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Allosteric Adenosine Modulation to Reduce Allodynia

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Background: **Adenosine and adenosine agonists reduce hypersensitivity following inflammation and peripheral nerve injury models of chronic pain. Because inhibitors of adenosine reuptake or metabolism are also effective at reducing hypersensitivity, it is likely that there is a tonic release of spinal adenosine in these models. One approach to avoid adverse effects from direct agonists is to enhance the effect of the endogenous ligand by administering a positive allosteric modulator of its receptor.**

Methods: **Rats with mechanical hypersensitivity after spinal nerve ligation received intrathecal injections of adenosine, the allosteric adenosine receptor modulator T62, or their combination, or received systemic T62 alone or with intrathecal injection of a specific A1 adenosine antagonist.**

Results: **Both adenosine and T62 reduced hypersensitivity alone, with 50% maximal doses (ED_{50}) of 14 ± 5.9 and 3.7 ± 0.8 μg, respectively. They interacted in an additive manner as determined by isobolography. T62 also reduced mechanical hypersensitivity after systemic administration (15 mg/kg), and this effect was blocked by intrathecal injection of 9 μg of the A1-specific adenosine receptor antagonist 8-cyclopentyl-1, 3-dipropylxanthine.**

Conclusions: **These results add to previous studies that suggest ongoing spinal release of adenosine, which is antiallodynic, in this animal model of neuropathic pain. Positive allosteric modulation of the adenosine receptor reduces hypersensitivity by a spinal mechanism involving A1 adenosine receptor stimulation. Although obvious adverse effects were not observed in this investigation, further study is required to determine the feasibility of the use of such modulators in the treatment of chronic pain associated with hyperalgesia and allodynia.**

PERIPHERAL nerve injury and inflammation can result in chronic pain associated with hypersensitivity to normally nonpainful or painful stimuli, termed allodynia and hyperalgesia, respectively. These elicited pains are frequently difficult to treat with standard analgesics, and the response to nontraditional agents, such as antidepressants, local anesthetics, and antiepileptics, is often incomplete or not realizable because of a high incidence of adverse effects. For this reason, there is a need for development of novel and effective analgesics for these types of pain.

Considerable recent work has demonstrated a remarkable neuronal plasticity associated with peripheral nerve injury, which may underlie hypersensitivity. This is reflected in the loss of efficacy of some classes of traditional agents, such as opioids;[1] increased efficacy of some classes of analgesics, such as α_2-adrenergic agonists;[2] and expression of efficacy of agents that are not analgesic in normal animals, such as the tricyclic antidepressants or gabapentin.[3] Adenosine is an example of the latter group in that intrathecal injection of adenosine has little or no effect in the normal animal but reduces mechanical hypersensitivity after surgery or spinal nerve ligation in rats.[4,5] Pharmacologic studies are consistent with an action of adenosine on spinal A1 receptors to produce this effect.[6]

A common problem with administration of direct agonists, like adenosine, are adverse effects caused by stimulation of all receptors, not just those involved in the therapeutic effect. One approach to obviate this problem is the use of positive allosteric modulators, as exemplified by the use of benzodiazepines to enhance the effect of γ-aminobutyric acid. Because we previously observed evidence for tonic spinal adenosine release after spinal nerve ligation,[5] the purpose of the current study was to test the efficacy and mechanism of action of a positive allosteric modulator of adenosine receptors in this model of hypersensitivity.

Methods

Animal Preparation

After Animal Care and Use Committee approval, mechanical hypersensitivity was generated in male Sprague-Dawley rats as previously described.[7] Briefly, under general anesthesia with inhalational halothane, the left L5 and L6 spinal nerves were identified through a small laminotomy and were tightly ligated. Approximately 1 week later, a polyethylene-10 intrathecal catheter was placed under general anesthesia by insertion under direct vision of a polyethylene catheter through a small slit in the dura at the cisterna magnum and advanced 8.5 cm such that the catheter tip resided in the lower lumbar intrathecal space. Proper location was confirmed by bilateral motor blockade after injection of 5 μl lidocaine, 2%. Animals were studied approximately 1 week later.

Ligation of the lumbar spinal nerves results in a primarily unilateral increase in the sensitivity to light touch on the side of surgery. Sensitivity was assessed *via* application of calibrated von Frey filaments. The withdrawal threshold was determined using an up–down method previously described.[8] In general, withdrawal threshold is 35 g or greater in animals before surgery,

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which is markedly reduced to less than 4 g within 1 week of spinal nerve ligation, and this hypersensitivity is stable for several weeks thereafter.

Drug Treatment

Animals were randomized to receive a single intrathecal injection of 1, 3, or 5 μg of the allosteric adenosine receptor modulator T62 or 5, 10, or 30 μg adenosine. Doses of T62 and adenosine were determined in pilot experiments or from previously completed studies to encompass the therapeutically effective range. Animals received six injections by the same individual, each separated by a minimum of 4 days to prevent development of tolerance. The withdrawal threshold to mechanical stimulation with von Frey filaments was determined before and at intervals for 3 h after intrathecal injection and then again at 20 h after injection. A dose response was determined using the time of peak effect (2 h for each compound), and the ED_{50} was determined by linear regression.

To determine the type of interaction between intrathecal adenosine and T62, an isobolographic approach was used. In this design, adenosine and T62 were combined in the ratio of their ED_{50}s, and this combination was administered in different doses in a constant ratio (adenosine:T62 ratio of 7:1.8, 14:3.5, or 24:6 μg). The ED_{50} for the mixture (total of each component combined) was determined by linear regression, an isobologram was constructed, and the observed ED_{50} was compared with the theoretical ED_{50} by a *t* test as previously described.[9]

To determine the site of action of T62, two experiments were performed. First, the effect of intraperitoneal injection of T62, 15 mg/kg, was tested and was noted to be effective to reduce hypersensitivity (see Results). The effect of systemic T62 was then tested 5 min after intrathecal injection of the A1 receptor-preferring antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 9 μg. This dose has been previously demonstrated to block the effect of adenosine to reduce hypersensitivity in rats after spinal nerve ligation.[6]

Adenosine A1 agonists produce behavioral sedation and motor blockade when administered in high doses.[10] To screen for these effects, animals receiving drug treatment were observed for changes in normal exploratory behavior when placed on an open surface and were observed for signs of abnormal ambulation.

Materials

The drugs used were adenosine (Adenocard; Fujisawa, Deerfield, IL), DPCPX (RBI, Natick, MA), and T62 (Medco Research, Inc., Cary, NC). T62 was dissolved in 45% 2-hydroxypropyl-ω-cyclodextrin (RBI). Drugs were administered intrathecally in a 5-μl volume followed by 10 μl saline to flush the dead space of the catheter.

Data Analysis

Data are presented as median ± 25th and 75th percentiles (for raw withdrawal thresholds) or by mean ± SE. Linear regression was used to calculate the ED_{50} for each drug alone and for the fixed ratio combination. The ED_{50} was determined for each animal rather than using a probit analysis of the entire data set. The effect of each agent alone and the combination on withdrawal threshold over time was tested by Kruskal-Wallis analysis followed by Wilcoxon rank sum test. $P < 0.05$ was considered significant.

Results

Intrathecal Administration Studies

Intrathecal injection of T62 produced dose- and time-dependent increases in withdrawal threshold, returning sensitivity to preligation baseline (35 g, 25th and 75th percentiles = 32.2 and 38.7 g) after the 5-μg dose (fig. 1, molecular structure inset, top). The ED_{50} of T62 was 3.7 ± 0.8 μg. Similarly, intrathecal adenosine produced a dose- and time-dependent increase in withdrawal threshold, returning sensitivity to preligation baseline at the 30-μg dose (fig. 1, middle). The ED_{50} of adenosine was 14 ± 5.9 μg, similar to that which we previously reported.[5] Based on these results, adenosine and T62 were combined in a 4:1 ratio. This combination also produced dose- and time-dependent increases in withdrawal threshold, returning it to preligation baseline at the 30-μg combined dose (fig. 1, bottom). The ED_{50} of this combination was 15 ± 2.9 μg (12 ± 2.3 μg adenosine plus 3 ± 0.6 μg T62). Injection of vehicle, either saline or cyclodextrin, had no effect on withdrawal threshold (data not shown). Adenosine, 30 μg, resulted in sedation, as evidenced by lack of exploration of an open environment, but no abnormal gait indicating motor blockade. T62 or the combination had no effect on exploration or walking behavior.

An isobologram is constructed by plotting the ED_{50} of one drug alone on the x-axis and the other drug alone on the y-axis. The ED_{50} of the combination is plotted and compared with the line connecting the individual drug ED_{50}. Combination ED_{50} values that lie to the left of and below this line indicate synergy, whereas those to the right of and above the line indicate antagonism. The isobologram for the T62–adenosine interaction demonstrates additivity in that the combination value does not differ significantly from the line of additivity (fig. 2).

Systemic Administration Studies

Intraperitoneal T62, 15 mg/kg, produced an increase in withdrawal threshold, peaking 90 min after injection, and was still significantly above the allodynic baseline 6 h after injection (fig. 3). Animal behavior was apparently normal after T62 treatment. The antiallodynic ef-

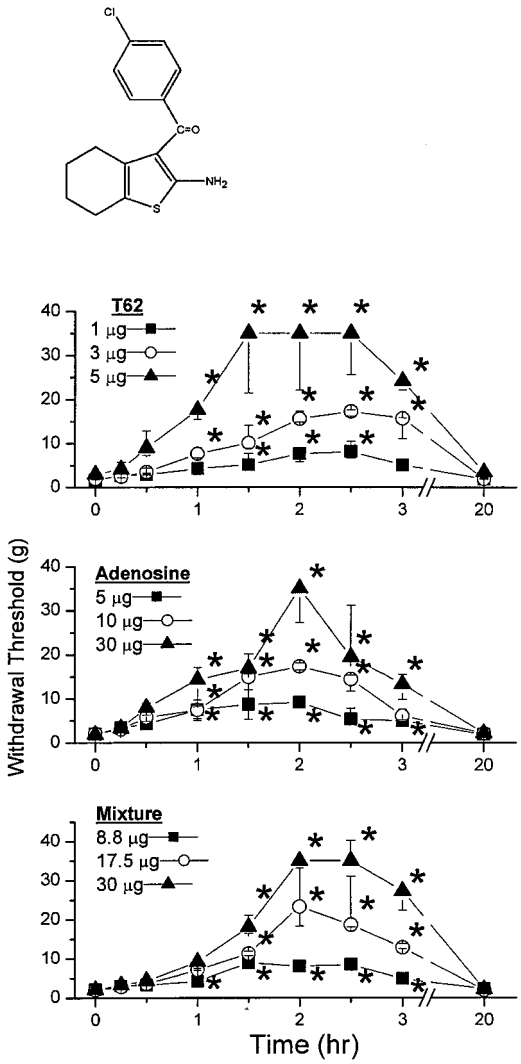

Fig. 1. Hind paw withdrawal threshold *versus* time after intrathecal injection of a low (■), an intermediate (○), or a high (▲) intrathecal dose of the allosteric adenosine receptor modulator T62 (*top, molecular structure inset*), adenosine (*middle*), or a 1:4 fixed ratio combination of adenosine and T62 (*top*). Doses are indicated in the figure. Data are presented as median ± 25th and 75th percentiles. **P* < 0.05 compared with preinjection value.

fect of T62 was blocked by intrathecal pretreatment with the A1 adenosine antagonist DPCPX (fig. 3).

Discussion

The first adenosine receptor modulators were characterized by Bruns and Fergus in 1990[11] and are considered to act by enhancing A1 agonist binding and slowing A1 agonist dissociation. To our knowledge, no modulator has been studied in neuropathic pain models. All the known modulators, including T62, are derivatives of the 2-amino-3-benzoylthiophenes first described by Bruns and Fergus.[11] The chemical name of T62 is [2-Amino-4,5,6,7-tetrahydrobenzo[b]thiophen-3-yl) (4-chlorophenyl) methanone] (fig. 1). The *in vitro* pharmacology of T62 has been described by Bruns *et al.*[12] and Van der Klein *et al.*[13] In these studies, T62 was found to be an enhancer with EC_{50} values of 15.3 and 6.8 μM, respectively. Because the benzoylthiophenes only selectively retard the dissociation of the receptor-ligand complex when an agonist radioligand is used, the benzoylthiophenes must bind to a site different from the agonist recognition site. This putative site is termed the allosteric site, and presumably, compounds that bind to this site and enhance the agonist effect are termed allosteric enhancers. This is the first examination of an allosteric modulator of adenosine receptors to treat pain.

The rationale for use of an adenosine receptor modulator assumes there is ongoing exposure of the receptor to agonist. Indeed, the benefit of this approach assumes there is a site and tissue-specific release of agonist such that administration of the modulator results in enhanced activity at the therapeutic site but not at others that result in unwanted effects. There is only indirect evidence that adenosine is tonically released in the spinal cord after peripheral nerve injury. For example, we[5] and others[14–19] have demonstrated efficacy of inhibitors of adenosine reuptake or metabolism in various pain models in rodents, consistent with ongoing release. Similarly, one small clinical trial in poorly characterized patients with chronic pain demonstrated analgesic efficacy from dipyridamole, an inhibitor of adenosine reuptake.[20]

However, the current study, as a previous one,[6] failed to demonstrate worsening hypersensitivity after spinal nerve ligation with intrathecal injection of an A1 adenosine antagonist. Although this could argue against ongoing spinal adenosine release, the extreme hypersensitivity to mechanical stimulation may have limited our

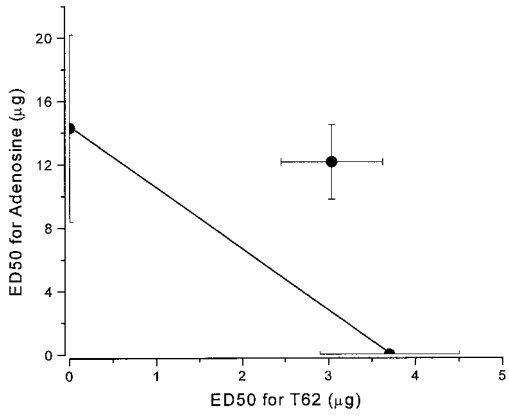

Fig. 2. Isobologram at the 50% maximum effective dose (ED_{50}) level of the interaction between intrathecal adenosine and T62. The value for the ED_{50} (and their 95% confidence limits) for each agent alone are depicted on the axes, with the line between them representing the line of additivity. The value for the ED_{50} of the combination does not differ significantly from the theoretical additive point.

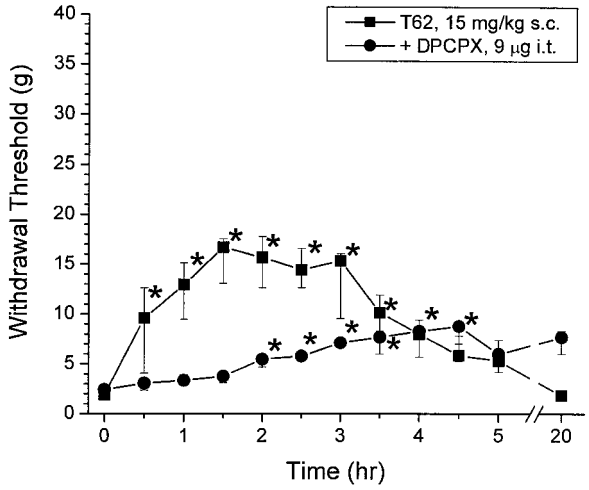

Fig. 3. Hind paw withdrawal threshold over time in rats receiving intraperitoneal injections of the allosteric adenosine receptor modulator T62, 15 mg/kg, alone (■) or preceded by intrathecal (i.t.) injection of 9 μg of the A1 adenosine receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, ●). Data are median ± 25th and 75th percentiles. *$P < 0.05$ compared with preinjection value. Groups differ by two-way analysis of variance. s.c. = subcutaneous.

ability to observe increased hypersensitivity (yet lower withdrawal threshold) in this model.

The additive interaction between T62 and adenosine suggests that this mixture might be useful, at least after intrathecal injection, in the treatment of chronic pain. Mechanistic considerations of modulators do not necessarily exclude additive, as opposed to synergistic interactions. Indeed, one might predict that the combination of an allosteric modulator with a direct agonist would result in an additive rather than synergistic interaction because both are acting by a final common pathway—G protein activation from A1 adenosine receptor stimulation. Figure 2 suggests that there could be active antagonism of the effect of adenosine by T62 because the combination group was to the right of the line of additivity. Although such antagonism has been observed with high concentrations of other drugs in the same family as T62, this has not been observed with this compound.[12,13]

Combination therapy is only useful if efficacy is enhanced or adverse effects are diminished compared with single agent approaches. The primary purpose of the current study was not to determine the nature of interaction between the allosteric modulator and adenosine itself in this regard. Adverse effects from intrathecal adenosine in humans include primarily transient headache and back or leg pain. Although no obvious behavioral toxicity was observed in these animals, the sensitivity of this model to determine these types of adverse effects is probably quite low, limiting the certainty of conclusions regarding these adverse effects.

Adenosine receptors can modulate pain and sensory processing at several peripheral and central sites. Thus, others have observed pain relief in patients with chronic pain after systemic adenosine infusion[21] or intrathecal injection.[22] That T62 demonstrated efficacy with systemic administration suggests that the simpler systemic route might be applicable in the treatment of clinical pain. However, the DPCPX experiment suggests that the primary site of action of this compound, even after systemic administration, is in the spinal cord. This is further supported by the large discrepancy in dose required to reduce hypersensitivity, being approximately three orders of magnitude greater after systemic administration than after intrathecal administration. Finally, T62 probably produces antiallodynia in this model by increased activity of A1 adenosine receptors because it is reversed by a small dose of a specific A1 adenosine antagonist.

The predictive value of the spinal nerve ligation model to treatment of patients with neuropathic pain is uncertain. However, studies demonstrate a similar pharmacology of several classes of agents in this model and in patients with chronic pain. Similarly, initial experience with intrathecal adenosine in patients with neuropathic pain suggests that it is effective,[22] similar to the effectiveness observed in the animal model. As such, one would predict, based on these results, that T62 would relieve hypersensitivity and elicited pain in patients with neuropathic pain after intrathecal administration.

Conclusion

In conclusion, intrathecal injection of a positive allosteric modulator of adenosine A1 receptors reduces mechanical hypersensitivity in a rat model of neuropathic pain, consistent with ongoing spinal adenosine release in this model. There is an additive interaction between the modulator and adenosine itself, as predicted by a final common mechanism of action. Antagonist studies are consistent with a spinal site of action on A1 adenosine receptors.

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