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Original article

Neuroprotective effects of a brain permeant 6-aminoquinoxaline derivative in cell culture conditions that model the loss of dopaminergic neurons in Parkinson disease

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ABSTRACT

Parkinson disease is a neurodegenerative disorder of aging, characterized by disabling motor symptoms resulting from the loss of midbrain dopaminergic neurons and the decrease of dopamine in the striatum. Current therapies are directed at treating the symptoms but there is presently no cure for the disease. In order to discover neuroprotective compounds with a therapeutical potential, our research team has established original and highly regioselective methods for the synthesis of 2,3-disubstituted 6-aminoquinoxalines. To evaluate the neuroprotective activity of these molecules, we used midbrain cultures and various experimental conditions that promote dopaminergic cell loss. Among a series of 11 molecules, only compound **MPAQ** (2-methyl-3-phenyl-6-aminoquinoxaline) afforded substantial protection in a paradigm where dopaminergic neurons die spontaneously and progressively as they mature. Prediction of blood–brain barrier permeation by Quantitative Structure–Activity Relationship studies (QSARs) suggested that **MPAQ** was able to reach the brain parenchyma with sufficient efficacy. HPLC-MS/MS quantification in brain homogenates and MALDI-TOF mass spectrometry imaging on brain tissue sections performed in **MPAQ**-treated mice allowed us to confirm this prediction and to demonstrate, by MALDI-TOF mass spectrometry imaging, that **MPAQ** was localized in areas containing vulnerable neurons and/or their terminals. Of interest, **MPAQ** also rescued dopaminergic neurons, which (i) acquired dependency on the trophic peptide GDNF for their survival or (ii) underwent oxidative stress-mediated insults mediated by catalytically active iron. In summary, **MPAQ** possesses an interesting pharmacological profile as it penetrates the brain parenchyma and counteracts mechanisms possibly contributive to dopaminergic cell death in Parkinson disease.

1. Introduction

Parkinson disease (PD) is characterized by a progressive loss of dopamine (DA) neurons within the *substantia nigra* (SN) and as a consequence by a decrease in striatal DA, at the nerve terminal level [1]. The depletion in striatal DA accounts for the typical motor symptoms of the disease. In the current state of knowledge, there is no definite answer about the mechanisms causing DA cell death even if several hypotheses have been put forward to explain the molecular basis of neuronal damage. In particular, it has been suggested that mitochondrial dysfunction, oxidative stress, calcium dyshomeostasis and neuroinflammatory processes involving glial cells could all contribute actively to DA cell demise [2,3].

Drugs that replace DA or activate its receptors can alleviate motor symptoms with efficacy, but such treatments are unable to stop or even slow down disease progression and they also induce

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side effects that become invalidating [4]. Neuroprotective/neurorestorative therapies, which are still theoretical in PD, are based on the concept that vulnerable DA neurons in the SN can somehow be protected from the degenerative process that leads to their demise. Those treatments should be, however, given as early as possible to be effective in PD patients, as motor symptoms appear when the depopulation in DA cell bodies exceeds 50% [5].

Glial-cell derived neurotrophic factor (GDNF) has been proposed as a therapeutic agent to delay the development of PD [6], but clinical trials have been disappointing, probably due to inherent drawbacks associated with the use of peptides applied as drugs, including pleiotropic effects, short half-life and inability to cross the blood–brain barrier (BBB), thus imposing repeated transcranial injections, with undesirable side-effects [7,8]. Substantial efforts have been made, therefore, to develop non-peptidic small molecules with protective/restorative activities for DA neurons [9].

With the aim of discovering new compounds having protective/neuritogenic effects for DA neurons, we synthetized initially natural product hybrids with an indole or a quinoline core structure [10,11]. Some of these compounds presented interesting neurotrophic properties in a model system of rat DA neurons in culture but the hit compound was found only in scarce amounts in brain tissue after systemic administration, precluding therefore any *in vivo* studies.

Quinoxaline derivatives represent another interesting family of compounds having a broad spectrum of biological activities that could possibly be used for therapeutical purposes [12–15]. Even if some of these compounds are known to provide protection against acute neuronal excitotoxic lesions [16,17], to our knowledge the properties of quinoxalines have not yet been evaluated thoroughly in a chronic degenerative context. We were interested, here, to characterize the protective potential of quinoxalines for DA neurons. Thus, we designed and synthesized some new substituted 6-aminoquinoxaline derivatives taking advantage of original and highly regioselective methods of synthesis that we developed in-house [18]. To assess the activity of these compounds, we used midbrain cultures and different experimental settings that model the loss of DA neurons in PD [19]. Among a series of 11 molecules evaluated, we found a single hit compound exhibiting not only interesting protective properties for DA neurons but also physicochemical characteristics allowing sufficient penetration into the brain parenchyma for *in vivo* use.

2. Results

2.1. Chemical syntheses

Quinoxalines **5b**–**c** are the expected products that can be prepared from a common amino-quinoxaline after organolithium reagent addition and oxidation, followed by *N*-alkylation [14]. First, 1,2-diamino-5-nitrobenzene is reacted with a ketoaldehyde to give the 2-substituted-6-nitroquinoxaline as the major regioisomer **1b** (ratio 90:10) [20], which is then reduced by H_2 in EtOH to give the corresponding aminoquinoxaline **2b**. **1a**, obtained from glyoxal, can be reduced under same reaction conditions to yield **2b**. In a second step, an addition of one equivalent of organolithium reagent followed by rearomatization with manganese oxide led to the expected non-symmetrical 2,3-disubstituted 6-aminoquinoxalines **3b**–**c**. In another approach, 2,3-disubstituted quinoxalines **3b**–**d** could be obtained in a one-pot reaction by reaction of 6-aminoquinoxaline **1a** with one equivalent of organolithium reagent at −78 °C, followed by addition of a second equivalent of a second organolithium reagent at 0 °C and then manganese oxide oxidation [18]. Then *N*-acylated quinoxalines **4a**–**c** were obtained by reaction with the corresponding carboxylic acid chlorides to give the expected products in good yields. Acylated compounds were then reduced into the corresponding *N*-alkyl products through $LiAlH_4$ reduction to give compounds **5b**–**c** (Fig. 1).

2.2. Neuroprotection of DA neurons using midbrain cultures

All synthesized compounds (**3a**–**d**, **4a**–**c**, **5b**–**c**) were evaluated for their protective potential for DA neurons using a culture system in which neurodegeneration occurs spontaneously and selectively as a consequence of a mechanism involving immature astrocytes and calcium dyshomeostasis [21,22]. Cultures were maintained for 10 days in the presence or absence of the different test compounds, and then neuroprotection was evaluated by counting TH immunopositive (TH^+) neurons. The uptake of [^{3}H]-DA, an active process, which occurs for the most part at the level of neuritic extensions [23], was also evaluated in order to explore the function and the degree of differentiation of DA neurons rescued by drug treatment. The radioactivity was counted by liquid scintillation spectrometry. An estimation of the neuritogenic action of the test compounds was obtained by normalizing DA uptake values to the total number of TH^+ neurons counted in sister cultures exposed to the same treatments.

Consistent with previous results, we observed that a large number of TH^+ neurons were lost after 10 days of culture in the absence of any treatment [21,22] Among a series of 11 molecules evaluated, we found only two active molecules: compounds **3c** and **5b**. Quinoxaline **3c**, named **MPAQ** (2-**M**ethyl-3-**P**henyl-6-**A**mino-**Q**uinoxaline) showed a significant neuroprotective effect at 100 μM with an efficacy, however, lower than that provided by the lipophilic analog of cAMP, dbcAMP, used as a positive control (Fig. 2A). We also used DA uptake to assess the function and the state of differentiation of DA neurons. We observed an effect of **MPAQ** on DA uptake at 50 and 100 μM (Fig. 2C). In addition, at 100 μM the rate of DA uptake per TH^+ neuron was significantly increased *vs* control cultures (Fig. 2D) The latter observation suggested that DA neurons exposed to **MPAQ** were functional and also that they were more differentiated. Consistent with this possibility, microscopical examination of the cultures revealed that neurons treated with **MPAQ** had generally a more developed neuritic network by comparison to controls (Fig. 2B).

Compound **5b**, which was tested only at 10 μM because of it was less soluble than other 6-aminoquinoxaline derivatives, had an effect on DA uptake but not on DA survival (see Fig. 3A and C). This effect was even more prominent when DA uptake was expressed per TH^+ neuron (Fig. 3D). Consistent with a possible neuritogenic effect of **5b**, we also observed that TH^+ neurons exposed to this compound were more differentiated in comparison to corresponding controls (see Fig. 3B).

In conclusion, the phenyl group at position 3 on the quinoxaline ring seems to be important for the neuroprotective activity since other related compounds (**2a**, **2b**, **3a**, **3b**, **3d**) were not active (data not shown). So this compound could be an interesting hit to perform further structure activities studies. Compound **5b**, which possesses only neuritogenic effect, is also interesting because it exerted its effects at lower concentration than **MPAQ**.

2.3. Penetration of the BBB

Physicochemical properties of compounds **MPAQ** and **5b** including log*P*, Polar Surface Area (PSA) and aqueous solubility were estimated or measured. These parameters entering the 'Lipinski's rule of five' and most of one-dimensional PSA/log*P*-based Quantitative Structure–Activity Relationship studies (QSARs) methods are commonly evaluated to predict BBB penetration of compounds by passive diffusion [24,25] Prediction of BBB