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Letter

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Identification of Aminopyridazine-Derived Antineuroinflammatory Agents Effective in an Alzheimer's Mouse Model

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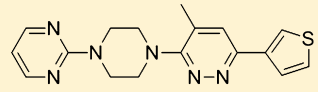
Supporting Information

ABSTRACT: Targeting neuroinflammation may be a new strategy to combat Alzheimer's disease. An aminopyridazine **1b** previously reported as a novel antineuroinflammatory agent was considered to have a potential therapeutic effect for Alzheimer's disease. In this study, we further explored the chemical space to identify more potent antineuroinflammatory agents and validate their in vivo efficacy in an animal model. Compound **14** was finally identified as an effective agent with comparable in vivo efficacy to the marketed drug donepezil in counteracting spatial learning and working memory impairment in an Aβ-induced Alzheimer's mouse model.

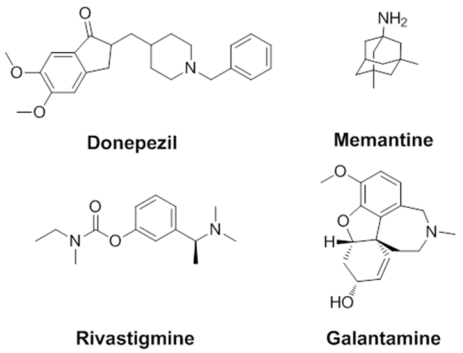


14

in vitro, BV-2 cell IC_{50} (IL-1β) = 2.4 μM; in vivo, comparable to donepezil

KEYWORDS: *Alzheimer's disease, neuroinflammation, antineuroinflammatory agent, animal model, Morris water maze*

Alzheimer's disease (AD)[1] is considered one of the most devastating, life-threatening diseases among the aged. It is also the most common form of neurodegenerative senile dementia, characterized by memory loss and progressive impairment of cognitive functions.[2] Until now, there have been very few approved AD drugs on the market (e.g., Figure 1), and these only provide minimal symptomatic relief rather than altering the disease progression. Thus, there is still a great need for disease-modifying therapies for AD.

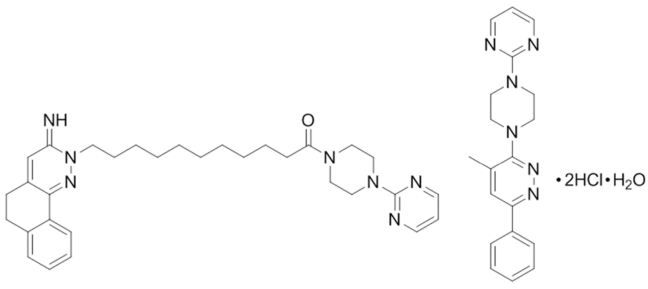


Donepezil | Memantine | Rivastigmine | Galantamine

Figure 1. Structures of marketed drugs to treat AD.

Accumulating evidence suggests that excessive microglial activation and up-regulation of proinflammatory cytokine production by activated microglia play a previously underappreciated role in the pathophysiology of AD.[3−7] Neuroinflammation stimulated by β-amyloid (Aβ) aggregation and intracellular neurofibrillary tangles leads to the overexpression of proinflammatory factors such as interleukin 1 β (IL-1β) and tumor necrosis factor α (TNF-α). These proinflammatory factors impose immunological insult to neurons and cause neuronal damage or death, which can also induce microglial activation, facilitating the propagation of a localized, detrimental cycle of neuroinflammation.[8−10] Given the significant role of neuroinflammation in variety of effects linked to AD mechanisms, neuroinflammatory inhibition using small-molecule inhibitors is an important and direct strategy for therapeutic intervention in AD and other neurodegenerative diseases.

Compound **1a** (Figure 2) was reported to be a novel antineuroinflammatory agent by the Watterson lab.[11] It can selectively block increased IL-1β, TNF-α, and nitric oxide (NO) production by activated glia without inhibition of



1a | **1b (Minozac)**

Figure 2. Structures of reported antineuroinflammatory agents.

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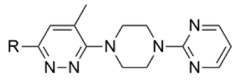
potentially beneficial glial functions. Although endowed with this favorable biological function, the intrinsic deficiency of druglikeness of compound **1a** eliminated it from possible clinical use because AD drugs must be orally available and able to cross the blood–brain barrier. Follow-up studies that mainly concentrated on the physical property-driven optimization led to compound **1b** (Minozac, under drug development, Figure 2), a water-soluble oral drug candidate.[12,13] However, the insufficient in vitro activity of compound **1b** limits its potential for anti-AD drug development, and its in vivo efficacy has not been well established using a standard animal model.

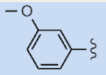
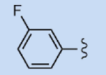
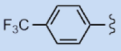
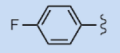
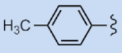
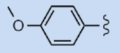
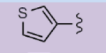
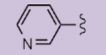
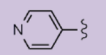
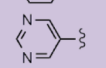
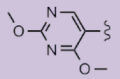
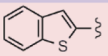
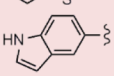
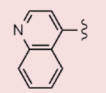
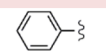
In this paper, we further investigate the unexplored chemical space of Minozac, attempt to identify inhibitors with higher in vitro activity, and validate in vivo efficacy using the Morris water maze AD animal model. Previous structural modifications focused on the improvement of molecular properties by diversifying the C-3 and C-4 positions of the aminopyridazine while leaving C-5 and C-6 unchanged.[4,5,11−14] The preliminary structure–activity relationship (SAR) study also showed that the in vitro function might be sensitive to substituents at C-6 position, prompting us to study this SAR further. This series of neuroinflammation suppressors have been reported to have a potential therapeutic effect in neurodegenerative disorders such as AD, but there is no concrete animal model evidence revealed in the literature to date. Several clinical candidates to treat AD had been discontinued because of the absence of data on behavioral effects in AD animal models,[15] suggesting that such studies are important criteria for the evaluation of AD drug candidates. Thus, we decided to employ the Morris water maze, a frequently used laboratory tool in behavioral neuroscience to investigate spatial learning and working memory[16−18] and assess the behavioral effects of our compounds in an AD mouse model.

To validate our hypothesis, a series of pyridazine compounds (Table 1) based on compound **1b** with changes at the C-6 position were designed and synthesized. The benzene ring was replaced with wide range of substituents. All synthesized compounds were screened for their inhibition of IL-1β production in lipopolysaccharide (LPS)-stimulated BV-2 mouse microglia cells. The synthetic approach to construct target compounds **5**–**21** is presented in Scheme 1. Briefly, the commercially available starting material 3,6-dichloro-4-methylpyridazine was treated with acetic acid[19] to afford 6-chloro-4-methylpyridazin-3(2*H*)-one (**2**) in 57% yield. Compound **2** was then transformed to the triflate intermediate **3**. Condensation of **3** with 1-(2-pyrimidyl) piperazine in dimethylformamide provided the key intermediate **4**. Hydrogen reduction with Pd/C converted compound **4** to the unsubstituted analogue **5**. Compounds **6** and **7** were finally obtained via iron-catalyzed cross-coupling reactions[20] with acceptable yields. Compounds **8**–**21** (Scheme 1 and Table 1) were prepared using Suzuki–Miyaura cross-coupling[21] reactions with intermediate **4**.

To retain druglikeness, all of the compounds designed here (Table 1) strictly obey Lipinski's rules of five. The IC_{50} of Minozac **1b** in our BV-2 cell-based assay was 11.4 μM. As compared with this positive control, the replacement of 6-phenyl with H (**5**), Cl (**4**), CH_3 (**6**), and even isopropyl (**7**) led to a loss in activity, unsurprisingly, confirming that in vitro activity is sensitive to the C-6 position and may require larger or aromatic functional groups. Thus, we decided to modify the 6-phenyl ring with different substituents, such as fluoro, methyl, and methoxy groups. The 3-methoxy analogue **8** exhibited superior activity over Minozac **1b**. The attachment of an aromatic heterocyclic ring (**15**–**21**) at the C-6 position led to a great loss in activity, whereas compound **14** also exhibited higher activity than Minozac (2.4 and 11.4 μM, respectively). Because our focus was to validate in vivo efficacy of these aminopyridazine-derived neuroinflammation suppressors against AD, further SAR studies were not pursued in the paper, and compound **14** was immediately selected to probe in vivo efficacy in the AD animal model.

Table 1. Structures and the IL-1β Synthesis Inhibition Activity of Compounds 4–21



Compd	Pyridazine 6-substituent	IC_{50}[a]
5	H–	>100
4	Cl–	>100
6	H_3C–	>100
7		>100
8		2.6±1.0
9		33.5±1.0
10		37.4±4.9
11		76.3±5.6
12		17.2±1.4
13		5.2±0.9
14		2.4±1.1
15		>100
16		>100
17		>100
18		>100
19		24.9±2.0
20		85.1±6.9
21		66.5±4.1
1b		11.4±1.2

[a]Concentration (μM) for 50% inhibition of IL-1β release in BV-2 cells. The IC_{50} values are the mean ± SEM for at least three experimental determinations.

The Morris water maze test was employed to evaluate the in vivo function of compound **14** in an AD mouse model by examining the effect of attenuating spatial learning and working memory impairment. The dosage was set at 2.5 mg/kg based on the previous study by Watterson, and the top marketed AD drug donepezil served as a reference at its regular dosage (0.65

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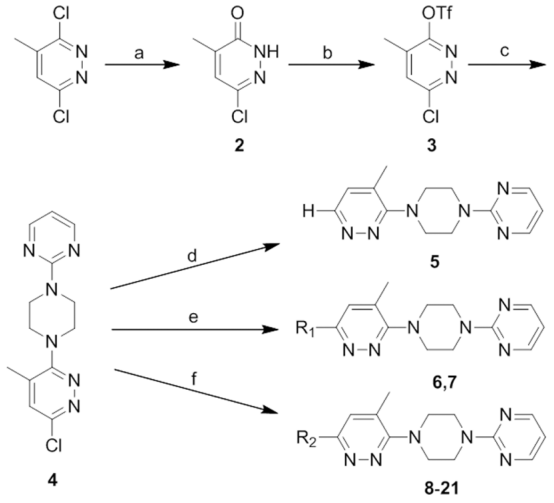
Scheme 1. Synthesis of Target Compounds 5−21[a]



[a]Reagents and conditions: (a) Acetic acid, reflux (57%). (b) Trifluoromethanesulfonic anhydride, triethylamine, DCM, −10 to 0 °C. (c) 1-(2-Pyrimidyl) piperazine, DMF, 0 °C (77% over two steps). (d) H_2, Pd/C, MeOH, rt, 24 h (64%). (e) R_1 = Me (**6**), MeMgCl, NMP, ferric acetylacetonate, Et_2O, THF, 0.5 h, rt (77%); R_1 = *i*-Pr (7), *i*-PrMgCl, NMP, ferric acetylacetonate, Et_2O, THF, 0.5 h, rt (84%). (f) $R_2B(OH)_2$, $Pd(PPh_3)_4$, K_2CO_3, DME (32−80%), R_2 as defined in Table 1 (**8**−**21**).

mg/kg). The Aβ model mouse group received interacerebroventricular (icv) injection with aggregated Aβ_{1-42} (equivalent to 410 pmol/mouse). As a control, the sham-operated group mice were injected icv with the vehicle (saline) only. The Aβ + compound **14** group and the Aβ + donepezil group received daily single dose per oral (po) administration of compound **14** at 2.5 mg/kg and donepezil at 0.65 mg/kg, respectively. The Aβ model group and sham-operated group received po administration of the same volume of 0.5% CMC−saline solution. All compounds were systemically administrated in a volume of 0.1 mL/10 g body weight from the day of icv Aβ until the end of behavioral testing. The Morris water maze was performed as previously described.[22] The reference memory test was conducted three times a day for 4 consecutive days. After the 12th training trial of the reference memory test on day 13 following the Aβ_{1-42} injection, probe tests were carried out, and the swimming percentage of path length and the time spent in the target quadrant where the platform was located during training were recorded.

Changes of escape latency and swimming distance are shown in Figures 3 and 4. Starting from day 3 of the reference memory test, Aβ_{1-42} model mice showed significantly prolonged swimming distance ($p < 0.05$) and escape latency to the safe platform ($p < 0.001$) as compared with the sham-operated group. Treatment with compound **14** significantly reversed the impairments in spatial recognition memory for Aβ_{1-42} model mice at day 3 ($F_{3,83} = 8.121$, $P = 0.001$) and day 4 ($F_{3,83} = 16.989$, $P = 0.0001$). The donepezil group exhibited a pattern similar to the compound **14** group in terms of swimming time and escape latency.

One day after the last training trial, we performed a probe test to measure the maintenance of memory function. The results of the probe test showed significant memory impairment in the Aβ_{1-42} model mice. As compared with the sham-operated group, Aβ_{1-42} model mice decreased in both the

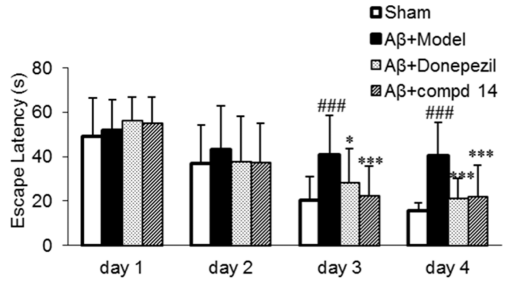


Figure 3. Effect of compound **14** on escape latency in the Morris water maze test for mice with icv Aβ_{1-42} (n = 7−8, mean ± SD). $^{###}P < 0.001$ vs sham group; $^{*}P < 0.05$ or $^{***}P < 0.001$ vs model group. Day 1 ($F_{3,83} = 1.278$, $P = 0.288$), day 2 ($F_{3,83} = 0.466$, $P = 0.707$), day 3 ($F_{3,83} = 8.121$, $P = 0.001$), and day 4 ($F_{3,83} = 16.989$, $P = 0.0001$).

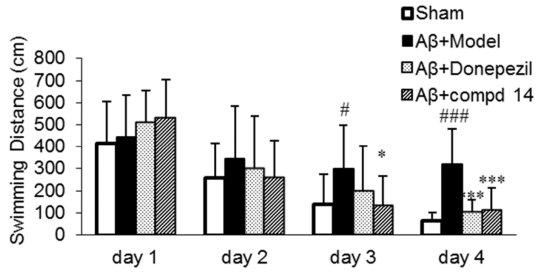


Figure 4. Effect of compound **14** on swimming distances in the Morris water maze test for mice with icv Aβ_{1-42} (n = 7−8, mean ± SD). $^{#}P < 0.05$, $^{###}P < 0.001$ vs sham group; $^{*}P < 0.05$ or $^{***}P < 0.001$ vs model group. Day 1 ($F_{3,83} = 2.080$, $P = 0.109$), day 2 ($F_{3,83} = 0.848$, $P = 0.472$), day 3 ($F_{3,83} = 4.216$, $P = 0.008$), and day 4 ($F_{3,83} = 24.142$, $P = 0.0001$).

swimming percentage of path length ($p < 0.05$; Figure 5) and the swimming time ($p < 0.05$; Figure 6) spent in the target

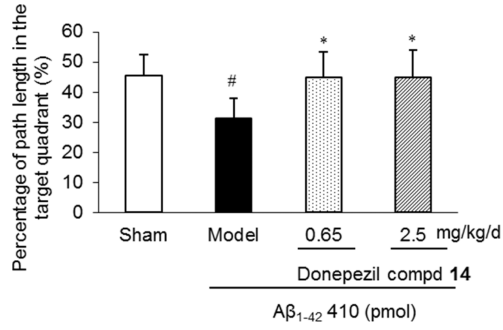


Figure 5. Effect of compound **14** on swimming percentage of path length spent in the target quadrant in the probe test for mice with icv Aβ_{1-42} (n = 7−8, mean ± SD). $^{#}P < 0.05$ vs sham group; $^{*}P < 0.05$ vs model group.

quadrant. Compound **14** treatment significantly increased the swimming percentage of path length ($F_{3,26} = 4.574$, $P = 0.025$) and the swimming time ($F_{3,26} = 4.576$, $P = 0.012$) spent in the target quadrant. This effect was also reversed by donepezil treatment, suggesting the improvement of spatial memory.

In the experiment, a single icv injection of a picomolar dose of Aβ_{1-42} effectively impaired learning and memory behavior in mice. Compound **14** prevented Aβ_{1-42}-induced spatial recognition memory impairment in the Morris water maze test. This study demonstrates that the antineuroinflammatory

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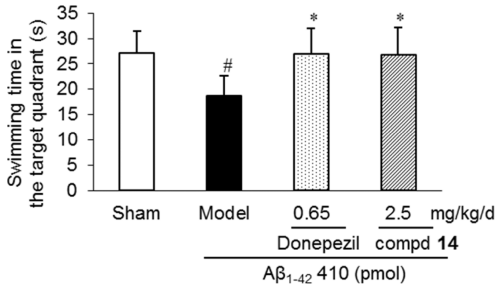

Figure 6. Effect of compound **14** on the swimming time spent in the target quadrant in the probe test for mice with icv $A\beta_{1-42}$ (n = 7−8, mean ± SD). $^{\#}P < 0.05$ vs sham group; $^{*}P < 0.05$ vs model group.

agent **14** has an effect on the memory impairment induced by icv injection of $A\beta_{1-42}$ in mice comparable to donepezil.

As a potent antineuroinflammatory agent targeted in central nervous system (CNS), compound **14** displayed rapid brain uptake (T_{max} = 0.33 h, $C_{max,brain}/C_{max,plasma}$ = 2.86%, and AUC_{brain}/AUC_{plasma} = 0.56%) after an orally administrated with therapeutic dose of **14** (2.5 mg/kg). This compound is oral bioavailable but not good enough to be considered as a drug candidate (17.4% of oral bioavailability in rats). So, a further pharmacokinetic driven optimization while keeping its desired in vitro activity may provide a compound with a better in vivo efficacy.

In summary, a series of aminopyridazine analogues were designed, synthesized, and screened for their inhibition against IL-1β synthesis in LPS-activated BV-2 mouse microglia cells. Some compounds exhibited enhanced inhibition (compounds **8**, **13**, and **14**), and the best compound, **14**, was selected for further in vivo evaluation. To our knowledge, this is the first demonstration of aminopyridazine-derived antineuroinflammatory agents, exemplified by compound **14**, that counteract spatial learning and working memory impairment in the Aβ-induced AD mouse model. In general, this study not only proves that countering neuroinflammation is indeed a potential therapeutic strategy for AD but also provides a good lead compound with efficacy comparable to donepezil for further oral anti-AD drug discovery and development.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures for the synthesis, characterization, and in vitro assay protocols of compounds **1b** and **4**−**21** as well as the in vivo study and the brain uptake assay of compound **14**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AD, Alzheimer's disease; Aβ, β-amyloid; TNF-α, tumor necrosis factor α; IL-1β, interleukin 1 β; SAR, structure−activity relationship; LPS, lipopolysaccharide; NO, nitric oxide; icv, interacerebroventricular; po, per oral; CNS, central nervous system

■ REFERENCES

(1) Alzheimer, A. Über eigenartige Krankheitsfälle des späteren Alters. *Z. Ges. Neurol. Psychiatr.* **1911**, *4*, 356−385.
(2) Walsh, D. M.; Selkoe, D. J. Deciphering the molecular basis of memory failure in Alzheimer's disease. *Neuron* **2004**, *44*, 181−193.
(3) Griffin, W. S.; Sheng, J. G.; Royston, M. C.; Gentleman, S. M.; McKenzie, J. E.; Graham, D. I.; Roberts, G. W.; Mrak, R. E. Glial-neuronal interactions in Alzheimer's disease: the potential role of a 'cytokine cycle' in disease progression. *Brain Pathol.* **1998**, *8*, 65−72.
(4) Munoz, L.; Ranaivo, H. R.; Roy, S. M.; Hu, W.; Craft, J. M.; McNamara, L. K.; Chico, L. W.; Van Eldik, L. J.; Watterson, D. M. Novel p38 alpha MAPK inhibitor suppresses brain proinflammatory cytokine up-regulation and attenuates synaptic dysfunction and behavioral deficits in an Alzheimer's disease mouse model. *J. Neuroinflammation* **2007**, *4*.
(5) Ranaivo, H. R.; Craft, J. M.; Hu, W. H.; Guo, L.; Wing, L. K.; Van Eldik, L. J.; Watterson, D. M. Glia as a therapeutic target: Selective suppression of human amyloid-beta-induced upregulation of brain proinflammatory cytokine production attenuates neurodegeneration. *J. Neurosci.* **2006**, *26*, 662−670.
(6) Craft, J. M.; Van Eldik, L. J.; Zasadzki, M.; Hu, W. H.; Watterson, D. M. Aminopyridazines attenuate hippocampus-dependent Behavioral deficits induced by human beta-amyloid in a murine model of neuroinflammation. *J. Mol. Neurosci.* **2004**, *24*, 115−122.
(7) Craft, J. M.; Watterson, D. M.; Frautschy, S. A.; Van Eldik, L. J. Aminopyridazines inhibit beta-amyloid-induced glial activation and neuronal damage in vivo. *Neurobiol. Aging* **2004**, *25*, 1283−1292.
(8) Gonzalez-Scarano, F.; Baltuch, G. Microglia as mediators of inflammatory and degenerative diseases. *Annu. Rev. Neurosci.* **1999**, *22*, 219−240.
(9) Akiyama, H.; Barger, S.; Barnum, S.; Bradt, B.; Bauer, J.; Cole, G. M.; Cooper, N. R.; Eikelenboom, P.; Emmerling, M.; Fiebich, B. L.; Finch, C. E.; Frautschy, S.; Griffin, W. S. T.; Hampel, H.; Hull, M.; Landreth, G.; Lue, L. F.; Mrak, R.; Mackenzie, I. R.; McGeer, P. L.; O'Banion, M. K.; Pachter, J.; Pasinetti, G.; Plata-Salaman, C.; Rogers, J.; Rydel, R.; Shen, Y.; Streit, W.; Strohmeyer, R.; Tooyoma, I.; Van Muiswinkel, F. L.; Veerhuis, R.; Walker, D.; Webster, S.; Wegrzyniak, B.; Wenk, G.; Wyss-Coray, T. Inflammation and Alzheimer's disease. *Neurobiol. Aging* **2000**, *21*, 383−421.
(10) Wyss-Coray, T. Inflammation in Alzheimer disease: Driving force, bystander or beneficial response? *Nat. Med.* **2006**, *12*, 1005−1015.
(11) Mirzoeva, S.; Sawkar, A.; Zasadzki, M.; Guo, L.; Velentza, A. V.; Dunlap, V.; Bourguignon, J. J.; Ramstrom, H.; Haiech, J.; Van Eldik, L. J.; Watterson, D. M. Discovery of a 3-amino-6-phenyl-pyridazine derivative as a new synthetic antineuroinflammatory compound. *J. Med. Chem.* **2002**, *45*, 563−566.
(12) Hu, W.; Ranaivo, H. R.; Craft, J. M.; Van Eldik, L. J.; Watterson, D. M. Validation of the neuroinflammation cycle as a drug discovery target using integrative chemical biology and lead compound development with an Alzheimer's disease-related mouse model. *Curr. Alzheimer Res.* **2005**, *2*, 197−205.
(13) Hu, W. H.; Ranaivo, H. R.; Roy, S. M.; Behanna, H. A.; Wing, L. K.; Munoz, L.; Guo, L.; Van Eldik, L. J.; Watterson, D. M. Development of a novel therapeutic suppressor of brain proinflammatory cytokine up-regulation that attenuates synaptic dysfunc-

tion and behavioral deficits. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 414−418.
(14) Craft, J. M.; Watterson, D. M.; Frautschy, S. A.; Van Eldik, L. J. Aminopyridazines inhibit beta-amyloid-induced glial activation and neuronal damage in vivo. *Neurobiol. Aging* **2004**, *25*, 1283−92.
(15) Imbimbo, B. P.; Giardina, G. A. Gamma-secretase inhibitors and modulators for the treatment of Alzheimer's disease: disappointments and hopes. *Curr. Top. Med. Chem.* **2011**, *11*, 1555−1570.
(16) Yamada, K.; Nabeshima, T. Animal models of Alzheimer's disease and evaluation of anti-dementia drugs. *Pharmacol. Ther.* **2000**, *88*, 93−113.
(17) D'Hooge, R.; De Deyn, P. P. Applications of the Morris water maze in the study of learning and memory. *Brain Res. Rev.* **2001**, *36*, 60−90.
(18) Chen, G. Q.; Chen, K. S.; Knox, J.; Inglis, J.; Bernard, A.; Martin, S. J.; Justice, A.; McConlogue, L.; Games, D.; Freedman, S. B.; Morris, R. G. M. A learning deficit related to age and beta-amyloid plaques in a mouse model of Alzheimer's disease. *Nature* **2000**, *408*, 975−979.
(19) Johnston, K. A.; Allcock, R. W.; Jiang, Z.; Collier, I. D.; Blakli, H.; Rosair, G. M.; Bailey, P. D.; Morgan, K. M.; Kohno, Y.; Adams, D. R. Concise routes to pyrazolo[1,5-a]pyridin-3-yl pyridazin-3-ones. *Org. Biomol. Chem.* **2008**, *6*, 175−186.
(20) Furstner, A.; Leitner, A.; Mendez, M.; Krause, H. Iron-catalyzed cross-coupling reactions. *J. Am. Chem. Soc.* **2002**, *124*, 13856−13863.
(21) Kotha, S.; Lahiri, K.; Kashinath, D. Recent applications of the Suzuki-Miyaura cross-coupling reaction in organic synthesis. *Tetrahedron* **2002**, *58*, 9633−9695.
(22) Morris, R. Developments of a Water-Maze Procedure for Studying Spatial-Learning in the Rat. *J. Neurosci. Methods* **1984**, *11*, 47−60.