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Deciphering the Enigma: NAP (CP201) the Active ADNP Drug Candidate Enters Cells by Dynamin-Associated Endocytosis

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The Discovery

NAP (NAPVSIPQ) was identified by screening a cDNA expression library from neuroglial origin with antibodies prepared against activity-dependent neurotrophic factor (ADNF-14/9), which then revealed a new protein to science, activity-dependent neuroprotective protein (ADNP) (Bassan et al. 1999), (Zamostiano et al. 2001). NAP showed higher in vitro and in vivo neuroprotective efficacy, compared with ADNF-9 (Bassan et al. 1999) constituting a future drug candidate (AL-108, davunetide, CP201).

Mechanism of Action

Discovering neuroprotective compounds requires complete understanding of the mechanism of action. Here, from protein binding point of view, NAP interacts with microtubules (MT). As such, ADNP, as well as NAP, have shown MT co-localization (Divinski et al. 2004; Furman et al. 2004; Oz et al. 2014), (Divinski et al. 2006). NAP protects against the MT-associated protein Tau (MAPT)–related impairments in mouse models including ADNP deficiency ($Adnp^{+/-}$ mice) (Vulih-Shultzman et al. 2007), Alzheimer's disease (AD) tauopathy, and 3xTg-AD with human double mutant Tau protein and additional Aβ human pathology, respectively (Matsuoka et al. 2007, 2008; Shiryaev et al. 2009). Protection against tauopathy is extended to mouse models of Parkinson's disease synucleinopathy (Magen et al. 2014) and copper zinc superoxide dismutase 1, amyotrophic lateral sclerosis (ALS) (Jouroukhin et al. 2013).

NAP protects axonal transport against MT disruption by colchicine and stimulates axonal transport in an ALS mouse model (Jouroukhin et al. 2013) and in a Drosophila model of tauopathy in which axonal transport defects are prominent (Quraishe et al. 2013). NAP has also been identified as a neurotrophic factor, stimulating neurite outgrowth (Smith-Swintosky et al. 2005; Gozes and Divinski 2007; Pascual and Guerri 2007) and synapse formation (Hacohen-Kleiman et al. 2018; Sragovich et al. 2019).

Originally, tubulin, the structural subunit of MTs (e.g., Gozes and Littauer 1978; Gozes and Sweadner 1981; Gozes and Barnstable 1982), was suggested as a NAP-binding ligand, since tubulin in brain extracts binds to NAP-affinity columns (Divinski et al. 2004). Furthermore, NAP protects MTs against degradation induced by MT disrupting agents (Divinski et al. 2004, 2006; Gozes and Divinski 2007; Zemlyak et al. 2009; Oz et al. 2012). Likewise, it has been shown that NAP affects Tau-MT interaction and prevents zinc-related dissociation of Tau from MTs in vitro (Divinski et al. 2004; Oz et al. 2012). Zinc was used as Tau-MT–dissociating agent, as previous studies have shown that Tau hyperphosphorylation and MT destruction are caused by zinc toxicity (Boom et al. 2009).

Looking at sequence interactions, both NAP (NAPVSIPQ) and the previously described ADNP-9 (SALLRSIPA) show neuroprotective activities and share a SIP motif that is a variation of the SxIP domain, providing direct interaction with MT end-binding proteins (EBs) (Honnappa et al. 2009; Oz et al. 2014; Gozes et al. 2016; Quraishe et al. 2016). EBs, referred to as part of the MT plus-end tracking protein (+TIPs) family, decorate polymerized MT plus-ends (Seetapun et al. 2012) and can directly affect MT dynamics (Mohan et al. 2013). There are three mammalian end-binding proteins: EB1, EB2, and EB3 (Gouveia and Akhmanova 2010). EB1 and EB3 proteins generate homo- and heterodimers, an essential feature required for the plus-end tracking

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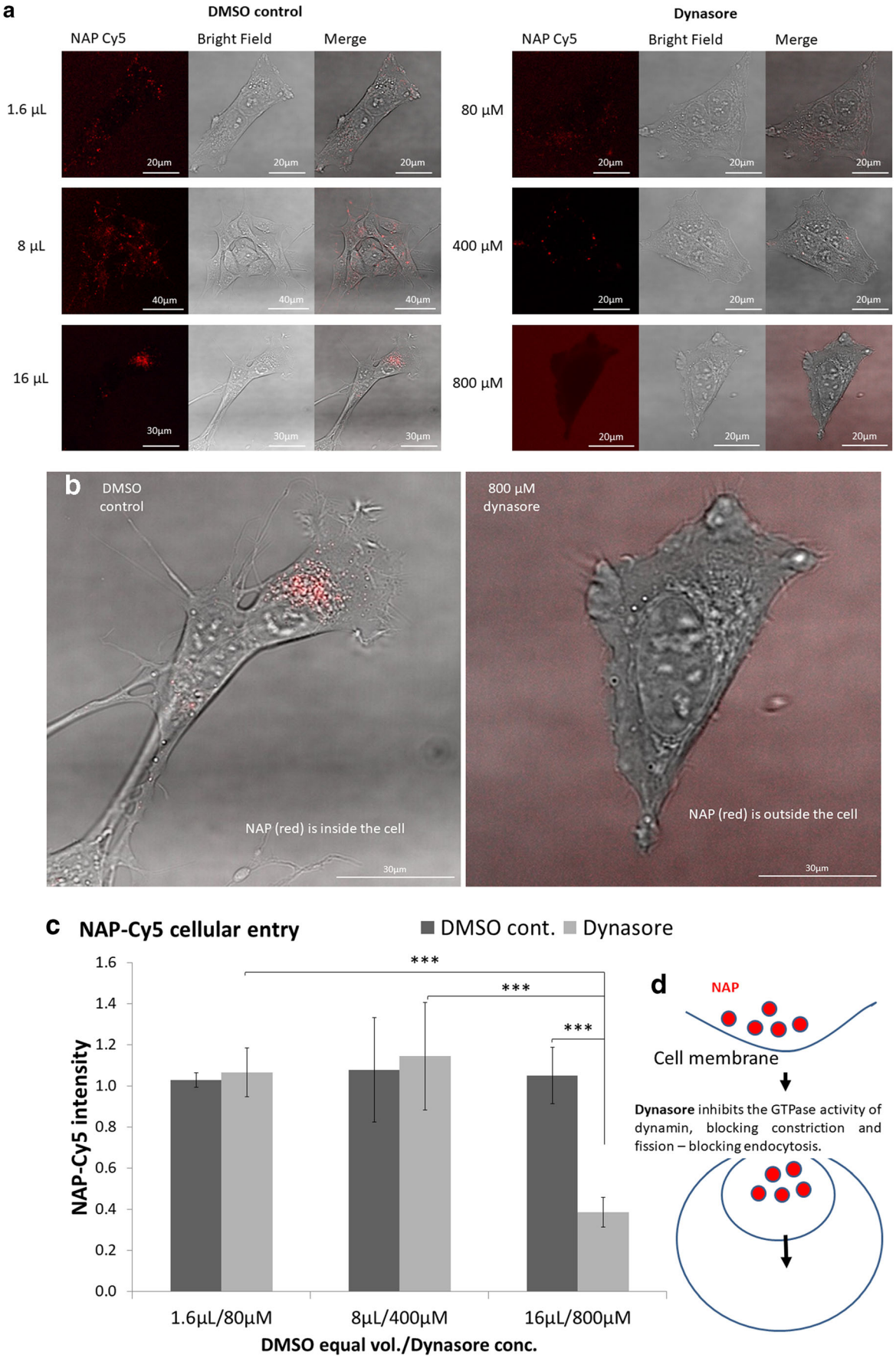
a
DMSO control
Dynasore
NAP Cy5
Bright Field
Merge
1.6 µL
8 µL
16 µL
80 µM
400 µM
800 µM
20µm
40µm
30µm
b
DMSO control
800 µM dynasore
NAP (red) is inside the cell
NAP (red) is outside the cell
30µm
c
NAP-Cy5 cellular entry
DMSO cont.
Dynasore
NAP-Cy5 intensity
0.0
0.2
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0.6
0.8
1.0
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1.6
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1.6µL/80µM
8µL/400µM
16µL/800µM
DMSO equal vol./Dynasore conc.
d
NAP
Cell membrane
Dynasore inhibits the GTPase activity of dynamin, blocking constriction and fission – blocking endocytosis.

◀ **Fig. 1** Human neuroblastoma SH-SYS5 cells, a neuronal model (ECACC, Public Health England, Porton Down, Salisbury, UK; passage numbers from 14 to 16), were maintained in Ham's F12: minimum essential media (MEM) Eagle (1:1), 2 mM glutamine, 1% non-essential amino acids, 15% fetal bovine serum (FBS) and 100 U/ml penicillin, 100 mg/ml streptomycin (Biological Industries, Beit Haemek, Israel). SH-SYS5 cells were plated on 35 mm dishes (81156, μ-Dish, Ibidi, Martinsried, Germany) at a concentration of 12,500 cells/dish, 48 h before the experiment. In the day of live imaging, cells were treated for 4 h with 80, 400, or 800 μM of dynasore (Abcam, ab120192) diluted in DMSO or with an equal volume of DMSO alone (DMSO control; 1.6, 8, and 16 μL, accordingly). Dishes were incubated at 37 °C with a 5% CO_2/95% air mixture in a thermostatic chamber placed on the stage of a Leica TCS SP5 confocal microscope (objective × 100 (PL Apo) oil immersion, NA 1.4). NAP was labeled with Cy5 as before (Ivashko-Pachima et al. 2017). Data are presented as the mean ± Stdev, $n = 5$ for each group. Statistical analysis was performed by one-way ANOVA test (Tukey post hoc test), IBM SPSS Statistics software version 23. *** $P < 0.001$. **a** In order to test the ability of NAP to enter into cells by endocytosis, we treated cells with increasing concentrations of the dynamin inhibitor—dynasore ("Dynasore") or with an equal volume of the dynasore solvent—dimethyl sulfoxide (("DMSO control"). NAP tagged with fluorescent dye Cy5 (red color in the pictures) was added to the cell culture media in a final concentration of 10^{-12} M, 15 min before live imaging. **b** Enlargement of merge images from panel **a**, represented DMSO control (16 μL) vs. Dynasore (800 μM). **c** Quantitative representation of the data—cellular intensity of the Cy5 fluorescent dye, reflecting the amount of NAP entry into the cell. The graph shows significant inhibition of NAP entry as a consequence of increasing concentrations of dynasore, while cells treated with DMSO did not show significant differences. **d** A cartoon of NAP cellular entry, mediated by dynamin-dependent endocytosis

behavior of the EBs (Sen et al. 2013). It has been further found that Tau directly associates with EB1 and EB3 and modulates the localization of these proteins on the MTs (Sayas et al. 2015). Our initial studies have shown a direct interaction of NAP and ADNP with the EB1 and the EB3 proteins (Oz et al. 2014) and recruitment of +TIPs to the MT growing end (Oz et al. 2014; Amram et al. 2016).

Through EB1/EB3 interactions, ADNP and NAP have an impact on MT dynamics and significantly enhance the elongation of MT growing ends (Ivashko-Pachima et al. 2017, 2019a). Regarding MT dynamicity, in a neuronal differentiation cellular model, NAP impacts the alpha-tubulin tyrosination cycle (a marker for MT dynamics), which has been correlated with increased MT network area (reflecting neurite outgrowth) (Oz et al. 2012). The area of dynamic MT invasion into the neuronal growth cone periphery (reflecting synapse plasticity and synapse formation) is also increased following NAP treatment (Oz et al. 2012).

Tau and EB1/3 were found to be required for NAP neuroprotective activity (Oz et al. 2014), (Ivashko-Pachima and Gozes 2018). Furthermore, NAP modulates Tau-EB interaction and recruits Tau and EBs to the MT lattice (Ivashko-Pachima et al. 2017, 2019a). Lastly, NAP exhibits a distinctive effect on Tau isoforms with 3 and 4 MT-binding sites (Tau3R and Tau4R, respectively), attributed to a protein region–specific Tau4R phosphorylation attenuating Tau-EB1 association (Ivashko-Pachima et al. 2019b). The molecular mechanism of NAP activity suggests an EB-mediated Tau involvement and a relatively reduced interaction with Tau4R compared to Tau3R. This may explain the observed inefficacy of NAP in the clinical study performed on progressive supranuclear palsy (PSP) patients (mostly Tau4R tauopathy)(Boxer et al. 2014), while the effectiveness of NAP treatment was clinically shown in patients with prodromal AD (mixed Tau3R/4R tauopathy)(Gozes et al. 2009; Morimoto et al. 2013).

Clinically, ADNP expression has been found to be dysregulated in schizophrenia (Dresner et al. 2011; Merenlender-Wagner et al. 2015), with NAP (davunetide) enhancing functional activities in schizophrenia patients (Javitt et al. 2012; Jarskog et al. 2013), Parkinson's disease (PD) (Chu et al. 2016), and Alzheimer's disease (AD)(Yang et al. 2012; Malishkevich et al. 2016).

Importantly, ADNP was found to be mutated de novo in an autism spectrum disorder (ASD) constituting the ADNP syndrome (O'Roak et al. 2012; Helsmoortel et al. 2014; Gozes et al. 2015, 2017b, c; Arnett et al. 2018; Levine et al. 2019; Van Dijck et al. 2019), with an estimated prevalence of 0.17% among ASD cases, making it a relatively frequent ASD identified gene (Helsmoortel et al. 2014). Indeed, a recent study evaluating thousands of ASD cases identified 102 ASD-associated genes, with ADNP being one of the 13 lead genes (Satterstrom et al. 2020). More than 30 different mutations have been found in the ADNP syndrome so far, which cause various manifestations in disease severity (Van Dijck et al. 2019). Even though neurodevelopmental processes have been linked to MT dynamic instability, the direct effect of de novo truncating mutations in ADNP on MTs is a new topic of investigation.

Our latest results discovered somatic ADNP syndrome-related and novel ADNP mutations in vulnerable parts of postmortem aging and AD brains. In the olfactory bulb, more than 100 disease-implicated genes have been found to be mutated, with related functionality to cytoskeletal mechanisms, ASD, and intellectual disability causing mutations (about 40% each) (Ivashko-Pachima et al. 2019a). Tauopathy represents a major hallmark of AD and related neurodegenerations (Gozes et al. 2017a; Yang and Wang 2018). Coupled with the interaction of ADNP and Tau, it became apparent that it was crucial to assess the direct effect of truncated forms of ADNP and autism/AD-related mutations on the neuronal MT cytoskeleton.

Our recent results showed that truncated ADNPs resulting from de novo ASD- and somatic AD–related mutations caused adverse effects on MT dynamics and MT-Tau association, which were ameliorated by NAP (Ivashko-Pachima et al. 2019a).

How Does NAP Enter Cells?

As the NAP targets EB1/EB3 are intracellular proteins (Oz et al. 2014), it is important to ascertain cellular bioavailability. Using fluorescently labeled NAP, we have shown rapid intracellular localization and target engagement of NAP even at low pH and low temperatures, coupled with NAP structure (NAPVSIPQ), which resembles cellular bioavailable, membrane-permeable peptides (Divinski et al. 2004). These results suggested no requirement for a cell surface receptor, for cellular internalization (Divinski et al. 2004; Oz et al. 2014; Ivashko-Pachima et al. 2017).

Current, live cell imaging suggested NAP cellular internalization by endocytosis (Ivashko-Pachima et al. 2017). Given the interactions of NAP-EB1/3-Tau and the reports on Tau pathology spreading, we focused on literature deciphering Tau cellular entry. A relatively recent article using human stem cell-derived neurons questioned the route of entry of monomeric and aggregated Tau into neurons and discovered that both forms of tau are efficiently taken up by human neurons by regulated endocytosis, with regulated endocytosis being dynamin-dependent (Evans et al. 2018).

Dynamins are fission proteins that mediate endocytic and exocytic membrane events. Dynamin II belongs to the dynamin family of large GTP-binding proteins. There are three mammalian classical dynamins: dynamin I, which is primarily expressed in brain, dynamin II which is ubiquitously expressed, and dynamin III which is expressed predominantly in neurons and testes. Dynamin proteins contain a number of conserved domains: a GTPase domain for GTP hydrolysis, a pleckstrin homology (PH) domain mediating lipid binding, a GTPase effector domain (GED), a middle domain which together with the GED domain controls self-assembly, and a proline-rich domain (PRD) (Kockx et al. 2014). Dynasore, a cell-permeable inhibitor of dynamin, inhibits the GTPase activity of dynamin as well dynamin-regulated endocytosis, which is required for numerous membrane fission events, including clathrin-mediated endocytosis (Kockx et al. 2014; Preta et al. 2015).

Here, we asked if the observed internalization of NAP into cells (Ivashko-Pachima et al. 2017) is through a dynamin-dependent process and hence inhibited by dynasore.

Figure 1 shows that dynasore dose-dependently inhibited fluorescent NAP (Cy5) cellular internalization, into human neuronal-like cells, with 800 μM providing a highly significant ~ 70% inhibition (***$p < 0.001$). Thus, we have now solved the conundrum of NAP (CP201) entry into cells, providing a fast forward development path for clinical exploitation, targeting first the ADNP syndrome.

Compliance with Ethical Standards

Conflict of Interest Professor Illana Gozes serves as the Chief Scientific Officer of Coronis Neurosciences developing CP201 for the ADNP syndrome.

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