Perspectives

Using Plants as a Source of Potential Therapeutics for the Treatment of Alzheimer’s Disease

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Alzheimer’s disease (AD) is the most common form of dementia with the numbers expected to increase dramatically as our society ages. There are no treatments to cure, prevent, or slow down the progression of the disease. Age is the single greatest risk factor for AD. However, to date, AD drug discovery efforts have generally not taken this fact into consideration. Multiple changes associated with brain aging, including neuroinflammation and oxidative stress, are important contributors to disease development and progression. Thus, due to the multifactorial nature of AD, the one target strategy to fight the disease needs to be replaced by a more general approach using pleiotropic compounds to deal with the complexity of the disease. In this perspectives piece, our alternative approach to AD drug development based on the biology of aging is described. Starting with plants or plant-derived natural products, we have used a battery of cell-based screening assays that reflect multiple, age-associated toxicity pathways to identify compounds that can target the aspects of aging that contribute to AD pathology. We have found that this combination of assays provides a replicable, cost- and time-effective screening approach that has to date yielded one compound in clinical trials for AD (NCT03838185) and several others that show significant promise.

INTRODUCTION

Alzheimer’s disease (AD) is the most common form of dementia. The main pathological characteristics are the accumulation of extracellular neuritic plaques containing amyloid beta (Aβ) peptide and intracellular neurofibrillary tangles containing tau [1]. The primary clinical symptom is a progressive loss of cognitive function which eventually results in an inability to perform the activities of daily living [2,3]. The currently approved therapies only modestly and transiently reduce the clinical symptoms but fail to alter the course of disease progression [4,5]. Although there have been a large number of clinical trials in recent years with drug candidates designed to directly or indirectly reduce the amyloid plaque load, all of these trials have failed [6,7].

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Abbreviations: AD, Alzheimer’s disease; ADME, absorption, distribution, metabolism and excretion; Aβ, amyloid beta; APP, amyloid precursor protein; ATF4, activating transcription factor 4; CNS, central nervous system; ERK, extracellular regulated kinase; FAD, familial Alzheimer’s disease; GSH, glutathione; HBD, hydrogen bond donor; HPLC, high performance liquid chromatography; IAA, iodoacetic acid; LTP, long term potentiation; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Nrf2, nuclear factor erythroid-derived 2-like 2; tPSA, total polar surface area.

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Old age is by far the greatest risk factor for AD [8,9]. However, AD drug discovery and AD clinical trials have largely focused on targets related to the familial form of AD (FAD) which only accounts for a small percentage (<5%) of the total cases [9]. The vast majority of AD cases are sporadic and may be quite distinct from the genetic form of the disease. There are a number of pathophysiological changes that occur in the aging brain that potentially contribute to the nerve cell damage and death that is characteristic of AD including increases in oxidative stress, alterations in energy metabolism, loss of neurotrophic support, alterations in protein processing leading to the accumulation of protein aggregates, dysfunction of the neurovascular system and immune system activation [10,11]. Given this wide range of age-related changes that occur in the brain, it seems unlikely that developing drugs that only modulate a single pathological change will prove effective at preventing the development and progression of AD. In addition, there is a strong possibility that the relative contributions of each of these changes will vary among individuals. Indeed, as Dale Bredesen [12] and others have pointed out, AD is pathologically heterogeneous and may require a “personalized” approach for treatment. Moreover, these age-associated changes in the brain are modulated by lifestyle as well as environmental and genetic risk factors. Thus, it is likely it will be necessary to use therapies directed against different targets in order to effectively prevent these multiple age-related changes to the brain. One approach now used in cancer therapeutics involves combinations of drugs. However, this approach is subject to a number of pharmacokinetic and bioavailability challenges because drugs for central nervous system (CNS) diseases need to get across the blood brain barrier as well as the potential for long-term adverse drug-drug interactions. A better approach is to identify small molecules that have multiple biological activities that can impact the multiplicity of age-associated pathophysiological changes in the brain that contribute to AD development and progression [13]. We have implemented this approach by using a novel battery of phenotypic screening assays that reflect multiple age-associated toxicity pathways as described below.

TOPICS

Approaches to Drug Discovery for Neurodegenerative Diseases

A mixture of molecular and structural biology, combinatorial chemistry, and high throughput screening has dominated the drug discovery process since the 1990s [14]. Although this approach provides a rapid process for the discovery of drug candidates with high selectivity and high affinity for a specific molecular target, it has not produced the successes that were initially expected. This is especially true for complicated indications such as neurodegenerative diseases, including AD. Prior to the development of this target-based drug discovery approach, new drugs were typically identified by an approach called phenotypic screening. Phenotypic screening involves evaluating compounds against observable characteristics or phenotypes in biological systems such as animals or cells. Although phenotypic screening is no longer favored by the pharmaceutical industry, it still continues to be more successful than target-based approaches for the identification of novel small molecule drugs [15]. It has been argued that this is because while phenotypic screening can incorporate the complexities of biological systems, target-based discovery cannot and also requires many more a priori assumptions that may not reflect the situation in vivo [10,15].

Many of the natural product-based, first in class drugs were originally discovered using the ultimate end user-humans [16,17]. However, this approach is no longer appropriate for drug discovery. Furthermore, while laboratory animals are often used for preclinical testing, their use for the initial screening of potential drug candidates is impractical due to cost and time constraints as well as ethically questionable. Thus, a better alternative is to create cell-based assays that reflect the molecular toxicity pathways that are relevant to age-associated neurodegeneration and select for further investigation potential drug candidates that work in multiple assays, not just one [10]. By using this approach, the cell-based assays should have disease relevance as well as reproducibility and reasonable throughput. Moreover, since arguments can be made against the relevance of any single cellular screening assay, based on the cell type or the nature of the toxic insult, phenotypic screening approaches for neurodegenerative diseases should combine multiple assays that address the different toxicities associated with the aging brain. Therefore, the assembly of our assay pipeline was based on the following considerations: 1) All assays are associated with pathology and molecular changes that are significantly altered in the normal aging brain relative to young brains; 2) The assays reflect conditions that are more robust in diseased brains relative to age-matched controls; 3) Different assays use distinct types of CNS cells in order to ensure that a candidate compound that is beneficial for one cell type does not have detrimental effects on the other; 4) Assays are conducted when the cell is physiologically stressed, either by a toxin or loss of trophic support. Ideal drug candidates should have no adverse effects on normal unstressed cells.

The assays that my colleagues and I have developed and characterized are described below and in Table 1. While the suitability of any one of these assays can be questioned on theoretical grounds, in combination, we
have found that they make reliable predictions about the neuroprotective effects of compounds in vivo that hold true across several dementia models, and are thus of significant practical use [10,13]. Moreover, the use of cell-based assays reduces the potential for animal toxicity and avoids false positive results attributed to pan assay interference compounds (PAINS) in single target assays [18,19]. In addition, we have found that this combination of assays provides a replicable, cost- and time-effective screening approach that has to date yielded one compound in clinical trials for AD (NCT03838185).

**Phenotypic Screening Assays**

1. **Oxytosis/ferroptosis:** This assay tests the ability of compounds to rescue cells from oxidative stress-induced cell death caused by glutathione (GSH) depletion [20]. A reduction in GSH is seen in the aging brain and is accelerated in many CNS diseases including AD [21]. Importantly, GSH loss in the brain is associated with impairments in cognitive function [21-23]. High levels of glutamate inhibit cystine uptake by the cystine/glutamate antiporter, system x, thereby leading to GSH depletion. Experimentally, oxytosis can be investigated in isolation from excitotoxicity in neuronal cell lines or immature primary cultured neurons devoid of NMDA receptors. The depletion of GSH from cells leads to reactive oxygen species production, lipoxygenase activation, lipid peroxidation, and calcium influx which initiates a form of regulated cell death with features of both apoptosis and necrosis [20]. All of these changes are implicated in the nerve cell damage and death seen in AD [24]. Oxytosis appears to be very similar, if not identical, to another recently described form of cell death called ferroptosis [25]. We have found that most, if not all, compounds that inhibit oxytosis also inhibit ferroptosis [26] while the ferroptosis inhibitor ferrostatin-1 can also inhibit oxytosis [27]. This is of relevance because oxytosis/ferroptosis has been implicated in a number of pathological processes including neurodegenerative diseases [20,28-30]. Because of the generality of the toxicity pathway in oxytosis and its mechanistic association with aging and age-associated neurodegenerative diseases such as AD, it is used as our primary screen. In this assay, HT22 hippocampal neuronal cells or primary cortical neurons are treated with 5 mM glutamate for 24 hr and cell survival assayed by the MTT assay. In the absence of a neuroprotective compound ≥ 90% of the cells die under these conditions. The most effective compounds are protective when added at the same time as the glutamate or even several hours afterwards.

2. **In vitro ischemia:** A breakdown in neuronal energy production leading to ATP loss is associated with nerve cell damage and death in AD [31]. Therefore, maintenance of ATP levels is an important but overlooked therapeutic target. In order to induce ATP loss, we use the compound iodoacetate acid (IAA), a well-known, irreversible inhibitor of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase [32], in combination with the HT22 mouse hippocampal neuronal cell line. IAA, which has been used in a number of other studies to induce in vitro ischemia [33-37] causes a rapid loss of ATP [32,38]. We have shown that this assay can identify compounds which can maintain ATP levels in the presence of stress [38] and has been used as a primary screen to identify novel and highly potent derivatives of the flavonoid fisetin [39]. In this assay, HT22 hippocampal neuronal cells are treated with 20 µM IAA for 2 hr which results in 90-95% cell death 20 hr later as determined by the MTT assay. The compounds are included during the IAA treatment and in the fresh medium added after the 2 hr IAA treatment.

3. **Inhibition of microglial activation:** Inflammation is a major feature of AD as well as essentially all other neurological disorders (for reviews see [40,41-43]). Microglia are the resident immune cell population of the CNS, comprising 5-10% of the total cell population (for reviews see [44-46]). Their presence can have both beneficial and detrimental effects on the brain. Classically activated microglia are implicated in the pathogenesis of a variety of neurological disorders including AD. These microglia produce a wide array of pro-inflammatory and cytotoxic factors including cytokines, free radicals, excitatory neurotransmitters and eicosanoids that may work in concert to promote neurodegeneration. In the context of the AD brain, there are thought to be multiple stimuli that

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**Table 1. Phenotypic Screening Assays.**

| Assay                        | Age-Related Toxicity                      |
|------------------------------|-------------------------------------------|
| Oxytosis/ferroptosis         | oxidative stress, lipid peroxidation       |
| In vitro ischemia            | energy loss                                |
| Microglial activation        | inflammation                              |
| Trophic factor withdrawal    | loss of trophic factors                   |
| PC12 neurites                | loss of neuronal connections               |
| Intracellular Aβ toxicity    | intracellular protein aggregation          |

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Further details on the experimental setup and outcomes can be found in the original paper.
generate an inflammatory response in the microglia including Aβ [43]. Thus, inhibiting the generation of classically activated microglia is another important therapeutic target for AD. In this assay, the ability of a compound to inhibit the classical activation of the mouse microglial cell lines BV2 or N9 by bacterial lipopolysaccharide (LPS) is tested using the increased production of NO as a primary read-out [39,47].

4. Trophic Factor Withdrawal: As the brain ages, there is a loss of the trophic factors that maintain neuronal integrity [48,49]. A simple assay to identify compounds that protect from the loss of trophic factors that promote nerve cell survival involves plating rat embryonic cortical neurons at low density in standard tissue culture medium. Freshly plated cells die within 24 hr, while if the cells are plated in the presence of both fibroblast growth factor and brain-derived neurotrophic factor they survive [50]. Importantly, in the presence of compounds that can substitute for the absence of survival-promoting trophic factors, the cells will also survive.

5. PC12 Differentiation: Connections between nerve cells are also altered in AD. Thus, compounds that can promote the regeneration of these connections might be of particular benefit, thereby promoting the recovery of higher neuronal function. As a model for this property, we use neurite outgrowth in PC12 cells, a well-studied model system of neuronal differentiation. In response to neurotrophic factors such as nerve growth factor, PC12 cells undergo a series of physiological changes culminating in a phenotype resembling that of sympathetic neurons (for review see [51]). These changes are the result of the activation of a coordinated series of signaling pathways and include the cessation of cell division, the expression of genes encoding nerve cell-specific proteins and the extension of neuritic processes. In this assay, PC12 cells are treated with compounds in N2 medium and neurite outgrowth scored after 24 hr as described [52].

6. Intracellular amyloid toxicity: Many now consider the accumulation of intracellular Aβ as being a primary toxic event in AD [53-56]. The human nerve cell line MC65 conditionally expresses the C99 fragment of the amyloid precursor protein (APP) leading to the accumulation of intracellular Aβ. The MC65 cells are routinely grown in the presence of tetracycline and, following its removal, the expression of C99 is induced and the cells die within 4 days because of the accumulation of intracellular, toxic protein aggregates [57,58] which is associated with a pro-inflammatory response [59]. Cell death is not caused by the secretion of Aβ and extracellular toxicity, nor by secreted toxins [57]. Death is inhibited by γ-secretase inhibitors [60]. Cell death is easily measured by the MTT assay as there is complete cell lysis.

Together, this combination of phenotypic screening assays enables the identification of potent, disease-modifying compounds for preclinical testing in animal models of neurodegenerative diseases. Importantly, by means of these assays, we have successfully identified compounds with distinct targets that show beneficial therapeutic efficacy in in vivo models of AD [13,58,61-63]. Moreover, one of these compounds, the curcumin derivative J147, is in a phase 1 clinical trial for AD (NCT03838185) and another, the fisetin derivative CMS121 [13,39,63], is in IND-enabling studies for the treatment of AD. However, as noted earlier, AD is a heterogeneous disease so even if multi-target drugs are identified, it is likely that multiple drugs will be needed to successfully treat all of the patients with the disease. In addition, most drug candidates do not make it past clinical trials into the clinic so it is essential to have multiple candidates against distinct drug targets in the pipeline. Thus, we believe that our phenotypic screening paradigm should be very useful for identifying additional, new drug candidates for the treatment of AD and other age-related neurodegenerative diseases. With these ideas in mind, what should be screened?

WHY START WITH PLANTS?

One of the best sources of multi-target compounds are plants, the original pharmacopeia. Records describing the use of plants for medicinal purposes date back to 2600-2900 BC [64]. Even today, ~25% of all prescribed drugs are thought to be derived from plants [16]. Plants synthesize a huge array of compounds called secondary metabolites that are not required for plant development, growth, or reproduction but are needed for survival. Surprisingly, these compounds are derived from a limited number of basic chemical scaffolds which are modified by specific types of chemical substitutions. It has been suggested [16,65] that these compounds, as well as receptors, enzymes, and regulatory proteins, originated from a relatively small number of parental molecules which may have co-evolved to interact with one another. Although their biological functions and structures have since diverged, structural features shared from their common past may be the reason that they interact with medically relevant targets. Since plants have been used for millennia for medicinal purposes, one good starting point for compound selection is the literature on traditional medicine [17,64]. Traditional Chinese medicine in particular provides a very rich source of information about plants used to treat a variety of diseases including those involving the brain [66,67]. As described below, we have taken several different approaches to selecting the compounds or plants that we have screened. With all of these approaches, we were able to identify interesting compounds that show promise for the treatment of AD and are continuing to identify more. Three examples are described below.
Examples from Our Lab

Example #1: We originally identified the flavonoid fisetin (3,7,3',4' tetrahydroxyflavone) in our screening assay for compounds that can prevent oxytosis/ferroptosis [68]. Of the ~30 flavonoids and related polyphenols tested in this study only two, fisetin and quercetin, were able to maintain GSH levels in the presence of oxidative stress, indicating that this is not a common property of flavonoids and related polyphenols. Further screening with many additional flavonoids and related polyphenols have confirmed this observation. Additional studies showed that fisetin also possessed neurotrophic activity, promoting the differentiation of PC12 cells via activation of the Ras-ERK cascade [52]. Again, this was a property that distinguished fisetin from almost all of the other ~30 flavonoids tested. Together, these observations suggested that fisetin had multiple properties that might make it useful for the treatment of AD. Further studies have demonstrated that fisetin can protect nerve cells from multiple toxic insults including Aβ toxicity, ischemia, and hyperglycemia. It has both direct antioxidant activity and maintains the levels of GSH under conditions of stress by inducing the transcription factors, Nrf2 and ATF4 [69]. Fisetin is also able to maintain ATP levels under ischemic conditions [38]. Moreover, fisetin was shown to facilitate long term potentiation (LTP) in hippocampal slices via modulation of ERK and CREB phosphorylation and oral administration of fisetin promoted learning and memory in mice using the object recognition test [70]. Fisetin is also effective in two different models of stroke [38,71]. Using three different models of Huntington’s disease (mutant huntingtin-expressing PC12 cells, mutant huntingtin-expressing Drosophila, and the R6/2 mouse) we found that fisetin was able to reduce the impact of mutant huntingtin in each of these disease models [72]. Most relevant to this article, fisetin prevents learning and memory deficits in both APPsw/PS1dE9 (huAPP/PS1) double transgenic AD mice [62] and rapidly aging SAMP8 mice [73]. Work from other laboratories has confirmed and extended these studies both in additional preclinical models of AD [74] as well as preclinical models of Parkinson’s disease [75] and amyotrophic lateral sclerosis [74]. Moreover, two recent studies showed that fisetin also has anti-aging properties [73,76] consistent with the idea that aging and neurodegenerative diseases are tightly linked.

However, fisetin’s relatively high EC50 in cell based assays (2-5 µM) as well as its low lipophilicity (cLogP 1.24), high tPSA (107Å) and high number of hydrogen bond donors (HBD = 5) suggested that there was room for medicinal chemical improvement. Using structure-activity relationship (SAR)-driven iterative chemistry, we synthesized more than 160 derivatives of fisetin based on several different chemical scaffolds. We used a multi-tiered approach to screening that allowed us to identify fisetin derivatives with significantly enhanced neuroprotective activity in our in vitro neuroprotection assays while at the same time maintaining other key actions including anti-inflammatory activity [39]. While all of the fisetin derivatives had improved medicinal chemical properties more consistent with those of known CNS drugs, ~20 had greatly enhanced neuroprotective activity [39]. ADME and pharmacokinetic studies on the six most promising derivatives showed that several had peak brain levels following a single oral dose of 20 mg/kg that greatly exceeded their average EC50 in the in vitro neuroprotection assays as well as good oral bioavailability. Based on these and other results we selected the fisetin derivative CMS121 for further testing in preclinical mouse models of aging and AD [63].

Example #2: In a proof of principle study from the lab [77], extracts from five different species of plants – Voacanga africana, Sacosperma paniculatum, Psychotria subobliqua, Psychotria principensis, and Tarenna nitiduloides – from São Tomé and Príncipe were tested in our panel of phenotypic screening assays. Three of the species were selected on the basis of ethnopharmacological data while the other two had no reported ethnopharmacological relevance and were chosen as negative controls. Briefly, our workflow consisted of testing hydro-ethanolic extracts from the different candidate species and selecting the extract that performed best in all of the different assays for subsequent fractionation. The resultant individual fractions were tested in the oxytosis/ferroptosis assay and the predominant compound of the most active fraction was purified, structurally determined, and its efficacy tested in the complete phenotypic screening assay panel. An extract from the bark of Voacanga africana was more protective than any other plant extract in all of the assays (average EC50 < 2.4 µg/ml). The HPLC fraction from the extract that was most protective contained the alkaloid voacamine as the dominant compound. To confirm this activity, voacamine was purified and tested and proved to be very effective in all five assays (average EC50 < 3.4 µM).

Example #3: More recently we screened a commercial library of extracts from plants with known ethnopharmacological uses to identify additional potential new drug candidates for the treatment of AD [78]. All plant extracts were first tested in the oxytosis/ferroptosis assay described above. Extracts that were positive in this assay were then screened in the additional assays that are also described above. As already mentioned, these assays reflect multiple, age-associated neurotoxicity/survival pathways directly relevant to AD, such as increased oxidative stress and GSH depletion, reduced energy metabolism, accumulation of misfolded, aggregated proteins, loss of neurotrophic support and inflammation [10]. Using this approach, we identified an extract from Yerba santa...
implementation of this platform with additional libraries of natural products not restricted to plants and including the vast amount of information available on traditional medical practices in China and elsewhere has the potential to identify new compounds for the treatment of AD and related neurodegenerative diseases. Although these compounds will need to be tested in animal models of AD, as we have done for fisetin \[62,73\], a major advantage of natural products is that in the US they generally don’t need to undergo the rigorous and expensive Investigational New Drug approval process. Thus, if funding is available, they can move much more quickly into small scale clinical trials. With this approach, we believe it is possible to develop a battery of drugs to successfully treat AD within the time frame of that proposed by the National Alzheimer’s Project Act (NAPA) whose goal is to find effective interventions to treat and prevent AD and related dementias by 2025.

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**CONCLUSIONS AND OUTLOOK**

There are no effective treatments for age-dependent neurodegenerative conditions such as AD. To address this major public health crisis, one or more effective drugs are required. Since AD and related dementias are diseases that occur with age, aging must be incorporated into the drug candidate identification strategy. Thus, we have developed an age-related phenotypic screening platform that has already yielded compounds that are effective in pre-clinical models of AD and one of which is now in AD clinical trials (Figure 1). We believe that further implementation of this platform with additional libraries of natural products not restricted to plants and including the vast amount of information available on traditional medical practices in China and elsewhere has the potential to identify new compounds for the treatment of AD and related neurodegenerative diseases. Although these compounds will need to be tested in animal models of AD, as we have done for fisetin \[62,73\], a major advantage of natural products is that in the US they generally don’t need to undergo the rigorous and expensive Investigational New Drug approval process. Thus, if funding is available, they can move much more quickly into small scale clinical trials. With this approach, we believe it is possible to develop a battery of drugs to successfully treat AD within the time frame of that proposed by the National Alzheimer’s Project Act (NAPA) whose goal is to find effective interventions to treat and prevent AD and related dementias by 2025.

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