Brief Articles

Structure−Activity Relationship Studies on *N*′-Aryl Carbohydrazide P2X$_7$ Antagonists

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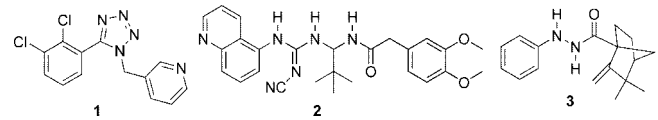
N′-Aryl acyl hydrazides were identified as P2X$_7$ receptor antagonists. Structure−activity relationship (SAR) studies evaluated functional activity by monitoring calcium flux inhibition in cell lines expressing recombinant human and rat P2X$_7$ receptors. Selected analogs were assayed in vitro for their capacity to inhibit release of cytokine IL-1β. Compounds with potent antagonist function were evaluated in vivo using the zymosan-induced peritonitis model. A representative compound effectively attenuated mechanical allodynia in a rat model of neuropathic pain.

Introduction

Recently, the physiology and pharmacology of the P2X family of ligand-gated ion channels have been studied extensively.[1–7] The ATP-sensitive, homomeric subtype P2X$_7$ receptor has received considerable attention because it is localized on cells of hematopoietic origin, and recent data indicate a role in the onset and persistence of certain pain states.[8] Specifically, the P2X$_7$ receptor is expressed on macrophages and epidermal Langerhans cells.[9] In addition, P2X$_7$ receptors are expressed on microglia[10] and astrocytes[11] in the central nervous, but expression on neurons remains uncertain.[12] Activation of P2X$_7$ by extracellular ATP leads to ion flux,[13] caspase-1 activation,[14] release of the cytokine IL-1β,[15,16] p38 MAP kinase activation,[17] and, ultimately, reversible cell membrane pore formation that may lead to lysis and cell death.[4] P2X$_7$ receptor activation has also been linked to glutamate release and inhibition of glutamate uptake.[18] The impact of P2X$_7$ receptor activation on cytokines and glutamate regulation contributes to the mechanistic rationale for its role in the development and progression of several disease states or conditions including inflammation,[19] neurodegeneration,[20] and neuropathic pain.[8,21] The phenotype of P2X$_7$-knockout mice, which showed reduction in symptom severity in an arthritis model and resistance to the development of inflammatory and neuropathic pain, is consistent with a significant role of P2X$_7$ in nociceptive signaling.[22]

Several different classes of P2X$_7$ receptor antagonists have been identified,[8] but many of the early antagonist structures lacked properties suitable for therapeutic use. Efforts from our laboratories have focused on the development of small-molecule P2X$_7$ receptor antagonists for the treatment of pain. Two structurally distinct classes of potent and selective P2X$_7$ receptor antagonists have been disclosed previously. The disubstituted tetrazole **1**[23] and cyanoguanidine-containing structure **2**[24] represent selective, reversible P2X$_7$ antagonists that competitively block the ATP binding site. Compound **1** inhibited Bz-ATP-stimulated IL-1β release in human THP-1 cells and effectively reversed mechanical allodynia in the Chung model of neuropathic pain.



1 **2** **3**

Our program has identified other distinct structures that function as P2X$_7$ receptor antagonists. A high-throughput screen (HTS) of the Abbott corporate compound library revealed the terpene-derived acyl hydrazide **3** as a novel P2X$_7$ antagonist, as measured by the inhibition of calcium flux (hP2X$_7$ pIC$_{50}$ = 7.3, rP2X$_7$ pIC$_{50}$ = 7.3). Herein, we describe the SAR studies around analogs of **3** that identified key substitutions influencing P2X$_7$ functional potency for this pharmacophore. An example from the new series of P2X$_7$ receptor antagonists exhibited efficacy in a behavioral model of neuropathic pain as well as a model of induced peritonitis.

Chemistry

The acyl hydrazide core of structure **3** differs from other reported pharmacophores that serve as P2X$_7$ receptor antagonists and presents new opportunities to probe the structural requirements for receptor activation.[25] The SAR studies involved manipulation of three different moieties within the acyl hydrazide pharmacophore: the aryl system bound to the terminal hydrazide nitrogen, the groups substituted on the nitrogen atoms, and the lipophilic acyl substituent. Synthesis of the acyl hydrazide compounds was accomplished by three different routes as shown in Scheme 1. Treatment of an aryl hydrazine or the corresponding hydrochloride salt with an acyl chloride in the presence of triethylamine generated the acyl hydrazides (route 1). Alternately, the aryl hydrazines coupled with carboxylic acids using tetramethylbenzotriazole uronium tetrafluoroborate (TBTU) in the presence of triethyl amine (route 2).[26] Selected libraries of acyl hydrazides were prepared by the high throughput organic synthesis (HTOS) group using microwave-

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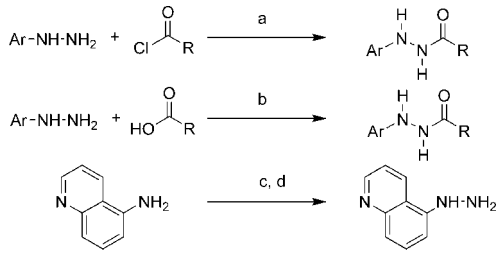
Scheme 1[a]



[a] Reagents and conditions: (a) NEt_3, THF, rt; (b) TBTU, NEt_3, MeCN, rt or carbodiimide resin, HOBt, microwave; (c) $NaNO_2$, aq. HCl; (d) $SnCl_2/2H_2O$.

assisted coupling reactions between aryl hydrazine salts and carboxylic acids using resin-bound carbodiimide reagents (route 3).[27]

Several aryl hydrazines required for the present study were commercially available, but brief syntheses of selected hydrazines were necessary. The quinoline and isoquinoline hydrazines were prepared by literature methods involving generation of a diazonium salt from the corresponding aminoquinoline derivative followed by reduction using tin(II) chloride.[28] For the chlorinated quinoline hydrazine required for the synthesis of **5h**, the 5-amino-2-chloroquinoline precursor was prepared by known procedures[29] and transformed into the hydrazinoquinoline as described above. The precursors of the lipophilic acyl substituents were commercially available as either the acid chlorides or the carboxylic acids.

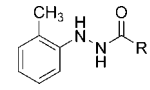
Biology

In Vitro. Functional $P2X_7$ receptor activity was measured using two different methods: (1) inhibition of Ca^{2+} flux in cell lines expressing recombinant human or rat $P2X_7$ receptor, and (2) inhibition of IL-1β release in differentiated THP-1 cells. Inhibition of Ca^{2+} flux was measured with a Fluorometric Imaging Plate Reader (FLIPR) using Fluo-4 as the calcium sensing dye and benzoylbenzoylATP (BzATP) as the agonist.[30] The FLIPR experiments utilized a 5 min pretreatment with the antagonist. Previous work has demonstrated that increased pretreatment times of 15, 30, and 60 min resulted in no change in the measured IC_{50} values.[23] Recombinant human and rat $P2X_7$ were functionally expressed in stably transfected human 1321N1 astrocytoma cells devoid of endogenous P2X receptor function. An assay for inhibition of IL-1β release was established in human THP-1 cells that had been differentiated with LPS and IFNγ then stimulated with BzATP for 30 min in the presence of test compounds.[31] Measurement of IL-1β release was performed using commercially available ELISA kits.

In Vivo. The in vivo efficacy of the novel $P2X_7$ antagonists in the acyl hydrazide series was evaluated in two different animal models. A model of zymosan-induced peritonitis determined compound-dependent modulation of cytokines including interleukin-1 (IL-1β) in rats.[32] In these experiments, acyl hydrazides were administered intraperitoneally or subcutaneously 30 min prior to intraperitoneal administration of a saline suspension of 2 mg zymosan. Four hours later, the animals were euthanized by CO_2 inhalation and the peritoneal cavities lavaged (2×15 mL) with ice cold phosphate buffered saline (w/o Ca^{2+} and Mg^{2+}) with 10 units heparin/mL. For cytokine determination, the samples were spun at 10000 $\times$ g in a refrigerated microfuge (4 °C). The supernatants were removed and frozen until IL-1β levels were determined by ELISA techniques.

Reversal of neuropathic pain was evaluated using the L5/L6 spinal nerve tight ligation (SNL) model.[33] In these experiments, spinal nerve ligation was performed 7−14 days prior to the behavioral test. Mechanical allodynia was evaluated using von Frey monofilaments. Rats were tested before compound administration. Rats with paw withdrawal threshold <5 g were kept for compound testing. Rats were then tested again, 30 min following intraperitoneal administration of the P_2X_7 antagonist.

Table 1. Functional Potency of Acyl Hydrazide $P2X_7$ Antagonists with Modified Acyl Groups



cmpd	R	hP2X$_7$ Ca^{2+} flux pIC_{50}[a,c]	rP2X$_7$ Ca^{2+} flux pIC_{50}[a,c]
4a	*n*-hexyl	6.39 ± 0.05	6.71 ± 0.06
4b	*iso*-pentyl	6.83 ± 0.07	6.47 ± 0.02
4c	cyclohexyl	6.76 ± 0.03	6.61 ± 0.17
4d	1-methylcyclohexyl	7.15 ± 0.02	6.83 ± 0.06
4e	cyclohexylmethyl	7.41 ± 0.04	6.82 ± 0.01
4f	cyclopentyl	7.47 ± 0.07[b]	7.31 ± 0.03[b]
4g	1-phenylcyclopentyl	6.88 ± 0.02	6.82 ± 0.02
4h	1-adamantyl	7.93 ± 0.01[b]	7.55 ± 0.09[b]

[a] Number of determinations = 2, unless otherwise indicated. [b] Number of determinations ≥ 3. [c] Standard error of measurement shown.

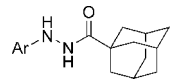
Result and Discussion

The initial structure−activity relationship studies of the acyl hydrazide compounds evaluated the spatial requirements for the lipophilic acyl moiety. Compounds containing acyclic and cyclic alkyl groups of varying size and flexibility indicated trends in the functional potency as measured by the FLIPR calcium flux assay (Table 1). Potency improved as larger, more rigid alkyl groups were incorporated. Cycloalkyl groups containing rings of 6−7 carbon atoms generated antagonists with comparable potency at both the human and rat receptors. Two of the acyl substitution patterns, specifically the benzylic groups with the spirocyclopentane moiety in the benzylic position (**4g**) and the adamantyl group (**4h**), afforded opportunities for rapid exploration and optimization of the structural properties. In addition, the rigid, three-dimensional adamantyl ring system distinguished the acyl hydrazides from the structures of the other antagonists under investigation in our laboratories.[34]

Following the survey of acyl substituents that could replace the terpene-derived moiety in **3**, attention focused on the aryl portion of the pharmacophore. In the next cycle of SAR investigations, the acyl moiety was maintained as the adamantyl group while the aryl group was modified. The data are summarized in Table 2. For *N*′-phenyl hydrazides, substitution at the *ortho* position of the phenyl ring resulted in more potent antagonists (**5d**, **5g**, **5h**). Compounds with simple aromatic heterocycles bound to the terminal nitrogen of the hydrazide as exemplified by the *N*′-pyridyl hydrazides (**5j**, **5k**) afforded $P2X_7$ receptor antagonists with only modest potency. However, the *N*′-(5-isoquinolinyl) (**5l**) and *N*′-(5-quinolinyl)hydrazides (**5m**) proved more potent. The quinolinyl hydrazide also represented a core structure with more desirable physicochemical properties and additional opportunities for SAR explorations.

The third iteration of SAR studies employed the *N*′-5-quinolyl moiety in conjunction with a selection of cyclic acyl moieties. Compounds **6a**−**e** evaluated the impact of modifications to the adamantyl ring system (Table 3). Contraction to the noradamantyl moiety (**6a**) caused a decrease in functional potency at the human receptor that eliminated some of the species discrepancy observed for adamantyl analog **5m**. Insertion of a methylene spacer between the adamantyl group and the carbonyl

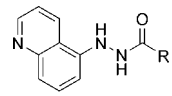
Table 2. Functional Potency of Acyl Hydrazide P2X$_7$ Antagonists with Modified Aryl Groups



cmpd	Ar	hP2X$_7$ Ca^{2+} flux pIC_{50}[a,c]	rP2X$_7$ Ca^{2+} flux pIC_{50}[a,c]
5a	4-methoxyphenyl	6.01 ± 0.04	5.61 ± 0.03
5b	2,5-dimethylphenyl	7.24 ± 0.06	6.87 ± 0.03
5c	phenyl	6.96 ± 0.05[b]	6.56 ± 0.07[b]
5d	2-fluorophenyl	7.40 ± 0.08[b]	6.69 ± 0.01[b]
5e	3-fluororphenyl	6.49 ± 0.20	6.33 ± 0.10
5f	4-fluorophenyl	6.55 ± 0.19[b]	5.97 ± 0.15[b]
5g	2-chlorophenyl	7.94 ± 0.07[b]	7.47 ± 0.04[b]
5h	2,3-dichlorophenyl	7.46 ± 0.05[b]	7.29 ± 0.06[b]
5i	2,4-dichlorophenyl	6.43 ± 0.27	>5
5j	2-pyridyl	6.66 ± 0.05[b]	6.37 ± 0.02
5k	3-pyridyl	6.08 ± 0.02	6.01 ± 0.09
5l	5-isoquinolyl	7.47 ± 0.07[b]	6.82 ± 0.08[b]
5m	5-quinolyl	7.99 ± 0.05[b]	7.35 ± 0.06[b]
5h	2-chloro-5-quinolyl	7.29 ± 0.14[b]	6.76 ± 0.01

[a] Number of determinations = 2, unless otherwise indicated. [b] Number of determinations ≥ 3. [c] Standard error of measurement shown.

Table 3. Functional Potency of Quinoline Derived Acyl Hydrazide P2X$_7$ Antagonists



cmpd	R^1	hP2X$_7$ Ca^{2+} flux pIC_{50}[a,c]	rP2X$_7$ Ca^{2+} flux pIC_{50}[a,c]
6a	3-noradamantyl	7.56 ± 0.08[b]	7.28 ± 0.08[b]
6b	1-adamantylmethyl	7.24 ± 0.01	6.22 ± 0.02[b]
6c	3-chloro-1-adamantyl	7.99 ± 0.04[b]	7.45 ± 0.06[b]
6d	3-ethyl-adamantyl	7.64 ± 0.17	6.84 ± 0.07
6e	3-hydroxy-1-adamantyl	6.56 ± 0.0.09	5.65 ± 0.07
6f	1-(4-methoxyphenyl)cyclohexyl	7.28 ± 0.04[b]	7.29 ± 0.10[b]
6g	1-(2-fluorophenyl)cyclohexyl	6.72 ± 0.06	6.49 ± 0.0.07
6h	1-(3-fluorophenyl)cyclohexyl	6.85 ± 0.10[b]	6.59 ± 0.17[b]
6i	1-(4-fluorophenyl)cyclohexyl	7.15 ± 0.09[b]	6.76 ± 0.02[b]
6j	2,2,3,3-tetramethylcyclopropyl	6.70 ± x0.11[b]	6.81 ± 0.04
6k	1,2,2,3-tetramethylcyclopentyl	−7.56 ± 0.06[b]	6.91 ± 0.02[b]
6l	2-methyl-5-norbornen-2-yl	7.73 ± 0.12[b]	7.50 ± 0.08[b]
6m	7,7-dimethyl-2-oxonorborn-1-yl	5.86 ± 0.03	5.82 ± 0.13

[a] Number of determinations = 2, unless otherwise indicated. [b] Number of determinations ≥ 3. [c] Standard error of measurement shown.

group in analog **6b** decreased functional potency. Halogen and alkyl substituents at the C(3) bridgehead of the adamanyl system (**6c**, **6d**) caused little change in antagonist potency, but the hydroxyl group was not well tolerated (**6e**). Compounds **6f**−**i** evaluated the influence of electron-donating and electron-withdrawing groups on the phenyl ring of the spirocyclic benzylic groups. The fluoro group was tolerated at the *ortho*, *meta*, and *para* positions, but the 4-methoxyphenyl substitution pattern of **6f** provided the greatest potency. Derivative **6f** also demonstrated comparable functional potency at both the human and rat receptors. Alternate lipophilic acyl moieties were also explored, and antagonist function was retained for a range of differently substituted cycloalkyl and polycyclic hydrocarbon groups (**6j**−**6m**).

Selected acyl hydrazide antagonists were evaluated for their capacity to inhibit IL-1β release in vitro using human THP-1 cells (Table 4). The *N*′-quinoline acyl hydrazide **5m** inhibited IL-1β release efficiently, and the pIC(50) value for IL-1β inhibition was comparable to the potency in the FLIPR assay. Compound **6c** was highly potent in the in vitro assay for inhibition of IL-1β release, and the IC_{50} was below the detection limit of the assay. Selected acyl hydrazides were also evaluated using an in vivo model of zymosan-induced peritonitis in which inhibition of production IL-1β was measured directly. Prophylactic administration of selected acyl hydrazide P2X$_7$ receptor antagonists caused a substantial inhibition of IL-1β release. Intraperitoneal administration of 20 mmol/kg of the acyl hydrazides **5m**, **5g**, and **4h** produced >60% reduction in IL-1β release.

Table 4. Functional Potency of Acyl Hydrazide P2X$_7$ Antagonists in the Inhibition of Human IL-1β Release In Vitro and Efficacy In Vivo in the Zymosan Model

cmpd (A-number)	hIL-1β release pIC_{50}[a,b]	zymosan (i.p.; % effect at 20 μmol/kg)[a]
5m	8.3 ± 0.2	63
6a	6.8 ± 0.1	55
6c	>9.0	49
5g	NT[c]	63
4h	NT	66

[a] Number of determinations = 3, unless otherwise indicated. [b] Standard error of measurement shown. [c] NT = not tested.

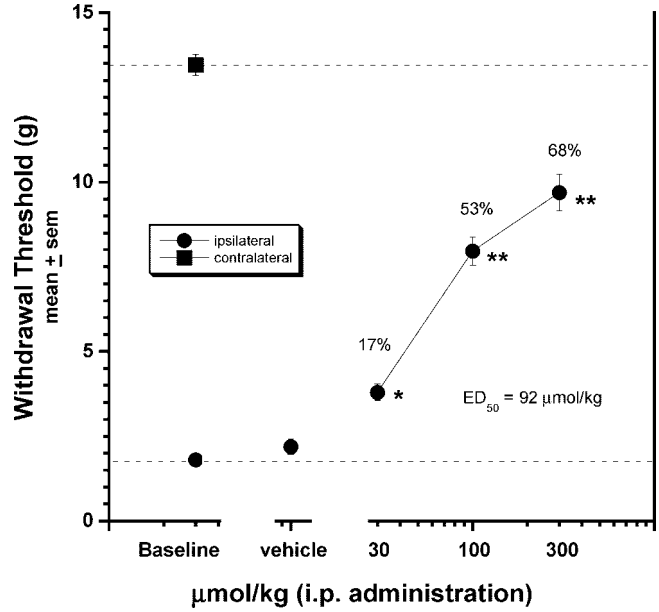


Figure 1. Effects of **5m** on mechanical allodynia observed in the SNL model of neuropathic pain.

Table 5. Pharmacokinetic Profile of **5m** in Rats[a]

route	V_β (L/kg)	Cl_p (L/hr·kg)	C_{max} (μg/mL)	T_{max} (hr)	AUC (μg·hr/L)	$t_{1/2}$ (hr)	F (%)
IV	0.90	1.7			0.94	0.40	
IP			0.44	0.50	0.39	0.60	42

[a] All animals administered doses of 5 μmol/kg in 10% DMSO/90%PEG.

Previous work with other classes of P2X$_7$ receptor antagonists demonstrated a correlation between inhibition of IL-1β release and efficacy in behavioral models of neuropathic pain,[24] although a mechanistic rationale for this relationship was not established. Behavioral effects of compound **5m** were evaluated in the spinal nerve ligation (SNL) model of neuropathic pain. Intraperitoneal (ip) administration of **5m** (30−300 μmol/kg) resulted in a dose-related reversal of mechanical allodynia on the injured (ipsilateral) side with an ED_{50} value of 92 μmol/kg and a maximum response of 68% relative to the noninjured (contralateral) side (Figure 1). The pharmacokinetic parameters of **5m** (Table 5) indicate plasma exposure levels consistent with the observed behavioral effects.

The above results demonstrate the potential of the acyl hydrazide pharmacophore to reduce pain transmission and cytokine production. The efficacy of **5m** in the SNL model of neuropathic pain (Figure 1) is consistent with previous reports of antinociception using other structurally distinct $P2X_7$ antagonists.[24] The mechanistic basis for the analgesic effects has not been determined, but compound **5m** was found to have no significant interactions for concentrations up to 10 μM at other P2 receptors ($P2X_3$, $P2X_4$, and $P2Y_2$). In addition, a screen of compound **5g** against a variety of over 70 other receptors, enzymes, and ion channels revealed no substantial interactions using a concentration of 10 μM.

Conclusion

We have identified a novel series of acyl hydrazide $P2X_7$ receptor antagonists. Examples of this new pharmacophore exhibited functional potency using in vitro assays that monitor distinct steps along the cascade initiated by $P2X_7$ receptor activation. The SAR studies conducted in this work revealed preferred substitution patterns for the N' and acyl termini of the hydrazide structure. For the N'-aryl moiety, *ortho*-substituted phenyl groups produced potent $P2X_7$ receptor antagonists, as did 5-substituted quinoline ring systems. Replacement of the N−H bonds in the hydrazide was not tolerated. In the acyl moiety, rigid polycyclic hydrocarbon ring systems exemplified by the adamantyl group generated potent antagonist function. Similarly, acyl groups derived from 1-phenylcyclohexanecarboxylic acid imparted a favorable balance of potency and physicochemical properties. The primary in vitro FLIPR assay utilized in this study revealed minor discrepancies between functional potency at the recombinant human and rat receptors, but certain substitution patterns minimized the differences. Finally, compound **5m** was found to possess antinociceptive efficacy in a model of neuropathic pain, providing additional evidence for the potential of the $P2X_7$ receptor as a molecular target for pain. Compound **5m** also reduced the release of the inflammatory cytokine IL-1β in a zymosan-induced model of peritonitis demonstrating that $P2X_7$ receptor antagonists can inhibit inflammatory pathways.

Supporting Information Available: Synthetic procedures and characterization data for intermediates and final products. Complete descriptions of biological protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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