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Research paper

Discovery of efficient stimulators for adult hippocampal neurogenesis based on scaffolds in dragon's blood

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ABSTRACT

Reduction of hippocampal neurogenesis caused by aging and neurological disorders would impair neural circuits and result in memory loss. A new lead compound (*N-trans*-3′,4′-methylenedioxystilben-4-yl acetamide **27**) has been discovered to efficiently stimulate adult rats' neurogenesis. In-depth structure-activity relationship studies proved the necessity of a stilbene scaffold that is absent in highly cytotoxic analogs such as chalcones and heteroaryl rings and inactive analogs such as diphenyl acetylene and diphenyl ethane, and validated the importance of an NH in the carboxamide and a methylenedioxy substituent on the benzene ring. Immunohistochemical staining and biochemical analysis indicate, in contrast to previously reported neuroprotective chemicals, *N*-stilbenyl carboxamides have extra capacity for neuroproliferation-type neurogenesis, thereby providing a foundation for improving the plasticity of the adult mammalian brain.

1. Introduction

Adult neurogenesis in mammals was documented in the 1960s, which overturned the long-held dogma that no new neurons are added in the adult mammalian brain [1]. The reservoirs of neural stem cells or neural progenitor cells (NPCs) normally exist within two regions: the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) and the subventricular zone (SVZ) of the lateral ventricles. Neurogenesis in the hippocampus plays an important role in improving learning and memory [2]. Unfortunately, adult neurogenesis continuously and severely declines with age and during neurodegenerative disease progress [3–5], especially in AD (Alzheimer's disease) patients [6]. Currently, no medical treatments can halt or reverse AD progression, which is characterized by prominent amyloid plaques, neurofibrillary tangles, and the progressive loss of neurons. Promoting adult neurogenesis in situ doesn't have to require invasive transplantation and suppression of host rejection [7], so it is a highly attractive strategy to reverse the loss of functional neurons that occurs in aging and AD progress, especially in view of the background of several failures in clinical trials targeted at hot spot β-amyloid.

Unlike traditional targets such as enzymes and receptors, stemistry, namely controlling the fate (proliferation and differentiation) of stem cells in situ by chemicals, lacks an efficient strategy to discover new leads [8]. Despite great efforts on the discovery of the promoters [9], only a short list of chemicals have survived in vivo neurogenesis screening, including donepezil [10], galantamine, memantine [11] curcumin [12], nodakenin [13], asarone [14], allantoin [15], tetrahydrohyperforin [16], 4'-dimethylaminoflavone [17], isoxazole [18,19], spinosin [20], isoquinoline-dione [21] and aminopropyl carbazole [22,23]. Due to the presence of the Blood-Brain Barrier (BBB), the promotion of neurogenesis by small molecules has not been established to occur at low doses of sub-milligram per kg. Most of the reported promotion of neurogenesis is heavily dependent on the neuroprotective mechanism. In

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other words, the promoters exert their neurogenesis effect by protecting newborn neurons from month-long apoptosis instead of directly stimulating proliferation of NPCs [24]. Therefore, the search for direct stimulators, instead of neuroprotective agents, with the capacity to promote efficient neuroproliferation-type neurogenesis is still an open challenge.

Recently, we found that an extract of Dragon's Blood (a bright red resin derived from *Dracaena cochinchinensis*) is capable of stimulating adult neurogenesis and has beneficial effects on improvement of learning ability (Unpublished data). Dragon's Blood contains a mixture of phenolic compounds of the stilbene and chalcone series such as resveratrol, pterostilbene, loureirin A, loureirin B and the like. It would be interesting to define the effective promoters in the extract. Thus far, no attempts have been made on stilbene and analogous scaffolds because of negative neurogenesis results from resveratrol. Resveratrol has been proved to reduce adult neurogenesis in a dose-dependent manner and it significantly impaired learning and memory in healthy adult mice [25]. Instead of searching for neuroprotective polyphenol radical scavengers that have limited potential because they are PAINS compounds (pan-assay interference compounds) [26], the present study looked for novel scaffolds that could efficiently promote adult neurogenesis.

Based on the scaffolds identified in Dragon's Blood, a pool of analogs was rationally designed and screened that consisted of stilbene, diphenyl acetylene, diphenylethane, chalcone, and other modified scaffolds that included heteroatomic linkers and heteroaryl rings. We iteratively designed compounds according to a set of rules for CNS-permeation [27,28] and determined the efficacy to illuminate SAR by employing a cell proliferation marker (BrdU) and a mature neuron marker (NeuN), which finally led to discovery of a novel series of efficient stimulators *N*-stilbenyl carboxamides that featured a crucial methylenedioxy group on the benzene ring not containing the carboxamide. None of the series of compounds presented in Fig. 1 has been disclosed (accessed by SCIfinder on Oct. 1, 2016). The advantages of the scaffold include achirality and low-cost synthetic accessibility. This report documents significant efficacy ($P < 0.001$) with potency at sub-milligram per kg.

2. Results

We designed a variety of stilbenes (Fig. 2, compounds **1**–**47**), diphenyl acetylenes (Fig. 3, compounds **48**–**58**), diphenyl ethanes (Fig. 4, compounds **59**–**60**), and a variety of hetero linkers and heteroaryl substitutions in the stilbene scaffold (Fig. 5, compounds **61**–**73**). For details see the Supporting Information (the known compounds are labeled with CAS codes). About 37 out of 73 compounds for in vitro tests are unknown, and 21 out of 32 compounds for in vivo assays are novel.

Long-term preventive treatment of AD definitely requires a strict demand for low cytotoxicities of the candidates. We examined the survival of cells exposed to three concentrations (100, 500 and 1000 μM) of the stilbene analogs. The lowest concentration (100 μM) is far higher than the level of the potent neuroprotective agent P7C3-A20 detected in the brain tissue (0.9 μM, after an oral dose of 20 mg/kg) [22]. A strategy of in vitro cytotoxicity evaluation prior to SAR-based in vivo screening was adopted to pinpoint a lead. Compounds with very low cytotoxicity were selected for further in vivo evaluation. However, some moderately to highly cytotoxic compounds were also selected in order to comprehensively understand the SAR. Next, 11-week old rats were treated intraperitoneally for 28 consecutive days with compounds followed by two BrdU injections on the next day. The newborn cells were immunohistochemically labeled by the BrdU marker.

2.1. In vitro cytotoxicity screening and structure-cytotoxicity relationships

The cytotoxicity data of all the compounds are listed in Table S1 (Supporting Information). Human neuroblastoma cells were used for evaluating the cytotoxicity. Due to low solubility, **62** was not tested. Generally, the introduction of a hydroxyl (**12**–**21**) or an amino group (**23**, **24** and **25**) on the stilbene resulted in higher cytotoxicity, particularly at the medium and the high dose. Methylation of the hydroxyl groups (**2** vs. **15**; **7** vs. **12**) and acetylation of the amino groups (**27** vs. **24**; **30** vs. **23**) minimized the unfavorable effects. In contrast, some hydrophobic groups such as methyl and trifluromethyl groups also have negative effects on survival of the cells (**6**, **16**, **20**, **50**, **51** and **54**). Substitution of the methyl groups with hydrophilic methoxy groups (**6** vs. **10**; **16** vs. **22**; **54** vs. **48**) and shrinkage of long alkyl groups to shorter ones (**34**, **28** vs. **27**; **56**, **55** vs. **48**; **52** vs. **53**) are beneficial for improving the survival ratio. Therefore, it is clear that low stilbene analog toxicity requires a balance of hydrophilicity and hydrophobicity.

Replacement of the substituted benzene rings with heteroaryl groups led to high toxicity (**71**, **72**, and **73**). The position of substitution on the phenyl ring of the stilbene is also an important factor. The para position is much more favorable than the meta position (**27** vs. **33**; **32** vs. **31**; **7** vs. **44**) or the ortho position (**7** vs. **9**). But this trend was not observed in the framework of diphenyl acetylene (**58** vs. **57**). The variation of the linkers between the two phenyl groups generally has a slight impact on the toxicity (**27** vs. **60**; **48** vs. **7**; **22** vs. **59**), but sometimes it has great influence (**27** vs. **58**; **57** vs. **33**). Compared to the stilbene analogs, the insertion of a carbonyl group into the stilbene, resulting in a chalcone, appeared to dramatically increase the toxicity (**43** vs. **66**; **27** vs. **67**; **22** vs. **65**). Generally, chalcones (**63**, **64**, **65**, **66**, **67** and **68**) are highly toxic with the exception of **69**. A direct ring closure of **69** to the dihydroflavone **70** resulted in low survival of the cells. The presence of an unsubstituted amide is necessary for the stilbene to maintain low toxicity in contrast to an acetyl group (**27** vs. **41**) or an N-methylacetamide (**27** vs. **40**).

Neuroblastoma cell viability data for stilbenes that stimulate neurogenesis are listed in Fig. 6. Most of the stilbenes produce dose-dependent cytotoxicity with the exception of **22** (3,5-*O*-dimethylresveratrol; known as pterostilbene) and **10** (4-*O*-acetylpterostilbene). In contrast, structure-cytotoxicity relationships show that the compounds sharing the common 3,4-

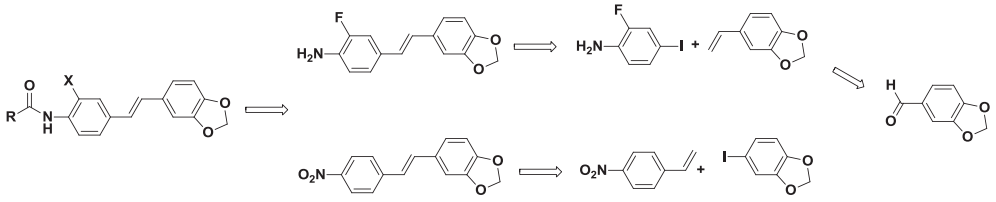

Fig. 1. Structures and retrosynthetic analysis of novel *N*-stilbenyl carboxamides.