ABT-089 [2-Methyl-3-(2-(S)-pyrrolidinylmethoxy)pyridine]: I. A Potent and Selective Cholinergic Channel Modulator with Neuroprotective Properties

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ABSTRACT

Accumulating preclinical and clinical evidence data suggests that compounds that selectively activate neuronal nicotinic acetylcholine receptor (nAChR) subtypes may have therapeutic utility for the treatment of several neurological disorders. In the present study, the in vitro pharmacological properties of the novel cholinergic channel modulator ABT-089 [2-methyl-3-(2-(S)-pyrrolidinylmethoxy)pyridine], are described. In radioligand binding studies, ABT-089 was shown to display selectivity toward the high-affinity (--)cytisine binding site present on the \( \alpha_2\beta_2 \) nAChR subtype (\( K_i = 16 \) nM) relative to the \([^{125}I]\alpha\)-bungarotoxin binding site present on the \( \alpha_7 \) (\( K_i \geq 10,000 \) nM) and \( \alpha_1\beta_1\delta_2 \) (\( K_i > 1,000 \) nM) nAChR subtypes. In cation flux and channel current studies, ABT-089 displayed a more complex profile than (--)nicotine having agonist, partial agonist and inhibitory activities depending on the nAChR subtype with which it interacts. ABT-089 differentially stimulated neurotransmitter release. The compound displayed a similar potency and efficacy to (--)nicotine to facilitate ACh release (ABT-089, EC\( _{50} \) = 3 \( \mu \)M; (--)nicotine, EC\( _{50} \) = 1 \( \mu \)M), but was markedly less potent and less efficacious than (--)nicotine to stimulate dopamine release (ABT-089, EC\( _{50} \) = 1.1 \( \mu \)M; (--)nicotine, EC\( _{50} \) = 0.04 \( \mu \)M). Additionally, ABT-089 was neuroprotective against the excitotoxic insults elicited by exposure to glutamate in both rat cortical cell cultures (EC\( _{50} \) = 10 \( \pm \) 3 \( \mu \)M) and differentiated human IMR32 cells (EC\( _{50} \) = 3 \( \pm \) 2 \( \mu \)M). The differential full agonist/partial agonist profile of ABT-089, as compared with (--)nicotine and ABT-418, illustrates the complexity of nAChR activation and the potential to target responses at subclasses of the neuronal and peripheral receptors.

Signaling through neuronal nAChRs is being recognized increasingly as playing an important role in neurotransmission in the central nervous system (Arneric et al., 1996a; McGehee et al., 1996a, 1995; Bertrand and Changeux, 1995). Accumulating preclinical and clinical evidence suggests that alterations in either the levels and/or activity of this ligand gated ion channel receptor family may play a role in several CNS disorders including Alzheimer’s disease and Parkinson’s disease (Williams et al., 1994; Arneric et al., 1996a). Modulation of nAChRs can elicit several functional responses including enhancement of fast excitatory neurotransmission (McGehee et al., 1995), facilitation of neurotransmitter release (Sacaan et al., 1995), the neurogenic control of cerebral blood flow (Linville et al., 1993) and a diversity of behavioral responses including cognitive enhancement (Levin, 1992), neuroprotection (Donnelly-Roberts et al., 1996), anxiolytic (Brioni et al., 1993) and analgesic activity (Sullivan and Bannon, 1996). Rapid advances in the molecular biology and pharmacology of nAChRs in the past decade have revealed that a diversity of nAChRs may mediate the wide spectrum of behavioral effects of nAChR ligands (McGehee and Role, 1995; Arneric et al., 1996a; Brioni et al., 1996). Eleven gene products (\( \alpha_2\-o_9 \), \( \beta_2\-\delta_4 \)) have been identified to date in brain, sensory systems and autonomic ganglia (Deneris et al., 1991; Sargent, 1993; Elgoyhen et al., 1994). Functional responses can be elicited either in Xenopus oocytes injected with pair-wise combinations of \( \alpha \) and \( \beta \) subunits (Deneris et al., 1991; Papke and Heineman, 1993), or in cell lines stably expressing the \( \alpha_4\beta_2 \) and \( \alpha_3\beta_4 \) subtypes (Gopalakrishnan et al., 1996; Papke and
Heinemann, 1993; Whiting et al., 1991; Wong et al., 1995) confirming biochemical observations which suggest that many native nAChRs consist of αβ heterooligomers. Further, more complex combinations that include αδ as part of the complex (i.e., α3δβδ) may also occur (Role and Berg, 1996). The α7, α8 and α9 gene products, however, differ from other members of the nAChR superfamily in that they can form functional receptors when expressed as homo-oligomers in oocytes (Seguela et al., 1993; Gerzanich et al., 1994; Elgoyhen et al., 1994; Briggs et al., 1995) or cell lines (Gopalakrishnan et al., 1985).

Although it is not clear which subunit combinations form nAChRs in situ, the pharmacology of the putative nAChR subtypes and selectivity of known nAChR ligands is beginning to emerge from in vitro heterologous expression studies. For example, studies in transfected cell lines indicate that the high-affinity (−)-nicotine binding site in brain corresponds to the α4β2 subunit combination (Whiting et al., 1991; Flores et al., 1992) whereas the distribution of the α7 subunit largely coincides with the distribution of high-affinity [125I]-α-BgT binding sites in rat brain (Seguela et al., 1993; Clarke et al., 1985). Furthermore, recent studies demonstrating (−)-nicotine to be an antagonist at α9 nAChRs together with the potent inhibitory effects of cytisine on β2-containing subunit combinations indicate that the pharmacological profile of classical nAChR ligands is more complex than previously recognized (Elgoyhen et al., 1994; Papke and Heinemann, 1993).

The historical precedent and pharmacology has led to the conventional nomenclature of calling functional combinations of these novel neuronal gene products related to the neuromuscular receptor, i.e., “nicotinic receptors.” This nomenclature has carried over to describe compounds that interact with nAChRs as either nicotinic agonists or nicotinic antagonists. An alternative nomenclature that refers to nAChRs as “cholinergic channels” serves not only to highlight mechanistic distinctions versus muscarinic receptors, but also to acknowledge the nonexclusive interactions of nicotine at the various subtypes (see above). The term “cholinergic channel modulators” (ChCMs) then defines the broad class of agents that includes competitive activators, allosteric activators and allosteric facilitators (collectively, cholinergic channel activators), as well as cholinergic channel inhibitors, which may act through any of at least four likely mechanisms: competitive antagonism, noncompetitive (allosteric) inhibition, ion channel blockade or receptor inactivation (e.g., “desensitization”). The term cholinergic channel modulator further emphasizes that it is possible for a compound to possess one set of properties (e.g., activate) at one subtype of nAChR and a different set of properties (e.g., inhibit) at a different subtype or different properties at the same subtype depending on the conditions (e.g., either activate or desensitize depending on concentration of the cholinergic channel modulator).

ChCMs that possess appropriate combinations of modulatory properties at cholinergic channel subtypes may be able to selectively influence central neurotransmission without having the side-effect liabilities associated with (−)-nicotine. Therapeutically, ChCMs lacking cardiovascular or CNS side effects associated with (−)-nicotine may represent a potential strategy to ameliorate many of the CNS deficits accompanying Alzheimer’s disease or related disorders. Accordingly, there has been a flurry of medicinal chemistry efforts in this area targeted at the development of novel and safe ChCMs (Holladay et al., 1995).

The concept that site- and/or subtype-selective modulation of nAChR function is possible has led to the identification and characterization of ABT-418 ([S]-3-methyl-5-[1-methyl-2-pyrrolidinyl]isoxazole) and SIB 1508Y ([S]-5-ethynylnicotine), analogs of (−)-nicotine, and GTS-21 [3-(2,4)-dimethoxybenzylidine], an anabaseine analog, as novel ChCMs (Arneric et al., 1995; Lloyd et al., 1995; Meyer et al., 1994). Relative to (−)-nicotine, ABT-418 displays selectivity toward α4β2 compared with the α7 nAChR. Further, ABT-418 is less potent and efficacious than (−)-nicotine to activate human ganglionic nAChRs (Arneric et al., 1995). In contrast, GTS-21 is a weak α7 agonist which may display greater efficacy at the α7 nAChR than the α4β2 subtype (deFiebre et al., 1995; Briggs et al., 1997). Both compounds demonstrate cognitive enhancing activities in rodents and primates and are neuroprotective in in vitro models of excitotoxicity (Arneric et al., 1995; Martin et al., 1994; Woodruff-Pak et al., 1994). Acute administration of ABT-418 has been shown to improve cognitive performance in patients with Alzheimer’s disease (Newhouse et al., in press, 1997). However, as has been observed with many ChCMs, both ABT-418 and GTS-21 are poorly (<10%) bioavailable in primates (Arneric et al., 1995; Briggs et al., 1997).

ABT-089 (fig. 1), a ChCM derived from a series of pyridyl ether compounds (Lin et al., 1997), has high oral bioavailability, excellent safety and behavioral efficacy comparable with ABT-418 and GTS-21 (Arneric et al., 1996b; Decker et al., 1997, companion paper). In the present and accompanying paper, the initial pharmacological characterization of ABT-089 is described. Herein, ABT-089 is shown to potently and selectively interact with neuronal nAChRs. In in vitro preparations that differentially express nAChR subtypes, ABT-089 is shown to display a complex profile having agonist, partial agonist and inhibitory activities depending on the neuronal nAChR subtype with which it interacts. Additionally, after acute and subacute exposure, ABT-089 is shown to be neuroprotective against an excitotoxic insult. In the accompanying paper, ABT-089 is shown to display cognitive enhancing activity in rodents and primates (Decker et al., 1997, companion paper).

**Materials and Methods**

Materials

ABT-089 [2-methyl-3-(2-((S)-pyrrolidinylmethoxy)pyridine dihydrochloride], and its enantiomer A-94224 [2-methyl-3-(2-(R)-pyrrolidinylmethoxy)pyridine dihydrochloride] were synthesized at Ab-

![Fig. 1. Structure of ABT-089.](Image 341x59 to 531x164)
bott Laboratories as described (Lin et al., 1997). DHβE and MLA were purchased from Research Biochemicals International (Natick, MA). (−)-Nicotine (hydrogen tartrate salt), morphine sulfate, atropine and mepacrine hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO). All radioligands were obtained from DuPont-NEN (Boston, MA). α-BgtW was obtained from BioToxins Inc. (Miami, FL).

Cells of the IMR-32 human neuroblastoma and TE 671 human medulloblastoma clonal cell lines (ATCC, Rockville, MD) were maintained in a log phase of growth according to established procedures (Lukas, 1993). Cell lines stably expressing the human α4β2 and α7 subtypes (K177 and K28, respectively) were maintained as described (Gopalakrishnan et al., 1995, 1996).

Animals were treated according to a protocol approved by Abbott's Institutional Animal Care and Use Committee.

Membrane Preparation

Rat cerebral cortical membranes were prepared from male Sprague-Dawley rats as described by Enna and Snyder (1977) with some modifications as described (Sullivan et al., 1994). Brains were rapidly removed after decapitation, homogenized in 15 volumes of 0.32 M sucrose and centrifuged at 1000 × g for 10 min at 4°C. The supernatants were removed and centrifuged at 20,000 × g for 20 min at 4°C. The resultant P2 pellets were homogenized with a Polytron (10 s, setting of 6) in ice-cold water and spun at 8,000 × g for 20 min. The supernatant and loose buoyy coat were carefully removed and centrifuged at 40,000 × g for 20 min. The membrane pellet was washed with ice-cold H2O and recentrifuged at 40,000 × g before storage at −50°C. Before use, the frozen membrane pellets were slowly thawed, washed and resuspended in 30 volumes of assay buffer (composition, 120 mM NaCl, 5 mM KCl, 2 mM CaCl2, 2 mM MgCl2 and 50 mM Tris-Cl, pH 7.4 at 4°C).

Confluent K177 cells stably expressing the human α4β2 subunit combination were rinsed with ice-cold binding buffer (composition, mM: Tris HCl, 50; NaCl, 120; KCl, 5; MgCl2, 1 and CaCl2, 2.5; pH 7.4 at 4°C), mechanically disaggregated and homogenized using a polytron for 10 s. The homogenate was centrifuged at 45,000 × g for 20 min at 4°C, and the pellet was resuspended in ice-cold buffer at a concentration of 40 to 50 μg protein. Confluent K28 cells stabil lying the human α7 subunit were rinsed with ice-cold binding buffer (composition: 118 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 20 mM HEPES, pH 7.5), mechanically disaggregated and homogenized with a polytron for 10 s. The homogenate was centrifuged at 45,000 × g for 20 min at 4°C, and the pellet was resuspended in ice-cold buffer at a concentration of 40 to 50 μg protein.

[3H](-)-Cytisine binding. Binding conditions were as described previously (Anderson et al., 1995; Gopalakrishnan et al., 1996). Samples containing 20 to 200 μg of protein, 0.7 nM [3H](-)-cytisine (30 Ci/mmol) and the indicated concentrations of drug were incubated in a final volume of 500 μl for 75 min at 4°C in triplicate. Nonspecific binding was determined in the presence of 10 μM (-)-nicotine.

[35S]Ia-Bgt binding. [35S]Ia-Bgt binding was determined in membranes prepared from rat brain and K28 cell membranes and from Torpedo californica electroplax.

α-Bgt binding to rat brain and K28 cell membranes was determined as described previously (Gopalakrishnan et al., 1995). A line-phase binding assay with a 96-well microtiter plate was used to measure the binding of [35S]Ia-Bgt (106 Ci/mmol) to the α-Bgt nAChR isolate from T. californica electroplax (Sullivan et al., 1994).

Additional receptor selectivity binding studies. To further assess the selectivity of ABT-089 as an nAChR ligand, the compound was evaluated in a PROFILE receptor binding selectivity screen by NovaScreen (Oceansix, Hanover, MD) by standard receptor binding protocols that are documented in table 2. ABT-089 was tested in 45 binding assays for several peptides, channel proteins, peptide factors, reuptake sites, second messenger systems and neurotransmitters. ABT-089 was evaluated at three concentrations (1, 100 and 10,000 nM) in duplicate.

Data analysis. In competition experiments, the drug concentration producing 50% inhibition (IC50) of radioligand binding and the Hill coefficient (nH) were determined from plots of log (Bmax − B/Bmax versus log (concentration of drug), where Bmax and B are specific binding in the absence and presence of competitor, respectively, with a four-parameter logistics program in RS/1 (Bolt, Beraneck and Newman Inc. Cambridge, MA). Inhibition constant (Ki) values were determined with the Cheng-Prusoff equation (Cheng and Prusoff, 1972).

86Rb+ Efflux from K177, IMR 32 and TE 671 Cells

Cells of the IMR-32 human neuroblastoma clonal cell line (ATCC, Rockville, MD) and the TE 671 human medulloblastoma cell line were maintained in a log phase of growth according to established procedures (Lukas, 1993). K177 cells stably expressing the human α4β2 nAChR subunit combination were maintained as described (Gopalakrishnan et al., 1996). Experimental cells were seeded at a density of 500,000 cells/ml into a 24-well tissue culture dish. Plates were allowed to proliferate for at least 48 h before loading with 2 μCi/ml of 86Rb+ (35 Ci/mmol) overnight at 37°C. The 86Rb+ efflux assays were performed according to previously published protocols (Sullivan et al., 1994; Gopalakrishnan et al., 1995) except serum-free DMEM was used during the 86Rb+ loading, rinsing and agonist-induced efflux steps. Data were analyzed by a two-way ANOVA with StatView II (Abacus Concepts, Inc., Berkeley, CA). The criterion of statistical significance was P < .05.

86Rb+ Efflux from Mouse Thalamic Synaptosomes

The ability of ABT-089 and (-)-nicotine to activate ion channels was investigated by measuring efflux of 86Rb+ from mouse thalami with a slight modification of the method of Marks et al. (1993) as described previously (Arneric et al., 1994).

A 3% fraction equivalent to two thalami was incubated for 30 min at 21°C in 35 ml of perfusion buffer containing 4 μCi of 86Rb+ (35 Ci/mmol). At the end of the incubation period, tissue was harvested and separated from the incubation medium by filtration onto 6-mm diameter glass fiber filters (Type GC50, Microfiltration Systems, Dublin, CA) under gentle vacuum (~0.2 atm) followed by three washes at room temperature with perfusion buffer. The filter containing the 86Rb+ loaded synaptosomes was placed on a 13-mm glass fiber filter (Type GC50, Microfiltration Systems, Dublin, CA) and perfused continuously at 21°C. After an initial average wash period of 8 min, fractions were collected every 30 s by use of a Retriever II fraction collector (ISCO, Inc., Lexington, KS). Exposure to ABT-089 and (-)-nicotine usually occurred 3 min into a 10-min collection period. In any experiment, five concentrations of each ligand were tested and the tissue on each filter stimulated only once. (-)-Nicotine (10 μM) was included in each experiment as control to normalize values between experiments. Radioactivity was measured with a Packard Auto-Gamma counter (Packard, Naperville, IL) and the magnitude of the 86Rb+ response amplitude calculated by determining the increase in radioactivity above the base line after stimulation of the tissue. The average base line underlying the peak was calculated by averaging the radioactivity present in the tubes immediately before and after the peak. Peak size was determined by subtracting the average base-line value from each fraction in the peak. To correct for differences in total tissue content and base-line release, the response was normalized by dividing by the amount of 86Rb+ present in the tissue at the time of stimulation. EC50 values and the maximum response obtained for stimulation of 86Rb+ efflux were calculated by use of Inplot™ (Graphpad, San Diego, CA). Data were analyzed by a two-way ANOVA with use of StatView II (Abacus Concepts, Inc., Berkeley, CA). The criterion of statistical significance was P < .05.
Channel Currents in Xenopus Oocytes Expressing the Human α7 nAChR

The ability of ABT-089 to activate the human α7 nAChR expressed in Xenopus laevis oocytes was determined as described previously (Briggs et al., 1995). Recordings were made using a two-electrode voltage clamp at a holding potential of −60 mV. Experiments were done in modified Barth’s solution (90 mM NaCl, 1 mM KCl, 0.66 mM NaNO₃, 0.74 mM CaCl₂, 0.82 mM MgCl₂, 2.4 mM NaHCO₃, 2.5 mM sodium pyruvate and 10 mM Na-HEPES buffer, pH 7.55) containing 10 mM BaCl₂ in place of CaCl₂ and MgCl₂ to prevent a secondary activation of Ca²⁺-dependent Cl⁻ current by Ca²⁺ influx through the α7 nicotinic channels. The duration of agonist exposure generally was 1.25 to 2.5 s, but was increased up to 20 s to elicit a plateau in the slower responses. The interval between agonist applications was 5 min. Response stability was assessed through multiple applications. Antagonists were superfused in the bathing solution for 3 to 8 min before testing the response and were applied with agonist so that the only change was the addition of agonist to the superfusion. Current responses were quantified by measuring the peak amplitude of the channel current to baseline before and after agonist exposure, which were normalized to the average response to 100 μM (-)-nicotine determined in the same oocyte to account for variability in receptor expression among oocytes.

Striatal [³H]Dopamine Release

nAChR-evoked release of [ring-2,5,6-³H]dopamine (24.4 Ci/mmol) was measured in superfused rat striatal slices. Slices were dissected from two male Sprague-Dawley rats per experiment and sliced 0.35 × 0.25 mm by a McIlwain Tissue Chopper (Brinkman Instrument Co., Westbury, NY). After two washes with Krebs-HEPES buffer (137 mM NaCl, 4.7 mM KCl, 1 mM MgSO₄, 2.5 mM CaCl₂, 1.25 mM NaH₂PO₄, 10 mM glucose, 15 mM HEPES-NaOH, pH 7.4, containing 10 μM pargyline and 10 μM ascorbic acid), slices were preincubated for 10 min at 37°C under 95%/5% O₂/CO₂. After removing the buffer, slices were labeled with 100 nM [³H]dopamine for 25 min in Krebs-HEPES at 37°C. Aliquots of slices were placed in 18 superfusion chambers of a Brandel SP2000 superfusion apparatus (Brandel, Gaithersberg, MD). After 47 min of washout, slices were superfused in Kreb's buffer as 20-s pulses, replacing the buffer, slices were labeled with 100 nM [³H]dopamine (diluted with unlabeled dopamine; specific activity, 2 Ci/mmol) and counted in 15 ml of Ecolume. Tissue was recovered from superfusion chambers, solubilized with 1 ml of Solvable (DuPont-NEN) and counted in 15 ml of Ecolume.

Fractional release of [³H]dopamine was calculated from radioactivity above base line as a fraction of total radioactivity. Relative potencies were calculated by the release evoked by 100 nM (-)-nicotine as a standard. EC₅₀ values were determined by nonlinear least squares regression analysis with Inplot.

Hippocampal [³H]ACh Release

nAChR-evoked release of [³H]ACh was measured in superfused rat hippocampal synaptosomes. The F4 synaptosomal fraction was washed twice in Krebs/bicarbonate buffer (NaCl, 118.5 mM; NaHCO₃, 24.9 mM; KCl, KH₂PO₄, 1.2 mM; CaCl₂, 2.5 mM; MgSO₄, 2.5 mM; glucose, 10 mM gassed with 95%/5% O₂/CO₂, to give pH 7.4), and resuspended to a protein concentration of 1 mg/ml. The synaptosomes were loaded with [³H]choline (Amersham International, Buckinghamshire, England) by incubation for 30 min with 0.8 μM [³H]choline (specific activity 6 nM, respectively. ABT-089 and subsequent was normalized with reference to (-)-nicotine-evoked [³H]ACh release. EC₅₀ values were determined by nonlinear least squares regression analysis with Inplot.

Neurotoxicity Studies

Primary cortical cultures were prepared from Sprague-Dawley rats (Charles River; Wilmington, MA) at day 18 of gestation as described previously (Donnelly-Roberts et al., 1996). Dissected cortices were placed on ice in Hanks’ Balanced Salt Solution (Gibco/BRL; Gaithersburg, MD) and meninges and blood vessels removed. The cortex was disaggregated by mechanical trituration through a fire–narrowed Pasteur pipette, then plated onto poly-L-lysine coated 96-well culture dishes at a density of about 50,000 cells per well in DMEM/10% FCS (reduced to 1% FCS 24 h after plating) at 33 mM glucose/2 mM glutamine/50 U/ml pen/strep/B27 supplement. Cultures were maintained at 36°C in a humidified atmosphere of 10% CO₂. The IMR 32 cells were maintained in proliferative growth phase following routine protocols (Lukas, 1993). After 7 to 14 days in vitro, cells were pretreated with test compound diluted in DMEM/N2 supplement (Gibco/BRL) for 2 h. This pretreatment solution was replaced by Hanks’ Balanced Salt Solution (without magnesium, but containing 3 mM calcium chloride) containing 1-glutamate (300 μM) and coapplied with the test compound for an additional 15 min. This compound/glutamate solution was removed and replaced with fresh DMEM/N2 supplement for 24 h. Neuronal damage was assessed by measuring the levels of the cytosolic enzyme LDH released into the medium by the damaged cells using a Cytotoxicity assay kit (Promega; Madison, WI). Basal LDH release was typically between 8 and 10% of the LDH released after lysis of the cells with 0.8% Triton X-100, whereas GLU treatment typically resulted in a 3- to 4-fold increase in release over basal levels. The final levels of LDH release, both from control and treated cells, varied from plate to plate because of variability in cell density. Therefore, to facilitate plate-to-plate comparison all values were normalized to the 300 μM GLU-induced maximal LDH release (assigned 100%). Data were analyzed by a two-way ANOVA with StatView II (Abacus Concepts, Inc., Berkeley, CA). The criterion of statistical significance was P < .05.

Results

Receptor Binding

[³H](-)-Cytisine has been shown to bind with high affinity to the α4β2 subtype of nAChRs, a major subtype in brain (Flores et al., 1992). ABT-089 displaced [³H](-)-cytisine binding to the human α4β2 subunit combination stably expressed in the K177 cell line (Gopalakrishnan et al., 1996) in a concentration-dependent manner with a Kᵢ value of 16 ± 2 nM and a Hill coefficient value of 0.99 ± 0.04 (n = 3; table 1). The Kᵢ-antagonist of ABT-089, A-94224, displaced binding to this subtype with a Kᵢ value of 35 ± 5 nM (n = 3). ABT-089 and A-94224 displaced [³H](-)-cytisine binding to rat brain with Kᵢ values of 17 ± 3 nM and 39 ± 6 nM, respectively. (-)
Nicotine displaced \[^{3}H\](-)-cytisine binding to K177 cells and rat brain with \(K_i\) values of 1.0 ± 0.1 nM and 1.0 ± 0.1 nM, respectively.

In contrast to its activity at the \(\alpha_4\beta_2\) nAChR subtype, ABT-089 was >500-fold less potent (\(K_i \geq 10,000\) nM, \(n = 3\)) in displacing \[^{125}\]I\(^{-}\)-Bgt binding from the \(\alpha\)-Bgt-sensitive nAChR subtype present in rat brain and the \[^{125}\]I\(^{-}\)-Bgt binding site present on the human \(\alpha_7\) nAChR subtype (\(K_i > 10,000\) nM, \(n = 3\)) (table 1). A-94224, the R-enantiomer of ABT-089, also displayed very weak affinity for the rat brain and human \(\alpha_7\) nAChR subtype (\(K_i > 10,000\) nM, \(n = 3\)). (-)-Nicotine displaced \[^{125}\]I\(^{-}\)-Bgt binding from rat brain and human \(\alpha_7\) with \(K_i\) values of 6000 ± 876 nM and 2000 ± 178 nM, respectively. ABT-089 and (-)-nicotine were also weak inhibitors (\(K_i > 1000\) nM) of the binding of \[^{125}\]I\(^{-}\)-Bgt to the \(\alpha_1\beta_1\gamma\) nAChR subtype found on Torpedo electroplax membranes (table 1).

ABT-089 was also examined in 45 other receptor binding and enzyme activity assays (table 2) and showed negligible affinity (\(K_i > 10\) μM) for muscarinic, 5-HT\(_3\), and the benzodiazepine receptors as well as other members of the ligand-gated ion channel superfamily, including \(\gamma\)-aminobutyrlic acid\(_A\), benzodiazepine, N-methyl-D-aspartate, MK 801, quisqualate, kainate; \(L\), \(N\), and \(T\) calcium, chloride, and potassium channel proteins; members of G-protein-coupled receptor superfamily, adenosine, \(\alpha\)-1, \(\alpha\)-2, \(\beta\)-1 and \(\beta\)-2 adrenergic, 5-hydroxytryptamine, bradykinin, endothelin, neuropeptide Y, opioid, vasoactive intestinal peptide; choline, norepinephrine, serotonin, and dopamine uptake sites; and did not inhibit the activity of acetylcholinesterase, protein kinase C or monoamine oxidase A and B (table 2).

### Ion Flux Studies

**Mouse thalamus.** nAChR-mediated \(^{86}\)Rb\(^{+}\) efflux from mouse thalamic synaptosomes has been proposed to reflect an activation of the \(\alpha_4\beta_2\) subtype (Marks et al., 1993). ABT-089 was less potent (EC\(_{50} = 5 \pm 2\) μM; \(n = 3\)) and significantly (\(P < 0.05\)) less efficacious (34% of (-)-nicotine) than (-)-nicotine (EC\(_{50} = 1 \pm 0.4\) μM) in evoking \(^{86}\)Rb\(^{+}\) efflux from mouse thalamic synaptosomes, an effect that was sensitive to the noncompetitive nAChR antagonist, mecamylamine (10 μM) (fig. 2).

**Human \(\alpha_4\beta_2\).** ABT-089 did not activate \(^{86}\)Rb\(^{+}\) efflux from the human \(\alpha_4\beta_2\) subtype at concentrations up to 300 μM (fig. 3A). However, ABT-089 did inhibit (-)-nicotine-induced cation efflux with an IC\(_{50}\) value of 176 μM (fig. 3A, inset; \(n = 3\)), whereas the R-enantiomer, A-94224, displayed negligible activity as either an agonist or antagonist at concentrations up to 300 μM. In contrast, (-)-nicotine (EC\(_{50} = 4.6 \pm 0.7\) μM; \(n = 5\)) potently activated cation efflux from this cell line.

**Human IMR 32 cells.** nAChR-mediated cation efflux from the human neuroblastoma IMR 32 is thought to reflect the activation of a ganglionic nAChR subtype possibly n3β4 (Lukas, 1993). ABT-089 was significantly (\(P < 0.05\)) less potent (EC\(_{50} > 300\) μM) and less efficacious (<15%) than (-)-nicotine (EC\(_{50} = 21 \pm 4\) μM, \(n = 3\)) in activating the efflux of \(^{86}\)Rb\(^{+}\) through this nAChR subtype (fig. 3B), a finding that agrees with the greatly diminished ability of this agent to elicit depressor/pressor responses in anesthetized dogs (Arneric et al., 1996b). When evaluated for its ability to block nAChR-mediated ion flux in IMR 32 cells, ABT-089 was found to inhibit (-)-nicotine-elicited cation efflux with an IC\(_{50}\) of 100 μM (fig. 3B, inset).

**Human TE 671 cells.** ABT-089 was more potent (EC\(_{50} = 30\) μM) but less efficacious (60%) than (-)-nicotine (EC\(_{50} = 180\) μM) to stimulate cation efflux from the human medulloblastoma cell line TE 671, effects that were completely blocked by mecamylamine (100 μM) and tubocurarine (100 nM) (fig. 4). The R-enantiomer of ABT-089, A-94224, activated cation efflux in this cell line at concentrations in excess of 300 μM. At a concentration of 1 mM, A-94424 was 40% as efficacious as (-)-nicotine. The nAChR subunits involved in mediating the effects of ABT-089 in this cell line are unclear at present (see discussion below).

### Channel Currents

**Human \(\alpha_7\).** The activity of ABT-089 at the human \(\alpha_7\) homo-oligomeric nAChR was determined electrophysiologically in *Xenopus laevis* oocytes injected with \(\alpha_7\) nAChR RNA. In this preparation, (-)-nicotine acts as an agonist with an EC\(_{50}\) of 83 μM (Briggs et al., 1995). In contrast, ABT-089 acted as a very weak agonist (fig. 5). At a concentration of 1 mM, the efficacy of ABT-089 was only 1.5 ± 0.2% (\(n = 9\)) of that found for (-)-nicotine (100 μM). This effect was concentration-dependent, because 100 μM ABT-089 elicited smaller responses (0.6 ± 0.1%, \(n = 4\)). The response to 1 mM ABT-089 was blocked reversibly by the \(\alpha_7\) nAChR antagonist, MLA (10 nM, not shown). A-94224, the R-enantiomer of ABT-089, was a slightly weaker agonist; 100 μM and 1 mM A-94224 elicited responses of 0.5 ± 0.2% (\(n = 3\)) and 1.0 ± 0.2% (\(n = 3\)) of the maximal (-)-nicotine response, respectively. The response to 1 mM A-94224 was 58 ± 6% as large as the response to 1 mM ABT-089 in the same oocytes (\(n = 3\)).

---

**TABLE 1**

Cholinergic binding properties of ABT-089, A-94224 and (-)-nicotine

<table>
<thead>
<tr>
<th>Compound</th>
<th>[^{[3]}H](-)-Cytisine</th>
<th>[^{[25]}I]α-BgtT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat brain</td>
<td>Human (\alpha_4\beta_2)</td>
</tr>
<tr>
<td>ABT-089</td>
<td>17 ± 2</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>A-94224</td>
<td>39 ± 5</td>
<td>35 ± 3</td>
</tr>
<tr>
<td>(-)-Nicotine</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.09</td>
</tr>
</tbody>
</table>
In Alzheimer’s disease, the most consistent neurochemical abnormality is the decrease in cholinergic neurotransmission (Coyle et al., 1983). Concentration-response curves for nAChR-mediated enhancement of ACh release from rat hippocampal synaptosomes by ABT-089 and (-)-nicotine are shown in figure 6. ABT-089 (EC50 0.43 μM administered to cultures of either rat cortical cells or IMR 32 cells for 15 min elicited a significant increase in [3H]ACh release. In contrast to its effects on nAChR-mediated ACh release, ABT-089 was 30% less efficacious and 25-fold less potent (EC50 = 1.1 ± 0.3 μM) than (-)-nicotine (EC50 = 0.04 ± 0.02 μM) in stimulating the release of [3H]ACh from rat striatal slices (fig. 7), an effect blocked by the competitive nAChR antagonist, DHβE (10 μM). The R-enantiomer of ABT-089, A-94224, was 10-fold less potent than ABT-089, but equally efficacious, to stimulate the release of [3H]dopamine.

Neuroprotective Effects of ABT-089

The ability of ABT-089 to protect against glutamate-induced neurotoxicity was investigated in primary cultures of rat cortical cells and in differentiated human IMR 32 cells. L-Glutamate (300 μM) administered to cultures of either rat cortical cells or IMR 32 cells for 15 min elicited a significant (P < .05) increase in the levels of LDH above basal levels (basal, 8–10%; glutamate-treated, 30–35%) assessed 24 h later. Pretreatment of either the IMR 32 or rat cortical cells with ABT-089 protected against the excitotoxic insult in a concentration-dependent manner (fig. 8, A and B). EC50 values for ABT-089 were 3 ± 2 μM (IMR 32 cells) and 10 ± 3 μM (rat cortical cells). The neuroprotection elicited by ABT-089 was time-dependent; maximal effects were observed after a 2-h pretreatment with ABT-089 (data not shown). The R-
Discussion

The present study describes the in vitro pharmacological properties of a novel ChCM, ABT-089. In radioligand binding studies, ABT-089 was shown to display selectivity toward the high-affinity (−)-cytisine binding site present on the α4β2 nAChR subtype ($K_i = 16$ nM) relative to the $[^{125}]$-α-BgT binding site present on the α7 ($K_i > 10,000$ nM) and α1β1γ (K = 1000 nM) nAChR subtypes. In cation flux and electrophysiological studies ABT-089 displayed a more complex profile than (−)-nicotine having agonist, partial agonist and inhibitory activities depending on the nAChR subtype with which it interacts. ABT-089 differentially stimulated neurotransmitter release; the compound displayed a potency and efficacy similar to (−)-nicotine to facilitate ACh release but was markedly less potent and less efficacious than (−)-nicotine to stimulate DA release. ABT-089 also displayed neuroprotective properties in primary cultures of rodent cortical cells, as well as human neuroblastoma cells, which suggested the potential to prevent the process of neurodegeneration. The differential full agonist/partial agonist profile of ABT-089, as compared with (−)-nicotine and ABT-418, illustrates the complexity of nAChR activation and the potential to target responses at subclasses of neuronal and peripheral receptors.

The cloning of rat, chick and human brain cDNAs for nAChRs indicates a diversity of receptor subtypes in mammalian brain (Sargent, 1993; Role and Berg, 1996). The widespread distribution, yet regionally selective combination of α2, α3, α4, α7 and β2 transcripts in the brain suggests that subtypes of neuronal nAChRs may be selectively localized, although the physiological functions that these serve are currently unknown. Nonetheless, the use of binding and functional assays to design compounds that selectively interact with these various nAChRs represents a logical approach to delineating the effects of cholinergic channel modulators.
Two major classes of nAChRs have been identified in brain by radioligand binding studies; a high-affinity (-)-nicotine binding site that likely corresponds to the \( \alpha_4\beta_2 \) subtype and a high-affinity \( \alpha \)-BgT binding site on the \( \alpha_7 \) subtype and also on the \( \alpha_8 \) and \( \alpha_9 \) nAChR subtypes (Clarke et al., 1985; Elgoyhen et al., 1994; Gerzanich et al., 1994; Seguela et al., 1993). In the present study the interaction of ABT-089 with the high-affinity (-)-nicotine and \( \alpha \)-BgT binding sites in rodent brain and on the human \( \alpha_4\beta_2 \) and \( \alpha_7 \) subtypes stably expressed in mammalian cell lines was investigated. ABT-089 was significantly more potent at the \( \alpha_4\beta_2 \) subtype than at the \( \alpha_7 \) nAChR. Comparison of the radioligand binding data obtained in this study with those recently reported for ABT-418 and GTS-21 (Arneric et al., 1995; Briggs et al., 1997), indicates that ABT-089 (\( K_i = 16 \) nM) is equipotent to GTS-21 but is approximately 5-fold less potent than ABT-418 (\( K_i = 3 \) nM) at the \( \alpha_4\beta_2 \) subtype. At the \( \alpha \)-BgT-sensitive binding site on the human \( \alpha_7 \) subtype, ABT-089 is less potent (\( K_i > 10,000 \) nM) than either ABT-418 (\( K_i = 4000 \) nM) or GTS-21 (\( K_i = 2000 \) nM). The lack of stereoselectivity of ABT-089 versus its enantiomer, A-94224, observed in the radioligand binding studies differentiates this agent from nicotine and other classical nAChR ligands. However, as newer ligands for nAChRs appear in the literature, it is becoming apparent that enantioselectivity is not a prerequisite property of ChCMs. For example, the enantiomers of epibatidine display similar potency (\( \sim 50 \) pM) in their interaction with the high-affinity-nicotine binding site present on the \( \alpha_4\beta_2 \) nAChR (Sullivan and Bannon, 1996).

Functionally, the effects of ABT-089 and (-)-nicotine were compared on neurotransmitter release, ion flux and current flow measured electrophysiologically. In contrast to (-)-nicotine and ABT-418, which have primarily cholinergic channel activator activity, ABT-089 demonstrates a complex pattern of activity with agonist activities at some subtypes of nAChRs and inhibitory activities at others.
ABT-089 was evaluated in two assay preparations thought to reflect an interaction with the \( \alpha 4 \beta 2 \) nAChR subtype. The human \( \alpha 4 \beta 2 \) subunit combination stably expressed in a mammalian cell line (K177) has recently been shown to have pharmacological properties similar to those of the avian \( \alpha 4 \beta 2 \) subtype and that found in rodent brain (Whiting et al., 1991; Flores et al., 1992; Gopalakrishnan et al., 1996). ABT-089 was found to display negligible agonist activity at this subtype at concentrations up to 300 \( \mu M \) but did weakly inhibit \((-\))-nicotine-evoked cation efflux (\( IC_{50} = 176 \mu M \)). As noted above, ABT-089 potently interacts with the agonist binding site on the \( \alpha 4 \beta 2 \) subtype in radioligand binding experiments thought to reflect an interaction with the desensitized state of the receptor (Lippiello et al., 1987). Thus, the very weak functional activity of ABT-089 at this subtype may be suggestive of a preferential interaction with a desensitized non-conducting state of this receptor.

In contrast to its effects on the human \( \alpha 4 \beta 2 \) subtype, ABT-089 was a relatively potent (\( EC_{50} = 5 \mu M \)) (34\% of \((-\))-nicotine) partial agonist to stimulate cation efflux from mouse thalamic synaptosomes. As noted above, nAChR-mediated stimulation of cation efflux from mouse thalamic synaptosomes has been proposed to reflect the activation of the \( \alpha 4 \beta 2 \) subtype (Marks et al., 1993). The differences in activity of ABT-089 in these two \( \alpha 4 \beta 2 \) preparations may reflect species differences in the effects of the ligand or differences in the methodology used to assess cation efflux. Unfortunately, amino acid sequence information is not yet available for the murine \( \alpha 4 \) subunit preventing analysis of the contribution, if any, of species differences. Alternatively, it maybe that in mouse thalamus ABT-089 is modulating cation efflux via interactions with nAChR subunits in addition to \( \alpha 4 \beta 2 \) because \( \alpha 3 \) and \( \alpha 5 \) are expressed in this region (Wada et al., 1989; Sargent, 1993).

The \( \alpha 7 \) nAChR will self-assemble to form functional homomeric nAChRs in Xenopus oocytes (Seguela et al., 1993; Briggs et al., 1995), and in a mammalian cell line stably expressing this subunit (Gopalakrishnan et al., 1995). In this...
preparation ABT-089 acted as a very weak agonist. Indeed, the responses to ABT-089 could appear “negligible” when plotted on the same scale as the control response to (−)-nicotine. However, the effect of ABT-089 could be measured when the recorded data were plotted on an expanded scale (see fig. 5). These small responses were blocked by the selective antagonist MLA (10 nM), confirming their being caused by activation of the α7 nAChR.

nAChR-mediated activation of cation efflux in the IMR 32 neuroblastoma cell line may be mediated via a “ganglionic-like” nAChR subtype, most likely containing α3 and β4 nAChR subunits and possibly an αδ subunit (Lukas, 1993). Thus, ChCMs with reduced activity relative to (−)-nicotine in this preparation might be expected to have an enhanced cardiovascular safety profile compared with the (−)-nicotine. Indeed, it was recently reported that ABT-418 and (−)-epibatidine, ChCMs with reduced and enhanced potency, respectively, relative to (−)-nicotine in this preparation display diminished and increased cardiovascular effects, respectively, relative to (−)-nicotine at equivalent doses in anesthetized dog (Arneric et al., 1995; Sullivan and Bannon, 1996). In the present study, ABT-089 was found to have negligible activity at this putative human ganglionic subtype. This finding agrees with the greatly diminished ability of this agent to elicit adverse cardiovascular effects in anesthetized dog (Arneric et al., 1996b).

Cells of the human medulloblastoma clonal line TE 671 have been shown to express the human α1, β1, δ and γ subunits that form high-affinity α-Bgt binding sites with pharmacological properties similar to the neuromuscular junction subtype (Lukas, 1986). Recently, we have found with use of reverse transcriptase-polymerase chain reaction and radioligand binding techniques that this cell line also expresses human neuronal subunits (L.M. Monteggia, manuscript in preparation). This finding raises the possibility that the β1, δ and γ subunits may combine with various neuronal nAChR subunits to form subtypes with unique pharmacological properties. ABT-089 is more potent (EC50 = 30 μM) but less efficacious (60%) than (−)-nicotine (EC50 = 180 μM) to stimulate cation efflux in this cell line (fig. 4), effects that are blocked by mecamylamine (100 μM). Thus, ABT-089 is more potent than (−)-nicotine to activate this human nAChR in contrast to its effects at the human α4β2, α7 and α3βx subtypes. Experiments elucidating the subunit combination responsible for the ABT-089 mediated cation efflux in this human cell line are ongoing and may provide additional insight into the mechanism of action of this compound.

nAChRs have a modular role on several neurotransmitter systems including cholinergic, noradrenergic, serotonergic, GABAergic and dopaminergic systems (Wonnacott et al., 1990; Sacaan et al., 1995; Wonnacott, 1997). A decrease in cholinergic transmission, arising from the degeneration of the basal forebrain cholinergic system, is the most consistent neurochemical abnormality in patients with Alzheimer’s disease (Coyle et al., 1983). Agents that act to selectively enhance cholinergic transmission have been a major focus of pharmaceutical research for the past decade (Arneric et al., 1996a). In the present study, ABT-089 was found to be a selective modulator of ACh release compared with (−)-nicotine. ABT-089 was found to be slightly less potent (EC50 = 3 μM), but equally efficacious compared with (−)-nicotine (EC50 = 1 ± 0.23 μM) to stimulate [3H]ACh release from rat hippocampal synaptosomes (fig. 6). Although the subtype(s) mediating this effect are uncertain at present, this finding suggests that ABT-089 is a full agonist at some nAChR subtypes.

In contrast to its effects on nAChR-mediated ACh release, ABT-089 is 30% less efficacious and 25-fold less potent (EC50 = 1.1 μM) than (−)-nicotine (EC50 = 0.04 μM) in stimulating the release of [3H]dopamine release from rat striatal slices (fig. 7). It is thought that the addiction liability and locomotor stimulant effects associated with chronic exposure to (−)-nicotine are mediated via dopamine release. The decreased potency of ABT-089 to stimulate dopamine release, compared with (−)-nicotine, thus suggests it may have less dependence potential.

Neurodegeneration caused by excitotoxic damage has been implicated in the etiology of both Alzheimer’s disease and Parkinson’s disease. Recent studies suggest that ChCMs can block N-methyl-D-aspartate-receptor-mediated glutamate toxicity in rat cortical cells and in differentiated neuroblastoma cell lines (Akaike et al., 1994; Marin et al., 1994; Donnelly-Roberts et al., 1996). Activation of either rat cortical or differentiated IMR 32 nAChRs by (−)-nicotine and ABT-418 afforded in vitro neuroprotection against glutamate toxicity (Donnelly-Roberts et al., 1996). In the present study, ABT-089 was found to protect rat cortical neurons and differentiated IMR 32 cells from an excitotoxic insult (fig. 8).

The effects of ABT-089 were time-dependent, with maximal neuroprotection being observed when ABT-089 was administered 2 h before the glutamate insult, which suggests that some early immediate gene response process may be involved in mediating the beneficial effects of the compound. Further, the potency of ABT-089 to elicit neuroprotection was dramatically increased after subacute (7-day) treatment. In a behavioral paradigm assessing cognition, the Morris water maze, the potency and efficacy of ABT-089 is enhanced after subacute treatment (Decker et al., 1997, companion paper). It is noteworthy that the minimally effective neuroprotective concentrations of ABT-089 (0.01–0.1 μM) after subacute exposure approximate the cognitive-enhancing plasma and brain levels of ABT-89 after subacute treatment in rodents (Decker et al., 1997, companion paper).

The neuroprotective effects of ABT-089 appear to be mediated via an interaction with nAChRs and may involve the α7 subtype, because the neuroprotective effects of this compound were attenuated by α-Bgt, a selective antagonist of the α7 subtype relative to the α4β2 and α3βx nAChRs. Similar results have recently been reported for (−)-nicotine and ABT-418 (Akaike et al., 1994; Donnelly-Roberts et al., 1996). α-Bgt was not neuroprotective by itself (Donnelly-Roberts et al., 1996). There are however several issues that need to be addressed before concluding that the α7 nAChR is mediating the neuroprotective effects of ABT-089. First, the neuroprotective potency of ABT-089 is similar to that of (−)-nicotine whereas the latter is at least 5-fold more potent to displace [125I]-βGt, a selective antagonist of α7 nAChRs. Second, the potency or efficacy of these compounds to activate homomeric α7 nAChRs does not correlate with their neuroprotective effects. Indeed, the EC50 values for neuroprotection are at least 10-fold lower than the corresponding EC50 to activate α7 channel currents. The explanation for this apparent paradox is not known. However, at the neuroprotective concentrations, ABT-089, ABT-418 and (−)-nicotine can inhibit the
functional activation of the α7 by ACh, possibly via desensitization of the receptor (C.A. Briggs, manuscript in preparation). Thus, it is possible that the neuroprotective effects of cholinergic channel modulators are mediated via the homeric α7 nAChR through mechanisms more complex than simple activation of channel currents, via a subunit combination that includes α7 and/or via a novel α-Bgt-T-sensitive nAChR. In the case of the latter, it is noteworthy that in situ α7 nAChR subunits may contribute to heteromeric nAChR assemblies containing other proteins that are not yet well defined (Listerud et al., 1991). Further, a second high-affinity α-Bgt-T-sensitive nAChR, α6, has been identified in chick brain (Gerzanich et al., 1994) but no evidence has been presented to date for its existence in rodent or human tissues.

The intracellular mechanism(s) underlying the temporal effects of ABT-089 in this in vitro model of neurototoxicity are unclear at present. Possibilities include an up-regulation of the α7 nAChR subtype and consequent changes in intracellular calcium levels which may trigger changes in the levels/activity of neurotrophic factors, intracellular messengers, transcriptional/translational factors (i.e., c-fos) or genes that prevent apoptotic processes (i.e., Bcl-x). It remains to be determined whether the neuroprotective effects of ABT-089 reported here extend to other forms of neurototoxicity (i.e., Aβ- or gp120-induced toxicity).

Compounds that selectively modulate nAChR subtypes to elicit beneficial effects on cognition, attentional processes and anxiety states and that have reduced side-effect liabilities compared with (−)-nicotine have started to appear in the literature in recent years. In particular, ABT-418 and GTS-21 represent novel ChCMs developed for the treatment of Alzheimer's disease. Comparison of the in vitro pharmacological properties of ABT-089 with those of ABT-418 and GTS-21 suggest that the profile of activity of ABT-089 is more similar to that found for GTS-21. Both modulators display negligible activity at α4β2 and human ganglionic receptors but do weakly activate the human α7 subtype. In vivo, both compounds have cognitive enhancing effects in rodents and primates (Woodruf-Pak et al., 1995; Briggs et al., 1997; Decker et al., 1997, companion paper). However, ABT-089 is substantially more bioavailable than GTS-21 and ABT-418 after oral administration and has potential as a once-a-day formulation (Arneric et al., 1996b).

In conclusion, the findings of the present paper suggest that ABT-089 is a novel ChCM that differentially interacts with nAChR subtypes to modulate neurotransmitter release and elicit neuroprotective effects.

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