

Characterization of Antiallodynic Actions of ALE-0540, a Novel Nerve Growth Factor Receptor Antagonist, in the Rat¹

JOSHUA B. OWOLABI, GEIHAN RIZKALLA, ASHOK TEHIM, GREGORY M. ROSS, RICHARD J. RIOPELLE, RAJENDER KAMBOJ, MICHAEL OSSIPOV, DI BIAN, SANDARA WEGERT, FRANK PORRECA, and DAVID K. H. LEE

Allelix Biopharmaceuticals Inc., Mississauga, Canada (J.B.O., G.R., A.T., R.K., D.K.H.L.); Department of Pharmacy, University of Arizona Health Science Center, Tucson, Arizona (M.O., D.B., S.W., F.P.); and Department of Medicine, Queen's University, Kingston, Canada (G.M.R., R.J.R.)

Accepted for publication February 17, 1999 This paper is available online at <http://www.jpet.org>

ABSTRACT

There is growing evidence that nerve growth factor (NGF) may function as a mediator of persistent pain states. We have identified a novel nonpeptidic molecule, ALE-0540, that inhibits the binding of NGF to tyrosine kinase (Trk) A or both p75 and TrkA (IC_{50} $5.88 \pm 1.87 \mu M$, $3.72 \pm 1.3 \mu M$, respectively), as well as signal transduction and biological responses mediated by TrkA receptors. ALE-0540 was tested in models of neuropathic pain and thermally-induced inflammatory pain, using two routes of administration, a systemic i.p. and a spinal intrathecal (i.th.) route. Morphine was also tested for comparison in the antiallodynia model using mechanical stimuli. We show that either i.p. or i.th. administration of ALE-0540 in rats produced antiallodynia in the L5/L6 ligation model of neuropathic pain. The calculated A_{50} values (and 95% confidence intervals) for ALE-

0540 administered i.p. and i.th. were 38 (17.5–83) mg/kg and 34.6 (17.3–69.4) μg , respectively. ALE-0540 given i.th., at doses of 30 and 60 μg , also blocked tactile allodynia in the thermal sensitization model. Although morphine displayed greater potency [A_{50} value of 7.1 (5.6–8.8) mg/kg] than ALE-0540 in anti-allodynic effect when given i.p. to L5/L6-ligated rats, it was not active when administered i.th. These data suggest that a blockade of NGF bioactivity using a NGF receptor antagonist is capable of blocking neuropathic and inflammatory pain and further support the hypothesis that NGF is involved in signaling pathways associated with these pain states. ALE-0540 represents a nonpeptidic small molecule which can be used to examine mechanisms leading to the development of agents for the treatment of pain.

Nerve growth factor (NGF) is a trophic molecule essential for the survival of sympathetic and small diameter primary sensory neurons (Crowley et al., 1994) and regulates the specification of the nociceptive phenotype (Ritter et al., 1991). Although NGF does not appear to be necessary for neuronal survival in maturity, there is evidence that it may regulate the levels of Substance P (SP) and calcitonin gene-related peptide (CGRP) in mature NGF-sensitive neurons (Diamond et al., 1987). There is also a wealth of evidence to suggest that NGF can interact with pain-signaling systems in adult animals (Lewin and Mendell, 1993). This includes the selective expression of high-affinity NGF receptors [tyrosine kinase (Trk) A receptors] on nociceptive primary afferents (Averill et al., 1995) and the hyperalgesic effects of exogenously administered NGF in animals (Lewin et al., 1993) and humans (Petty et al., 1994). In a variety of animal models of inflammation, including those produced by Freund's adjuvant (Donnerer et al., 1992), s.c. carrageenin (Aloe et al., 1992), and in a rat model of cystitis (Andreev et

al., 1995; McMahon et al., 1995), NGF expression is increased.

In the absence of pharmacological antagonists, several studies have reported the effects of neutralizing anti-NGF antibodies on hyperalgesic states. These include attenuation of behavioral sensitivity, the up-regulation of neuropeptides SP and CGRP, and inflammation-induced expression of the immediate early gene *c-fos* in dorsal horn neurons (Woolf et al., 1994; Lewin et al., 1994; Woolf and Doubell, 1994). Recently, administration of a novel TrkA-IgG fusion molecule that sequesters endogenous NGF was shown to produce a sustained thermal and chemical hypoalgesia in the carrageenin model of inflammation (McMahon et al., 1995). These studies suggest that peripherally produced NGF normally acts to maintain the sensitivity of nociceptive sensory neurons and that an up-regulation of NGF is responsible for alterations in pain-related behavior. Therefore, blockade of NGF production or action has been proposed to provide a novel class of non-anti-inflammatory analgesics with a specific action on the sensory hypersensitivity produced by inflammation.

Received for publication October 22, 1998.

¹ This work was supported by Allelix Biopharmaceuticals, Inc.

ABBREVIATIONS: NGF, nerve growth factor; BDNF, brain-derived growth factor; Trk, tyrosine kinase; ET, endothelin; i.th., intrathecal; i.c.v., intracerebroventricular; CGRP, calcitonin gene-related peptide; CI, confidence interval; PE, polyethylene tubing; L5/6, fifth and sixth lumbar nerves; DRG, dorsal root ganglia; 5-HT, hydroxytryptamine.

Compared to the strong evidence for involvement of NGF in inflammatory pain, there is relatively little known about its role in neuropathic pain. Following nerve injury, damaged axons degenerate and trigger changes in the endoneurium known collectively as Wallerian degeneration (WD). Prominent among these changes is the production of NGF by fibroblasts, Schwann cells, and macrophages, triggered by cytokines released from endogenous or exogenous phagocytes (Heumann et al., 1987). In a recent study, the hypothesis that NGF is involved in the genesis of neuropathic pain was tested by comparing behavioral responses to mechanical and thermal pain stimuli following chronic constriction injury in three strains of mice: C57BL/Ola, which have delayed Wallerian degeneration; C57BL/J, "normals"; and C57BL transgenics overexpressing the NGF gene driven by a glial factor activated protein promoter. For both behavioral tests, it was reported that Ola mice show minimal sensitivity, C57BL/J mice show a modest increase, whereas glial factor activated protein-NGF transgenics show a greater increase (Ramer et al., 1995). Thus, NGF is also implicated in the development of pain-related behaviors in this rodent model of neuropathic pain. In a partially damaged nerve, intact nociceptive axons could be exposed to elevated levels of NGF, much as occurs in inflammation, but with the difference that the source of the NGF is from adjacent, degenerating regions of the endoneurium.

Although neutralizing anti-NGF antibodies or TrkA-IgG may represent new agents in the treatment of some chronic pain states, they have very limited potential as therapeutics. Some anti-NGF antibodies have been tested to have cross-reactivity to other neurotrophins (Murphy et al., 1993). The supply of TrkA-IgG fusion protein, as any recombinant protein, may require complex manufacturing and purification processes. The discovery and development of orally active small molecule-based drugs which antagonize the action of NGF could also solve issues of delivery. In the present study, we report the discovery of ALE-0540, a nonpeptidic heterocyclic molecule that inhibits the binding of NGF to p75 and TrkA, as well as signal transduction and biological responses mediated by TrkA receptors. A preliminary assessment of potential clinical utility of ALE-0540 in neuropathic and thermally induced inflammatory pain states is also presented.

Materials and Methods

Binding of ^{125}I -NGF to PC12 Cells. Mouse NGF (2.5S) obtained from Cedarlane Laboratories (Toronto, Ontario, Canada) was used for all studies. The iodination of NGF was performed as described previously (Sutter et al., 1979) and the labeled NGF was purified by size exclusion chromatography on a PD10 column (Pharmacia, Uppsala, Sweden) pre-equilibrated with HKR buffer (10 mM HEPES, pH 7.35) containing 125 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl_2 , 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 , 1 mg/ml BSA, and 1 mg/ml glucose). PC12 cells were grown in RPMI 1640 with 10% heat-inactivated donor horse serum and 5% fetal calf serum. Cells were harvested for binding by washing off the media with calcium-magnesium-free balanced salt solution (Gey's solution). Cells were pelleted by centrifugation and suspended in HKR buffer. The binding assay was set up using 96-well microtiter plates, each well containing ^{125}I -NGF (final concentration of 0.5 nM), 400,000 cells (for a final concentration of 10^6 cells/ml), and HKR buffer (for total binding) or brain-derived nerve factor (BDNF; at 40 nM for TrkA binding) or NGF (at 50 nM

for nonspecific binding). The plates were incubated with shaking for 2 h at 4°C and 100- μl aliquots were transferred to 400- μl microcentrifuge tubes containing 200 μl of 10% glycerol in HKR. The tubes were centrifuged for 1 min at 5,000 rpm, the tip containing the cell pellet was cut off, and the radioactivity bound to the cells was determined in a gamma counter. Specific binding was calculated as the difference between the amount of ^{125}I -NGF bound in the absence (total) and presence (nonspecific binding) of 50 nM unlabeled NGF. TrkA binding was determined similarly except 10 nM BDNF was added in all reactions.

Other Receptor Radioligand-Binding Assays. The affinity of ALE-0540 for various receptors was determined in radioligand-binding assays by CEREP (Celle L'Evescault, France) using standard published methods and experimental conditions. All experiments were performed in triplicate. In each experiment, the respective reference compound was simultaneously tested at eight concentrations in duplicate to obtain a competition curve to validate the assay. Following incubation, membranes or cells in suspension were rapidly filtered under vacuum through Whatman GF/B or Packard GF/C filters. The filtered membranes were washed several times with an ice-cold buffer, and bound radioactivity was measured using a liquid scintillation counter. Results are expressed as a percentage of inhibition of specific binding obtained in the presence of ALE-0540.

Neurite Outgrowth. Dissociated cells enriched for sensory neurons were prepared from embryonic day 8 chick dorsal root ganglia (DRG) as described (Dostaler et al., 1996). Neurons were seeded at a density of 800 to 1000 cells/well in Ham's F-12 containing 5% fetal calf serum and NGF at 10 pM into wells of Falcon microculture plates treated with poly-D-lysine. The cells were incubated with the test compound (at concentration ranging from 0.1 to 50 μM) at 37°C in a 5% CO_2 atmosphere. The cells on the entire lower horizontal surface of the well were scored for neurite outgrowth at 18 to 20 h using an inverted microscope fitted with phase-contrast optics. A neurite was scored if its caliber from the origin to terminal was constant and its length was equal or greater than 1.5 cell body diameters. Neurite growth was corrected for background (no NGF).

TrkA Phosphorylation Assay. TrkA phosphorylation was determined by a modification of the methods described by Ross et al. (1998). Briefly, PC12 cells were used at 10^6 cells/ml and incubated with 40 pM NGF with or without ALE-0540 (at indicated concentrations) for 15 min at 37°C. At the conclusion of the reaction, cells were washed, lysed, and immunoprecipitated with the anti-TrkA antibody. Isolated phosphoproteins were resolved using 6% SDS-polyacrylamide gel and Western blot analysis performed with antiphosphotyrosine 4G10 (Upstate Biotechnology Inc., Lake Placid, NY) visualized with enhanced chemiluminescence (Amersham, Piscataway, NJ).

Nerve Ligation Injury. Nerve ligation injury was performed according to the method described previously (Kim and Chung, 1992). This technique produces signs of tactile allodynia and thermal hyperalgesia. Rats were anesthetized with halothane and the L5 and L6 spinal nerves were exposed, carefully isolated, and tightly ligated with 4-0 silk suture distal to the DRG. After ensuring homeostatic stability, the wounds were sutured, and the animals were allowed to recover in individual cages. Sham-operated rats were prepared in an identical fashion except that the L5 and L6 spinal nerves were not ligated.

Intrathecal Catheter Placement. Two routes of administration, a systemic i.p. and a spinal intrathecal (i.th.) route, were used to explore the activity of compounds. For the spinal route, test compounds were injected through indwelling i.th. catheters in the manner described by Yaksh and Rudy (1976). While under anesthesia, polyethylene tubing 10 tubing (8 cm) was inserted through an incision made in the atlanto-occipital membrane to the level of the lumbar enlargement of the rat and secured. Drug injections were made in a volume of 5 μl followed by a 9- μl saline flush.

Thermal Sensitization. Rats were lightly anesthetized with ether. The left hindpaw was placed in a water bath maintained at

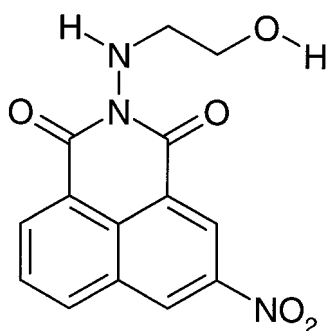


Fig. 1. Structure of ALE-0540.

50°C for 1 min. Inflammation suggested by rubor of the paw developed immediately. The rats were allowed to recover from anesthesia and tactile testing was begun 2 h after thermal sensitization. This procedure has produced signs of thermal hyperalgesia and tactile allodynia that persisted for over 12 h.

Evaluation of Tactile Allodynia. Mechanical allodynia was determined in the manner described previously (Chaplan et al., 1994). The paw withdrawal threshold was determined in response to probing with calibrated von Frey filaments. The rats were kept in suspended cages with wire mesh floors and the von Frey filaments were applied perpendicularly to the planar surface of the paw of the rat until it bent slightly, and was held for 3 to 6 s, or until the paw is withdrawn. A positive response was indicated by a sharp withdrawal of the paw. The 50% paw withdrawal threshold was determined by the nonparametric method (Dixon, 1980). Data were converted to percentage of antiallodynia by the formula:

$$\% \text{ Antiallodynia} = 100$$

$$\times (\text{test value} - \text{control value}) / (15 \text{ g} - \text{control value})$$

Results

Pharmacological Characterization of ALE-0540. The cell line PC12 (Greene and Tischler, 1976) expresses both types of NGF receptor, with p75 representing approximately 90% and TrkA representing approximately 10% of the NGF-binding sites. A low-volume screening of commercial compound libraries was completed using the PC12 cells in ligand-binding experiments, as described in *Materials and Methods*. From our screening efforts, we identified a compound, ALE-

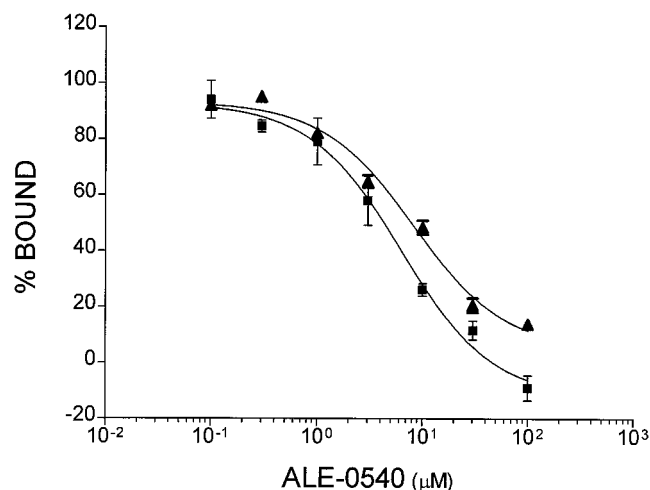


Fig. 2. Inhibition of ^{125}I -NGF binding to PC12 cells. ■, inhibition in the presence of BDNF; ▼, inhibition in the absence of BDNF.

TABLE 1

Competition by ALE-0540 for ligands binding at selected receptors

Receptor	IC ₅₀ , μM (mean \pm S.E.M.)
TrkA	5.88 \pm 1.87
P75 + TrkA	3.72 \pm 1.3
% Inhibition, 1 μM	
A3	14
$\alpha 2c$	17
CB2	<10
ETA	<10
H1	<10
δ	<10
κ	<10
μ	<10
% Inhibition, 10 μM	
A1	14
5HT2A	<10

0540 (*N*-{5-nitro-1*H*-benz[de]isoquinoline-1,3(2*H*)-dione}-2-aminoethanol; Fig. 1) that displayed significant ($\geq 50\%$) inhibition of ^{125}I -NGF binding at 100 μM . A typical competition binding isotherm of ALE-0540 for ^{125}I -NGF binding to PC12 cells in the presence or absence of BDNF is shown in Fig. 2. ALE-0540 displaced ^{125}I -NGF binding in a concentration-dependent manner, in four independent experiments, with IC₅₀ (mean \pm S.E.M.) of 5.88 \pm 1.87 μM at the TrkA and 3.72 \pm 1.3 μM for both TrkA and p75.

ALE-0540 was assessed for its binding to a battery of receptors at 1 or 10 μM concentration (Table 1). As can be seen, ALE-0540 was mostly inactive; less than 20% inhibition at 1 or 10 μM at A3, cannabinoid 2, endothelin (ET) A, H1, opioid (δ , κ , and μ) and 5-hydroxytryptamine (5-HT) type 2A receptors.

Functional Characterization of ALE-0540. Effects of ALE-0540 on NGF-mediated neurite outgrowth of DRG neurons were quantitated. ALE-0540 showed significant inhibition of neurite extension in a concentration-dependent manner (Fig. 3). NGF induces a rapid increase in phosphorylation of tyrosine residues within TrkA receptor as part of signal transduction cascade. Using conditions where a rapid increase in TrkA phosphorylation is induced by NGF, ALE-0540 prevented phosphorylation of this receptor dose-depen-

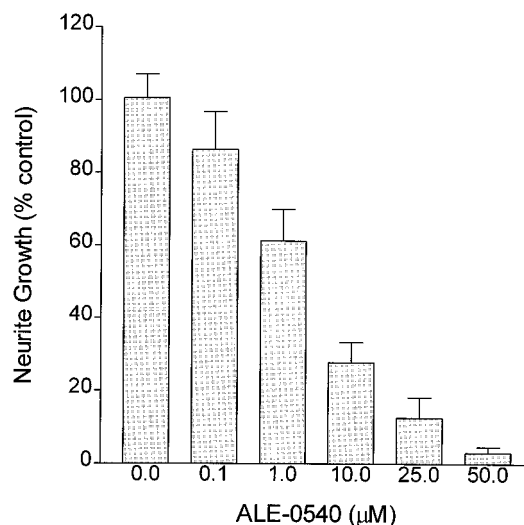


Fig. 3. Effects of ALE-0540 on neurite outgrowth. The ability of ALE-0540 to inhibit NGF-induced neurite outgrowth in DRG neurons was evaluated in the presence of varying concentrations of ALE-0540. Error bars, S.E.M.

dently and exhibited an EC_{50} value of $28 \pm 21 \mu\text{M}$ (n 3) (Figs. 4 and 5).

Antiallodynic Activity of ALE-0540. The L5/L6 nerve-ligation model is commonly accepted as representing some aspects of neuropathic pain and critically does not represent inflammatory pain. In the nerve-injury experiments, ALE-0540 was tested for mechanical antiallodynic activity compared to actions in sham-operated animals. ALE-0540 produced dose-dependent antiallodynia after either i.p. or i.th. injection in rats with L5/L6 nerve ligations (Fig. 6). The calculated A_{50} value [and 95% confidence intervals (CIs)] for ALE-0540 administered i.p. was 38 (17.5–83) mg/kg, indicating a reduced potency compared to i.p. morphine which displayed an A_{50} (and 95% CIs) of 7.1 (5.6–8.8) mg/kg; however, both were equally efficacious. In contrast to morphine given i.th., which was inactive up to 100 μg , ALE-0540 produced concentration-dependent antiallodynia with an A_{50} (and 95% CIs) of 34.6 (17.3–69.4) μg (Fig. 6B). ALE-0540 did not block tactile allodynia at a concentration of 100 μg when given directly into a lateral cerebral ventricle (i.c.v.) (data not shown).

ALE-0540 blocked tactile allodynia produced by thermal sensitization of the hindpaw of rats at doses of 30 and 60 μg i.th. (Fig. 7). Antiallodynia ranged from 55 ± 21 to 64 ± 23 (mean \pm S.E.M.) percentage of antiallodynia at these doses.

Discussion

This report describes the discovery of a nonpeptidic molecule, ALE-0540, capable of inhibiting NGF binding to PC12 cells in the presence and absence of BDNF. Inhibition of NGF binding to PC12 cells by ALE-0540 appears to be through blockade of its receptors as shown by the IC_{50} values at TrkA and both p75 and TrkA. Preliminary experiments also demonstrate that ALE-0540 does not bind to NGF, within the detection limits of an NGF-affinity chromatography column (G.M.R. and R. Riopelle, unpublished observations). Therefore, we hypothesize that ALE-0540 is unlike PD 90780 (Speigel et al., 1995) or kynurenic acid derivatives (Jaen et

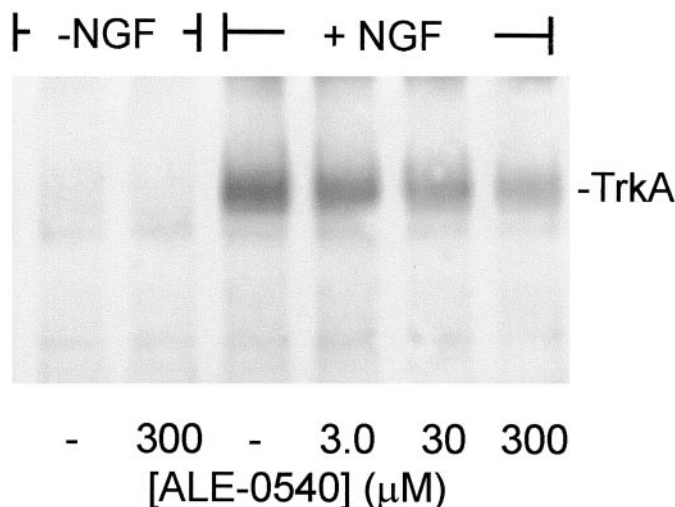


Fig. 4. Effects of ALE-0540 on TrkA phosphorylation. The ability of ALE-0540 to prevent NGF-induced phosphorylation of the TrkA receptor was evaluated by antiphosphotyrosine Western blotting of the TrkA receptor isolated by immunoprecipitation. ALE-0540 prevented phosphorylation observed in the presence of 40 pM NGF. Densitometry of the gels revealed an EC_{50} $28 \pm 21 \mu\text{M}$ (n 3).

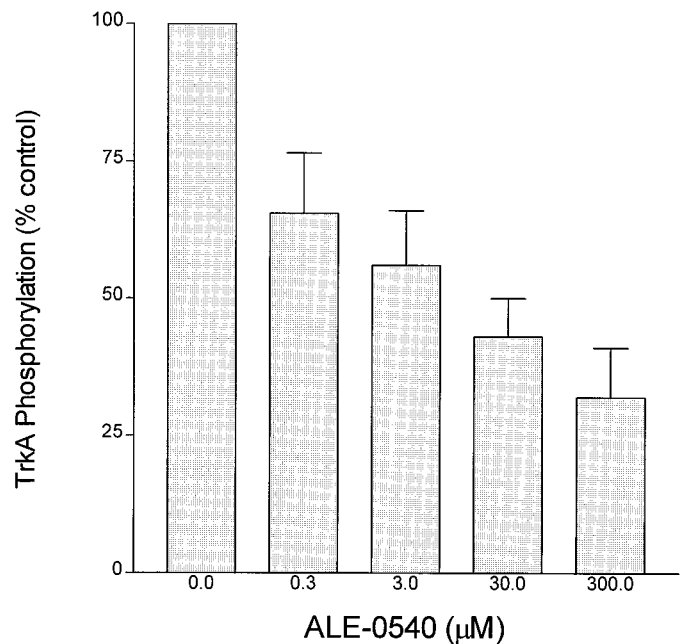


Fig. 5. Inhibition of NGF-induced TrkA phosphorylation by ALE-0540. PC12 cells (at 10^6 cells/ml) were incubated with 40 pM NGF with or without ALE-0540 (at indicated concentrations) for 15 min at 37°C . Afterwards, cells were washed, lysed, and immunoprecipitated with anti-TrkA antibody. Isolated phosphoproteins were resolved using a 6% SDS-polyacrylamide gel and Western blot analysis performed with antiphosphotyrosine 4G10 visualized with enhanced chemiluminescence. Results are expressed as mean \pm S.E.M. (n 3).

al., 1995) which have been shown to prevent binding of NGF to p75 by specifically binding to the NGF molecule.

Consistent with the ability of ALE-0540 to inhibit binding of NGF to its receptor sites is the demonstration that it inhibited NGF-induced neurite outgrowth of DRG neurons (Dostaler et al., 1996). Accompanying the loss in NGF binding and biological function is the inhibition of TrkA phosphorylation induced by NGF, a key initial event in the signal transduction pathway mediated by this receptor (Kaplan et al., 1991). This study did not determine the effects of ALE-0540 on p75-mediated signaling.

Noiceptive responses to experimentally induced pain are the most important measures in laboratory animal tests of the analgesic efficacy of novel drugs. As indicated above, there is selective expression of TrkA receptors on nociceptive primary afferents (Averill et al., 1995). In the absence of pharmacological antagonists, NGF neutralizing antibodies or TrkA-IgG fusion protein have attenuated hyperalgesia in several models of inflammation (Woolf et al., 1994; Lewin et al., 1994; McMahon et al., 1995). The goal of testing ALE-0540 in models of inflammatory pain and neuropathic pain was to assess its potential clinical utility in these different pain states. ALE-0540 was effective in inducing antiallodynic activity after i.p. and i.th. injections following L5/L6 nerve injury in rats. Although systemic (i.p.) morphine was more effective than ALE-0540 in inducing antiallodynia, it was not active by the i.th. route of administration. These results confirm previous findings that morphine is less effective in inducing antinociception at the spinal level after peripheral nerve injury in rats (Xu and Wiesenfeld-Hallin, 1991; Nichols et al., 1995). The mechanism for a lack (or reduced) effect of spinal morphine on neuropathic pain is not fully understood,

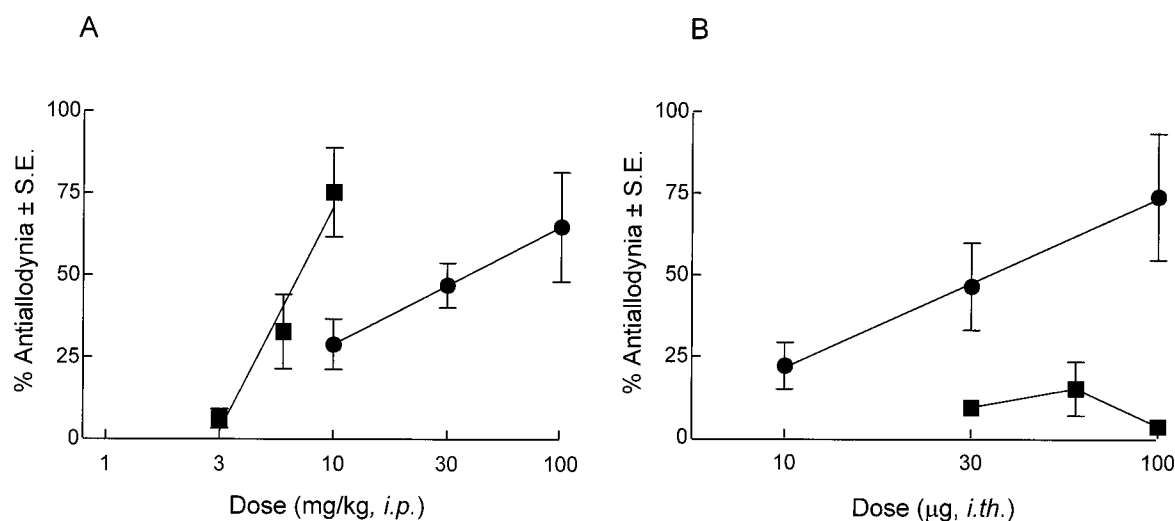


Fig. 6. Antiallodynic effects of ALE-0540 or morphine in L5/L6 nerve-ligated rats. A, i.p. route of administration. B, i.th. route of administration. ●, ALE-0540; ■, morphine. Results are expressed as mean \pm S.E.M.

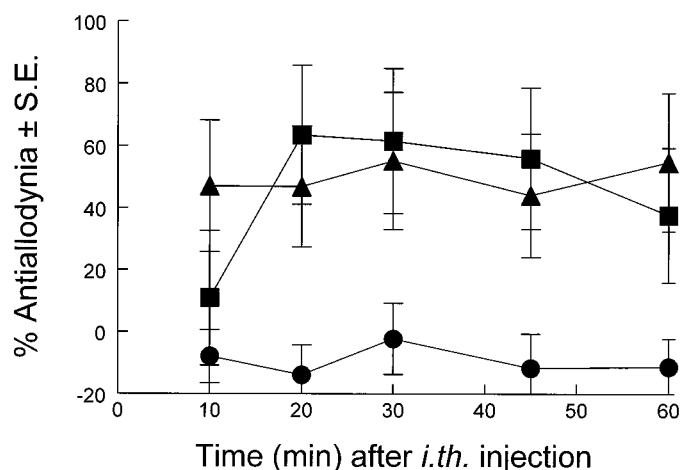


Fig. 7. Effect of ALE-0540 given i.th. on tactile allodynia in rats with thermal sensitization of the hindpaws. ●, saline/dimethyl sulfoxide; ■, 30 μ g; ▲, 60 μ g. Results are expressed as mean \pm S.E.M.

but some studies are emerging which indicate that it may share some features with morphine tolerance (Wiesenfeld-Hallin and Xu, 1996) or it may be due to a reduction in available μ receptors from degeneration of primary efferents following nerve injury (Nichols et al., 1995). It is significant that i.th. administration of ALE-0540 attenuated mechanical allodynia in animals with nerve ligation injury, which suggests a principally spinal site of action. This is also reinforced by the relative lack of efficacy of ALE-0540 after i.c.v. administration. If ALE-0540 acts mainly through the spinal route, it may produce analgesia without side effects associated with centrally acting analgesics (Porreca et al., 1983; Max et al., 1988).

The mechanism by which ALE-0540 attenuated mechanical allodynia in the L5/L6-ligated rats is still a matter of future investigations. It is clear, however, that it must interfere in some manner with injury-induced sensitization of the dorsal horn neurons which is fundamental to the development of hyperalgesia and allodynia associated with neuropathic pain (Woolf et al., 1994). Drawing on recent studies by Chung et al. (1993) and Ramer and Bisby (1997), both chronic constriction injury and L5/L6 spinal ligation models produce

rapid sympathetic sprouting into the DRG coincident with behavioral signs of neuropathic pain. In these animal models, mechanisms such as inflammation and/or a cytokine role for NGF cannot be ruled out. We propose that ALE-0540 may act by blocking NGF-dependent sprouting of TrkA-expressing sensory and sympathetic axons.

To our knowledge, ALE-0540 serves as the first nonpeptidic NGF receptor antagonist molecule to be discovered to have antiallodynic effects in thermally induced inflammatory and neuropathic pain models. Its profile indicates lack of significant interaction with known analgesic targets including α_2 , A1, H1, endothelin A, 5-hydroxytryptamine type 2, cannabinoid 2, and opioid (μ , δ , and κ) receptors (Pertovaara et al., 1990; Karlsten et al., 1991; Mazzari et al., 1996; Suh et al., 1996; May et al., 1996). As a first generation NGF receptor antagonist, it may be useful as an agent to examine the roles of p75 and TrkA receptor antagonists as antinociceptive agents. Coupled with previous findings that NGF can induce hyperalgesia (Andreev et al., 1995; Ramer et al., 1995), our present findings further support the hypothesis that NGF is involved in signaling pathways associated with persistent pain states.

References

- Aloe L, Turveri MA, Carcassi U and Levi-Montalcini R (1992) Nerve growth factor in synovial fluid of patients with chronic arthritis. *Arthritis Rheumat* **35**:351–355.
- Andreev NY, Dimitrova N, Koltzenburg M and McMahon SB (1995) Peripheral administration of nerve growth factor in the adult rat produces a thermal hyperalgesia that requires the presence of sympathetic post-ganglionic neurones. *Pain* **63**:109–115.
- Averill S, McMahon SB, Clary DO, Reichardt LF and Priestly JV (1995) Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. *Eur J Neurosci* **7**:1484–1494.
- Chaplan SR, Bach FW, Pogrel JW, Chung JM and Yaksh TL (1994) Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* **53**:55–63.
- Chung K, Kim HJ, Na HS, Park MJ and Chung JM (1993) Abnormalities of sympathetic innervation in the area of an injured peripheral nerve in a rat model of neuropathic pain. *Neurosci Lett* **162**:85–88.
- Crowley C, Spencer SD, Nishimura MC, Chen KS, Pittsmeek S, Armanini MP, Ling LH, McMahon SB, Shelton DL, Levinson AD and Phillips HS (1994) Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. *Cell* **76**:1001–1011.
- Diamond J, Coughlin M, Macintyre L, Holmes M and Visheau B (1987) Evidence that endogenous NGF is responsible for the collateral sprouting, but not the regeneration, of nociceptive axons in adult rats. *Proc Natl Acad Sci USA* **84**:6596–6600.
- Dixon WJ (1980) Efficient analysis of experimental observations. *Annu Rev Pharmacol Toxicol* **20**:441–462.
- Donnerer J, Schuligoi R and Stein C (1992) Increased content and transport of substance-P and calcitonin gene-related peptide in sensory nerves innervating

- inflamed tissue—Evidence for a regulatory function of nerve growth factor *in vivo*. *Neuroscience* **49**:693–698.
- Dostaler SM, Ross GM, Myers SM, Weaver DF, Ananthanarayanan V and Riopelle RJ (1996) Characterization of a distinctive motif of the low molecular weight neurotrophin receptor that modulates NGF-mediated neurite outgrowth. *Eur J Neurosci* **8**:870–879.
- Greene LA and Tischler AS (1976) Establishment of a noradrenergic clonal cell line of rat pheochromocytoma cells which respond to nerve growth factor. *Proc Natl Acad Sci USA* **73**:2424–2432.
- Heumann R, Korshing S, Bandtlow C and Thoenen H (1987) Changes of nerve growth factor synthesis in non-neuronal cells in response to sciatic nerve transection. *J Cell Biol* **104**:1623–1631.
- Jaen JC, Laborde E, Busch RA, Caprathe BW, Sorenson RJ, Fergus J, Spiegel K, Marks J, Dickerson MR and Davis RE (1995) Kynurenic acid derivatives inhibit the binding of nerve growth factor(NGF) to the low-affinity p75 receptor. *J Med Chem* **38**:4439–4445.
- Kaplan DR, Matin-Zanca D and Parada LF (1991) Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF. *Nature (Lond)* **350**:158–160.
- Karlsten R, Post C, Hide I and Daly J (1991) The antinociceptive effect of intrathecally administered adenosine analogs in mice correlates with the affinity for the A1 adenosine receptor. *Neurosci Lett* **121**:267–270.
- Kim SH and Chung JM (1992) An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain* **50**:355–363.
- Lewin GR and Mendell LM (1993) Nerve growth factor and nociception. *Trends Neurosci* **16**:353–359.
- Lewin GR, Rueff A and Mendell LM (1994) Peripheral and central mechanisms of NGF-induced hyperalgesia. *Eur J Neurosci* **6**:1903–1912.
- Lewin GR, Ritter AM and Mendell LM (1993) Nerve growth factor-induced hyperalgesia in the neonatal and adult rat. *J Neurosci* **13**:2136–2148.
- Max MB, Schafer SC, Culnane M, Dubner R and Gracely RH (1988) Association of pain relief with drug side effects in postherpetic neuralgia: A single-dose study of clonidine, codeine, ibuprofen, and placebo. *Clin Pharmacol Ther* **43**:363–371.
- May A, Gijssman HJ, Wallnofer A, Jones R, Diener HC and Ferrari MD (1996) Endothelin antagonist bosentan blocks neurogenic inflammation, but is not effective in aborting migraine attacks. *Pain* **67**:375–378.
- Mazzari S, Canella R, Petrelli L, Marcolongo G and Leon A (1996) *N*-(2-hydroxyethyl) hexadecanamide is orally active in reducing edema formation and inflammatory hyperalgesia by down-regulating mast cell activation. *Eur J Pharmacol* **300**:227–236.
- McMahon SB, Bennett DLH, Priestly JV and Shelton DL (1995) The biological effects of endogenous nerve growth factor on adult sensory neurons revealed by trkA-IgG fusion molecule. *Nat Med* **1**:774–780.
- Murphy RA, Acheson A, Hodges R, Haskins J, Richard C, Reklow E, Chlumecky V, Barker P, Alderson RF and Lindsay RM (1993) Immunological relationships of NGF, BDNF, and NT-3: Recognition and functional inhibition by antibodies to NGF. *J Neurosci* **13**:2853–2862.
- Nichols ML, Bian D, Ossipov MH and Porreca F (1995) Modulation of opioid efficacy in a model of neuropathic pain in rats. *Soc Neurosci Abstr* **21**:1172.
- Pertovaara A, Kauppila T and Tuveva T (1990) Effect of medetomidine, an α_2 -adrenoceptor agonist, in various pain tests. *Eur J Pharmacol* **179**:323–328.
- Petty BG, Cornblath DR, Adornato BT, Chaudhry V, Flexner C., Wachsman M, Sinicropi D, Burton LE and Perouka SJ (1994) The effect of systematically administered recombinant human nerve growth factor in healthy subjects. *Ann Neurol* **36**:244–246.
- Porreca F, Mosberg HI, Hurst R, Hruby VJ and Burks TF (1983) A comparison of the analgesic and gastrointestinal transit effects of [D-Pen², L-Cys⁵]enkephalin after intracerebroventricular and intrathecal administration to mice. *Life Sci* **33**:457–460.
- Ramer MS and Bisby MA (1997) Rapid sprouting of sympathetic axons in dorsal root ganglia of rats with a chronic constriction injury. *Pain* **70**:237–244.
- Ramer MS, French GD, Fahnestock M and Bisby MA (1995) Strain differences in neuropathic pain behavior in mice following chronic constriction injury: Correlation with NGF production in injured nerve. *Soc Neurosci Abstr* **21**:897.
- Ritter AM, Lewin GR, Kremer NE and Mendell LM (1991) Requirement for nerve growth factor in the development of myelinated nociceptors *in vivo*. *Nature (Lond)* **350**:500–502.
- Ross GM, Shamovsky IL, Lawrence G, Solc M, Dostaler SM, Weaver DF and Riopelle RJ (1998) Reciprocal modulation of TrkA and p75NTR affinity states is mediated by direct receptor interactions. *Eur J Neurosci* **10**:890–898.
- Spiegel K, Agraftiotis D, Caprathe B, Davis RE, Dickerson MR, Fergus JH, Hepburn TW, Marks JS, Van Dorf M, Wieland DM and Jaen JC (1995) PD 90780, a non peptide inhibitor of nerve growth factor's binding to the p75 receptor. *Biochem Biophys Res Commun* **217**:488–494.
- Suh HW, Song DK, Choi YS and Kim YH (1996) Effects of intrathecally injected histamine receptor antagonists on the antinociception induced by morphine, beta-endorphin, and U50,488H administered intrathecally in the mouse. *Neuropeptides* **30**:485–490.
- Sutter A, Riopelle RJ, Harris-Warrick RM and Shooter EM (1979) Nerve growth factor receptors. *J Biol Chem* **254**:5972–5982.
- Wiesenfeld-Hallin Z and Xu X-J (1996) The role of cholecystokinin in nociception, neuropathic pain and opiate tolerance. *Regul Pept* **65**:23–28.
- Woolf CJ and Doubell TP (1994) The pathophysiology of chronic pain-increased sensitivity to low threshold A β -fiber inputs. *Curr Opin Neurobiol* **4**:525–534.
- Woolf CJ, Safiehgarabedian B, Ma QP, Crilly P and Winter J (1994) Nerve growth factor contributes to the generation of inflammatory sensory hypersensitivity. *Neuroscience* **62**:327–331.
- Xu X-J and Wiesenfeld-Hallin Z (1991) The threshold for the depressive effect of intrathecal morphine on the spinal nociceptive flexor reflex is increased during antotomy after sciatic nerve section in rats. *Pain* **46**:223–229.
- Yaksh TL and Rudy TA (1976) Chronic catheterization of the spinal subarachnoid space. *Physiol Behav* **17**:1031–1036.

Send reprint requests to: Joshua B. Owolabi, Ph.D., 81 Hinchleywood Grove, Brampton, Ontario, Canada L6V 4B1. E-mail owolabi@sprint.ca
