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Research paper

Neuroprotective profile of pyridothiazepines with blocking activity of the mitochondrial Na^+/Ca^{2+} exchanger

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ABSTRACT

The mitochondrial Na^+/Ca^{2+} exchanger plays an important role in the control of cytosolic Ca^{2+} cycling in excitable cells, essential for the regulation of a plethora of Ca^{2+}-dependent physio-pathological events, such as apoptosis in the presence of a Ca^{2+} overload. There are very few pharmacological tools available to study both physiological and pathological implications of the mitochondrial Na^+/Ca^{2+} exchanger, where the benzothiazepine CGP37157 is the best-known ligand, used since the 1980s. However, it is not an efficient blocker and lacks of selectivity, as also blocks several other cellular Ca^{2+} transporters. Moreover, CGP37157 is a very lipophilic drug, showing very poor water solubility, what has hindered its therapeutic use. Attempting to improve its pharmacokinetic profile as well as its potency and selectivity, we herein describe the synthesis of new CGP37157 analogs, where the benzene-fused ring has been replaced by a pyridine. On top of a better water solubility and lower log P value, some of these new pyridothiazepine derivatives also presented a higher capacity to regulate the mitochondrial Ca^{2+} clearance, while keeping the neuroprotective properties presented in the head compound CGP37157.

1. Introduction

Mitochondria are the energy-producing factory of eukaryotic cells [1]. In addition, they play a fundamental role in the clearance of the cytosolic Ca^{2+} ($[Ca^{2+}]_c$) transients occurring during cell activation. Upon cell stimulation, mitochondria are capable of accumulating vast amounts of Ca^{2+} in their matrix through the Ca^{2+} uniporter, which uses the driving force of the electrical potential across the mitochondrial membrane [2]. When the stimulus ceases, the Ca^{2+} accumulated in the mitochondrial matrix is released back to the cytosol through antiporters, being the principal the Na^+/Ca^{2+} exchanger (mNCX) [3]. By this way, a mitochondrial-controlled Ca^{2+} cycling is generated, which was firstly observed in isolated cardiac mitochondria [4]. Later on, it was also demonstrated in hippocampal neuronal cultures [5]. Thus, mitochondria are now considered not only the cell bioenergetic plant, in which ATP production is controlled by Ca^{2+}-dependent matrix enzymes, but also essential players in shaping the $[Ca^{2+}]_c$ transients produced during cell stimulation, as a consequence of the intervention of different Ca^{2+} channels and Ca^{2+} transporters [6].

Although a minimal $[Ca^{2+}]_c$ level is required to maintain neuronal viability, when the physiological $[Ca^{2+}]_c$ level is highly altered, below or above a critical point, apoptosis is rapidly induced and the death of neurons occurs [7,8]. In the frame of this Ca^{2+} set-

Abbreviations: ALS, amytrophic lateral sclerosis; AD, Alzheimer's disease; AUC, area under the curve; CALHM1, Ca^{2+} homeostasis modulator 1; $[Ca^{2+}]_c$, cytosolic Ca^{2}; ER, endoplasmic reticulum; IP3, inositol triphosphate; LDH, lactate dehydrogenase; LiHMDS, lithium bis(trimethylsilyl)amide; mNCX, mitochondrial Na^+/Ca^{2+} exchanger; mit_AEQ, mitochondria-targeted aequorin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NCLX, Na^+/Ca^{2+} or Li^+/Ca^{2+} exchanger; NMDA, N-methyl-D-aspartate; R/O, rotenone plus oligomycin A; SEM, standard error of the mean; TFA, trifluoroacetic acid; TTX, tetrodotoxin; VGCC, voltage-gated Ca^{2+} channels.

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point hypothesis, we found that a mild sustained elevation of $[Ca^{2+}]_c$ elicited by the Ca^{2+} promoter ITH4012 is associated with neuroprotection [9,10]. We also explored the possibility that the mitigation of the rate of mitochondrial Ca^{2+} efflux by the mNCX blocker CGP37157 [11] (**1**, Fig. 1) could afford neuroprotection against neurotoxicity elicited by cell Ca^{2+} overload; we confirm this firstly in chromaffin cells [12] and, subsequently, in hippocampal slices [13,14].

However, **1** also blocks voltage-gated Na^+ channels [12], voltage-gated Ca^{2+} channels (VGCC) [12,15], the plasmalemmal NCX [16], and the recently discovered channel Ca^{2+} homeostasis modulator 1 (CALHM1) [17], among other targets. Thus, it remains unclear whether the neuroprotective actions of **1** are due to the blockade of mNCX or to the intervention over its other pharmacological targets. Given the high relevance of the mNCX as a potential target to develop new neuroprotective agents [18] and the recent discovery of the gene that expresses the long sought mNCX, namely NCLX [19], we anticipate that newly synthesized compounds targeting the mNCX with higher selectivity and potency could find therapeutic interest as neuroprotectants in neurodegenerative diseases and stroke, and could also serve as novel and more specific pharmacological tools to clarify the biological functions of the mNCX at different cell systems. For this reason, we aim the synthesis and evaluation of new **1** derivatives, with the goal of finding more selective and potent ligands of mNCX. We have recently reported a family of **1** derivatives by means of modifying the substituent at the pending phenyl ring, finding out an improvement in the neuroprotective properties together with an slight enhancement of the blockade of mNCX [20]. Attempting to optimize the pharmacokinetic profile of potential new drugs based on **1**, we paid attention to the recognized liposolubility of this type of 4,1-benzothiazepines [14], what would be beneficial for crossing the blood–brain barrier, but detrimental for an appropriate water solubility. Indeed, some of those benzothiazepine derivatives were barely dissolved in buffer media at concentrations above 5 μM [20]. A successful drug, able to reach its intended target and to present therapeutic efficacy, must possess an adequate pharmacokinetic profile, for which lipophilicity and solubility play an essential role [21]. The lipophilicity, or permeability across membranes such as the gut wall or the blood brain barrier, can be expressed as the logarithm of the partition coefficient (log P). Prediction of log P [22,23] for **1** indicated that its value would be close to the highest limit (log P = 5, Table S1, Supplementary data) according to the Lipinski's rules to predict druggability of chemical compounds [21]. This fact prompted us to search for structural alternatives to improve water solubility and to reduce log P values, but without hindering CNS penetration. We considered to replace the benzene-fused ring by pyridine, which would not only provide an additional ability to form hydrogen bonds with the biological target, but also an increase in polarity, as well as the possibility to prepare, for instance, their hydrochloride salts. With this slight structural change, the predicted log P diminished to values between 3 and 4 (Table S1, Supplementary data). Hence, in this work, we have synthesized new pyridothiazepines, analogs to **1**, and evaluated their ability to down-regulate the release of Ca^{2+} from mitochondria through mNCX, comparing with the best-known blocker CGP37157 **1**, and whether such blocking effect had consequences in their neuroprotective profiles, in several *in vitro* models of neurodegeneration.

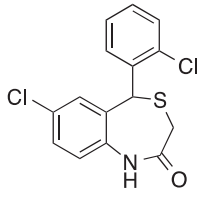

Fig. 1. Chemical structure of CGP37157 (**1**).

2. Results and discussion

2.1. Chemistry

Firstly, preparation of pyridine-fused analogs to **1** was performed according to previous experimental conditions, used for the synthesis of 4,1-benzothiazepines [20]. However, we observed that the *ortho*-lithiation of the Boc-protected 2-amino-4-chloropyridine and the subsequent nucleophilic addition to aldehydes were accompanied of a huge amount of by-products. Then, the further cyclization to form pyridothiazepines **6**, only provided the Boc-protected open intermediates analogs to **5**. All the attempts to optimize both reactions were unsuccessful. We therefore moved to synthetic alternatives, selecting pivaloyl chloride as a protecting group for 2-amino-5-chloropyridine. Compound **2** [24] reacted with ^{t}BuLi to promote the *ortho*-lithiation reaction. The generation of the aryllithium intermediate needed higher temperatures (0 °C) than the regular conditions for such a metalation. When the addition to the corresponding arylaldehyde at −78 °C was completed, reaction was stirred at room temperature for several hours (Scheme 1) yielding the intermediates **3**. Since the pivaloyl group is not removed under the acidic environment elicited by TFA, a deprotection step under basic conditions (KOH) was necessary, to obtain the corresponding amines **4** in good yields. Then, reaction with methyl thioglycolate and TFA only afforded open intermediates **5**, as free amines at C2 of the pyridine ring were not nucleophile enough to attack the methyl ester in the TFA/CH_2Cl_2 medium. Such cyclization was further achieved by lithium hexamethyldisilylamide (LiHMDS), which deprotonates the amine, thus obtaining the desired pyridothiazepines **6** (Scheme 1).

2.2. Pharmacology

2.2.1. Blockade of the mitochondrial Ca^{2+} clearance

To assess the blocking effect of the mNCX by the new pyridothiazepines, we have used HeLa cells transfected with the mitochondria-targeted aequorin (mit_AEQ), a bioluminescent dye with low affinity for Ca^{2+} ions, what allows to register dramatic Ca^{2+} oscillations into mitochondria. The reconstituted bioluminescent dye mit_AEQ suffers a chemical reaction in presence of three Ca^{2+} ions [25], producing photons that correlate with the concentration of Ca^{2+} within the mitochondria. The HeLa cell line is a reproducible model for the study of the mNCX, because it lacks of VGCC, plasmalemmal NCX or CALHM1, i.e. the other main biological targets blocked by the head compound **1**, so the possible direct effect of the new compounds on mNCX would be more accurately observed. Ca^{2+} uptake by mitochondria is stimulated by histamine. The exposure to histamine triggers the synthesis of IP_3 after the binding to its plasmalemmal histamine receptors; the IP_3 interacts with its own receptor in the endoplasmic reticulum (ER), inducing the release of Ca^{2+} from ER to cytosol. This Ca^{2+} pool is quickly taken up by mitochondria, given that both organelles are intimately coupled [26]. Finally, as mitochondria are unable to store Ca^{2+}, mNCX releases it back to cytosol. Thus, a typical register of mitochondrial Ca^{2+} oscillations, as schematized in the Fig. S2 (Supplementary data), shows a Gauss-type curve that can be characterized with the subsequent parameters: area under the curve (AUC), maximal peak of Ca^{2+} reached, rise rate (τ_{on}), and decay rate (τ_{off}). In this scenario, the presence of a compound able to reduce the mitochondrial Ca^{2+} clearance through the mNCX should modify