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# Analysis of 3-(4-Hydroxy, 2-Methoxybenzylidene)Anabaseine Selectivity and Activity at Human and Rat *Alpha*-7 Nicotinic Receptors[1]

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## ABSTRACT

3-(2,4-dimethoxybenzylidene)anabaseine (GTS-21) is a selective partial agonist for rat *alpha*-7 nicotine receptors with reportedly much lower efficacy for human *alpha*-7 receptors. Because this drug improves memory-related performance in nonhuman primates, and is presently in a clinical trial for Alzheimer's disease, we investigated the potential effects of its primary human metabolite, 3-(4-hydroxy, 2-methoxy-benzylidene)anabaseine) on human as well as rat nicotinic acetylcholine receptor. 4OH-GTS-21 exhibited a similar level of efficacy for both rat and human *alpha*-7 receptors expressed in *Xenopus* oocytes. It displaced high affinity [$^{125}$I]$\alpha$-bungarotoxin binding to human SK-N-SH cell-membranes ($K_i$ 0.17 $\mu$M) and rat PC12 cell-membranes ($K_i$ 0.45 $\mu$M). GTS-21 also displaced [$^{125}$I]$\alpha$-bungarotoxin binding to PC12 cell membranes with high potency ($K_i$ 0.31 $\mu$M), but was much less potent in this regard in SK-N-SH cells (23 $\mu$M). 4OH-GTS-21 produced less residual inhibition of either the human or rat AChR subtypes than GTS-21 did. To compare the neuroprotective efficacies of GTS-21 and 4OH-GTS-21 in both species, an amyloid-toxicity model (A$\beta$ 25-35) was used. 4OH-GTS-21 was protective in both human and rat cell lines, although GTS-21 was effective only in the latter. These studies suggest that the efficacy of GTS-21 in primates may depend on a pro-drug function.

Molecular, biochemical and physiological studies demonstrate the presence of multiple nicotinic receptor (AChR) subunits in brain and other tissues (Alkondon and Albuquerque, 1993; Deneris *et al.,* 1991; Papke, 1993). One of the predominant nicotinic receptor subtypes in the brain contains the *alpha*-7 subunit, especially in telencephalic regions such as hippocampus and neocortex, based on high affinity to BTX binding studies (Clarke *et al.,* 1985; Marks *et al.,* 1986). These receptors function as homo-oligomers when expressed in oocytes, where they demonstrate characteristic high affinity binding to $\alpha$-BTX, high calcium-permeability and rapid desensitization (Couturier *et al.,* 1990; Seguela *et al.,* 1993; de Fiebre *et al.,* 1995).

The identification of agents selective for the *alpha*-7 AChR subtype has drawn attention to their roles in neuronal survival and memory-related behaviors (de Fiebre *et al.,* 1995). One of these compounds, GTS-21 (also known as DMXB), is presently in a clinical trial for Alzheimer's disease, based on its behavioral actions and ability to protect rat neurons from various apoptotic and necrotic insults, both *in vivo* and *in vitro* (Martin *et al.,* 1994; Meyer *et al.,* 1998a, Shimohama *et al.,* 1998). These insults include amyloid-exposure, removal of nerve growth factor from differentiated PC12 cells, NMDA-induced toxicity and axotomy of septal-hippocampal cholinergic neurons. Recently, several selective *alpha*-7 agonists with differing residual *alpha*-7 receptor-antagonist activities were compared relative to their ability to protect differentiated PC12 cells from trophic factor deprivation (Meyer *et al.,* 1998b). This apparent residual inhibition of *alpha*-7 receptors most likely represents some form of channel block by agonist or a form of desensitization that is unique from the rapidly reversible desensitization that occurs with the application of high concentrations of ACh (de Fiebre *et al.,* 1995; Papke *et al.,* 1997). Agonists with significant residual antagonism were not cytoprotective, suggesting that an analysis of both agonist and antagonist activities may be necessary for predicting the cytoprotective efficacy of *alpha*-7 AChRs.

GTS-21 has also been shown to improve memory-related behaviors in nonhuman primates (Briggs *et al.,* 1997), aged rabbits (Woodruff-Pak *et al.,* 1994), nucleus-basalis lesioned rats (Meyer *et al.,* 1994) and aged rats (Arendash *et al.,* 1995). The improvement in delayed pair-matching behavior

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**ABBREVIATIONS:** GTS-21, 3-(2,4-dimethoxybenzylidene)anabaseine; 4OH-GTS-21, 3-(4-hydroxy, 2-methoxybenzylidene)anabaseine; ACh, acetylcholine; AChR, nicotinic acetylcholine receptor; BTX, bungarotoxin; PC12, pheochromocytoma 12.

in aged primates observed after GTS-21 administration is particularly interesting because this drug reportedly is only very weakly efficacious at human *alpha*-7 receptors. Although this result may indicate that nonnicotinic receptors underlie at least some actions of GTS-21 *in vivo,* it is also conceivable that one or more metabolites of GTS-21 possesses nicotinic agonist activity in humans.

An initial pharmacokinetic study of GTS-21 in the rat indicated that only minor amounts of this compound were excreted in an unaltered form (Mahnir *et al.,* 1998). Both methoxy substituents on the benzylidene ring of GTS-21 were potential sites of primary hepatic metabolism by *O*-demethylation (Kem *et al.,* 1996). The principal human primary metabolite is 3-(4-hydroxy,2-methoxybenzylidene)-anabaseine, or 4OH-GTS-21 (Azuma *et al.,* 1996). Because little is known about the properties of this metabolite relative to nicotinic receptors or their biological functions, we investigated these properties in several systems. First, to determine whether 4OH-GTS-21 was an agonist or antagonist at human and rat nicotinic receptor subtypes, its effects were measured on different AChR subunit combinations in the *Xenopus* oocyte system. Its binding to *alpha*-7 receptors was characterized by displacement of high affinity $\alpha$-BTX binding in human (SK-N-SH) and rat (PC12) cell lines. Finally, its biological activity was compared to GTS-21 relative to cytoprotection against amyloid-induced toxicity in both cell lines. An amyloid model for neurotoxicity was chosen because of its sensitivity to nicotinic receptor-mediated cytoprotection (Kihara *et al.,* 1997; Zamani *et al.,* 1997) and its ability to affect PC12 cells (Fagarasan and Efthimiopoulos, 1996); in addition to the well established involvement of amyloid-deposition in Alzheimer's disease. Our results indicate that 4OH-GTS-21 is an *alpha*-7-selective partial agonist with at least 10-fold greater efficacy for both human and rat $\alpha$7 AChRs than for any $\beta$-subunit containing AChR. It is also cytoprotective in human and rat cells, unlike GTS-21, which was protective only in the rat cell line.

## Methods

**Animals.** Female *Xenopus laevis* were housed in aquarium tanks maintained precisely at 18°C to reduce potential problems with seasonal variability in oocyte viability. Frogs were fed frog brittle (Nasco) and also kept on a 12 hr light:dark cycle.

**Cell cultures.** Rat PC12 cells and human SK-N-SH cells were purchased from American Tissue Culture Co. (Rockville, MD). PC12 cells were cultured by modifications of Greene and Tischler (1976). They were plated at 20 to 30% confluence on 35-mm plates precoated with poly-L-lysine (10 g/liter), then grown in Dulbecco's modified Eagle medium containing 10% heat-inactivated horse serum, 5% fetal bovine serum, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin and 0.5 mM L-glutamine. SK-N-SH cells were cultured and grown similarly, except without poly-L-lysine precoated plates. Cultures were maintained at 37°C, 94% $O_2$/6% $CO_2$ and 90 to 92% humidity. Nearly 100% confluent cultures were used for binding studies, although cytoprotection studies used 40 to 50% confluent plates.

**Xenopus oocyte expression and recording.** Preparation of *in vitro* synthesized cRNA transcripts and oocyte injection were described previously (de Fiebre *et al.,* 1995, Papke *et al.,* 1997). The measurement of human $\alpha4\beta2$ responses was as described (Gerzanich *et al.,* 1995), although all other conventional recordings were as in Papke *et al.* (1997). Recordings were made 2 to 7 days after mRNA injections. Current responses to drug administration were studied under two electrode voltage clamp at a holding potential of −50 mV. Recordings were made using a Warner Instruments oocyte amplifier interfaced with National Instruments' (Dallas, Texas) LabView software. Current electrodes were filled with 250 mM CsCl, 250 mM CsF and 100 mM EGTA, pH 7.3 and had resistances of 0.5 to 2.0 MΩ. Voltage electrodes were filled with 3 M KCl and had resistances of 1 to 3 MΩ. Because the general health of the oocytes, their ability to express the heterologous receptors and to maintain stable holding potential were all correlated with the cell's initial resting potential, a criterion for minimal initial resting potential was set at −30 mV. For analysis of concentration-responses relationships, oocytes were placed in a Lucite recording chamber with a total volume of 0.6 ml and were perfused at room temperature with frog Ringer's (115 mM NaCl, 2.5 mM KCl, 10 mM HEPES pH 7.3, 1.8 mM $CaCl_2$) plus 1 $\mu$M atropine to block potential muscarinic responses. The bath solution maintains calcium at a physiologically relevant concentration because extracellular calcium is an important modulator of neuronal nAChR function (Mulle *et al.,* 1992, Vernino *et al.,* 1992); calcium will also have indirect effects on agonist-evoked responses via calcium-dependent chloride channels at this concentration. However, we have previously shown that calcium-dependent effects produce only linear amplification of peak responses and do not distort the concentration-responses relationships over a wide range of agonist concentrations (Papke *et al.,* 1997).

Drugs were diluted in perfusion solution and applied at a rate of 6 ml/min for all concentrations and AChR subtypes. This represents an agonist application protocol typical for oocyte-expression experiments (Briggs *et al.,* 1997; Luetje and Patrick, 1991; Papke and Heinemann, 1991; Papke *et al.,* 1997). Responses were normalized for the level of channel expression in each cell by measuring the response to an initial ACh application 5 min before presentation of the test concentration of ACh or experimental agonist. These control ACh applications were: 1 $\mu$M ACh for muscle type AChRs; 10 $\mu$M ACh for $\alpha2\beta2$, $\alpha3\beta2$, and $\alpha4\beta2$ AChRs; 30 $\mu$M for $\alpha3\beta4$ AChRs and 300 $\mu$M ACh for $\alpha$7 AChRs. Means and S.E.M. were calculated from the normalized responses of at least four oocytes for each experimental concentration. Comparisons of the GTS-21 and 4OH-GTS-21 evoked responses of non-*alpha*-7 rodent AChRs were expressed relative to ACh-evoked maximum responses. The comparisons were based on the internal ACh controls for each cell and every subtype tested. The ACh control values in the 4OH-GTS-21 challenged cells were then compared to the ACh maximums reported in previous oocyte studies that relied on identical methods and materials for the mouse muscle (Francis and Papke, 1996), and rat neuronal (Papke, *et al.,* 1997) nAChR. *Alpha*-7 AChR responses typically display an increase after the initial application of agonist that subsequently stabilizes (de Fiebre *et al.,* 1995); therefore, *alpha*-7-expressing oocytes receive two control applications of ACh separated by 5 min at the start of recording, with the second response used for normalization.

After the application of experimental drug solutions, cells were washed with control Ringer's solution for 5 min and then evaluated for potential inhibition by measuring the response to another application of the ACh. These second control responses were normalized to initial ACh responses measured 10 min earlier. If the second ACh response showed a difference of ≥25% from the initial ACh control response, the oocyte was not used for further evaluations. Otherwise, the second ACh application served to normalize the response of any subsequent drug application.

Complete concentration-response relationships for ACh and the experimental anabaseine compounds were calculated for both human and rat $\alpha$7 AChR subtypes using standard methods. To compare responses to experimental compounds directly with responses evoked by ACh, the concentration-response relationships for GTS-21 and 4OH-GTS-21 were scaled by the ratio of the maximum ACh response to the control ACh concentration response for each specific subtype. These scaled values are plotted in the figures. The plots were generated in Kaleidagraph, and the curves were generated

using the following modified Hill equation (Luetje and Patrick, 1991):

$$\text{Response} = \frac{I_{max}[\text{agonist}]^n}{[\text{agonist}]^n + (EC_{50})^n}$$

where $I_{max}$ denotes the maximal response for a particular agonist/subunit combination and n represents the Hill coefficient. Unless otherwise noted, $I_{max}$, $EC_{50}$ and n were all free parameters and optimized by the curve-fitting procedure.

***α*-BTX binding measurements.** Cultured cells were homogenized in 10 volumes of iced cold Krebs-Ringer's HEPES (KRH) buffer (NaCl, 118 mM; KCl, 4.8 mM; $MgSO_4$, 1.2 mM; $CaCl_2$, 2.5 mM; and HEPES, 20 mM; pH adjusted to 7.5 with NaOH). These homogenates were centrifuged at 20,000 × *g* for 20 min at 4°C, and the resulting pellets resuspended in Krebs-Ringer's HEPES. Binding of [$^{125}$I]-BTX was measured using the method of Marks *et al.* (1986). The final incubation contained 200 to 400 μg protein/250 μl with 2 nM [$^{125}$I]α-BTX; this 2-hr incubation was at 37°C. Binding was terminated by diluting with 3 ml of ice-cold KRH buffer, followed immediately by filtration through glass fiber filters (Whatman GFB; Springfield Mill, UK) soaked in buffer containing 0.5% polyethylenimine for 30 min at room temperature. Nonspecific binding was determined with 1 mM unlabeled nicotine. Each condition was measured in triplicate, and each assay conducted with at least three separate preparations of tissue. $K_i$ values were calculated by the equation of Cheng and Prusoff (1973) using $K_d$ values calculated for each cell type.

**Neuroprotection assay.** PC12 or SK-N-SH cells were exposed to 20 μM Aβ 25 to 35 in fresh DMEM for 24 hr in the presence of specified drug treatments. Medium was replaced 10 min before addition of amyloid, with drugs added with the new medium. Cell density was estimated using the NIH Image program version 1.55 (Martin *et al.,* 1994). A Nikon inverted microscope (100× magnification) was attached to a Mac II computer via a monochrome video camera (Cohu, Inc., San Diego, CA). Four random areas were counted per plate and there were four plates per treatment group, unless otherwise indicated. Values were compared by one-way analysis of variance using the Statview II program.

**Chemicals.** GTS-21 was provided by Taiho Pharmaceuticals (Tokushima, Japan), and 4OH-GTS-21 by Dr. Bill Kem at the University of Florida. All other chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

## Results

**GTS-21 and 4OH-GTS-21 concentration-response relationships for human and rat *alpha*-7 AChR.** Data on the full concentration responses relationships for ACh, GTS-21 and 4OH-GTS-21 were generated for human and rat *alpha*-7 AChR with regard to both activation and the residual inhibition produced to subsequent ACh applications (fig. 1). To compare these results to previous studies (Briggs *et al.,* 1997; de Fiebre *et al.,* 1995; Gopalakrishnan *et al.,* 1995; Hunter *et al.,* 1994; Papke *et al.,* 1997, Sands *et al.,* 1993; Séguéla *et al.,* 1991), responses were plotted as a function of applied concentration. The resulting values are in reasonable agreement with those of previous studies and represent the relative scaling of potencies and efficacies.

Our results indicate that 4OH-GTS-21 had a good activity profile for both human and rat AChRs, producing relatively large responses and having only small residual inhibitory effects. The values obtained from the curve fits are given in table 1. After the application of GTS-21, and to a far lesser extent, after the application of 4OH-GTS-21, subsequent control ACh responses were decreased. This residual inhibition was quantified, and the results are displayed in figure 1, C and D, with $IC_{50}$ values for inhibition provided in table 2. We previously reported (de Fiebre *et al.,* 1995) that ACh produced no residual inhibition of control ACh responses over a similar concentration range. Responses that were inhibited by GTS-21 were not much recovered after two wash periods. Recovery time-constants from inhibition were not determined because they were too slow compared to the time scale of an ordinary experiment (20–60 min).

***α*-BTX binding in rat and human cell lines.** Binding studies with [$^{125}$I]α-BTX indicated that both rat PC12 cells and human SK-N-SH cells had similar $K_d$s for the toxin, 1.7 and 2.2 nM, respectively. Based on an α-BTX binding concentration of 2 nM, $K_i$ values for the inhibition of α-BTX binding to PC12 cells were 0.31 ± 0.02 μM and 0.17 ± 0.02 μM (mean ± S.E.M. of three experiments) for GTS-21 and 4OH-GTS-21, respectively (fig. 2). For SK-N-SH cells, the $K_i$ values for the inhibition of [$^{125}$I]α-BTX binding were 0.45 ± 0.03 μM and 23 ± 2 μM for 4OH-GTS-21 and GTS-21, respectively.

**Selectivity of 4OH-GTS-21 for *α*7 AChRs.** The low relative efficacy of GTS-21 for a variety of rat neuronal nicotinic AChRs (*i.e.,* α2β2, α2β4, α3β2, α3β4, α4β2 and α4β4) has previously been reported (Meyer *et al.,* 1997). Specifically, 100 μM GTS-21 produced responses in these AChRs that were less than 1% of the maximum response obtainable with ACh on all these AChR subtypes. To test the hypothesis that the 4OH-GTS-21 derivative would show a similar selectivity for *alpha*-7-type receptors compared to other nAChRs, the activity of 100 μM 4-OH-GTS-21 was measured on mouse muscle AChR and rat neuronal AChR. Responses to 4OH-GTS-21 were less than 1% of the ACh maximum for α1β1γδ, α2β2, α3β4 and α4β2 AChR, and 4 ± 1% the ACh maximum for α3β2 AChR (data not shown).

We previously observed that there was a small but significant inhibition of rat α4β2 AChR responses following the application of 100 μM GTS-21 (de Fiebre *et al.,* 1995). However, our data indicate that after a 100 μM application of 4OH-GTS-21, there was no significant residual inhibition of any of the rodent AChR subtypes tested (*i.e.,* postapplication controls were all ±25% of preapplication controls; data not shown).

**Effects of GTS-21 and 4OH-GTS-21 on the human *α4β2* AChRs.** Because the α4β2 subunit combination represents the predominant high affinity nicotine receptor in the brain (Flores *et al.,* 1992; Nakayama *et al.,* 1991; Whiting and Lindstrom, 1988), it was also of interest to evaluate the activity of the two anabaseine compounds on the human form of this AChR. Consistent with the results obtained with rodent AChRs, both GTS-21 and 4OH-GTS-21 appeared to be only very weak partial agonists on the human α4β2 AChRs, with relative efficacies compared to ACh of no more than 5 or 1%, respectively (fig. 3).

At a concentration of 3.0 μM, GTS-21 produced responses that were approximately about 2.5% of the ACh maximum response, which corresponded to 50% of the maximum response that could be elicited by this drug. Coapplication of the GTS-21 and 4OH-GTS-21 with ACh inhibited responses compared to ACh alone (fig. 3, B and D for GTS-21 and 4OH-GTS-21, respectively). The inhibitory potency of 4OH-GTS-21 was nearly 10 times higher than for GTS-21 ($IC_{50}$ values of 2.13 ± 0.15 μM and 19.7 ± 1.3 μM, respectively). However, no residual inhibition of subsequent responses to

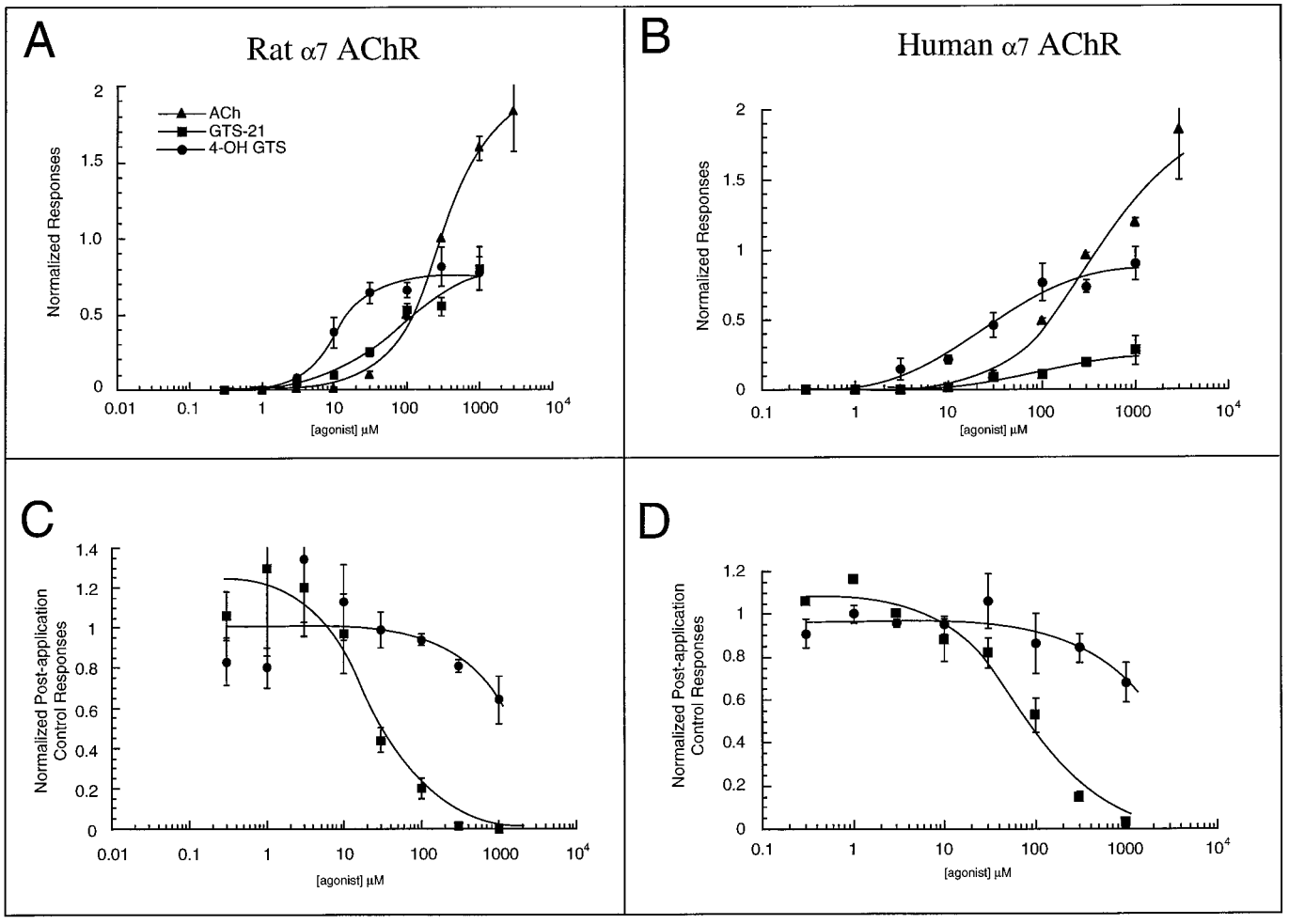


**Fig. 1.** Rat and human *alpha*-7 AChR responses to ACh, GTS-21 and 4OH-GTS-21. **A,** The concentration-response relationships for the peak agonist activated currents of oocytes injected with RNA coding for the rat *alpha*-7 subunit to ACh, GTS-21 and 4OH-GTS-21. All responses were initially measured relative to the individual oocyte's response to 300 $\mu$M ACh, applied 5 min before the experimental application. **B,** The concentration-response relationships for the peak agonist activated currents of oocytes injected with RNA coding for the human *alpha*-7 subunit to ACh, GTS-21 and 4OH-GTS-21. All responses were initially measured relative to the individual oocyte's response to 300 $\mu$M ACh, applied 5 min before the experimental application. To obtain fits that were satisfactory by eye, the maximum responses for ACh and GTS-21 were constrained to the maximum responses achieved experimentally. **C,** The residual inhibition of control (300 $\mu$M) ACh responses of rat *alpha*-7 injected oocytes after the application of ACh, GTS-21 or 4OH-GTS-21 at the indicated concentrations. All responses are expressed relative to the individual oocyte's response to 300 $\mu$M ACh, applied 5 min before the application of the experimental agonist. **D,** The residual inhibition of control (300 $\mu$M) ACh responses of human *alpha*-7 injected oocytes after the application of ACh, GTS-21 or 4OH-GTS-21 at the indicated concentrations. All responses are expressed relative to the individual oocyte's response to 300 $\mu$M ACh, applied 5 min before the application of the experimental agonist.

TABLE 1

Efficacy and potency of GTS-21 and 4OH-GTS-21 on rat and human $\alpha$7 AChR

| Agonist | Rat $\alpha$7 AChRs | | Human $\alpha$7 AChRs | |
|---|---|---|---|---|
| | Efficacy compared to ACh | $EC_{50}$ | Efficacy compared to ACh | $EC_{50}$ |
| ACh | 100% | 274 ± 20 $\mu$M | 100% | 322 ± 54 $\mu$M |
| GTS-21 | 50% | 81 ± 39 $\mu$M | <20% | 109 ± 20 $\mu$M |
| 4OH GTS-21 | 50% | 10 ± 2 $\mu$M | 40% | 26 ± 7 $\mu$M |

Efficacy values were calculated from ratios of the $I_{max}$ parameters generated in the curve fits in Figure 1 (see "Methods"). Similarly, the $EC_{50}$ values were those generated by the curve fits from figure 1.

control ACh applications was observed when either compound was applied in the absence of ACh to human $\alpha4\beta2$ AChRs at concentrations $\leq$5 times their $IC_{50}$ values in co-application experiments.

**Cytoprotective effects of GTS-21 and 4OH-GTS-21 on PC12 and SK-N-SH cells.** A$\beta$ 25-35 reduced the viability of both PC12 cell and SK-N-SH cells to a similar extent at a 20 $\mu$M concentration over a 24-hr interval (fig. 4). GTS-21 and 4OH-GTS-21 both protected against this A$\beta$ 25-35 induced toxicity in PC12 cells at a 10 $\mu$M concentration; however, only 4OH-GTS-21 protected SK-N-SH cells at this concentration. The cytoprotective activity of 4OH-GTS-21 in SK-N-SH cells was blocked by mecamylamine, a nicotinic antagonist, indicating the role of nicotinic receptors in its cytoprotective action (fig. 5). Mecamylamine alone had no effect on cell viability, indicating that the endogenous agonist choline, which is generated by cell plasma membranes, had no apparent effect on cell viability.

## Discussion

Nicotine has been studied as a potential treatment for Alzheimer's disease because of its ability to improve a variety of spatial memory and nonspatial avoidance tasks in animals

TABLE 2
Residual inhibition of α7 AChRs (from fig. 1)

| Agonist | *Rat α7 AChRs* $IC_{50}$ | *Human α7 AChRs* $IC_{50}$ |
|---|---|---|
| ACh | ≫3 mM | ≫3 mM |
| GTS-21 | 22 μM | 76 μM |
| 4OH GTS-21 | >1 mM | >1 mM |

$IC_{50}$ values for GTS-21 inhibition of rat and human α7 receptors were generated from the curve fits in figure 1, C and D. No $IC_{50}$ values could be calculated for 4OH-GTS-21 or ACh inhibition of rat and human α7 receptors due to the relatively low amounts of inhibition produced by these compounds. However, after the application of 1 mM 4OH-GTS-21 (the highest concentration tested), a small but statistically significant inhibition was observed. No inhibition by ACh was observed, even after the application of 3 mM ACh, the highest concentration tested. $n = 4$ or more experiments/value.

and to enhance delayed recall, attention and other memory-related behaviors in humans. Unfortunately, the toxic side effects associated with this drug reduce its practicality as a therapeutic agent, which has led to the search for novel agents with more selectivity for the nicotinic receptors underlying these behaviors. The observations that GTS-21 and related 3-substituted anabaseine compounds were highly selective *alpha*-7 agonists, and that these agents improved a variety of memory related behaviors in primates as well as several rodent species, suggested that they may be useful along these lines (Briggs *et al.,* 1997; Woodruff-Pak *et al.,* 1994; Meyer *et al.,* 1994; Arendash *et al.,* 1995). However, GTS-21 was also found to be a very weak agonist at human *alpha*-7 receptors, arguing that its behavioral activity in primates may depend on some other mechanism of action (Briggs *et al.,* 1997).

Our results demonstrate that the principal metabolite of GTS-21 in humans, 4OH-GTS-21, is a selective partial agonist for both human and rat *alpha*-7 receptors. It retains the cytoprotective activity of GTS-21 previously reported in rat cell lines, but is the first *alpha*-7 AChR selective agonist demonstrated to be cytoprotective in human cells. Our results also extend the concept that benzylidine-anabaseine derivatives are selective for *alpha*-7 AChRs (de Fiebre *et al.,* 1995). The specific substitutions on the benzylidine ring influence this selectivity in terms of regulating the potency, efficacy and the balance between agonist and antagonist properties. The 4OH-metabolite of GTS-21 (Azuma *et al.,* 1996) appears to have a favorable activity profile for the human *alpha*-7 AChR. The selective retention of agonist activity by 4OH-GTS-21, compared to GTS-21, is consistent

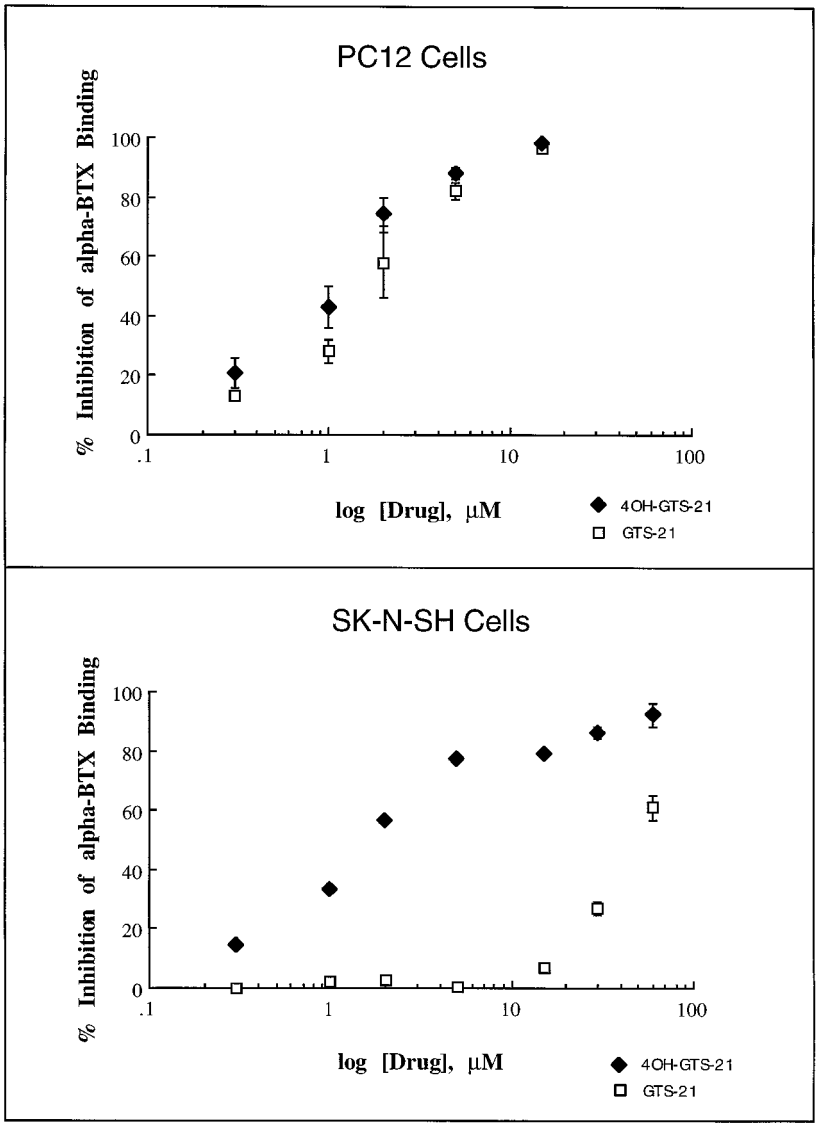


**Fig. 2.** Effects of GTS-21 and 4OH-GTS-21 on high affinity α-BTX binding in membranes from rat PC12 cells and human SK-N-SH cells. Membranes from PC12 cells (top) or SK-N-SH cells (bottom) were assayed for nicotine-displaceable, high affinity binding of α-BTX (2 nM) in the presence of specified concentrations of GTS-21 or 4OH-GTS-21. Each value is the mean ± S.E.M. of three plates per group, each assayed in triplicate.

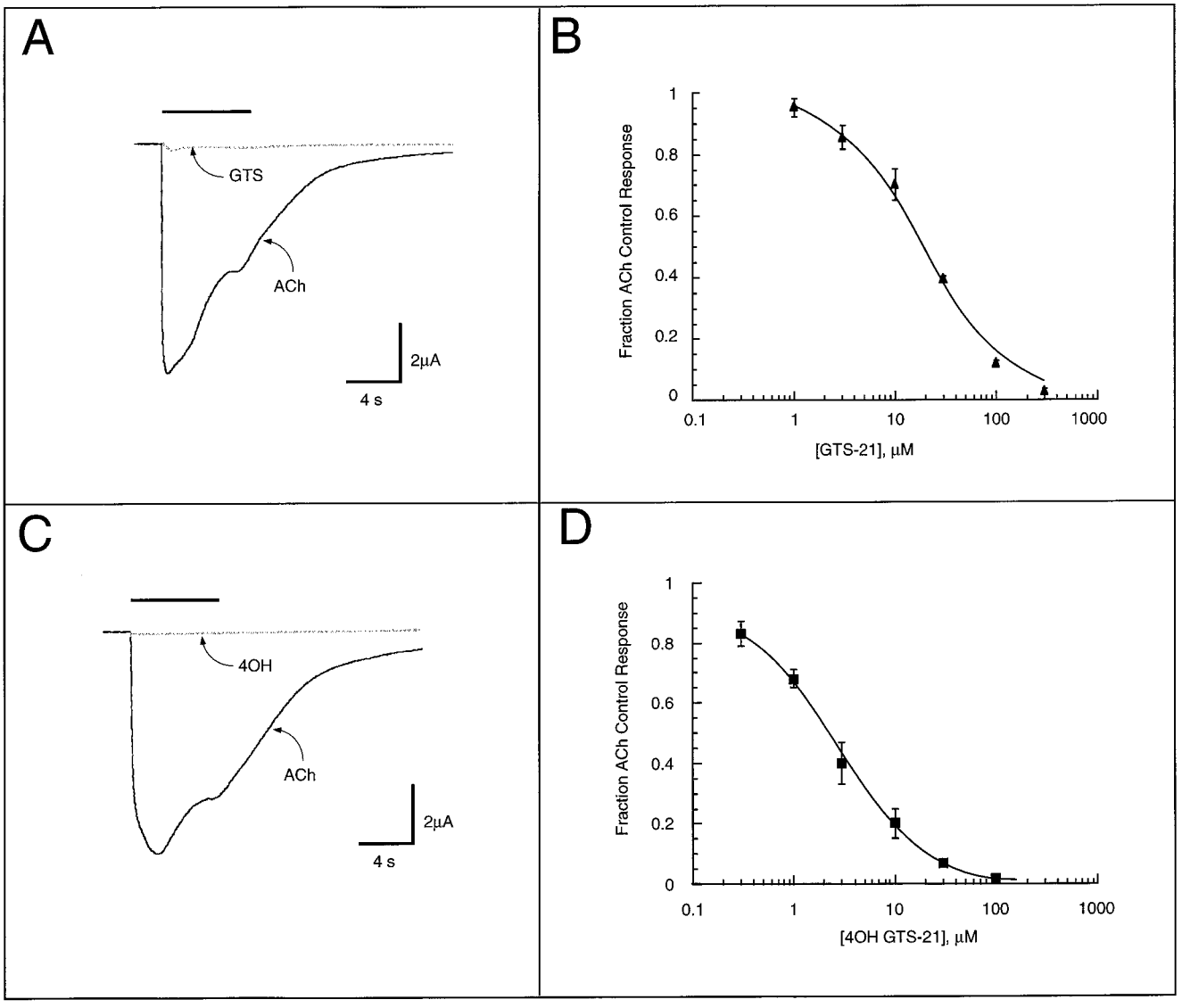


**Fig. 3.** The effects of GTS-21 and 4OH-GTS-21 on human $\alpha4\beta2$ AChRs. **A,** Responses of an oocyte expressing human $\alpha4\beta2$ AChR to either 10 $\mu$M GTS-21 (dotted line) or 10 $\mu$M ACh (solid line). The duration of drug application is indicated by the bar above the trace. **B,** Concentration response relationship for the inhibition of human $\alpha4\beta2$ responses to 10 $\mu$M ACh by increasing concentration of GTS-21. **C,** Responses of an oocyte expressing human $\alpha4\beta2$ AChR to either 100 $\mu$M 4OH-GTS-21 (dotted line) or 10 $\mu$M ACh (solid line). The maximum response to 4OH-GTS-21 was less than 1% of the ACh maximum. The duration of drug application is indicated by the bar above the trace. **D,** Concentration response relationship for the inhibition of human $\alpha4\beta2$ responses to 10 $\mu$M ACh by increasing concentrations of 4OH-GTS-21.

with binding experiments on human neuroblastoma SK-N-SH cells and rat PC12 cells. In human cells, GTS-21 produced no consistent concentration-dependent displacement of [$^{125}$I]$\alpha$-BTX at concentrations of up to 10 $\mu$M, although similar concentrations of 4OH-GTS-21 produced nearly total displacement of [$^{125}$I]$\alpha$-BTX binding. These observations suggest that GTS-21 itself may function as a pro-drug in therapeutic applications, gaining activity once it is demethylated to 4OH-GTS-21. The activity of the metabolite may therefore have contributed to the efficacy demonstrated for GTS-21 in delayed match to sample experiments with monkeys (Briggs *et al.,* 1997).

Studies with a variety of *alpha*-7 agonists indicate that agonist and residual inhibitory activities are both important for predicting receptor function (Meyer *et al.,* 1998b; de Fiebre *et al.,* 1995). Residual inhibitory activity appears to interfere particularly with the neuroprotective effects of *alpha*-7 agonists, and has less effect on memory-related behavioral improvements (Meyer *et al.,* 1998b). 4OH-GTS-21 is only a 50% partial agonist for human and rat *alpha*-7 AChRs, but has little inhibitory activity these receptors. This profile might permit 4OH-GTS-21 to cause more receptor-activation over extended intervals than seen with full agonists that induce greater inhibition, *e.g.,* DMAC (de Fiebre *et al.,* 1995). The lack of inhibitory activity would also appear to be important for the neuroprotective activity of 4OH-GTS-21.

Amyloid-induced neurotoxicity has been characterized extensively because of the accumulation of this protein in Alzheimer's disease. Mutations in this gene product that increase expression of aggregatable forms of amyloid predispose individuals to the disease, presumably because of aggregating capacity of this A$\beta$ 1-41 form (Hardy, 1997). The peptide fragment A$\beta$ 25-35 also aggregates to form fiber-like structures similar to those formed by A$\beta$ 1-41; however, this

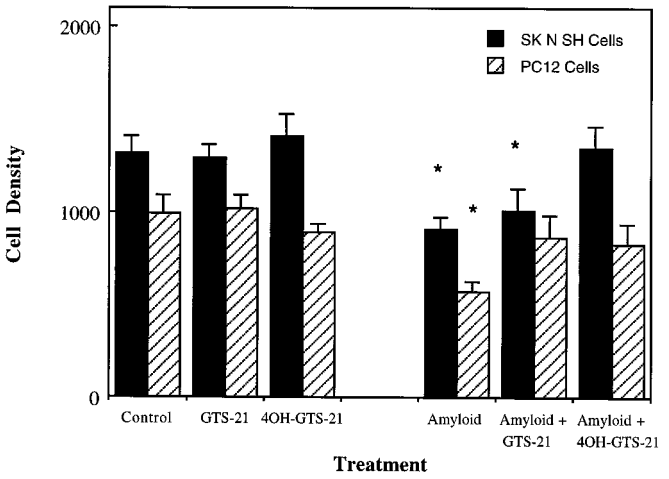


**Fig. 4.** Effects of GTS-21 and 4OH-GTS-21 on PC12 and SK-N-SH cell viability in the presence of A$\beta$ 25-35. Cell cultures were incubated for 10 min with the specified drug (10 $\mu$M each) before the addition of 20 $\mu$M A$\beta$ 25-35. Cell viability was then measured 24 hr later using an NIH image system, with four random areas counted/plate. Each value is the mean $\pm$ S.E.M. of cell density (cells/6 mm$^2$ counting area) for at least four plates per group. *P $<$ .05 compared to untreated control group (one-way analysis of variance).

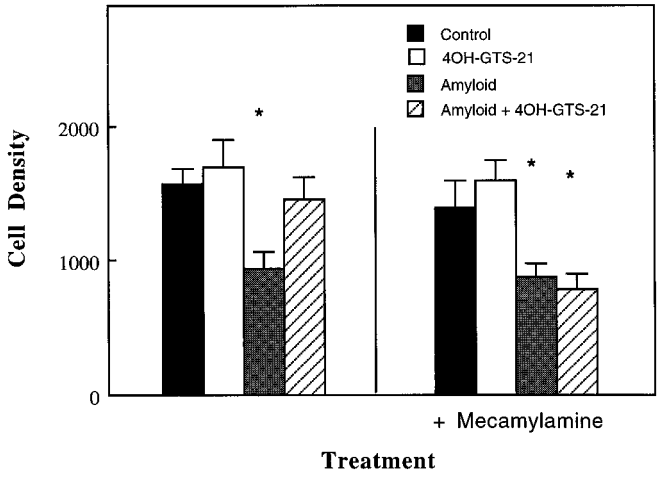


**Fig. 5.** Effects of mecamylamine on 4OH-GTS-21-induced neuroprotection against A$\beta$ 25-35 in SK-N-SH cells. SK-N-SH cells were treated as described in figure 4, with mecamylamine (1 mM) added to the medium prior to the 4OH-GTS-21 when specified. Each value is the mean $\pm$ S.E.M. of at least four plates per group of cell density (cells/6 mm$^2$ counting area). *P $<$ .05 compared to corresponding control value (with or without mecamylamine), one-way analysis of variance.

peptide does not require several hours of preincubation for the aggregation to occur as A$\beta$ 1-41 does (Cafe *et al.,* 1996; Cotman, 1997). Exposure to either A$\beta$ 25-35 or A$\beta$ 1-41 causes cell death through a process that appears to involve membrane peroxidation and increased intracellular calcium accumulation (Fukuyama *et al.,* 1994; Richardson *et al.,* 1996). Nonselective nicotinic receptor activation as well as GTS-21 have been shown previously to reduce this amyloid-induced toxicity in rodent cells (Kihara *et al.,* 1997; Zamani *et al.,* 1997). Our results extend this observation to human cells for the first time for 4OH-GTS-21. It therefore seems very likely that the neuroprotective activity of this class of compound indeed derives from the ability to activate of this class of receptor, because the former was much more potent at these receptors in oocyte and binding studies.

Nicotinic receptor activation was found previously to exert a neuroprotective action in other model systems for cell viability as well, including trophic factor deprivation in sympathetic ganglia (Koike *et al.,* 1989) or differentiated PC12 cells (Martin *et al.,* 1994), lesioned brain dopamine neurons (Janson, 1988), NMDA-induced toxicity in primary neocortical neurons (Akaike *et al.,* 1994), and ischemia *in vivo* (Shimohama *et al.,* 1998). Each of these neuroprotective actions was found to be sensitive to antagonists of *alpha*-7 receptors, including mecamylamine (Martin *et al.,* 1994), which was recently confirmed to inhibit these receptors (Meyer *et al.,* 1997). The antiapoptotic activity of nicotinic receptor activation seems to occur through a protein kinase C-dependent pathway, at least in part (Wright *et al.,* 1993). Nicotinic receptors, particularly highly calcium permeant *alpha*-7 containing subtypes, may therefore trigger this calcium-sensitive transduction pathway to elicit a neuroprotective action. Studies investigating this neuroprotective transduction process are currently in progress, focusing on the apparent role of protein kinase C activity.

In addition to providing evidence that GTS-21 may serve as a pro-drug for clinical conditions sensitive to *alpha*-7 activation, our study indicates that species differences in *alpha*-7 receptor structure may provide important clues for elucidating ligand-receptor interactions among potential agonists. The sequence for *alpha*-7 subunits is well conserved between rat and human, with 94% sequence identity. Of the 30 nonidentical residues, one site, f222, a tyrosine to phenylalanine difference in the human, stands out as a likely element for species specific effects. This site is nine amino acids downstream from cysteines known to be essential for agonist binding and is conserved as a tyrosine in other putative vertebrate nicotinic *alpha*-7 subunits cloned to date. Our results suggest that an investigation of the influence of the tyrosine to phenylalanine conversion, along with other subtle differences between these species, is likely to lead to the design of better therapeutic agents for nicotinic receptors.

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