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J. Med. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jmedchem.6b00297 • Publication Date (Web): 24 Jun 2016

New 6-Aminoquinoxaline Derivatives with Neuroprotective Effect on Dopaminergic Neurons in Cellular and Animal Parkinson Disease Models

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KEYWORDS

Neurodegenerative diseases, phenotypic screening, medicinal chemistry, heterocycle synthesis, primary neuronal cultures.

ABSTRACT

Parkinson disease (PD) is a neurodegenerative disorder of ageing characterized by motor symptoms that result from the loss of midbrain dopamine neurons and the disruption of dopamine-mediated neurotransmission. There is currently no curative treatment for this disorder. To discover druggable neuroprotective compounds for dopamine neurons, we have designed and synthesized a second-generation of quinoxaline-derived molecules based on structure–activity relationship studies, which led previously to the discovery of our first neuroprotective brain penetrant hit compound **MPAQ (5c)**. Neuroprotection assessment in PD cellular models of our newly synthesized quinoxaline-derived compounds has led to the selection of a better hit compound, **PAQ (4c)**. Extensive *in vitro* characterization of **4c** showed that its neuroprotective action is partially attributable to the activation of reticulum endoplasmic ryanodine receptor channels. Most interestingly, **4c** was able to attenuate neurodegeneration in a mouse model of PD, making this compound an interesting drug candidate for the treatment of this disorder.

INTRODUCTION

Parkinson disease (PD) is a neurodegenerative disorder of ageing, characterized by motor symptoms resulting from the loss of dopamine (DA) neurons in the *substantia nigra* (SN) and the depletion of the neurotransmitter DA in the striatum.[1] The molecular mechanisms at the origin of the disease are not yet completely understood, but several hypotheses are currently considered. In particular, mitochondrial dysfunction, oxidative and excitotoxic stresses, protein misfolding, calcium dyshomeostasis and neuroinflammation processes have been

reported to be involved in disease progression.[2,1,3,4] Most likely, these pathological processes interact with one another to lead to DA cell demise.

The pharmacological treatment of PD can be divided into neuroprotective and symptomatic therapies. In practice, nearly all of the available treatments are symptomatic ones, as they act principally, if not uniquely, on the motor symptoms of the disease.[5] Neuroprotective agents, having the potential to slow down or reverse the natural course of the disease, are therefore greatly needed. Many agents developed for neuroprotection in PD have shown great promise in preclinical assays, but none of them resulted in a treatment for patients until now.[6] This is probably partly due to the fact that a large number of these compounds have poor brain bioavailability. Another reason is our limited understanding of PD pathomechanisms.[7] Although the number of mechanisms targeted by PD drug candidates is steadily increasing, the search for an effective neuroprotective agent is continuing.

In the search for neuroprotective molecules for PD treatment, our laboratory has synthesized small non-peptide chemicals, based on structure–activity relationship (SAR) studies of molecules with neuroprotective/neuritogenic effects.[8,9] We initially synthesized hydrids of melatonin and fatty acids, i.e., natural products previously described for their antioxidative, neuroprotective or neurotrophic effects.[10,11] As a result, we obtained an in-house collection of *N*-acylated tryptamine derivatives that was screened in a PD cellular model of DA cell death. This work led to the identification of good antioxidative and neuroprotective/neuritogenic compounds, but, unfortunately, these products did not possess all the physicochemical properties expected for a good blood-brain barrier (BBB) permeation.[9] Therefore, *N*-alkyl-6-aminoquinoxaline derivatives were synthesized in order to obtain better BBB permeability. Interestingly, a compound with no aliphatic side chain exerted both neuroprotective and neuritogenic activities on DA neurons in midbrain cultures, through a mechanism of action, that was apparently indirect, as it involved astroglial cells.[12]

This product, **5c**, had almost all the physicochemical prerequisites for good BBB permeation.[12] The ability of this product to cross the BBB was confirmed by two complementary analytical methods: matrix-assisted laser desorption/ionization/time-of-flight (MALDI-TOF) mass spectrometry imaging of brain tissue sections and high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) quantification of brain tissue homogenates. Interestingly, this compound also exerted antioxidant effects.[12]

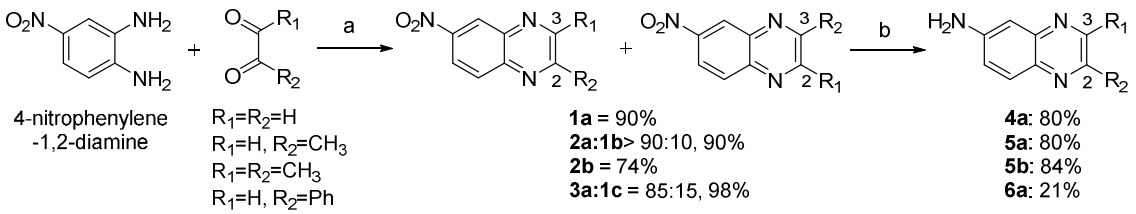
In order to improve the neuroprotective activity of compound **5c**, we designed and synthesized derivatives and carried out their screening using the same PD cellular model as before.[12] For that, several structural modifications were designed based on our previous studies, which reported that the phenyl ring in position 3 of the quinoxaline core structure is important for neuroprotection.[12] In addition, the presence of a C16 aliphatic chain on an *N*-alkyl-6-aminoquinoxaline derivative was correlated with a neuritogenic activity.[12] On this basis, we prepared several **5c** derivatives with either an aryl or a heteroaryl ring in position 3 of the quinoxaline. The role of the methyl substituent in position 2 was also studied. We also synthesized 6-amino-quinoxaline derivatives functionalized by a chlorine and/or bromine atom in position 5 and 7, as halogenation of quinolines can be associated with neuroprotection, as previously reported in the case of clioquinol.[13] *N*-alkyl derivatives of **5c** were also synthesized since a number of *N*-alkyl-6-aminoquinoxaline derivatives and propargylamine-derived compounds, such as rasagiline exhibit interesting pharmacological features; rasagiline is a monoamine oxidase inhibitor used as symptomatic treatment for PD and this compound is also believed to delay disease progression .[14,15]

After characterization of the neuroprotective activity of our derivatives on DA cells, we also studied the activity of our new hit compounds on glial cells, because neuroprotection can be indirect in this model system as in the case of **5c**.[12] The protective effect of compound **4c**, one of our hit compounds that was closely related structurally to **5c**, had no effect on glial cells, but seemed to operate directly on DA neurons. Its effect was due in part to the activation of ryanodine receptor (RyR) calcium release channels. Such a mechanism of action has been previously described for paraxanthine, the main neuroprotective metabolite of caffeine, which demonstrated *in vivo* neuroprotection.[16,17] Interestingly, **4c** also exerted substantial neuroprotection against DA cell death triggered by trophic support withdrawal or by induction of low-level oxidative stress, suggesting that this compound may be effective against different PD-related mechanisms. Most interestingly, these neuroprotective properties were still observed *in vivo* as **4c** exerted a robust neuroprotective action towards nigrostriatal DA neurons in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD.

RESULTS

Chemistry. Di-substituted 6-aminoquinoxalines were obtained by various methods. Hinsberg condensation between a dianiline (e.g. 4-nitrophenylene-1,2-diamine) and a 1,2-dicarbonyl compound in refluxing water gave the corresponding quinoxaline in good yield. Ketoaldehyde (e.g. pyruvaldehyde, phenylglyoxal) gave preferentially the 2-substituted-6-aminoquinoxaline (**Scheme 1**).[18] After reduction of the nitro group to the corresponding aniline (H_2, Pd/C in EtOH), the major regioisomer was obtained as a pure product after column chromatography.

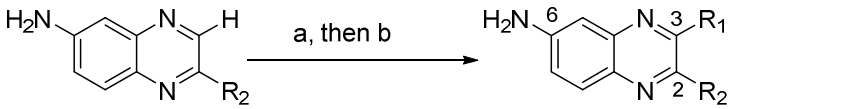
Scheme 1. Synthesis of 6-aminoquinoxaline and 2-substituted-6-aminoquinoxalines by Hinsberg condensation.



Reagents and conditions: (a) H_2O, reflux, (b) H_2, Pd/C in EtOH, 60 °C, then purification by flash chromatography.

Starting from 2-substituted-6-aminoquinoxalines, which are prepared by Hinsberg condensation, various 2,3-disubstituted quinoxalines were obtained by addition of an organolithium reagent at –78 °C and subsequent oxidation by manganese dioxide[19] (**Scheme 2**). Yields are low to moderate, depending on the nature of the organolithium reagent; aryllithium gave the expected compounds in low yields. Indeed, the reactivity of the organolithium reagent in this reaction is correlated with its basicity, which explains these contrasting results.

Scheme 2. Synthesis of 2,3-disubstituted quinoxalines.



4a, R_2 = H

5a, R_2= CH_3

6a, R_2= Ph

4e, R_1=4-F-Phenyl, 7%

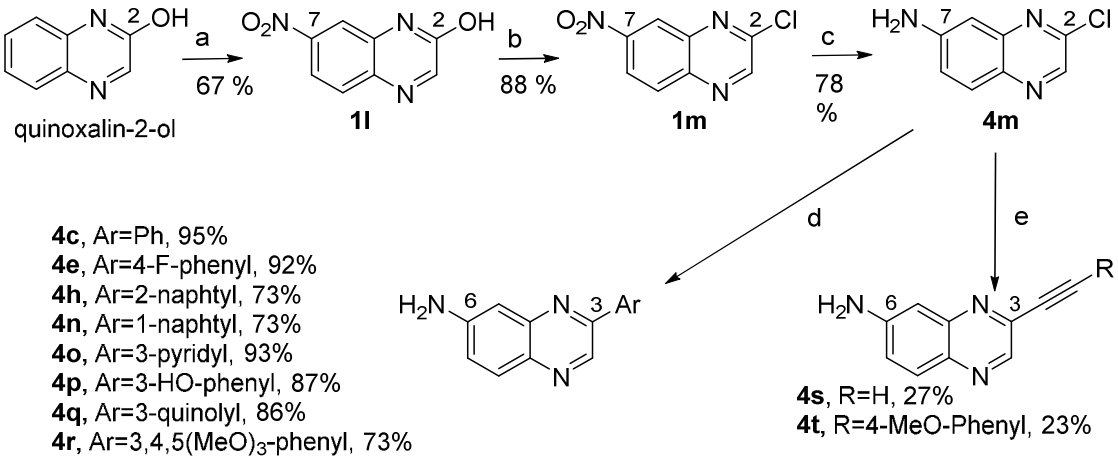
5c, R_1=Ph, 45%
5d, R_1=4(MeO)-phenyl, 34 %
5e, R_1=4-F-Phenyl, 35%
5f, R_1=4-Cl-Phenyl, 70%
5g, R_1=4-Me-Phenyl, 56%
5h, R_1=2-naphtyl, 48%
5i, R_1=3,4-$(MeO)_2$-phenyl, 31%
5j, R_1=3,4-Cl_2-phenyl, 45%
5k, R_1=4-binaphtyl, 30%
5r, R_1=3,4,5-$(MeO)_3$-phenyl, 21%

6d, R_1=4-MeO-Phenyl, 2%

Reagents and conditions: (a) R_1Li (2.5 equiv.), THF, –78 °C or *t*BuLi (8 equiv.), R_1Br (4 equiv), –78 °C, Et_2O/THF if R_1 is aryl (b) MnO_2 (5 equiv.), $CHCl_3$, reflux.

Because of the weak reactivity of aryl lithium reagents, 3-aryl-6-aminoquinoxalines were produced by an alternative approach. Regioselective nitration at position 7 of quinoxalin-2-ol was performed by treatment with nitric acid in acetic acid. Then, 7-nitroquinoxalinol **1l** was treated with $POCl_3$ to yield 2-chloro-7-nitroquinoxaline **1m**.[20] $SnCl_2$ mediated reduction of nitro function gave 2-chloro-7-aminoquinoxaline **4m**. Finally, palladium-catalyzed cross-coupling reaction of the latter with diverse arylboronic acids led to the expected 3-aryl-6-aminoquinoxalines in excellent yields. Alternatively, an acetylene function was introduced in position 3 through a Sonogashira cross coupling reaction, albeit in a low yield. These steps are presented in **Scheme 3**.

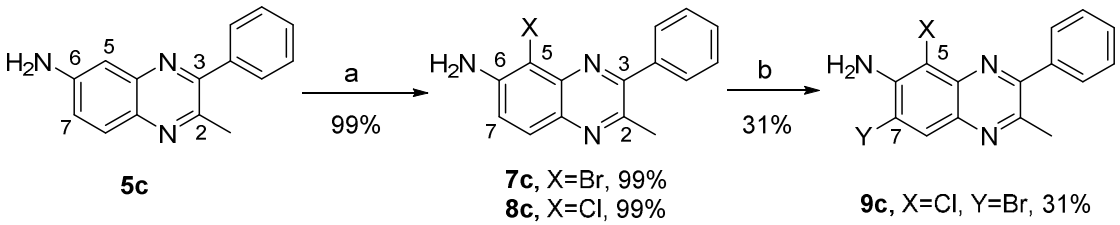
Scheme 3. Synthesis of 3-aryl-6-aminoquinoxalines from quinoxalin-2-ol.



Reagents and conditions: (a) HNO_3 (2 equiv.), acetic acid, rt. (b) $POCl_3$, reflux. (c) $SnCl_2$, EtOAc, reflux. (d) $ArB(OH)_2$ or ArB(MIDA) (1.3 equiv.), $(PPh_3)_2PdCl_2$ (0.03 equiv.), K_2CO_3 (2.6 equiv.), dioxane/water, reflux. (e) TMS-acetylene or 4-methoxy-phenylacetylene, $PdCl_2(PPh_3)_2$, CuI, Et_3N, THF, reflux. When R = TMS subsequent basic treatment (K_2CO_3, MeOH, reflux 10 min) furnishes compound **4s**. MIDA = *N*-methyliminodiacetic acid.

Starting from the above synthesized 6-aminoquinoxalines, several other derivatives were obtained. For instance, regioselective bromination in position 5 of compound **5c** was prepared in almost quantitative yield by reaction of **5c** with one equivalent of bromine in AcOH or with a slight excess of NBS in CH_2Cl_2 at room temperature (rt) to give **7c**. Chlorination of **5c** was very effective as well by using NCS in CH_2Cl_2, at rt, to give regioselectively, compound **8c**. Position 7 of **8c** could be halogenated (e.g. bromine in excess) with more difficulties to give for instance compound **9c** (**Scheme 4**).

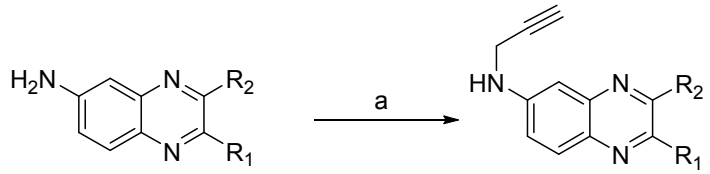
Scheme 4. Regioselective halogenation of 6-aminoquinoxaline 5c.



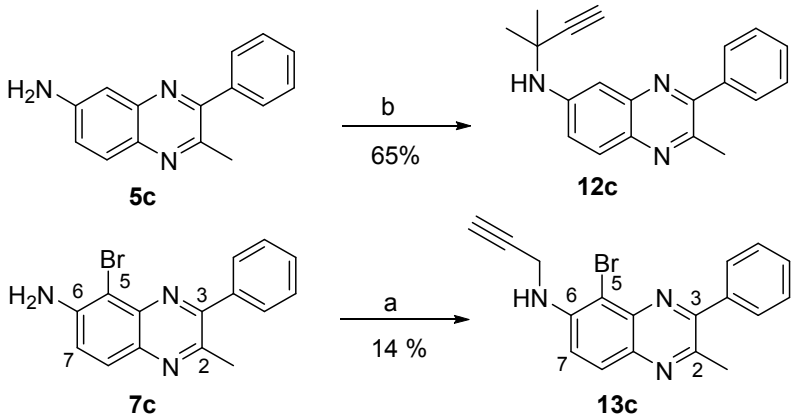
Reagents and conditions: (a) Br_2 (1 equiv.), AcOH, rt., 1 h or NCS / NBS (1.2 equiv.), DCM, rt., 2 h. (b) Excess Br_2, AcOH, rt, 2 h.

N-propargyl-6-aminoquinoxaline derivatives have been also prepared. Thus, *N*-propargyl-6-aminoquinoxaline derivatives **10c**, **10e**, **11c**, **11d**, **11e**, **11f**, **11g**, **11h**, **11n**, **11r**, were respectively obtained by reaction of compounds **4c**, **4e**, **5c**, **5d**, **5e**, **5f**, **5g**, **5h**, **5j**, **5r**, with propargyl-bromide and K_2CO_3 in hot DMF. In a similar manner, propargyl derivative **12c** was prepared from **5c** and 3-chloro-3-methyl-1-butyne in presence of a catalytic amount of CuCl and trimethylamine.[21] *N*-propargylation of 5-bromo-2-methyl-3-phenyl-6-aminoquinoxaline derivative **7c** was also performed, affording compound **13c**. These steps are presented in **Scheme 5**.

Scheme 5. Synthesis of N-propargyl-6-aminoquinoxaline derivatives.



4c, R_1= H, R_2=Ph	**10c**, 49%
4e, R_1=H, R_2=4-F-phenyl	**10e**, 41%
5c, R_1=Me, R_2=Ph	**11c**, 40%
5d, R_1=Me, R_2=4-MeO-phenyl	**11d**, 71%
5e, R_1=Me, R_2=4-F-phenyl	**11e**, 28%
5f, R_1=Me, R_2=4-Cl-phenyl	**11f**, 34%
5g, R_1=Me, R_2=4-Me-phenyl	**11g**, 40%
5h, R_1=Me, R_2=2-Naphtyl	**11h**, 28%
5n, R_1=Me, R_2=3,4-Cl_2-phenyl	**11n**, 50%
5r, R_1=Me, R_2=3,4,5-$(MeO)_3$-phenyl	**11r**, 42%



Reagents and conditions: (a) propargyl bromide (1.5 equiv.), KI (1 equiv.), K_2CO_3 (1 equiv.), DMF, 70 °C. (b) 1,1-dimethyl-propargyl chloride (1.4 equiv.), CuCl (cat.), Et_3N (1,4 equiv.), THF/water, rt.

Neuroprotective potential of newly synthesized compounds using a culture system that models DA cell loss in PD. All synthesized compounds (**4c, 4e, 4h, 4m, 4n, 4o, 4p, 4q, 4r, 4s, 4t, 5a-k, 6a, 6d, 7c, 8c, 9c, 10c, 10e, 11c-h, 11n, 11r, 13c, 12c)** were evaluated for their neuroprotective potential using a PD culture model in which DA neurons die spontaneously and selectively as a consequence of a mechanism involving immature astrocytes and calcium dyshomeostasis.[22,23] Cultures were maintained for 10 days in the presence or absence of the

different test compounds, and neuroprotection was then evaluated by counting surviving TH immunopositive (TH^+) neurons.

Consistent with previous results, we observed that more than 60% of TH^+ neurons were generally lost after 10 days of culture in the absence of any treatment.[22,23] *N*-(6), 2'-O-dibutyryladenosine 3':5' cyclic monophosphate (DbcAMP), a lipophilic analog of cAMP that is commonly used as a reference molecule in this model system[24] exhibited robust protective effects for DA neurons in the present study (**Figure 1**). Among a series of 25 6-aminoquinoxaline derivatives (**4c, 4e, 4h, 4m, 4n, 4o, 4p, 4q, 4r, 4s, 4t, 5b-k, 6a, 6d, 7c, 8c, 9c**), 14 of them showed a significant neuroprotective effect at 50 µM (**4m, 4c, 4p, 4r, 4e, 4h, 4n, 4o, 4q, 5e, 5j, 6a, 7c, 8c**). This effect was either lower or higher than that provided by **5c**, used at 100 µM. Based on these results, we noticed that all synthesized compounds with a 2-hydrogen-3-aryl-6-aminoquinoxaline core structure (**4c**, **4e**, **4h**, **4n,,4o**, **4p**, **4q**, **4r**) were significantly neuroprotective, thus suggesting that the lack of a substituent at position 2 of the quinoxaline ring was always associated with neuroprotection. When comparing **6a** to its regioisomer **4c**, we found that these two compounds had strong and similar neuroprotective activities (compound **4c** at 50 µM: 226.0 ± 9.4 % *vs* compound **6a** at 50 µM: 187.3 ± 6.1 %). This suggests that a lack of substitution in position 2 is not a necessary requirement for neuroprotection. Instead, to be optimally effective 6-aminoquinoxalines need to be mono-substituted in position 2 or 3 by an aryl group.

Halogenation of the quinoxaline ring (**7c**, **8c**) or substitution of the phenyl ring in position 3 (**4e**, **5e**, **5j**) with a halogen group (chlorine or bromine) led generally to active compounds. Replacement of the phenyl ring by a chlorine atom led also to an active compound (**4m**), while replacement of the phenyl ring by an alkyl or alkyne group led to inactive (**5b**) or even toxic compounds (**4s**, **4t**, data not shown). The effects of 6-aminoquinoxaline derivatives, which are protective for DA neurons at 50 µM, are described in **Figure 1**. The effects of the

other test compounds (**4s**, **4t**, **5b**, **5d**, **5f**, **5g**, **5h**, **5i**, **5k**, **6d**, **9c**) with no protective activity are not shown. Chemical structures of all test compounds (active and inactive) are depicted in **Chart 1**.

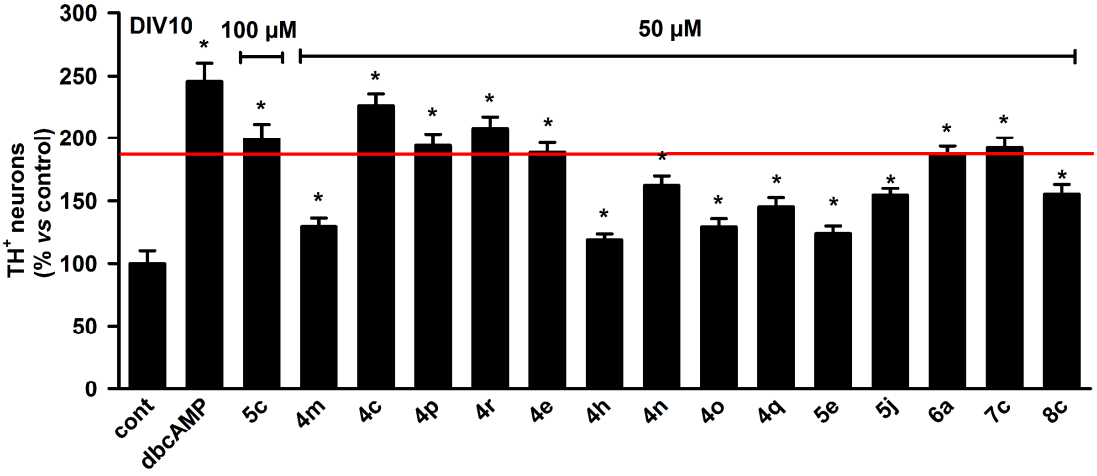


Figure 1. Screening of 6-aminoquinoxaline derivatives in a cellular system that models selective DA cell death in midbrain cultures. Number of TH^+ neurons surviving after 10 days *in vitro* (DIV) in midbrain cultures treated or not with 6-aminoquinoxalines (50 µM, except (**5c**) 100 µM). Note that **5c** is inactive at 50 µM.[12] DbcAMP (1 mM) is used as reference protective molecule for DA neurons. Inactive 6-aminoquinoxaline derivatives (**4s**, **4t**, **5b**, **5d**, **5f**, **5g**, **5h**, **5i**, **5k**, **6d**, **9c**) are not included in the figure. A 185 % increase in survival at 50 µM, is our cut-off for the selection of the most active compounds (above red line). *, $p< 0.05$ *vs* controls.

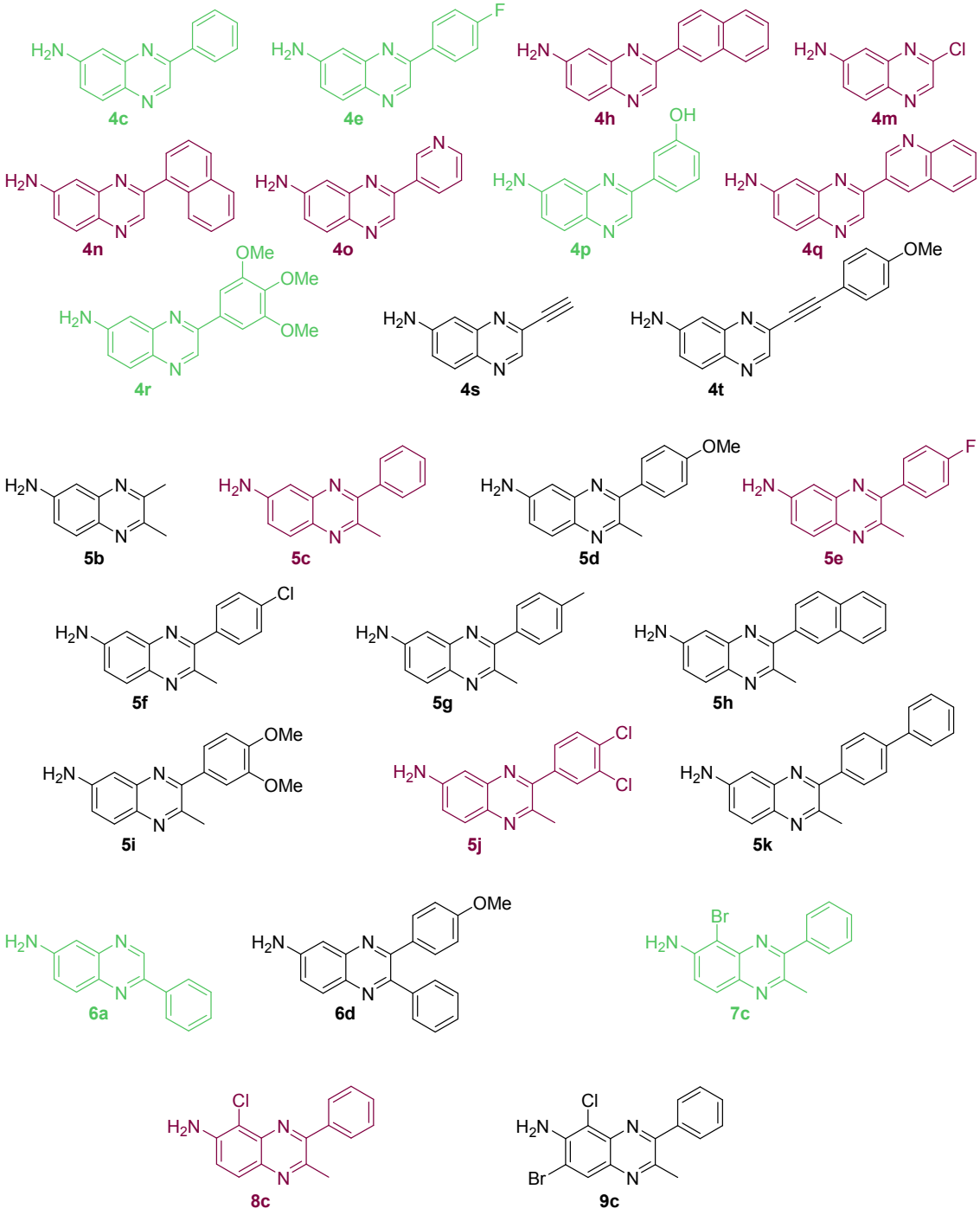


Chart 1. Chemical structures of all 6-aminoquinoxaline derivatives tested in the cellular PD model of spontaneous DA cell death. Compounds having significant protective effects

towards DA neurons at 50 µM are colored in purple. Protective compounds selected for further investigations (see **Figure 1** for selection criteria) are colored in green.

In order to find 6-aminoquinoxaline derivatives more potent and efficient than **5c**, we selected 6 compounds of the present series (**4c, 4p, 4r, 4e, 6a, 7c**) on the basis of their efficacy to protect DA neurons, at 50 µM. More specifically, we selected compounds improving the survival rate of DA neurons at 10 DIV by more than 185 % in comparison to corresponding control cultures, a protection similar or superior to that provided by **5c**, at 100 µM. The selected compounds were then tested at 10 µM, using the same cellular assay (see **Figure 2**).

Only compounds **4c**, **4e** and **6a** remained neuroprotective at 10 µM. These compounds possessed similar efficacy at 10 µM (compound **4c** *vs* **4e** vs **6a**: 151.5 ± 6.2 % *vs* 144.2 ± 7.0 % *vs* 134.4 ± 4.9 %), with, however, a slightly better protective effect for **4c**. Interestingly compound **4c,** which has a core structure very close to that of **5c** (**4c**: 3-phenyl-6-aminoquinoxaline *vs* **5c**: 2-methyl-3-phenyl-6-aminoquinoxaline), was significantly more effective than **5c** at 100 µM (**5c** 100 µM: 199.2 ± 11.8 % *vs* **4c** 50 µM: 226.0 ± 9.4 %). This confirms the idea that a lack of substituent in position 2 of the quinoxaline ring is beneficial for neuroprotection.

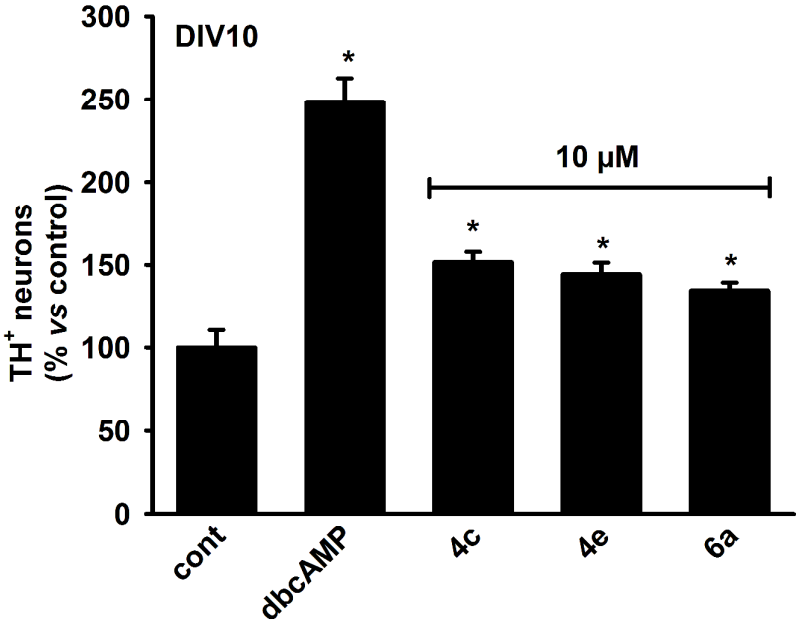


Figure 2. Evaluation of previously selected 6-aminoquinoxaline derivatives for their efficacy at a concentration of 10 µM. Selected 6-aminoquinoxaline derivatives were applied to midbrain cultures at 10 µM and the survival rate of DA neurons was assessed at 10 DIV. Derivatives not providing protection at 10 µM (**4p**, **4r**, **7c**) are not shown in this figure. DbcAMP (1 mM) was used as reference protective molecule for DA neurons. *, $p< 0.05$ *vs* controls.

Among another series of 12 *N*-propargyl-6-aminoquinoxaline derivatives (**10c**, **10e**, **11c**, **11d**, **11e**, **11f**, **11g**, **11h**, **11n**, **11r**, **13c**, **12c**), 5 compounds showed a significant neuroprotective effect at 50 µM (**10c**, **10e**, **11c**, **11e**, **13c**). The protective effect was either lower or higher than that provided by **5c**, at 100 µM. The activity of *N*-propargyl-6-aminoquinoxaline derivatives at 50 µM and their chemical structures are described in **Figure 3**. The effects of the other test compounds (**11f**, **11g**, **11h**, **11n**, **12c**) with no protective activity are not shown. Chemical structures of all test compounds (active and inactive) are presented in **Chart 2**.

Consistent with previous results obtained with 2,3-disubstituted-6-aminoquinoxalines, we noticed that among five *N*-propargyl-6-aminoquinoxaline derivatives (**10c**, **10e**, **11c**, **11e**, **13c**) that are neuroprotective for DA neurons, two are not substituted in position 2 (**10c**, **10e**), which confirms our hypothesis that lack of substitution at position 2 of the quinoxaline ring is beneficial for neuroprotection.

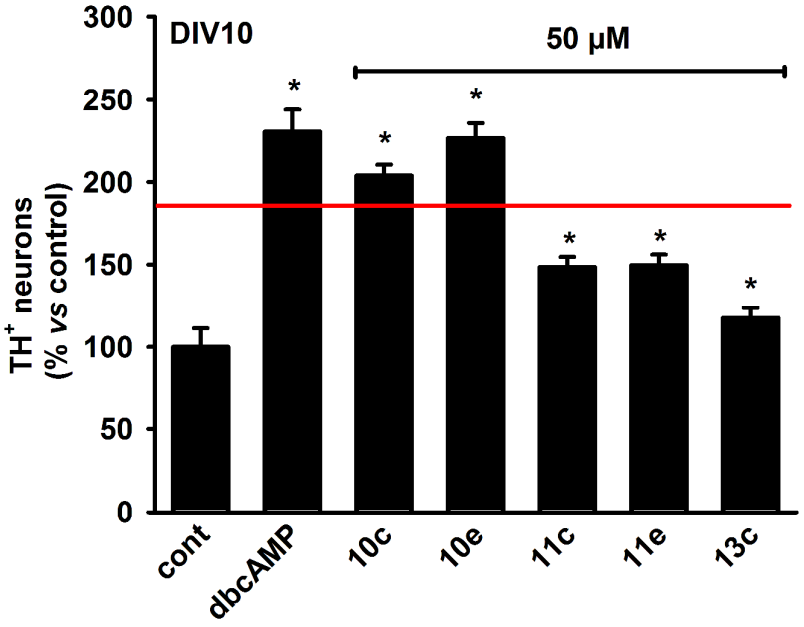


Figure 3. Screening of *N*-propargyl-6-aminoquinoxaline derivatives in a cellular system that models selective DA cell death in midbrain cultures. Number of TH^+ neurons surviving after 10 DIV in cultures treated or not with *N*-propargyl-6-aminoquinoxaline derivatives (50 µM). DbcAMP (1 mM) is used as reference protective molecule for DA neurons. Inactive *N*-propargyl-6-aminoquinoxaline derivatives (**11d**, **11f**, **11g**, **11h**, **11n**, **11r**, **12c**) are not included in this figure. A 185 % increase in survival at 50 µM, is our cut-off for active compounds (above red line). *, $p< 0.05$ *vs* controls.

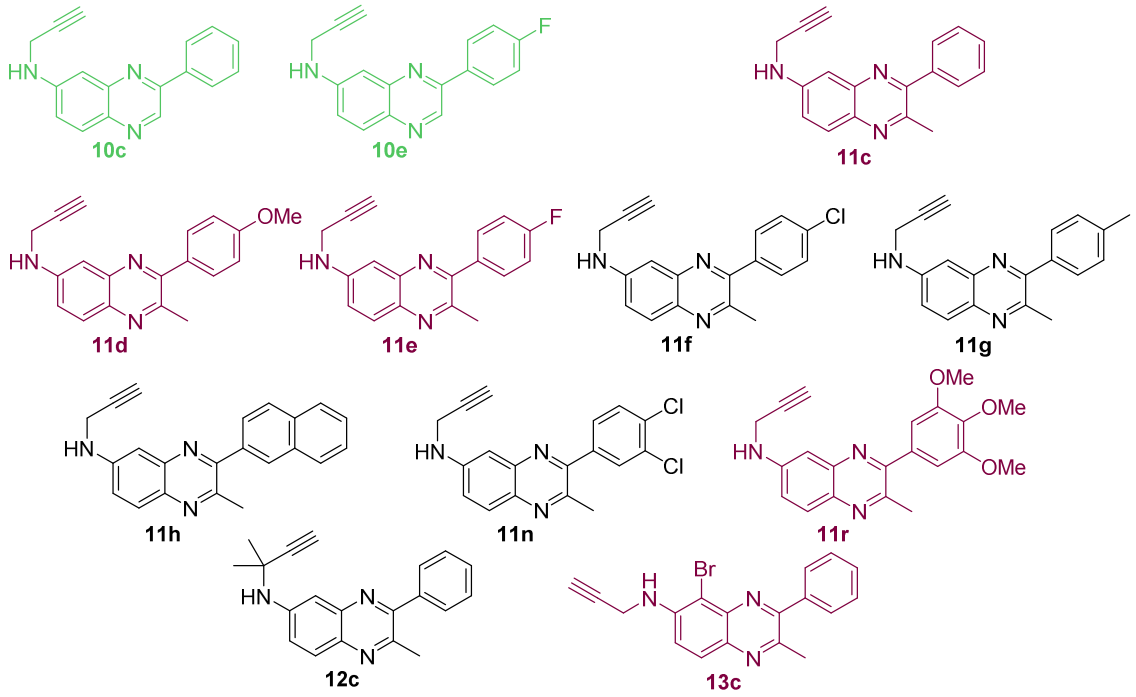


Chart 2. Chemical structures of all *N*-propargyl-6-aminoquinoxaline derivatives synthesized and tested in the cellular PD model of spontaneous DA cell death. Compounds having significant protective effects towards DA neurons at 50 µM are colored in purple. Protective compounds selected for further investigations (see **Figure 3** for selection criteria) are colored in green.

In order to find *N*-propargyl-6-aminoquinoxaline derivatives more potent and efficient than **5c**, we selected 2 compounds of this series (**10c**, **10e**) on the basis of their efficacy to protect DA neurons, at 50 µM. More specifically, we selected compounds improving the survival rate of DA neurons at 10 DIV by more than 185 % in comparison to corresponding control cultures, a protection similar or superior to that provided by **5c**, at 100 µM. The selected compounds were then tested at 10 µM, using the same cellular assay (see **Figure 4**).

Compounds **10c** and **10e** conserved a significant neuroprotective activity at 10 µM. Compound **10c** had a better efficacy than **10e** and a similar efficacy to dbcAMP, at 1mM (dbcAMP 1 mM: 225.3 ± 13.1 % *vs* **10c** 10 µM: 211.6 ± 7.6 %). Interestingly, compound **10c,** which has a core structure very close to **5c**, like **4c**, was significantly more potent and efficient than **5c** (**5c** 100 µM: 199.2 ± 11.8 % *vs* **10c** 10 µM: 211.6 ± 7.6 %).

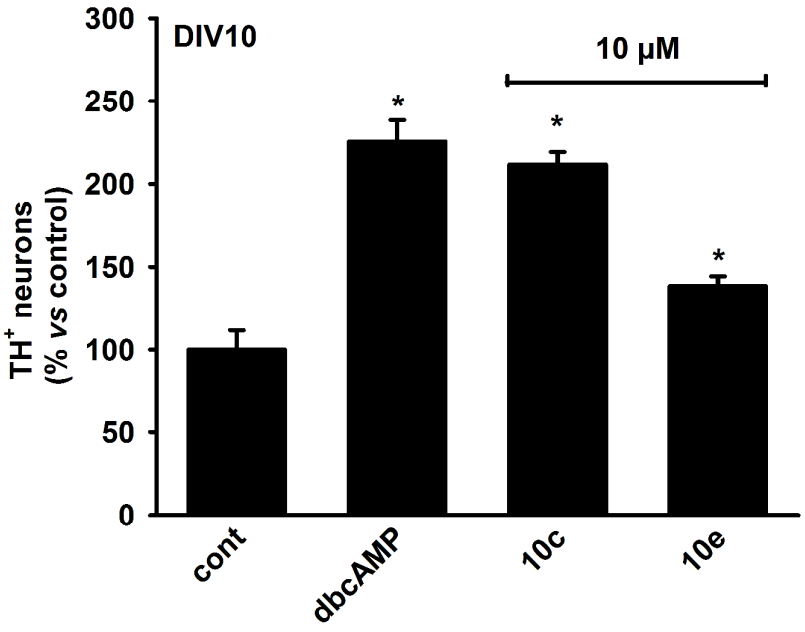


Figure 4. Evaluation of previously selected *N*-propargyl-6-aminoquinoxaline derivatives for their efficacy at 10 µM. Selected *N*-propargyl-6-aminoquinoxaline derivatives were applied to midbrain cultures at 10 µM and the survival rate of DA neurons was assessed at 10 DIV. DbcAMP (1 mM) is used as reference protective molecule for DA neurons. *, $p < 0.05$ *vs* controls.

Effect of quinoxaline derivatives on glial cell proliferation. In the cellular model used herein, we reported previously that neuroprotection could result from a repressive effect on

dividing glial cells, in particular on astrocytes and their precursor cells, which represent the bulk of proliferating cells.[25,26,27] In particular, we have shown that the reference compound dbcAMP and **5c** were protective by decreasing glial cell proliferation.[12,28] Therefore, we wished to have first a rough estimate of the impact that 6-aminoquinoxaline derivatives may have on glial cells. For that, we determined semi-quantitatively whether test compounds had an effect on the density and morphology of glial cells that were labelled with the glial marker protein, vimentin, using fluorescence microscopic examination of the cultures. We tested at two concentrations (10 and 50 µM) the three 6-aminoquinoxaline and the two *N*-propargyl-6-aminoquinoxaline derivatives that were selected on the basis of their neuroprotective effect for DA neurons at 10 µM. Data are presented in **Table 1**.

	Semi-quantitative effect of test treatments on the density and morphology of vimentin^{+} glial cells	
Compound	10 µM	50 µM
-	-	
dbcAMP[a]	+++	
5c	-	++[b]
4c	-	+/-
4e	-	+
6a	-	+/-
10c	+	++
10e	+/-	++

Table 1: Impact of 6-aminoquinoxaline and *N*-propargyl-6-aminoquinoxaline derivatives on the density and the morphology of labeled glial cells with glial cell marker vimentin. (+/-) no

effect or only weak effects, (+) weak effects, and (++) marked effects on vimentin^{+} cells. [a] DbcAMP (1 mM) is used as reference molecule. [b] tested at 100 μM.

Almost all the neuroprotective compounds had at least a weak effect on glial cells. Similar to **5c** at 100 μM, *N*-propargyl derivatives exerted the strongest effects on the astrocytic glial cell population. Thus, we may assume that neuroprotective effects of compounds **10c** and **10e** resulted from a glial-dependent mechanism, as observed with the first generation compound, **5c**. By contrast, compounds **6a** and **4c**, which possess a strong neuroprotective effect at 50 μM and 10 μM, did not seem to exert an effect on glial cells. This result is surprising for compound **4c**, since this product is structurally very close to the previous hit compound **5c**.

Overall **4c** presents a number of interesting features; (i) it is very effective for neuroprotection, (ii) it is apparently inactive towards glial cells, which suggests that it may operate *via* a direct effect on DA neurons and (iii) it is the closest structural homolog of **5c,** which is a druggable candidate in particular due to its capability to penetrate the brain parenchyma.[12] To further demonstrate that **4c** had no significant effects on glial cells, we used [methyl-^{3}H]-thymidine as a marker of proliferation of glial cells. The amount of incorporated [methyl-^{3}H]-thymidine was measured as previously described.[28] As shown in **Figure 5**, **4c** did not significantly affect the incorporation of tritium-labeled thymidine in midbrain cultures (93.8 ±1.7 % *vs* control 100.0 ± 1.5 %), which is consistent with our previous semi-quantitative analysis. This result suggests that compound **4c** may be protective through a direct effect on DA neurons. This is a key advantage, as it may reduce the risk of side effects possibly associated with systemic drug administration in an animal model of PD and ultimately in PD patients.

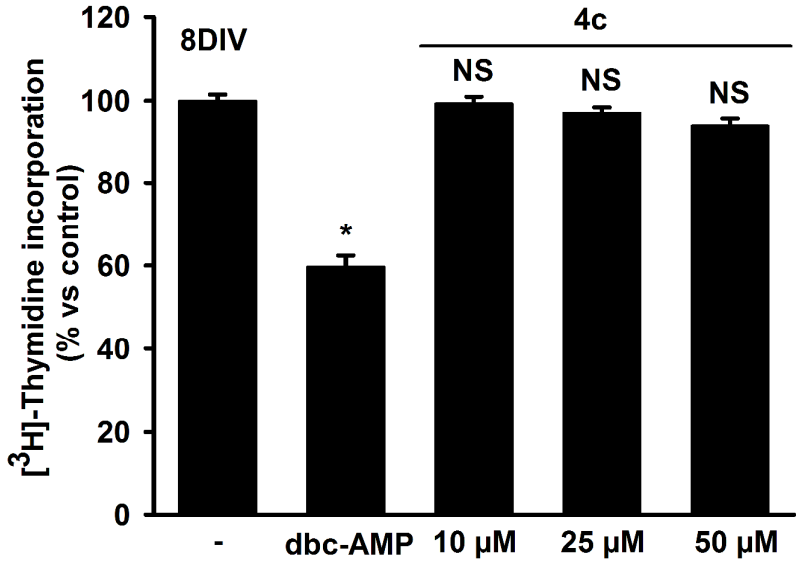


Figure 5. 4c does not reduce glial cell proliferation in midbrain cultures. Quantification of [^{3}H]-thymidine incorporation in 8 DIV cultures, treated or not with **4c** (10-50 µM). DbcAMP at 1 mM was used as a positive control. NS: no significant effect.

Characterization of the effects of 4c towards DA neurons: morphological and functional effects. We wished to further characterize the effect of **4c** towards DA neurons. In particular, we were interested to determine (i) whether rescued DA neurons remained functional and (ii) whether **4c** exerted neuritogenic effects on these neurons. To characterize these effects, we performed experiments in which midbrain cultures treated with different concentrations of **4c** (10, 25, 50 µM) were processed either for TH immunodetection or for tritium-labeled DA uptake, a marker of both DA cell function and differentiation.[29]

The protective effects of **4c** observed between 10-50 µM were associated with an increase of DA uptake in the same range of concentrations (see **Figure 6A, B, C**), suggesting that **4c**-treated DA neurons were fully functional. Also, the rate of DA uptake per TH$^+$ neuron was significantly increased in **4c**-treated cultures (see **Figure 6D**). This latter observation

suggested that DA neurons rescued by **4c** were not only functional but that they were also more differentiated, as DA uptake sites are preferentially localized on neuritic extensions.[29] Consistent with this observation, microscopic examination of the cultures revealed that TH^+ neurons treated with **4c** had generally a more developed neuritic network in comparison to control neurons (**Figure 6B**). Note that TH^+ cell survival and DA uptake were increased in similar proportions in cultures treated with dbcAMP. This is in agreement with the absence of neuritogenic effects of this compound on TH^+ neurons (**Figure 6B**).

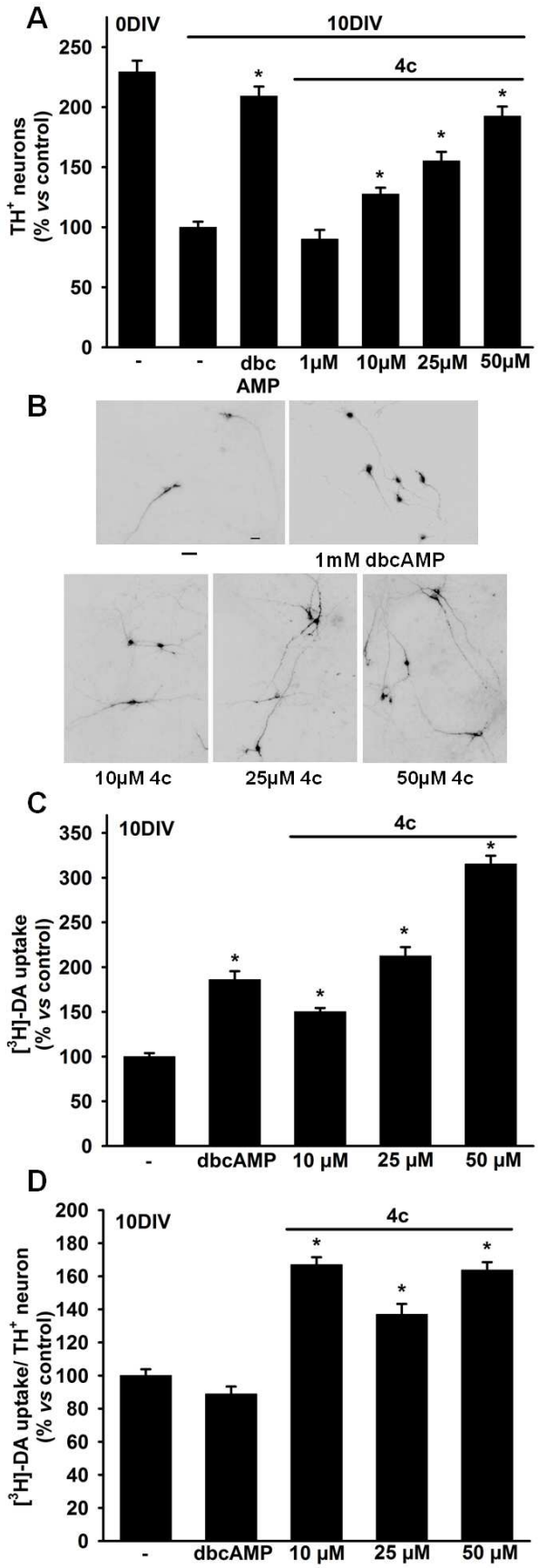

A
0DIV
10DIV
4c
TH+ neurons (% vs control)
250
200
150
100
50
0
-
-
dbc AMP
1μM
10μM
25μM
50μM
B
1mM dbcAMP
10μM 4c
25μM 4c
50μM 4c
C
10DIV
4c
[3H]-DA uptake (% vs control)
350
300
250
200
150
100
50
0
-
dbcAMP
10 μM
25 μM
50 μM
D
10DIV
4c
[3H]-DA uptake/ TH+ neuron (% vs control)
200
180
160
140
120
100
80
60
40
20
0
-
dbcAMP
10 μM
25 μM
50 μM

Figure 6. Neuroprotective and neuritogenic effects of **4c** onto DA neurons in midbrain cultures. (A) Number of TH^+ neurons, in 10 DIV cultures, treated or not with **4c** (1-50 μM). (B) Illustration showing TH^+ neurons, in 10 DIV cultures, treated or not with **4c** (10-50 μM). Illustrations of the effects of **4c** on TH^+ cells are presented under an inverted format. Scale bars = 60 μm. In (A), (B), (C) and (D) DbcAMP (1mM) was used as reference protective molecule. (C) Quantification of [^{3}H]-DA uptake, in 10 DIV cultures, treated or not with **4c** (10-50 μM). (D) Rate of DA uptake per TH^+ neuron in 10 DIV cultures treated or not with **4c** (10-50 μM). *P < 0.05 *vs* control cultures.

Mechanism of action of 4c: role of intracellular calcium stores. Previous studies have shown that DA neurons can be rescued in this culture system by compounds that have a repressive effect on glial cells, i.e., immature astrocytes.[30] Such a mechanism is unlikely for **4c**, as this compound does not prevent glial cell proliferation, as shown in **Figure 5**. Another set of neuroprotective molecules is represented by depolarizing compounds that have the ability of maintaining cytosolic calcium levels within a neuroprotective range of concentrations.[23] To explore the possible impact of **4c** on intracellular calcium levels, we exposed **4c**-treated cultures to molecules having the capacity to prevent Ca^{2+} influx at the plasma membrane. We used blockers of L-type (nifedipine) and N-type (ω-conotoxin MVIIA) voltage gated calcium channels. None of these compounds caused a decrease of **4c** neuroprotective effects. However, the blockade of endoplasmic reticulum calcium release channels, namely ryanodine receptor channels (RyRs), by dantrolene led to a substantial reduction of the effects of **4c**. This indicates that RyRs may represent a potential target for neuroprotection by **4c** (see **Figure 7**). This is reminiscent of previous observations showing that activation of this receptor by paraxanthine, the primary metabolite of caffeine, is protective for DA neurons in experimental PD models.[31,32,16]

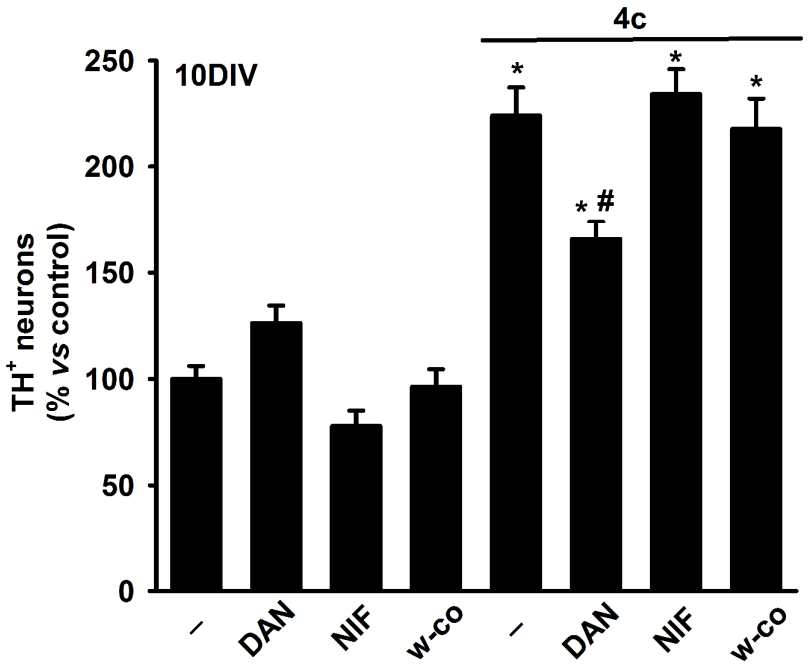


Figure 7. Neuroprotection by **4c** requires calcium mobilization through RyRs. Number of TH^+ neurons in 10 DIV cultures chronically exposed or not to **4c** (50 μM) in the presence or not of nifedipine (NIF, 20μM), ω-conotoxin MVIIA (ω-CON, 0.5μM) or dantrolene (DAN, 30 μM). *P < 0.05 vs. no treated cultures. #P < 0.05 *vs* **4c** treated cultures.

The neuroprotective effect of 4c on DA neurons is independent of Glial cell-Derived Neurotrophic Factor (GDNF). GDNF is a trophic peptide that exerts potent neuroprotective and neuritogenic effects on DA neurons.[33] Interestingly, we observed that **4c** had about the same efficacy as GDNF in rescuing DA neurons in midbrain cultures. Furthermore, like GDNF, **4c** promoted neuritogenesis. This led us to postulate that **4c** could operate by stimulating the endogenous production of GDNF. To test this possibility, we assessed the activity of **4c** in the presence of an anti-GDNF antibody (AB-212-NA; 10 μg/ml) that neutralizes the biological activity of the neurotrophic peptide, as previously described.[12,22] Whereas the antibody was sufficient to prevent the increase in DA cell survival resulting

from a treatment with GDNF, it failed to reduce neuronal survival in the presence of 50 μM of **4c** (**Figure 8**). This indicates that the effects of **4c** are unrelated to that of GDNF.

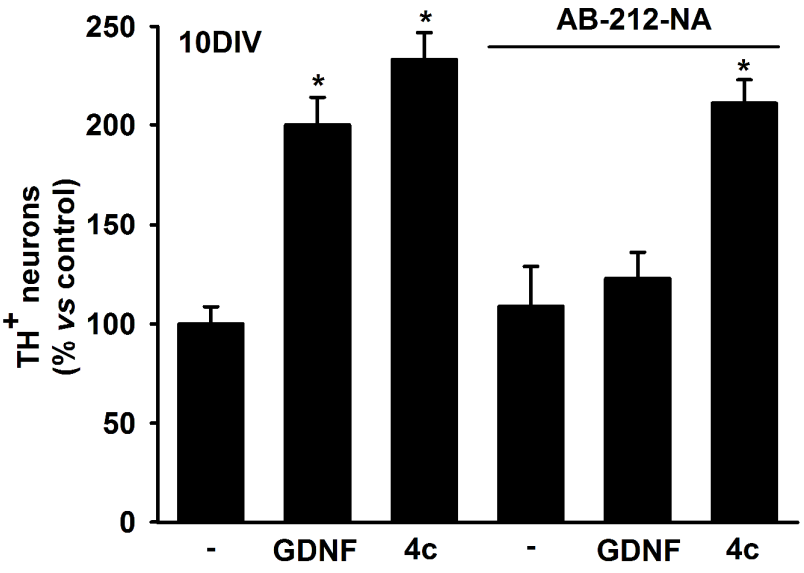


Figure 8. The protective effect of **4c** is unrelated to that of GDNF. Number of TH^+ neurons, in 10 DIV cultures, treated or not with **4c** (50 μM), in presence or not of an anti-GDNF antibody (AB-212-NA, 10 μg/mL). GDNF at 20 ng/ml was used as a positive control. *P < 0.05 *vs* control cultures. $^{\#}P < 0.05$ *vs* GDNF-treated cultures.

Mature DA neurons deprived of GDNF can be efficiently rescued by 4c

To verify that the effect of **4c** was not restricted to a short developmental period after plating, we used midbrain cultures in which the spontaneous death of DA neurons was prevented by chronic application of 20 ng/mL trophic peptide GDNF.[12,22] Removal of GDNF from these cultures at 10 DIV, leads to a massive loss of TH^+ neurons within the next 5 days.[22] We confirmed this finding and established that **4c** was potently protective for DA neurons in this particular setting. DA cell rescue was already highly significant at 10 μM and reached an

optimum between 25-50 µM. The neuroprotective effect of **4c** following GDNF withdrawal is depicted in **Figure 9**.

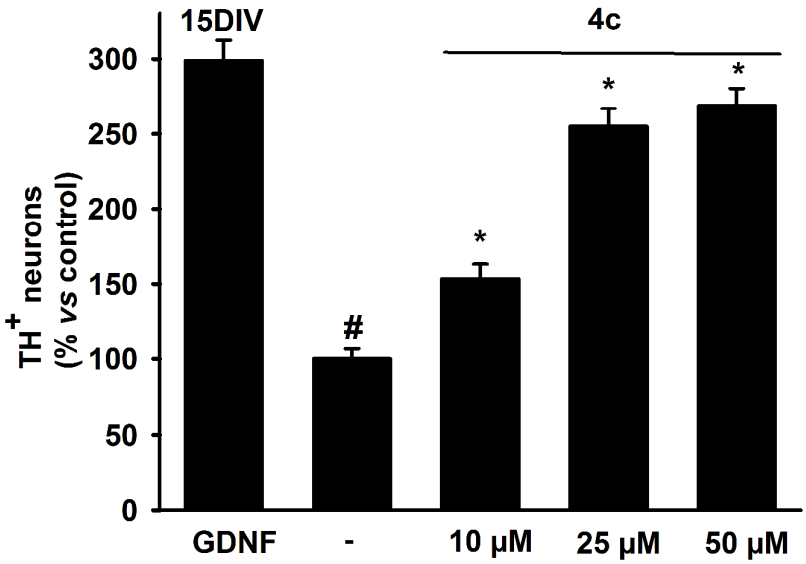


Figure 9. Mature DA neurons dependent on GDNF for their survival can be rescued by **4c**. Rescuing effect of **4c** (10-50 µM) in midbrain cultures exposed to 20 ng/ml GDNF for 10 days and then deprived of the trophic peptide between 11 DIV and 15 DIV. Comparison to cultures maintained continuously with GDNF up to 15 DIV. *P < 0.05 *vs* GDNF-treated cultures. #P < 0.05 *vs* GDNF-deprived cultures.

Antioxidant potential of 4c for DA neurons. 5c, a close structural analog of **4c** is a potent antioxidant molecule.[12] Thus, we tested whether **4c** could also operate *via* an antioxidant effect.[34] For this purpose, we used a culture setting where the death of DA neurons is caused by a Fenton-type reaction that is generated by the presence in the culture medium of catalytically active iron (i.e., ferrous iron) reacting with molecular oxygen.[35] Like **5c**, we found that **4c** was strongly protective for DA neurons. Optimal effects were obtained at 5 µM

(see **Figure 10**); **4c** at 5 μM was as effective as desferrioxamine (10 μM), a molecule with iron chelating properties that has a clinical use.[36,37]

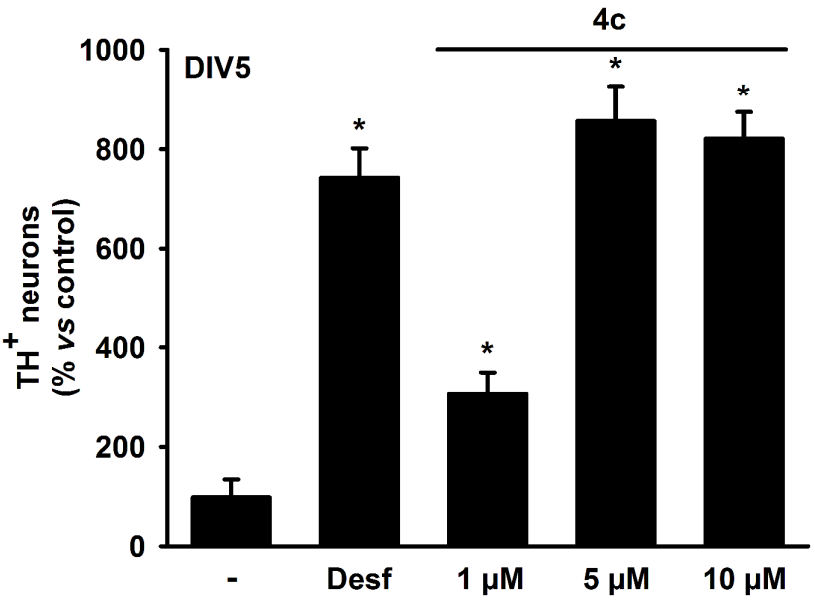
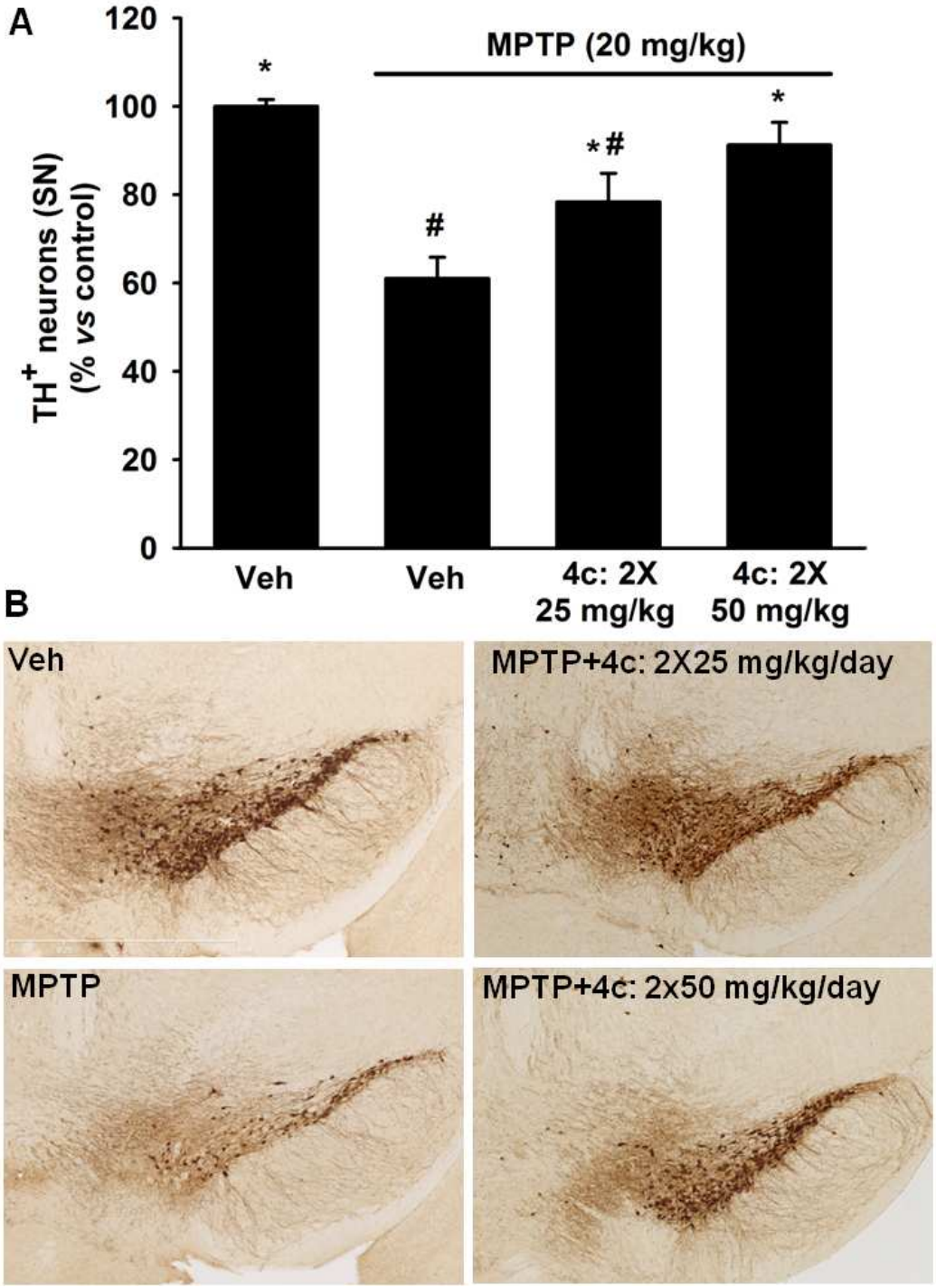


Figure 10. Antioxidant effect of **4c**. Neuroprotective effect of **4c** (1-10 μM) for DA neurons in a model system of chronic oxidative stress. Desferrioxamine (Desf 10 μM) is used as a positive control. *P < 0.05 *vs* control cultures.

Neuroprotective effects of 4c on SN DA neurons in the MPTP mouse model of PD. One of the most commonly used animal models for DA depletion in PD is achieved through systemic administration of the neurotoxin MPTP in mice. This PD model reproduces a number of pathological processes, such as oxidative stress, mitochondrial dysfunction, and inflammation that are thought to participate in disease progression.[38,39] Herein, C57BL/6 mice received a sub-chronic MPTP treatment in order to induce the degeneration of nigrostriatal DA neurons and a depletion in striatal DA and related metabolites.[40]

SN DA neurons were detected by immunostaining the TH enzyme using an anti-TH antibody, followed a revelation step with the chromogenic substrate 3,3-diaminobenzidine (DAB). In line with previous studies,[40,41] a sub-chronic MPTP treatment, in C57BL/6 mice, led to a massive degeneration of nigrostriatal DA neurons (MPTP/vehicle: 61 ± 5% *vs* vehicle: 100 ± 2% TH^+ neurons). Following 11 days of **4c** treatment, at the dose of 2x25 mg/kg/day (*per os*), there was a partial but substantial rescue of TH^+ neurons in comparison to MPTP/vehicle-treated mice (MPTP/**4c** (2x25 mg/kg/day): 78 ± 7% *vs* MPTP/vehicle: 61 ± 5% TH^+ neurons). At the highest dosage (2x50 mg/kg/day), **4c** prevented almost entirely the toxic effect of MPTP on DA neurons (MPTP/**4c** (2x50 mg/kg/day): 91 ± 5% *vs* vehicle: 100 ± 2% TH^+ neurons, no significant difference; $P>0.05$). The neuroprotective effect of **4c** onto DA neurons in the SN of MPTP-treated mice is depicted in **Figure 11**. Importantly, we found that **4c** was only a poor inhibitor of MAO-B, the enzyme that converts MPTP into its active metabolite MPP^+ within the brain.[38,39] More specifically, IC_{50} values for the reference MAO-B inhibitor selegiline and **4c** were estimated to be 1.86 and 513 μM, respectively. This signifies that the neuroprotective effect of **4c** against MPTP is unlikely to result from a reduced bioavailability of MPP^+ for target DA neurons.


A
120
100
80
60
40
20
0
TH+ neurons (SN)
(% vs control)
MPTP (20 mg/kg)
*
#
*#
*
Veh
Veh
4c: 2X
25 mg/kg
4c: 2X
50 mg/kg
B
Veh
MPTP+4c: 2X25 mg/kg/day
MPTP
MPTP+4c: 2x50 mg/kg/day

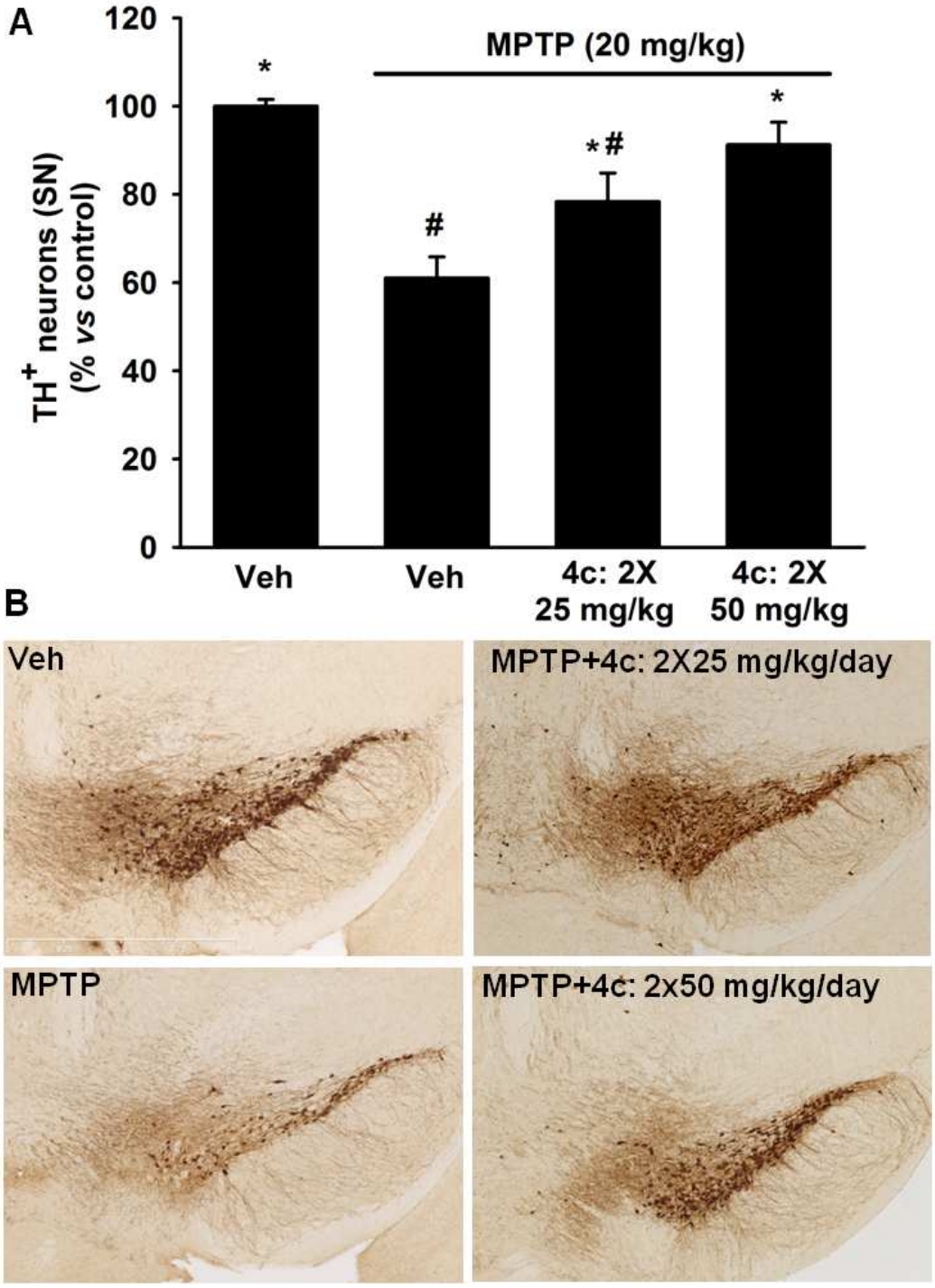


Figure 11. Neuroprotective effects of **4c** onto DA neurons in the SN of MPTP-treated mice. (A) Neuroprotective effect of 11 days of treatment with **4c** (2x25, 2x50 mg/kg/day; *p.o.*) on the number of TH^+ neurons in the SN of MPTP-treated mice (one hemisphere analyzed by mouse). Data expressed as mean ± SEM (n=9-12). *P<0.05 *vs* vehicle + MPTP (20

mg/kg/day) (one-way ANOVA with Dunnett's multiple comparisons). #P<0.05 *vs* vehicle (one-way ANOVA with Dunnett's multiple comparisons). (B) Illustration showing the impact of **4c** (2x25, 2x50 mg/kg/day; *p.o.*) on the number of DA neurons in the SN of MPTP or vehicle-treated mice. High-resolution bright-field digital images of the labeled sections were acquired using a NanoZoomer Digital Scanner (Hamamatsu Photonics France, Massy, France. objective × 40) equipped with the NDP software (Hamamatsu Photonics France, Massy, France. magnification x 4.5). Scale bar = 1 mm. Veh: vehicle.

Partial restoration of striatal DA in the MPTP mouse model of PD. Following 11 days of treatment, **4c** (2x25 mg/kg/day, *per os*), partially restored striatal DA (MPTP/**4c** (2x25 mg/kg/day): 39 ± 2 % *vs* MPTP/vehicle: 28 ± 2 %) and homovanillic acid (HVA) (MPTP/**4c** (2x25 mg/kg/day): 64 ± 2 % *vs* MPTP/vehicle: 53 ± 3 %) levels and reduced the elevation of the 3,4-dihydroxyphenylacetic acid (DOPAC)+HVA)/DA ratio (an index of DA turnover) by 36 % in MPTP-treated mice (see **Figure 12**). At the highest dosage (2x50 mg/kg/day), **4c** produced quite similar effects. There was no significant effect of **4c** treatment on DOPAC levels. These data show that **4c** treatment not only robustly counteracted nigral DA cell loss and attenuated striatal DA depletion but also limited the rise in DA turnover caused by MPTP exposure.

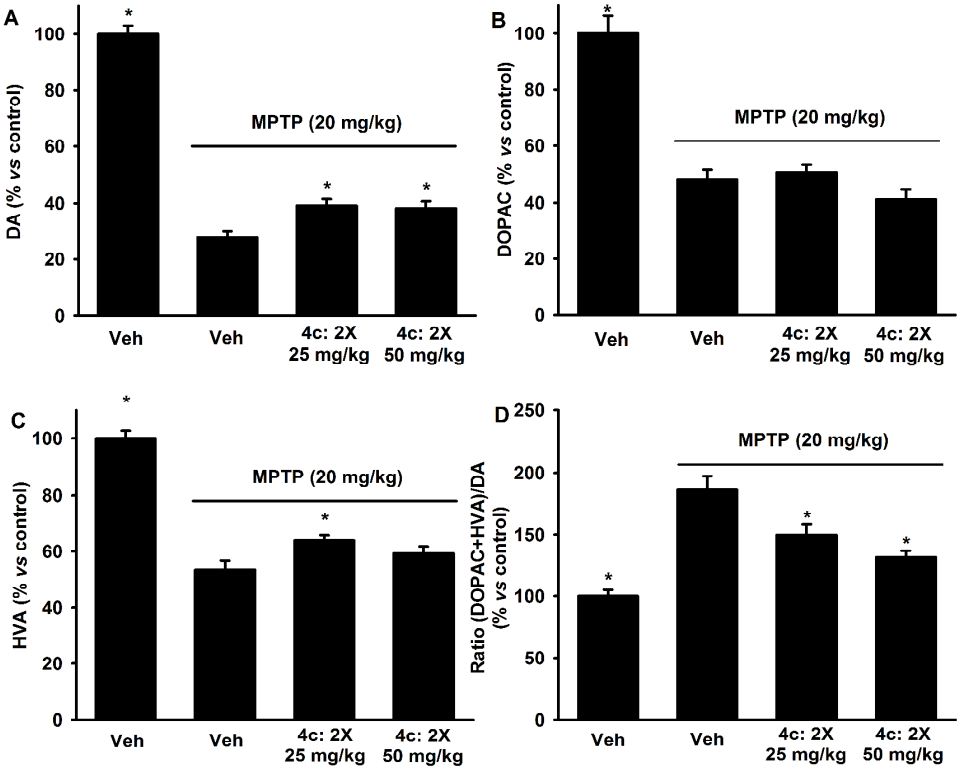


Figure 12. (A), (B), (C) Effect of 11 days of treatment with **4c** (2x25, 2x50 mg/kg/day; *p.o.*) on the level of DA (A) and its metabolites (DOPAC: (B), HVA: (C)) in the striatum of MPTP-treated mice. (D) Effect of 11 days of treatment with **4c** (2x25, 2x50 mg/kg/day; *p.o.*) on the ratio of (DOPAC+HVA)/DA in the striatum of MPTP-treated mice. Data expressed as mean ± SEM (n=9-12). *P<0.05 *vs* vehicle + MPTP (20 mg/kg) (one-way ANOVA with Dunnett's multiple comparisons). Veh: vehicle.

DISCUSSION

The present study reports on the synthesis of 6-aminoquinoxalines and their *N*-propargyl derivatives through an efficient, high-yielding strategy that relies on palladium-catalyzed Suzuki cross-coupling of arylboronic acids with 2-chloro-6-aminoquinoxaline and aryllithium addition to 2-methyl-6-aminoquinoxaline. The desired compounds were obtained

in moderate to good yields. Propargylation of the 6-aminoquinoxalines gave the expected mono-alkylated products in moderate yields. Some halogenated derivatives were also prepared in reasonable yields using NCS or NBS, or alternatively bromine.

The synthesized compounds were tested in a PD cellular model of selective and spontaneous DA cell death.[22,30] Several test compounds were more effective in rescuing DA neurons than our first generation hit compound **5c**. Most importantly, these compounds were also active at a lower concentration than **5c**. Note, however, that among 3-aryl-6-aminoquinoxalines, the presence of a methyl at position 2 of the quinaxoline ring resulted in less active compounds, compared to non-substituted products. Overall, three products provided neuroprotection at 10 μM, with 3-phenyl-6-aminoquinoxaline, **4c** being the most active one. Among the *N*-propargyl-3-aryl-6-aminoquinoxalines, the 2-non-methylated substituted products seemed also to be slightly more active than the *N*-propargyl-2-methyl-3-aryl-6-aminoquinoxalines. At 10 μM, *N*-propargyl-3-phenyl-6-aminoquinoxaline **10c** was as effective as the reference neuroprotectant dbcAMP, at 1mM. Thus, propargylation of compound **4c**, leading to **10c**, significantly improved neuroprotection for DA neurons.

In this cellular model, neuroprotection can possibly result from a direct effect on target DA neurons or from an indirect effect on dividing glial cells.[22,30] Indeed, we have shown before that the first generation hit compound **5c** was protective by decreasing glial cell proliferation.[12] Therefore, we evaluated the potential of second generation hit compounds (i.e., **4c**, **4e**, **6a**, **10c**, **10e**) to operate *via* a glial-dependent mechanism, at two concentrations that were found neuroprotective. These compounds had generally a weak to strong effect on glial cells based on fluorescent microscopic examination. In particular, *N*-propargyl-6-aminoquinoxalines had a strong effect on astrocytic cells comparable to that of the reference compound dbcAMP. 6-Aminoquinoxaline **4c**, which was the only compound with no obvious effect on glial cells based on microscopic criteria, had also no impact on the incorporation of

[methyl-^{3}H]-thymidine used as a marker of glial cell proliferation. This means that **4c** was neuroprotective presumably *via* a direct effect on DA neurons. Taking this into account, **4c** was selected for further investigations.

First, we established that **4c**-treated DA neurons were functional by showing that they efficiently accumulated tritiated DA *via* the active transport system for the neurotransmitter.[29] Noticeably, the ratio DA uptake/DA neurons was also substantially increased, suggesting that the rescued neurons had a more developed neuritic network as DA uptake sites are preferentially localized on neuritic extensions of DA neurons.[29] In accordance with this hypothesis, microscopic examination of **4c**-treated cultures revealed that TH^{+} neurons were more differentiated. This means **4c** did not only afford protection to DA neurons but also provided neurotrophic support to them.

A number of neuroprotective compounds, characterized in this model system, were reported to exert their rescuing effect by promoting slight elevations of cytosolic calcium levels through the modulation of ion channels or receptor/channels.[42] This is in particular the case for the alkaloid nicotine and the caffeine metabolite paraxanthine.[23,16] To address the possibility that **4c** could operate also through a calcium-dependent mechanism, we co-incubated **4c**-treated cultures with molecules having the capacity to block calcium influx through voltage-gated calcium channels. We found that neither nifedipine, a L-type Ca^{2+} channel blocker, nor ω-conotoxin MVIIA, a N-type Ca^{2+} channel blocker, were able to reduce the neuroprotective effect of **4c** for DA neurons. However, dantrolene, a blocker of RyRs (an endoplasmic reticulum calcium release channel) significantly reduced the protective effect of **4c**. This suggests that **4c** may operate by activation of this receptor/channel. This is coherent with previous observations showing that RyRs may represent a potential target for neuroprotection in PD.[16,17] Note, however, that part of the survival promoting effect of **4c** was resistant to dantrolene treatment, which suggests that a

mechanism unrelated to RyR activation was also involved in neuronal rescued mediated by **4c**. The nature of this mechanism remains to be clarified.

Besides exerting pure protective effects for DA neurons, **4c** also provided trophic support for these neurons. This led us to hypothesize that **4c** could provide protection and neurotrophic effects by stimulating the endogenous production of GDNF, a neurotrophic factor for DA neurons. In contradiction with this possibility, we found that a GDNF antibody failed to reduce neuronal rescue by **4c**, whereas it neutralized efficiently the biological activity of GDNF as previously reported.[22] Indirectly, this suggests that the protective component of **4c** that is unrelated to RyR stimulation, cannot be explained by stimulation of endogenous GDNF synthesis and release.

To further explore the neuroprotective potential of **4c**, we used two other experimental settings that model DA cell death. Firstly, we found that **4c** was strongly protective in a situation where DA neurons had acquired dependency on GDNF for their survival. Noticeably, **4c** (25-50 μM) was almost as protective as GDNF (20 ng/mL) in this experimental context, suggesting that the effect of **4c** was well preserved in more mature cultured DA neurons. Secondly, in another culture setting where DA neurons die as a result of low-level oxidative stress generated by Fenton-type reaction catalyzed by trace amounts of ferrous iron in the culture medium,[35] **4c** (5 μM) afforded the same level of protection for DA neurons than the iron chelator desferrioxamine (10 μM).[37] Overall, this signifies that **4c** has the potential to counteract different mechanisms possibly contributive to DA cell death in PD.

Since **4c** is structurally closely related to **5c**, which readily diffuses through the BBB,[12] we wished to study the *in vivo* activity of **4c** in the most commonly used animal model for DA depletion in PD, the MPTP-lesioned mouse model.[39,40] When C57BL/6 mice received a MPTP sub-chronic treatment, we observed a substantial loss of nigrostriatal DA neurons.

This led to reduced levels of DA and its metabolites DOPAC and HVA in the striatum and to an increased DA turnover reflected by a rise in the DOPAC+HVA/DA ratio. After 11 days of **4c** treatment in MPTP-treated mice, we observed a robust neuroprotective effect at the level of DA cell bodies in the SN. This was correlated to a partial restoration of striatal DA levels and to a reduction of DOPAC+HVA/DA ratios at both doses of **4c** (2x25 and 2x50 mg/kg/day). Noticeably, TH^+ neurons had also a well-preserved dendritic network in midbrain tissue sections from MPTP-treated mice receiving **4c**. The conversion of MPTP into its active metabolite MPP^+ within the brain requires the MAO-B.[38,39] Thus, we tested whether MAO-B inhibition could explain the neuroprotection provided by **4c** against MPTP. Excluding this possibility, we found that **4c** was only a very weak MAO-B inhibitor in comparison to the reference compound selegiline. This means that **4c** did not afford protection by reducing MPP^+ bioavailability in MPTP-intoxicated mice.

Overall, present results suggest that **4c** has a very interesting neuroprotective potential and presumably good central nervous system bioavailability after oral treatment. Further experiments will be required, however, for the evaluation of pharmacokinetic parameters. Protective effects of **4c** in MPTP-intoxicated mice, is also an indication that the PD cellular models used in this study are relevant for the screening of neuroprotective drugs for Parkinson disease. Concentrations reported to be protective *in vitro* were, however, relatively high and therefore not necessarily predictive of active-site concentrations in the brain.

In conclusion, we have designed and synthesized new 6-aminoquinoxalines and their *N*-propargyl derivatives. After testing them in a cellular PD model system of selective and spontaneous DA cell death, we selected one of the most active one, **4c**. This compound seemed to operate through a direct effect on DA neurons *via* a mechanism that is partially dependent on RyRs. Finally, we demonstrated that orally administered **4c** had the potential to reduce substantially DA loss and striatal DA depletion in the MPTP mouse model of PD.

These results are of great interest, as they offer a new strategy for PD treatment. However, additional studies are warranted to further establish the neuroprotective potential of **4c** for PD-vulnerable DA neurons.

EXPERIMENTAL SECTION

Chemistry. General. Tetrahydrofuran was distilled over sodium/benzophenone under nitrogen atmosphere prior to use. All reactions involving moisture-sensitive reactants were performed under nitrogen atmosphere using oven-dried glassware. Routine monitoring of reactions was performed using Riedel-deHaën, S, 0.063 mm, 0.032 silica gel plates, which were dipped in a sulfomolybdic acid solution or o-vanillin in $EtOH/H_2SO_4$ (95:5) and then heated to stain or dipped few min in a mixture of silica and iodide. Flash chromatography was performed using Riedel-deHaën, S, 0.063 mm, 0.032 silica under moderate pressure with the appropriate solvent giving us a migrating front (Rf) in the range of 0.2-0.3. ^{1}H NMR spectra were recorded on BRUKER AR 200 P (200 MHz), BRUKER AVANCE 300 (300 MHz) and AM-400 (400 MHz) spectrometers as solutions in $CDCl_3$. Chemical shifts are expressed in parts per million (ppm, δ) downfield from tetramethylsilane (TMS) and are referenced to $CHCl_3$ (7.26 ppm) as internal standard. ^{13}C NMR spectra were recorded on BRUKER AR 200 P (50 MHz) and AM-400 (100 MHz) spectrometers as solutions in $CDCl_3$. Chemical shifts are expressed in parts per million (ppm, δ) downfield from tetramethylsilane (TMS) and are referenced to $CDCl_3$ (77.0 ppm) as internal standard. The attribution of the different carbons (C, CH, CH_2, CH_3) was determined by ^{13}C to ^{1}H polarization transfer (J modulation). Mass spectra (MS) were measured on a Nermag- Sidar R10-10C spectrometer with a quadrupole filter. High Resolution Mass Spectra (HRMS) were measured on a Xevo Q-Tof Waters® spectrometer. Analytical HPLC was performed for compound purity

determination using Xbridge C18 3.5 µm 2.1x150mm columns and UV detection. The solvent system used for HPLC analyses was water+0.1% formic acid : acetonitrile with a gradient from 99:1 to 100:0 over 20 min at 1.0 mL/min. The purity of all the tested compounds is higher than 95% unless noted.

*6-Nitroquinoxaline (**1a**).* An aqueous solution (30 mL) of 4-nitro-phenylene-1,2-diamine (1.53 g, 10 mmol, 1 equiv.) and glyoxal (1.2 mL, 10 mmol, 1 equiv., 40% in water) was heated at reflux during 4 hours. After cooling, the precipitate was filtered, washed with water and dried at 100°C overnight to give a yellow/brown powder (1.58 g, 90% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.01 (s, 3H), 8.52 (dd, J = 9.2, 2.4 Hz, 1H), 8.26 (d, J = 9.2 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 147.9. 147.6, 147.0, 145.3, 141.9, 131.3, 125.9, 123.4. MS (ESI) *m/z*: 176 ($[M+H]^+$, 23). HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_8H_8N_3$: 146.0718, found : 146.0712.

*7-Nitroquinoxalin-2-ol (**1l**).* To a suspension of 2-quinoxalinol (20.0 g, 0.137 mol, 1 equiv.) in acetic acid (200 mL) was added drop-wise a solution of nitric acid at 70% (17.4 mL, 0.274 mol, 2 equiv.) in acetic acid (20 mL). The reaction mixture was stirred at room temperature for 18 h and the bright yellow precipitate filtered and washed with water. The solid obtained was dried *in vacuo* over P_2O_5 during 48 h (17.6 g, 67% yield). ^{1}H NMR (300 MHz, DMSO-d6): δ 12.79 (brs, 1H, OH), 8.33 (s, 1H), 8.05 (d, J = 2.4Hz, 1H), 8.03 (dd, J = 8.6, 2.4 Hz, 1H), 7.96 (d, J = 8.6 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 155.2, 154.2, 147.2, 132.0, 129.9, 117.1, 110.9. HRMS (ESI) calcd *m/z* for $[M-H]^-$ $C_8H_4N_3O_3$: 190.0253, found : 190.0259.

*2-Chloro-7-nitroquinoxaline (**1m**).* To a solution of compound **1l** (17.568 g, 91.50 mmol, 1 equiv.) in $POCl_3$ (80 mL) was added DMF (100 µL). The reaction mixture was heated at reflux for 3 h. After cooling the dark reaction mixture was poured carefully into a 500 mL

beaker full of crushed ice. The grey precipitate was filtered, washed with water and dried *in vacuo* over P_2O_5 for 48 h (17.02 g, 88% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.93 (s, 1H), 8.91 (d, J = 2.4 Hz, 1H), 8.55 (dd, J = 9.2, 2.4 Hz, 1H), 8.29 (d, J = 9.2 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 149.8, 148.0, 143.2 (2C), 141.1, 131.1, 124.8, 123.7. MS (EI) *m/z*: 211 ($Cl^{37}M^{\bullet+}$, 31), 209 ($Cl^{35}M^{\bullet+}$, 97), 181 (33), 179 (100), 165 ($Cl^{37}M^{\bullet+}$–NO_2, 29), 163 ($Cl^{35}M^{\bullet+}$–NO_2, 89), 163 ($Cl^{35}M^{\bullet+}$–NO_2, 89).

*2-Methyl-6-nitroquinoxaline (**2a**).* An aqueous suspension (50 mL) of 4-nitro-phenylene-1,2-diamine (3.06 g, 20 mmol, 1 equiv) and pyruvic aldehyde (3.65g, 30 mmol, 1.5 equiv, 40% in water) was heated at reflux for 1.5 hours. After cooling, the beige precipitate was filtered, washed with water and dried at 100°C overnight to give a purple/brown powder (3.40 g, 90% yield) as a 90:10 mixture of regioisomers. ^{1}H NMR (400 MHz, $CDCl_3$, *major regioisomer*): δ 8.92 (s, 1H), 8.87 (s, 1H), 8.45 (d, J = 8.0 Hz, 1H), 8.10 (d, J = 8.4 Hz, 1H), 2.82 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, *major regioisomer*): δ 157.3, 148.1, 147.0, 144.5, 139.7, 130.2, 125.5, 123.3, 22.8. MS (ESI) *m/z*: 190 ($[M+H]^+$, 100). HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_9H_8N_3O_2$: 190.0619, found : 190.0617.

*2,3-Dimethyl-6-nitroquinoxaline (**2b**).* An aqueous suspension (50 mL) of 4-nitro-phenylene-1,2-diamine (1.530 g, 10 mmol, 1 equiv.) and butanedione (1.0 mL, 11.8 mmol, 1.2 equiv.) was heated at reflux during 30 min. The formation of an orange precipitate was immediately observed. After cooling and water addition, the precipitate was filtered, washed with water and dried at 100°C overnight to give compound **2b** (1.50g, 74% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.85 (d, J = 2.5 Hz, 1H), 8.41 (dd, J = 9.2, 2.5 Hz, 1H), 8.07 (d, J = 9.2 Hz, 1H), 2.78 (s, 6H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 157.2, 156.3, 147.2, 143.7, 139.9, 129.9, 124.9, 122.4, 23.5, 23.3. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{10}H_{10}N_3O_2$: 204.0773, found : 204.0765.

*2-Phenyl-6-nitroquinoxaline (**3a**).* A suspension of 4-nitro phenylene-1,2-diamine (1.52 g, 10.0 mmol, 1equiv.) and phenylglyoxal monohydrate (1.52 g, 10.0 mmol, 1 equiv.) in water (40 mL) was heated at reflux for 1 h. After cooling, the precipitate was filtered and washed with water. The orange solid was dried in an oven for 24 h to give a mixture of compound **3a** and **1c** in a 3:1 mixture of regioisomers. (2.46g, 98% yield). ^{1}H NMR (400 MHz, $CDCl_3$, *major regioisomer*) : δ 9.49 (s, 1H), 9.02 (d, J = 2.4 Hz, 1H), 8.55 (dd, J = 9.3, 2.3 Hz, 1H), 8.24-8.29 (m, 3H), 7.61 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, *major regioisomer*): δ 154.3, 147.4, 145.5, 144.9, 140.3, 135.6, 131.6, 131.4, 131.4, 131.2, 129.4, (2C), 127.9 (2C), 125.6, 123.8. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{14}H_{10}N_3O_2$: 252.0773, found : 252.0771.

*6-Aminoquinoxaline (**4a**).* To a solution of 6-nitroquinoxaline (**1a**) (6.48 g, 42.6 mmol, 1 equiv) in ethanol (150 mL) and EtOAc (100 mL) was added Pd/C 10% (10% w/w, 650 mg). The mixture was stirred at 60 °C under hydrogen atmosphere for 8 h. After cooling, the reaction was filtered on celite® then washed with ethanol and concentrated *in vacuo*. Recrystallisation (Dioxane /H_2O) afforded a yellow solid (4.16 g, 80% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.62 (s, 1H), 8.52 (s, 1H), 7.84 (d, J = 8.8 Hz, 1H), 7.15 (dd, J = 8.8, 2.4 Hz, 1H), 7.12 (d, J = 2.4 Hz, 1H), 4.10 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 148.1, 144.9, 140.9, 137.9, 130.3, 122.0, 107.8. MS (ESI) *m/z*: 146 ($[M+H]^+$, 100). HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_8H_8N_3$: 146.0718, found : 146.0712.

3-Phenyl-6-aminoquinoxaline (**4c**). A suspension of quinoxaline **4m** (1.7 g, 9.5 mmol, 1 equiv.), K_2CO_3 (3.41 g, 24.7 mmol, 2.6 equiv.), phenylboronic acid (1.5 g, 12.35 mmol, 1.3 equiv.) and $PdCl_2(PPh_3)_2$ (200 mg, 0.285 mmol, 0.03 equiv.) in a dioxane:H_2O (20 mL: 10 mL) mixture was heated at reflux for 3 h. Column chromatography on silica gel (CH_2Cl_2 : EtOAc, 85 :15) afforded compound **4c** as bright yellow crystals (1.98 g, 95% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.04 (s, 1H), 8.14 (dd, J = 7.2, 1.2 Hz, 2H), 7.89 (d, J = 8.9 Hz, 1H), 7.49-7.57 (m, 3H), 7.21 (d, J = 2.5 Hz, 1H), 7.16 (dd, J = 8.9, 2.5 Hz, 1H), 4.21 (brs, 2H

NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 151.1, 147.2, 143.2, 138.5, 136.3, 135.7, 129.1, 128.8, 128.0 (2C), 126.5 (2C), 120.5, 107.5. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{14}H_{12}N_3$: 222.1031, found : 222.1030.

*3-(4-Fluorophenyl)-6-aminoquinoxaline (**4e**).* To a solution of 1-Bromo-4-Fluorobenzene (1.3 mL, 12.2 mmol, 4 equiv.) in anhydrous Et_2O (6 mL) at –78 °C was added drop-wise *t*-BuLi (13 mL, 24.4 mmol, 8 equiv., 1.9 M in hexane). After 1 h at –78 °C, a solution of quinoxaline (0.44 g, 3.05 mmol, 1 equiv.) in THF (8 mL) was added drop-wise to the reaction mixture and the reaction mixture was stirred at this temperature for 2 h and then allowed to warm to rt. The reaction mixture was quenched by the addition of a saturated aqueous solution of $NaHCO_3$. The aqueous layout was extracted by ethyl acetate and the combined organic layers were washed with a saturated aqueous solution of NaCl, dried over $MgSO_4$ and concentrated *in vacuo*. The residue obtained was dissolved in $CHCl_3$ (10 mL), then MnO_2 (1.3 g, 15.3 mmol, 5 equiv.) was added and the reaction mixture was heated at reflux during 2h. The reaction mixture was filtered on celite®, concentrated *in vacuo* and the residue was then purified by silica gel flash chromatography (EtOAc:Pet. Ether (50:50) to give compound **4e** (48.0 mg, 7% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.99 (s, 1H), 8.14 (m, 2H), 7.88 (d, J = 9.0 Hz, 1H), 7.13-7.27 (m, 4H), 4.2 (brs, 2H, NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 164.0, 152.7, 150.1, 148.3, 144.2, 139.0, 136.6 (d, J = 3.3 Hz), 130.1, 129.4 (d, J = 8.5 Hz, 2C), 116.0 (d, J = 22.8 Hz, 2C), 121.6, 108.3. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{14}H_{11}FN_3$: 240.0935, found : 240.0937.

*3-(Naphthalen-2-yl)quinoxalin-6-amine (**4h**).* For experimental protocol, see compound **4c**. Yield: 73% (110.0 mg). ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.19 (s, 1H), 8.61 (s, 1H), 8.31 (dd, J = 8.6, 1.6 Hz, 1H), 8.01 (m, 2H), 7.91 (m, 2H), 7.57 (t, J = 3.5 Hz, 1H), 7.54 (t, J = 3.5 Hz, 1H), 7.18 (dd, J = 9.0, 2.6 Hz, 1H), 7.26 (d, J = 2.6 Hz, 1H), 4.2 (brs, 2H, NH_2). ^{13}C NMR (75 MHz, Acetone-d_6): δ 151.93, 151.59, 145.51, 139.31, 137.31, 135.89, 135.08, 134.58,

130.76, 129.80, 129.53, 128.69, 128.00, 127.86, 127.52, 125.53, 122.88, 107.51. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{18}H_{14}N_3$: 272.1188, found : 272.1192.

*3-Chloroquinoxalin-6-amine (**4m**).* To a suspension of 2-chloro-7-nitroquinoxaline (**1m)** (14.36 g, 68.5 mmol, 1 equiv.) in EtOAc (300 mL) was added $SnCl_2.2H_2O$ (45.5g, 239.9 mmol, 3.5 equiv.) and the reaction mixture refluxed for 2h. After cooling to rt, NaOH (480 mmol, 6 equiv., 50% in water) was added drop-wise at 0°C and the reaction mixture filtered on a pad of silica gel, then eluted with hot acetone. After concentration, the yellow residue was purified by recrystallisation (dioxane/H_2O) to afford 3-chloroquinoxalin-6-amine (**4m**) as a bright yellow solid (9.65g, 78% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.47 (s, 1H), 7.85 (d, J = 8.8 Hz, 1H), 7.15 (dd, J = 8.8, 1.7 Hz, 1H), 7.03 (d, J = 1.7 Hz, 1H), 4.30 (brs, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 149.1, 147.7, 144.2, 140.3, 135.98, 130.3, 121.7, 107.2. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_8H_7N_3Cl$: 180.0329, found : 180.0326.

*3-(Naphthalen-1-yl)quinoxalin-6-amine (**4n**).* For experimental protocol, see compound **4c**. Yield: 73% (220.7 mg). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.86 (s, 1H), 8.16 (dd, J = 7.3, 1.8 Hz, 1H), 7.96 (dt, J = 9.9, 5.3 Hz, 2H), 7.95 (m 1H), 7.73 (dd, J = 7.1, 1.1 Hz, 1H), 7.61 (dd, J = 8.0, 7.1 Hz), 7.54 (td, J = 6.7, 1.5 Hz, 1H), 7.50 (td, J = 6.7, 1.5 Hz, 1H), 7.26 (d, J = 2.4 Hz, 1H), 7.26 (dd, J = 7.2, 2.4 Hz, 1H), 4.25 (brs, 2H, NH_2). ^{13}C NMR (75 MHz, $CDCl_3$): δ 154.33, 148.32, 144.10, 142.61, 136.41, 135.65, 133.98, 131.24, 130.23, 129.73, 128.48, 128.15, 126.89, 126.16, 125.33, 125.29, 121.83, 108.38. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{18}H_{14}N_3$: 272.1188, found : 272.1193.

*3-(Pyridin-3-yl)quinoxalin-6-amine (**4o**).* For experimental protocol, see compound **4c**. Yield: 93% (115.0 mg). ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.36 (dd, J = 2.3, 0.8 Hz, 1H), 9.03 (s, 1H), 8.73 (dd, J = 4.9, 1.6 Hz, 1H), 8.45 (ddd, J = 8.0, 2.3, 1.6 Hz, 1H), 7.90 (m, 1H), 7.46 (ddd, J = 8.0, 4.9, 0.8 Hz, 1H), 7.20 (d, J = 2.6 Hz, 1H), 7.18 (dd, J = 6.5, 2.4 Hz, 1H),

4.29 (brs, 1H, NH_2). ^{13}C NMR (75 MHz, $CDCl_3$): δ 150.62, 149.50, 148.63, 148.53, 144.32, 138.67, 137.06, 134.75, 132.98, 130.24, 123.75, 122.18, 108.14. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{13}H_{11}N_4$: 223.0984, found : 223.0978.

3-(7-Aminoquinoxalin-2-yl)phenol ***(4p)***. For experimental protocol, see compound **4c**, Yield: 87% (115.0 mg). ^1H NMR (300 MHz, DMSO-d6): δ 9.66 (s, 1H, OH), 8.98 (s, 1H), 7.74 (d, J = 9.0 Hz, 1H), 7.65 (d, J = 1.2 Hz, 1H), 7.64 (m, 1H), 7.35 (t, J = 8.0 Hz, 1H), 7.22 (dd, J = 7.0, 0.5 Hz, 1H), 6.95 (d, J = 2.2 Hz, 1H), 6.91 (dt, J = 9.0, 2.2 Hz, 1H), 6.07 (brs, 2H, NH_2). ^{13}C NMR (75 MHz, DMSO-d6): δ 157.9, 150.8, 150.4, 143.9, 138.1, 137.4, 135.4, 135.4, 130.0, 129.3, 122.0, 117.8, 116.9, 113.6, 105.11. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{14}H_{12}N_3O$: 238.0980, found : 238.0983.

3-(Quinolin-3-yl)quinoxalin-6-amine ***(4q)***. For experimental protocol, see compound **4c**, however the reaction was conducted with *N*-methyliminodiacetic acid (MIDA) boronate in conjunction with sodium hydroxide rather than K_2CO_3. Yield: 86% (130.0 mg). ^1H NMR (300 MHz, DMSO): δ 9.73 (d, J = 2.1 Hz, 1H), 9.28 (s, 1H), 9.21 (d, J = 2.1 Hz, 1H), 8.16 (d, J = 8.3 Hz, 1H), 8.10 (d, J = 8.3 Hz, 1H), 7.84 (td, J = 6.9, 1.3 Hz, 1H), 7.79 (d, J = 8.9 Hz, 1H), 7.69 (ddd, J = 8.0,7.0, 1.0 Hz, 1H), 7.26 (dd, J = 8.9, 2.4 Hz, 1H), 7.02 (d, J = 2.4 Hz, 1H), 6.17 (brs, 2H, NH_2). ^{13}C NMR (75 MHz, DMSO): δ 151.14, 149.13, 148.52, 147.82, 144.06, 137.61, 135.65, 134.25, 134.12, 130.55, 129.49, 128.95, 128.78, 127.32, 127.28, 122.62, 104.95. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{18}H_{13}N_4$: 273.1140, found : 273.1148. Purity (HPLC/UV λ at 250 nm): 88%.

3-(3,4,5-Trimethoxyphenyl)quinoxalin-6-amine ***(4r)***. For experimental protocol, see compound **4c**. Yield: 73% (127 mg). ^1H NMR (300 MHz, $CDCl_3$): δ 8.98 (s, 1H), 7.88 (d, J = 8.8 Hz, 1H), 7.36 (s, 2H), 7.20 (d, J = 2.4 Hz, 1H), 7.15 (dd, J = 8.9, 2.5 Hz, 1H), 4.24 (s, 2H), 4.00 (s, 6H), 3.92 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 153.8, 151.7, 148.3, 144.1,

139.9, 139.3, 136.6, 132.9, 130.1, 121.5, 108.3, 104.8, 61.0, 56.4. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{17}H_{18}N_3O_3$: 312.1348, found : 312.1350.

*3-Ethynylquinoxalin-6-amine (**4s**).* To a solution of compound of compound **4m** (100 mg, 0.557 mmol, 1 equiv.) in THF (5 mL), was added trimethylsilylacetylene (314 µL, 2.23 mmol, 4 equiv.), CuI (10.0 mg, 0.056 mmol, 0.1 equiv.), $PdCl_2(PPh_3)_2$ (13.0 mg, 0.022 mmol, 0.04 equiv) then Et_3N (3 mL). The reaction mixture was refluxed under argon atmosphere for 48 h, then filtered on a pad of silica gel, and washed with EtOAc. The filtrate was concentrated, then diluted in MeOH (10 mL), K_2CO_3 was added (500 mg), and the reaction mixture refluxed for 10 min. The reaction mixture was then diluted with water, extracted with EtOAc and washed with brine. The organic layer was dried over $MgSO_4$, filtered, concentrated, and the residue then purified by silica gel column chromatography (EtOAc:CH_2Cl_2, 8:92 to 15:85) to give orange crystals (25.6 mg, 27% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.63 (s, 1H), 7.86 (d, J = 8.9 Hz, 1H), 7.19 (dd, J = 8.9, 2.5 Hz, 1H), 7.10 (d, J = 2.4 Hz, 1H), 4.27 (brs, 2H), 3.36 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 148.57, 144.03, 143.26, 138.40, 136.51, 130.24, 122.85, 107.60, 81.38, 80.45. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{10}H_8N_3$: 170.0718, found : 170.0713.

*3-(2-(4-Methoxyphenyl)ethynyl)quinoxalin-6-amine (**4t**).* To a solution of compound of compound **4m** (100 mg, 0.557 mmol, 1 equiv.) in THF (5 mL), was added trimethylsilylacetylene (314 µL, 2.23 mmol, 4 equiv.), CuI (10.0 mg, 0.056 mmol, 0.1 equiv.), $PdCl_2(PPh_3)_2$ (13.0 mg, 0.022 mmol, 0.04 equiv) and then Et_3N (3 mL).The reaction mixture was refluxed under argon atmosphere for 48 h, then filtered on a pad of silica gel, washed with EtOAc. The filtrate was then concentrated and then purified by silica gel column chromatography (EtOAc:CH_2Cl_2, 10:92 to 15:85) to give orange crystals (36.0 mg, 23% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.67 (s, 1H), 7.84 (d, J = 8.8 Hz, 1H), 7.60 (d, J = 8.9 Hz, 2H), 7.15 (dd, J = 8.8, 2.5 Hz, 1H), 7.11 (d, J = 2.3 Hz, 1H), 6.91 (d, J = 8.9 Hz, 2H),

4.25 (m, 2H), 3.84 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 160.60, 148.46, 144.11, 143.47, 139.89, 135.89, 133.94, 130.15, 122.10, 114.17, 113.66, 107.69, 93.38, 86.48, 55.34. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{17}H_{14}N_3O$: 276.1145, found : 276.1137.

*2-Methyl-6-aminoquinoxaline (**5a**).* To a solution of 2-methyl-6-nitroquinoxaline (**2a**) (3.18 g, 20 mmol, 1 equiv.) in ethanol (100 mL) was added Pd/C 10% (10% w/w, 318 mg). The mixture was stirred at 60 °C under hydrogen atmosphere for 4 hours. After cooling, the reaction was filtered on celite®, then washed with ethanol and finally concentrated *in vacuo* to afford a yellow solid (2.54 g, 80% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.52 (s, 1H), 7.73 (d, J = 4.2 Hz, 1H), 7.09-7.11 (m, 2H), 4.22 (s, 2H), 2.62 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 149.3, 147.1, 145.7, 142.5, 141.7, 136.8, 129.4, 121.7, 108.1, 121.8. MS (ESI) *m/z*: 160 ($[M+H]^+$, 100). HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_9H_{10}N_3$: 160.0876, found : 160.0876.

*2,3-Dimethyl-6-aminoquinoxaline (**5b**).* For experimental protocol, see compound **5g**. Yield: 84% (1.08 g). ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.67 (d, J = 8.4 Hz, 1H), 6.98 (m, 2H), 4.11 (s, 2H), 2.59 (s, 3H), 2.58 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 153.2. 149.0, 147.0, 142.7, 135.8, 129.1, 120.5, 107.9, 23.0, 22.5. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{10}H_{12}N_3$: 174.1032, found 174.1031.

*2-Methyl-3-phenyl-6-aminoquinoxaline (**5c**).* To a solution of 2-methyl-6-aminoquinoxaline (**5a**) (4.0 g, 25.15 mmol, 1 equiv.) in THF (65 mL), under N_2 at –78 °C was slowly added 48.9 mL of PhLi (48.9 mL, 88.0 mmol, 1.8 M in dibutyl ether, 3.5 equiv.). The reaction mixture turned to dark red and was stirred at –78 °C during 3 hours, then hydrolyzed by a saturated aqueous solution of NH_4Cl and extracted three times with ethyl acetate. The combined organic layers were washed with a saturated aqueous solution of NaCl, dried over Na_2SO_4, filtered and concentrated *in vacuo*. The residue was dissolved in $CHCl_3$ (600 mL),

then MnO_2 (10.940 g, 125.74 mmol, 5 equiv.) was added and the mixture heated at reflux for 4 hours, cooled down, filtered on celite® then concentrated *in vacuo*. The residue was then purified by flash chromatography on silica gel (cyclohexane : EtOAc, 90: 10) to give a yellow powder (2.66 g, 45% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.83 (d, J = 8.7 Hz, 1H), 7.61 (m, 2H), 7.48 (m, 3H), 7.16 (m, 2H), 3.88 (s, 2H), 2.68 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 154.8, 148.0, 147.3, 142.6, 139.4, 136.2, 129.2, 128.9, 128.7, 128.4, 121.7, 108.4, 23.8. HRMS (ESI) Calcd *m/z* for $[M+H]^+$ $C_{15}H_{14}N_3$: 236.1188, found : 236.1187.

*2-Methyl-3-(4-methoxyphenyl)-6-aminoquinoxaline (**5d**)*. For experimental protocol, see compound **5g**. Yield: 34% (168.2 mg). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.79 (d, J = 9.1 Hz, 1H). 7.58 (d, J = 9.1 Hz, 2H), 7.14 (s, 1H), 7.09 (d, J = 9.1 Hz, 1H), 7.00 (d, J = 8.4 Hz, 2H), 4.16 (brs, 2H), 3.85 (s, 3H), 2.68 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 160.0, 154.4, 148.1, 147.3, 142.7, 135.9, 131.8, 130.3 (2C), 129.1, 121.4, 113.8 (2C), 108.3, 55.3, 23.8. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{16}H_{16}N_3O$: 266.1293, found : 266.1291.

*2-Methyl-3-(4-fluorophenyl)-6-aminoquinoxaline (**5e**)*. For experimental protocol, see compound **5g**. Yield: 35% (352.2 mg). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.85 (d, J = 9.2 Hz, 1H), 7.63 (d, J = 8.4 Hz, 1H), 7.61 (d, J = 8.4 Hz, 1H), 7.16-7.21 (m, 4H), 3.90 (brs, 2H, NH), 2.68 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.1 (d, J = 250.4 Hz), 153.6, 147.8, 147.5, 142.6, 136.2, 135.3 (d, J = 2.5 Hz), 130.9 (d, J = 8.1 Hz), 129.2, 122.0, 115.5 (d, J = 21.5 Hz), 108.1, 23.7. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{15}H_{13}FN_3$: 254.1094, found : 254.1088. Purity (HPLC/UV λ at 254 nm): 92%.

*2-Methyl-3-(4-chlorophenyl)-6-aminoquinoxaline (**5f**)*. For experimental protocol, see compound **5g**. Yield: 70% (1.128 g). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.83 (d, J = 9.1 Hz, 1H), 7.58 (d, J = 7.9 Hz, 2H), 7.47 (d, J = 7.9 Hz, 2H), 7.16 (d, J = 7.5 Hz, 1H), 7.15 (s, 1H), 4.15 (brs, 2H, NH), 2.67 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 153.5, 147.8, 147.4,

142.7, 137.9, 136.4, 134.9, 130.4 (2C), 129.3, 128.7 (2C), 122.0, 108.2, 23.7. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{15}H_{13}ClN_3$: 270.0798, found : 270.0795.

*2-Methyl-3-p-tolyl-6-aminoquinoxaline (**5g**).* To a solution of 4-bromotoluene (24.0 mmol, 4 equiv.) in anhydrous ether (60 mL), under nitrogen at –78 °C was slowly added *tert*-BuLi (10 mL, 1.6 M in pentane, 8 equiv.). After 1 h of stirring at –78 °C, compound **5a** (0.954 g, 6 mmol, 1 equiv.) in anhydrous THF (15 mL) was added slowly. The mixture was stirred at –78 °C during 2 h and then warmed to room temperature. After 24 hours, the mixture was hydrolyzed by an aqueous solution of $NaHCO_3$ then extracted with ethyl acetate. The combined organic layers were washed by a saturated aqueous solution of NaCl, dried over Na_2SO_4, filtered and concentrated *in vacuo*. The residue was dissolved in $CHCl_3$ (50 mL) then MnO_2 (2.607 g, 30.0 mmol, 5 equiv.) was added. The mixture was heated to reflux for 4 h, cooled down filtered over celite® then concentrated *in vacuo*. The residue was then purified by flash chromatography on silica gel (CH_2Cl_2: MeOH, 98: 2) to give a yellow powder (851.7 mg, 56% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.84 (d, J = 8.9 Hz, 1H), 7.56 (d, J = 7.6 Hz, 2H), 7.32 (d, J = 7.6 Hz, 2H), 7.20 (d, J = 2.1 Hz, 1H), 7.12 (dd, J = 8.9, 2.1 Hz, 1H), 4.28 (brs, 2H, NH), 2.72 (s, 3H), 2.45 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 154.6, 147.9, 147.3, 142.6, 138.5, 136.4, 135.9, 128.9 (3C), 128.7 (2C), 121.5, 108.0, 23.7, 21.2, HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{16}H_{16}N_3$: 250.1344, found : 250.1346.

*2-Methyl-3-(naphtalen-2-yl)-6-aminoquinoxaline (**5h**).* For experimental protocol, see compound **5g**. Yield: 48% (531.8 mg). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.10 (s, 1H), 7.97 (d, J = 8.9 Hz, 1H), 7.92 (d, J = 9.5 Hz, 1H), 7.86 (d, J = 8.9 Hz, 1H), 7.74 (dd, J = 8.1, 1.8 Hz, 1H), 7.91 (d, J = 9.5 Hz, 1H), 7.52-7.57 (m, 2H), 7.21 (d, J = 2.5 Hz, 1H), 7.16 (dd, J = 8.9, 2.5 Hz, 1H), 4.14 (brs, 2H, NH_2), 2.74 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 154.8, 148.3, 147.4, 142.8, 136.9, 136.3, 133.2, 133.1, 129.3, 128.5, 128.4, 128.1, 127.7, 126.7 (2C), 126.4,

121.7, 108.4, 23.9. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{19}H_{16}N_3$: 286.1344, found : 286.1343. Purity (HPLC/UV λ at 254 nm): 88%.

*3-(3,4-Dimethoxyphenyl)-2-methyl-6-aminoquinoxaline (**5i**).* For experimental protocol, see compound **5g**. Yield: 31% (310.0 mg). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.87 (d, J = 8.8 Hz, 1H), 7.27 (s, 1H), 7.22 (d, J = 6.0 Hz, 1H), 7.21 (s, 1H), 7.17 (d, J = 8.8 Hz, 1H), 6.99 (d, J = 8.8 Hz, 1H), 3.96 (s, 3H), 3.95 (s, 3H), 3.94 (brs, 2H, NH_2), 2.74 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 154.5, 149.6, 148.9, 148.2, 147.3, 142.7, 136.1, 132.1, 129.2, 121.7, 121.5, 112.3, 110.9, 108.4, 56.0 (2C), 23.8. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{17}H_{18}N_3O_2$: 296.1401, found : 296.1399.

*2-Methyl-3-(3,4-dichlorophenyl)-6-aminoquinoxaline (**5j**).* For experimental protocol, see compound **5g**. Yield: 45% (813.9 mg). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.83 (d, J = 8.9 Hz, 1H), 7.18 (dd, J = 9.1 and 2.3 Hz, 1H), 7.75 (d, J = 2.0 Hz, 1H), 7.58 (d, J = 8.4 Hz, 1H), 7.47 (dd, J = 8.4, 2.0 Hz, 1H), 7.14 (d, J = 2.0 Hz, 1H), 4.17 (brs, 2H, NH_2), 2.68 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 152.2, 147.6, 147.4, 142.7, 139.4, 136.5, 133.1, 132.8, 131.1, 130.4, 129.3, 128.3, 122.3, 108.1, 23.7. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{15}H_{12}Cl_2N_3$: 304.0408, found : 304.0405.

*2-Methyl-3-biphenyl-6-aminoquinoxaline (**5k**).* For experimental protocol, see compound **5g**. Yield: 30% (219.3 mg). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.84 (d, J = 8.7, 1H), 7.71 (m, 4H), 7.66 (d, J = 7.5 Hz, 2H), 7.47 (t, J = 7.5 Hz, 2H), 7.38 (t, J = 7.5 Hz, 1H), 7.18 (d, J = 1.8 Hz, 1H), 7.12 (dd, J = 8.7, 1.8 Hz, 1H), 4.23 (brs, 2H, NH_2), 2.77 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 154.3, 127.5, 147.9, 147.4, 142.7, 141.4, 140.4, 138.2, 136.0, 129.3 (2C), 129.0, 128.8 (2C), 127.0 (4C), 121.7, 108.1, 23.7. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{21}H_{18}N_3$: 312.1501, found : 312.1505. Purity (HPLC/UV λ at 254 nm): 92%.

*2-Methyl-3-(3,4,5-trimethoxyphenyl)-6-aminoquinoxaline (**5r**).* For experimental protocol, see compound **5g**. Yield: 21 % (317.4 mg). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.83 (d, J = 8.1 Hz, 1H), 7.18 (s, 1H), 7.17 (dd, J = 8.8, 2.4 Hz, 1H), 6.84 (s, 2H), 4.28 (brs, 2H, NH), 3.92 (s, 9H), 2.72 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 154.4, 153.1 (2C), 147.8, 147.5, 142.4, 138.5, 136.1, 134.9, 129.1, 121.7, 108.0, 106.2 (2C), 60.8, 56.1 (2C), 23.7. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{18}H_{20}N_3O_3$: 326.1505, found : 326.1498.

*2-Phenyl-6-aminoquinoxaline (**6a**).* To a solution of compound **3a** (9.8 mmol) in EtOH (150 mL), was added Pd/C (245 mg, 10% w/w) under nitrogen and the reaction mixture stirred at 60 °C under hydrogen atmosphere for 24 h. After cooling, the reaction was filtered over celite® then rinsed with EtOAc and the filtrate was then concentrated *in vacuo*. The residue was then purified on a silica gel flash chromatography (CH_2Cl_2: MeOH, 98: 2) to give compound **6a** (453.3 mg, 21% yield) and its isomer **4c** (79.5 mg, 4% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.15 (s, 1H), 8.11 (dd, J = 7.3, 1.6 Hz, 2H), 7.9 (d, J = 8.9 Hz, 1H), 7.52 (td, J = 7.4, 1.6 Hz, 2H), 7.45 (tt, J = 7.4, 1.6 Hz, 1H), 7.18 (dd, J = 8.9, 2.6 Hz, 1H), 7.17 (brs, 1H), 3.99 (brs, 2H NH_2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 148.1, 147.7, 143.2 (2C), 137.1 (2C), 130.5, 129.2, 128.9 (2C), 126.8 (2C), 122.2, 107.8. MS (ESI) *m/z*: 222.2 ($[M+H]^+$, 100). HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{14}H_{12}N_3$: 222.1026, found : 222.1026.

*3-(4-Methoxyphenyl)-2-phenyl-6-aminoquinoxaline (**6d**).* For experimental protocol, see compound **5g** from compound **6a**. The crude material was then purified by semi-preparative HPLC chromatography (SunFire (Waters C18, 150x19m; H_2O:MeOH, 30:70) to give the compound **6d**. Yield: 2% (8.3 mg). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.94 (d, J = 8.9 Hz, 1H), 7.46-7.49 (m, 2H), 7.44 (d, J = 8.8 Hz, 2H), 7.32 (m, 3H), 7.22 (d, J = 2.2 Hz, 1H), 7.16 (dd, J = 8.8, 2.1 Hz, 1H), 6.83 (d, J = 8.8 Hz, 2H), 3.81 (s, 3H), 3.63 (brs, 2H, NH_2). ^{13}C NMR (75 MHz, $CDCl_3$): δ 160.0, 152.9, 149.6, 148.0, 143.0, 139.7, 136.1, 131.7, 131.3 (2C),

130.2, 129.7 (2C), 128.2 (2C), 128.1, 121.7, 113.6 (2C), 107.9, 55.3. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{21}H_{18}N_3O$: 328.1447, found : 328.1450.

*5-Bromo-2-methyl-3-phenyl-6-aminoquinoxaline (**7c**).* To a solution of compound **5c** (401.0 mg, 1.7 mmol, 1 equiv.) in CH_2Cl_2 (10 mL) was added *N*-bromosuccinimide (303.7 mg, 1.7 mmol, 1 equiv.). After 2 h, the reaction mixture was quenched by the addition of 20 mL of a solution of NaOH 5%. The reaction mixture was then extracted with ethyl acetate, dried over Na_2SO_4 then concentrated *in vacuo* to give compound **7c** (553.7 mg, 99% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.73 (m, 3H), 7.46 (m, 3H), 7.13 (d, J = 8.7 Hz, 1H), 4.83 (brs, 2H, NH_2), 2.73 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 154.5, 147.7, 145.3, 140.0, 138.6, 135.7, 129.3 (2C), 128.8, 128.2 (2C), 127.4, 120.9, 102.9, 23.3. MS (ESI) *m/z*: 314 (^{79}Br), 316 (^{81}Br) ($[M+H]^+$, 70), 336 (^{79}Br); 338 (^{81}Br) ($[M+Na]^+$, 80), 651 ($[2M+Na]^+$, 100). HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{15}H_{13}N_3Br$: 314.0291, found : 314.0293.

*5-Chloro-2-methyl-3-phenyl-6-aminoquinoxaline (**8c**).* To a solution of compound **5c** (280.0 mg, 0.89 mmol, 1 equiv.) in CH_2Cl_2 (10 mL) was added of *N*-chlorosuccinimide (118.0 mg, 1.07 mmol, 1.2 equiv.) at room temperature and the reaction mixture was heated at reflux overnight. After cooling, the reaction mixture was hydrolyzed by a solution of NaOH at 5%, and then extracted with CH_2Cl_2. The combined organic layer were washed with brine, dried over Na_2SO_4 then concentrated under nitrogen to give compound **8c (**279.0 mg, 99% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.71 (dd, J = 8.6, 1.3 Hz, 2H), 7.48 (m, 3H), 7.45 (d, J = 9.2 Hz, 1H). 7.19 (d, J = 9.2 Hz, 1H), 4.60 (brs, 2H, NH_2), 2.73 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 154.6, 148.3, 143.6, 139.1, 139.0, 136.4, 129.3 (2C), 128.9, 128.3 (2C), 127.3, 120.8, 111.1, 23.7. MS (ESI) *m/z*: 270.2 ($[^{35}$Cl-M+H$]^+$, 25), 272.2 ($[^{37}$Cl-M+H$]^+$, 7.5). HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{15}H_{13}N_3Cl$: 270.0798, found : 270.0798.

*5-Chloro-7-bromo-2-methyl-3-phenyl-6-aminoquinoxaline (**9c**)*. To a solution of compound **8c** (50.0 mg, 0.186 mmol, 1 equiv.) in AcOH (3 mL) was added Br_2 (100 µL, 0.809 mmol, 4.4 equiv.) at room temperature. After 2 h of mixing, the reaction mixture was neutralized by the addition of 10 mL of a solution of NaOH 10% followed by a solution of $Na_2S_2O_3$ 10% (10 mL). The reaction mixture was extracted with ethyl acetate, dried on Na_2SO_4, and concentrated *in vacuo*. The residue was then purified by flash chromatography on silica gel (cyclohexane: ethyl acetate, 9:1 to 7:3) to give of compound **9c** (553.7 mg, 31% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.15 (s, 1H), 7.53-7.54 (m, 3H), 7.72 (dd, J = 8.0, 1.5 Hz, 2H), 5.01 (brs, 2H, NH_2), 2.74 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 155.1, 149.6, 141.5, 138.8, 138.2, 135.7, 130.0, 129.3 (2C), 129.1, 128.4 (2C), 114.6, 111.7, 23.9. MS (ESI) *m/z*: 348 ($[M+H]^+$, 80), 350 ($[M+H]^+$, 100), 352 ($[M+H]^+$, 20). HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{15}H_{12}N_3{}^{35}Br^{79}Cl$: 347.9903, found 347.9903 / calcd *m/z* for $[M+H]^+$ $C_{15}H_{12}N_3{}^{35}Br^{81}Cl$: 349.9884, found 349.9883 / calcd *m/z* for $[M+H]^+$ $C_{15}H_{12}N_3{}^{37}Br^{81}Cl$: 351.9861, found : 351.9853.

*3-Phenyl-N-propargyl-6-aminoquinoxaline (**10c**).* To a suspension 3-phenyl-6-aminoquinoxaline **4c** (500mg, 2.262 mmol, 1 equiv) and K_2CO_3 (469 mg, 3.39 mmol, 1.5 equiv.) in dry DMF (5 mL) was added propargyl bromide (380µL, 3.39 mmol, 1.5 equiv., 80 % in toluene) and the reaction mixture was heated at 80 °C for 18 h. The reaction mixture was then allowed to cool down to rt, quenched with water and was extracted with EtOAc. The combined organic layers were washed with brine, dried over $MgSO_4$, filtered and concentrated. The residue was purified by chromatography on silica gel (CH_2Cl_2: EtOAc, 90:10 to 80:20) to give yellow crystals (290 mg, 49% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.04 (s, 1H), 8.15 (dd, J = 8.0, 1.5 Hz, 2H), 7.90 (d, J = 8.5 Hz, 1H), 7.60-7.66 (m, 3H), 7.16 (s, 1H), 7.15 (m, 1H), 4.43 (brs, 1H, NH), 4.11 (brm, 2H), 2.29 (t, J = 2.5 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 152.1, 148.1, 144.4, 141.1, 139.4, 136.9, 129.9, 129.8, 129.0 (2C), 127.5

(2C), 121.5, 105.5, 79.8, 72.0, 33.5. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{17}H_{14}N_3$: 260.1183, found 260.1188. Purity (HPLC/UV λ at 254 nm): 93%.

*3-(4-Fluorophenyl)-N-propargyl-6-aminoquinoxaline (**10e**).* For experimental protocol, see compound **10c**. Yield: 41% (18.0 mg). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.99 (s, 1H), 8.15 (dd, J = 8.8, 5.6 Hz, 2H), 7.89 (d, J = 9.5 Hz, 1H), 7.21 (t, J = 9.0 Hz, 2H), 7.14 (dd, J = 9.5, 2.5 Hz, 2H), 7.12 (brs, 1H), 4.48 (brs, 1H, NH_2), 4.11 (brs, 2H), 2.29 (t, J = 2.0 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 164.0 (d, $J_{C\text{-}F}$ = 250.3 Hz), 151.0, 148.2, 144.4, 138.9, 136.8, 133.5, 129.7 (d, $J_{C\text{-}F}$ = 31.8 Hz, 2C), 129.4, 121.7, 116.1 (d, $J_{C\text{-}F}$ = 21.7 Hz, 2C), 105.3, 79.8, 72.1, 33.5, HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{17}H_{13}N_3F$: 278.1095, found : 278.1094.

*2-Methyl-3-phenyl-N-propargyl-6-aminoquinoxaline (**11c**).* To a solution of 2-methyl-3-phenyl-6-aminoquinoxaline **5c** (200 mg, 0.85 mmol, 1 equiv.), K_2CO_3 (118.0 mg, 0.85 mmol, 1 equiv.), KI (141 mg, 0,85 mmol, 1 equiv.) in anhydrous DMF (23 ml) under inert atmosphere was added propargyl bromide (0.2 mL, 1.7 mmol, 2 equiv.) drop-wise. The mixture was heated at 70 °C for 24 h, then cooled down to rt, hydrolyzed by saturated aqueous solution of NH_4Cl and extracted with ethyl acetate. The combined organic layer was washed with brine, dried over Na_2SO_4, filtered and concentrated *in vacuo*. The residue was then purified by flash chromatography on silica gel (cyclohexane: EtOAc, 7:3 to 6:4) to give dipropargylated product **11c'** (14 mg, 5%, not described) followed by mono-propargylated product **11c** (90.1 mg, 40% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.81 (d, J = 8.8 Hz, 1H), 7.61 (dd, J = 8.8, 1.6 Hz, 2H), 7.50 (m, 3H), 7.43-7.12 (d, J = 2.6 Hz, 1H), 7.09 (dd, J = 8.8, 2.6 Hz, 1H), 4.56 (brs, 1H, NH), 4.01 (brs, 2H), 2.67 (s, 3H), 2.23 (t, J = 2.4 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 154.6, 147.9, 147.3, 142.8, 139.4, 136.3, 128.9, 128.8 (2C), 128.6, 128.3 (2C), 121.6, 105.2, 79.9, 71.7, 33.3, 23.6. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{18}H_{16}N_3$: 274.1344, found : 274.1342.

*2-Methyl-3-(4-methoxyphenyl)-N-propargyl-6-aminoquinoxaline (**11d**).* For experimental protocol, see compound **11c**. Yield: 71% (280.0 mg). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.81 (d, J = 8.7 Hz, 1H), 7.60 (d, J = 8.7 Hz, 2H), 7.12 (d, J = 2.5 Hz, 1H), 7.02 (d, J = 8.7 Hz, 2H), 7.09 (dd, J = 8.7, 2.5 Hz, 1H), 4.44 (brs, 1H, NH), 4.03 (d, J = 2.4 Hz, 2H), 3.86 (s, 3H), 2.70 (s, 3H), 2.24 (t, J = 2.4 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 160.0, 154.4, 148.1, 147.2, 142.9, 136.1, 131.9, 130.4 (2C), 128.9, 121.4, 113.8 (2C), 105.3, 80.0, 71.8, 55.3, 33.4, 23.9. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{19}H_{18}N_3O$: 304.1450, found : 304.1458.

*2-Methyl-3-(4-fluorophenyl)-N-propargyl-6-aminoquinoxaline (**11e**).* For experimental protocol, see compound **11c**. Yield: 28% (74.0 mg). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.97 (d, J = 9.2 Hz, 2H), 7.64 (dd, J = 8.8, 5.6 Hz, 1H), 7.51 (dd, J = 9.2, 2.7 Hz, 1H), 7.42 (d, J = 2.7 Hz, 1H), 7.20 (t, J = 8.8 Hz, 2H), 4.27 (s, 1H, NH), 4.27 (d, J = 2.5 Hz, 2H), 2.71 (s, 3H), 2.26 (t, J = 2.5 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.1 (d, $J_{C\text{-}F}$ = 248.0 Hz), 154.0, 148.8, 148.2, 142.9, 136.5, 135.6 (d, $J_{C\text{-}F}$ = 2.0 Hz), 130.9 (d, $J_{C\text{-}F}$ = 8.5 Hz, 2C), 128.9, 121.8, 115.5 (d, $J_{C\text{-}F}$ = 22.4 Hz, 2C), 109.9, 80.0, 73.1, 40.4, 23.8. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{18}H_{15}N_3F$: 292.1250, found : 292.1250. Purity (HPLC/UV λ at 260 nm): 94%.

*2-Methyl-3-(4-chlorophenyl)-N-propargyl-6-aminoquinoxaline (**11f**).* For experimental protocol, see compound **11c**. Yield: 34% (160.0 mg). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.83 (d, J = 8.7 Hz, 1H), 7.58 (d, J = 8.4 Hz, 2H), 7.48 (d, J = 8.4 Hz, 2H), 7.13 (dd, J = 8.7, 2.5 Hz, 1H), 4.10 (d, J = 2.5 Hz, 1H), 7.44 (brs, 1H, NH), 4.05 (brs, 2H), 2.68 (s, 3H), 2.25 (t, J = 2.5 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 153.5, 147.7, 147.4, 142.9, 137.9, 136.5, 134.9, 130.3 (2C), 129.1, 128.7 (2C), 121.9, 105.2, 79.9, 71.9, 33.4, 23.7. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{18}H_{15}ClN_3$: 308.0955, found : 308.0959.

*2-Methyl-3-p-tolyl-N-propargyl-6-aminoquinoxaline (**11g**).* For experimental protocol, see compound **11c**. Yield: 40% (207.0 mg). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.81 (d, J = 8.9 Hz,

1H), 7.53 (d, *J* = 8.0 Hz, 2H), 7.30 (d, *J* = 8.0 Hz, 2H), 7.12 (d, *J* = 2.5 Hz, 1H), 7.10 (dd, *J* = 8.9 and 2.5 Hz, 1H), 4.48 (brs, 1H, NH), 4.02 (brs, 2H), 2.69 (s, 3H), 2.42 (s, 3H), 2.23 (t, *J* = 2.5 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 154.8, 148.1, 147.2, 142.9, 138.6, 136.6, 136.3, 129.1 (2C), 128.9, 128.8 (2C), 121.5, 105.4, 80.0, 71.8, 33.4, 23.8, 21.3. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{19}H_{18}N_3$: 288.1501, found : 288.1501.

2-Methyl-3-(naphthalen-2-yl)-N-propargyl-6-aminoquinoxaline ***(11h)***. For experimental protocol, see compound **11c**. Yield: 28% (80.5 mg). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.11 (s, 1H), 7.97 (d, *J* = 8.4 Hz, 1H), 7.92 (t, *J* = 4.7 Hz, 2H), 7.86 (d, *J* = 8.5 Hz, 1H), 7.75 (dd, *J* = 8.5, 1.7 Hz, 1H), 7.51-7.57 (m, 2H), 7.17 (d, *J* = 2.5 Hz, 1H), 7.12 (dd, *J* = 9.0, 2.5 Hz, 1H), 4.48 (t, *J* = 5.5 Hz, 1H), 4.04 (dd, *J* = 5.5, 2.5 Hz, 2H), 2.74 (s, 3H), 2.25 (t, *J* = 2.5 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 154.6, 148.1, 147.3, 142.9, 136.8, 136.4, 133.1, 133.0, 129.0, 128.5, 128.4, 128.1, 127.7, 126.6, 126.4 (2C), 121.7, 105.3, 79.9, 71.8, 33.4, 23.8. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{22}H_{18}N_3$: 324.1501, found : 324.1472. Purity (HPLC/UV λ at 220 nm): 90%.

2-Methyl-3-(3,4-dichlorophenyl)-N-propargyl-6-aminoquinoxaline ***(11n)***. For experimental protocol, see compound **11c**. Yield: 50% (196.0 mg). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.85 (d, *J* = 9.0 Hz, 1H), 7.77 (d, *J* = 2.0 Hz, 1H), 7.59 (d, *J* = 8.2 Hz, 1H), 7.49 (dd, *J* = 8.2 and 2.0 Hz, 1H), 7.17 (dd, *J* = 9.0, 2.5 Hz, 1H), 7.10 (d, *J* = 2.5 Hz, 1H), 4.40 (t, *J* = 5.7 Hz, 1H, NH), 4.07 (dd, *J* = 5.7, 2.5 Hz, 2H), 2.70 (s, 3H), 2.27 (t, *J* = 2.5 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 152.1, 147.5, 147.4, 142.9, 139.4, 136.8, 133.1, 132.8, 131.1, 130.4, 129.2, 128.3, 122.3, 105.1, 79.8, 72.0, 33.5, 23.7, HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{18}H_{14}Cl_2N_3$: 342.0565, found : 342.0565. Purity (HPLC/UV λ at 264 nm): 94%.

2-Methyl-3-(3,4,5-trimethoxyphenyl)-N-propargyl-6-aminoquinoxaline ***(11r)***. For experimental protocol, see compound **11c**. Yield: 42% (118.7 mg). ^{1}H NMR (400 MHz,

$CDCl_3$): δ 7.78 (d, J = 9.6 Hz, 1H), 7.08-7.11 (m, 2H), 6.80 (s, 2H), 4.64 (brs, 1H, NH), 4.00 (brs, 2H), 3.87 (s, 9H), 2.67 (s, 3H), 2.21 (t, J = 2.5 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 154.4, 153.1 (2C), 147.7, 147.4, 142.6, 138.5, 136.3, 134.9, 128.8, 121.7, 106.2 (2C), 104.9, 79.9, 71.6, 60.8, 56.1 (2C), 33.2, 23.6. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{21}H_{22}N_3O_3$: 364.1661, found : 364.1659. Purity (HPLC/UV λ at 254 nm): 93%.

*5-Bromo-2-methyl-3-phenyl-N-propargyl-6-aminoquinoxaline (**13c**).* For experimental protocol, see compound **11c**. Yield: 14% (62.0 mg). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.94 (d, J = 9.1 Hz, 1H), 7.75 (dd, J = 8.0, 1.5 Hz, 2H), 7.48-7.54 (m, 3H), 7.39 (d, J = 9.1 Hz, 1H), 5.24 (t, J = 5.9 Hz, 1H, NH), 4.19 (dd, J = 5.9, 2.3 Hz, 2H), 2.29 (t, J = 2.5 Hz, 1H), 2.77 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 155.0, 148.7, 144.8, 139.8, 139.0, 136.2, 129.5 (2C), 128.4 (2C), 129.0, 128.2, 116.9, 105.2, 79.9, 72.1, 33.6, 23.8. MS (ESI) *m/z*: 352.1 (^{79}Br) et 354.1 (^{81}Br) ($[M+H]^+$, 65 et 70), $[2M(^{79}Br)Na]^+$: 727 ($[M+H]^+$, 100). HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{18}H_{15}N_3Br$: 352.0447, found : 352.0449.

*2-Methyl-3-phenyl-N-(2,2-dimethyl)-propargyl-6-aminoquinoxaline (**12c**).* To a solution of 2-methyl-3-phenyl-6-aminoquinoxaline (**5c**) (500.0 mg, 2.13 mmol, 1 equiv.) in a mixture of THF/water (10.5 / 1 mL) with of CuCl (20 mg) under inert atmosphere was added triethylamine (0.41 mL, 2.98 mmol, 1.4 equiv.) and of 3-chloro-3-methyl-1-butyne (0.34 mL, 2.98 mmol, 1.4 equiv.). The mixture was stirred at room temperature during 12 h, hydrolyzed by a saturated aqueous solution of K_2CO_3, then extract with CH_2Cl_2. The combined organic layer was washed with brine, dried over Na_2SO_4 then concentrated *in vacuo*. The residue was purified by flash chromatography on silica gel (CH_2Cl_2: EtOAc, 85:15) to obtain compound **12c**. Yield: 65% (414.0 mg). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.81 (d, J = 9.0 Hz, 1H), 7.61 (d, J = 8.3 Hz, 2H), 7.56 (d, J = 2.5 Hz, 1H), 7.41-7.50 (m, 3H), 7.16 (dd, J = 9.0, 2.5 Hz, 1H), 4.32 (brs, 1H, NH), 2.66 (s, 3H), 2.40 (s, 1H), 1.68 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 154.6, 147.8, 145.8, 142.6, 139.6, 136.1, 128.8 (2C), 128.6, 128.5, 128.4 (2C),

123.2, 108.0, 86.4, 71.2, 47.8, 30.0 (2C), 23.6. HRMS (ESI) calcd *m/z* for $[M+H]^+$ $C_{20}H_{20}N_3$: 302.1657, found : 302.1656.

BIOLOGICAL ASSAYS

Ethic statement. Animals were housed, handled, and cared for in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH Publication no. 85-23, revised 1996) and with the European Union Council Directives (86/609/CEE). The protocol was approved by the Committee on the Ethics of Animal Experiments Charles Darwin n°5 (reference number N° 5012099-A). Animals were manipulated according to protocols that have been validated by the scientific community; sacrifice was performed under sodium pentobarbital anesthesia and all efforts were made to minimize suffering. Animals were housed and maintained at a constant temperature (22 ± 1 °C) and in a humidity-controlled (55 ± 20%) environment. A 12 h/12 h light-dark cycle was kept constant, with lights turned on at 08:00 a.m. During the acclimatization period (3 days) and throughout all the study, the animals had free access to food and water.

Paradigms of DA cell death in midbrain cultures. At gestational day 15.5, embryos were collected from the uterine horns of Sprague-Dawley female rats (Janvier LABS, Le Genest St. Isle, France) that had been deeply anesthetized, and then decerebrated. The ventral mesencephalon was dissected out and cultures were prepared using N5 medium and 1 mg/ml polyethylenimine as a coating as previously described.[12] Under these conditions, the loss of DA neurons is spontaneous and selective.[22] When needed some sets of cultures were also maintained with 20 ng/mL of the trophic peptide GDNF, until 10 DIV to postpone

neurodegeneration. Then, these cultures were deprived of GDNF until 15 DIV to evaluate neuroprotection.

To study the antioxidant potential of test compounds, mesencephalic cells were seeded in a culture medium consisting of equal volumes of Dulbecco's minimal essential medium and Ham's F12 nutrient mixture (DMEM-F12, Life Technologies, Saint Aubin, France) as previously described.[12,22] The presence of trace amounts of ferrous iron in the culture medium generates a Fenton-type reaction that is causing progressively the death of DA neurons.[35]

Treatments of the cultures. When using spontaneous DA cell death paradigms in either N5 or DMEM-F12 culture media, test treatments were applied after culture plating and then every day after 3 DIV until completion of the cultures. When using the GDNF deprivation model, midbrain cultures were maintained initially with the trophic peptide at 20 ng/ml for 10 DIV, and then substitutive treatments were applied each day between 11 and 15 DIV. Treatments were renewed by exchanging 70% of the culture medium.

TH immunolabeling. To detect DA neurons, TH immunofluorescence was used. After fixation with a 4% formaldehyde solution in Dulbecco's phosphate buffered saline medium (PBS) for 12 min, cultured cells were washed three times with PBS and incubated overnight at 4°C with a mouse anti- TH monoclonal antibody (1:5000, Euromedex, Souffelweyersheim, France). Subsequent incubations were performed, at rt, with a secondary anti-mouse IgG Alexa Fluor 488 (1:500, Life Technologies, Paisley, UK). Images were acquired with the HCI software (Hamamatsu Corp., Bridgewater, NJ) using an inverted fluorescent microscope (TE-300, Nikon, Tokyo, Japan) coupled to an ORCA-ER digital camera from Hamamatsu. TH^+ cells were uniquely DA neurons in our culture conditions[43] and represented ~2-3% of the total number of neurons present in these cultures at the time of plating.[16] Cell counting

was performed at 200x magnification using a 20x objective matched with a 10x ocular. The number of TH^+ neurons in each culture well was estimated after counting all visual fields distributed along the x-and y-axes.

Vimentin immunolabeling. Vimentin immunofluorescence was used to detect astrocytic cells. After fixation with a 4% formaldehyde solution in PBS medium for 12 min, cultured cells were washed three times with PBS and incubated overnight at 4 °C with a mouse anti-vimentin monoclonal antibody (1:100, Sigma, Saint Quentin Fallavier, France). Subsequent incubations were performed, at rt, with a secondary anti-mouse IgG Alexa Fluor 555 (1:500, Life Technologies, Paisley, UK). Semi-quantitative scoring of the effects of test compounds on glial cell density and differentiation was performed by visual inspection of the fluorescent signal (20x objective) in treated and untreated cultures.

Uptake of [2,5,6-^{3}H]-DA. The integrity and synaptic function of DA neurons were evaluated by their ability to accumulate tritiated DA.[44,45] Uptake was initiated by addition of 50 nM [2,5,6-^{3}H]-DA (40 Ci/mmol) (PerkinElmer, Boston, USA) to cultures maintained at 37 °C in 500 mL of PBS containing 5 mM glucose. Blanks values were obtained by treating some cultures with 1 µM of GBR-12909 (Sigma-Aldrich, Saint Quentin Fallavier, France), a selective inhibitor of the DA transporter. Uptake was stopped after 20 min by removing the incubation mixture, followed by two rapid washes with ice-cold PBS. Then, cells were scraped off culture wells using 500 mL distilled water and the radioactivity accumulated by DA neurons assessed by liquid scintillation spectrometry.

Incorporation of [methyl-^{3}H]-thymidine. A marker of DNA synthesis, [methyl-^{3}H]-thymidine (Perkin Elmer, Boston, USA), was used to measure glial cell proliferation in the spontaneous DA cell death paradigm with N5 medium.[12] Note that the proliferation rate was quantified after 8 DIV, i.e., at a stage where DA cell death is still ongoing in this

experimental setting. Briefly, midbrain cultures were washed once with PBS and then incubated in N5 medium lacking serum supplementation. Then 1μCi [methyl-^{3}H]-thymidine (84.4 Ci/mmol) was added to each culture well and the incubation was carried out at 37 °C for 2 h. Blanks values were obtained by treating some of the cultures with 1 mM unlabeled thymidine. Finally, the incubation mixture was removed and after two rapid washes with PBS. Cells were then scraped off culture wells using 500 μL distilled water and the radioactivity accumulated by dividing cells assessed by liquid scintillation spectrometry.

Subchronic MPTP mouse model of PD. C57BL/6 male mice (Janvier labs, Le Genest-Saint-Isle, France, 22-28 g) were divided into 4 groups (n=12/group) for testing the effects of compound **4c** *in vivo*. One group received MPTP hydrochloride (20 mg/kg, *i.p.*; once daily) injected for 5 consecutive days, a treatment which was followed by a washout period of 4 days. Two groups received MPTP together with **4c,** administrated *per os* in 1% carboxymethylcellulose and 0.5% of tween 80 (2x25 or 2x50 mg/kg/day;10 mL/kg), the administration starting 3 days before initiation of MPTP treatment and continuing until sacrifice time. The control group received vehicles used for MPTP and **4c** administration and the MPTP group the vehicle used for **4c** treatment, only. Six hours after the last administration of **4c** or vehicle, all mice were sacrificed by cervical dislocation. The striatums were dissected out, weighted and snap frozen separately. Tissues samples were stored at –80 °C and used for HPLC analysis of DA and its metabolites.

The caudal part of the brain (containing the SN) was placed in PFA 4% (in 0.1 M PBS pH 7.4) for 5 days and then, transferred to 20% sucrose (20% in 0.1 M PBS) for cryoprotection. Tissues were stored at –80 °C until processing for TH^+ immunohistochemistry.

HPLC measurement of striatal DA and its metabolites. The extent of striatal DA depletion was assessed by measuring levels of DA, DOPAC and HVA in the striatum, using HPLC with an electrochemical detection system. The system consisted of a MD 150 x 3.2mm

3µm C18 column (ESA), a dual electrode 5014B micro-dialysis cell (ESA) with the electrode potentials set at -150 mV and +375 mV. DA and its metabolites were detected at the second electrode, combined with a CoulArray detector (ESA). A 5020 guard cell (ESA) with a potential of +350 mV was placed before the autosampler. The mobile phase contained 90 mM sodium dihydrogen phosphate, 50 mM citric acid, 1.7 mM 1-octane sulphonic acid, 50 µM EDTA, 10% acetonitrile, pH 3.0 at a flow rate of 0.5 mL/min.

The striatums from the left hemispheres (stored at –80 °C) were weighted and homogenized (Bandelin UW70 ultrasonic probe) in 10 volumes (w/v) of 0.1 N perchloric acid. Homogenates were centrifuged at 15,000 rpm for 7 min at 0 °C before 10 µL of supernatant (or calibration standard) was injected into the HPLC-ECD system.

The calibration standards contained DA (Sigma-Aldrich, Saint Quentin Fallavier, France), DOPAC (Sigma-Aldrich, Saint Quentin Fallavier, France) and HVA (Sigma-Aldrich, Saint Quentin Fallavier, France). A calibration curve was created from a range of standards (24.4 – 1562.5 ng/mL).

TH^+ immunohistochemistry of SN. Twenty micrometer coronal sections of the mesencephalon were obtained with a freezing microtome and collected in 10 regularly spaced series. All sections were stored in PBS/0.4% sodium azide solution at 4 °C until use. For TH^+ immunohistochemistry the sections were rinsed in PBS 0.1M, treated with 0.3% H_2O_2/PBS for 30 min to remove endogenous peroxide activity, and blocked in 5% goat serum (Sigma-Aldrich, Saint Quentin Fallavier, France) in PBS/0.15% Triton X-100 (PBST) for 1 h. Sections were incubated with the following primary antibodies for 24 h at 4 °C: mouse anti-TH (1/500; US Biologicals, Salem, USA) antibodies. After incubation with appropriate anti-mouse secondary antibodies diluted 1/250 (Vector Laboratories, Burlingame, USA) for 1 h, the antibodies were revealed by the ABC method according to manufacturer's instructions

(Vector Laboratories, Burlingame, USA) with DAB (Sigma-Aldrich, Saint Quentin Fallavier, France) as the peroxidase substrate.

Quantification of SN DA neurons. Quantification of DA cell loss was done stereologically on regularly spaced DAB-stained sections covering the whole mesencephalon from the rostral pole of the SN to the caudal part of the structure as previously reported.[46] High-resolution bright-field digital images of the labeled sections were acquired using a NanoZoomer Digital Scanner (Hamamatsu Photonics France, Massy, France. objective × 40) equipped with the NDP software (Hamamatsu Photonics France, Massy, France.). One side of the SN was delineated at ×4 magnification for each section. TH^+ cell bodies were subsequently quantified stereologically in magnified images (× 20) of the selected regions using the ImageJ software (W.S. Rasband, ImageJ, US National Institutes of Health, Bethesda, MD, USA). Total cell numbers were estimated by integration along the rostrocaudal extent of the structures.[47] The quantification was performed by an experimenter blinded to the treatment groups. The total number of SN TH^+ neurons in one hemisphere varied from 4850 to 5535 in saline-treated mice.

Assessment of MAO-B inhibition. The potential of **4c** to inhibit MAO-B activity**,** was assessed using a modification of a protocol published by Scorza and colleagues.[48] Briefly, **4c** or the reference MAO-B inhibitor selegiline, were added to an ammonium acetate buffer solution (pH 7.4) containing a crude rat brain mitochondrial extract, the MAO-B specific substrate 4-dimethylaminophenethylamine, aldehyde dehydrogenase and the pyridine nucleotide cofactor β-NAD. After 30 min of incubation at 37°C, $HClO_4$ was added to stop the reaction and the supernatant recovered by centrifugation for the dosage of the reaction product 4-dimethylaminophenylacetic acid, using HPLC-HRMS.

Data analysis. All statistical analyses were performed using SigmaPlot 11.0. All data are presented as mean ± the standard error of the mean (SEM). For *in vitro* experiment, experimental values expressed as mean ± SEM were derived from at least triplicates of 3 independent experiments. Multiple comparisons against a single reference group were made by one-way analysis of variance (ANOVA) followed by Dunnett's or Bonferroni's post-hoc tests. A P value of less than 0.05 was considered significant.

ASSOCIATED CONTENT

Supporting Information.

Molecular formula strings and the associated biological data . This material is available free of charge via the Internet at http://pubs.acs.org.

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AUTHOR CONTRIBUTIONS

G.L.D., L.F. and B.F., designed the chemistry synthesis; G.L.D., P.P.M. and R.R.V., designed the biological research; G.L.D. L.F. and B.S.M. performed the chemistry synthesis; G.L.D., M.A, J.E.S.D., A.H. performed the biological research; G.L.D., L.F., P.P.M., R.R.V. and B.F., wrote the paper. All authors have given approval to the final version of the manuscript.

FUNDING SOURCES

The authors gratefully thank the Instituts Carnot and the LERMIT LabEx for the financial support of part of this study. This work was also supported by ANR through the ANTIPARK (ANR-201-EMMA-006) program and by Medicen. The research leading to these results has received further funding from the program "Investissements d'avenir" ANR-10-IAIHU-06 and from ANR (ANR-10-LABX-33).

NOTES

The authors declare no competing financial interest.

ACKNOWLEDGMENT

We thank Motac for the *in vivo* experiments. We thank Alexis Genin, director of research applications at ICM for his help in the valorization of our compounds. We thank Prof. T. Morton (UCR, Riverside, USA) for fruitful discussions and proof reading our manuscript.

ABBREVIATIONS USED

Ar: aryl; BBB: blood–brain barrier; CNS: central nervous system; ω-CON: ω-conotoxin MVIIA; DA: dopamine or dopaminergic; DAB: 3,3-diaminobenzidine; DAN: dantrolene; dbcAMP: *N*-(6),2'-O-dibutyryladenosine 3':5' cyclic monophosphate; Desf: Desferrioxamine; DIV: day *in vitro*; DOPAC: 3,4-Dihydroxyphenylacetic acid; EtOH: ethanol; GDNF: glial cell line-derived neurotrophic factor; HPLC: High-Performance Liquid Chromatography;

HVA: Homovanillic acid; MALDI-TOF: matrix-assisted laser desorption/ionization/time-of-flight; MPAQ: 2-methyl-3-phenyl-6-aminoquinoxaline; MIDA: *N*-methyliminodiacetic acid; MPP^+: 1-methyl-4-phenylpyridinium; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NBS: N-bromosuccinimide; NCS: N-chlorosuccinimide; NIF: nifedipine; NS: no significant; PBS: Dulbecco's phosphate buffered saline; PAQ: 3-phenyl-6-aminoquinoxaline; PD: Parkinson disease; QSARs: Quantitative Structure–Activity Relationship; rt: room temperature; RyRs: ryanodine receptor channels; SAR: Structure-activity relationship; SN: *substantia nigra*; TH: tyrosine hydroxylase; THF: tetrahydrofuran; TMS: tetramethylsilane; Veh: vehicle.

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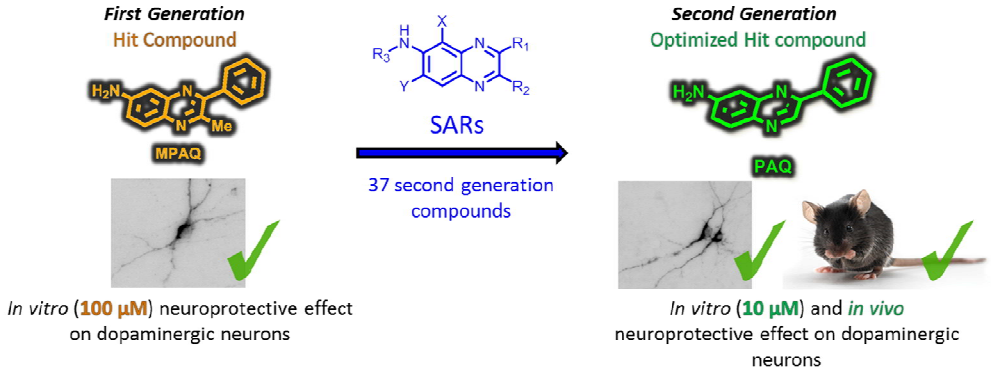
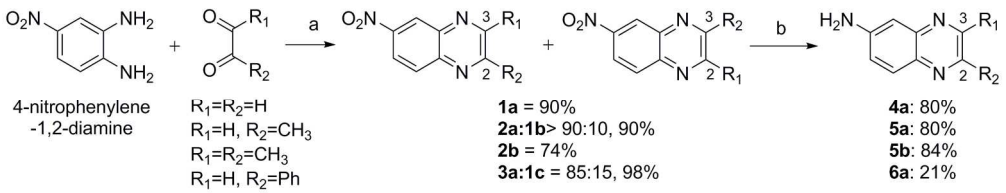
Table of Contents Graphic





Scheme 1. Synthesis of 6-aminoquinoxaline and 2-substituted-6-aminoquinoxalines by Hinsberg condensation.

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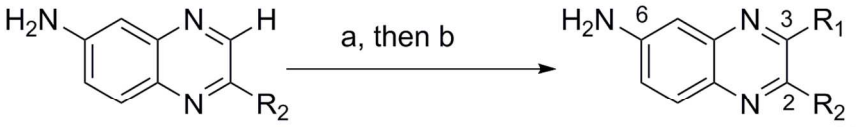

4a, R_2 = H

5a, R_2= CH_3

6a, R_2= Ph

4e, R_1=4-F-Phenyl, 7%

5c, R_1=Ph, 45%
5d, R_1=4(MeO)-phenyl, 34 %
5e, R_1=4-F-Phenyl, 35%
5f, R_1=4-Cl-Phenyl, 70%
5g, R_1=4-Me-Phenyl, 56%
5h, R_1=2-naphtyl, 48%
5i, R_1=3,4-$(MeO)_2$-phenyl, 31%
5j, R_1=3,4-Cl_2-phenyl, 45%
5k, R_1=4-binaphtyl, 30%
5r, R_1=3,4,5-$(MeO)_3$-phenyl, 21%

6d, R_1=4-MeO-Phenyl, 2%

Scheme 2. Synthesis of 2,3-disubstituted quinoxalines.
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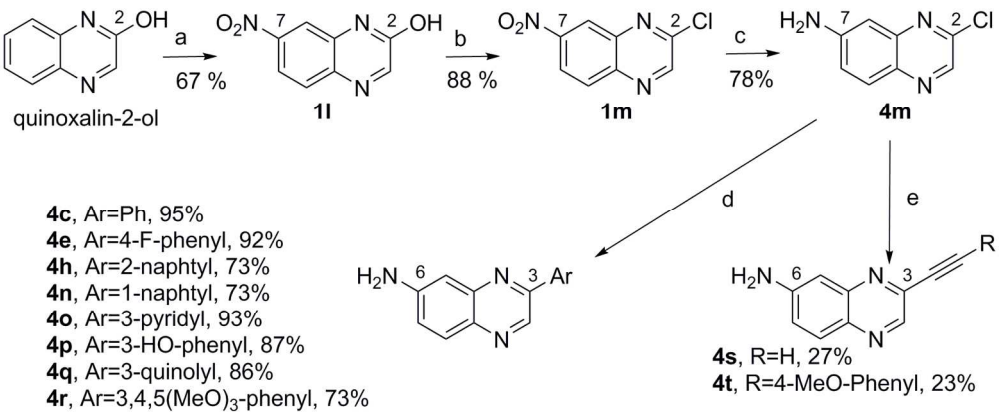

Scheme 3. Synthesis of 3-aryl-6-aminoquinoxalines from quinoxalin-2-ol.
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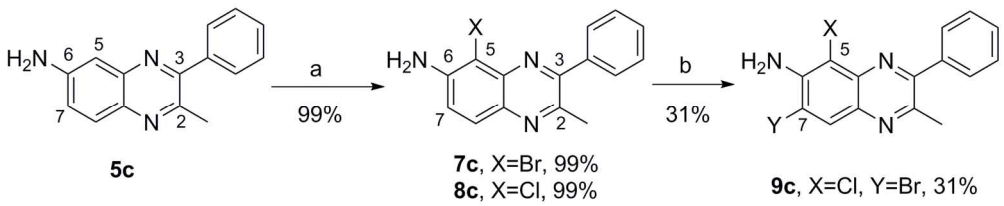

Scheme 4. Regioselective halogenation of 6-aminoquinoxaline 5c (MPAQ).
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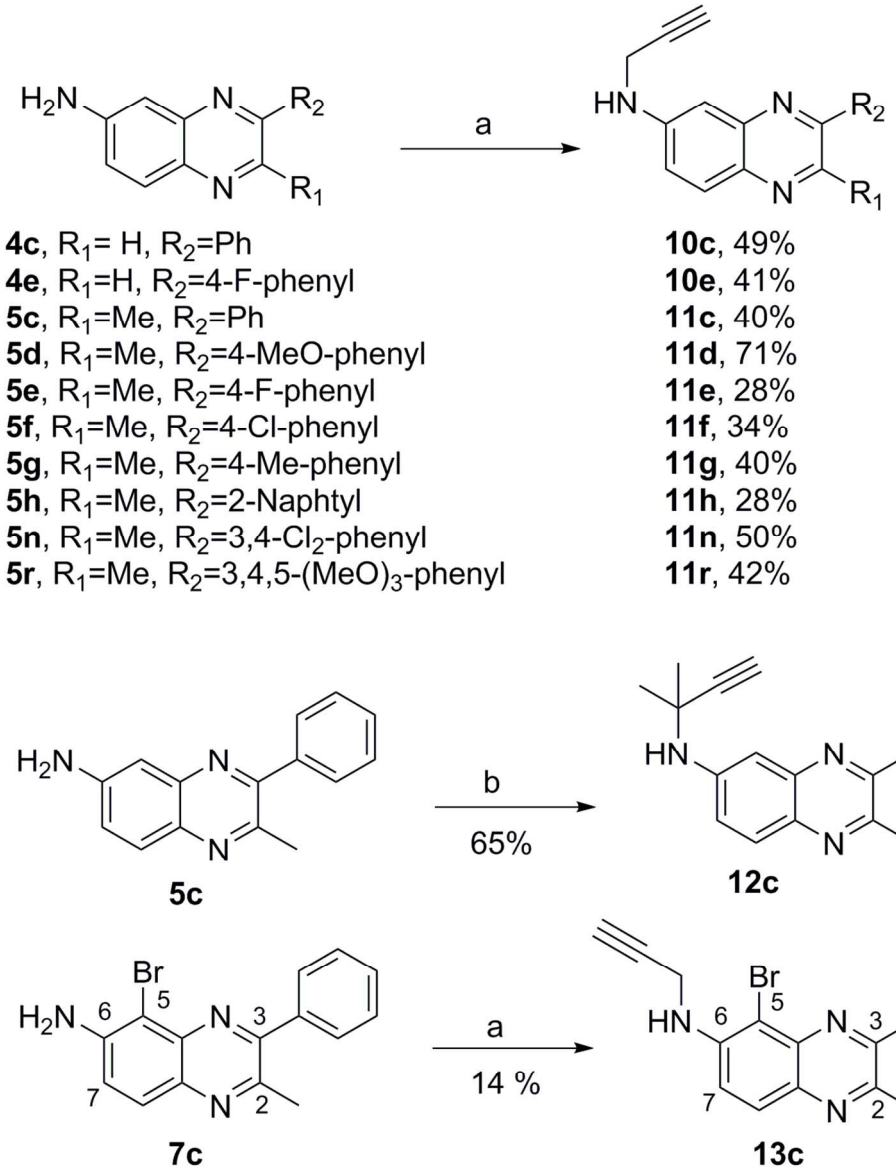

Scheme 5. Synthesis of N-propargyl-6-aminoquinoxaline derivatives.
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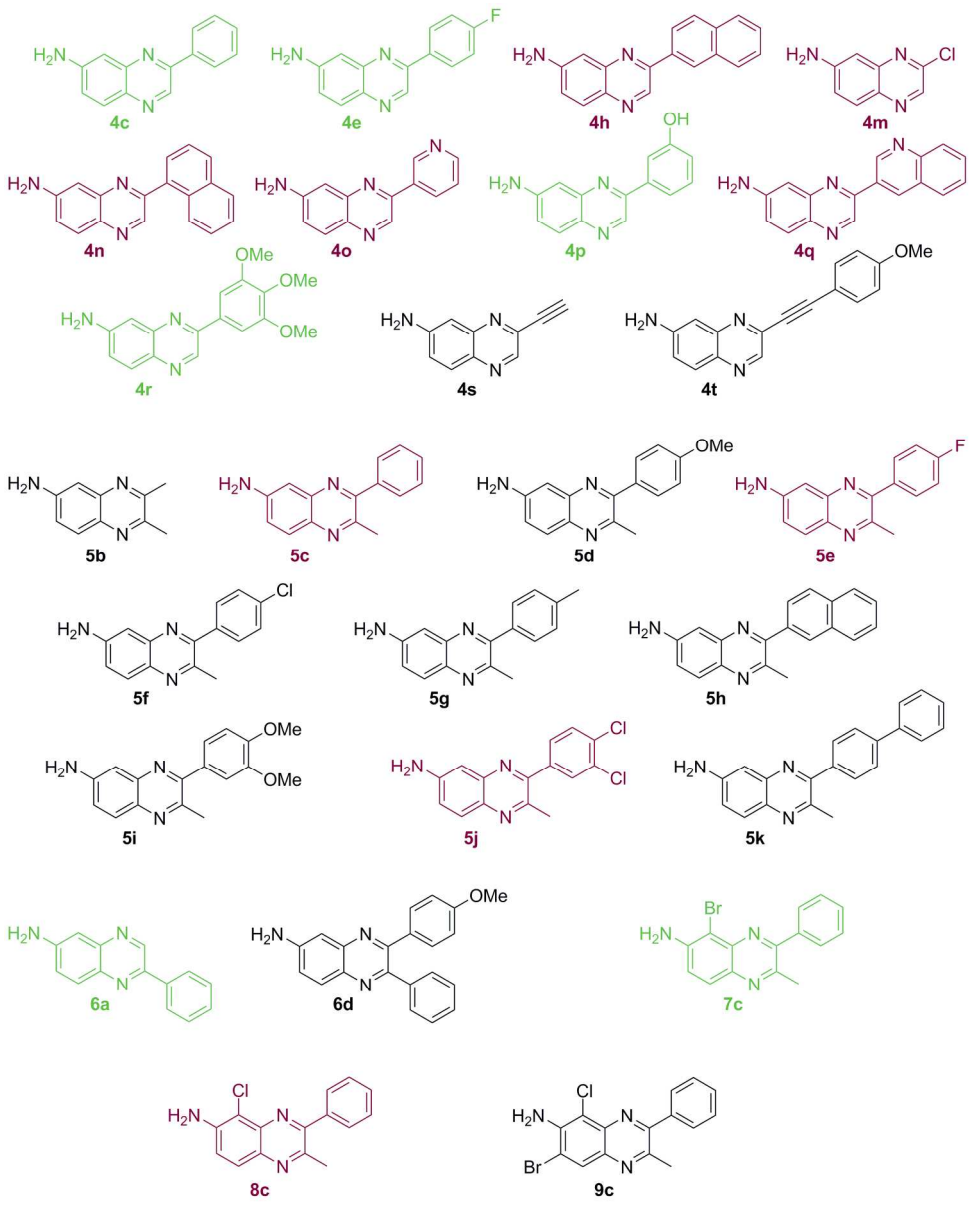

Chart 1. Chemical structures of all 6-aminoquinoxaline derivatives tested in the cellular PD model of spontaneous DA cell death. Compounds having significant protective effects towards DA neurons at 50 µM are colored in purple. Protective compounds selected for further investigations (see **Figure 1** for selection criteria) are colored in green.
191x238mm (300 x 300 DPI)

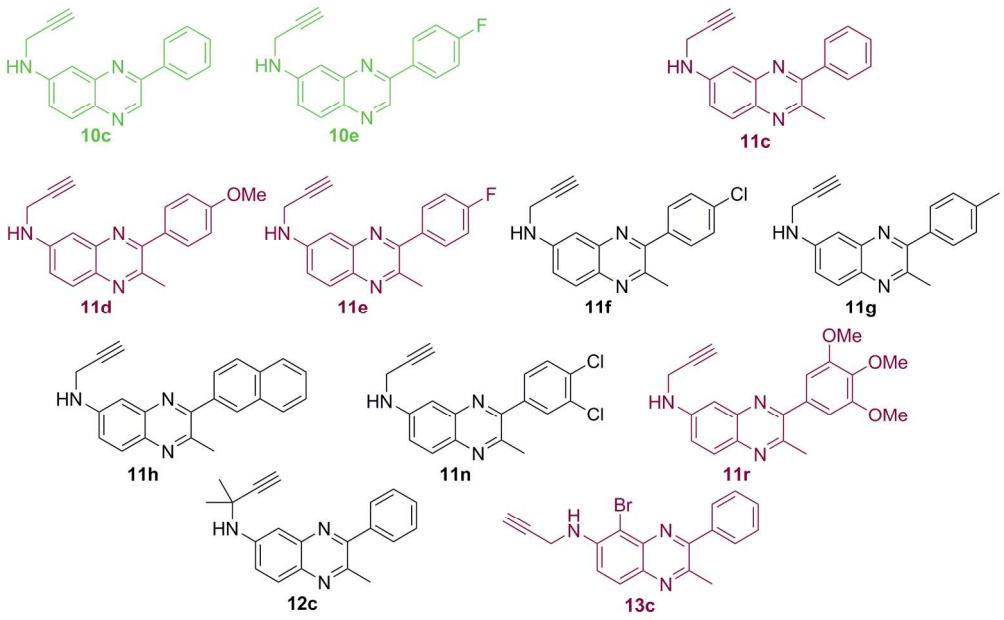

Chart 2. Chemical structures of all N-propargyl-6-aminoquinoxaline derivatives synthesized and tested in the cellular PD model of spontaneous DA cell death. Compounds having significant protective effects towards DA neurons at 50 μM are colored in purple. Protective compounds selected for further investigations (see Figure 3 for selection criteria) are colored in green.
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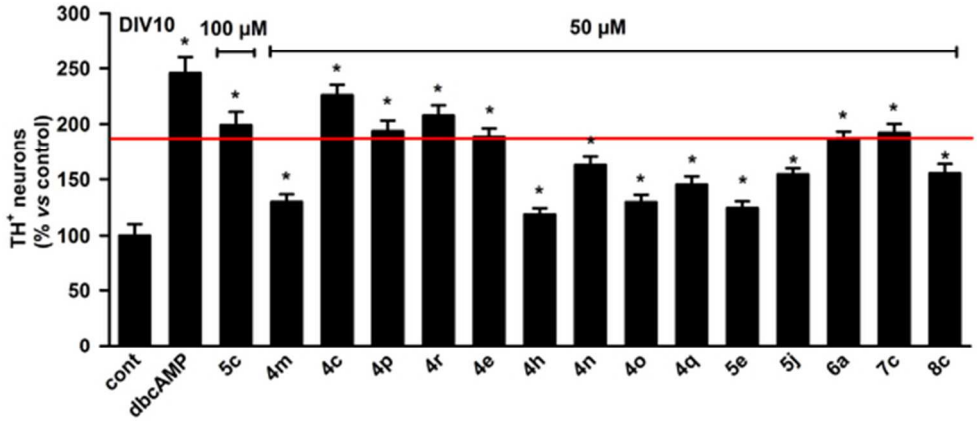

Figure 1. Screening of 6-aminoquinoxaline derivatives in a cellular system that models selective DA cell death in midbrain cultures. Number of TH+ neurons surviving after 10 days in vitro (DIV) in midbrain cultures treated or not with 6-aminoquinoxalines (50 µM, except MPAQ (**5c**) 100 µM). Note that **5c** is inactive at 50 µM.12 DbcAMP (1 mM) is used as reference protective molecule for DA neurons. Inactive 6-aminoquinoxaline derivatives (**4s**, **4t**, **5b**, **5d**, **5f**, **5g**, **5h**, **5i**, **5k**, **6d**, **9c**) are not included in the figure. A 185 % increase in survival at 50 µM, is our cut-off for the selection of the most active compounds (above red line). *, p< 0.05 vs controls.
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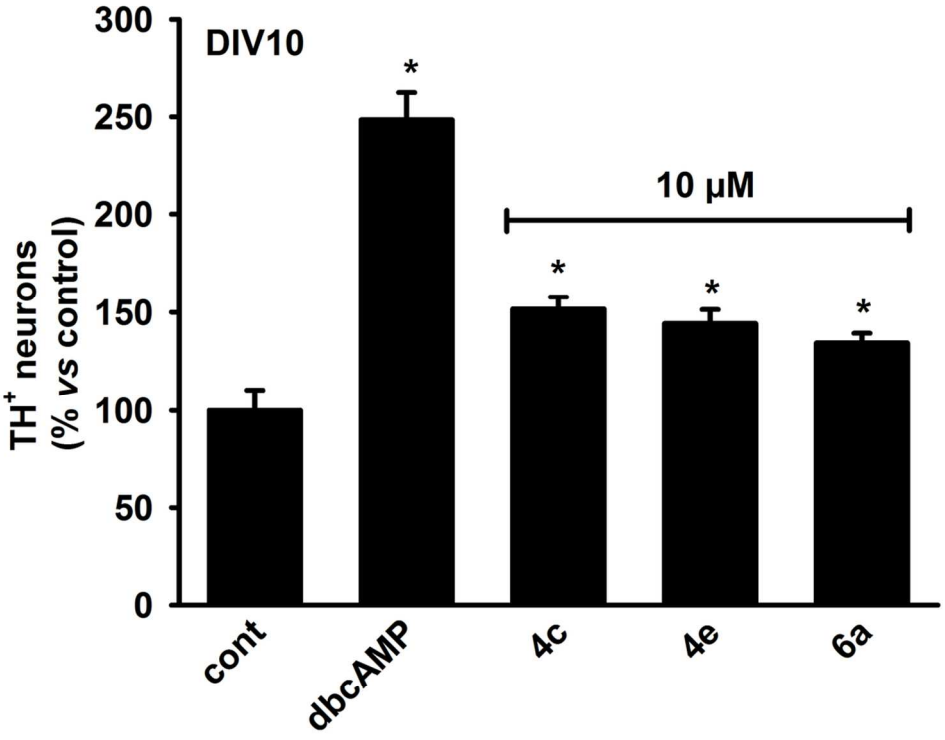

Figure 2. Evaluation of previously selected 6-aminoquinoxaline derivatives for their efficacy at a concentration of 10 µM. Selected 6-aminoquinoxaline derivatives were applied to midbrain cultures at 10 µM and the survival rate of DA neurons was assessed at 10 DIV. Derivatives not providing protection at 10 µM (**4p**, **4r**, **7c**) are not shown in this figure. DbcAMP (1 mM) was used as reference protective molecule for DA neurons. *, $p < 0.05$ vs controls.
104x91mm (300 x 300 DPI)

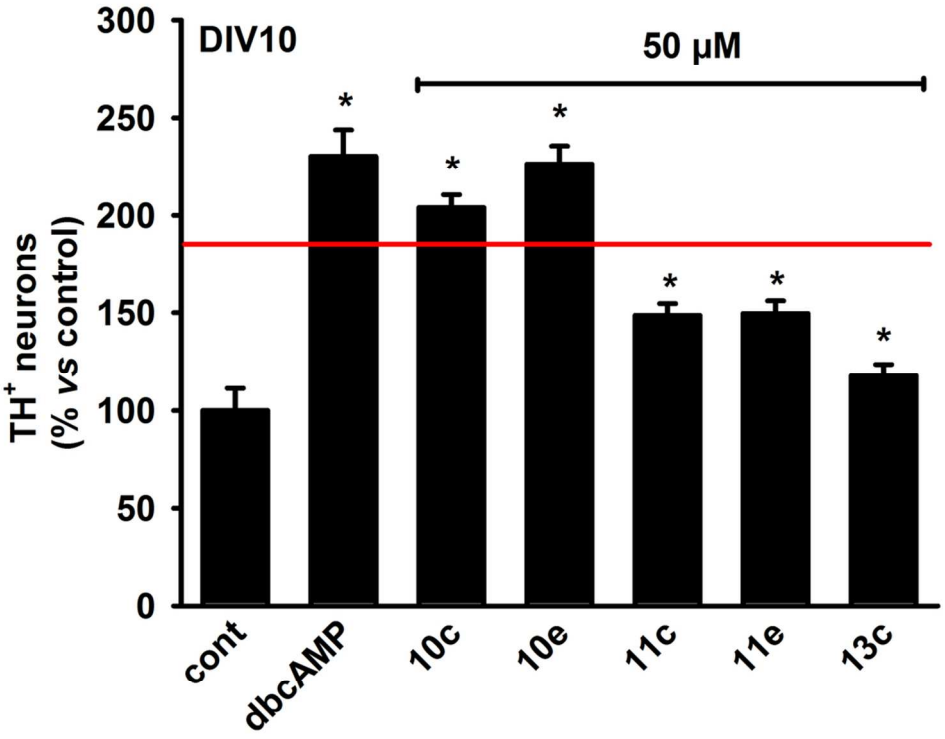

Figure 3. Screening of N-propargyl-6-aminoquinoxaline derivatives in a cellular system that models selective DA cell death in midbrain cultures. Number of TH+ neurons surviving after 10 DIV in cultures treated or not with N-propargyl-6-aminoquinoxaline derivatives (50 µM). DbcAMP (1 mM) is used as reference protective molecule for DA neurons. Inactive N-propargyl-6-aminoquinoxaline derivatives (**11d**, **11f**, **11g**, **11h**, **11n**, **11r**, **12c**) are not included in this figure. A 185 % increase in survival at 50 µM, is our cut-off for active compounds (above red line). *, p< 0.05 vs controls.
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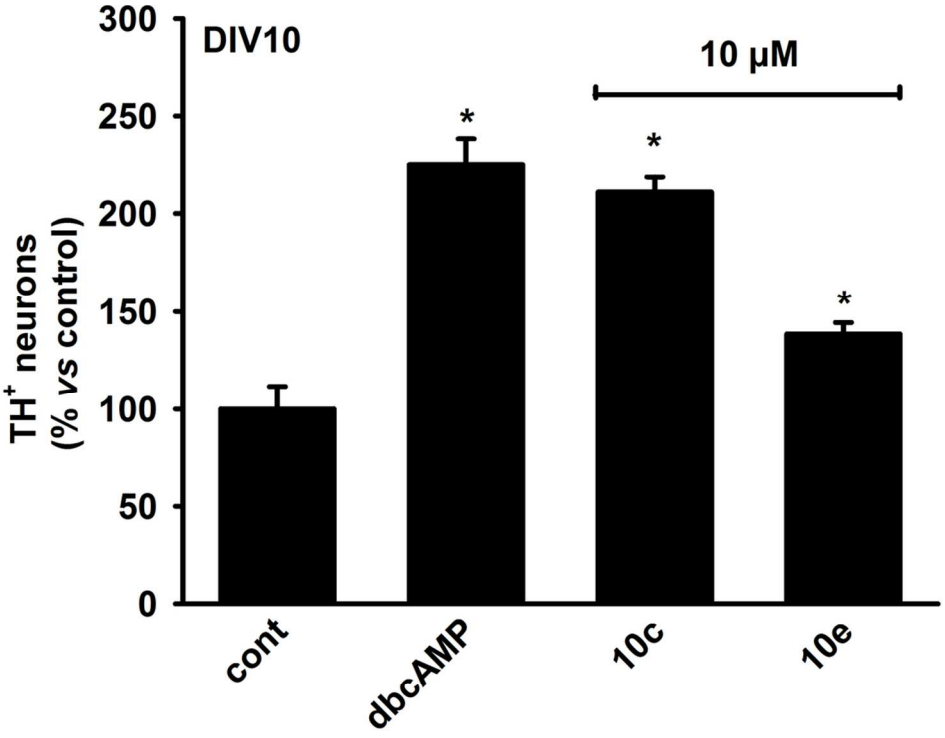

Figure 4. Evaluation of previously selected N-propargyl-6-aminoquinoxaline derivatives for their efficacy at 10 µM. Selected N-propargyl-6-aminoquinoxaline derivatives were applied to midbrain cultures at 10 µM and the survival rate of DA neurons was assessed at 10 DIV. DbcAMP (1 mM) is used as reference protective molecule for DA neurons. *, $p < 0.05$ vs controls.
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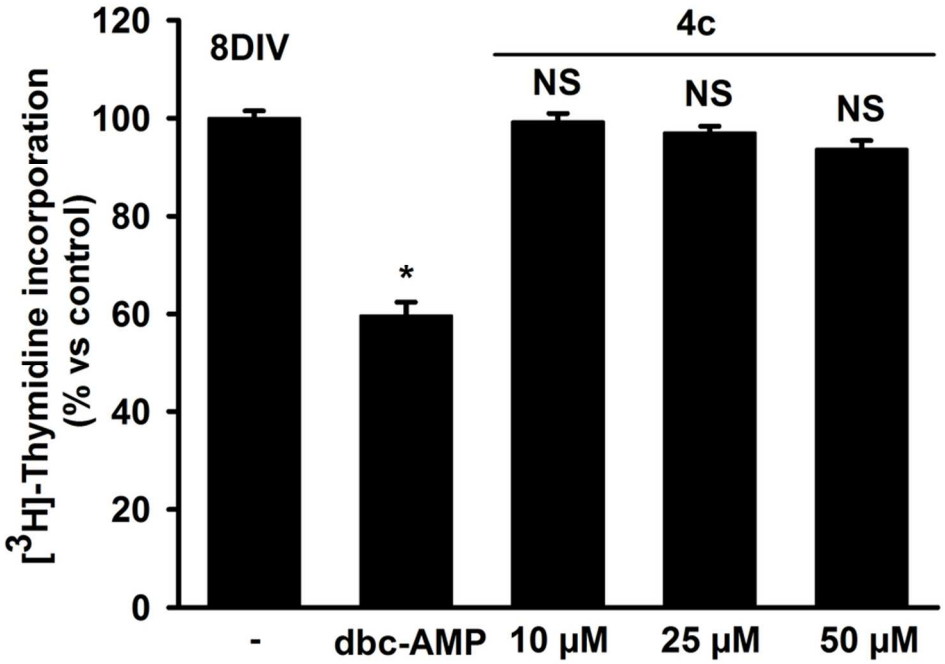

Figure 5. 4c (**PAQ**: 3-Phenyl-6-AminoQuinoxaline) does not reduce glial cell proliferation in midbrain cultures. Quantification of [3H]-thymidine incorporation in 8 DIV cultures, treated or not with **4c** (10-50 µM). DbcAMP at 1 mM was used as a positive control. NS: no significant effect.
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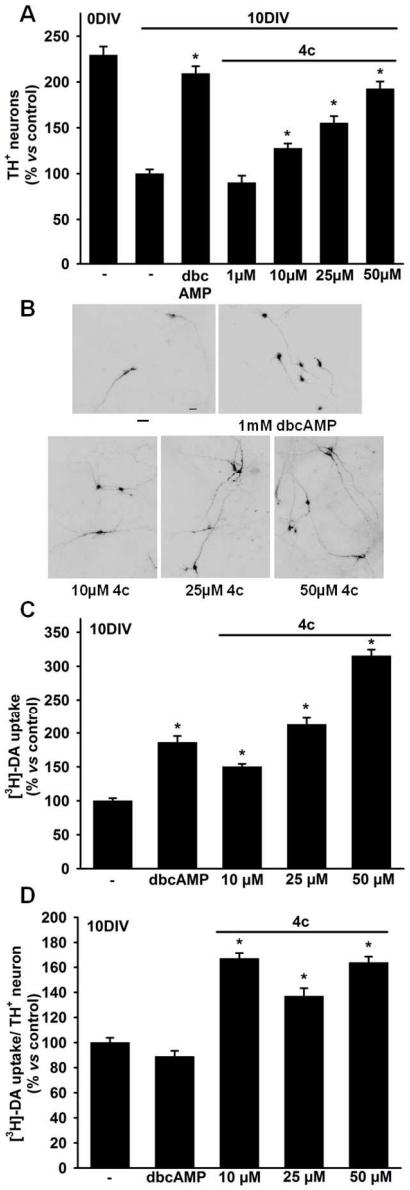

Figure 6. Neuroprotective and neuritogenic effects of 4c onto DA neurons in midbrain cultures. (A) Number of TH+ neurons, in 10 DIV cultures, treated or not with **4c** (1-50 µM). (B) Illustration showing TH+ neurons, in 10 DIV cultures, treated or not with **4c** (10-50 µM). Illustrations of the effects of **4c** on TH+ cells are presented under an inverted format. Scale bars = 60 µm. In (A), (B), (C) and (D) DbcAMP (1mM) was used as reference protective molecule. (C) Quantification of [3H]-DA uptake, in 10 DIV cultures, treated or not with **4c** (10-50 µM). (D) Rate of DA uptake per TH+ neuron in 10 DIV cultures treated or not with **4c** (10-50 µM). *P < 0.05 vs control cultures.
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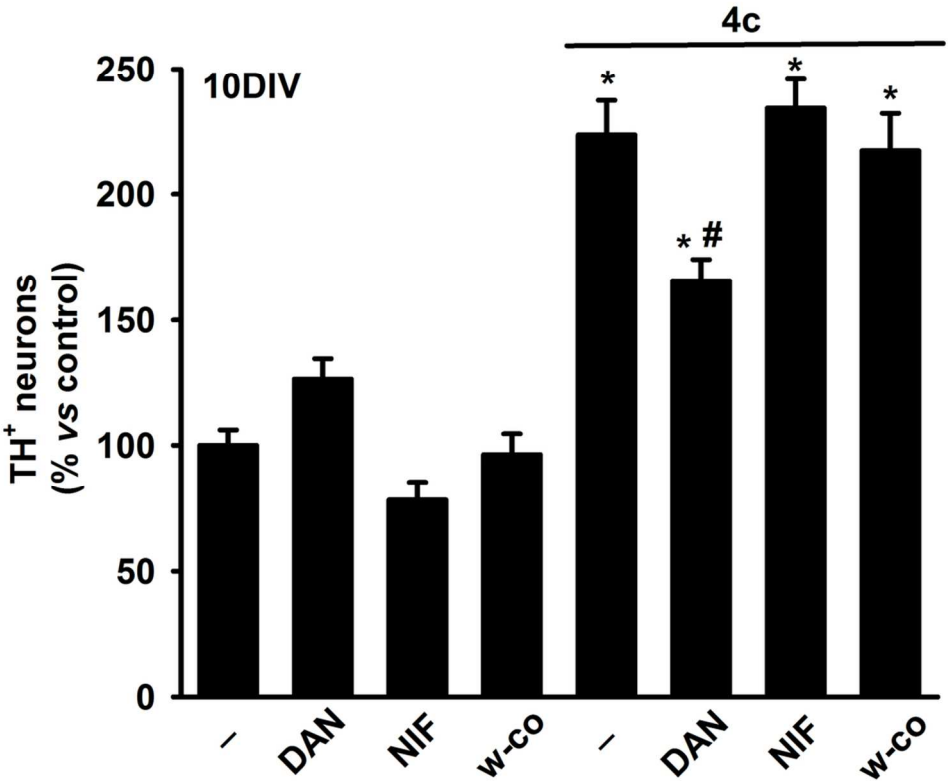

Figure 7. Neuroprotection by **4c** requires calcium mobilization through RyRs. Number of TH+ neurons in 10 DIV cultures chronically exposed or not to **4c** (50 μM) in the presence or not of nifedipine (NIF, 20μM), ω-conotoxin MVIIA (ω-CON, 0.5μM) or dantrolene (DAN, 30 μM). *P < 0.05 vs. no treated cultures. #P < 0.05 vs **4c** treated cultures.
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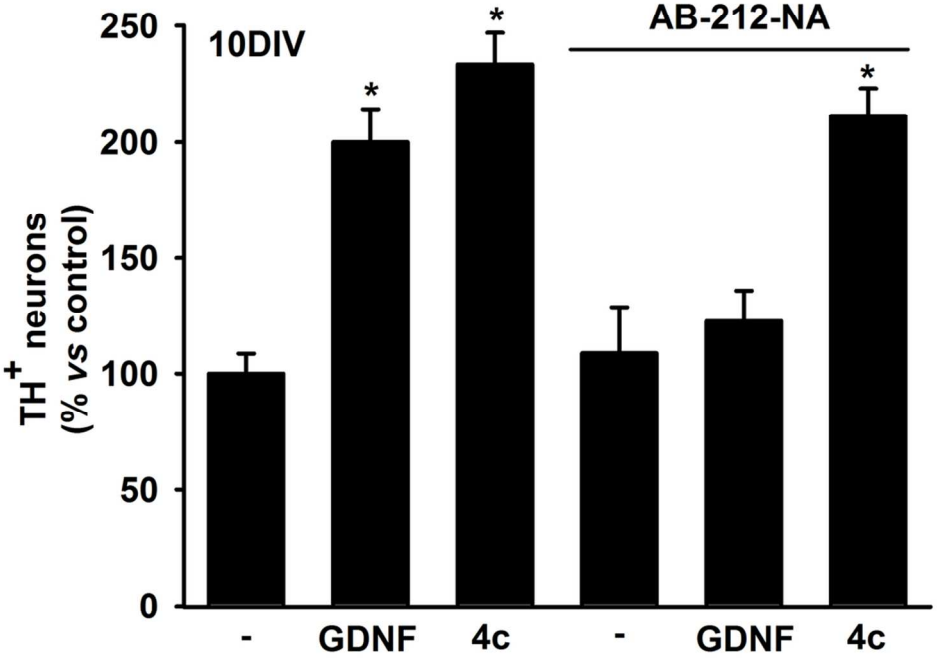

Figure 8. The protective effect of **4c** is unrelated to that of GDNF. Number of TH+ neurons, in 10 DIV cultures, treated or not with **4c** (50 μM), in presence or not of an anti-GDNF antibody (AB-212-NA, 10 μg/mL). GDNF at 20 ng/ml was used as a positive control. *P < 0.05 vs control cultures. #P < 0.05 vs GDNF-treated cultures.
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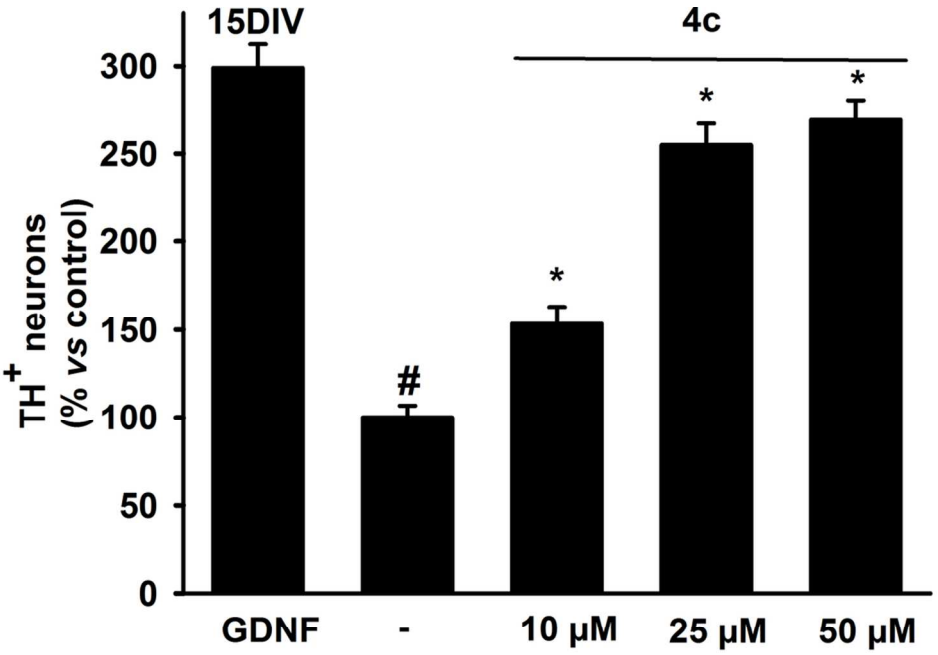

Figure 9. Mature DA neurons dependent on GDNF for their survival can be rescued by **4c**. Rescuing effect of **4c** (10-50 µM) in midbrain cultures exposed to 20 ng/ml GDNF for 10 days and then deprived of the trophic peptide between 11 DIV and 15 DIV. Comparison to cultures maintained continuously with GDNF up to 15 DIV. *P < 0.05 vs GDNF-treated cultures. #P < 0.05 vs GDNF-deprived cultures.
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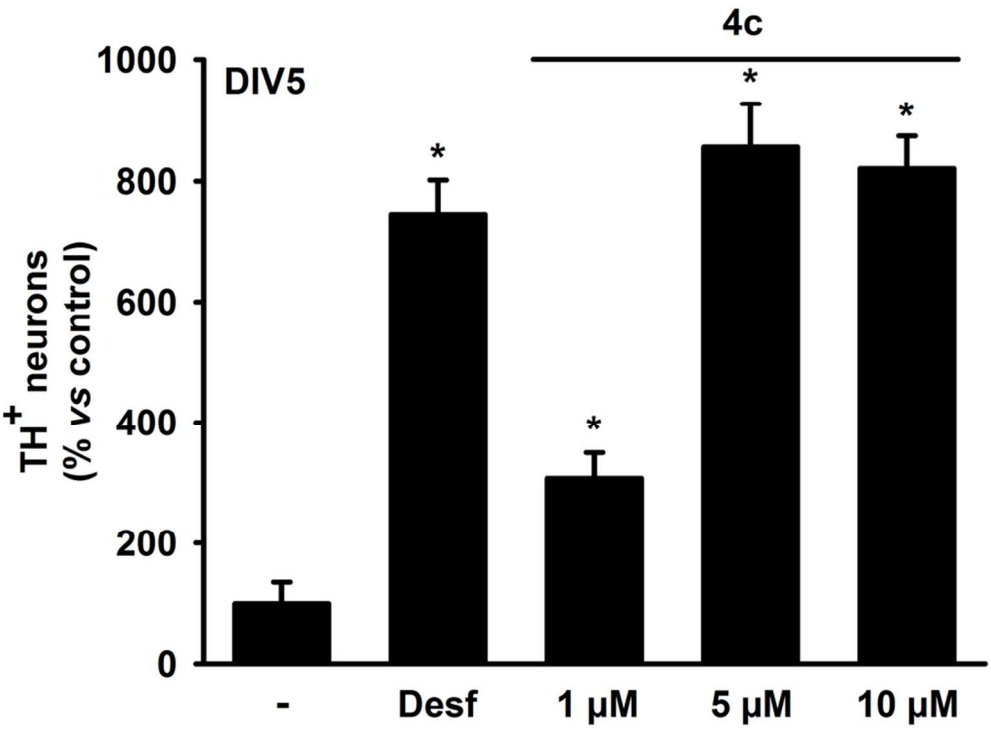

Figure 10. Antioxidant effect of **4c**. Neuroprotective effect of **4c** (1-10 µM) for DA neurons in a model system of chronic oxidative stress. Desferrioxamine (Desf 10 µM) is used as a positive control. *P < 0.05 vs control cultures.
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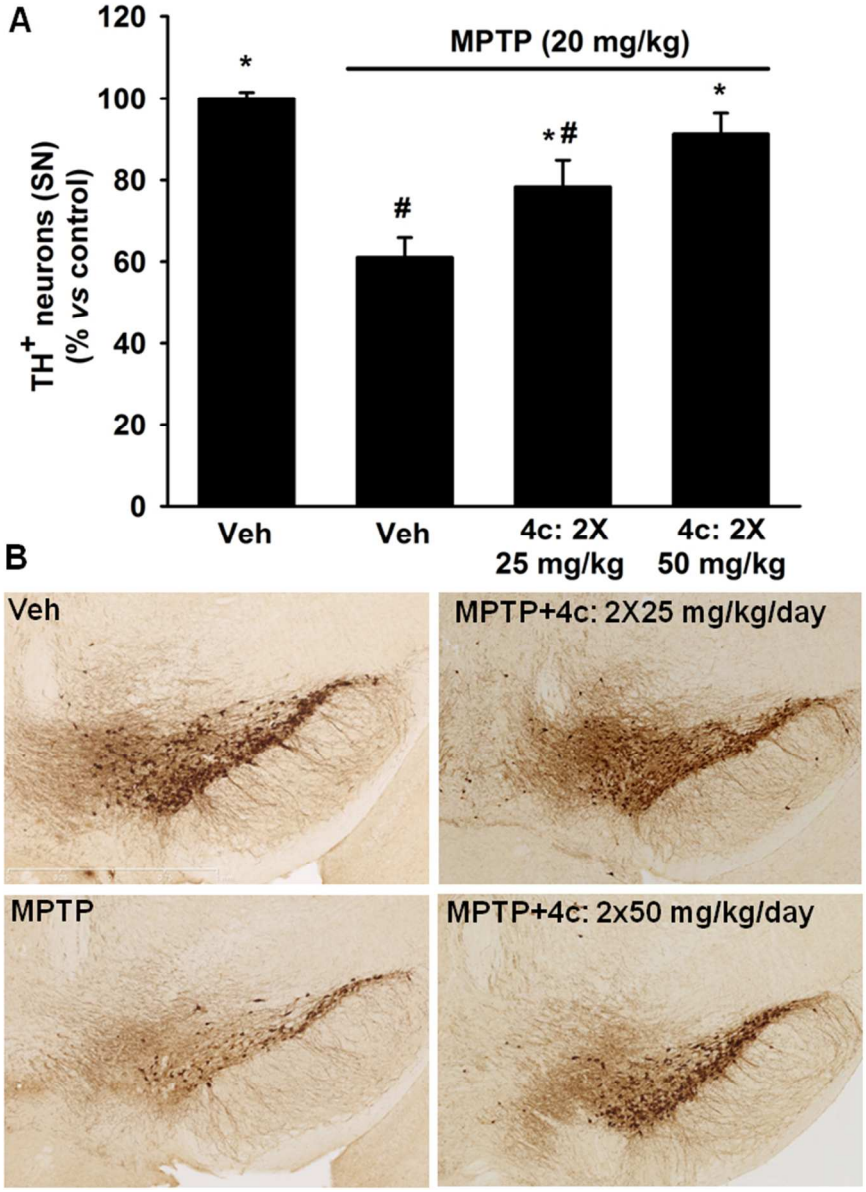


Figure 11. Neuroprotective effects of **4c** onto DA neurons in the SN of MPTP-treated mice. (A) Neuroprotective effect of 11 days of treatment with **4c** (2x25, 2x50 mg/kg/day; *p.o.*) on the number of TH+ neurons in the SN of MPTP-treated mice (one hemisphere analyzed by mouse). Data expressed as mean ± SEM (n=9-12). *P<0.05 vs vehicle + MPTP (20 mg/kg/day) (one-way ANOVA with Dunnett's multiple comparisons). #P<0.05 vs vehicle (one-way ANOVA with Dunnett's multiple comparisons). (B) Illustration showing the impact of **4c** (2x25, 2x50 mg/kg/day; *p.o.*) on the number of DA neurons in the SN of MPTP or vehicle-treated mice. High-resolution bright-field digital images of the labeled sections were acquired using a NanoZoomer Digital Scanner (Hamamatsu Photonics France, Massy, France. objective × 40) equipped with the NDP software (Hamamatsu Photonics France, Massy, France. magnification x 4.5). Scale bar = 1 mm. Veh: vehicle.
210x267mm (96 x 96 DPI)

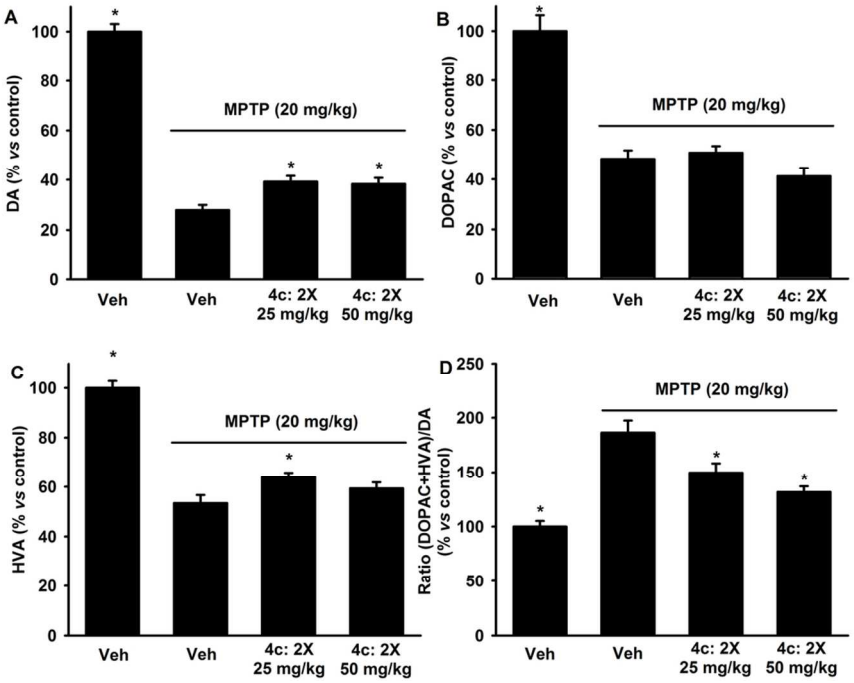
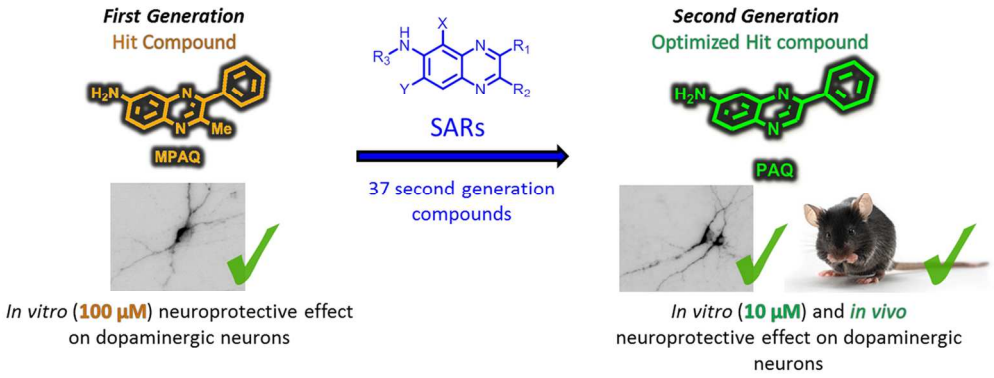


Figure 12. (A), (B), (C) Effect of 11 days of treatment with **4c** (2x25, 2x50 mg/kg/day; *p.o.*) on the level of DA (A) and its metabolites (DOPAC: (B), HVA: (C)) in the striatum of MPTP-treated mice. (D) Effect of 11 days of treatment with **4c** (2x25, 2x50 mg/kg/day; *p.o.*) on the ratio of (DOPAC+HVA)/DA in the striatum of MPTP-treated mice. Data expressed as mean ± SEM (n=9-12). *P<0.05 vs vehicle + MPTP (20 mg/kg) (one-way ANOVA with Dunnett's multiple comparisons). Veh: vehicle.
104x85mm (300 x 300 DPI)



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