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Towards Development of Disease-Modifying Therapy for Alzheimer's Disease Using Redox Chemical Biology Pathways

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Abstract

Redox modifications are described that can be harnessed for the treatment of neurodegenerative disorders, including Alzheimer's disease (AD). The approach has shown potential therapeutic efficacy in AD in both transgenic mouse and hiPSC cerebral organoids models. In this review, two such redox targets are highlighted. First, protein S-nitrosylation of the NMDA-type of glutamate receptor is described as a potential therapeutic target. Second, an S-alkylation reaction of critical, redox-active cysteine thiol(s) on the protein KEAP1 to activate the anti-oxidant/anti-inflammatory transcription factor NRF2 is proposed. In both approaches, we utilize compounds described as Pathologically Activated Therapeutics (or "PAT" drugs), which can only be activated by the disease process that they then combat. Thus, PAT drugs remain relatively innocuous and therefore clinically-tolerated in normal tissue in the absence of disease, thereby avoiding severe side effects both systemically and in the brain.

Introduction

Developing a truly disease-modifying therapy for Alzheimer's disease (AD) has proven to be elusive, in part because of the multiple pathways involved in neuronal and synaptic damage in the disease, and in part due to the difficulty in interacting pharmacologically with central nervous system (CNS) targets without interfering with their normal function and creating unacceptable clinical side effects. In our laboratory group, we have taken a

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DECLARATION OF INTEREST

The author discloses that is an inventor of worldwide patents for the use of memantine and NitroSynapsin for neurodegenerative and neurodevelopmental disorders. Per Harvard University guidelines, he participates in a royalty-sharing agreement with his former institution, Boston Children's Hospital/Harvard Medical School, which licensed the FDA-approved drug memantine (Namenda®) to Forest Laboratories, Inc./Actavis/Allergan/AbbVie. He is a scientific founder of Adamas Pharmaceuticals, Inc., which developed or comarkets FDA-approved forms of memantine- or amantadine-containing drugs (NamendaXR®, Namzaric®, and Gocovri®), and of EuMentis Therapeutics, Inc., which licensed NitroSynapsin and related aminoadamantane nitrates.

The author further discloses that he is a patent holders for the use of carnosic acid congeners for degenerative diseases. Scripps Research, his current home institute, has also filed for patent protection for the composition of matter and use of carnosic acid derivatives in a variety of neurodegenerative disorders and other conditions. These compounds are being licensed to Infla*MED*, LLC, of which the author is a scientific founder.

different approach to drug development for AD and related disorders, striving to develop what we have termed Pathologically Activated Therapeutics or "PAT" drugs, i.e., a pat being a gentle tap, reflecting our relatively low affinity (but highly selective) agents that *(i)* target only pathological conformations of the target, or *(ii)* are only activated by the pathological process itself, which they then combat.

Building on our experience and success in developing the FDA- and EMA-approved drug, memantine (in the form of Namenda®, NamendaXR®, and Namzaric®), which represents a PAT drug in that it only blocks *N*-methyl-D-aspartate (NMDA)-type glutamate receptor-operated channels when they are excessively open, our group has designed and synthesized a variety of new drugs using these principles. Here, I describe our recent efforts toward improved therapeutics for AD using a non-pharmacological approach in order to broaden the number of targets available. We chose these targets because of emerging evidence that redox posttranslational modifications of these proteins affect a large number of neurodegenerative disorders, including AD (Nakamura et al., 2013, 2016, 2017, 2021a, 2021b; Satoh et al., 2013; Satoh and Lipton, 2017). Interaction with these targets, mainly represented by specific cysteine residues whose sulfhydryl or thiol groups undergo redox reactions, involve covalent reactions rather than pharmacological/ligand binding, and thus chemically invoke sharing or transfer of electrons. These relatively long-lasting reactions need to be carefully targeted to pathological situations since they may not be as readily reversible as pharmacological agents, but many examples of such interactions have proven useful in drug development.

Targeted Protein S-Nitrosylation of the NMDA Receptor to Combat Excessive Electrical Activity and Protect Synapses in AD

As an initial foray toward targeting a reactive cysteine residue in this fashion, we built upon our success with memantine, which we had shown only enters excessively activated (primarily extrasynaptic) NMDA receptor-associated ion channels to block aberrant activity. Both the group of Lennart Mucke and our group, and subsequently others, had demonstrated that both transgenic AD mice and AD hiPSCs and cerebral organoids manifest abnormally increased electrical activity, similar to human AD patient brains (Palop and Mucke, 2009, 2010, 2016; Talantova et al., 2013; Ghatak et al., 2019, 2021a). Due to its Uncompetitive mechanism of open-channel block with a relatively Fast Off-rate (so-called "UFO" mechanism of action), memantine-like drugs bind preferentially to channels that are excessively open and underlie at least in part the aberrant hyperelectrical activity observed in AD. Thus, like a guided missile, the aminoadamantane structure of memantine can be used to target another inhibitory "warhead" to the NMDA receptor. Indeed, after synthesizing many series of such drugs, we accomplished this in our lead candidate, NitroSynapsin, which offers dual allosteric inhibition of the NMDA receptor by first blocking the ion channel with a memantine-like moiety, which then targets a nitro group to the protein S-nitrosylation sites on the receptor to offer additional inhibition (Fig. 1). This dual allosteric mechanism is necessary to improve the relatively modest effect of memantine itself in abating excessive electrical activity – this shortcoming of memantine is mainly due to the fact that at physiological pH the drug is positively charged and thus repelled from the ion channel as positive charges rush into the neuron during excessive excitation (Lipton, 2006; Talantova et al., 2013; Ghatak et al., 2021a). Another effect of pH in the context of redox

reactions on the NMDA receptor is that decreasing pH by itself can inhibit current flux through the NMDA receptor. Concurrently, lowering pH can often stabilize nitrosothiols, thus prolonging the effect of S-nitrosylation on the NMDA receptor in inhibiting its function.

To date, NitroSynapsin has shown excellent efficacy in electrophysiological, histochemical, and behavioral paradigms in multiple transgenic models of AD and in human cerebral organoid models of AD (Talantova et al., 2013; Ghatak et al., 2021a). Intriguingly, our group and others have shown that neurodevelopmental disorders, including various forms of autism spectrum disorder (ASD)/intellectual disability (ID) also manifest increased electrical activity and consequently excitatory/inhibitory (E/I) imbalance that is also corrected by NitroSynapsin to a far greater degree than memantine (Tu et al., 2017; Okamoto et al., 2019; Trudler et al., 2020; Ghatak et al., 2021b). Currently, NitroSynapsin is completing investigational new drug (IND)-enabling studies for an FDA-approved human clinical trial for ASD. This work was recently funded by a small biotech company in the Boston area, EuMentis Therapeutics, Inc., of which the author is the scientific founder.

Pro-Electrophilic Drugs (PEDs) for Activation of the Anti-Oxidant/Anti-Inflammatory NRF2 Transcriptional Pathway

As a second example of a redox chemical biology approach to AD therapeutics, our group in collaboration with our Japanese collaborator Takumi Satoh characterized a series of plant diterpene metabolites, represented by the compound carnosic acid, found in the herbs rosemary and sage. Our groups reported that carnosic acid in a pro-drug, in this case a pro-electrophilic drug (PED), that is converted to the active *ortho*-quinone form by oxidative/inflammatory environments – incredibly, conditions that the activated form of carnosic acid then combats, as described below. We demonstrated that the active (quinone) form of carnosic acid accomplishes this by undergoing nucleophilic attack of its electrodeficient carbon by thiol groups, with the primary thiol target located on KEAP1 (Kelch-like ECH-associated protein 1) (Fig. 2) (Satoh et al., 2011, 2013, 2015, 2017). We showed that one reason that KEAP1 thiols are so susceptible to this reaction compared to other cell thiols is that the major source of thiol groups, glutathione (GSH), has already been depleted by the oxidative insult (Satoh et al, 2008, 2013).

When characterizing the reactive thiol groups of KEAP1, dipyridyl disulfide and 4,4′-dipyridyl disulfide have been used as spectroscopic titration reagents for the thiol groups of Keap1 because their reductions by thiols are unidirectional and relatively pH-insensitive. These experiments indicated that a single cysteine residue in Keap1 is most reactive, with others less reactive, among the 25 cysteines of KEAP1 (Dinkova-Kostova et al., 2002).

Mechanistically, degradation of KEAP1, initiated by reaction with the quinone form of carnosic acid at a critical cysteine residue, contributes to activation of the transcription factor, NRF2 (erythroid derived 2-related factor 2). NRF2 then stimulates transcription of a wide variety of phase II anti-inflammatory and anti-oxidant genes (Johnson and Johnson, 2015). These gene products counteract a large number of inflammatory pathways, including the NLRP3 inflammasome (recently reviewed in Satoh et al., 2022). Many groups have now shown that activation of NRF2 is neuroprotective and improves behavioral outcomes in a

variety of neurodegenerative disease models, and, in particular, administration of carnosic acid can trigger this pathway to neuroprotection in AD transgenic mice (Lipton et al., 2016).

Another potentially neuroprotective target of the quinone form of carnosic acid is HSF-1 (heat shock factor-1), which regulates chaperone protein transcription, an important factor for handling misfolded proteins in neurodegenerative disorders and hence another important protective pathway (Satoh et al., 2011. 2015). Additionally, activity-based protein profiling by mass spectrometry with Ben Cravatt's laboratory at Scripps Research revealed that while other cysteine targets of carnosic acid exist, all appeared to be much less frequent that the KEAP1/NRF2 pathway when considered in conjunction with our prior data (Satoh et al, 2008, 2013, 2017).

Importantly, our collaboration with Takumi Satoh also demonstrated that carnosic acid, which is a catechol, had excellent blood-brain barrier permeability in a variety of animal models as well as a lack of toxicity, even at very high doses (Satoh et al., 2008; Rezaie et al., 2012). Thus, carnosic acid itself is relatively innocuous, and conversion to the active quinone form occurs only under oxidative stress and neuroinflammatory conditions, thus targeting the effect to damaged tissue and hence avoiding systemic side effects. This is a primary reason that other NRF2 activators, which stimulate the NRF2 pathway under basal or normal conditions, have failed in the clinic (reviewed in Satoh et al., 2017). In contrast, carnosic acid has a well-known safety profile in humans (reviewed in Petiwala and Johnson, 2015) and represents about 20% of rosemary extract, which is on the FDA generally regarded as safe (GRAS) list for human consumption. There is, however, room for improvement in carnosic acid as a potential therapeutic. Along these lines, our group in collaboration with Scripps chemist Phil Baran has synthesized novel congeners of carnosic acid with improved pharmacokinetic (PK) and other drug properties, and these new compou0nds are currently in testing.

Conclusions

This review highlights two examples of drug discovery for neurodevelopmental and neurodegenerative disorders, including AD, based upon chemical redox modifications discovered in our laboratory. These modifications include *(i)* targeted protein S-nitrosylation, (ii) nucelophilic attack on plant metabolites by specific cell thiol-containing proteins. In the first instance, downregulation of excessive neuronal electrical activity and thus neuroprotection can be provided by S-nitrosylation of the NMDA subtype of glutamate receptor, facilitated by targeted interaction via open-channel block of overactive receptor-operated ion channels with an aminoadamantane moiety that bears a "nitro warhead" for nitrosylation and thus provides further inhibition of receptor activity. In the second example presented, nucleophilic attack by KEAP1 thiol on an electron-deficient carbon center of the *ortho*-quinone form of carnosic acid. The conversion of the basal catechol form of carnosic acid to the *ortho*-quinone is mediated by oxidative/inflammatory stress, and this very stress is then combatted via the resulting activation of the NRF2 transcriptional pathway after *ortho*-quinone reaction with KEAP1. Optimization of these compounds is currently underway and will soon lead to human clinical trials for AD and ASD.

ACKNOWLEDGMENTS

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Dual allosteric mechanism of action of NitroSynapsin on NMDA-type Glutamate Receptors

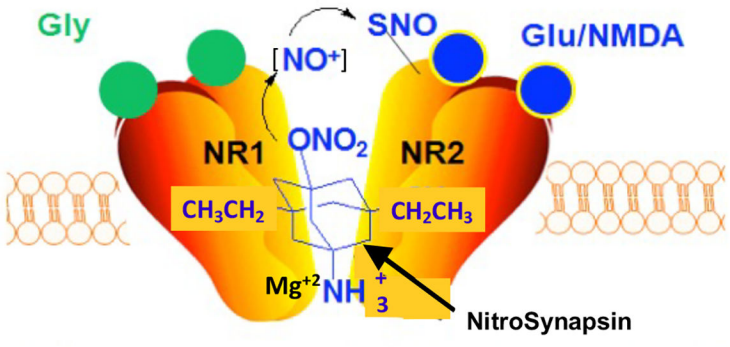

Fig. 1. Protonated aminoadamantane (memantine-like) nitrate compound binds at the NMDA receptor Mg^{2+} site, which then targets protein S-nitrosylation of regulatory cysteine residues on receptor to inhibit excessive activity.

NR1 (or GluN1) and NR2 (or GluN2) are the major NMDA receptor subunits, four or which form a functional channel. Gly (glycine) and glutamate (or exogenous NMDA) are co-agonists for the receptor.

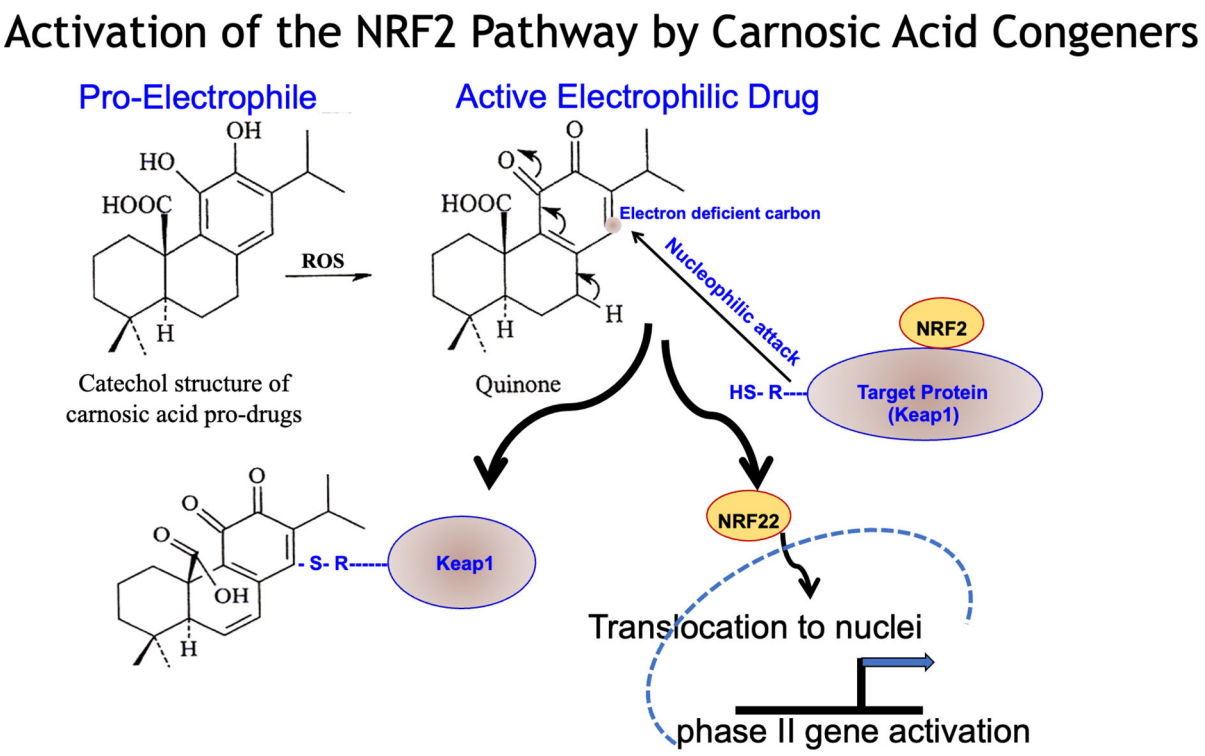

Fig. 2. Carnosic acid acts as a pro-electrophilic drug (PED), acting as a pathologically-activated therapeutic (PAT), via NRF2 stimulation.

Carnosic acid (CA), which can be synthesized or extracted from *Rosmarinus officinalis,* is a catechol-type pro-electrophilic drug (PED). Oxidative activation of the pro-electrophilic state to the electrophilic state is accomplished by electron acceptors, including ROS. The resulting quinone form then reacts with cysteine thiols. For this reaction, a cysteine thiol triggers nucleophilic attack of the electrophilic compound to form an adduct. Carnosic acid thus transforms from a non-active (pro-electrophilic) state to an active (electrophilic) state under oxidative stress. The outcome is that carnosic acid is activated only in tissue undergoing oxidative and inflammatory stress, and in turn then protects the tissue from such stress, as occurs in neurodegenerative disorders such as AD. This protection is achieved by activation of the KEAP1/NRF2 pathway by the quinone form of carnosic acid. The NRF2/KEAP1 pathway represents one of the major cellular defense systems against oxidative stress and inflammatory processes. NRF2 is a transcription factor that induces phase II anti-oxidant/anti-inflammatory enzymes. Under normal conditions, KEAP1 protein binds to NRF2 and functions as an adaptor protein for cullin 3 (encoded by Cul3 in humans) E3 ubiquitin ligase, which polyubiquitinates NRF2. Consequently, NRF2 is ubiquitinated and degraded by the proteasome. Hence, transcriptional activity of NRF2 is potently inhibited under normal conditions. KEAP1 contains critical cysteine thiols that react with CA after electrophilic conversion, as described above. This reaction prevents KEAP1 from inducing ubiquitination and degradation of NRF2. NRF2 thus dissociates from the cytoplasmic complex with KEAP1, enters the nucleus, and binds to AREs (anti-oxidant response elements) in the promoters of target phase II genes, which encode a coordinated system of anti-oxidant and anti-inflammatory enzymes. These proteins include enzymes that generate the major cellular antioxidant, glutathione (GSH). Thus, NRF2 activators protect

various cell types including neurons via chemical redox regulation. In the brain, activation of NRF2 occurs mainly in astrocytes and microglial cells (adapted from Satoh et al., 2022).