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42. The concept, design, and construction of the aerosol apparatus was carried out in collaboration with J. C. Hemminger, F. S. Rowland, and D. W. Toohey and was supported by NSF (grant ATM-9512181) and the University of California, Irvine. We are also grateful to NSF (grant ATM-9741817), especially J. L. Moyers, and the U.S. Department of Energy (grant DE-FG-03-94ER61899) for support. We thank P. Poyner, L. Moritz, and J. Meyer for assistance with the chamber design and construction, J. N. Pitts Jr., J. Stutz, E. G. Chapman, C. M. Berkowitz, and W. C. Keene for helpful discussions, and C. W. Spicer for advice on API-MS and for providing the annual denuder.

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Broad-Spectrum, Non-Opioid Analgesic Activity by Selective Modulation of Neuronal Nicotinic Acetylcholine Receptors

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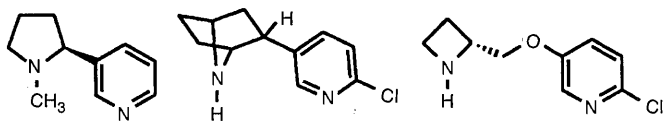
Development of analgesic agents for the treatment of severe pain requires the identification of compounds that are devoid of opioid receptor liabilities. A potent (inhibition constant = 37 picomolar) neuronal nicotinic acetylcholine receptor (nAChR) ligand called ABT-594 was developed that has antinociceptive properties equal in efficacy to those of morphine across a series of diverse animal models of acute thermal, persistent chemical, and neuropathic pain states. These effects were blocked by the nAChR antagonist mecamylamine. In contrast to morphine, repeated treatment with ABT-594 did not appear to elicit opioid-like withdrawal or physical dependence. Thus, ABT-594 may be an analgesic that lacks the problems associated with opioid analgesia.

Systemic administration of opioid analgesics such as morphine remains the most effective means of alleviating severe pain across a wide range of conditions that includes acute, persistent inflammatory, and neuropathic pain states (*1*). Despite the broad-spectrum analgesic actions of the opioids, their clinical use is limited by side effects such as respiratory depression, constipation, and physical dependence as well as scheduling constraints and perceived abuse liabilities (*2*). Efforts to develop new generations of analgesics for the treatment of moderate to severe pain states based on advances in the understanding of endogenous opiate systems have resulted in only modest incremental improvements (*3*). Thus, the challenge in developing therapies for the management of severe pain has been the identification of compounds that are devoid of opioid receptor interactions and consequently of opioid-related liabilities, yet have a defined mechanism of action that is related to pain-processing mechanisms.

The antinociceptive activity of (−)−nicotine was reported as early as 1932 (*4*). However, (−)−nicotine has not been developed as an analgesic agent because of its poor spectrum of antinociceptive activity, low intrinsic activity relative to that of the opioids, and poor side-effect profile (*5*). The potent, broad-spectrum antinociceptive actions of epibatidine (*6*), an alkaloid isolated from the skin of Ecuadorian frogs, are also mediated via a neuronal nicotinic acetylcholine receptor (nAChR) mechanism (*7*). However, these antinociceptive actions are accompanied by adverse effects (for example, hypertension, neuromuscular paralysis, and seizures) at or near the doses required for antinociceptive efficacy (*8*), and these dose-limiting in vivo actions have precluded the development of epibatidine as an analgesic agent. Because these effects are mediated via interactions with distinct ganglionic, neuromuscular, and central nervous system (CNS) nAChR subtypes, the design of compounds selective for distinct nAChR subtypes is an approach to identifying analgesic agents with reduced side effects as compared to those of (±)−epibatidine. In the rodent CNS, the predominant nAChR subtypes are α4β2 and the homooligomer α7 (*9*). These differ from the $\alpha_1\beta_1\delta\gamma(\varepsilon)$ and α3-containing nAChR subtypes found at the neuromuscular junction (*10*) and sympathetic ganglia (*11*), respectively, that mediate many of the undesired functional effects of (±)−epibatidine. A number of nAChR ligands have been reported that are selective for neuronal nAChR subtypes and may have potential in the treatment of Alzheimer's disease and Parkinson's disease (*12*).

ABT-594 [(*R*)-5-(2-azetidinylmethoxy)-2-chloropyridine] (Fig. 1) was synthesized as a potential neuronal nAChR ligand and identified as a potential analgesic agent in a mouse hot plate screen (*13*). The activity of ABT-594, (−)−nicotine, and (±)−epibatidine at α4β2 neuronal nAChRs was determined with the use of [^{3}H]cytisine binding to rat brain membranes (Table 1) (*14*). The inhibition constant (K_i) values for the three nAChR ligands were 37 pM, 1 nM, and 42 pM, respectively. In cell membranes from *Torpedo californica* electroplax (that is, neuromuscular nAChRs), ABT-594 and (−)−nicotine were ineffective in displacing [^{125}I]α-bungarotoxin (α-bgt) binding (K_i > 10 μM), whereas (±)−epibatidine had a K_i value of 2.4 nM. Thus, while ABT-594 and (±)−epibatidine have similar affinity for



(-)-Nicotine (±)-Epibatidine ABT-594

Fig. 1. The chemical structures of (−)−nicotine, (±)−epibatidine, and ABT-594 [(*R*)-5-(2-azetidinylmethoxy)-2-chloropyridine]. Like epibatidine, ABT-594 possesses a 2-chloro-5-pyridyl group and a basic nitrogen atom, but it differs structurally in several respects (*13*), including (i) the azacycle moiety encompassing the basic nitrogen atom (azetidine versus 7-azabicyclo[2.2.1]heptane); (ii) elements linking the pyridyl group to the azacycle moiety (oxymethylene versus a single bond); (iii) the smallest number of contiguous bonds intervening between the pyridine moiety and the basic nitrogen atom (four versus three); (iv) the number of freely rotatable internal bonds in the molecule (three versus one); and (v) the number of chiral centers (one versus three).

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α4β2 neuronal nAChRs, ABT-594 had approximately 4000 times less affinity for neuromuscular nAChRs than did (±)−epibatidine. Morphine was inactive (K_i > 10 μM) at both nAChR subtypes studied. ABT-594 had low affinity (K_i > 1000 nM) for 70 other drug targets, including other ligand-gated ion channels, heterotrimeric GTP-binding protein–coupled receptors (including opioid and muscarinic receptor subtypes), amine uptake sites, channel proteins, second messenger system proteins, and isoforms of cyclooxygenase (*15*). The preferential selectivity of ABT-594 for neuronal α4β2 nAChRs thus provided a basis for an improved therapeutic index relative to (±)−epibatidine.

ABT-594, (−)−nicotine, and morphine were compared in animal models of acute thermal (rat hot box) (*16*) and persistent chemical (formalin test) (*17*) pain. In the hot box assay, morphine and (−)−nicotine are effective in attenuating the response to pain (*18*). ABT-594 was, however, 30 to 70 times more potent in eliciting a dose-dependent antinociceptive effect, with an efficacy similar to that seen with morphine (Fig. 2A). The analgesic effects of ABT-594 [0.1 μmol per kilogram of body weight (μmol/kg), administered intraperitoneally (ip)] in the hot box model were attenuated by pretreatment with the nAChR antagonist mecamylamine (5 μmol/kg, ip) (Fig. 2B) but not by the opioid antagonist naltrexone (*19*). In the formalin test, the second phase of the biphasic nociceptive response is thought to be mediated, in part, by a sensitization of neuronal function at the level of the spinal cord (*20*) and may reflect the clinical observation of hyperalgesia associated with tissue injury. Nociceptive responding during phase 2 was blocked by ABT-594 in a dose-dependent manner with a potency nearly 70 times greater than that of morphine when ABT-594 was administered before the injection of 5% formalin into the paw (Fig. 2C). The antinociceptive effects of ABT-594 (0.3 μmol/kg, ip) in the formalin test were attenuated by pretreatment with mecamylamine (5 μmol/kg, ip) (Fig. 2D) but not by naltrexone (*19*). (−)−Nicotine (0.62 to 6.2 μmol/kg, ip) was ineffective in this model, with higher doses being toxic. In contrast to the restricted activity of (−)−nicotine, ABT-594 was an effective antinociceptive agent in both an acute thermal model of pain (the hot box) and a persistent chemical model of pain (the formalin test), with efficacy equivalent to that of morphine. Thus, based on these behavioral endpoints, ABT-594 was effective in reducing the nociceptive input that is known to be encoded primarily by C-fiber afferents.

In neuropathic pain models, nerve injury results in neuroplastic changes that lead to allodynia, a condition characterized by nocifensive behavioral responses to what are normally nonnoxious stimuli conducted by Aβ fibers. In the Chung model of neuropathic pain, allodynia is produced in the hind limb ipsilateral to the ligation of the L5 and L6 spinal nerves (*21*). ABT-594 produced a dose-dependent antiallodynic effect (Fig. 2E) in this model that was blocked by mecamylamine pretreatment (Fig. 2F). (−)−Nicotine and morphine treatment also produced antiallodynic effects but had potencies 20 and 30 times lower, respectively, than that seen with ABT-594. Thus, ABT-594 appears to interact with nAChRs to achieve antinociception, equal in efficacy to and greater in potency than morphine, in three mechanistically diverse animal models of pain. ABT-594 is able to reduce nociceptive behaviors regardless of whether they are encoded by C fibers (for example, acute

Table 1. Binding assays for nAChR subtypes (*8*). The values represent the mean ± SEM.

Compound	[^{3}H]cytisine K_i (nM) (rat brain, α4β2)	[^{125}I]α-bgt K_i (nM) (*T. californica* electroplax, neuromuscular junction)
(−)−Nicotine	1.0 ± 0.1	>10,000
(±)−Epibatidine	0.042 ± 0.001	2.4 ± 0.5
ABT-594	0.037 ± 0.003	>10,000
Morphine	>10,000	>10,000

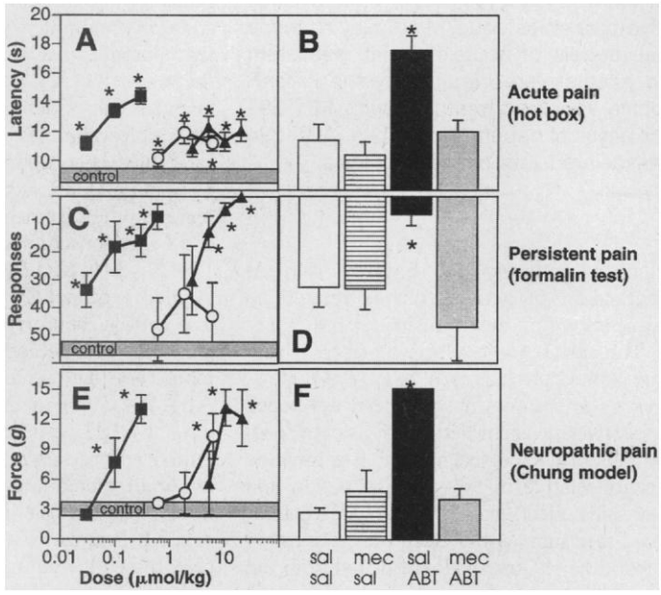

Fig. 2. (**A**), (**C**), and (**E**) show the effects of ABT-594 (squares), (−)−nicotine (circles), and morphine (triangles) in preclinical models of acute, persistent, and neuropathic pain. All compounds were administered ip. Each compound was tested independently, but for graphical presentation, control (that is, saline-treated animal) values were pooled from each experiment. Values shown represent the mean ± SEM. Statistical significance (*) represents different from control within each experiment with the use of analysis of variance (ANOVA), followed by Fisher's protected least-significant difference ($P < 0.05$). (**B**), (**D**), and (**F**) show the blockade of the antinociceptive effect of ABT-594 by pretreatment with the nAChR antagonist mecamylamine (mec) in these same three models; (*) represents statistically different from saline/saline (sal/sal) and mec/ABT-594 groups with the use of ANOVA, followed by Fisher's protected least-significant difference ($P < 0.05$). (A) Effects in the rat hot box, a model of acute thermal pain ($n = 8$ per treatment group). The latency for the animal to remove its paw from a thermal stimulus was used as the dependent measure. Data were collapsed from measures taken 15, 30, and 45 min after ABT-594 treatment. (B) Mecamylamine pretreatment (5 μmol/kg, ip) attenuated the antinociceptive effect of ABT-594 (0.1 μmol/kg, ip) in the rat hot box ($n = 8$ per group). Data were collapsed from measures taken 15, 30, and 45 min after ABT-594 treatment. (C) Effects in the formalin test, a model of persistent pain ($n = 8$ per treatment group). The number of nocifensive responses 30 to 50 min after injection of 5% formalin (phase 2) into the dorsal surface of the hindpaw was used as the dependent measure, and drugs were administered 5 min before the injection of formalin. Note the reversed *y* axis on the graph. Statistical significance (*) represents different from control within each experiment with the use of ANOVA, followed by Fisher's protected least-significant difference ($P < 0.05$). (D) Mecamylamine pretreatment (5 μmol/kg, ip) attenuated the antinociceptive effect of ABT-594 (0.3 μmol/kg, ip) in the formalin test ($n = 8$ per group). (E) Effects in the Chung model, a model of neuropathic pain ($n = 6$ per treatment group). Allodynia was measured with calibrated Von Frey filaments according to the method of Chaplan *et al.* (*34*). (F) Mecamylamine pretreatment (5 μmol/kg, ip) attenuates the anti-allodynic effect of ABT-594 (0.3 μmol/kg, ip) in the Chung model ($n = 6$ per group). The data were obtained 15 min after ABT-594 treatment. Initial treatment (with saline or mecamylamine) was administered 15 min before the second treatment (with saline or ABT-594).

pain) or Aβ fibers (for example, neuropathic pain).

Activation of primary afferent pain fibers by noxious stimuli can induce the release of neurotransmitters such as calcitonin gene-related peptide, glutamate, and substance P (SP) from nerve terminals in the dorsal horn of the spinal cord (*22*). These then activate secondary neurons in the dorsal horn to facilitate nociceptive transmission to supraspinal levels. The analgesic effects of opioids such as morphine are mediated, in part, by decreasing the release of these neurotransmitters in the dorsal horn (*1*). Selective depolarization of C fibers by 1 μM capsaicin can be used to stimulate SP release from spinal cord slices (*23*). ABT-594 dose-dependently reduced capsaicin-induced release (1 to 30 μM), with maximal effects observed at 30 μM concentration. The effect of 30 μM ABT-594 was blocked by pretreatment with mecamylamine (100 μM) (*24*).

Electrophysiological studies were also conducted in anesthetized rats to determine whether ABT-594 selectively affected afferent sensory neuron activation after non-noxious (that is, Aβ-fiber activation) and noxious (for example, C-fiber activation) stimuli. Extracellular recordings were made from convergent neurons in the dorsal horn that responded to both non-noxious and noxious stimuli (*25*). An antinociceptive dose of ABT-594 [0.3 μmol/kg, administered subcutaneously (sc)] reduced activity in neurons activated by noxious mechanical or thermal stimuli but did not alter the activity of these dorsal horn neurons when activated by non-noxious mechanical and thermal stimuli (*26*). This suggests that ABT-594 can selectively inhibit afferent pain signal transmission without affecting other sensory modalities such as touch.

In separate electrophysiological studies, intradermal injection of ABT-594 (2.7 to 29.7 nmol in 50 μl) into the hindpaw reduced the spinal neuronal responses to noxious stimuli in a dose-dependent manner (*25*). The maximal effects of ABT-594 (60 to 70%) on the spinal neuronal responses to noxious heat and mechanical stimuli applied to the hindpaw were reversible by mecamylamine (250 μg in 50 μl) given at the same site. Because the expression of the nAChRs on the central terminals of C fibers is likely to be paralleled by a peripheral location, it is likely that inhibition of transmitter release and activity by ABT-594 may occur at both ends of C fibers.

At the supraspinal level, the primary mechanisms for inhibiting nociceptive transmission include activation of the brainstem-descending pain inhibitory systems that arise from monoaminergic cell groups such as the nucleus raphe magnus (NRM) and the locus coeruleus (*27*). Activation of these areas can gate transmission of afferent impulses at the level of the spinal cord and thus prevent nociceptive input from reaching higher centers. Injection of (−)−nicotine into the NRM of rats can produce an antinociceptive effect in both the tail-flick and hot plate assays (*28*). Using expression of the immediate early gene *c-fos* as a marker of neuronal activation (*29*), antinociceptive doses of ABT-594 were shown to increase *c-fos* immunoreactivity in the NRM (Fig. 3A) (*30*). To establish the fact that local activation of these neurons was antinociceptive, very low doses of ABT-594 (0.004 to 0.04 nmol per rat) were microinjected directly into the NRM and produced a significant antinociceptive effect in the hot box model (Fig. 3B) (*31*). Thus, the antinociceptive activity of ABT-594 could result, in part, from activation of neurons in the NRM, which in turn provides a critical descending pain-inhibitory mechanism.

Studies were also conducted in rats to determine if, like morphine, ABT-594 produces overt physical dependence with repeated administration and elicits withdrawal signs when discontinued (*32*). Decreases in food intake in response to compound discontinuation have been interpreted as a sign of opioid withdrawal. Rats were treated twice a day for 10 days with vehicle, ABT-594, or morphine at doses that were approximately four times the maximally effective antinociceptive dose. Treatment was stopped after day 10 and animals were monitored for an additional 8 days. Decreases in baseline food intake were observed during treatment in both morphine- and ABT-594–treated rats (Fig. 3C). Upon discontinuation of treatment (that is, withdrawal), animals given morphine showed an additional decrease in food intake that peaked at day 2. In contrast, food intake in animals treated with ABT-594 returned rapidly to control levels after cessation of treatment, which suggests that ABT-594 does not produce opioid-like withdrawal effects. Clinical study of ABT-594 will help determine whether or not there are nicotinelike dependence liabilities, as observed in users of tobacco products.

The nAChR ligand ABT-594, but not

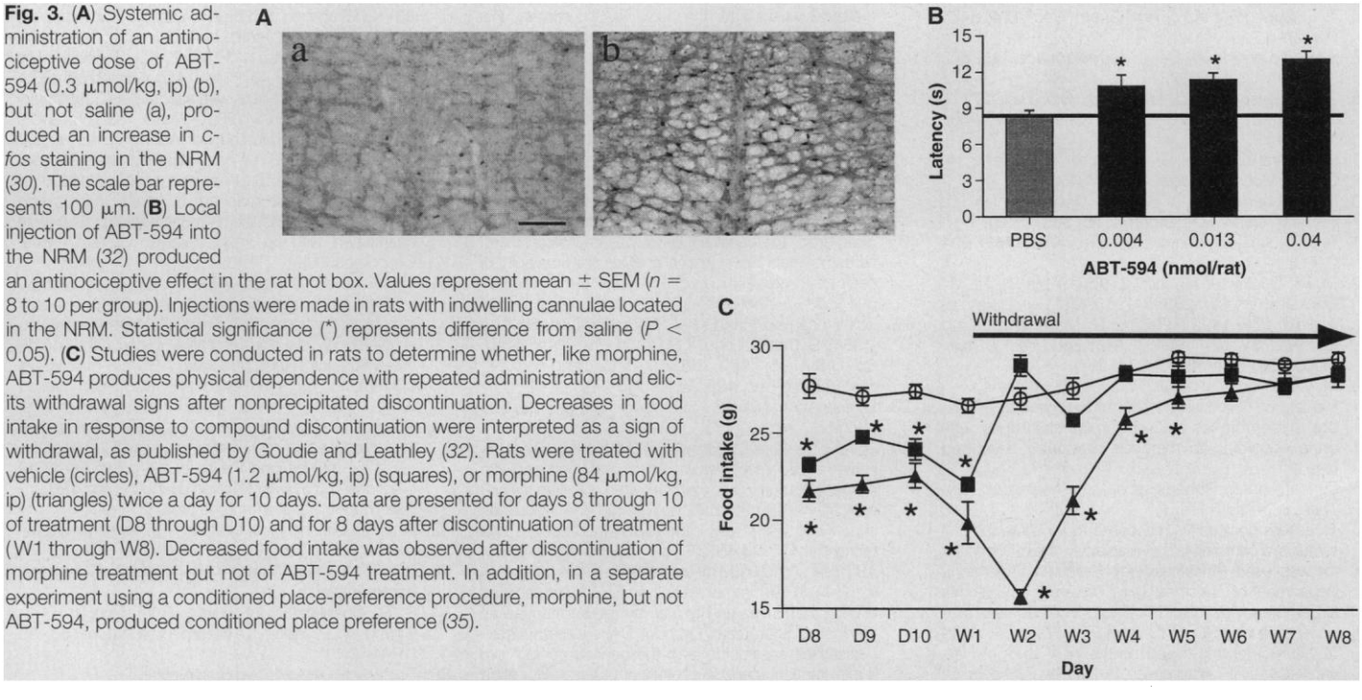

Fig. 3. (**A**) Systemic administration of an antinociceptive dose of ABT-594 (0.3 μmol/kg, ip) (b), but not saline (a), produced an increase in *c-fos* staining in the NRM (*30*). The scale bar represents 100 μm. (**B**) Local injection of ABT-594 into the NRM (*32*) produced an antinociceptive effect in the rat hot box. Values represent mean ± SEM (n = 8 to 10 per group). Injections were made in rats with indwelling cannulae located in the NRM. Statistical significance (*) represents difference from saline ($P <$ 0.05). (**C**) Studies were conducted in rats to determine whether, like morphine, ABT-594 produces physical dependence with repeated administration and elicits withdrawal signs after nonprecipitated discontinuation. Decreases in food intake in response to compound discontinuation were interpreted as a sign of withdrawal, as published by Goudie and Leathley (*32*). Rats were treated with vehicle (circles), ABT-594 (1.2 μmol/kg, ip) (squares), or morphine (84 μmol/kg, ip) (triangles) twice a day for 10 days. Data are presented for days 8 through 10 of treatment (D8 through D10) and for 8 days after discontinuation of treatment (W1 through W8). Decreased food intake was observed after discontinuation of morphine treatment but not of ABT-594 treatment. In addition, in a separate experiment using a conditioned place-preference procedure, morphine, but not ABT-594, produced conditioned place preference (*35*).

(−)−nicotine, has both peripheral and central antinociceptive effects in preclinical models of acute thermal, persistent chemical, and neuropathic pain states. The cardiovascular liablities associated with nAChR ligands such as epibatidine are reduced with ABT-594 (*13*). To date, only systemic treatment with opioids such as morphine has been reported to have broad-spectrum analgesic activity. Like the opioids, ABT-594 can selectively modulate pain transmission by inhibiting SP release from C fibers at the level of the dorsal horn and by activating the brainstem centers that provide descending inhibitory pathways that are known to gate painful stimuli. In contrast to morphine, repeated treatment with ABT-594 did not appear to produce opioid-like withdrawal effects at termination of treatment, which suggests an absence of physical dependence liabilities. Also in contrast to morphine, at antinociceptive doses, ABT-594 did not decrease gastric motility in rats (*33*). Compounds such as ABT-594 that can selectively modulate neuronal nAChR function and possess broad-based antinociceptive activity may provide a therapeutic approach to pain management that avoids the liabilities typically associated with opioid analgesics.

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14. Binding of [^{3}H]cytisine in the rat brain and of [^{125}I]α-bgt to membranes of *T. californica* electroplax was done according to methods previously described [see (*9*)].
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16. To assess nociceptive responses to an acute thermal stimulus, a commercially available paw thermal stimulator was used (Anesthesiology Research Laboratory, Department of Anesthesiology, University of California at San Diego, La Jolla, CA). This device has been previously described [D. M. Dirig and T. L. Yaksh, *Pain* **62**, 321 (1995)] and is based on the initial work of Hargreaves *et al.* [K. Hargreaves, R. Dubner, F. Brown, C. Flores, J. Joris, *ibid.* **32**, 77 (1988)]. Briefly, rats were placed in Plexiglas cubicles that were located on a glass surface of the apparatus. The surface of the glass was maintained at 30°C. A thermal stimulus was applied to the bottom of the rear foot of the rat by means of a movable focused projection bulb. The stimulus current was maintained at 4.8 A. The latency until the animal moved its foot from the stimulus was recorded automatically by photodiode motion sensors. In the current studies, a 20-s cutoff was employed to limit possible tissue damage after exposure to the stimulus. For measurements, the following protocol was used. Six animals were used in each run. For each measure (that is, each time point), one foot of each of the six animals was tested and then the process was repeated for the opposite foot. Mean values for the response were then computed based on the two scores.
17. The method used for the formalin test was based on a modified version of a previously published method [D. Dubuisson and S. G. Dennis, *ibid.* **4**, 161 (1977)]. After a 20-min period of acclimation to individual cages, 50 μl of a 5% formalin solution was injected sc into the dorsal aspect of one of the rear paws, and the rats were then returned to the clear observation cages suspended above mirror panels. Only phase 2 of the formalin test was scored, and phase 2 was defined as the 20-min period of time from 30 to 50 min after formalin injection. The investigator recorded nocifensive behaviors in the injected paw of four animals during the session by observing each animal for one 15-s observation period during each 1-min interval. Nocifensive behaviors recorded included flinching, licking, or biting the injected paw. In dose-response studies, ABT-594 (or saline) was administered 5 min before the injection of formalin. In antagonist studies, the antagonists or saline were administered 10 min before ABT-594 treatment.
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24. Spinal cord slices (250 μm thick) of the dorsal half of the lumbar enlargement were continuously superfused with oxygenated buffer, and fractions were collected every 3 min. After a 36-min equilibrium period, the tissue was superfused with 1 μM capsaicin in the absence and presence of 30 μM ABT-594 (n = 4 per group) or 30 μM ABT-594 and 100 μM mecamylamine (n = 4 per group) for 6 min. SP-like immunoreactivity (SP-LI) was assayed in both perfusates and lysates by radioimmunoassay (Peninsula Laboratories, Belmont, CA). Release was expressed as a percentage of the peptide content per 3-min fraction [(peptide content in perfusate)/(peptide content in perfusate + peptide content in tissue lysate)]. Release detected both during (6 min) and 3 min after capsaicin treatment was significantly higher than the baseline value. For this reason, data are expressed as the sum of SP-LI content measured in the three fractions (9 min) immediately following treatment. The value for the capsaicin treatment alone was 0.2 ± 0.03%. The value for the capsaicin treatment + 30 μM ABT-594 was 0.07 ± 0.02%; which is significantly different from capsaicin treatment alone ($P < 0.05$). In the capsaicin + 30 μM ABT-594 + mecamylamine (100 μM) group, the percent of SP-LI measure was 0.14 ± 0.3, which indicates blockade of the effect of ABT-594 by mecamylamine.
25. Electrophysiological studies were conducted in anesthetized Sprague-Dawley rats as previously described [A. Diaz and A. H. Dickenson, *Pain* **69**, 93 (1997)]. Briefly, for extracellular recordings, a tungsten electrode was lowered into the dorsal horn of the spinal cord, and recordings were made from convergent neurons (n = 5) within the dorsal horn that responded to both noxious thermal (4°C) and mechanical (50*g*) stimuli, as well as to non-noxious thermal (40°C) and mechanical (5*g*) stimuli. The depth of the cells from the spinal cord surface was established with the use of a SCAT microdriver (Digitimer, Hertfordshire, UK), and the cells used in this experiment were located deep in the dorsal horn with a mean depth ranging from 741 to 817 μm. During recording, animals were spontaneously breathing, and the level of halothane was kept at 2 to 2.5% to maintain complete areflexia.
26. ABT-594 treatment inhibited responses to noxious mechanical and thermal stimuli by 70 and 61%, repectively. ABT-594 treatment had no effect on responses of these neurons to non-noxious mechanical and thermal stimuli.
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30. Immunohistochemistry of free-floating sections (anti-Fos staining) was as follows. After overnight incubation in 20% sucrose–phosphate-buffered saline (PBS), brains from saline- and ABT-594–treated rats were cut on a cryostat (in 40-μm coronal sections). Free-floating sections were immunostained for Fos protein with a three-step ABC-peroxidase technique, beginning with a 30-min incubation with blocking serum. Sections were next incubated with antibody (Ab) to Fos (sheep polyclonal immunoglobulin G, 1:2000; Genosys, The Woodlands, TX) for 48 hours at 4°C, washed with PBS, and incubated for 1 hour with a biotinylated secondary sheep Ab solution (1:200). Finally, sections were washed in PBS, incubated with ABC reagent (Vector), and then developed in a peroxidase substrate solution. The sections were mounted and cover-slipped, then examined and photographed with a light microscope (Leica, DMRB). Fos-like immunoreactivity (FLI) was quantitated with an image analysis system (Leica, Quantimet 500).
31. Rats were anesthetized with sodium pentobarbital (55 mg/kg, ip). Animals were placed in a David Kopf student stereotaxic instrument (Tujunga, CA) with the skull on an even horizontal plane. For NRM cannula, the coordinates from intra-aural zero were AP −2.5 mm, lateral 0.0 mm, and −0.5 mm deep. Animals were implanted with a C315G (26-gauge) guide cannula (Plastics One, Roanoke, VA) cut to 7 mm. The guide cannula was implanted so that the injector cannula (33 gauge) extended 4 mm beyond the tip of the guide. For NRM injections, 0.3 μl was delivered over 60 s, and the cannula was left in place for another 40 s.
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36. All experiments involving animals were conducted under protocols approved by the Institutional Animal Care and Use Committee of Abbott Laboratories. The authors acknowledge the synthetic contributions of J. Lynch, J. Wasicak, and H. Bai; the biological contributions of D. Anderson and D. J. B. Kim; and J. Daly for his pioneering work in the field and his thoughtful input to this manuscript.

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Inhibition of the Hammerhead Ribozyme Cleavage Reaction by Site-Specific Binding of Tb(III)

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Terbium(III) [Tb(III)] was shown to inhibit the hammerhead ribozyme by competing with a single magnesium(II) ion. X-ray crystallography revealed that the Tb(III) ion binds to a site adjacent to an essential guanosine in the catalytic core of the ribozyme, approximately 10 angstroms from the cleavage site. Synthetic modifications near this binding site yielded an RNA substrate that was resistant to Tb(III) binding and capable of being cleaved, even in the presence of up to 20 micromolar Tb(III). It is suggested that the magnesium(II) ion thought to bind at this site may act as a switch, affecting the conformational changes required to achieve the transition state.

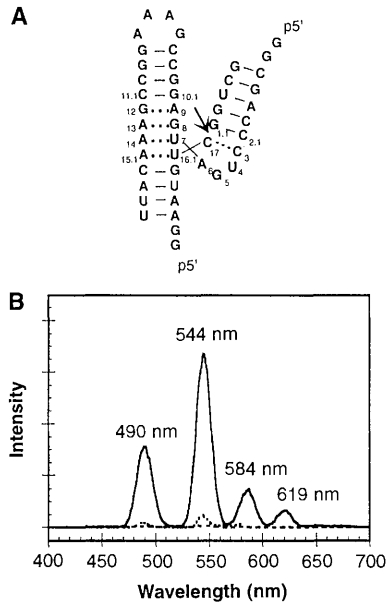

Fig. 1. (**A**) Schematic representation of the hammerhead ribozyme as well as the standard numbering scheme for the residues (*38*). The sequence shown is that of hammerhead 8 (HH8), a construct that has been previously characterized (*39*) and was used for most of the inhibition studies. (**B**) Sensitized luminescence spectrum of 10 μM $TbCl_3$ in the presence of 10 mM $MgCl_2$ and 1 μM HH16 (solid line). Control luminescence spectra of 1 μM HH16 and 10 mM $MgCl_2$ with no added $TbCl_3$ and of 100 μM $TbCl_3$ in water (dashed and dotted lines, respectively) are also shown. Excitation was at 260 nm.

RNA enzymes require divalent metal ions for activity, either to promote folding or for direct participation in catalysis. The hammerhead ribozyme (Fig. 1A), a self-cleaving RNA found naturally in plant viroids and virusoids, is an excellent system in which to study metal ion–RNA interactions because of the extensive structural and mechanistic data available (*1–4*). Two independent crystal structures of the hammerhead ribozyme have revealed divalent metal ions binding to six different sites on the molecule (*5–7*). Biochemical methods available to evaluate the role of these metal ions in ribozyme function are limited. The most common approach is to introduce a phosphorothioate modification into the RNA and to examine its effect on the metal specificity of the catalytic reaction (*8–13*). We present an approach to studying metal binding to ribozymes based on the observation that ions that compete efficiently for critical Mg-binding sites can thereby inhibit catalysis. The powerful enzymatic and spectroscopic tools originally developed for use with protein metalloenzymes can then be applied to RNA systems such as the hammerhead ribozyme.

Interactions between lanthanide ions and RNA molecules have been studied (*14, 15*). The luminescence properties of Tb(III) made it an attractive choice from a list of potential inhibitors (*16*). Irradiation (excitation wavelength, 260 nm) of a 1 μM solution of hammerhead 16 (HH16) (*17*) in the presence of 10 μM Tb(III) and 10 mM Mg(II) resulted in sensitized emission from the $^5D_4$ state of the Tb ion (Fig. 1B). This signal was absent from control samples lacking either RNA or Tb(III). These data indicate that the Tb ion binds to the RNA, resulting in energy transfer from the RNA to the lanthanide ion. We therefore investigated the effects of Tb(III) binding on the hammerhead-catalyzed reaction, a site-specific cleavage of a phosphodiester bond to form 2′,3′-cyclic phosphate and 5′-hydroxyl termini.

Terbium(III) proved an efficient inhibitor of hammerhead cleavage (*18*). For example, Tb(III) inhibited the HH8 single-turnover cleavage reaction with an apparent inhibition constant ($K_{i,app}$) of 2.0 ± 0.3 μM at 25 mM Mg(II) (Fig. 2A), and similar values were obtained under multiple-turnover conditions. Two other well-characterized hammerheads, HH16 (*19*) and HHα1 (*20*), showed $K_{i,app}$ values for Tb(III) of 1.1 ± 0.4 and 0.57 ± 0.08 μM, respectively, at 10 mM Mg(II) (*21*). Because all three ribozymes were inhibited similarly by Tb(III) despite sequence differences in the peripheral base-paired regions and loops, the data indicate that the binding event that underlies inhibition results from the interaction of Tb(III) with a site in the conserved catalytic core.

The $K_{i,app}$ values for Tb(III) increased with increasing Mg(II) concentrations, indicating that the two ions compete for a site (Fig. 2B). Competition was confirmed by a chase experiment (Fig. 2C); when Tb(III) was added to a cleavage reaction after it had already started, the reaction stopped rapidly and completely. When even higher concentrations of Mg(II) were later added to the same reaction, cleavage resumed. The reversibility of this inhibition implied that the Tb(III) adduct rapidly equilibrates with the fully hydrated ion and does not irrevocably damage the hammerhead. This implication was confirmed by direct analysis of the RNA by gel electrophoresis after incubation with Tb(III) for up to 4 hours (*22*).

Two crystallographic experiments were performed to determine the location of Tb(III) binding. In one experiment, 2 mM $TbCl_3$ was allowed to soak into already-formed crystals of the hammerhead; in the other, the ribozyme was crystallized in the presence of 2 mM $TbCl_3$ (Table 1). The high concentration was required to offset the ionic strength of the mother liquor. In both instances, the overall structures were identical to the native hammerhead with the exception of localized regions of positive electron density indicative of metal binding. The difference electron density map from the cocrystallization experiment (Fig. 3A) revealed three bound ions. The site with the highest occupancy was adjacent to residues G5 and A6 in the catalytic core. This Tb ion was the only one observed in the soak experiment. One of the two binding sites with lower occupancy identified in the cocrystallization experiment

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