Published in final edited form as:

Anesth Analg. 2012 September ; 115(3): 713–720. doi:10.1213/ANE.0b013e31825a3c72.

The Novel Small Molecule $\alpha 9\alpha 10$ Nicotinic Receptor Antagonist Prevents and Reverses Chemotherapy-Evoked Neuropathic Pain in Rats

Elzbieta P. Wala, PhD,

Department of Anesthesiology, College of Medicine, University of Kentucky, Lexington, Kentucky

Peter A. Crooks, PhD,

Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, Kentucky

J. Michael McIntosh, MD, and

Departments of Psychiatry and Biology, University of Utah, Salt Lake City, Utah

Joseph R. Holtman Jr., MD, PhD

Departments of Anesthesiology and Molecular Pharmacology and Therapeutics, Loyola School of Medicine, Maywood, Illinois

Abstract

Background—Peripheral neuropathy is a common dose-limiting side effect of chemotherapy. There are no clinically proven analgesics for the treatment of this condition. Drugs from different classes have been tested with mixed results. Identification of novel molecular targets for analgesic(s) is important. Antagonism of the $\alpha 9\alpha 10$ nicotinic acetylcholine receptor (nAChR) subtype (absent in brain) is thought to underlie analgesic efficacy of peptide α -conotoxins. We found novel non-peptide small molecule analogs from a family of *tetrakis*-, *tris*- and *bis*-azaaromatic quaternary ammonium salts (high potency with selectivity as antagonists at the $\alpha 9\alpha 10$ nAChRs) to produce dose-related analgesia in rat models of nerve injury-evoked neuropathy and persistent inflammatory pain. No tests were done in a model of neuropathy induced by drug administration (i.e., chemotherapy).

Corresponding Author: Elzbieta P. Wala, PhD, University of Kentucky, College of Medicine, Department of Anesthesiology, UKMC, Rose Street, N-202, Lexington, KY 40536-0293, Phone: 858-257-4487, FAX: 859-323-1080, epwala@uky.edu.

The authors declare no conflicts of interest.

DISCLOSURES:

Name: Elzbieta P. Wala, PhD

Contribution: This author helped design the study, conduct the study, analyze the data, and write the manuscript

Attestation: Elzbieta P. Wala has seen the original study data, reviewed the analysis of the data, approved the final manuscript, and is the author responsible for archiving the study files

Name: Peter A. Crooks, PhD

Contribution: This author helped design the study, conduct the study, and write the manuscript

Attestation: Peter A. Crooks has seen the original study data and approved the final manuscript

Name: J. Michael McIntosh, MD

Contribution: This author helped design the study and write the manuscript

Attestation: J. Michael McIntosh has seen the original study data and approved the final manuscript

Name: Joseph R. Holtman, Jr., MD, PhD

Contribution: This author helped design the study and write the manuscript

Attestation: Joseph R. Holtman, Jr. has seen the original study data, reviewed the analysis of the data, and approved the final manuscript

Methods—In this study, a lead *bis*-analog, ZZ1-61c, was characterized in a rat model of vincristine-evoked neuropathy. Male Sprague-Dawley rats were repeatedly dosed with the vinca-alkaloid, vincristine (100 µg/kg/day IP, days 1–5 and 8–12). ZZ1-61c (100 µg/kg/day IP) was given either along with or after completion of vincristine (commencing by day 15 when neuropathy was maximum). Responsiveness was assessed with von Frey hairs and the paw-pressure test. The effects of ZZ1-61c on motor function (rotarod) and muscle strength (grip test) were characterized in naïve rats.

Results—The development of neuropathy was demonstrated with repeated dosing of vincristine (pain hypersensitivity in response to mechanical stimulation). ZZ1-61c showed both preventive and restorative effects on this condition: 1) vincristine-evoked sensitivity to pressure was reduced by co-administration of ZZ1-61c; 2) established neuropathy was diminished by ZZ1-61c after cessation of chemotherapy. ZZ-1-61c did not cause motor dysfunction (rotarod) or muscular weakness (the grip test).

Conclusions—This study suggests that ZZ1-61c, a novel compound with a unique mechanism of antagonistic action at the $\alpha 9\alpha 10$ nAChR, may be a potential drug candidate for prevention and attenuation of neuropathic pain resulting from chemotherapy. Such a strategy may provide effective treatment which circumvents toxicity of centrally acting agonists at nAChR.

Introduction

Vincristine, an antineoplastic drug, is used for solid tumors and hematologic malignancies. Its use as a chemotherapeutic drug is limited as a result of the development of a dose-related polyneuropathy characterized by painful paresthesias and dysesthesias. No effective treatment of chemotherapy-induced neuropathy is currently available. Drugs typically used for neuropathic pain, including anticonvulsants (gabapentin) and antidepressants (amitriptyline), are of limited efficacy for chemotherapy-evoked neuropathy. There is a need for new therapeutic drugs for the prevention and treatment of this pain state.^{1,2}

One potential target for new analgesics effective for neuropathy is the neuronal nicotinic acetylcholine receptor (nAChR).^{3–6} A variety of nAChR subtypes, the homomeric and heteromeric combinations of $\alpha 2$ – $\alpha 10$ and $\beta 2$ – $\beta 4$ subunits, have been identified so far, but their specific role in analgesia has not been fully elucidated. Significant effort has focused on the $\alpha 4\beta 2$ nAChR found in the central nervous system (CNS), and thought to be involved in analgesia via activation of multiple descending inhibitory pathways originating in the brainstem. The efficacy of several agents acting as agonists at the $\alpha 4\beta 2$ nAChR subtype (nicotine, epibatidine, ABT-894) has been established in preclinical models of pain: nociception, inflammatory pain, neuropathy resulting from nerve trauma or diabetes.^{7,8} A novel drug from this class, ABT-594, also alleviated allodynia resulting from chemotherapy in rats.⁹ The $\alpha 4\beta 2$ nAChR is widespread in the CNS and thus, centrally mediated side effects make these agents less desirable analgesic drugs.

A unique approach to avoid side effects related to drug activity at CNS nicotinic receptors has been to focus on the heteromer assembled from two different α subunits, $\alpha 9$ and $\alpha 10$. Gene transcripts for $\alpha 9\alpha 10$ have been identified in diverse yet limited numbers of tissues but importantly not in the brain; thus, it is unlikely that this subunit may be significantly

involved in mediation of CNS-related side effects.¹⁰ The α -conotoxins, venom peptides which have been the source of selective antagonists for $\alpha 9\alpha 10$ nAChR subtype, demonstrated an interesting pharmacological profile, including effectiveness in rodent models of neuropathic and inflammatory pain; a likely peripheral mechanism of action (no effect after intrathecal administration); ability to accelerate functional recovery from nerve injury; no significant side effects; ability to reduce migration of macrophages, lymphocytes, and ACh-producing cells into the neural and perineural areas of nerve injury.^{11–13} Modification of the nicotine molecule by quaternization of the pyridine-N atom with a lipophilic substituent (in order to afford N-substituted analogs) yielded a family of novel non-peptide small molecule *bis*-, *tris*-, *tetrakis*-azaaromatic quaternary ammonium analogs.^{14–16} A number of these analogs also showed high affinity and selectivity for $\alpha 9\alpha 10$ nAChR expressed in *Xenopus oocytes* while they have significantly less potency on other subtypes.^{17,18} These analogs demonstrated effectiveness against persistent inflammatory pain and posttraumatic neuropathy at doses well below those that produced motor dysfunction in rats.^{17,19} However, they were not investigated in chemotherapy-evoked pain. In order to test this possibility the most potent and $\alpha 9\alpha 10$ -selective agent from the *bis*-group, ZZ1-61C, was characterized in a rat model of vincristine-induced neuropathy.

Materials and Methods

Male Sprague-Dawley rats (250 g; Harlan, Indianapolis, IN) were housed individually in transparent cages with a sawdust-covered floor in a humidity- and temperature-controlled Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility (a 12 h alternative light/dark cycle) with free access to standard laboratory chow and tap water. Body weights (BW) were determined on the day of experimentation. At the end of the experiment, rats were euthanized with medical grade pentobarbital, 150 mg/kg (Vortech, Deaborn, MI) given by the intraperitoneal route (IP). Rats were allowed to habituate to the housing facility for at least one week. Next, they were familiarized (3 times) with the experimental environment/apparatus before the experiments began. In addition, rats were accustomed to the experimental room for at least 30 min before testing. All experiments were conducted during the light phase of the cycle (0800–1700). The experiments were performed in accordance with a protocol approved by the University of Kentucky Institutional Animal Care and Use Committee and the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals (1996) prepared by the National Academy of Sciences' Institute for Laboratory Animal Research.

Vincristine sulfate (Tocris Bioscience, Ellisville, MO) was administered IP 100 μ g/kg/day (diluted to 1 ml in 0.9% saline) in two 5-day cycles with 2 days break between cycles (10 injections, 1 mg/kg cumulative dose). ZZ1-61c, *N,N'*-[(1,1'-biphenyl)-4,4'-di-(1-propyn-3-yl)]-*bis*-(3,4-dimethylpyridinium) dibromide, was synthesized as previously described.¹⁸ ZZ1-1-61c was dissolved in saline and administered 100 μ g/kg/day IP either along with or after completion of vincristine treatment.

The experimental design used one group of rats per treatment condition with each rat in each group tested using all pain assays. Each treatment was given in two 5-day cycles with 2 days

break between cycles (days 1–5 and 8–12). Group 1 (n=6 rats) was treated with vincristine, 100 µg/kg/day IP alone to validate a rodent model of chemotherapy-induced neuropathic pain. Group 2 (n=6 rats) was treated with vincristine, 100 µg/kg/day IP, and ZZ1-61c, 100 µg/kg/day IP in order to determine the prophylactic effects of ZZ1-61c on inhibition of vincristine-induced neuropathic pain. Control rats were injected with ZZ1-61c alone, 100 µg/kg/day IP (group 4, n=6 rats) or saline vehicle, 1 ml/kg/day IP (group 4, n=6 rats). Groups 1–4 were not drawn from a single pool of subjects. In addition, in order to assess the treatment effects of ZZ1-61c on vincristine-induced neuropathic pain, another group of rats (n = 12) was exposed to vincristine, 100 µg/kg/day IP, on days 1–5 and 8–12. Once neuropathic pain was evident (day 15 post-vincristine initiation) the rats were randomly divided into two groups and then treated with either ZZ1-61c, 100 µg/kg/day IP (group 5A, n=6 rats) or saline, 1 ml/kg/day (group 5B, n=6 rats) starting on day 15 and ending on day 33.

Each rat was tested before initiation of the treatment (day 1, baseline, assessed twice, 15 min apart) and then every other day (starting at day 3 after the first injection) across the time of study (33 days). Control rats (ZZ1-61c, saline) were also assessed every other day (days 1–15). Responsiveness was measured before each injection (except rats in groups 5A and 5B; testing before and 15 min after administration of ZZ1-61c or saline). Two well-established tests based on the use of mechanical stimuli were used. First, rats were placed on the elevated wire mesh floor and then von Frey monofilaments (VFH) with bending forces 4, 8, 15 g (Stoelting, Wood Dale, IL) were applied in ascending order to the mid-plantar side of the hindpaw (five times/paw) and held for approximately 5 s.²⁰ The number of withdrawals was recorded for each paw, averaged, and expressed as an overall percentage response (paw withdrawal, %PW) in each rat. Next, rats were tested by the paw pressure technique²¹ using the Basile Analgesimeter (UGO Milan, Italy). Briefly, increasing pressure was applied to the dorsal side of the paw with vocalization used as the end point (32 g/s; cut-off 300 g). Each paw was tested 3 times; responses were averaged and presented as a vocalization threshold (VT, g). The observer was blinded to treatment.

The effect of ZZ1-61c on motor coordination was determined using the rotarod performance test. Naïve rats were trained to run on the Rat Rota Rod (Ugo Basile, Comeno, Italy) at a constant speed (10 rev/ min) for 180 s (cut off). Thereafter, they were injected with ZZ1-61c 100 µg/kg/day IP for three subsequent days. Latency to fall off the drum was recorded before (baseline) and at 15, 30, 45, 60 min after injection.

The effect of ZZ1-61c on hindlimb weakness was assessed in the grip strength test using a Digital Grip Strength Meter (Columbus Instruments, Columbus, OH). The rat grasped the bar with both forepaws and then was steadily pulled backwards by the tail until its grip was broken. Each rat was tested before (baseline) and 15, 30, 45, 60 min (3 measurements per time) after repeated administration of ZZ1-61c 100 µg/kg/day IP on days 1–3.

All data are expressed as mean ± SEM for n rats. The time courses (%PW or VT) were generated for each treatment. The areas under the curves (AUC_{0-t}) were calculated by the trapezoidal rule. One-way analysis of variance (ANOVA), post hoc Dunnett's method, *t*-

paired test, *t*-test, and Wilcoxon signed rank test were used (SigmaPlot for Windows version 11.0, Systat Software, Inc.).

Results

The daily injections of vincristine (100 µg/kg/day IP, days 1–5 and 8–12) resulted in a decrease in thresholds (compared to day 1 pretreatment baseline). This was indicated by exaggerated paw withdrawal responsiveness to monofilament fibers (4, 8, 15 g) and vocal responses in the paw pressure test ($F_{14,89} = 28.4, 208.6, 22.8, 59.5$, respectively; $P < 0.001$, one-way ANOVA with Dunnett's test). Significant pain sensitivity was first noted after the 5th vincristine injection (1st cycle), it peaked after the 10th injection (2nd cycle), and gradually declined when dosing ceased (Figure 1A–D; group 1).

Responsiveness to VFH and paw pressure was still elevated (relative to day 1 pretreatment baseline) in the presence of ZZ1-61c (co-administration of drugs; 100 µg/kg/day IP of each; days 1–5 and 8–12) ($F_{14,89} = 4.9, 5.2, 3.2, 13.1$ for 4, 8, 15 g VFH and VT, respectively; $P < 0.001$, one-way ANOVA with Dunnett's test) (Figure 1A–D; group 2). Nevertheless, pain hypersensitivity was markedly less (lower %PW, higher VT) for a vincristine and ZZ1-61c combination therapy versus vincristine. The $AUC_{1-15\text{day}}$ values were significantly different (*t*-test) for vincristine plus ZZ1-61c versus vincristine alone: 421.7 ± 42.2 versus 628.2 ± 37.5 (4g VFH, $P = 0.004$); 600.8 ± 32.4 versus 871.6 ± 29.8 (8g VFH, $P < 0.001$); 875 ± 44.8 versus 1122.2 ± 20.9 (15g VFH, $P < 0.001$) and 3356.6 ± 43.8 versus 1653.4 ± 9.8 (VT, $P < 0.001$). Similar differences (*t*-test) were observed when the recovery period also was included ($AUC_{1-33\text{day}}$): 910.8 ± 54.6 versus 1174.8 ± 12.7 ($P < 0.001$); 1422.3 ± 79.0 versus 1812.0 ± 42.2 ($P = 0.001$); 1998.3 ± 113.1 versus 2411.2 ± 23.9 ($P = 0.005$) and 4472.0 ± 42.2 versus 4261.9 ± 13.7 ($P < 0.001$) for 4g, 8g, 15g VFH and VT, respectively. No significant effects (relative to baseline) were seen after administration of ZZ1-61c or saline in control rats (Figure 1A–D; group 3 and 4, respectively).

Pain sensitivity was significantly enhanced after vincristine treatment (measured on day 15 after the initiation of vincristine) as compared to naïve values (day 1, pre-vincristine) ($P < 0.001$, paired *t*-test) regardless of noxious stimuli used (Figure 2A–D). These rats were subsequently treated with ZZ1-61c or saline during the post-vincristine recovery period (commencing on day 15 and continued through day 33 of the study). Overall, thresholds were higher (decreased %PW, increased VT) in rats treated with ZZ1-61c compared to rats which were injected with saline (Figure 2A–D, group 5A and 5b, respectively). The $AUC_{17-33\text{day}}$ for ZZ1-61c versus saline (*t*-test) were: 390.0 ± 63.4 versus 407.5 ± 29.4 (4g VFH, nonsignificant); 656.7 ± 63.4 versus 755.8 ± 21.4 (8g VFH, $P = 0.075$); 947.5 ± 43.2 versus 1049.2 ± 7.9 (15g VFH, $P < 0.05$) and 2212.4 ± 11.7 versus 2506.8 ± 30.0 (VT, $P < 0.001$). It is notable that the immediate effect of ZZ1-61c (15 min postinjection versus preinjection pain threshold) was of statistical significance only after the first injection (day 15) but not with the subsequent dosing (Figure 3A–D).

The next experiment was conducted to determine the side effects of ZZ1-61c. No changes in motor coordination (rotarod performance test) or muscle strength (grip strength test) were

seen during repeated administration of ZZ1-61c, 100 µg/kg/day IP, in naïve rats (Figure 4A,B).

Vincristine caused a moderate BW loss in rats. This effect was inhibited by coadministration of ZZ1-61c. At maximum (day 12), BW (% of initial value) = $95.5\% \pm 0.5$ versus $86.7\% \pm 1.1$ ($P < 0.01$, *t*-test) for vincristine plus ZZ1-61c versus vincristine alone. At the end of a study, BW gain also was greater in rats previously exposed to combination therapy than vincristine alone (day 33): BW = $128.5\% \pm 1.5$ versus $115.3\% \pm 2.5$ ($P < 0.001$, *t*-test). Administration of ZZ1-61c after completion of vincristine treatment had no effect on BW gain (day 33): BW = $116.0\% \pm 1.7$. Naïve rats repeatedly dosed with ZZ1-61c gained weight normally (day 15): BW = $104.1 \pm 0.47\%$ (ZZ1-61c) versus $105.0 \pm 0.62\%$ (saline). No mortality or apparent morbidity occurred when vincristine was given alone or in combination with ZZ1-61c (e.g., no obvious changes noted when rats were observed in their home cages for motor activity, guarding, gait, and abnormally formatted stool).

Discussion

The ability of a novel non-peptide small molecule $\alpha 9\alpha 10$ nAChR antagonist, *bis*-quaternary ammonium salt ZZ1-61c, to prevent and/or correct vincristine-evoked neuropathic pain was characterized in the present work. Partial but significant inhibition/reversal of pain hypersensitivity was demonstrated at a dose that did not result in overt side effects. ZZ1-61c's potential as a new drug for chemotherapy-induced neuropathy may be suggested.

Painful neuropathy was repeatedly demonstrated after repeated bolus injections or continuous IV infusion of vincristine in rodents.^{22–24} The present data confirmed previous findings²⁵ that intermittent systemic exposure to vincristine (100 µg/kg/day IP on a 5-day-on, 2-day-off, 5-day-on schedule) was sufficient enough to reduce nociceptive thresholds in rats. This effect was demonstrated using VGH of different bending forces or an increasing pressure to the paw. Pain sensitization could not be attributed to repeated testing because it was not seen in control rats. As previously reported, naïve rats rarely withdraw paws from the 4g filament (normally innocuous stimuli) while only 10–20% of rats withdraw from the 15g force (noxious stimuli) so enhanced responses to these stimuli are thought to be indicative of mechano-allodynia and mechano-hyperalgesia, respectively.^{20,26} Naïve baseline responsiveness was noticeably higher, in our study. This was a consistent observation.²⁷ The reason for these differences is not clear. Increased sensitivity to VFH (withdrawal threshold) was evident starting with the 5th dose of vincristine and lasted for a period of ~20 days. The present data also showed marked changes in responsiveness to the paw pressure test. Nociceptive thresholds (a vocal response) also were reduced at day 5 of vincristine dosing but remained lowered for the whole study (33 days). The discrepancy in time courses of hypersensitivity to pressure may likely be explained by facilitation of inputs from different fibers (progressive versus sharp mechanical stimulation).²⁸

The present data clearly showed efficacy of ZZ1-61c. First, it inhibited the development of neuropathy resulting from vincristine (alleviation of pain sensitivity during chemotherapy). Second, it reversed established neuropathy (restoration of pain sensitivity after chemotherapy). Third, the acute effect of ZZ1-61c (pre- versus postinjection responses) was

weak and transient, thereby suggesting the persistent effect rather than the immediate effect. Fourth, ZZ1-61c was not an active analgesic agent on its own (naïve rats). Fifth, no motor dysfunction or muscle weakness was seen at the tested dose of ZZ1-61c. Vincristine (100 µg/kg) was ineffective in the rotarod²⁹ and grip strength³⁰ tests; however, potential interaction between these drugs cannot be excluded. In our study, responsiveness was not confounded by motor impairment because an increase in pain thresholds was seen regardless of whether ZZ1-61c was given along with or after discontinuation of vincristine. In agreement with the earlier data,^{30,31} vincristine caused a moderate BW loss in rats. Notably, this effect also was hampered by coadministration of ZZ1-61c. The present findings may suggest that ZZ-1-61C has disease modification/prevention properties, i.e., ability to preempt and/or correct neuropathy caused by vincristine. Whether ZZ161c interferes with chemotherapeutic activity of vincristine needs to be further investigated.

Because this work was merely of an exploratory nature, its limitations must be considered. First, a dose of ZZ1-61c was based on maximum efficacy in a formalin model of persistent inflammatory pain.¹⁸ Caution must be taken when comparisons are made between different pain models; a wide range of doses ought to be implemented in a further study. Second, since ZZ1-61c acts as antagonist at nAChRs^{17,18} and has no efficacy on its own desensitization of receptors is less likely to occur. Even so, it is of interest to determine if efficacy remains consistent with repeated dosing (a cumulative dose paradigm). Third, no positive control was used. A number of drugs from different classes were characterized earlier in a similar paradigm.³² For example, the nAChR agonist, ABT-594⁹ and selective T-type calcium channel blocker, ethosuximide,²⁰ reversed vincristine-induced neuropathy in a dose-dependent manner. The positive effects were noted with anticonvulsant,^{33,34} neurosteroids,³⁵ cannabinoids,³⁶ and drugs that enhance mitochondrial function.²⁶ Mixed results were demonstrated with common analgesic drugs such as opioids and nonsteroidal antiinflammatory drugs.^{33, 37–39} Fourth, strikingly, the pain-enhancing effect of vincristine was comparable in magnitude to that seen after chronic constriction nerve injury (reduction VT ~ 80 g, the paw pressure test).^{19,27,40} Even though behavioral outcomes appear similar, the mechanisms are thought to be somewhat distinct in these pain models (i.e., axonal degeneration in peripheral nerves accompanies painful neuropathy secondary to nerve trauma but not chemotherapy).^{22–24} From a pain treatment perspective, drugs that modify cellular calcium levels were effective against pain sensitization caused by toxic but not traumatic nerve insult.⁴¹ On the contrary, effectiveness of drugs acting as antagonists at the N-Methyl-d-Aspartate receptors was well-established in the later model yet was not seen in a former model of neuropathy.²⁰ In this context, it is remarkable that analogs from a family of *tetrakis-* *tris-* and *bis-* quaternary ammonium salts (lead compounds highly selective for $\alpha 9\alpha 10$ nAChR) alleviated signs of pain hypersensitivity that was derived from neurotoxicity or nerve trauma; in that, partial reversal of pain hypersensitivity in vincristine treated rats (present study) was comparable to what was previously seen in rats with chronic nerve injury.^{19,42} They also blocked, in a dose-related manner, formalin-evoked flinching (a late phase, which is thought to involve central sensitization) in a rat model of persistent inflammatory pain. None of them was active in a rat model of acute nociception (tail-flick test) suggesting a limited role of $\alpha 9\alpha 10$ in this type of pain.^{17,19,42}

Activation of central $\alpha 4\beta 2$ (and to lesser extent $\alpha 7$) subunits nAChR have been associated with analgesic effects of nicotinic agonists.³⁻⁶ Nevertheless, peptides α -conotoxins (Vc1, RgIA) that act as antagonists at $\alpha 9\alpha 10$ nAChR also show analgesic activities in rodent models of neuropathy (chronic constriction nerve injury, spinal nerve ligation, diabetes) and inflammation (complete Freund's adjuvant). Although the mechanism of action is unknown, it has been thought that blockade (antagonism of $\alpha 9\alpha 10$ nAChR) may reduce the number of macrophages and T-cells at the site of injury (both $\alpha 9$ and $\alpha 10$ are expressed in a variety of immune cells), which in turn contributes to the cumulative analgesic and restorative effects of α -conotoxins.^{10,11} The antagonism of $\alpha 9\alpha 10$ nAChRs in sensory nerves (expression of $\alpha 9$ and $\alpha 10$ in sciatic nerve is unknown),^{11,13} interaction with $\alpha 3(\alpha 5)\beta 2$ and $\alpha 3(\alpha 5)\beta 4$ nAChR⁴³ and action via G-protein-coupled gamma-aminobutyric acid_B receptors^{44,45} have also been proposed as mechanisms of action. Novel nonpeptide small molecule quaternary ammonium analogs also potently blocked $\alpha 9\alpha 10$ and discriminated between $\alpha 9\alpha 10$ and $\alpha 7$ nAChR (IC₅₀ similar to those for α -conotoxins). In this regard, *bis*-analog ZZ1-61c (used in our study) showed 75-fold greater selectivity for the $\alpha 9\alpha 10$ subtype over the $\alpha 7$ subtype (IC₅₀= 16 versus 1190 nM) and even lower antagonist activities at other nAChR subtypes (e.g., IC₅₀= 4000 nM for the ganglion type $\alpha 3\beta 4$). Lead agents from *tris*- (GZ5-56A) or *tetrakis*- (ZZ1-04G) group also demonstrated preference as antagonists for the $\alpha 9\alpha 10$ versus the $\alpha 7$ (50- and 10-fold, respectively) or the $\alpha 3\beta 4$ (26- and 1220-fold, respectively) subtype.^{17,18,42} Discrimination between $\alpha 9\alpha 10$ and $\alpha 7$ nAChR is important, whereas antagonism of the former receptor is desirable, antagonism of the latter receptor would not be. The antiinflammatory effectiveness (i.e., suppression of release of proinflammatory cytokines from macrophages) appears to be achieved with both $\alpha 9\alpha 10$ -selective antagonists and $\alpha 7$ -selective agonists at nicotinic receptors. The expression of $\alpha 9\alpha 10$ appears to overlap with that of $\alpha 7$ nAChR (e.g., dorsal root ganglion neurons, lymphocytes); however, the $\alpha 9\alpha 10$ subunit nAChR has restricted distribution in tissues while the $\alpha 7$ nAChR is abundant in the CNS. The mechanism of action of quaternary ammonium analogs is expected to be predominantly peripheral rather than central (no $\alpha 9\alpha 10$ receptors in brain). In addition, the quaternary structure of these compounds makes them less likely to cross the blood-brain barrier and enter the CNS. Although highly speculative, it is likely that antagonism of $\alpha 9\alpha 10$ nAChR and reduction of inflammation at the site of injury (decreased accumulation of microphage and T-cells) may underlie efficacy of these agents in rat models of pain with inflammatory components such as the formalin test and chronic constriction nerve injury; however, other mechanism(s) cannot be excluded. The mechanism of action against vincristine-evoked neuropathy seems even more complex, i.e., mononuclear inflammatory cells were not evident in neuronal specimens in this type of pain condition. Notably, there is a line of evidence that chemotherapy-induced neuropathy may likely result from inflammatory mediators involving proinflammatory cytokines produced by non-neuronal cells in peripheral nerves (Schwann cells).²³ Therefore, more studies are needed to determine whether antagonism of $\alpha 9\alpha 10$ nAChR has immune consequences that may play a role in the prophylactic and symptomatic effects against vincristine-evoked pain.

In summary, efficacy of $\alpha 9\alpha 10$ nAChR antagonist, a nonpeptide *bis*-quaternary ammonium analog ZZ1-61c, was shown in a rat model of chemotherapy-evoked neuropathy.

The present preliminary findings may represent a new lead in the search for a novel class of peripherally acting nicotinic antagonists. Such a strategy may provide effective treatments, which circumvent toxicity of centrally acting nicotinic receptor agonists.

Acknowledgements

This study was supported in part by KMFS (EPW) and MH-53631 (JMM). The Department of Anesthesiology also provided funding for this research. Technical help of Mitchell Elliott and Jonathan Walter (Univ. of Kentucky) is greatly appreciated.

References

1. Windebank AJ, Grisold W. Chemotherapy-induced neuropathy. *J Peripheral Nervous System*. 2008; 13:27–46.
2. Wolf S, Barton D, Kottschade L, Grothey A, Loprinzi C. Chemotherapy-induced peripheral neuropathy: prevention and treatment strategies. *Eur J Cancer*. 2008; 44:1507–1515. [PubMed: 18571399]
3. Arneric SP, Holliday M, Williams M. Neuronal nicotinic receptors: a perspective on two decades of drug discovery research. *Biochem Pharmacol*. 2007; 74:1092–1101. [PubMed: 17662959]
4. Buccafusco JJ. Neuronal nicotinic receptors subtypes: defining therapeutic targets. *Mol Interv*. 2004; 4:285–295. [PubMed: 15471911]
5. O'Hoedt, d; Bertrand, D. Nicotinic acetylcholine receptors: an overview on drug discovery. *Expert Opin Ther targets*. 2009; 13:395–411. [PubMed: 19335063]
6. Vincler M. Neuronal nicotinic receptors as targets for novel analgesics. *Expert Opin Investig Drugs*. 2005; 14:1191–1198.
7. Decker MW, Rueter LE, Bitner RS. Nicotinic acetylcholine receptors agonists: a potential new class of analgesics. *Current Topics Medicinal Chemistry*. 2004; 4:369–384.
8. Lippello PM, Bencherif TA, Hauser KG, Jordan SR, Letchworth SR, Mazurow AA. Nicotinic receptors as targets for therapeutic discovery. *Expert Opin Drug Discov*. 2007; 2:1185–1203. [PubMed: 23496128]
9. Lynch JJ, Wade CL, Mikusa JP, Decker MW, Honore P. ABT-594 (a nicotinic acetylcholine agonist): anti-allodynia in a rat chemotherapy-induced pain model. *Eur J Pharmacol*. 2005; 509:43–48. [PubMed: 15713428]
10. Vincler M, McIntosh JM. Targeting the $\alpha 9\alpha 10$ nicotinic acetylcholine receptor to treat severe pain. *Expert Opin Ther Targets*. 2007; 11:891–897. [PubMed: 17614758]
11. McIntosh JM, Absalom N, Chebib M, Elgoyhen AB, Vincler M. Alpha9 nicotinic acetylcholine receptors and the treatment of pain. *Biochem Pharmacol*. 2009; 78:693–702. [PubMed: 19477168]
12. Satkunathan N, Livett b, Gayler K, Sandall D, Down J, Khalil Z. Alpha-conotoxin Vc1.1. alleviates neuropathic pain and accelerates functional recovery of injured neurones. *Brain Research*. 2005; 1059:149–158. [PubMed: 16182258]
13. Vincler M, Wittenauer S, Parker R, Ellison M, Oliviera BM, McIntosh JM. Molecular mechanism for analgesia involving specific antagonism of $\alpha 9\alpha 10$ acetylcholine receptors. *Proc Natl Acad Sci USA*. 2006; 103:17880–17884. [PubMed: 17101979]
14. Ayers JT, Dwoskin LP, Deaciuc AG, Grinevich VP, Zhu J, Crooks PA. *Bis*-azaaromatic quaternary ammonium analogues; ligands for $\alpha 4\beta 4$ and $\alpha 7$ subtypes of nicotinic receptors. *Bioorg Med Chem Lett*. 2002; 12:3067. [PubMed: 12372503]
15. Zhang Z, Zheng G, Pivovarchyk M, Deaciuc AG, Dwoskin LP, Crooks PA. *Tetrakis*-azaaromatic quaternary ammonium salts: novel subtype-selective antagonists at neuronal nicotinic receptors that mediate nicotine-evoked dopamine release. *Bioorg Med Chem Lett*. 2008; 18:5753–5757. [PubMed: 18851914]
16. Zheng G, Sumithran SP, Deaciuc AG, Dwoskin LP, Crooks PA. *Tris*-azaaromatic quaternary ammonium salts: novel templates as antagonists at nicotinic receptors mediating nicotine-evoked dopamine release. *Bioorg Med Chem Lett*. 2007; 17:6701–6706. [PubMed: 17977723]

17. McIntosh JM, Dwoskin LP, Crooks PA, Holtman JR Jr. GZ5-556A and ZZ2-04G are novel small molecule antagonists of $\alpha 9\alpha 10$ nAChRs and are analgesic in rats. *Biochem Pharmacol Suppl.* 2009; 78:921.
18. Zheng G, Zhang Z, Dowell C, Wala E, Dwoskin LP, Holtman JR, McIntosh JM, Crooks PA. Discovery of non-peptide, small molecule antagonists of $\alpha 9\alpha 10$ nicotinic acetylcholine receptors as novel analgesics for the treatment of neuropathic and tonic inflammatory pain. *Biorg Med Chem Lett.* 2011; 21:2476–2479.
19. Holtman JR Jr, Dwoskin LP, Dowell C, Wala EP, Crooks PA, McIntosh JM. Novel small molecule $\alpha 9\alpha 10$ nicotinic receptor antagonists for pain. *J Pain Suppl.* 2010; 11:S33.
20. Flatters SJ, Bennett GJ. Ethosuximide reverses paclitaxel- and vincristine-induced painful peripheral neuropathy. *Pain.* 2004; 109:150–161. [PubMed: 15082137]
21. Randall, Selitto. A method for measurement of analgesic activity of inflamed tissue. *Arch Int Pharmacodyn Ther.* 1957; 111:409–419. [PubMed: 13471093]
22. Bennett G. Pathophysiology and animal models of cancer-related painful peripheral neuropathies. *The Oncologists.* 2010; 15:9–12.
23. Cata JP, Weng HR, Lee BN, Reuben JM, Dougherty PM. Clinical and experimental findings in humans and animals with chemotherapy-induced peripheral neuropathy. *Minerva Anesthesiol.* 2006; 72:151–169.
24. Polomano RC, Bennet GJ. Chemotherapy-evoked painful peripheral neuropathy. *Pain Medicine.* 2001; 2:8–14. [PubMed: 15102312]
25. Weng HR, Cordewilla JV, Dougherty PM. Changes in sensory processing in the spinal dorsal horn accompanies vincristine-induced hyperalgesia and allodynia. *Pain.* 2003; 103:131–138. [PubMed: 12749967]
26. Flatters SJ, Bennett GJ. Studies on peripheral sensor nerves in paclitaxel-induced painful peripheral neuropathies. *Pain.* 2006; 109:150–161. [PubMed: 15082137]
27. Holtman JR Jr, Crooks PA, Johnson-Hardy J, Wala EP. Antinociceptive effects and toxicity of morphine-6-O-sulfate sodium salt in rat. *Eur J Pharmacol.* 2010; 648:87–94. [PubMed: 20828549]
28. Le Bars D, Gozariu M, Cadden SW. Animal models of nociception. *Pharmacol Rev.* 2001; 53:597–652. [PubMed: 11734620]
29. Aley KO, Reichling DB, Levine JD. Vincristine hyperalgesia in the rat: a model of painful vincristine neuropathy in human. *Neuroscience.* 1996; 73:259–265. [PubMed: 8783247]
30. Authier N, Gillet JP, Fialip J, Eschaliere A, Coudore F. A new animal model of vincristine-induced nociceptive peripheral neuropathy. *NeuroToxicology.* 2003; 24:797–805. [PubMed: 14637374]
31. Nozaki-Taguchi N, Chaplan SR, Higuera ES, Ajakwe RC, Yaksh TL. Vincristine-induced allodynia in the rat. *Pain.* 2001; 93:69–76. [PubMed: 11406340]
32. Sang CN, Bennett GJ. Novel therapies for the control and prevention of neuropathic pain. *Neurotherapeutics.* 2009; 6:607–608. [PubMed: 19789065]
33. Lynch JJ, Wade CL, Zhong CM, Mikusa JP, Honore P. Attenuation of mechanical allodynia by clinically utilized drugs in a rat chemotherapy-induced neuropathic pain model. *Pain.* 2004; 110:56–63. [PubMed: 15275752]
34. Xiao W, Boroujerdi A, Bennett GJ, Luo ZD. Chemotherapy-evoked painful peripheral neuropathy: analgesic effects of gabapentin and effects on expression of the $\alpha 2\text{-delta}$ type-1 calcium channel subunit. *Neuroscience.* 2007; 144:714–720. [PubMed: 17084535]
35. Meyer L, Patte-Mensah C, Taleb O, Mensah-Nyagan AG. Cellular and functional evidence for a protective action of neurosteroids against vincristine chemotherapy-induced painful neuropathy. *Cell Mol Life Sci.* 2010; 67:3017–3034. [PubMed: 20431905]
36. Rahn EJ, Makriyannis A, Hohmann AG. Activation of cannabinoid CB1 and CB2 receptors suppresses neuropathic nociception evoked by the chemotherapeutic agent vincristine in rats. *Br J Pharmacol.* 2007; 152:765–777. [PubMed: 17572696]
37. Cata JP, Weng HR, Dougherty PM. Cyclooxygenase inhibitors and thalidomide ameliorate vincristine-reduced hyperalgesia in rats. *Cancer Chemother Pharmacol.* 2004; 54:391–397. [PubMed: 15235822]

38. Pascual D, Goicoechea C, Burgos E, Martin MI. antinociceptive effect of three common analgesic drugs on peripheral neuropathy by paclitaxel in rats. *Pharmacol Biochem Behav.* 2010; 95:331–337. [PubMed: 20184918]
39. Xiao W, Naso L, Bennett GJ. Experimental studies of potential analgesics for the treatment of chemotherapy-evoked painful peripheral neuropathies. *Pain Med.* 2008; 9:505–517. [PubMed: 18777607]
40. Holtman JR Jr, Crooks PA, Johnson-Hardy J, Wala EP. The analgesic and toxic effects of nornicotine enantiomers alone and in interaction with morphine in rodent models of acute and persistent pain. *Pharmacol Biochem Behav.* 2010; 94:352–362. [PubMed: 19800911]
41. Siau C, Bennett GJ. Dysregulation of cellular calcium homeostasis in chemotherapy-evoked painful peripheral neuropathy. *Anesth Analg.* 2006; 102:1485–1490. [PubMed: 16632831]
42. Holtman JR Jr, Dwoskin LP, Dowell C, Wala EP, Zhang Z, Crooks PA, McIntosh JM. The novel small molecule $\alpha 9\alpha 10$ nicotinic acetylcholine receptor antagonist ZZ-204G is analgesic. *Eur J Pharmacol.* 2011; 670:500–508. [PubMed: 21944926]
43. Clark RJ, Fisher H, Nevin ST, Adams DJ, Craik DJ. The synthesis, structural characterization, and receptor specificity of the α -conotoxin Vc1.1. *J Biol Chem.* 2006; 281:23254–23263. [PubMed: 16754662]
44. Callaghan B, Haythornthwaite A, Berecki G, Clark RJ, Craik DJ, Adams DJ. Analgesic α -conotoxin Vc1.1 and Rg1A inhibit N-type calcium channels in rat sensory neurons via GABA_B receptor activation. *J Neurosci.* 2008; 28:10943–10951. [PubMed: 18945902]
45. Nevin ST, Clark RJ, Klimis H, Christe MJ, Craik DJ, Adams DJ. Are $\alpha 9\alpha 10$ nicotinic acetylcholine receptors a pain targets for α -conotoxin. *Molecular Pharmacol.* 2007; 72:1406–1410.

Figure 1.

Responsiveness to von Frey filaments (VFH) and the paw pressure test in rats which were treated on days 1–5 and 8–12 with: vincristine alone, 100 µg/kg/day (group 1, n=6 rats), vincristine in combination with ZZ1-61c, 100 µg/kg/day of each (group 2, n=6 rats), ZZ1-61c alone, 100 µg/kg/day (group 3, n=6 rats), saline, 1 ml/kg/day (group 4, n=6 rats). Percentage of paw withdrawal (%PW): [A] 4g VFH; [B] 8g VFH; [C] 15g VFH and [D] vocalization threshold (VT g). Data are mean ± SEM (n=6 rats/group). Day 1 indicates response level before initiation of a treatment (pretreatment baseline). Bars above the abscissa indicate time

of treatment (days 1–5 and 8–12). * $P < 0.05$, one-way ANOVA with post hoc Dunnett's test comparing to pretreatment baseline.

Figure 2.

Responsiveness to von Frey filaments (VFH) and the paw pressure test in rats which were treated with vincristine, 100 µg/kg/day on days 1–5 and 8–12 followed by either ZZ1-61c, 100 µg/kg/day (group 5A) or saline, 1ml/kg (group 5B) starting at day 15 and ending on day 33 after vincristine initiation. Percentage of paw withdrawal (%PW): [A] 4g VFH; [B] 8g VFH; [C] 15g VFH and [D] vocalization threshold (VT g). Data are mean ± SEM (n rats). Day 1 indicates pre-vincristine baseline (response level before initiation of vincristine; n=12 rats). Day 15 indicates post-vincristine baseline (response level after completion of

vincristine treatment/ before initiation of ZZ1-61c or saline; n=12 rats). Days 17–33 show response levels (before each injection) during the course of administration of ZZ1-61c (n=6 rats) or saline (n=6 rats). * $P<0.001$, t -paired test comparing to pre-vincristine baseline; $^+P<0.05$, $^{++}P<0.01$; $^{+++}P<0.005$, $^{++++}P<0.0001$, t -test comparing ZZ1-61c with saline at each time point. Bars above the abscissa mark the time of administration of vincristine (days 1–5, 8–12) and ZZ1-61c or saline (days 15–33).

Figure 3.

Responsiveness to von Frey filaments (VFH) and the paw pressure test assessed before and 15 min after administration of ZZ1-61c (100 μ g/kg/day on days 15–33) in rats which were previously exposed to vincristine (100 μ g/kg/day on days 1–5 and 8–12). Percentage of paw withdrawal (%PW): [A] 4g VFH; [B] 8g VFH; [C] 15g VFH and [D] vocalization threshold (VT g). Mean $\pm$ SEM (n=6 rats). Significantly different from pre-ZZ1-61c response, * $P=0.032$ (Wilcoxon signed rank test)

Figure 4. The effects of ZZ1-61c (100 µg/kg/day) on [A] rotarod performance test and [B] grip strength test. Area under the curve (AUC_{0-60 min}): ZZ1-61c (day 1, 2, 3) and saline (control). Mean ± SEM (n=4 rats)