

**Allosteric Adenosine Receptor Modulation Reduces Hypersensitivity Following  
Peripheral Inflammation by a Central Mechanism**

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**Running Title:** T62 Reduces inflammation induced hypersensitivity

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Abstract:

Activation of adenosine A1 receptors by endogenous adenosine or synthetic agonists produces antinociception in animal models of acute pain and also reduces hypersensitivity in models of inflammatory and nerve-injury pain. Allosteric adenosine modulators facilitate and potentate the action of adenosine agonists at the A1 receptors. The purpose of the current study was to examine the effect and site of action for an allosteric adenosine modulator, T62 [2-Amino-3-(4-chlorobenzoyl)-5,6,7,8-tetrahydrobenzothiothiophene], in rat models of acute pain and inflammation. Intrathecal (i.t.) T62 did not change the withdrawal latency or threshold of normal rats to acute heat, or to acute paw pressure. In contrast, i.t.T62 reversed thermal hypersensitivity in carrageenin-inflamed rats. Subcutaneous (s.c.) injection of T62 into the inflamed paw had no such effect. To investigate a potential site of action on nociceptors, single unit afferent activity to mechanical stimuli on A $\delta$  and C fibers was examined in normal or carrageenin-inflamed rats before and after intravenous (i.v.) T62 administration. I.v. T62, 3 mg/kg had no significant effect in either normal or inflamed conditions. These results support previous studies to suggest that adenosine receptor modulators lack efficacy to acute nociceptive stimuli in the normal condition, but reduce hypersensitivity during inflammation through a central mechanism.

Adenosine receptor agonists and analogs produce antinociception in a variety of pain models (Keil and DeLander, 1992), including acute (Post, 1984; Fastbom et al., 1990; Sosnowski et al., 1989; Sollevi, 1997), neuropathic (von Heijne et al., 1999; Karlsten and Gordh, Jr., 1995; Sollevi et al., 1995; Sjolund et al., 1998) and inflammatory pain (Malmberg and Yaksh, 1993; Poon and Sawynok, 1999; Sawynok et al., 1998) in human and animals. However, unlike direct adenosine receptor agonists such as R-phenylisopropyl-adenosine (R-PIA) (Sosnowski et al., 1989) or 5'-N-ethylcarboxamide adenosine (NECA) (Post, 1984), intrathecal injection of adenosine itself does not produce antinociception to acute stimuli in normal animals (Eisenach et al., 2002). As an adenosine receptor modulator, T62 [2-Amino-3-(4-chlorobenzoyl)-5,6,7,8-tetrahydrobenzothiothiophene] has been previously shown to reduce mechanical allodynia in rats with spinal nerve ligation via a mechanism involving spinal A1 adenosine receptors (Pan et al., 2001; Li et al., 2002). However, the effects of adenosine receptor modulators in inflammation-induced hypersensitivity have not previously been reported.

The purpose of the current study was therefore to evaluate the efficacy of the adenosine modulator, T62, in normal and inflamed rats and to test, using different methods of administration, its site of action. Both thermal (Hargreaves et al., 1988; Dirig et al., 1997), and mechanical withdrawal thresholds (Randall-Sellito) were determined. In carrageenin-inflamed rats, thermal threshold was determined after i.t., iv., and s.c administration of T62. Finally, in both normal and inflamed rats, the effect of i.v T62 on single-unit afferents was determined in normal and inflamed conditions.

## MATERIALS AND METHODS

**Intrathecal catheter insertion.** After Animal Care and Use Committee approval, male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 250 g were used. An intrathecal catheter was inserted as previously described (Yaksh and Rudy, 1976) under halothane anesthesia by insertion of polyethylene tubing through a small hole in the cisterna magnum and advancement caudad 8 cm such that the tip of the catheter lay in the intrathecal space at the lumbar enlargement. After surgery, 90% animals showed normal neurologic behavior. Rats showing neurologic deficits were immediately euthanized by an overdose of pentobarbital; the other animals were allowed to recover 4-5 days before drug testing.

**Thermal testing in normal rats.** To examine the effect of T62 in normal rats to noxious heat, a plantar withdrawal method (Dirig et al, 1997) was used, with a lamp intensity set at 5.0 and 5.25 A. Following habituation to the environment of a clear plastic box on a raised floor of a clear heat-tempered glass for 30–45 min, a projection bulb was illuminated and focused on the plantar surface of one hind paw. Latency to withdrawal was determined and a cut off value of 30 sec was not exceeded. Rats were intrathecally injected with vehicle alone or one of 4 doses of T62 (0.5, 2, 10 or 40  $\mu$ g), n=6 per group. The experiments were executed by mixed and double blinded drug dosing. Each rat received a maximum of four drug injections with experiments separated by at least one week (drug dose applied randomly). After drug or vehicle treatment, the withdrawal latency (an average of two tests at each time point, the same for the following inflamed rats) was determined before and 30, 60, 90, 120, 150, 180, 210, 240, 270, and 300 min after injection. Withdrawal latency data were analyzed by two-way analysis of

variance followed by Fisher's PLSD test (Shapira et al., 1994).  $P < 0.05$  was considered significant.

**Mechanical testing in normal rats.** One week after i.t. catheter implantation, a group of 18 rats was used to test the effect of T62 against noxious acute mechanical stimuli, using a Randall-Sellito device (Ugo Basile, Type 7200, Italy). Vehicle or one of 3 i.t. doses of T62 (1, 2 or 20  $\mu\text{g}$ ) was injected, with  $n=6$  per group. The experiments were executed by mixed and double blinded drug dosing. Each rat received a maximum of four drug injections with experiments separated by at least one week (drug dose applied randomly). After drug or vehicle injection, the withdraw threshold (an average of two tests at each time point) was determined before and 30, 60, 90, 120, 150, 180, 210, 240, 270, and 300 min after T62 injection. Mechanical withdrawal threshold data were analyzed by two-way analysis of variance followed by Fisher's PLSD test (Shapira et al, 1994).  $P < 0.05$  was considered significant.

**Carrageenin-inflammation: I.t. T62.** One week after i.t. catheter implantation, 34 rats were used to test the efficacy of i.t T62 following carrageenin-inflammation (Eisenach and Gebhart, 1995). Briefly, the rat was habituated to the testing environment, and baseline hind paw withdrawal latencies were obtained to radiant heat, as described as above (Dirig et al, 1997). Unilateral inflammation was induced by intraplantar injection in the right hind paw of 2 mg freshly prepared carrageenin in 0.1 ml normal saline. Three hours after carrageenin injection, vehicle or T62 (0.2, 2 or 10  $\mu\text{g}$ ;  $n=7-11$  per group) was injected intrathecally in less than 20  $\mu\text{l}$  total volume with 5-10  $\mu\text{l}$  of saline for flush. In all studies, baseline withdrawal latencies were obtained immediately before carrageenin injection, 3 hr after carrageenin, then at 30-min intervals after drug injection for another 3

hours. Hind paw thickness at the mid-plantar level was determined before and at the end of each experiment involving carrageenin injection using a calibrated micrometer. Each rat was studied only once and was killed at the end of the experiment by an overdose of pentobarbital.

**Carrageenin-inflammation: I.v. T62.** Under halothane anesthesia, polyethylene tubing was inserted into right jugular vein and the catheter was externalized for drug administration. After surgery, all rats were allowed to rest for a week before study. On the study day, rats were injected with 2 mg freshly prepared carrageenin. Three hours later, a single dose of T62 at 3 mg/kg was injected intravenously, and the withdrawal latency data were recorded for 300 min as described above, (n=6).

**Carrageenin-inflammation: Local T62 administration.** On the study day, 4 rats were injected with 2 mg freshly prepared carrageenin. Three hours later, a single dose of T62, 25 µg, was injected subcutaneously into the inflamed hind paw, and the withdrawal latency was recorded for 300 min as described above.

**Single-unit afferent recording.** Under halothane anesthesia (1-1.5% halothane through out the experiment), the left carotid artery was cannulated for monitoring blood pressure. The trachea was cannulated and the rat was mechanically ventilated. The right jugular vein was cannulated with polyethylene tubing for drug injection. At the end of preparative surgery, the rat was paralyzed with pancuronium bromide (1mg/kg), with supplementary doses given at approximately 1-h intervals. Rectal temperature was maintained in the range of 37-38°C with circulating water heating pad and heat lamps throughout the experiment. The left sciatic nerve at the middle and distal part was exposed through a restricted skin incision over the posterior hindlimb, and the overlying

fascia and sheath were carefully removed. The nerve was then draped on a platform and covered with warm mineral oil. Small nerve filaments were transected in the middle of the sciatic nerve, teased gently from the nerve segment under an operating microscope (model M900; D.F. Vasconcellos S.A., São Paulo, Brazil), and connected to the recording electrode of a bipolar stainless electrode. Reference electrodes were placed on the surrounding tissues. The nerve filaments were then dissected gradually until single-unit activity of afferents was detected. The action potential of the afferent was amplified 10,000-30,000x by an AC differential amplifier (DAM80, World Precision Instruments, Sarasota, IL), and output to an audio amplifier (AM8; Grass Instrument, W. Warwick, RI) and displayed on an oscilloscope (450; Gould, Cleveland, OH). The neurogram was recorded on a thermal-sensitive recorder (K2G; Astro-Med, W. Warwick, RI). A single unit was identified initially by examining the wave form and the spike amplitude on the oscilloscope at a rapid sweep speed as well as by checking the recorded sound frequency related to each spike activity. Furthermore, the signals were digitized at a sampling rate of 20 kHz and fed into a PC compatible computer through an analog-to-digital interface card for subsequent off-line analysis. An amplitude threshold was set for recording firing frequency. When nerve activity was detected, the associated wave form (6 ms) would be extracted and displayed continuously in a separate software oscilloscope window (DataWave Technology, Inc., Longmont, CO). Single-unit recording was ensured by checking the constancy of the shape and polarity of the displayed spike wave form. Discharge frequency was quantified by using data acquisition and analysis software (DataWave Technology), and a histogram was created for each filament. Accurate counting of the afferent discharge frequency (an average of 4 seconds) was verified for

each afferent by comparing the constructed histogram with the hard copy, which was recorded simultaneously.

**Isolation, identification, and classification of fibers.** To search for units we used two approaches: (1) electrical stimulation of sciatic nerve fibers at a site between the recording site and the fiber's receptive field (RF); (2) mechanical stimulation of their hindpaw receptive fields. Electrical stimulation was performed with transcutaneous needle electrodes that were placed in the heel area to permit multiunit stimulation of plantar and sural nerve fibers. Stimulus duration was 0.2 msec for A $\beta$ - and A $\delta$ -fibers and 0.5-0.75 msec for C-fibers. The amplitude of stimuli was usually set at 1.5 times the fiber response threshold, whereas the frequency of stimuli was usually 1 Hz. Responses to natural stimulation of their receptive fields and conduction velocity (CV) of fibers were the main criteria used for physiological characterization and classification. Two types of mechanical stimulation of receptive fields were used: (1) non-calibrated search stimuli such as tapping, stroking, or moderately firm pressure applied with a cotton-tipped swab to plantar surfaces; (2) stimuli using calibrated von Frey hairs (Stoelting, Wood Dale, IL) for more precise physiological characterization of units. Conduction velocities were calculated by dividing the distance between the stimulating and recording electrodes by the latency of the electrically evoked spike. Units with CVs >15 m/sec were identified as A $\beta$ -fibers, units with CVs of 2.5-15 m/sec were identified as A $\delta$ -fibers, and units with CVs <2.5 m/sec were identified as C-fibers.

**Afferent activity in normal animals.** After obtaining a stable single-unit afferent recording, the baseline discharge and response to a set of stimuli from calibrated von Frey filaments (10 sec application with 1 min interval) were recorded in duplicate over 15

to 30 min. These measurements were repeated at 30 and 60 min after i.v. T62 injection, 3 mg/kg. After the last stimulus, the conduction velocity of the recorded afferents was measured using electrical stimulation. Finally, the rats were sacrificed by an overdose of pentobarbital.

**Afferent activity following inflammation.** After obtaining a single-unit afferent recording, the baseline discharge and response to a set of stimuli from von Frey filaments were recorded as described above. Two mg freshly prepared carrageenin solution, 0.1 ml, was injected into the RF. Three hours after the carrageenin injection, the baseline discharge and response to von Frey filament stimulation in the RF were recorded again, followed by i.v. T62 injection, 3 mg/kg. Then, the baseline discharge and response to von Frey filament stimulation in the RF were recorded 30 and 60 min after injection.

Electrophysiological data are presented as mean  $\pm$  S.E.M. Discharge activity of afferents was averaged before and after each T62 treatment. The T62 effect on afferent activity was determined by analysis of variance followed by the Dunnett's post hoc test.  $P < 0.05$  was considered to be statistically significant.

**Materials.** T62 was provided by King Pharmaceuticals (Cary, NC). Pancuronium Bromide was purchased from Abbott Laboratories (North Chicago, IL). Carrageenin, dimethyl sulfoxide (DMSO) and remaining chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Hydroxypropyl- $\beta$ -cyclodextrin was dissolved as a 45% solution in saline as drug vehicle. T62 was gradually dissolved into cyclodextrin solution to a final concentration of 0.2  $\mu\text{g}/\mu\text{l}$  as a stock solution for i.t. injection and 1  $\mu\text{g}/\mu\text{l}$  for i.v. injection, or dissolved in DMSO/cyclodextrin (1/100) mixture at 1  $\mu\text{g}/\mu\text{l}$  as a stock solution for 10  $\mu\text{g}$  i.t. injection.

## Results

**I.t. T62 in normal rats.** Baseline withdrawal latency to thermal testing for normal rats was 11 sec. The vehicle, cyclodextrin, gradually increased withdrawal latency after i.t. injection, reaching a plateau of 17 sec at 210 min after injection (Fig. 1). Withdrawal latency also increased over a 300 min period in animals receiving i.t T62. However, the withdrawal latency increased after i.t T62 injection was not significantly different from that of i.t vehicle, and there was no dose dependency to the effect of T62 over the range of doses used.

Baseline withdrawal threshold to paw pressure using the Randall-Sellito apparatus in normal rats was 126 g. I.t. injection of vehicle did not significantly affect withdrawal threshold to this mechanical stimulus. Similarly, no dose of i.t. T62 affected withdrawal threshold (Fig. 2).

**T62 in carrageenin inflammation.** After receiving 2 mg freshly prepared intraplantar carrageenin, the withdrawal latency to thermal testing was reduced from 10 s to 3.5 s (Fig. 3). I.t T62 reversed this hypersensitivity to pre-carrageenin levels in a time- and dose-dependent manner over the 300 min observation period. Although thermal hypersensitivity slowly diminished (withdrawal latency increased) over this 300 min period in vehicle controls, the effect of T62 was nonetheless obvious and significant. In contrast, i.t T62 did not affect paw edema induced by carrageenin injection (Fig. 4).

I.v. T62, 3 mg/kg, produced a modest increase in withdrawal latency to the thermal stimulation in carrageenin-inflamed rats (Fig. 5). In contrast, subcutaneous injection of T62, 25  $\mu$ g into the inflamed hind paw failed to affect withdrawal latency during carrageenin inflammation (Fig. 6).

**Effect of i.v. T62 on single unit afferent activity in normal and inflamed rats.**

Data were obtained from four A $\delta$  (average CV at 4.4 m/s) and six C (average CV at 0.84m/s) fibers in normal rats (Fig. 7), and five A $\delta$  (average CV at 6.6 m/s) and five C (average CV at 1.6 m/s) fibers (Fig.8) in carrageenin inflamed rats. Mechanical stimulation with calibrated von Frey filaments increased the unit firing frequency of both A $\delta$  and C fibers in a stimulus-dependent manner. Neither A $\delta$  nor C fiber responses to mechanical stimulation were inhibited by i.v. T62 in normal rats. Similarly, T62 had no effect on response to mechanical stimulation in A $\delta$  nor C fibers after carrageenin inflammation (Fig. 8)

## Discussion

In accordance with previous studies using the endogenous ligand, adenosine, i.t. injection of the positive allosteric modulator of adenosine receptor, T62, failed to produce antinociception in normal animals, but reversed hypersensitivity in rats with inflammation from carrageenin injection. As an adenosine receptor modulator, T62, facilitates activation of the G-protein coupled adenosine A1 receptor by adenosine through binding to an allosteric site on A1 receptors (Bruns and Fergus, 1990). Previous studies using synthetic adenosine analogs, either nonselective or preferring the A1 receptor, demonstrate both antinociception in the normal animal to mechanical stimuli (Keil and DeLander, 1992), as well as reversal of the hypersensitivity in animals with inflammation (Malmberg and Yaksh, 1993). However, studies in humans suggest that i.t. adenosine itself does not cause antinociception to acute thermal stimuli (Eisenach et al, 2002). The results from current study are therefore in agreements with previous results with the endogenous ligand adenosine.

Single nerve fiber recording is a classical and useful method for studies of peripheral action since they preclude by their nature any effect in central nervous system (Pogatzki et al., 2002). The lack of effect of i.v. T62 to reduce afferent activity in either the normal or inflamed condition is consistent with a central, but not peripheral site of action of this drug. In contrast, 0.2  $\mu\text{g}$  i.t. T62 almost totally reversed the hypersensitivity from carrageenin inflammation, consistent with a spinal site of action. In agreement with this hypothesis, we previously demonstrated that the anti-hypersensitivity effect on mechanical stimulation of oral T62 following peripheral nerve injury was reversed by i.t. injection of A1 adenosine receptor antagonist (Li et al, 2002).

Furthermore, injection of T62 directly in the inflamed paw in the current study failed to affect thermal hypersensitivity or paw edema, suggesting an action for T62 at nerve terminals is unlikely. According to previous results (Segerdahl and Sollevi, 1998), central sites of actions most likely explain analgesic actions of adenosine through A1 receptor. Besides changes in receptive field area and sensitivity, carrageenin inflammation causes sensitization of spinal cord neurons (Neumann et al., 1996). However, the above results do not necessarily support an absence of A1 receptors at peripheral sites. Although A1 receptors have not been reported by immunohistochemistry in peripheral afferent terminals, they are present on dorsal root ganglion cell bodies (Macdonald et al., 1986) and central terminals of primary afferent neurons (Santicioli et al., 1993). In addition, peripheral A1 receptor activation by synthetic adenosine analogs (Karlsten et al., 1992) or metabolism inhibitors (Sawynok et al., 1998) reduces hypersensitivity in rats with peripheral inflammation. The lack of efficacy of peripherally applied exogenous adenosine or T62 in reducing hypersensitivity caused by inflammation is curious in this regard, and may reflect rapid absorption in the case of adenosine or minimal endogenous adenosine release at the site of inflammation in the case of T62 .

In addition, carrageenin paw injection is a commonly used model of inflammatory pain, resulting in hypersensitivity to both thermal and mechanical stimuli following intraplantar injection. The inflammation caused by carrageenin results in an increase in receptive field size and spontaneous nerve fiber activity (Woolf and King, 1990; Neumann et al., 1996). In addition, adenosine A2 receptor activation produces anti-inflammatory effect in rats after carrageenin (Cronstein et al., 1995). In the current study,

T62 did not alter inflammatory edema, consistent with the notion that T62 does not act on A2 receptors.

In a summary, intrathecal and intravenous, but not local injection of the positive allosteric modulator of A1 adenosine receptors, T62, reverse hypersensitivity from carrageenin-induced inflammation. These results coupled with the lack of effect of T62 in normal animals and its lack of effect on afferent activity, suggest that central, likely spinal changes in the sensitization uncover the analgesic potential of adenosine and adenosine modulators.

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## Legends for figures

- Figure 1:** Withdrawal latency from thermal stimulation before and after intrathecal injection at time 0 of vehicle or T-62 effect in normal rats. . No significant differences within or between groups.
- Figure 2:** Withdrawal threshold to mechanical stimulation before and after intrathecal injection at time 0 of vehicle or T-62 effect in normal rats. No significant differences within or between groups.
- Figure 3:** Withdrawal latency to thermal stimulation before and after intrathecal injection, at time 0, of vehicle or T-62 effect in carrageenin inflamed rats. \$ P<0.05 between T-62 groups and vehicle by two way repeated measures ANOVA. \*, &, # or \*\* -- P < 0.05 within each T-62 group for change from time 0 by one way repeated measures ANOVA.
- Figure 4:** Rat hind paw thickness before and 3 hours after carrageenin inflammation in vehicle or intrathecal T-62 treated animals. No significant differences within or between groups.
- Figure 5:** Withdrawal latency to thermal stimulation before and after intravenous injection, at time 0, of T62, 3 mg/kg, in carrageenin inflamed rats.  
\* P<0.05 compared to time 0.
- Figure 6:** Withdrawal latency to thermal stimulation before and after subcutaneous injection, at time 0, of T62, 25 µg, into the paw of carrageenin inflamed rats. No significant differences compared to time 0.

**Figure 7:** Stimulus response of single afferent A fibers (A) or C fibers (B) to von Frey hair (VFH) testing before and at 30 and 60 min after intravenous injection of T62, 3 mg/kg, in normal rats. No significant differences between responses before and after T62 administration.

**Figure 8:** Stimulus response of single afferent A fibers (A) or C fibers (B) to von Frey hair (VFH) testing before and at 30 and 60 min after intravenous injection of T62, 3 mg/kg, in carrageenin inflamed rats. No significant differences between responses before and after T62 administration.



















