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NAP: Research and Development of a Peptide Derived from Activity-Dependent Neuroprotective Protein (ADNP)

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ABSTRACT

Activity-dependent neuroprotective protein (ADNP) is essential for brain formation. Peptide activity scanning identified NAP (NAPVSIPQ) as a small active fragment of ADNP that provides neuroprotection at very low concentrations. In cell culture, NAP has demonstrated protection against toxicity associated with the beta-amyloid peptide, N-methyl-D-aspartate, electrical blockade, the envelope protein of the AIDS virus, dopamine, H_2O_2, nutrient starvation and zinc overload. NAP has also provided neuroprotection in animal models of apolipoprotein E deficiency, cholinergic toxicity, closed head injury, stroke, middle aged anxiety and cognitive dysfunction. NAP binds to tubulin and facilitates microtubule assembly leading to enhanced cellular survival that is associated with fundamental cytoskeletal elements. A liquid-chromatography, mass spectrometry assay demonstrated that NAP reaches the brain after either intravenous or intranasal administration. In a battery of toxicological tests including repeated dose toxicity in rats and dogs, cardiopulmonary tests in dogs, and functional behavioral assays in rats, no adverse side effects were observed with NAP concentrations that were ~500-fold higher than the biologi-

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cally active dose. A Phase Ia clinical trial in the US assessed the tolerability and pharmacokinetics of intranasal administration of NAP in sequential ascending doses. The results supported the safety and tolerability of a single dose of NAP administered at up to 15 mg intranasally. Furthermore, dosing was recently completed for a second Phase I clinical trial in healthy adults and elderly volunteers with an intravenous formulation of NAP. NAP is poised for further clinical development targeting several indications, including Alzheimer's disease.

PHARMACOLOGY OF NAP

Background

The discovery of NAP arose from studies of vasoactive intestinal peptide (VIP) (24,31, 32). VIP, a 28 amino-acid peptide, was found to be a major brain peptide having neuroprotective activities (9,24,31). "Lipophilic fatty" derivatives of VIP cross the blood brain barrier, provide neuronal protection, and accelerate learning and memory in animal models of Alzheimer's disease (AD) (22,23,34).

The neuroprotective activity of VIP is mediated through glial cells, including a subpopulation of astrocytes. It has been demonstrated that the neuroprotective effect of VIP is modulated, at least partially, by the hop2 PAC-1 receptor expressed on the astrocyte membrane and that VIP protects neurons by inducing the secretion of neuroprotective proteins from astrocytes (2). Activity-dependent neurotrophic factor (ADNF) is an isolated protein that protects neurons from death associated with blockade of electrical activity and is secreted by glial cells in response to VIP (10,25,26). A nine-amino-acid-long ADNF peptide was identified (ADNF-9 also known as SAL or AL-209). ADNF-9 activity surpassed that of the parent protein exhibiting increased potency and a broader range of effective concentrations (11,26). Furthermore, ADNF-9 exhibited protective activity in AD-related systems, e.g., β-amyloid (Aβ) toxicity (11), presenilin 1 mutation (38), apolipoprotein E (ApoE) deficiency (5). Recent comparative experiments suggested that ADNF-9 exhibits the highest potency in protection against Aβ-induced neurotoxicity when compared to several other known growth factors (39).

Antibodies prepared against ADNF induce neuronal apoptosis and prevent synapse formation (7), suggesting that ADNF-like molecules provide neurotrophic and neuroprotective activities under natural conditions (27). Similar antibodies were prepared against ADNF-9 and were used to screen a mouse neuroglial cDNA expression library, discovering the cross-reacting activity-dependent neuroprotective protein (ADNP). ADNP is another glial mediator of VIP-associated neuroprotection (5). NAP, an eight amino acid peptide derived from ADNP (sharing structural and functional similarities with ADNF-9). was identified as a potent neuroprotectant in an animal model of apolipoprotein deficiency (ApoE knockout mice) (5). Because the lipid carrier, ApoE, has been implicated as a risk factor in AD (5,22), and because the knockout mice exhibit short-term memory deficits that are ameliorated by chronic NAP treatment, this peptide holds promise for future treatment against short-term memory deficits associated with AD. In addition, NAP was efficacious in an animal model of cholinotoxicity, enhancing performance in the Morris water maze test following intranasal administration (33), an attractive drug application route. Further experiments identified NAP as a neurotrophic, stimulating neurite elongation (40,51,56). Collectively, these observations provide the basis for development of

two formulations of NAP, AL-108 for intranasal administration and AL-208 for intravenous administration.

Rationale for Development of an Agent as a Treatment for Alzheimer's Disease

The impairments of learning, memory and other aspects of cognition that are characteristic of Alzheimer's disease (AD), may result from deficits in cholinergic neurotransmission. It has been known for decades that neurons require neurotrophic factors for development and survival. In the absence of such factors, programmed cell death (apoptosis) occurs. Cholinergic neurons of the basal forebrain express nerve growth factor (NGF) receptors, and NGF treatment promotes survival of these neurons in experimental animal models. It is thus reasonable to consider that insufficient neurotrophic factors might contribute to premature neuronal degeneration of cholinergic neurons in AD.

There are, however, a number of obstacles to the therapeutic use of neurotrophic proteins, particularly issues of delivery to appropriate brain regions and adverse effects such as systemic toxicity or deleterious proliferation of glial cells. NGF administered systemically does not cross the blood-brain barrier. Intraventricular administration is possible, but requires surgical implantation of a delivery system, carrying the risk of serious infection and other significant adverse events. Gene therapy or transplantation with growth factor producing cells is also possible but requires genetic manipulations in addition to invasive surgical intervention (55).

An alternative approach to the development of neurotrophic therapies for AD involves the use of small peptides with neurotrophic/neuroprotective activity that can be readily delivered to brain tissue. In the case of NGF, the development of NGF peptide mimetics are in progress. However, these derivatives do not provide the activity of the entire protein (12,42).

This review summarizes research activities aimed at the development of a peptide-based neurotropic/neuroprotective therapy. Activity-dependent neuroprotective protein (ADNP) is a highly conserved gene product (5,62) that is crucial for brain formation (48) and maintenance (19,37,59). ADNP is regulated by VIP (5,65) and secreted from glial cells to provide neuroprotection (18). Peptide-activity scanning identified an eight amino acid peptide, NAP [Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (single-letter code: NAPVSIPQ)] as an important neuroprotective sequence in ADNP (5). NAP can be delivered to the brain via intranasal (33) or intravenous administration (41). NAP provides nerve cell protection at very low concentrations. This extraordinary potency offers new hope for the formulation of neuroprotective drugs for the treatment of AD (5,33). The interest in NAP is enhanced by its efficacy in neuronal cell cultures and in animal models, protecting against toxicity associated with Aβ (4), N-methyl-D-aspartate (NMDA) excitotoxicity (5), electrical blockade (tetrodotoxin) (5), GP-120 (the envelope protein of the AIDS virus) (5), H_2O_2 (53), glutathione deprivation (45), alcohol (57), dopamine (45), oxidative stress (5, 45,53,58,63), tumor necrosis factor-alpha (TNF_α) toxicity (6), nutrient deprivation (40, 63), and zinc toxicity (15). *In vivo* neuroprotection has been observed for ApoE deficiency (5), traumatic brain injury (6,60,61), stroke (41) fetal alcohol syndrome, severe oxidative stress (52,57), and cholinergic toxicity (33). In addition, human cortical neurons exposed to increased oxidative stress (H_2O_2) and human cortical neurons isolated from Down Syndrome brain tissue (that is overexpressing the amyloid precursor protein) are also protected by NAP (14,46).

Cellular localization studies using antibodies directed against the NAP epitope in ADNP identified decoration of microtubules (19). Further studies using NAP affinity chromatography identified tubulin, the major subunit of microtubules as the NAP binding protein (15). NAP can enter the cell and interact directly with the microtubular network as evidenced by cellular localization studies with fluoresceine labeled compound. Further experiments showed that NAP promoted tubulin assembly into microtubules in a cell free system and in cell cultures and increased the ratio of non-phosphorylated tau to phosphorylated tau (28).

It is well recognized that tau performs a critical function of stabilizing and maintaining the microtubular network that is essential for axonal transport in neurons. The formation of neurofibrillary tangles, which results from the hyperphosphorylation of tau, leads to microtubule breakdown and impaired axonal transport, which culminates in axonal and synaptic destruction. Electron microscopic studies of AD brain tissue have shown a decrease in microtubules as compared to normal brains. We have demonstrated that exposure to toxic zinc concentrations resulted in microtubule breakdown in astrocytes and neurons and that NAP protects these cells by promoting the reorganization of the microtubular network (15,28). Other studies have shown that NAP treatment facilitates neurite outgrowth (40,51,56), suggesting a neurotrophic/neuroprotective function that is associated with microtubule dynamics.

Lee, Trojanowski and colleagues (64) showed that taxol-based microtubule-binding drugs offset tau sequestration by stabilizing microtubules and reversing fast axonal transport deficits in a tauopathy model. They suggest a therapeutic potential for treating neurodegenerative tauopathies by microtubule-stabilizing compounds. Other studies have implied that deposition of Aβ enhances tau phosphorylation in neuronal cultures (13) and *in vivo* when Aβ is deposited in the brain. Furthermore, there is evidence indicating significant microtubule disruption in the presence of Aβ (54). Michaelis et al. demonstrated that microtubule stabilizing agents have protective effects on microtubule structure in the presence of Aβ (43).

In depth sequence characterization of NAP identified beta sheet breaker characteristics and *in vitro* experiments have demonstrated that the presence of NAP prevents Aβ aggregation (4). Furthermore, the presence of NAP promotes the dissolution of Aβ plaques in cell culture and, as indicated above, protects neuronal cells against this toxicity (4).

In conclusion, NAP demonstrates neuroprotection in a broad array of *in vivo* and *in vitro* studies. These include models of the primary AD pathologies — Aβ plaques and hyperphosphorylated tau that are both associated with microtubule breakdown and neuronal death. NAP provides neuroprotection at unprecedented low concentrations. It is bioavailable and can be easily delivered via both the intranasal and the intravenous routes. Preclinical toxicology studies indicated that NAP has a reasonable safety profile to be considered as a pharmaceutical candidate. Based on these results, a Phase I clinical trial was conducted in healthy volunteers and showed that a single dose of AL-108 (intranasal formulation of NAP) was well tolerated over five ascending doses.

The Effects of NAP on *In Vitro* Systems (Table 1)

Summary of in vitro *effects on oxidative and β-amyloid stress*

The results of the *in vitro* cell culture systems suggest that NAP protects against oxidative stress [H_2O_2 toxicity in PC12 cells (53) and human neurons (14)]. The mechanism may involve glutathione (GSH), a major cellular protector against free radicals (45,52).

NAP provides *in vitro* protection against Aβ peptide (amino acids 25-35) toxicity in rat cerebral cortical neurons in culture (5). NAP also protects Down syndrome neurons that overexpress the amyloid precursor protein (APP) (14,46). In an *in vitro*, cell-free system, femtomolar concentrations of NAP protected against aggregation of the Aβ peptide (amino acids 25-35) as assessed by electron microscopy and fluorescent dye assays (4). Finally, NAP stimulates neurite outgrowth and synaptic plasticity in rat hippocampal and cortical cell cultures (40,56). Selected *in vitro* studies related to oxidative and Aβ stress are highlighted in the following paragraphs.

Rat pheochromocytoma, PC12 cells

Challenging PC12 cells with 0.1 mM H_2O_2 results in death of about 70% of the cells. Cells are protected in the presence of $10^{-17} – 10^{-14}$ M NAP (53). PC12 cells were also protected from TNF_α toxicity in the presence of NAP, suggesting protection against inflammatory responses (6).

Rat cerebral cortical neurons

In mixed neuron-astrocyte cultures, NAP provides neuroprotection at sub-femtomolar concentrations against toxicity associated with the Aβ peptide (believed to be neurotoxic

TABLE 1. Summary of in vitro *pharmacology of NAP*

Model	Insults	Protective Concentrations	Reference
Cell lines			
Rat pheochromocytoma (PC12 cells)	Oxidative stress (H_2O_2)	$10^{-17} – 10^{-14}$ M	53
	Dopamine toxicity	$10^{-18} – 10^{-10}$ M	45
	Tumor necrosis factor-alpha (TNF_α) toxicity	10^{-14} M	6
Human neuroblastoma	Reduced glutathione (GSH); Buthionine sulfoximine (BSO)	$10^{-14} – 10^{-13}$ M; $10^{-10} – 10^{-7}$ M	45
Alcohol sensitive, human L1-transfected NIH/3T3 cells	Ethanol toxicity	$>10^{-16}$ M	57, 58
Primary cultures (rat)			
Cerebral cortical neurons in mixed neuronal-glial cultures	Alzheimer's disease related toxicity (beta amyloid peptide fragments)	$10^{-16} – 10^{-15}$ M	5
	AIDS-related toxicity (gp120, envelope protein of the virus)	$10^{-15} – 10^{-10}$ M	5
	Excitotoxicity (N-methyl-D-aspartate, NMDA)	$10^{-16} – 10^{-8}$ M	5
	Electrical blockade (tetrodotoxin)	$10^{-18} – 10^{-14}$ M; $10^{-11} – 10^{-9}$ M	5, 57
Neuron enriched cortical cultures	Glucose deprivation	10^{-12} M	57
Cortical astrocytes	Zinc toxicity	$10^{-15} – 10^{-10}$ M	15
Retinal ganglion cells	Serum free conditions	$10^{-14} – 10^{-10}$ M	40
Rat hippocampal cultures	Naturally slow neurite outgrowth	$10^{-16} – 10^{-12}$ M	51
Primary cultures (human)			
Embryonic cortical neurons	Down syndrome	$10^{-15} – 10^{-13}$ M	14
Embryonic cortical neurons	Oxidative stress (H_2O_2)	$10^{-15} – 10^{-13}$ M	14

in the brains of patients with AD), tetrodotoxin (electrical blockade). Protection by NAP against N-methyl-D-aspartate (excitotoxicity) occurred in a remarkably broad range of effective concentrations ($10^{-16} – 10^{-8}$ M) (5). In this model system, astrocytes are isolated from newborn rat cerebral cortex and maintained in culture. These astrocytes are further used to provide feeder layers to neurons from newborn rat cerebral cortex. Neuroprotection was also observed with NAP in isolated cortical neurons against glucose deprivation and Aβ peptide toxicity, albeit at higher NAP concentrations than those required for mixed cultures, $10^{-13} – 10^{-11}$ M vs. $10^{-16} – 10^{-15}$ M (5,63). Recent studies demonstrated that femtomolar concentrations of NAP provided protection to astrocytes against zinc toxicity (15). These recent results suggest a direct mechanism and an indirect (through astrocyte protection) route for NAP-mediated neuroprotection.

Human cortical cultures

NAP ($10^{-15} – 10^{-13}$ M) protects against oxidative stress (0.05 mM H_2O_2) in human embryonic cortical neurons grown in culture and prevents degeneration of Down syndrome neurons that normally exhibit increased generation of free radicals, increased lipid peroxidation and neuronal apoptosis (14,46). Analysis of cell viability revealed that 10^{-13} M and 10^{-15} M NAP almost doubled the number of Down syndrome surviving neurons during the second week in culture. Furthermore, RNA analysis suggested that ADNP expression may be compromised in Down syndrome brains (14).

Neurite outgrowth in rat hippocampal culture

ADNP is expressed in fetal and adult nervous tissue and may play a neurotrophic role in nervous system development and maintenance. The effect of NAP on neurite outgrowth was assessed in rat hippocampal cultures (51). Using MAP2-FITC labeling, NAP was found to promote neurite outgrowth in a concentration-dependent manner with maximal activity observed at femtomolar concentrations. NAP stimulated robust neurite outgrowth in hippocampal cells (~150% of control; $p < 0.01$). However, the outgrowth-promoting effect was abolished in the absence of serum, suggesting that soluble factors might be necessary for the neurotrophic activity. Finally, NAP increased synaptophysin expression in both rat hippocampal and cortical cultures. These results suggest that NAP might contribute to neuronal plasticity and synapse formation associated with development and repair after injury (51).

In summary, most *in vitro* studies demonstrate that NAP is effective in providing neuroprotection at concentrations that generally range between at 10^{-12} to 10^{-16} M.

Effects of NAP in Animal Models

Summary of in vivo *effects of NAP* (Table 2)

A broad spectrum of animal studies suggests that NAP protects against neurodegeneration and affects both reference memory and short-term memory as well as alertness. Additionally, NAP can improve the memory performance of mice not suffering from any cognitive impairment.

Cholinotoxicity

An intact cholinergic system is required for normal brain function, whereas AD is associated with death of cholinergic neurons. Rats treated with the cholinotoxin, ethylcholine aziridium (AF64A), a blocker of choline uptake, provide a model for testing the *in vivo* efficacy of drugs that protect against cognitive impairment resulting from cholinotoxicity

(16,23,33). In this animal paradigm, rats were subjected to two daily tests in the Morris water maze, including a hidden platform. The time required to reach the platform was recorded over a period of 4–5 days. After intracerebroventricular (i.c.v.) single injection of the cholinotoxin, animals were allowed to recover for a week followed by daily exposure to intranasal administration of NAP (0.5–1 mg/animal/day). After a week of peptide treatment, the animals were subjected to two daily tests in the water maze as above. During the test period, animals were given the peptide intranasally at one hour before the daily tests. The results demonstrate significant improvement in short-term spatial memory in NAP-treated animals (33). In another experiment, NAP-treated animals showed significant improvement in the water maze two days after NAP application, demonstrating its long-term protective effects beyond the immediate treatment period (33).

Apolipoprotein E (ApoE) knockout mice

ApoE knockout mice may serve as a model of the cognitive impairment observed in AD (22). Daily NAP injections to newborn ApoE-deficient mice for the first two weeks of life accelerate the acquisition of developmental reflexes, prevent short-term memory deficits (measured a week after cessation of treatment) and increase cholinergic activity. Comparative studies suggest that NAP is more efficacious than other neuroprotective peptides in the ApoE-deficiency model (5).

TABLE 2. Summary of in vivo *pharmacology of NAP*

Model	Species	Administration	Protective doses	Outcome	Reference
Cholinotoxicity	Rats	Chronic intra-nasal	2 μg/kg	Cognitive enhancement	33
Apolipoprotein E deficiency (gene knockout)	Mice	Chronic (developmental) subcutaneous	0.25–0.5 μg/g	Cognitive enhancement	5
Head trauma	Mice	Acute subcutaneous	0.25 to 0.3 μg/g	Reduced mortality and enhanced clinical recovery	6, 50
	Mice	Chronic (developmental) subcutaneous	0.25–0.5 μg/g	Enhanced clinical recovery and cognition	61
Stroke (middle cerebral artery occlusion)	Hypertensive rats	Acute intravenous	3, 30 μg/kg	Protection against apoptosis and enhanced clinical recovery	41
Middle age	Rats	Chronic intra-nasal	2 μg/kg	Cognitive enhancement	21
Middle age	Mice	Chronic intra-nasal	~12.5 μg/kg	Anxiolytic	1
Multiple sclerosis (autoimmune encephalomyelitis)	Mice	Chronic intra-nasal	20 μg/kg	Protection against paralysis	36
	Mice	Acute intravenous	0.3 μg/g	Protection against paralysis and axonal damage	
Fetal alcohol syndrome	Mice	Acute intraperitoneal	1–2 μg/g	Protection against fetal demise	52

Head trauma

Head trauma may be a risk factor for AD, as Aβ accumulates in the injured brain. NAP injection after injury reduces mortality and facilitates behavioral recovery in a mouse model of closed head injury (6). This protection may be reflected, in part, by the dramatic ~70% reduction in cerebral edema in NAP-treated mice. Magnetic resonance imaging (at two weeks after head trauma) demonstrates significant brain-tissue recovery in NAP treated animals (about two-fold better improvement as compared to sham treated controls). The NAP-treated animals also exhibit faster recovery of motor ability, balancing and alertness. Furthermore, NAP-treated mice also demonstrated significantly lower production of TNF_{α}, which is suspected to be one of the mediators of delayed brain damage (6).

A separate set of experiments showed that closed head injury (CHI) resulted in an upregulation of Mac-1, a molecule found on the cell surface of neutrophils, monocytes, macrophages and microglia. NAP administration to CHI-treated mice led to a reduction in Mac-1 mRNA to the basal level equivalent to that seen in sham-treated mice. Thus, Mac-1 could be used as a marker for the long-term outcome of head injury and as a potential target for NAP protective actions (50). Furthermore, newborn mice pretreated with NAP four months prior to CHI showed reduced level of Mac-1 αM chain mRNA expression. These results may be related to the neuroprotective abilities of NAP and to the inhibition of pro-inflammatory processes, mediating brain damage, secondary to the mechanical injury (61).

Recent studies showed that Mac-1-deficient mice are protected in part against adverse effects of traumatic head injury and that NAP can produce additional protection. The time course of increases in Mac-1 expression in the injured brain parallels an increase in ADNP (the NAP containing protein) expression which is not seen in Mac-1-deficient mice, suggesting an interaction between the regulation of the pro-inflammatory Mac-1 and the neuroprotective ADNP/NAP (59,60).

Finally, spatial memory and learning performance assessed by Morris water maze test showed that NAP-pretreatment offers significant protection against damaged working and spatial memory processes resulting from CHI (61).

Stroke animal model: in vivo *protection against apoptosis*

Stroke, like head trauma, may present a risk for AD. In a permanent mid-cerebral artery occlusion (PMCAO) hypertensive rat model, cerebral blood flow in the peri-infarct area dropped to 22.1 ± 1.2 and 23.3 ± 2.5% of baseline values in NAP- and vehicle-treated rats, respectively, after PMCAO and did not change after drug administration (24.2 ± 3.1 and 23.4 ± 2.2% of baseline values in the NAP and vehicle groups, respectively). Motor disability scores were significantly lower in NAP-treated than in vehicle-treated rats throughout 30 experimental days post PMCAO. Apoptotic death was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) and caspase-3 staining. Twenty-four hours after surgery, the percentages of TUNEL positive cells in the core and penumbra of NAP-treated animals represented at least half of vehicle-treated values. Further results showed that a single post-injury (up to 4 h) injection of NAP had durable protective effects (infarct size, sensory and motor reflexes) in a model of focal irreversible cerebral ischemia. NAP appeared to produce cerebroprotection by reducing apoptosis (41).

Learning and memory animal model

As aging represents a risk factor for AD, a study was designed to assess the effect of intranasal NAP administration in normal middle-aged rats. Control rats and rats treated with NAP were subjected to a 4-day Morris water maze test evaluating cognitive functions. All groups learned and significantly improved between the first and the fourth day in the first daily trial indicative of reference memory in the water maze test. However, in the second daily trial, which is indicative of short-term memory, only the NAP-treated rats showed significant improvements by the end of the testing period (21).

In summary, the animal models support the finding that NAP is effective in enhancing neuronal survival, improving mortality and providing neuroprotection against various forms of neurodegenerative diseases; its protective doses ranged from 0.2 to 20 mg/kg.

Mechanism of Action

NAP administration results in a broad spectrum of neuroprotective effects both *in vitro* and *in vivo*. An obvious issue is how can a compound provide such a wide range neuroprotection? Recent experiments have revealed the potential mechanism of NAP, which may provide the common neuroprotection across the different experimental systems.

As indicated above, NAP is derived from activity dependent neuroprotective protein (ADNP), a highly conserved protein in humans and rodents that is essential for brain formation and function (5,48,62). Antibodies directed against the NAP sequence motif in ADNP were shown to interact with the microtubular network which is known to deteriorate in brain cells of AD patients (19).

Affinity chromatography with NAP bound to a solid-support, identified tubulin, the major subunit of microtubules, as a NAP-binding protein (15). Additional studies indicated that NAP specifically binds to brain tubulin but not to fibroblastic tubulin and provides protection against oxidative stress to brain cells but not to fibroblasts (29). NAP promoted *in vitro* tubulin assembly into microtubules as measured by increases in turbidity and in cell cultures as measured by confocal microscopy. Structural changes in the microtubular network in astrocytes and in neurons that were induced by zinc intoxication were inhibited by NAP leading to increased cellular survival.

Further studies showed that NAP displaced paclitaxel (Taxol®) binding to tubulin, suggesting an association with the paclitaxol binding site (29). Paclitaxolis known to enhance microtubule polymerization and paclitaxol analogs have been implicated in neuroprotection *in vitro* (43) and *in vivo* (64). However, NAP in contrast to taxol does not interact with proliferating cells (30). Furthermore, our recent studies suggest that NAP does not affect the ability of paclitaxel to inhibit cell proliferation.

In association with tubulin polymerization into microtubules, NAP also influences tau dynamics by increasing the ratio of non-phosphorylated tau to phosphorylated tau (28), inferring a dynamic process of cellular maintenance of the microtubular network, which is essential for the survival of the cell.

The increased cellular survival in the presence of NAP is manifested by biochemical markers including, *in vitro* inhibition of injury associated increases in p53 (35), inhibition of caspase 3 activation and DNA *in vivo* fragmentation (41), increases in polyADP ribosylation of key proteins in a mechanism similar to classical neurotrophins (56) and increases in cGMP (3).

In summary, the neuroprotective mechanism of NAP may involve the dynamic maintenance of the microtubular network, ensuring transport and cellular integrity of the brain cells.

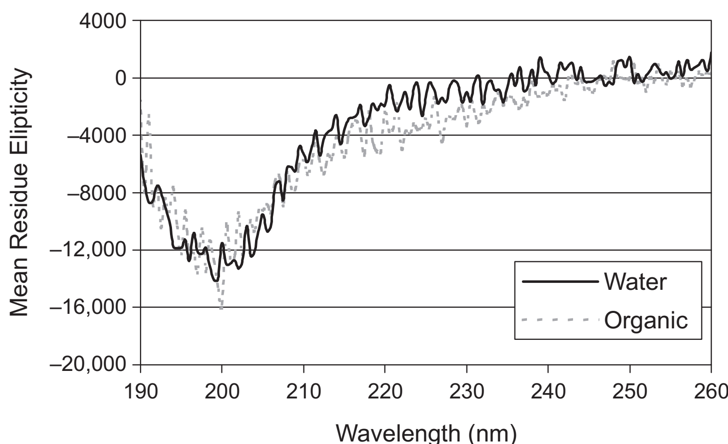

Fig. 1. Circular dichroism spectra of NAP. The CD spectra of NAP was acquired in either water (solid line) or 40% trifluroethanol (organic, dotted line).

CHEMISTRY

NAP (molecular weight 824.9) is an eight amino acid peptide with the following structure:

Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (single letter code: NAPVSIPQ)

The secondary structure of NAP was determined in either aqueous or organic solutions by a series of circular dichroism (CD) studies. CD spectra were recorded on an AVIV-202 circular dichroism spectrometer (Lakewood, NJ, USA). Duplicate scans over a wavelength range of 190–260 nm were taken at ambient temperature. Peptides were dissolved in double distilled water (DDW) and trifluroethanol/DDW (40/60), at a final concentration of 0.05 mM. A baseline was recorded and subtracted for each spectrum. Elipticity was calculated as the mean residue elipticity (Ø) in degrees-cm^2/dmol.

$$(Ø) = (Ø)_{OBS}(MRW/10lC),$$

where $(Ø)_{OBS}$ is the elipticity measured in millidegrees, MRW is the mean residue molecular weight of the peptide (molecular weight divided by the number of residues), C is the concentration of the sample in mg/mL, and l is the optical path length of the cell in cm.

NAP was found to be soluble and appears as a random coil in both aqueous and organic solutions (Fig. 1).

NAP was found to be stable for at least 24 months when stored at –20°C and for at least 3 months at 25°C/60% relative humidity and 40°C/75% relative humidity. No appreciable change in appearance, purity or related substances was detected.

The primary sequence exhibits the following structure-functional characteristics:

- It allows penetration through lipid membranes as in the case of the cellular membrane and the blood-brain barrier.
- It binds to tubulin and promotes proper microtubule assembly — as a peptide chaperone.
- It has intrinsic β sheet breaker characteristics, thus acting as a peptide chaperone to protect against toxic Aβ plaque associated with AD.

RELEVANCE TO ALZHEIMER'S DISEASE

The rationale for developing an agent for the treatment of Alzheimer's disease was outlined above. Results described below highlight recent findings.

Experiments including co-incubation of NAP and Aβ peptide fragments showed that NAP inhibited $A\beta_{(25–35)}$ and $A\beta_{(1–40)}$ fibril formation (4). Further analysis showed that NAP bound directly to $A\beta_{(25–35)}$ with high affinity. Taken together, in cell cultures, NAP protects against $A\beta_{(25–35)}$ toxicity in mixed neuro-glial preparation as well as in enriched neuronal populations. This protection could result from direct interaction with the toxic Aβ deposit as well as by direct cellular protection(4).

A comprehensive study using the AD triple transgenic mouse model expressing the APP_{swe}, tau_{P301L}, and $PS1_{M146V}$ transgenes (44) is currently underway to provide additional confirmatory *in vivo* data supporting the neuroprotective activity of NAP in AD-related neurodegeneration. The model takes advantage of the presence of both AD neuropathologies that include amyloid plaque formation and hyperphosphorylated tau/tangle formation. Intranasally administered NAP (0.5 μg NAP/day) significantly reduced levels of phosphorylated tau and Aβ in transgenic mice with early Aβ pathology. We are further investigating therapeutic potency of NAP using mice with advanced Aβ and tau pathology.

TOXICOLOGY

Immunogenic Potential

The immunogenic potential of NAP (NAPVSIPQ) was evaluated in rabbits. NAP was injected in phosphate-buffered saline at a concentration of 2 mg per 2.7 mL. Rabbits were boosted twice at a monthly interval. Serum was collected 10 days after the second boost and evaluated for the presence of antibodies to NAP by dot blot.

There were no detectable antibodies to NAP in either pre-immune serum or serum from immunized rabbits. As a control, a longer peptide (C**NAPVSIPQ**QSQSVKQLLPS) containing the NAPVSIPQ sequence was immobilized to nitrocellulose. As with NAP, no antibodies were detected. When the longer peptide was conjugated to Keyhole Limpet Hemocyanin and injected with complete Freund's adjuvant, an immune response was elicited and antibodies were generated that recognized both peptides, C**NAPVSIPQ**QSQSVKQLLPS and NAPVSIPQ.

It, therefore, appears that antibodies are not formed in rabbit when the unconjugated NAP is injected monthly for a total of 3 months.

Nonclinical Safety and Toxicity Profile

Numerous nonclinical safety and toxicity studies of NAP have been conducted. NAP was found to have minimal effect on vital organ function tests including cardiovascular and pulmonary assessments. By repeated intranasal or intravenous administration to rats or dogs NAP was well tolerated and there were no toxicological findings. Its initial toxicological studies were previously reviewed (35) and a summary is provided in Table 3.

INTRANASAL DELIVERY

Several peptides and peptide mimetics used in the clinic are delivered by the intranasal route. For example, DDAVP [the synthetic molecule 1-(3-mercaptopropionic acid)-D(8)-arginine vasopressin, also called desmopressin or dDAVP] is used intranasally in the treatment of diabetes insipidus and primary nocturnal enuresis. Recent publications by Frey et al., (17) Born et al. (8), and Gozes (20) describe protein and peptide availability to the brain by the intranasal route. An example of Born's work includes intranasal administration of atrial natriuretic peptide that acts as central nervous inhibitor of the hypothalamo-pituitary-adrenal stress system in humans (47). Another recent human study demonstrated that by intranasal administration insulin could improve visual working memory and visual search in patients with AD without raising plasma insulin, suggesting that by intranasal administration insulin may have therapeutic potential without the risk of peripheral hypoglycemia (49).

BIOAVAILABILITY

The pharmacodynamic compartment for NAP is the brain or the central nervous system (CNS). When NAP is administered it must be able to reach this target compartment at pharmacologically active concentrations. As indicated above, NAP is active *in vitro* at $\sim 10^{-15}$ M. Preclinical and Phase I clinical experiments (see below) demonstrated that intranasal administration of NAP to rat, dog or human results in measurable plasma levels of the drug. Specifically, after intranasal administration of [^{3}H]NAP to rats, radioactivity was detected in the blood and in the various organs of the body (33). Intact peptide was

TABLE 3. Summary of nonclinical safety and toxicity studies

Completed studies	Dose levels
Safety Pharmacology	
Rat Functional Observation Battery (intravenous)	1, 10, and 100 μg/kg
Dog Cardiopulmonary (intravenous)	1, 10, and 100 μg/kg
Single Dose Toxicity	
Dog Acute intravenous/Intranasal	IV: 0, 5, 50, 500 μg/kg; IN: 500 μg/day
Repeat Dose Toxicity	
Rat 14-day (Intranasal)	0, 1, 10, 40 μg/day
Rat 30-day (Intranasal)	0, 0.2, 2, 10, 100, and 1000 μg/day
Rat 90-day (Intranasal)	0, 100, 1000 μg/day
Dog 30-day (Intranasal)	0, 2, 14, 140, and 1400/925 μg/kg/day
Dog 90-day (Intranasal)	0, 1, 5, 12.5 μg/kg/day
Rat 14-day (intravenous)	0, 10, 100, 300 μg/kg/day
Dog 14-day (intravenous)	0, 20, 50, 150 μg/kg/day
Genotoxicity	
Ames Test	0, 50, 150, 500, 1500, and 5000 μg/plate
Mouse Lymphoma	0, 313, 625, 1250, 2500, and 5000 μg/mL
Mouse Micronucleus (Intra-peritoneal)	0, 500, 1000, and 2000 μg/kg

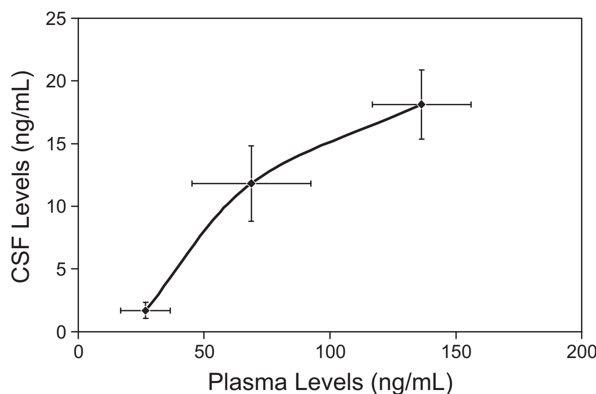

Fig. 2. Correlation between plasma and CSF levels of NAP. Note: 1 ng/mL NAP = ~1.2 nM.

identified in the rat cortex at 30 and 60 min after intranasal administration. In the permanent middle cerebral artery occlusion (PMCAO) rat model, intravenous administration of radioactive NAP resulted in measurable levels in the cerebellum and cortex at 15 min after injection and was maintained in the ischemic tissue for at least 30 min (41). Liquid chromatography mass spectrometry assays in rats and dogs corroborated and extended these results. Recent data from a pharmacokinetic study in rat suggested a correlation between plasma and CSF levels of NAP (Fig. 2). Furthermore, in a Phase Ia human safety and pharmacokinetic study (see below), a single intranasal dose of NAP gave rise to detectable plasma levels. Therefore, it is likely that following intranasal or intravenous administration, NAP reaches the target pharmacodynamic compartment.

PRELIMINARY CLINICAL STUDIES

Phase I Studies

A Phase I double-blind, placebo-controlled, randomized, single ascending intranasal dose study to evaluate the safety, tolerability and pharmacokinetics of the nasal formulation of NAP (AL-108) was conducted in 30 healthy subjects. The results of the study demonstrated that a single intranasal dose up to 15 mg of AL-108 was well tolerated. There were no clinically significant changes in ECG or vital signs measured in any subject during the observation period. Dosing was recently completed in a second Phase I study. NAP (AL-208, up to 300 mg) was administered intravenously to six dose groups totaling 64 volunteers, including 48 healthy adults and two additional dose groups totaling 16 healthy elderly adults in a single-dose, double-blind, placebo controlled, randomized, sequential and ascending study to evaluate primarily the safety and pharmacokinetics of the drug.

Future Plans

A series of Phase II studies are planned to explore the safety and tolerability of NAP in target populations, as well as the impact of NAP treatment on biomarkers of neurodegeneration. Disease indications that require neuroprotection include the chronic Alzheimer's

disease and the acute mild cognitive impairment following cardiac bypass surgery (a disease condition that was not elaborated upon in this review). The brain bioavailability of the intranasal (AL-108) and intravenous (AL-208) formulations makes NAP an attractive neuroprotective drug candidate for acute and chronic treatment procedures.

CONCLUSIONS

NAP, a highly potent neuroprotective peptide, shows promise as a disease-modifying treatment for Alzheimer's disease. Extensive testing using *in vitro* and *in vivo* models relevant to Alzheimer's disease pathophysiology support its therapeutic potential, and early human testing indicates that intranasal and intravenous administration of NAP is feasible. Safety and efficacy will be determined by randomized controlled trials now being planned.

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