more active (21.8%) than compound **12** (11.8%) with one methoxy at C4′, but somewhat less active than molecule **11** (25%) with the methoxy group located at C3′; thus, no additive group effect was observed in this case. Regarding the heterocycle containing compounds **15** and **16**, the most potent correlated by far to the 4′-pyridyl substituted molecule (**16**).

To assess a possible neuroprotective effect of these new compounds against [Ca^{2+}] overload, the usual protocol was followed (see Section 4). Nimodipine (**2**) (Fig. 1) was used as a reference compound. All the compounds, including nimodipine, were assayed at the concentration of 0.3 μM. The results are shown in Table 2. Most of the compounds induced a remarkable neuroprotective effect, with values ranging from 12.9% to 54.7%. Comparing 2-amino-4-aryl-3-cyano-7,7-dimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinolines (**17**–**21**) with 6-amino-4-aryl-5-cyano-1,4-dihydropyridines (**3**–**16**), the former group of molecules showed a higher protection with values in the range of 39.5–54.7%, the best neuroprotectant compound being compound **17**, with a 54.7% protection; note also that compounds **19** (4′-Me) and **20** (4′-MeO) with electron-donating groups were more potent than those bearing electron-withdrawing groups (**18** and **21**); this is just the opposite behavior of that described for the blockade of [Ca^{2+}] increase in SH-SY5Y cells for the same molecules (see Table 1). Note once more that nimodipine (**2**) was significantly less potent (35.9%) than the best neuroprotectant compound (**17**) analyzed in this study. Comparatively, the protection values recorded for 6-amino-4-aryl-5-cyano-1,4-dihydropyridines (**3**–**16**) (Table 2) were also stronger than those shown in Table 1 for the blockade of [Ca^{2+}] increase in SH-SY5Y cells for the same compounds; in this family, products bearing electron-withdrawing groups at the aromatic ring [(**8**) 4-NO_2 (45.7%), (**15**, **16**) (3′-, 4′-pyridyl) (43.4%, 43.3%), (**6**) 2′-NO_2 (42.6%)] showed higher protection than those bearing electron-donating groups. For a definite substituent, the nitro group for instance, the order of potency was displayed as compound **8** > **6** ≫ **7**, corresponding to the position of the group at C4′, C2′ and C3′, while for the methoxy group, the order of potency showed compound **11** > **10** ≫ **12**, referring to the position of the group at C3′, C2′ and C4′. In general, it is clear that the more electron-withdrawing the groups are, the more effective neuroprotection was observed (compare compound **8** with **4**), and the less electron-donating the groups are, the more potent neuroprotection was detected (compare compound **9** with **10**–**14**).

Furthermore, the effect of our compounds as neuroprotectants towards oxidative stress elicited by free-radical generation was evaluated. For this purpose, SH-SY5Y cells were exposed



Scheme 2. Synthesis of the 2-amino-7,7-dimethyl-5-oxo-1,4,5,6,7,8-hexahydro-3-quinolinenitrile (**21**).

Table 1
Effects of 6-amino-4-aryl-5-cyano-1,4-dihydropyridines (**3**–**16**) and 2-amino-4-aryl-3-cyano-7,7-dimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinolines (**17**–**21**) on [Ca^{2+}] increase elicited by 70 mM [K^+] in SH-SY5Y cells (% inhibition with respect to a control without any drug)

Compound	Substituent	% Blockade [Ca^{2+}] uptake
Nimodipine (**2**)	–	45.5 ± 6.1***
3	H	34.03 ± 6.6**
4	4′-F	7.2 ± 4.7 ns
5	2′-CF_3	30.4 ± 3.5**
6	2′-NO_2	16.1 ± 5.02 ns
7	3′-NO_2	27.8 ± 3.2**
8	4′-NO_2	17.6 ± 3.2 ns
9	4′-Me	16.9 ± 3.4 ns
10	2′-OMe	24.3 ± 5.6*
11	3′-OMe	25 ± 3.7**
12	4′-OMe	11.8 ± 6.3 ns
13	3′,4′-Di-OMe	21.8 ± 4.05*
14	4′-Phenyl	23.9 ± 3.7*
15	3′-Pyridyl	14.1 ± 4.6 ns
16	4′-Pyridyl	32.6 ± 4.3**
17	H	20.8 ± 4.3 ns
18	4′-F	63.9 ± 3.2***
19	4′-Me	28.6 ± 3.5**
20	4′-MeO	12.6 ± 3.7 ns
21	4′-Pyridyl	50.4 ± 6.7***

Data are expressed as means ± SEM of at least three different cultures in quadruplicate.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ns = not significant. All compounds were assayed at the concentration of 0.3 μM.

Table 2
Neuroprotective effects of 6-amino-4-aryl-5-cyano-1,4-dihydropyridines (**3**–**16**) and 2-amino-4-aryl-3-cyano-7,7-dimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinolines (**17**–**21**), expressed as reduction in the increase of LDH released in the presence of 70 mM [K^+], in neuroblastoma cells

Compound	Substituent	LDH[a] release (% control)	% Protection ([Ca^{2+}] overload)
Nimodipine (**2**)	–	75.00 ± 2.2***	35.9 ± 2.8
3	H	76.26 ± 2.4***	34.1 ± 3.2
4	4′-F	72.80 ± 2.9***	39.8 ± 4.03
5	2′-CF_3	72.11 ± 2.7***	40.2 ± 3.6
6	2′-NO_2	71.11 ± 3.6***	42.6 ± 5.2
7	3′-NO_2	79.4 ± 3.8*	30.3 ± 5.5
8	4′-NO_2	68.9 ± 2***	45.7 ± 2.6
9	4′-Me	72.3 ± 3.1***	40.8 ± 4.4
10	2′-OMe	78.2 ± 2.6**	31.5 ± 3.7
11	3′-OMe	77.9 ± 2.9**	32.1 ± 3.8
12	4′-OMe	89.9 ± 3.05 ns	14.4 ± 4.4
13	3,′4′-Di-OMe	80.5 ± 4.3*	31.2 ± 4.9
14	4′-Phenyl	90.9 ± 8.7 ns	12.9 ± 1.9
15	3′-Pyridyl	70.5 ± 2.6***	43.4 ± 3.5
16	4′-Pyridyl	70.5 ± 4.01***	43.3 ± 5.7
17	H	61.2 ± 1.8***	54.7 ± 2.6
18	4′-F	69.9 ± 2.2***	42.3 ± 3.05
19	4′-Me	68.4 ± 2.8***	44.4 ± 4.01
20	4′-MeO	69.3 ± 1.5***	43.2 ± 2.01
21	4′-Pyridyl	71.4 ± 1.8***	39.5 ± 2.4

Data are expressed as means ± SEM of at least three different cultures in quadruplicate.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ns = not significant. All compounds were assayed at the concentration of 0.3 μM.
[a] Lactic dehydrogenase activity.

for 24 h to 60 μM H_2O_2, following the usual protocol (see Section 4). The results are reported in Table 3. Nimodipine (**2**), and catalase as a reference antioxidant compound, were included. In general, protection afforded by these compounds was from low to moderate, with most of the values below 30%, but some of them showed more than 40% inhibition. Comparing 2-amino-4-aryl-3-cyano-7,7-dimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinolines (**17**–**21**) with 6-amino-4-aryl-5-cyano-1,4-dihydropyridines (**3**–**16**), the former group of molecules revealed less protection. Accordingly, only compounds **9**, **13** and **16** were more effective, with protection values of 42.4%, 40.8% and 46.7%, respectively. These compounds were more potent than nimodipine (**2**) (36.0%), but twofold less active than catalase. No obvious relationship was observed between the type of substituent and the neuroprotective effect; however, for a given substituent, regardless of its electronic nature, being at the C4′ position conferred to compounds, the best neuroprotective profile.

3. Conclusions

We have reported the synthesis of a number of 6-amino-1,4-DHPs (compounds **3**–**21**) and investigated for the first time their pharmacological activity, including the effect on cytosolic [Ca^{2+}] increase elicited by depolarization with high [K^+] of SH-SY5Y human neuroblastoma cells, the neuroprotective effects against [Ca^{2+}] overload, and their neuroprotective action on free-radical generation. First of all, most of the compounds blocked the [K^+]-induced [Ca^{2+}] increase in a statistically significant manner, and induced a remarkable neuroprotective effect at the concentration of 0.3 μM, with values ranging from 12.9% to 54.7% protection against cell death caused by [Ca^{2+}] overload, induced upon incubation of SH-SY5Y cells with 70 mM [K^+]. The antioxidant effect of the compounds was less pronounced, with protection values around 30% in most of the cases. As representative examples, compound **18** showed a 63.9% blockade for the increase in [Ca^{2+}] induced by high [K^+] in SH-SY5Y cells; molecule **17** displayed a 54.7% protection for cell viability in the presence of 70 mM [K^+]; finally, product **16** exhibited significant protection (46.7%) against the free-radical insult, while nimodipine (**2**), a well known calcium antagonist used as a reference compound, presented lower values in all these experiments when compared with the most active compound in each experiment.

4. Experimental part

4.1. General methods

Reactions were monitored by TLC using precoated silica gel aluminium plates containing a fluorescent indicator (Merck, 5539). Detection was done by UV (254 nm) followed by charring with sulfuric–acetic acid spray, 1% aqueous potassium permanganate solution or 0.5% phosphomolybdic acid in 95% EtOH. Anhydrous Na_2SO_4 was used to dry organic solutions during work-ups and the removal of solvents

Table 3
Cell viability for 6-amino-4-aryl-5-cyano-1,4-dihydropyridines (**3**–**16**) and 2-amino-4-aryl-3-cyano-7,7-dimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinolines (**17**–**21**), expressed as reduction in the increase of LDH released in the presence of 60 μM H_2O_2

Compound	Substituent	LDH[a] release (% control)	% Protection (free radicals)
Catalase	–	18.9 ± 1.8***	88.3 ± 2.8
Nimodipine (**2**)	–	75.4 ± 2.1***	36.03 ± 2.8
3	H	86.7 ± 1.7 ns	17.3 ± 2.1
4	4′-F	82.2 ± 2.8*	24.7 ± 3.8
5	2′-CF_3	78.6 ± 1.3**	28.2 ± 1.5
6	2′-NO_2	98.8 ± 2.2 ns	1.6 ± 3.1
7	3′-NO_2	84.5 ± 3.2 ns	21.2 ± 4.3
8	4′-NO_2	83.4 ± 1.8 ns	23.1 ± 2.4
9	4′-Me	69.7 ± 2.4***	42.4 ± 3.2
10	2′-OMe	88.4 ± 2 ns	15 ± 2.6
11	3′-OMe	86.1 ± 3.9 ns	27.7 ± 4
12	4′-OMe	77.7 ± 1.6***	31.3 ± 2.2
13	3′,4′-Di-OMe	70.2 ± 3.1 ns	40.8 ± 3.9
14	4′-Phenyl	80.3 ± 1.1**	26.2 ± 1.7
15	3′-Pyridyl	84.5 ± 3.8***	21.2 ± 4.8
16	4′-Pyridyl	66.7 ± 2.6***	46.7 ± 3.7
17	H	84.2 ± 1.4***	20.6 ± 1.8
18	4′-F	75.4 ± 3.1***	32.4 ± 4
19	4′-Me	88.6 ± 1.4 ns	14.9 ± 1.8
20	4′-MeO	77.8 ± 1.7***	29 ± 2.2
21	4′-Pyridyl	77.7 ± 2.5***	29.1 ± 3.1

Data are expressed as means ± SEM of at least three different cultures in quadruplicate.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ns = not significant. Catalase was assayed at the concentration of 50 units/mL. The rest of the compounds were assayed at the concentration of 0.3 μM.
[a] Lactic dehydrogenase activity.

was carried out under vacuum with a rotary evaporator. Flash column chromatography was performed using silica gel 60 (230–400 mesh, Merck). Melting points were determined on a Kofler block and are uncorrected. IR spectra were obtained on a Perkin–Elmer Spectrum One Spectrophotometer. ^{1}H NMR spectra were recorded with a Varian VXR-200S spectrometer, using tetramethylsilane as internal standard and ^{13}C NMR spectra were recorded with a Bruker WP-200-SY. All the assignments for protons and carbons were in agreement with 2D COSY, gHSQC, gHMBC, and 1D NOESY spectra. Values with (*) can be interchanged. Elemental analyses were carried out on a Carlo Erba EA 1108 apparatus.

4.2. Ethyl ester 2-[(4-biphenyl)methylene]-3-oxo-butanoic acid (*24*)

To a solution of ethyl acetoacetate (1.42 g, 10.9 mmol) in dry toluene (30 mL) biphenyl-4-carboxaldehyde (2.0 g, 10.9 mmol) and piperidine (15 drops) were added. After 4 h, the solvent was removed and the crude mixture was submitted to chromatography (hexane:AcOEt, 15%) to give compound **24** (2.45 g, 76%), as a mixture of *Z*/*E* isomers in a 67:33 ratio: oil; IR (KBr) ν 3072, 2985, 1716, 1662, 1623, 1603, 1487, 1232 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) (major *Z* isomer) δ 7.75–7.38 (m, 10H, C_6H_4–C_6H_5, HC=C), 4.35 (q, J = 6.9 Hz, 2H, OCH_2CH_3), 2.45 [s, 3H, CH_3(CO)], 1.56 (t, J = 6.9 Hz, 3H, $CO_2CH_2CH_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) (major *Z* isomer) δ 203.3 (C=O), 194.4 (CO_2), 140.6 (*C*H=C), 134.0 (CH=*C*), 131.6, 130.2, 130.0, 128.8, 128.7, 128.1, 127.9, 127.4, 126.8 (aromatic), 61.8 (OCH_2CH_3), 26.5 [CH_3(CO)], 13.8 (CH_3CH_2O); MS (API-ES+) *m/z*: $[M + 1]^+$ 295.2; $[M + Na]^+$ 317.0; $[2M + Na]^+$, 611.2. Anal. Calcd. for $C_{19}H_{18}O_3$: C, 77.53; H, 6.16. Found: C, 77.32; H, 5.97.

4.3. General method for the synthesis of 6-amino-4-aryl-5-cyano-1,4-dihydro-2-methyl-3-pyridinecarboxylic acid ethyl esters (*5*, *13*–*16*)

A solution of ethyl 2-cyanoacetimidate hydrochloride (1 equiv) and ammonium acetate (1–5 equiv), in methanol, was warmed at reflux for 15 min. Then, the corresponding ethyl ester 2-arylmethylene-3-oxo-butanoic acid (1 equiv) was added and the mixture was refluxed for 15 min, and cooled at 5 °C overnight. The solid was separated and recrystallized from methanol.

4.3.1. 6-Amino-5-cyano-1,4-dihydro-4-(2-trifluoromethylphenyl)-2-methyl-3-pyridinecarboxylic acid ethyl ester (*5*)

Following the method described in Section 4.3, the reaction of ethyl 2-cyanoacetimidate hydrochloride (**27**) (437.4 mg, 2.93 mmol) and ammonium acetate (733.5 mg, 9.51 mmol), in methanol (15 mL), with compound **22** [19] (700.0 mg, 2.44 mmol), in 30 min, gave product **5** (368.0 mg, 43%): mp 243–245 °C; IR (KBr) ν 3401, 3340, 3224, 2992, 2833, 2182, 1657, 1624, 1489, 1367, 1255 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.70 (s, 1H, NH), 7.58–7.42 (m, 4H, aromatic), 5.62 (s, 2H, NH_2), 4.79 (s, 1H, H4), 3.83 (q, J = 7.1 Hz, 2H, OCH_2CH_3), 2.28 [s, 3H, CH_3], 0.86 (t, J = 7.1 Hz, 3H, $CO_2CH_2CH_3$); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 166.8 (C=O), 151.8 (C2), 148.2–124.4 (aromatic), 146.6 (C6), 121.0 (CN), 101.4 (C3), 59.2 (OCH_2CH_3), 58.2 (C5), 35.9 (C4), 19.1 (CH_3), 14.1 (CH_3CH_2O); MS (API-ES$^+$) *m/z*: $[M + 1]^+$ 352.3; $[M + Na]^+$ 374.2; $[2M + Na]^+$ 723.5. Anal. Calcd. for $C_{17}H_{16}F_3N_3O_2$: C, 58.12; H, 4.59; N, 11.96. Found: C, 58.26; H, 4.35; N, 11.74.

4.3.2. 6-Amino-5-cyano-1,4-dihydro-4-(3,4-dimethoxyphenyl)-2-methyl-3-pyridinecarboxylic acid ethyl ester (*13*)

Following the method described in Section 4.3, the reaction of ethyl 2-cyanoacetimidate hydrochloride (**27**) (545.0 mg, 3.69 mmol) and ammonium acetate (915.9 mg, 11.8 mmol), in methanol (15 mL), with compound **23** [20] (850.0 mg, 3.05 mmol), after 35 min, afforded compound **13** (797.0 mg, 77%): mp 166–168 °C; IR (KBr) ν 3420, 3348, 3224, 2934, 2898, 2833, 2179, 1650, 1513, 1266 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.90 (s, 1H, NH), 6.86 (d, J = 8.3 Hz, 1H), 6.71 (d, J = 2.0 Hz, 1H), 6.64 (dd, J = 8.3, 2.0 Hz, 1H), 5.74 (s, 2H, NH_2), 4.31 (s, 1H, H4), 3.96 (q, J = 7.1 Hz, 2H, OCH_2CH_3), 3.70 (s, 6H, 2 × OCH_3), 2.25 [s, 3H, CH_3], 1.10 (t, J = 7.1 Hz, 3H, $CO_2CH_2CH_3$); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 166.6 (C=O), 150.4 (C2), 148.2–110.8 (aromatic), 147.2 (C6), 121.8 (CN), 100.9 (C3), 59.0 (OCH_2CH_3), 57.3 (C5), 55.4, 55.3 (2C, 2 × OCH_3), 39.7

(C4), 18.5 (CH_3), 14.0 (CH_3CH_2O); MS (API-ES$^+$) *m/z*: $[M+1]^+$ 344.0; $[M+Na]^+$ 382.1; $[2M+1]^+$ 687.5; $[2M+Na]^+$ 709.3. Anal. Calcd. for $C_{18}H_{21}N_3O_4$: C, 62.96; H, 6.16; N, 12.24. Found: C, 62.69; H, 6.25; N, 12.43.

4.3.3. 6-Amino-5-cyano-1,4-dihydro-2-methyl-4-(4-biphenyl)-3-pyridinecarboxylic acid ethyl ester (**14**)

Following the method described in Section 4.3, the reaction of ethyl 2-cyanoacetimidate hydrochloride (**27**) (303.89 mg, 2.03 mmol) and ammonium acetate (507.83 mg, 6.59 mmol), in methanol (15 mL), with compound **24** (500 mg, 1.69 mmol), after 35 min, gave compound **14** (363 mg, 60%): mp 217–219 °C; IR (KBr) ν 3405, 3346, 3225, 2876, 2179, 1630, 1662, 1494, 1369, 1270, 1221 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.08 (s, 1H, NH), 7.60 (d, $J=8.8$ Hz, 2H), 7.58 (d, $J=8.8$ Hz, 2H), 7.43 (t, $J=7.5$ Hz, 2H), 7.32 (t, $J=7.5$ Hz, 1H), 7.20 (d, $J=7.5$ Hz, 2H), 5.74 (s, 2H, NH_2), 4.39 (s, 1H, H4), 3.94 (q, $J=7.1$ Hz, 2H, OCH_2CH_3), 2.27 [s, 3H, CH_3], 1.08 (t, $J=7.1$ Hz, 3H, $CO_2CH_2CH_3$); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 166.5 (C=O), 150.4 (C2), 146.9–128.8 (aromatic), 145.2 (C6), 121.7 (CN), 100.6 (C3), 59.1 (OCH_2CH_3), 57.2 (C5), 39.8 (C4), 18.7 (CH_3), 14.0 (CH_3CH_2O); EM (API-ES$^+$) *m/z*: $[M+1]^+$ 360.1; $[M+Na]^+$ 382.1; $[2M+1]^+$ 719.2; $[2M+Na]^+$ 741.2. Anal. Calcd. for $C_{22}H_{21}N_3O_2$: C, 73.52; H, 5.89; N, 11.69. Found: C, 73.27; H, 5.90; N, 11.83.

4.3.4. 6-Amino-5-cyano-1,4-dihydro-2-methyl-4-(3-pyridyl)-3-pyridinecarboxylic acid ethyl ester (**15**)

Following the method described in Section 4.3, the reaction of ethyl 2-cyanoacetimidate hydrochloride (**27**) (883.2 mg, 5.9 mmol) and ammonium acetate (1.37 g, 17.9 mmol), in methanol (15 mL), with compound **25** [21] (1 g, 4.56 mmol), after 25 min, gave product **15** (855 mg, 66%): mp 226–228 °C; IR (KBr) ν 3401, 2971, 2920, 2182, 1661, 1491, 1327 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.74 (s, 1H, NH), 8.38–7.30 (m, 4H, aromatic), 5.77 (s, 2H, NH_2), 4.40 (s, 1H, H4), 3.94 (q, $J=7.1$ Hz, 2H, OCH_2CH_3), 2.28 [s, 3H, CH_3], 1.04 (t, $J=7.1$ Hz, 3H, $CO_2CH_2CH_3$); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 166.5 (C=O), 150.8 (C2), 148.4–124.0 (aromatic), 146.2 (C6), 121.7 (CN), 100.2 (C3), 59.5 (OCH_2CH_3), 57.1 (C5), 37.1 (C4), 18.7 (CH_3), 14.3 (CH_3CH_2O); MS (API-ES$^+$) *m/z*: $[M+1]^+$ 285.2; $[M+Na]^+$ 307.3; $[2M+1]^+$ 569.5; $[2M+Na]^+$ 591.5. Anal. Calcd. for $C_{15}H_{16}N_4O_2$: C, 63.37; H, 5.67; N, 19.71. Found: C, 63.07; H, 5.90; N, 20.02.

4.3.5. 6-Amino-5-cyano-1,4-dihydro-2-methyl-4-(4-pyridyl)-3-pyridinecarboxylic acid ethyl ester (**16**)

Following the method described in Section 4.3, the reaction of ethyl 2-cyanoacetimidate hydrochloride (**27**) (816.0 mg, 5.47 mmol) and ammonium acetate (1.37 g, 17.9 mmol), in methanol (15 mL), with compound **26** [21] (1 g, 4.56 mmol), after 30 min, gave product **16** (855 mg, 66%): mp 252–254 °C; IR (KBr) ν 3425, 3347, 3228, 2981, 2172, 1642, 1592, 1485, 1370, 1328, 1264, 1217 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.77 (s, 1H, NH), 8.47 (dd, $J=4.5$, 1.5 Hz, 2H), 7.12 (dd, $J=4.5$, 1.5 Hz, 2H), 5.81 (s, 2H, NH_2), 4.36 (s, 1H, H4), 3.93 (q, $J=7.1$ Hz, 2H, OCH_2CH_3), 2.29 [s, 3H, CH_3], 1.04 (t, $J=7.1$ Hz, 3H, $CO_2CH_2CH_3$); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 166.5 (C=O), 155.9 (C2), 151.0 (C6), 150.0–122.2 (aromatic), 121.6 (CN), 99.6 (C3), 59.5 (OCH_2CH_3), 56.4 (C5), 40.4 (C4), 19.0 (CH_3), 14.3 (CH_3CH_2O); MS (API-ES$^+$) *m/z*: $[M+1]^+$ 285.2; $[M+Na]^+$ 307.3; $[2M+1]^+$ 569.5; $[2M+Na]^+$ 591.5. Anal. Calcd. for $C_{15}H_{16}N_4O_2$: C, 63.37; H, 5.67; N, 19.71. Found: C, 63.24; H, 5.86; N, 19.70.

4.3.6. 2-Amino-7,7-dimethyl-5-oxo-4-(4-pyridyl)-1,4,5,6,7,8-hexahydro-3-quinolinecarbonitriles (**21**)

A solution of 3-amino-5,5-dimethyl-2-cyclohexenone (**29**) [24] (899.0 mg, 6.44 mmol) and ammonium acetate (1.48 g, 19.33 mmol), in methanol (20 mL), was warmed at reflux, for 15 min. Then, [2-(4-pyridyl)methylene]malononitrile (**28**) [23] (1.0 g, 6.44 mmol) was added, and the mixture was refluxed for 3 h 30 min. The mixture was cooled at 5 °C overnight, the solid was filtered, washed with cold methanol and recrystallized from methanol to yield compound **21** (1.35 g, 71%): mp 253–255 °C; IR (KBr) ν 3385, 3329, 3220, 2963, 2884, 2183, 1657, 1598, 1477, 1370, 1270 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.97 (s, 1H, NH), 8.43 (d, $J=$ 6.0 Hz, 2H), 7.11 (d, $J=6.0$ Hz, 2H), 5.86 (s, 2H, NH_2), 4.32 (s, 1H, H4), 2.45–2.29 (m, 2H, 2H6), 2.20–1.97 (m, 2H, 2H8), 1.05, 0.90 (s, s, 3H, 3H, $2\times CH_3$); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 194.3 (C5, C=O), 155.4 (C2), 150.9 (C8a), 150.7–122.5 (aromatic), 121.5 (CN), 107.8 (C4a), 57.5 (C3), 50.3 (C6), 40.1 (C8), 37.2 (C4), 32.4 (C7), 29.1, 27.0 (2C, $2\times CH_3$); MS (API-ES$^+$) *m/z*: $[M+1]^+$ 295.1; $[M+Na]^+$ 317.1; $[2M+Na]^+$ 611.3. Anal. Calcd. for $C_{17}H_{18}N_4O$: C, 69.37; H, 6.16; N, 19.03. Found: C, 69.50; H, 6.21; N, 18.91.

4.4. Pharmacological protocols

4.4.1. Culture of SH-SY5Y cells

SH-SY5Y cells, at passages between 3 and 16 after defreezing, were maintained in a Dulbecco's modified Eagle's medium (DMEM) containing 15 non-essential amino-acids (NEAAs) and supplemented with 10% fetal calf serum (FCS), 1 mM glutamine, 50 units/mL penicillin and 50 μg/mL streptomycin (reagents from Gibco, Madrid, Spain). Cultures were seeded into flasks containing supplemented medium and maintained at 37 °C in 5% CO_2/humidified air. Stock cultures were passaged 1:4 twice weekly. For assays, SH-SY5Y cells were sub-cultured in 24-well plates at a seeding density of 2×10^5 cells per well, or in 96-well plates at a seeding density of 8×10^4 cells per well. For the cytotoxicity experiments cells were treated with drugs before confluence, in DMEM free of serum.

4.4.2. Measurement of lactic dehydrogenase (LDH) activity

Extracellular and intracellular LDH activity was spectrophotometrically measured using a Cytotoxicity Cell Death

kit (Roche–Boehringer, Mannheim, Germany) according to the manufacturer's indications. Total LDH activity was defined as the sum of intracellular and extracellular LDH activities; released LDH was defined as the percentage of extracellular compared to total LDH activity.

4.4.3. Measurement of cytosolic [Ca^{2+}] concentrations

For these experiments, SH-SY5Y neuroblastoma cells were grown at confluence in 96-well black dishes. Cells were loaded with 4 μM fluo 4/AM for 1 h at 37 °C in DMEM. Then cells were washed twice with Krebs–Hepes solution and kept at room temperature for 30 min before the beginning of the experiment. Fluorescence was measured in a fluorescence microplate reader (FLUOstar Optima, BMG, Germany). Wavelengths of excitation and emission were 485 nm and 520 nm, respectively.

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