Cholinergic nervous system as therapeutic approach for the treatment of arthritis
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Therapeutic effect of stimulating the nicotinic acetylcholine receptor in the collagen-induced model of rheumatoid arthritis: a role for ion channel activity and penetration of the central nervous system?

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Submitted
Chapter 5

ABSTRACT

Background. The cholinergic antiinflammatory pathway can downregulate inflammation via the release of acetylcholine (ACh) by the vagus nerve. This neurotransmitter can bind to the α7 subunit of nicotinic acetylcholine receptors (α7nAChR), expressed on macrophages and other immune cells, and upon binding suppress the release of proinflammatory cytokines. We recently described for the first time the role of the α7nAChR in murine collagen-induced arthritis (CIA) showing that the α7nAChR could represent a new target for the treatment of rheumatic diseases. It is however at present unclear what the role of ion channel activity is.

Methods. We tested the pharmacological and functional profile of two novel compounds, CTI-15311 and CTI-15072, with different effects on ion channel activity and investigated the role of both compounds in modulating CIA. Both compounds were characterized with binding, electrophysiologic, and pharmacokinetic studies. For in vivo efficacy studies in the CIA model CTI-15311 and CTI-15072 were administered daily by oral gavage from day 20 till sacrifice on day 34. Disease progression was monitored by visual clinical scoring and measurement of paw swelling. Inflammation and joint destruction were examined by histology and radiology.

Results. While both compounds bind to α7nAChR with high affinity, CTI-15311 acts like a classical agonist of ion channel activity, and CTI-15072 can act as an antagonist of ion channel activation. However, treatment with both CTI-15311 and CTI-15072 resulted in significant amelioration of arthritis. The anti-arthritic effect can be observed despite limited penetration of the central nervous system.

Conclusions. CTI-15311 is more efficacious as an α7nAChR agonist and more potent in the relief of arthritis than CTI-15072. However, although CTI-15072 is not able to induce significant levels of ion channel activity, it can still improve arthritis. Moreover, CTI-15072 was clearly distinct from typical competitive antagonists, since it was able to synergize with the allosteric modulator PNU-120596, suggesting that it is a selective desensitizer of α7nAChR. These data provide direct evidence that the α7nAChR in immune cells does not require typical ion channel activation to exert its antiinflammatory effects.
INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, immune-mediated inflammatory disease of unknown etiology, characterized by nonspecific, often symmetric, inflammation of the peripheral joints. Hallmarks of the disease include inflammation of the synovium leading to destruction of cartilage and bone (1;2). Although the introduction of anti-tumor necrosis factor (TNF) therapy and other new biologicals has played a major role in improving patient outcomes, RA is still associated with long-term morbidity and early mortality (3). Thus, there is still a need for the identification of new pathways involved in the modulation of inflammation, which could help to increase the efficacy of the RA treatment.

In recent years, it has been demonstrated that the efferent vagus nerve may inhibit inflammatory responses. This process was first described by Tracey and colleagues and has been termed “the cholinergic antiinflammatory pathway” (4;5). The key mediator of the cholinergic antiinflammatory pathway, acetylcholine (ACh), may inhibit proinflammatory cytokine release via interaction with members of the nicotinic acetylcholine receptor family (nAChR), and in particular with the α7 subunit (α7nAChR). This receptor is not only expressed by neuronal cells but also by macrophages and other cells involved in the inflammatory response. In these cells stimulation of the α7nAChR by ACh or α7-specific agonists suppresses proinflammatory cytokine release (4). Another strategy for activating the cholinergic antiinflammatory pathway is by vagus nerve stimulation (VNS) using an electrical device. Activation of the cholinergic antiinflammatory pathway, either by VNS or through pharmacologic approaches, has been shown to significantly ameliorate disease in several animal models, including endotoxemic shock (4;6), septic peritonitis (7), colitis (8), pancreatitis (9), and ischemia-reperfusion injury (10;11).

The cholinergic antiinflammatory pathway may also be relevant in arthritis. Pharmacological or electrical stimulation of the vagus nerve decreases carrageenan-induced inflammation in the rat paw (12). Moreover, we have shown that unilateral cervical vagotomy exacerbates collagen-induced arthritis (CIA), whereas treatment with AR-R17779, an α7nAChR agonist, ameliorates arthritis activity (13). In addition, α7-deficient mice showed a marked increase in synovial inflammation compared with wild-type littermates (van Maanen M.A. et al., submitted). Underscoring the potential importance of α7nAChR in humans, it has been shown that leukocytes and fibroblast-like synoviocytes (FLS) in the RA synovium express α7nAChR and α7nAChR-specific agonists can, in vitro, modulate the inflammatory response of RA FLS (14;15).

The members of the nAChR family form homopentameric and heteropentameric receptors in neurons, which function as ligand-gated ion channels, and can in the case of the heteropentameric receptors, mediate fast signal transmission at synapses. However, it is at present controversial whether the α7nAChR in immune cells requires ion channel activity
to exert its antiinflammatory effects. In the present study, we describe the binding profile, biological properties, and pharmacological effects of two novel α7nAChR selective small molecules (CTI-15311 and CTI-15072).

**MATERIALS AND METHODS**

**Chemicals**

Experimental compounds CTI-15311 and CTI-15072 were synthesized by Cornerstone Therapeutics, Inc. (Cary, NC) and provided as a hydrochloride or fumerate salt, respectively. PNU-120596 was purchased from Tocris (Ellisville, MO). All other chemicals for electrophysiology were obtained from Sigma (St. Louis, MO).

**Binding studies with the rat α7nAChR**

Binding studies with rat α7nAChR was done using the rat pheochromocytoma cell line PC12 that endogenously expresses the α7nAChR (American Type Culture Collection, Manassas, VA). PC12 cells were maintained in Ham F-12 nutrient mixture, containing 15% horse serum, 2.5% fetal bovine serum (FBS), 2 mM L-glutamine, 1.5 g/L NaHCO₃, 100 units of penicillin, and 100 μg streptomycin.

For the binding assay, PC12 cells were resuspended in binding buffer (phosphate buffered saline with calcium and magnesium, containing 1% FBS and 0.02% NaN₃) at 1.5 to 2.7 x 10⁶ cells per ml and 55 μl (0.8-1.5 x 10⁵ cells per well) was added to a 96-well, v-bottom plate. Test compounds were diluted in binding buffer, to 2.2 times the desired final concentration, and 55 μl was added to the cells; 55 μl binding buffer, was added to the cells in the control wells (total binding, non-specific binding, and cell controls; n=1-3). Biotinylated α-bungarotoxin (BTx) (Invitrogen) was added to the cells (excluding the cell control) for a final concentration of 10 nM. An excess of unlabeled BTx was added to the non-specific binding (NSB) control at a final concentration of 1.5 μM. The samples were incubated at room temperature for 1.0 to 1.5 hour(s) and thereafter the cells were washed one time with binding buffer, to remove unbound BTx.

Phycoerythrin-labeled streptavidin (streptavidin-PE) (Becton-Dickinson, Franklin Lakes, NJ) was diluted in binding buffer and 50 μl was added to the cells (excluding the cell control) at a 1.0 μg/ml final concentration. The samples were incubated in the dark, at room temperature, for 15 minutes. Thereafter cells were washed one time with binding buffer, to remove the excess streptavidin-PE. The samples were resuspended in 120 μl binding buffer. BTx binding was quantified by fluorescence-activated cell sorting (FACS) analysis. For each concentration of test compound, the displacement of BTx from the α7nAChR was quantified by measuring the intensity of the fluorescent signal. Raw data units are in percent events (% events), which is equal to the percentage of cells in the total
Therapeutic effect of stimulating nAChR in CIA

cell population that has a fluorescent intensity greater than the background level. Percent inhibition (% inh) of BTx binding was calculated from the ratio of the % events measured in the sample to the total binding % events, with background (NSB) subtracted:

\[
% \text{inh} = (1-\{(\text{sample % events} - \text{NSB % events})/\{(\text{total binding % events} - \text{NSB % events})\}\}) \times 100
\]

Curve-fit analysis was done using GraphPad Prism (GraphPad Software, San Diego, CA), % inh values were plotted versus the log10 of the concentration. Curve-fit analysis was performed using a four-parameter logistic equation:

\[
y = (\text{Bottom} + (\text{Top}-\text{Bottom})/(1+10^{((\text{LogIC}_{50}-X)*\text{illSlope}))})
\]

with the “Top” parameter constrained at 100% and the “Bottom” parameter constrained at 0%. Ki values were calculated from IC\(_{50}\) values using the Cheng-Prusoff equation:

\[
\text{Ki} = \text{IC}_{50}/\left(1+\left(\frac{[\text{BTx}]}{\text{KD}}\right)\right)
\]

**Broad selectivity panel**

Broad selectivity was assessed at Cerep (Celle l’Evescault, France), as described in the Cerep catalog, though the determination of the effect of CTI-15311 and 15072 in *in vitro* radioligand receptor binding assays with 52 different receptors, channels, and transporters. The specific ligand binding to the receptors is defined as the difference between the total binding, and the nonspecific binding determined in the presence of an excess of unlabelled ligand. The results are expressed as a percent of control specific binding and as the mean percent inhibition of control specific binding obtained in the presence of 10 µM CTI-15311 or CTI-15072. Individual and mean values are presented in the results section. The IC\(_{50}\) values (concentration causing a half-maximal inhibition of control specific binding) and Hill coefficients (nH) were determined by non-linear regression as described above.

**Pharmacokinetics**

Pharmacokinetic studies were carried out at Cerep using non-cannulated, non-fasted CD1 mice (Charles River Laboratories, Wilmington, MA). CTI-15311 or CTI-15072 were formulated in phosphate buffered saline (PBS), pH 7.4, as a clear solution and 5 mg/kg was administered by bolus intravenous (IV) injection or by oral gavage. Plasma samples were obtained from 3 mice per time point at 15, 30, 60, 120, 240, 360, 480 and 1440 min post-dose. The plasma samples were processed using acetonitrile precipitation and analyzed by HPLC-MS or HPLC-MS/MS to determine the concentration of drug as compared to a plasma calibration curve (aliquots of drug-free plasma were spiked with the test compound at the specified concentration levels
and processed together with the unknown plasma samples using the same procedure). The processed plasma samples were stored frozen (-20°C) until the HPLC-MS/MS analysis. Peak areas were recorded, and the concentrations of the test compound in the unknown plasma samples were determined using the respective calibration curve. The reportable linear range of the assay was determined, along with the lower limit of quantitation (LLQ).

Plots of plasma concentration of compound versus time were constructed. The fundamental pharmacokinetic parameters of compound after oral and IV dosing (Cmax: maximum concentration, Tmax: time to maximum concentration, T1/2: terminal elimination half-life, AUC: area under the curve, CI: clearance, Vd: volume of distribution, and %F: bioavailability) were obtained from the non-compartmental analysis of the plasma data using WinNonlin (Pharsight, St. Louis, MO).

cDNA clones and preparation of RNA
Human nAChR receptor clones were obtained from Dr. Jon Lindstrom (University of Pennsylvania, Philadelphia, PA). The RIC-3 clone was obtained from Dr. Millet Treinin (Hebrew University, Jerusalem, Israel). Subsequent to linearization and purification of cloned cDNAs, RNA transcripts were prepared in vitro using the appropriate mMessage Machine kit from Ambion Inc. (Austin, TX).

Receptor expression in Xenopus oocytes
The preparation of Xenopus laevis oocytes for RNA expression was conducted as previously described (16). In brief, mature (> 9 cm) female Xenopus laevis African frogs (Nasco, Ft. Atkinson, WI) were used as a source of oocytes. Before surgery the frogs were anesthetized by placing them in a 1.5 g/l solution of MS222 (3-aminobenzoic acid ethyl ester) for 30 min. Oocytes were removed from an incision made in the abdomen.

In order to remove the follicular cell layer, harvested oocytes were treated with 1.25 mg/ml collagenase (Worthington Biochemical Corporation, Freehold, NJ) for 2 hours at room temperature in calcium-free Barth’s solution (88 mM NaCl, 1 mM KCl, 2.38 mM NaHCO3, 0.82 mM MgSO4, 15 mM HEPES pH 7.6, 12 mg/l tetracycline). Subsequently, stage 5 oocytes were isolated and injected with 50 μl (5-20 ng) of each subunit cRNAs. Recordings were conducted 2-5 days after injection. RNA coding for human α7nAChR was routinely co-injected with the cDNA for human RIC-3, an accessory protein that improves and accelerates α7nAChR expression without affecting the pharmacological properties of the receptors (17).

Electrophysiology
Experiments were conducted using OpusXpress6000A (Molecular Devices, Union City, CA). OpusXpress is an integrated system that provides automated impalement and voltage clamp of up to eight oocytes in parallel. Both the voltage and current electrodes were filled with 3 M KCl. The oocytes were bath-perfused with Ringer’s solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM
CaCl₂, 10 mM HEPES, 1 μM atropine, pH 7.2). Flow rates were set at 2 ml/min. Agonist solutions were delivered from a 96-well plate using disposable tips. The oocytes were clamped at a holding potential of -60 mV. Data were collected at 50 Hz and filtered at 20 Hz. ACh applications were for 12 seconds.

**Experimental protocols and data analysis**

Each oocyte received two initial control applications of 300 μM ACh, followed by the experimental drug application, and subsequent control application of 300 μM ACh. Responses to experimental drug applications were determined relative to the preceding ACh control responses in order to normalize the data, compensating for the varying levels of channel expression among the oocytes. Responses for α7nAChR were calculated as net charge (16), since peak currents inaccurately report the agonist concentration dependence of α7nAChR-mediated responses (18). For experiments measuring the effects of ACh and the experimental compounds on allosterically modulated receptors, following the acquisition of baseline responses, cells were given a 60 s application of 300 μM PNU-120596. We have previously reported, that due to the slow reversibility of PNU-120596’s effects in the oocyte system, that this protocol produces modulation which allows previously desensitized receptors to be reactivated and that a significant percentage of the receptors remain in the modulated state for at least 15 minutes (19). For all experiments, means and standard error of the mean (SEM) were calculated from the normalized responses of at least four oocytes for each experimental concentration. For concentration-response relations, data were plotted using Kaleidagraph 3.52 (Synergy Software, Reading, PA), and curves were generated from the Hill equation:

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

where \(I_{max}\) denotes the maximal response for a particular agonist/subunit combination, and \(n\) represents the Hill coefficient. \(I_{max}\), \(n\), and the EC₅₀ were all unconstrained for the fitting procedures except in the case of the ACh concentration-response curves. Because ACh is our reference full agonist, those data were normalized to the observed ACh maximum, and the \(I_{max}\) of the curve fits were constrained to equal 1.

**Animals**

Male DBA/1 mice (8-10 weeks of age) were purchased from Harlan (Horst, The Netherlands). They were housed under specific pathogen-free conditions at the animal facility of the Academic Medical Center, University of Amsterdam. Animals were fed ad libitum. The Institutional Animal Care and Use Committee of the Academic Medical Center approved all experiments.

**Induction and assessment of collagen-induced arthritis**

Bovine type II collagen (2 mg/ml in 0.05 M acetic acid; Chondrex, Redmond, WA) was mixed
in an equal volume of Freund’s complete adjuvant (2 mg/ml of *Mycobacterium tuberculosis*; Chondrex). The mice were immunized intradermally at the base of the tail with 100 μl of emulsion (100 μg collagen) on day 0. On day 20, mice received an intraperitoneal booster injection of 100 μg type II collagen in PBS.

The severity of arthritis was assessed using an established semiquantitative scoring system of 0-4, where 0 = normal, 1 = swelling in 1 joint, 2 = swelling in >1 joint, 3 = swelling in the entire paw, and 4 = deformity and/or ankylosis (13;20;21). The cumulative score for all 4 paws of each mouse (maximum possible score 16) was used to represent overall disease severity and progression. Hind paw ankle joint thickness was measured using a caliper (POCO SO247 test gauge; Kroeplin Längenmesstechnik, Schlüchtern, Germany). For the evaluation of incidence, mice were considered to have arthritis if the arthritis score increased by at least 1 point for 2 or more following days.

**Study design and evaluation of arthritis activity**

In study 1, we evaluated the role of the two novel α7nAChR-specific modulators in CIA. The receptors were stimulated by oral gavage of CTI-15311 (5 mg/kg; n=15) or CTI-15072 (5 mg/kg; n=17). The compounds were administered once a day from day 20 until the end of the experiment (day 34). Control mice received saline. In study 2, we evaluated the antiinflammatory effects of different dosages of the α7nAChR-specific agonists. CTI-15311 (2 or 10 mg/kg; n=15) and CTI-15072 (10 or 20 mg/kg; n=15) were administered in the same way as in study 1. In both studies, mice were inspected daily for signs of arthritis and thickness of hind paws was measured using a caliper from day 16 till sacrifice by 2 independent observers (MAvM and MJV) who were not aware of the treatment.

**Radiologic analysis**

Hind paws were used for radiographic evaluation. Joint destruction was scored on a scale of 0-4, where 0 = no damage, 1 = demineralization, 2 = 1 or 2 erosions, 3 = severe erosions, and 4 = complete destruction of the joints (13). The radiographs were scored by 2 independent observers (MAvM and MJV) in a blinded manner; minor differences in scoring between the observers were resolved by mutual agreement.

**Histologic analysis**

Hind paws were fixed for 24 hours in 10% buffered formalin and decalcified in 15% EDTA. The paws were then embedded in paraffin, and serial 5 μm sagittal sections of whole hind paws were cut and stained with hematoxylin and eosin (HE). Two independent observers (MAvM and MJV) assessed the tissue for the degree of synovitis by microscopic evaluation, under blinded conditions, as described previously (13;20;21). Synovitis was graded on a scale of 0 (no inflammation) to 3 (severely inflamed joint) based on the extent of infiltration of inflammatory cells into the synovium.
**Statistical analysis**

To evaluate the effects of different treatments, we determined the change in clinical arthritis scores in each mouse from the start of treatment until the end of the experiment. AUC for the change in arthritis scores were calculated. The significance of the differences in the mean changes in scores (clinical, radiologic and histologic) between groups was determined by Kruskal-Wallis test followed by Mann-Whitney U test (SPSS version 12.0.2; SPSS, Chicago, IL). Incidence was compared using Kaplan-Meier survival analysis (GraphPad Prism). Cytokine levels were compared by Mann-Whitney U test. *P* values less than 0.05 were considered statistically significant.

**RESULTS**

**Functional activity and selectivity of the compounds on α7nAChR and α4β2nAChR**

In a competitive binding assay, compounds CTI-15311 and CTI-15072 displaced the α7nAChR-specific agonist α-bungarotoxin from binding to cultured PC12 cells (derived from a pheochromocytoma of rat adrenal medulla) that endogenously express the α7-subunit. Both compounds showed potent binding to α7nAChR with Ki values of 0.9 nM or 6.9 nM, respectively (Table 1). CTI-15311 does have some affinity for α4β2nAChR (cytosine binding to rat brain membranes; Ki = 30 nM). Both CTI-15311 and CTI-15072 do not exhibit any affinity for the muscle receptor (α-bungarotoxin binding to TE671 cell membranes; Ki > 100,000 nM) (Table 1).

<table>
<thead>
<tr>
<th>nAChR</th>
<th>CTI-15311 (Ki)</th>
<th>CTI-15072 (Ki)</th>
</tr>
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<tbody>
<tr>
<td>α7nAChR</td>
<td>0.9 ± 0.2 nM</td>
<td>6.9 ± 1.4 nM</td>
</tr>
<tr>
<td>α4β2nAChR</td>
<td>30 nM</td>
<td>≥100,000 nM</td>
</tr>
<tr>
<td>α1β1nAChR</td>
<td>&gt;100,000 nM</td>
<td>&gt;100,000 nM</td>
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*α-btx binding to rat PC12 cell membranes*  
*cytosine binding to rat brain membranes*  
*α-btx binding to TE671 cell membranes*

**Table 1.** Binding affinities of CTI-15311 and CTI-15072 for α7nAChR, α4β2nAChR and α1β1nAChR

In addition, the binding selectivity of both compounds with respect to other nAChRs, as well as to a broader selection of targets was evaluated by testing for competition in radioligand binding assays with 52 pharmacologically important receptors, channels, and transporters. CTI-15311 shows very little interaction with the panel of targets, with the exception of the human serotonin
transporter (72% inhibition at 10 μM). CTI-15072 does also not show any significant interactions with any of these additional targets with the exception of the human serotonin transporter (Ki = 1800 nM). This includes the α4β2nAChR (cytosine binding to rat brain membranes; Ki > 100,000 nM), and muscle receptor (α-bungarotoxin binding to TE57 cell membranes; Ki > 100,000 nM) (data not shown).

Using voltage-clamp electrophysiological techniques, we examined the functional activity of both compounds at human α7nAChR, expressed in *Xenopus* oocytes in comparison with ACh responses. It is known that the maximal channel activation, measured as net charge, is achieved with the application of 300 μM ACh and that application of concentrations greater than 300 μM produce no further increase in response (16). Application of 3 μM CTI-15311 to *Xenopus* oocytes elicited a typical inward current (Figure 1B), consistent with opening of the α7nAChR.

**Figure 1.** Compound activity of CTI-15311 and CTI-15072 on α7nAChR. **A**, Competitive binding assay showed that CTI-15311 and CTI-15072 displaced the α7nAChR-specific agonist α-bungarotoxin from binding to PC12 cells. **B**, Voltage-clamp electrophysiological techniques revealed that application of CTI-15311 (3 μM) to *Xenopus* oocytes elicited a typical inward current. **C**, CTI-15311 showed a dose-dependent activation of α7nAChR with a maximum about 85% that of ACh. **D**, CTI-15072 does not show to be an agonist of α7nAChR ion channel activity. **E**, CTI-15072 is an antagonist of α7nAChR channel activity.
The efficacy of CTI-15311 for the activation of α7nAChR was dose-dependent and the maximum responses to CTI-15311 were about 85% compared to ACh with an EC₅₀ of about 200 nM (Figure 1C). Compound CTI-15072 did not appear to be an agonist of the classical α7nAChR ion channel activity in the *Xenopus* oocyte membrane current assay (Figure 1D), but by virtue of its binding to α7nAChR it could act as an antagonist of ACh-stimulated α7nAChR channel activity, with an IC₅₀ of 20-50 nM (Figure 1E).

Both compounds were also tested for potential effects on α4β2nAChR. As seen in the competitive binding assays CTI-15311 does interact with α4β2nAChR; in the ion channel assays it was shown to be a potent inhibitor of α4β2nAChR with an IC₅₀ below 1 μM (data not shown). The mechanism of inhibition is probably related to competition.

### Pharmacokinetics

The pharmacokinetic properties of both compounds in mouse are shown in Table 2. Following an oral dose of 5 mg/kg maximum plasma concentrations (Cₘₐₓ) were 2.5 μM (787 ng/ml) at 15 min and 0.94 μM (324 ng/ml) at 30 min for CTI-15311 and CTI-15072, respectively. The bioavailability (%F), used to describe the fraction of the orally administered dose of unchanged compound that reaches the systemic circulation, was 50% for CTI-15311 and 76% for CTI-15072. Moreover, both compounds had comparable relatively short plasma half-lives in mice. Brain penetration was measured 30 minutes after intravenous administration of the compounds. CTI-15311 showed 44% brain penetration whereas CTI-15072 only showed 6% of brain penetration.

<table>
<thead>
<tr>
<th></th>
<th>CTI-15311</th>
<th>CTI-15072</th>
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<tbody>
<tr>
<td>5 mg/kg oral</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cₘₐₓ</td>
<td>787 ng/ml (2.5μM)</td>
<td>324 ng/ml (0.94μM)</td>
</tr>
<tr>
<td>Tₘₐₓ</td>
<td>15 min</td>
<td>30 min</td>
</tr>
<tr>
<td>T½t</td>
<td>98 min</td>
<td>104 min</td>
</tr>
<tr>
<td>AUC</td>
<td>34633 min ng/mL</td>
<td>35259 min ng/mL</td>
</tr>
<tr>
<td>%F</td>
<td>50%</td>
<td>76%</td>
</tr>
<tr>
<td>5 mg/kg IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T½t</td>
<td>19 min</td>
<td>39 min</td>
</tr>
<tr>
<td>Cl</td>
<td>72 mL/min/kg</td>
<td>103 mL/min/kg</td>
</tr>
<tr>
<td>Vd</td>
<td>1936 mL/kg</td>
<td>5833 mL/kg</td>
</tr>
<tr>
<td>Brain penetration</td>
<td>44%</td>
<td>6%</td>
</tr>
</tbody>
</table>

*relative to plasma level 30 min after administration of 5 mg/kg IV

**Table 2.** Compound pharmacokinetics in mouse
Stimulation of the α7nAChR by CTI-15311 treatment ameliorates arthritis activity and reduces disease incidence

Mice were treated with CTI-15311 or CTI-15072 at 5 mg/kg. Both compounds were administered daily by oral gavage from day 20 until day 34 and all mice tolerated the drug treatment well. Control mice received saline. Treatment with CTI-15311 resulted in an amelioration of clinical signs of arthritis (Figure 2A). The AUC was decreased by 51% ($P < 0.05$) in mice treated with CTI-15311 compared to control mice (Figure 2B). This clinical effect was accompanied by a decrease

**Figure 2.** Amelioration of arthritis by CTI-15311. Arthritis was induced in mice by immunization with type II collagen, and mice were treated with CTI-15311, CTI-15072 or saline by oral gavage from day 20 until day 34. **A**, Clinical score; Mice treated with CTI-15311 (5 mg/kg) showed a decrease in arthritis scores compared to saline-treated mice. **B**, Area under the curve (AUC) (day 20 to day 34) was decreased in CTI-15311-treated mice versus control mice. **C**, Caliper score; Mice treated with CTI-15311 showed a decrease in hind paw thickness, measured daily with a caliper, compared to the control group. **D**, AUC of the caliper score was decreased in CTI-15311-treated mice compared with saline-treated mice. **E**, Disease incidence; CTI-15311 reduced the incidence and delayed the onset of arthritis. $^*$ $P < 0.05$. 

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in paw swelling in the mice treated with CTI-15311 showing a decrease of 39% compared to saline-treated mice \((P < 0.05)\) (Figure 2C and D). Moreover, treatment with CTI-15311 resulted in reduced disease incidence and delayed onset of disease \((P < 0.05)\) (Figure 2E). CTI-15072 did not significantly ameliorate arthritis activity but a trend towards reduced incidence of disease was seen (Figure 2A-E).

**CTI-15311 treatment reduces bone degradation and synovial inflammation in knee joints**

To examine the effects of α7nAChR-specific agonists CTI-15311 (5 mg/kg) and CTI-15072 (5 mg/kg) on bone degradation, radiographs of knee joints collected at the end of the experiment were evaluated. Consistent with the effect on arthritis activity, mice treated with CTI-15311 showed a significant reduction in joint destruction compared with saline-treated mice \((P < 0.01)\), whereas CTI-15072 did not reduce bone degradation (Figure 3A). Similarly, there was a significant reduction of synovial inflammation, assessed by HE staining of knee joints, in mice treated with CTI-15311 \((P < 0.05)\) (Figure 3B).

**Figure 3.** Inhibition of bone degradation and reduction of synovial inflammation in murine collagen-induced arthritis by CTI-15311. **A**, Semiquantitative scores of joint destruction. Joint destruction was decreased in mice treated with CTI-15311 (5 mg/kg). **P** < 0.01. **B**, Semiquantitative scores for synovial inflammation, assessed by hematoxylin and eosin staining of the knee joints, showed a decrease of synovitis in CTI-15311-treated mice. *P* < 0.05.

**Dose-response study of the effects of CTI-15311 and CTI-15072 on arthritis scores and paw swelling**

Having shown proof of concept that CTI-15311 treatment results in decreased arthritis activity, we next performed an independent dose-response study in mice with CIA. We tested the effects of CTI-15311 in 1 lower and 1 higher dosage than used in study 1: 2 mg/kg and 10 mg/kg. Because CTI-15072 showed a trend towards amelioration of clinical arthritis, we tested in the same experiment the effects of 2 higher doses: 10 mg/kg and 20 mg/kg. All of the animals tolerated the drug treatments well. To allow comparison with the results obtained in study 1, we calculated the percentages of the score compared to the control group. This experiment confirmed the beneficial effect of treatment with 5 mg/kg CTI-15311 (a reduction of 49% in clinical score compared to saline \((P < 0.05)\) (Figure 4A)). Arthritis scores
were also significantly lower after treatment with CTI-15311 at either 2 mg/kg or 10 mg/kg with a reduction of 40% and 39%, respectively, compared to saline-treated mice ($P < 0.01$) (Figure 4A). These results suggest that all dosages were in the therapeutic range and conceivably even lower dosages might be effective. This notion is supported by the fact that the most pronounced effect of treatment on paw swelling was observed after low dose treatment (Figure 4B). Of importance, treatment with CTI-15311 2 mg/kg also resulted in a significant reduction in joint destruction (Figure 5A) and synovial inflammation (Figure 5B) compared with saline-treated mice ($P < 0.01$).

Figure 5. Inhibition of bone degradation and reduction of synovial inflammation in murine collagen-induced arthritis at different doses of CTI-15311 and CTI-15072. A, Semiquantitative scores for joint destruction of the knee joints. Joint destruction was significantly decreased in mice treated with CTI-15311 2 mg/kg and 10 mg/kg. B, Semiquantitative scores for synovial inflammation, assessed by hematoxylin and eosin staining of the knee joints, showed a decrease of synovitis in mice treated with CTI-15311 2 mg/kg and CTI-15072 10 mg/kg. * $P < 0.05$ and ** $P < 0.01$.

We also confirmed a trend towards improvement after treatment with 5 mg/kg CTI-15072. Of importance, there was a reduction of 48% in arthritis scores compared to saline-treated mice after treatment with 10 mg/kg CTI-15072 ($P < 0.05$), but there was no improvement
with the higher dosage of 20 mg/kg (Figure 4A). The beneficial effect of 10 mg/kg CTI-15072 was also shown by a decrease in paw swelling ($P < 0.05$) (Figure 4B). In line with these clinical effects, CTI-15072 10 mg/kg treatment resulted in significantly lower scores for synovitis and a trend towards reduced joint destruction compared to saline-treated mice (Figure 5A and B). The dosages needed to achieve a clinical effect were higher for CTI-15072 compared to CTI-15311, which is consistent with differences in pharmacokinetics and in binding to the α7nAChR. In addition, the highest dosages appeared to be less effective than lower dosages, perhaps related to desensitization and loss of biologic response of the receptor due to sustained agonist stimulation.

**Effects of CTI-15311 and CTI-15072 on α7nAChR primed with the positive allosteric modulator PNU-120596**

PNU-120596 is an α7nAChR-selective type 2 positive allosteric (22;23) that appears to convert desensitized receptors into a conducting state and impede the reversion of receptors back to the desensitized state. Since PNU-120596 itself is not an agonist, the effects of PNU-120596 on the reactivation of desensitized receptors requires either the co-application of PNU-120596 with a desensitizing drug, or the priming of the receptors with an application of PNU-120596 which when applied alone produces no ion channel activation. In the oocyte system, the priming effect of a PNU-120596 application persists for more than 15 minutes (19). The enhancement of ACh evoked responses by PNU-120596 priming is shown in Figure 6A. ACh-evoked responses are increased both in amplitude and duration, since receptors are prevented from desensitizing. As shown in Figure 6B, responses of PNU-120596 primed cells to CTI-15311, which under normal conditions functions as a typical α7nAChR agonist, are similar to the responses of primed cells to ACh. In contrast (Figure 6C), CTI-15072, which does not produce detectable ion channel activation under normal conditions, is nonetheless able to activate large ion channel currents in PNU-120596 primed cells. This result suggests that although CTI-15072 is able to inhibit ACh-evoked responses in co-application experiments, it may not be a true antagonist, but rather, an α7nAChR-selective silent desensitizer (24). To confirm that true competitive α7nAChR antagonists do not produce ion channel currents in PNU-120596 primed cells, we applied the widely-used α7nAChR-selective competitive antagonist methyllycaconitine (MLA) to PNU-120596 primed cells (25). As shown in Figure 6D, not only did MLA fail to activate the primed cells, the MLA application had residual effects, inhibiting the potentiating of a subsequent ACh-evoked response.
The identification of α7nAChR as a potential therapeutic target for several diseases, including RA (26), has stimulated the development of α7nAChR-selective drugs (27). The present study investigated the pharmacological properties of 2 novel α7nAChR-specific compounds (CTI-15311 and CTI-15072) with high oral bioavailability in the mouse. In addition, we tested their therapeutic potential in the CIA model of RA.

Both compounds had an antiinflammatory effect in CIA. The dosages needed to induce improvement of arthritis are higher for CTI-15072, which is expected based on differences
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in binding to the α7nAChR. In spite of its relatively high affinity for the α7nAChR, CTI-15072 produced negligible ion channel activation. The prevailing hypotheses for how α7nAChR mediate the sorts of downstream signal transduction pathways that regulate chemokine release and effects are based on the assumption that the α7-mediated ion currents (in particular the calcium ion component of the currents) provide the crucial initiating step for all downstream effects. With this model, the failure of CTI-15072 to stimulate ion channel current would be consistent with a lack of antiinflammatory activity. However, CTI-15072 had an antiinflammatory effect in CIA at concentrations of 10 mg/kg, suggesting that either it has some off-target activity or that α7nAChR ion channel activity may not always be required for α7-mediated signal transduction that leads to down modulation of inflammation. Even under the most optimal conditions the steady state $P_\text{open}$ of α7nAChR is very low (less than $10^{-6}$, Williams and Papke, unpublished), and there are many examples where it has been shown that α7nAChR activate signal transduction pathways not associated with ion channel currents in non-neuronal cells (5;28-31). The pathways shown to be potentially activated by α7nAChR include Jak-STAT and NFκB (28-30), Toll receptor-mediated signaling (32), Bac-Bcl (33), HMGB1-TNF (31), phospholipase C/IP3 (34), and the Ras/Raf-1/MEK1/ERK pathway (28;35). In many cases, although clearly dependent on the presence of α7nAChR and putative agonists, the activation of the signal transduction mechanisms appear to be independent of α7nAChR ion channel activation (32;34). These observations support the hypotheses that α7nAChR may function in multiple ways and suggest that various ligands may differ in their ability to stimulate ion channel activation and/or signal transduction. Alternatively, the forms of α7-type receptors expressed in the non-neuronal cells which mediate antiinflammatory cholinergic effects may be intrinsically different from the ion-channel forms of α7nAChR that are expressed in neurons (36).

We hypothesize that, although CTI-15072 is functionally an antagonist of α7nAChR ion channel activation, it is nonetheless an agonist for ion channel-independent signal transduction. The α7nAChR-selective partial agonist GTS-21 (DMXBA) is also relatively ineffective at activating the α7-receptor’s ion channel and yet has been shown to be very effective in several models for suppressing peripheral inflammation (6;9;37-39). We have shown that a factor limiting the efficacy of GTS-21 is its tendency to preferentially induce a stable desensitized state of the receptor, an effect that can be revealed with the type 2 positive allosteric modulator PNU-120596 (19). We have hypothesized that the state in which the ion channel is desensitized may nonetheless be an active mediator of signal transduction. In this work we show that although CTI-15072 is ineffective at activating α7nAChR-mediated ion currents, it does modulate the expression of PNU-120596-sensitive desensitization.

In addition to differences in affecting ion channel activation, there were also other differential effects between CTI-15311 and CTI-15072. Binding studies showed that CTI-15311 is quite selective and had high affinity for rat α7nAChR, whereas it showed lower affinity for the other nAChR tested. Functional electrophysiological experiments using human nAChR expressed
in *Xenopus* oocytes confirmed that when CTI-15311 binds to α7nAChR, it functions as a conventional agonist, whereas its binding to other nAChR subtypes does not produce ion channel activation. Specifically, CTI-15311 acted as an antagonist of the α4β2nAChR (IC50 ≈ 20 nM, data not shown). CTI-15072 had a lower affinity for rat α7nAChR than CTI-15311, but it was more selective than CTI-15311 in binding to α7nAChR relative to α4β2nAChR. Of note, the previously described α7-selective agonist AR-R17779 also showed an antiinflammatory effect in CIA (13); the fact that AR-R17779 selectively activates α7nAChR without significant antagonism of α4β2nAChR (40) suggests that α4β2nAChR antagonist activity of CTI-15311 is not required for its efficacy in treating of CIA. This notion is supported by the antiinflammatory effect of CTI-15072 described here, since it is less effective in binding to α4β2nAChR than CTI-15311. Finally, CTI-15072 exhibited markedly lower brain penetration than CTI-15311. The data confirm that an effect on the central nervous system is not required to induce the antiinflammatory effect of α7nAChR ligand treatment. Collectively, the results of this study confirm and extend previous work showing that α7nAChR ligands may reduce arthritis activity and protect against joint destruction in the CIA model of RA. Of importance, we provide direct evidence that α7nAChR agonists may exert their antiinflammatory effect independent of ion channel activation and in the absence of penetration of the central nervous system.

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