Discovery of a 3-Amino-6-phenyl-pyridazine Derivative as a New Synthetic Antineuroinflammatory Compound

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Abstract: Excessive glial activation, with overproduction of cytokines and oxidative stress products, is detrimental and a hallmark of neurodegenerative disease pathology. Suppression of glial activation is a potential therapeutic approach, and protein kinases are targets of some antiinflammatory drugs. To address an unmet need for selective inhibitors of glial activation, we developed a novel 3-amino-6-phenylpyridazine derivative that selectively blocks increased IL-1β, iNOS, and NO production by activated glia, without inhibition of potentially beneficial glial functions.

Introduction. Neuroinflammation, a characteristic feature of disease pathology and progression in several neurodegenerative disorders that include Alzheimer's disease (AD) and stroke, is brought about by glial (astrocytes and microglia) activation.[1] Normally serving a beneficial role as part of an organism's homeostatic response to injury or developmental change, chronic or excessive activation of glia can contribute to disease through the increased production of proinflammatory cytokines and chemokines, oxidative stress-related enzymes, acute phase proteins, and various components of complement cascades.[1] For example, the deposition of β-amyloid (Aβ) and neurofibrillary tangles in AD is associated with glial activation, neuronal loss, and cognitive decline. There is increased expression of nitric oxide synthase (NOS) in glial cells surrounding amyloid plaques, peroxynitrite-mediated neuronal damage, and nitric oxide (NO) overproduction.[2] The isoform of NOS (iNOS or NOSII) induced as part of the glial activation response is an oxidative stress-related enzyme that generates NO, which can combine with superoxide to generate peroxynitrite. The proinflammatory cytokine interleukin-1β (IL-1β), which is also overexpressed in activated glia in AD brain, is involved in glial inflam-

matory and neuronal dysfunction responses, and IL-1 gene polymorphisms are associated with an increased risk of AD.[3]

The direct linkage of glial activation to disease pathology underscores the importance of understanding the signal transduction pathways that mediate these critical glial cellular responses and of the need for discovery of cell-permeable ligands that can modulate disease-relevant pathways. The potential for modulation of glial inflammation as a therapeutic approach is indicated by delayed onset or slower progression of neurodegeneration with use of antiinflammatory drugs.[1] Experimental antiinflammatory drugs inhibit iNOS production in activated glia, with some of the more effective drugs inhibiting phosphorylation of transcription factors that may regulate iNOS.[4] This may occur through modulation of relevant kinases, such as calmodulin dependent kinases (CaMKs), which have certain isoforms present in glia.[5] Some of the more promising experimental antiinflammatory compounds are natural product analogues that are not readily amenable to high throughput chemical diversification and that inhibit multiple protein kinases key to regulation of IL1-β and iNOS induction. Therefore, there is a need for new chemical classes of compounds that are cell-permeable suppressors of glial activation and are more selective for the protein kinases involved in regulation of IL-1β and iNOS expression.

We describe here a novel alkylated 3-amino-6-phenylpyridazine derivative (compound **1**) that selectively blocks the production of IL-1β, iNOS, and NO by activated glia. Remarkably, the inhibitor exerts its desired effects without diminishing the production of endogenous antiinflammatory glial proteins, such as apolipoprotein E (apoE), or functionally related response pathways, such as cyclooxygenase (COX)-2 induction. The mechanism of action of the inhibitor is clearly distinct from that of currently available inhibitors, which target p38 MAP kinase (MAPK), an enzyme that is important in peripheral inflammation. Although the mechanism of action of compound **1** remains to be established, the results suggest CaMK-dependent pathways as potential targets.

Chemistry and Assays. Compounds were made by standard procedures from commercially available starting materials and precursors described in the literature[6] or were obtained commercially. The reaction scheme for the parallel synthesis of selected derivatives (compounds **1**–**4**: Chart 1) that yielded the title compound **1** reported here is shown in Scheme 1. Additional details of syntheses are given in Supporting Information (Schemes 2–4). Briefly, the 3-amino-6-phenylpyridazine was prepared from the corresponding chloropyridazine derivative, which was converted to the hydrazine derivative by refluxing with aqueous hydrazine. The hydrazine derivative was reduced to the 3-amino-6-phenylpyridazine by reduction with hydrogen over a nickel–aluminum alloy catalyst. The intermediate 3-amino-6-phenylpyridazines alkylated at ring nitrogen 2 and the deprotection of the carboxylic acid were prepared by standard procedures, with typical condi-

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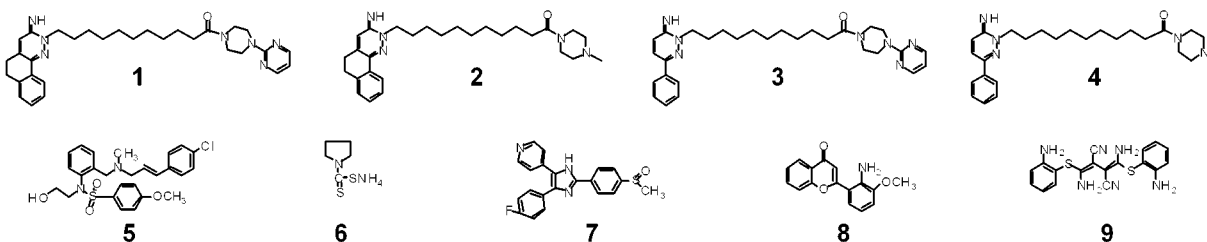† Department of Molecular Pharmacology & Biological Chemistry.
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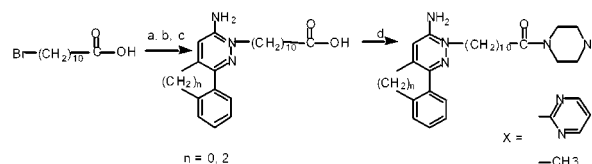⊥ Université Louis Pasteur.

Chart 1

Scheme 1[a]

[a] Reagents and conditions: (a) EtOH, 1 equiv of 4 N HCl, 48 h, 95%; (b) 0.75 equiv of 3-aminopyridazine compound, DMF, 80 °C, 94%; (c) 20% concentrated HCl in AcOH, 90 °C, 10 h, 90%; (d) 1 equiv of 1-(2-pyrimidyl)piperazine or 1-methylpiperazine, HOBt/EDC, DMF, 0–22 °C, 21 h, 60–98%.

tions given in the footnote to Scheme 1. The commercially available inhibitors, compounds **5**−**9** (Chart 1), were chosen on the basis of their selectivity for inhibiting various signal transduction pathways that converge on the regulation of IL-1β and iNOS expression.

Glial cells were maintained and treated as described previously[5] with one of two activating stimuli: bacterial endotoxin (lipopolysaccharide (LPS)) or Aβ1−42 peptide. Compounds were tested for their concentration-dependent effects on NO accumulation in conditioned media,[4] and proteins were detected by Western blots.[5] Reconstituted enzyme assays were done as described.[7] Elemental analysis of the title compound **1** gave the following: C, 54.87; H, 5.84; N, 12.43. Expected for $C_{31}H_{41}N_7O\cdot 2TFA$ was the following: C, 55.62; H, 5.73; N, 12.97. The mass was 528.6 (expected, 528.3). NMR analysis of compound **1** was consistent with the structure. ^{1}H NMR (CD$_3$OD): δ 1.4 (s, 16H), 1.98 (t, 2H), 2.41−2.44 (t, 2H), 3.03−3.06 (d d, 4H), 3.67−3.74 (t, 4H), 3.82−3.92 (d d 4H), 6.82 (t, 1H), 7.34 (t, 1H), 7.35−7.60 (m, 3H), 8.2 (d, 1H), 8.47−8.49 (d, 2H). Additional details of assays and characterization, including mass spectrometry and HPLC analysis of compounds **1**−**4**, are given in Supporting Information.

Results and Discussion. Alkylated 3-amino-6-phenylpyridazine derivatives have been described previously[6] as part of discovery efforts for central nervous system (CNS) drugs. Previously, we found[4,5] that several experimental antiinflammatory drugs and aminopyridazines that suppressed glial activation were also kinase inhibitors, consistent with the emerging theme of gene-regulating protein kinases being potential drug discovery targets in inflammation.[8] Therefore, we used a parallel synthesis and cell-based activity screen to search for selective inhibitors of glial activation within this class of compounds. The approach is outlined in Scheme 1 for the compounds reported here. The quantitative ability of the compounds to inhibit NO accumulation, a disease-linked surrogate end point, by activated glia is shown in Figure 1. The cell-based activity screen showed that compound **1** was the most active compound. The amine, 1-(2-pyrimidyl)piperazine, used in the production of compound **1** was one of several found in a structure-based search of the available chemicals database for aromatic heterocyclic compounds, with prioritization of choices for inclusion in parallel syntheses based on use in the synthesis of other drug-like compounds. The 1-(2-pyrimidyl) piperazine is present in various CNS active compounds and is thought to be an important contributor to the pharmacology of the clinically effective drugs of which it is a component.[9] Although the 1-(2-pyrimidyl)piperazine was chosen for its prior use in CNS-active drugs, the comparative

results obtained with compounds **2** and **4** show that the characteristic aromatic ring associated with its pharmacological activity with other CNS targets is not required for inhibition of glial activation in the cell-based assay. The presence of an aromatic ring, however, resulted in the best cellular activity. Synthetic precursors shown in Scheme 1 were inactive. Overall, the results indicate that the contribution of the pyridazine and piperazine to activity is context-dependent.

The cell-based assay results for compounds **5**−**9** (Figure 1) allow a tentative ranking of the quantitative importance of various gene-regulating protein kinase pathways that converge on a common biological end point. For example, compounds **5** (KN93), **6** (PDTC), **7** (SB203580), **8** (PD98059), and **9** (U0126) are inhibitors of CaMKII, NFκB, p38 MAPK, and MEK1/2 signaling pathways, respectively,[10] and each of these distinct intracellular signal transduction pathways converges at the level of transcription regulation in glial activation. However, the NFκB, p38 MAPK, and MEK1/2 inhibitors are less effective (Figure 1) than compound **1** or **5** (KN93), a widely used CaMKII-selective inhibitor. Overall, the results are consistent with all of these pathways contributing to NO production. However, the relative importance of p38 MAPK mediated pathways to the end point in glia might be less than previously thought from studies of peripheral tissues.

To confirm that compound **1** does not target p38 MAPK and to examine its potential selectivity for CaMKII, we examined in reconstituted enzyme assays the ability of compound **1** to inhibit several relevant protein kinases key to the intracellular pathways that converge on iNOS and IL-1β gene expression. As shown in Table 1, compound **1** does not inhibit p38 MAPK activity up to the highest concentration of compound tested (100 μM). In contrast, compound **1** inhibits CaMKII activity at concentrations similar to that of compound **5**, the current standard in the field. Compound **1** also does not inhibit the closely related CaM regulated protein kinase, myosin light chain kinase (MLCK), or other second messenger signal transducing kinases, such as protein kinase A (PKA) and protein
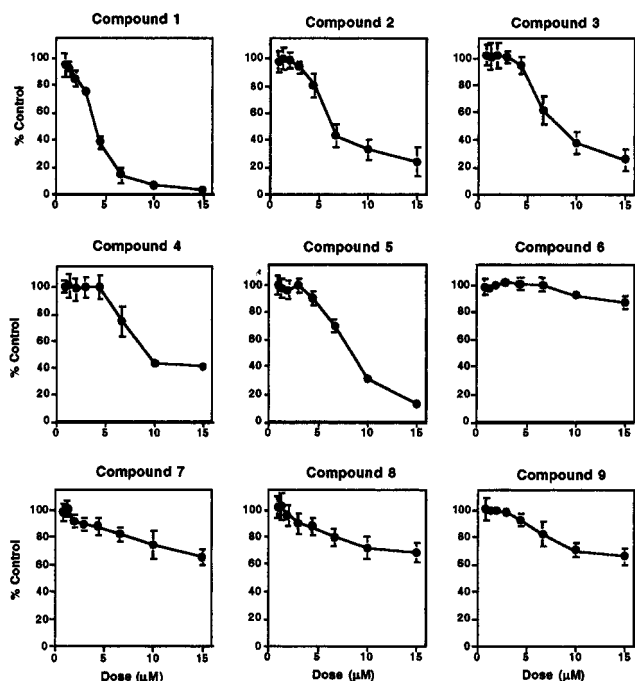
Figure 1. Cell-based screen for inhibition of LPS-stimulated NO release. BV-2 microglial cells were incubated with LPS in the presence of increasing concentrations of compounds **1**−**9**, and accumulation of NO in conditioned media was determined. Inhibition is expressed as percent control, where the control is the NO accumulation from cells stimulated with LPS alone.

**Table 1.** Selective Inhibition of Protein Kinase Activity[a]

compd	IC_{50} (μM)				
	CaMKII	p38 MAPK	MLCK	PKC	PKA
1	9.7 ± 1.9	>100	>100	>100	>100
5	8.4 ± 1.0	>100	9.8 ± 0.7	>100	>100

[a] Enzyme assays were done using γ-[^{32}P]-ATP and synthetic peptide substrates. IC_{50} values were calculated from the linear part of inhibition curves and represent the mean $\pm$ SEM obtained from at least two different experiments.

kinase C (PKC). Clearly, derivatives of compound **1** have the potential to provide more selective small-molecule inhibitors of CaMKs as well as new antineuroinflammatory compounds.

The comparatively robust effect of compound **1** prompted an examination of its selectivity on various glial activation responses. Figure 2A shows that compound **1** inhibits both IL-1β and iNOS production. The levels of the astrocyte marker protein, glial fibrillary acidic protein (GFAP), are not changed. The GFAP results provide a biological response control to demonstrate that compound **1** is not a general protein synthesis inhibitor and not cytotoxic under the assay conditions. Compound **1** also inhibits (Figure 2B) iNOS and IL-1β production in glia stimulated by Aβ1−42, a peptide that is involved as an initiating event in models of AD pathogenesis. In terms of selectivity for activation responses, there was no effect of compound **1** on the levels of COX-2, an inflammatory response enzyme that is also increased in activated glia, and no effect on the production of apoE, an endogenous antiinflammatory protein (Figure 2B).

The concentration-dependent effect (Figure 2A) of compound **1** on iNOS and IL-1β levels, its ability to
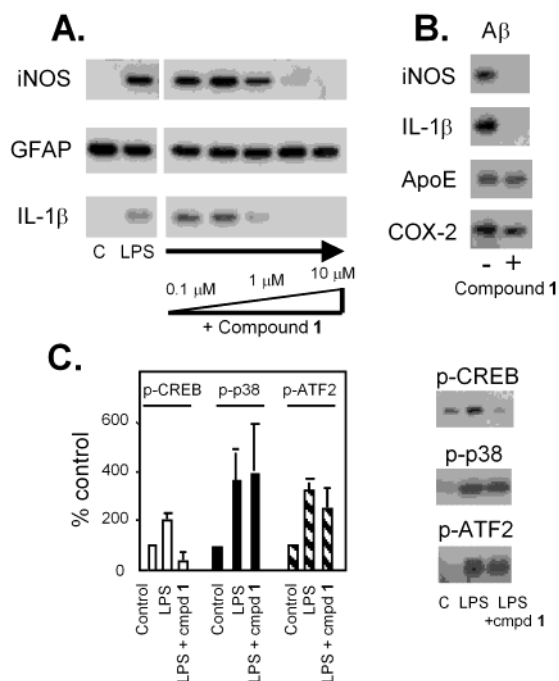
Figure 2. Selective effects of compound **1** on glial activation responses and gene-regulating protein kinase pathways. BV-2 cells and rat astrocytes were treated with control buffer (C) or stimulated with LPS or Aβ1−42 peptide in the presence or absence of compound **1**, and cell lysates were analyzed by Western blots. (A) Astrocytes treated with LPS and compound **1** show concentration-dependent inhibition of iNOS and IL-1β production but not GFAP. (B) Astrocytes treated with Aβ1−42 and compound **1** (10 μM) show inhibition of iNOS and IL-1β but not apoE and COX-2. (C) BV-2 cells treated with LPS and compound **1** (10 μM) show inhibition of phosphorylated (p-) CREB but not p-p38 and p-ATF2, as assayed with antibodies that are specific for the phosphorylated forms of the proteins. Data are expressed as percent control (mean $\pm$ SD; $n = 3$ determinations). The right panel shows a representative blot.

suppress the production of selected disease-linked end points in response to multiple activating stimuli (Figure 2B), and its robust activity with a quantifiable end point (Figure 1) raise the possibility that compound **1** works at a late step in intracellular signal transduction pathways, where multiple, interacting pathways are known to converge on common biological end points such as iNOS and IL-1β gene regulation. In addition, previous results[4] have shown that antiinflammatory drugs that block NO production can inhibit the phosphorylation of transcription factors such as CREB, a substrate of CaMKII isoforms that are capable of nuclear localization. As shown in Figure 2C, compound **1** inhibits the increased phosphorylation of CREB, but does not inhibit the increase in phosphorylated ATF2 (an endogenous p38 MAPK substrate) or the upstream increase in phosphorylated p38 MAPK itself (Figure 2C). These results demonstrate that the p38 MAPK pathway is activated in these cells, as expected, but there is no detectable down modulation of the pathway by compound **1**. The inhibition of CREB phosphorylation is consistent with the inhibition of one of its upstream kinases, such as CaMKII. Regardless of the details of mechanism, the effects of compound **1** fit the redundant paradigm of inhibition of transcription factor phosphorylation as a mechanism for antiinflammatory action.[8]

In the case of joint inflammation, the p38 MAPK pathway is quantitatively important and a viable target for drug discovery, whereas our results raise the possibility that other kinase-mediated pathways might be more viable targets for the CNS.

Compound **1** has key features that are attractive in early-generation compounds. These include its selective targeting of steps in the glial activation loop that are disease-linked without perturbation of related pathways. In addition, compound **1** does not target the same regulatory pathways that can be critical in peripheral inflammatory responses. This raises the possibility that future refinements of compound **1** structure and activity might allow avoidance of undesired side effects such as suppression of peripheral inflammatory responses. Finally, pyridazines are attractive starting points for future refinement because they have the potential for generating chemically diverse compounds as part of in-parallel syntheses, and slight modifications result in a range of pharmacological activities.

Conclusion. Compound **1** is a new glial activation inhibitor that has desirable functional properties, targets different pathways compared to currently available experimental drugs, and is amenable to rapid chemical diversification with facile chemistries, making it an attractive starting point for future refinement.

Supporting Information Available: Details of experimental procedures for the synthesis of compounds, activity assays, and analytical characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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