

A-317491, a novel potent and selective non-nucleotide antagonist of P2X₃ and P2X_{2/3} receptors, reduces chronic inflammatory and neuropathic pain in the rat

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P2X₃ and P2X_{2/3} receptors are highly localized on peripheral and central processes of sensory afferent nerves, and activation of these channels contributes to the pronociceptive effects of ATP. A-317491 is a novel non-nucleotide antagonist of P2X₃ and P2X_{2/3} receptor activation. A-317491 potently blocked recombinant human and rat P2X₃ and P2X_{2/3} receptor-mediated calcium flux ($K_i = 22\text{--}92$ nM) and was highly selective ($IC_{50} > 10$ μ M) over other P2 receptors and other neurotransmitter receptors, ion channels, and enzymes. A-317491 also blocked native P2X₃ and P2X_{2/3} receptors in rat dorsal root ganglion neurons. Blockade of P2X₃ containing channels was stereospecific because the *R*-enantiomer (A-317344) of A-317491 was significantly less active at P2X₃ and P2X_{2/3} receptors. A-317491 dose-dependently ($ED_{50} = 30$ μ mol/kg s.c.) reduced complete Freund's adjuvant-induced thermal hyperalgesia in the rat. A-317491 was most potent ($ED_{50} = 10\text{--}15$ μ mol/kg s.c.) in attenuating both thermal hyperalgesia and mechanical allodynia after chronic nerve constriction injury. The *R*-enantiomer, A-317344, was inactive in these chronic pain models. Although active in chronic pain models, A-317491 was ineffective ($ED_{50} > 100$ μ mol/kg s.c.) in reducing nociception in animal models of acute pain, postoperative pain, and visceral pain. The present data indicate that a potent and selective antagonist of P2X₃ and P2X_{2/3} receptors effectively reduces both nerve injury and chronic inflammatory nociception, but P2X₃ and P2X_{2/3} receptor activation may not be a major mediator of acute, acute inflammatory, or visceral pain.

The cloning and characterization of the P2X₃ receptor, a specific ATP-sensitive ligand-gated ion channel that is selectively localized on peripheral and central processes of sensory afferent neurons (1–3), has generated much interest in the role of this receptor in nociceptive signaling (4). The discovery of the P2X₃ receptor has provided a putative mechanism for previous reports that ATP, released from sensory nerves (5), produces fast excitatory potentials in dorsal root ganglion (DRG) neurons (6). These actions appear to be physiologically relevant because iontophoretic application of ATP to human skin elicits pain (7) and exogenously applied ATP enhances pain sensations in a human blister base model (8).

The P2X₃ receptor is natively expressed as a functional homomer and as a heteromultimeric combination with the P2X₂ (P2X_{2/3}) receptor (1, 2, 9). Both P2X₃-containing channels are expressed on a high proportion of isolectin IB4-positive neurons in DRG (3, 10). These receptors share similar pharmacological profiles (11), but differ in their acute desensitization kinetics (10, 12). Immunohistochemical studies have shown that P2X₃ receptor expression is up-regulated in DRG neurons and ipsilateral spinal cord after chronic constriction injury (CCI) of the sciatic nerve (13). Addi-

tionally, CCI results in a specific ectopic sensitivity to ATP that is not observed on contralateral (uninjured) nerves (14).

Recently, the phenotypic profile of P2X₃ receptor gene-disrupted mice has been reported (15, 16). P2X₃($-/-$) mice are viable and show no overt behavioral perturbations. However, P2X₃($-/-$) mice show reduced pain-related behaviors in response to intraplantar ATP or formalin administration, and ATP-mediated rapidly desensitizing inward currents in DRG neurons are absent in these mice. Although these observations support a role for P2X₃ receptor activation in pain, one group has reported a transient hyperalgesic response in P2X₃($-/-$) mice after the intraplantar administration of complete Freund's adjuvant (CFA) (16). The effects of P2X₃ receptor gene disruption on visceral pain and other models of acute and chronic nociception have not been reported.

There are numerous studies demonstrating that P2 receptor agonists and antagonists modulate nociceptive behaviors in rodents (17). However, determination of the specific role of P2X₃ receptor activation in different pain states has been hampered by the lack of useful receptor ligands for *in vivo* studies. Existing P2 receptor agonists nonselectively activate a variety of P2 receptor subtypes and are metabolically labile (18). P2 receptor antagonists such as suramin and pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid lack high affinity and specificity for individual P2 receptor subtypes (18).

The present studies were undertaken to characterize the antinociceptive effects of A-317491 (Fig. 1A), the first non-nucleotide antagonist that has high affinity and selectivity for blocking P2X₃ homomeric and P2X_{2/3} heteromeric channels. The chiral pure *S*-enantiomer, A-317491, was found to potently block P2X₃ and P2X_{2/3} receptor-activated calcium flux *in vitro*. After s.c. administration, A-317491 dose-dependently reduced nociception in neuropathic and inflammatory animal pain models, but generally lacked acute analgesic efficacy. The *R*-enantiomer, A-317344, showed markedly less activity at P2X₃ and P2X_{2/3} receptors *in vitro* and lacked antinociceptive effects in animal models.

Materials and Methods

Ca²⁺ Influx Assay. Stably transfected 1321N1 human astrocytoma cells expressing rat and human P2X receptors have been described (9–11). Activation of human and rat recombinant P2X receptors was determined on the basis of agonist-induced increases in cytosolic Ca²⁺ concentration as described with minor

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Abbreviations: CCI, chronic constriction injury; CFA, complete Freund's adjuvant; DRG, dorsal root ganglion.

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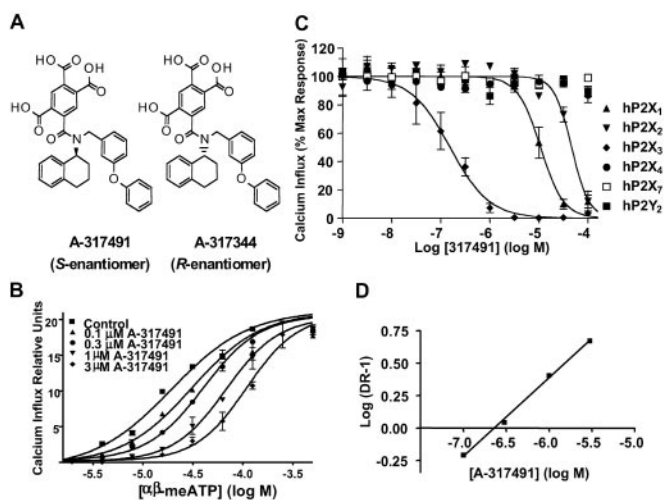


Fig. 1. (A) Structures of A-317491 and A-317344. (B) The selectivity of A-317491 to block activation of the human P2X₃ receptor, as compared with other P2X receptor subtypes. Representative concentration-effect curves were normalized to the agonist response (percentage maximal response) in the absence of A-317491. See Tables 1 and 2 for agonist concentrations and derived K_i values. (C) Representative concentration-effect curves for α,β -meATP in the absence (control) and presence of increasing concentrations of A-317491. Data represent mean \pm SEM from three separate experiments. (D) Schild plot.

modifications (11). The fluorescent Ca²⁺ chelating dye fluo-4 was used as an indicator of the relative levels of intracellular Ca²⁺ in a 96-well format by using a fluorescence imaging plate reader (Molecular Devices). P2X receptor-expressing cells were grown to confluence and plated in 96-well black-walled tissue culture plates \approx 18 h before the experiment. One to two hours before the assay, cells were loaded with fluo-4 AM (2.28 μ M; Molecular Probes) in Dulbecco's PBS (D-PBS) and maintained in a dark environment at room temperature. Immediately before the assay, each plate was washed twice with 250 μ l D-PBS per well to remove extracellular fluo-4 AM. Two 50- μ l additions of compounds (prepared in D-PBS) were made to the cells during each experiment. The first test compound (antagonist) addition was made, and incubation continued for 3 min before the addition of α,β -me-ATP (3 μ M final concentration). Measurements continued for 3 min after this final addition. Fluorescence data were collected at 1- or 5-s intervals throughout the course of each experiment. Concentration-response data were analyzed by using GRAPHPAD PRISM (San Diego). K_i values were estimated by the equation: $K_i = IC_{50}/(1 + [\text{agonist}]/\text{agonist EC}_{50})$ (19).

Electrophysiology. Whole-cell patch-clamp recordings were obtained as described (10) from stable cell lines or DRG neurons by using a modified extracellular saline consisting of 155 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM Hepes, 12 mM glucose, pH 7.4. The patch pipette solution consisted of 140 mM potassium aspartate, 20 mM NaCl, 10 mM EGTA, 5 mM Hepes. All cells were voltage-clamped at -60 mV, and series resistance was compensated 75–90% by using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA).

Rat DRG neurons were prepared as described (10). Lumbar (L4–6) DRG were dissected and placed in DMEM (HyClone) containing 0.3% collagenase B (Roche Molecular Biochemicals) for 60 min at 37°C. The collagenase was replaced with 0.25% trypsin (GIBCO/BRL) in Ca²⁺/Mg²⁺-free Dulbecco's PBS and further digested for 30 min at 37°C. Ganglia were washed in fresh DMEM, dissociated by trituration, and plated on polyethyleneimine-treated coverslips. Cells were plated in 1 ml DMEM supplemented with 10% FBS (HyClone), nerve growth factor

(50 ng/ml, Roche Molecular Biochemicals), and 100 units/ml penicillin/streptomycin.

Drugs were applied to the cells by using a piezoelectric-driven glass theta tube positioned near the cell. During experiments, agonists were usually applied every 3 min. A-317491 was both preapplied and coapplied to cells during agonist application. Responses were acquired and digitized at 3 kHz, and analyzed by using PCLAMP software (Axon Instruments). Current amplitudes were measured at the peak of the response.

Pharmacological Selectivity Studies. The activity of A-317491 (10 μ M) was evaluated in a number of assays to assess pharmacological selectivity relative to 86 other cell-surface receptors, ion channels, transport sites, and enzymes including the opioid receptor subtypes and cyclooxygenases 1 and 2, by use of standardized assay protocols (Cerep, Celle l'Evescault, France) as described (20).

In Vivo Studies: Subjects. In most experiments, male Sprague-Dawley rats (Charles River Breeding Laboratories) weighing 200–300 g were used. The abdominal constriction assay was conducted by using male 129J mice weighing 20–25 g (The Jackson Laboratories). The hotplate assay was conducted by using male CF-1 mice (Harlan Farms, Portage, MI) weighing 25–30 g. These animals were group-housed in American Association for the Accreditation of Laboratory Animal Care-approved facilities at Abbott Laboratories in a temperature-regulated environment with lights on between 0700 and 2000 hours. Food and water was available ad libitum except during testing. All animal handling and experimental protocols were approved by an institutional animal care and use committee.

Analgesia and Side-Effect Assays. A-317491 and A-317344 were evaluated in a number of well-characterized *in vivo* models to assess acute (noxious thermal, mechanical, and chemical stimulation), inflammatory (intraplantar formalin, carrageenan, and CFA), and neuropathic (CCI and L5/L6 nerve ligation) pain, as well as models of visceral (acetic acid-induced abdominal constriction, and normal and inflamed colonic distention) and postoperative pain (20–22). The specific methodologies for these nociceptive assays and the assessment of rat motor performance, hemodynamics, and general CNS function are described in detail in the *Supporting Text*, which is published as supporting information on the PNAS web site, www.pnas.org. Unless otherwise noted, all experimental and control groups contained at least six animals each, and data are expressed as mean \pm SEM. Data analysis was conducted by using ANOVA and appropriate post hoc comparisons ($P < 0.05$) as described (20, 21). ED₅₀ values were estimated by using least-squares linear regression.

Compounds. A-317491 and A-317344 were synthesized at Abbott Laboratories. Compounds were dissolved in sterile water for s.c. administration and administered in a final volume of 1–5 ml/kg, s.c. Except where noted, compounds were administered 30 min before nociceptive and side-effect testing.

Results

In Vitro Activities of A-317491. ATP and α,β -meATP are potent agonists at both P2X₃ and P2X_{2/3} receptors (11). Because α,β -meATP is a poor agonist for P2X₂ receptors (1, 12), it was used to activate calcium flux in 1321N1 cells expressing human and rat homomeric P2X₃ and heteromeric P2X_{2/3} receptors. A-317491 (S-enantiomer) was identified as a potent antagonist of α,β -meATP-activated recombinant rat and human P2X₃ and P2X_{2/3} receptors and was significantly more potent than the R-enantiomer, A-317344 (Table 1). A-317491 showed significantly higher affinity in blocking P2X₃ receptors (Table 1) compared with its ability to inhibit functional activation of other P2X receptors or the P2Y₂ receptor (Table 2 and Fig. 1C).

Table 1. *In vitro* activity of A-317491 and A-317344 at human and rat P2X₃ and P2X_{2/3} receptors

Calcium influx (K _i , nM)	A-317491 (S-enantiomer)	A-317344 (R-enantiomer)
Rat P2X ₃	22 ± 8	>7,300
Human P2X ₃	22 ± 3	>10,000
Rat P2X _{2/3}	92 ± 11	1,300 ± 30
Human P2X _{2/3}	9 ± 2	1,100 ± 200

K_i calculated by the method of Cheng–Prusoff (19) using the IC₅₀ values determined in the presence of 3 μM agonist (α,β-meATP); mean ± SEM (n = 3–10). pEC₅₀ values for α,β-meATP at rat and human P2X₃ receptors averaged 6.21 ± 0.05; pEC₅₀ values at rat and human P2X_{2/3} receptors averaged 5.65 ± 0.08 (ref. 11 and unpublished observations).

Because the fast-desensitizing properties of the homomeric P2X₃ receptors limit analysis of antagonist competitiveness (23), the nature of the antagonist actions of A-317491 was investigated by using P2X_{2/3} receptors. A-317491 was found to be a competitive antagonist of rat P2X_{2/3} receptors, with increasing concentrations of A-317491 producing rightward parallel shifts in the α,β-meATP dose–response curve (Fig. 1B). A Schild analysis (Fig. 1D) of these data yielded a pA₂ value of 232 nM, which is in agreement with the estimated K_i value of 92 nM at rat P2X_{2/3} receptors (Table 1).

Fig. 2 summarizes electrophysiological studies of the effects of A-317491 on α,β-meATP-induced currents in both stably transfected cells and DRG neurons. Application of α,β-meATP evoked rapidly desensitizing currents in cells expressing human P2X₃ receptors. A-317491 produced a concentration-dependent block of human P2X₃ currents with an IC₅₀ value of 97 nM (n = 3, K_i = 17 nM, Fig. 2A and B). Similar results were obtained with recombinant human P2X₃ receptors when ATP (3 μM) was used as the agonist (IC₅₀ = 99 nM, n = 3, K_i = 4 nM) and with human P2X_{2/3} receptors when α,β-meATP (10 μM) was used as the agonist (IC₅₀ = 169 nM, n = 2–6, K_i = 20 nM). A-317491 was equally effective at blocking rapidly desensitizing P2X₃-mediated currents in rat DRG neurons (Fig. 2C and D). A-317491 produced a concentration-dependent block of DRG currents with an IC₅₀ value of 15 nM (n = 3). At all receptor subtypes, the effects of A-317491 were reversible, and essentially complete block of current was observed at a concentration of 10 μM. At this concentration, no nonspecific effects of the compound were observed on cellular input resistance or voltage-clamp holding current.

A-317491 was also evaluated by Cerep for activity at 86 other receptors, enzymes, and ion channels (17). A-317491 was inactive (IC₅₀ > 10 μM) at most of these other proteins. The only interaction with an IC₅₀ < 10 μM was at the δ opioid receptor (IC₅₀ ≈ 5 μM); in contrast, 10 μM A-317491 caused <10% inhibition of binding to the κ opioid receptor and only 15% inhibition of binding to the μ opioid receptor. Preliminary pharmacokinetic studies in rats indicated that 10 μmol/kg A-317491 had high (≈80%) systemic bioavailability after s.c.

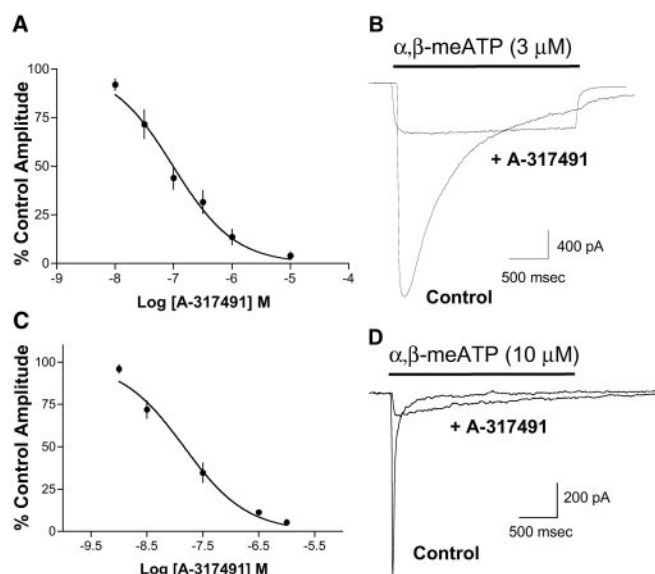


Fig. 2. (A) A-317491 concentration–response curve measured in stable cells expressing the recombinant human P2X₃ receptor. Currents were activated by 3 μM α,β-meATP (control), and the percentage control response was measured in the presence of increasing concentrations of A-317491 (n = 3). (B) Representative α,β-meATP-induced current traces recorded from a P2X₃-expressing cell before (control) and during application of 300 nM A-317491. (C) A-317491 concentration–response curve measured in rat DRG neurons. Currents were activated by 10 μM α,β-meATP, and the percentage control response was measured in the presence of increasing concentrations of A-317491 (n = 3). (D) Representative α,β-meATP-induced current traces recorded from a rat DRG neuron before (control) and during application of 300 nM A-317491.

dosing (estimated plasma concentration = 15 μg/ml, >99% protein bound) and a half-life in plasma of 11 h. A-317491 did not undergo any detectable metabolism (oxidation or glucuronidation) in *in vitro* assays using human and rat liver microsomes (unpublished observations).

Antinociceptive Activity of A-317491. To further characterize the nociceptive role of P2X₃ receptor activation, A-317491 was evaluated in a variety of animal pain models after s.c. administration. A-317491 was most potent in reducing mechanical allodynia and thermal hyperalgesia in the CCI model (ED₅₀ = 10 and 15 μmol/kg s.c., Fig. 3A and B) as compared with the other animal models of nociception tested (Table 3). A-317491 was fully effective in blocking nociception in this model, whereas the R-enantiomer, A-317344, was inactive (Fig. 3C). The antinociceptive effects of A-317491 in the CCI model were rapid in onset and persisted for 5 h after s.c. administration (Fig. 3D). In contrast to its full efficacy in the CCI model, A-317491 was only partially effective (50% reduction at 100 μmol/kg s.c.) in reducing tactile allodynia thresholds in the L5/L6 nerve ligation model (Table 3).

Table 2. Activity of A-317491 at other P2X and the P2Y₂ receptors

Agonist	Agonist conc., μM	Human P2X receptor	Agonist pEC ₅₀	A-317491 pIC ₅₀	A-317491 K _i , μM
ATP	0.2	P2X ₁	7.73 ± 0.27	4.97 ± 0.17	2.53
ATP	5.0	P2X ₂	6.11 ± 0.20	4.33 ± 0.13	4.13
ATP	4.0	P2X ₄	6.30 ± 0.17	<4	>2.7
BzATP	10.0	P2X ₇	5.86 ± 0.15	<4	>8.6
UTP	1.0	P2Y ₂	7.99 ± 0.31	<4	>2.0

Data represent means ± SEM from at least three separate experiments.

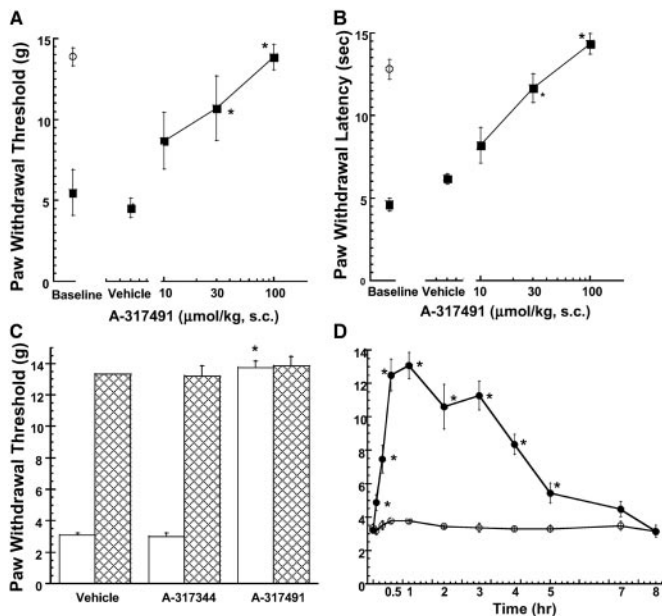


Fig. 3. A-317491 dose-dependently increases mechanical allodynia (von Frey hair) thresholds (A) and thermal paw withdrawal latencies (B) in the CCI model. ■, Paw responses ipsilateral to the nerve injury; and ○, paw responses of the contralateral paw (mean ± SEM). (C) A-317491 (100 µmol/kg s.c.), but not A-317344 (100 µmol/kg s.c.) attenuates mechanical allodynia (open bars) in the CCI model of neuropathic pain. Hatched bars represent responses of the contralateral paw. (D) Time course for the onset and duration of A-317491 (100 µmol/kg s.c.)-mediated antinociception (●) in the CCI model. ○, Mechanical allodynia thresholds of CCI animals treated with vehicle. *, $P < 0.05$ as compared with vehicle-treated animals ($n = 6$ per group).

After 48 h of inflammation induced by the intraplantar administration of CFA, A-317491 fully blocked thermal hyperalgesia (Fig. 4A). The antinociceptive effects of A-317491 were specific to the injured paw, as the paw withdrawal latencies for the uninjured paw were not significantly altered by A-317491 at the doses tested. The antinociceptive effects of A-317491 in this model were also stereospecific because the *R*-enantiomer,

A-317344, was inactive in this model (Fig. 4B). As was observed in the CCI model, the analgesic effects of A-317491 were rapid in onset and lasted for 8 h after s.c. administration (Fig. 4C). A-317491 produced an equivalent amount of antinociception after twice-daily administration for 4 days as was observed after acute administration (Fig. 4D).

A-317491 also dose-dependently reduced nociceptive responses in chemically induced pain models including the persistent phase of the formalin test and a murine model of abdominal pain, the acetic acid-induced abdominal constriction assay (ACA) (Table 3; Fig. 5). The *R*-enantiomer, A-317344, was completely inactive in the ACA assay. As shown in Table 3, A-317491 was significantly more effective in reducing nociception in the persistent phase of the formalin assay compared with its activity in the acute phase immediately after formalin administration. A-317491 was also generally ineffective at doses up to 100 µmol/kg s.c. in reducing nociception elicited by a variety of other acute noxious stimuli including thermal, mechanical, capsaicin, and acute inflammatory hyperalgesia (Table 3).

The effects of A-317491 were also evaluated in a model of postoperative pain induced by the incision of the skin, fascia, and plantaris muscle in the rat (22). In this model, A-317491 (100 µmol/kg s.c.) administered 30 min before or after the surgery, or locally (300 nmol) around the incision site 5 min before or after the surgery, did not decrease the mechanical allodynia observed 2 h after surgery (data not shown). In addition, these treatments did not modify the development of mechanical allodynia (von Frey hair sensitivity) 1 and 2 days after surgery (Table 3). A-317491 (100 µmol/kg s.c.) also had no effect on visceral pain in the rat as shown by its inability to attenuate either the visceromotor response observed after acute noxious colonic distension or inflammation-induced visceral hyperalgesia (Table 3).

Effects on Motor Activity, CNS, and Cardiovascular Function.

A-317491 had no significant effect ($P > 0.05$) on motor coordination at doses up to 300 µmol/kg s.c., as measured by the ability of rats to run on an accelerating rotating rod (rotorod assay, control latency = 59 ± 1 s, A-317491 300 µmol/kg latency = 53 ± 2 s). A-317491 also had no effect on spontaneous exploratory activity of rats in a novel open field at 100 µmol/kg s.c., but a statistically significant ($32 \pm 7\%$, $P < 0.05$) reduction

Table 3. Analgesic profile of A-317491

Model	ED ₅₀ , µmol/kg s.c.	% Effect, 100 µmol/kg s.c.
Neuropathic pain		
CCI		
Mechanical	15	100 ± 8*
Thermal	10	110 ± 7*
L5/L6 nerve ligation	100	50 ± 8*
Inflammatory pain		
Formalin test (persistent phase)	50	60 ± 3*
Chronic thermal hyperalgesia (CFA)	30	74 ± 11*
Acute thermal hyperalgesia (Carrageenan)	>100	15 ± 5
Visceral nociception		
Mouse abdominal constriction	27	78 ± 2*
Rat colonic distention	>100	0
Rat visceral hyperalgesia	>100	0
Postoperative somatic pain		
>100	>100	0
Acute nociception		
Rat acute thermal	>100	0 ± 10
Rat acute mechanical	>100	0 ± 4
Mouse hotplate	>100	-10 ± 10
Rat intraplantar capsaicin	>100	7 ± 5
Formalin (acute phase)	>100	25 ± 7

*Significantly different ($P < 0.05$) from vehicle-treated control animal responses ($n = 6$ per group).

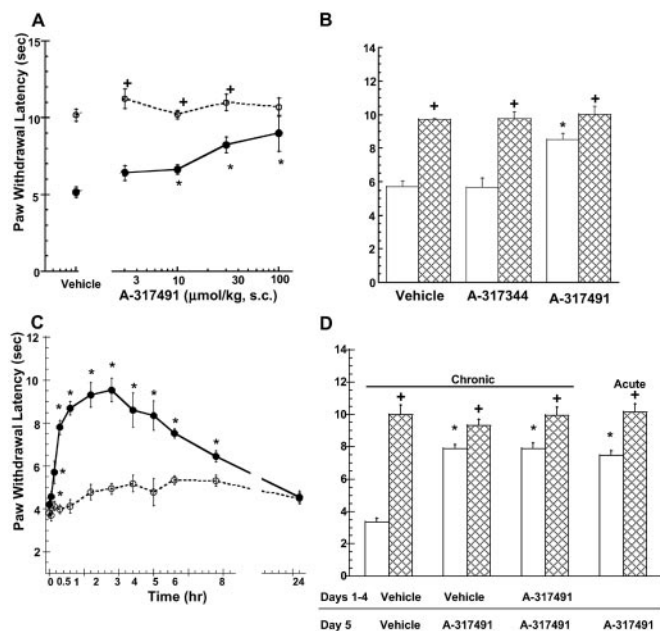


Fig. 4. (A) A-317491 dose-dependently increases paw withdrawal latencies 48 h after intraplantar administration of CFA. ●, Responses of CFA-injected paw. ○, Paw withdrawal latencies of uninjected contralateral paw (mean ± SEM). (B) A-317491 (100 μmol/kg s.c.), but not A-317344 (100 μmol/kg s.c.), attenuates CFA-induced thermal hyperalgesia (open bars) in the rat. Hatched bars represent responses of the contralateral paw. (C) Time course for the onset and duration of A-317491 (100 μmol/kg s.c.)-mediated antihyperalgesia (●) in the rat. ○, Paw withdrawal latencies of CFA-injected paws from animals treated with vehicle. (D) A-317491 was administered twice daily for 4 days. The antinociceptive effects of A-317491 (100 μmol/kg s.c., open bars) after repeated dosing in the CFA model were not significantly different as compared with vehicle-pretreated animals (chronic) or animals that received a single (acute) administration of A-317491. Hatched bars represent responses of the contralateral uninjected paw. *, $P < 0.05$ as compared with vehicle-treated animals ($n = 6$ per group). +, $P < 0.05$ as compared with CFA-treated paw.

of spontaneous exploratory activity was observed at 300 μmol/kg s.c. Rats were fully awake, were responsive to stimuli, and retained the righting reflex, consistent with their ability to perform the rotarod test at all doses tested.

A-317491 (10–300 μmol/kg s.c.) was also evaluated in a number of assays to assess general CNS function. No significant differences ($P > 0.05$) from vehicle-treated animals were noted for A-317491-treated mice in the Irwin test, the ethanol and barbital interaction assays, and the pentylenetetrazol-induced seizure assay (data not shown). Statistically significant, but dose-independent, anticonvulsant effects were found for A-317491 (100 μmol/kg s.c., 10% protection, $P < 0.05$) in the mouse electroconvulsive shock model, and a 0.4–0.6°C increase in rectal temperature was observed at 10 and 30 μmol/kg s.c. General CNS depression (respiration, sensory-motor deficits) and lethality were observed at a dose of 1,000 μmol/kg s.c. in mice. The cardiovascular effects of A-317491 were examined by using conscious, freely behaving rats instrumented with telemetry transmitters. After s.c. administration, A-317491 produced no statistically significant changes in mean arterial pressure or heart rate when administered to conscious rats at 300 μmol/kg.

Discussion

These data demonstrate that A-317491 is a potent and selective antagonist of P2X₃ and P2X_{2/3} receptors. Like the nucleotide-based antagonist 2',3'-O-2,4,6-trinitrophenyl (TNP)-ATP (22), A-317491 is a competitive antagonist of P2X_{2/3} receptors. However, unlike TNP-ATP, which also has high affinity for P2X₁

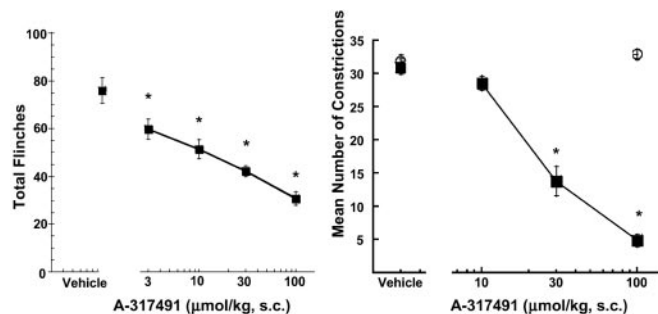


Fig. 5. (Left) Antinociceptive effects of A-317491 in the persistent phase of the formalin test. (Right) A-317491 (■), but not A-317344 (100 μmol/kg s.c., ○), dose-dependently reduces nociception in the mouse acetic acid-induced abdominal constriction assay. Data represent mean ± SEM. *, $P < 0.05$ as compared with vehicle-treated animals ($n = 6$ per group).

receptors (24), A-317491 exhibits >100-fold selectivity for P2X₃ and P2X_{2/3} receptors compared with its activity at other P2X receptor subtypes. A-317491 shows only very weak or no affinity for a large selection of other cell surface receptors, ion channels, and enzymes. A-317491, at concentrations up to 100 μM, also did not inhibit ectonucleotidase activity as measured by [³²P]ATP degradation (unpublished observations). The specificity of the antagonist actions of A-317491 for P2X₃ and P2X_{2/3} receptor blockade is further supported by the significantly weaker activity of the *R*-enantiomer, A-317344, as a P2X₃ receptor antagonist. Electrophysiological data from both recombinant and native P2X₃ receptor-mediated responses demonstrate that receptor block is rapid in onset, reversible, and devoid of nonspecific effects. A-317491 is also not susceptible to metabolic dephosphorylation like TNP-ATP (25). Thus, A-317491 represents the first non-nucleotide, potent and selective, antagonist of P2X₃-containing channels.

A-317491 effectively reduced nociception in the CFA-induced model of chronic inflammatory pain and was particularly potent in reducing both thermal hyperalgesia and tactile allodynia in the CCI neuropathic pain model. The enhanced antinociceptive efficacy of A-317491 in the CCI model is consistent with the previously documented up-regulation of P2X₃-containing channels in rat DRG and spinal dorsal horn in this model (13). Although less active, A-317491 also significantly reduced tactile allodynia thresholds in the L5/L6 nerve injury model. After L5/L6 nerve ligation, there is a significant decrease in the density of IB4-positive small diameter neurons in the L5/L6 DRG and a corresponding reduction in P2X₃ immunoreactivity (26). However, a subpopulation of IB4 negative larger diameter neurons in the L5/L6 DRG remain intact, show P2X₃ immunoreactivity, and demonstrate both fast (P2X₃-like) and slowly (P2X_{2/3}-like) desensitizing responses to ATP (26). Taken together, these data provide neurochemical and functional evidence that activation of P2X₃ and P2X_{2/3} receptors is modulated during chronic pain and blockade of these receptors can reduce nociception mediated by both small and larger diameter sensory neurons in chronic pain states.

The antinociceptive effects of A-317491 in both the CFA and CCI models were rapid in onset and pharmacologically specific because similar antinociceptive effects were not observed after systemic administration of A-317344, the less active *R*-enantiomer. The antinociceptive effects of A-317491 in the CFA model were also maintained after repeated administration twice daily for 4 days. Thus, A-317491 shows reduced potential to produce tolerance compared with morphine, which has significantly reduced antinociceptive activity after repeated dosing (21). The antinociceptive effects of A-317491 were not accompanied by side effects commonly associated with other analgesic

agents. A-317491 produced no significant effects on cardiovascular function and did not significantly alter motor performance at doses up to 100 $\mu\text{mol/kg}$ s.c. in the rat. Additional studies in the mouse indicated that A-317491 was generally devoid of dose-dependent effects on CNS function up to doses of 300 $\mu\text{mol/kg}$ s.c.

The antinociceptive effects of A-317491 in the CFA and CCI models are in agreement with other recent data demonstrating that intrathecal P2X₃ antisense (27) and P2X₃ receptor gene disruption (15, 16) reduce nociceptive sensitivity. Specifically, systemic administration of A-317491 was found to produce a similar reduction ($\approx 50\%$) of spontaneous formalin-induced persistent nociception as was produced by P2X₃ antisense or observed in P2X₃ gene-disrupted animals. However, in contrast to the reported hyperalgesic effects of P2X₃ receptor gene disruption (16), A-317491 produced significant antihyperalgesia in models of both chronic inflammatory hyperalgesia and neuropathic pain.

Unlike its analgesic effects in the chronic inflammatory and the neuropathic pain assays, A-317491 was ineffective in models of acute nociception involving a variety of noxious stimuli including heat, mechanical, and chemical (capsaicin, carrageenan, and formalin) stimulation. Although A-317491 was effective in reducing nociception in the acetic acid-induced mouse abdominal constriction assay, it was ineffective in reducing visceromotor responses after acute noxious colonic distension or inflammation-induced visceral hyperalgesia in the rat. A-317491 was also ineffective in reducing pain-related behaviors in a plantar incision model of postoperative somatic pain (22). In comparison to its analgesic efficacy in the CFA and neuropathic chronic pain models, these data suggest that activation of P2X₃ and P2X_{2/3} receptors may be more involved in specific aspects of chronic inflammatory hyperalgesia and nerve injury-induced allodynia than in acute or visceral pain states.

Although the reasons for the differential analgesic efficacy of A-317491 in various pain states remain unclear, this pattern of activity may reflect different relative contributions of glutamatergic neurotransmission in various pain states. For example, recent studies have shown that the pharmacology of the plantar

incision model of postoperative pain is different from the pharmacology of more classical models of inflammatory and neuropathic pain states (28). In particular, compounds that block spinal *N*-methyl-D-aspartate (NMDA) receptor and metabotropic glutamatergic receptor transmission have little antinociceptive effect in normal animals, but have antinociceptive properties in several models of chronic pain such as thermal hyperalgesia and mechanical allodynia observed in inflammatory (carrageenan, CFA) or neuropathic (sciatic nerve constriction, spinal nerve ligation) pain states (29, 30). Interestingly, it has recently been shown that spinal NMDA receptor and metabotropic glutamatergic receptor antagonists are ineffective at reducing incision-induced mechanical allodynia, suggesting a limited role of these receptors in this model of postoperative pain (28). Because previous data has shown that activation of P2X receptors facilitates the release of glutamate in spinal dorsal horn neurons (31), the analgesic effects of A-317491 may depend on glutamatergically mediated nociceptive processes.

Previous pharmacological studies of the nociceptive role of P2X₃-containing channels have been limited to the evaluation of low-affinity nonselective antagonists like suramin and pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (17) or more recently to the potent, but unstable antagonist, 2',3'-*O*-2,4,6-trinitrophenyl-ATP, after local (32) or intrathecal administration (33, 34). In the absence of selective ligands, molecular approaches like gene disruption (15, 16) and gene knockdown (antisense) (27) studies have provided some evidence that P2X₃-containing channels contribute to nociception. A-317491 represents an important advance in P2X₃ receptor pharmacology because of its high degree of P2X receptor selectivity and its metabolic stability *in vivo*. Both the analgesic profile of A-317491 and the P2X₃ gene disruption data (15, 16, 27) indicate that activation of P2X₃-containing ion channels may contribute to the processes of peripheral and central sensitization states associated with some forms of chronic inflammatory pain and to nociceptive states arising from nerve injury.

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