Anti-inflammatory and Anti-oxidant Neuroprotection in the Prevention of Alzheimer's Disease

Thesis submitted by

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Abstract

Current therapy for Alzheimer's disease addresses the symptoms of the disease and results in moderate improvements in cognitive functions. Acetylcholinesterase inhibitors, the main treatment of choice, increase the availability of acetylcholine in the brain, thereby enhancing synaptic transmission and improving cognition. This approach however, does not prevent or delay the onset of the disease. The new class of drug in the treatment of Alzheimer's disease, NMDA-receptor antagonists, do offer some protection of neurons against excitotoxic insult, but are only effective following diagnosis and do not possess anti-inflammatory properties.

The elucidation of the inflammatory processes responsible for Alzheimer's disease has demonstrated similarities to other inflammation-associated diseases. It is therefore not surprising that therapies used for the treatment of other medical conditions, namely Non-steroidal anti-inflammatory drugs, statins and antioxidants, may be of benefit in Alzheimer's disease. Closer inspection however, reveals that only selected drugs within these groups appear to provide neuroprotection. The aim of this study was to determine whether this effect translates to the *in vitro* situation and if so, the causes of the disparity between drugs within the same class.

A co-culture model of Alzheimer's disease was designed containing human microglia and fluorescent neurons, to allow determination of neuronal viability separately from microglial viability. The establishment of stably-expressing fluorescent neurons for this purpose required significant optimization of transfection and stable selection, resulting in a methods paper [1]. The primary outcome of this article is that although linearization of DNA can increase the production of stable clones, it is dependent on the site of restriction enzyme digestion and requires plasmid-specific optimization.

Initially, the aim of this study was to assay anti-inflammatory compounds in three distinct culture models of neurodegeneration. The development of these models highlighted important aspects of neurodegeneration *in vitro*. Transferral of activated microglial media to neurons and activation of microglia when separated from neurons in co-culture, both failed to induce neuron death in the presence of pro-inflammatory mediators Lipopolysaccharide and Interferon-γ. These mediators however, induced significant neuron death when microglia and neurons were co-cultured with direct cell to cell contact. Thus, neuron death *in vitro* is

dependent on microglial proximity and is likely to be due to short-lived toxic factors such as free radicals, as opposed to long-lived cytokines and other inflammatory mediators. Furthermore, inhibitors of Nitric oxide synthase were found to rescue neurons from microglial insult, indicating that the free radical nitric oxide is highly involved in the induction of neurodegeneration.

The direct co-culture system found to induce neuron death was utilized to assay numerous compounds, from the non-steroidal anti-inflammatory drug, statin and antioxidant classes, for their neuroprotective abilities. In agreement with *in vivo* studies, it was found that not all compounds within a drug class shared neuroprotective properties. Moreover, the neuroprotection conveyed by ibuprofen, indomethacin and sulindac sulphide in this *in vitro* system coincides with epidemiological observations that suggest these therapies provide greater protection against the onset of Alzheimer's disease compared to other non-steroidal anti-inflammatory drugs. Selected therapeutics from the statin and nutraceutical antioxidant classes also provided neuroprotection and although the pathways or targets responsible for neuroprotection were not determined, it is clear that the inhibition of Nitric oxide via direct or antioxidant mechanisms plays a role.

The findings in this study indicate that some currently available anti-inflammatory therapies protect neurons against inflammation-dependent degeneration *in vitro*. In the clinical setting, this neuroprotective action may translate to a delay in the onset and perhaps progression of Alzheimer's disease. The relatively safe toxicity profiles and ease of access to currently available anti-inflammatory and nutraceutical therapies renders them attractive as interim therapies until more specific therapies for Alzheimer's disease are developed. Furthermore, investigation into the shared targets of these anti-inflammatory therapies that are responsible for neuroprotection may assist in the identification of candidate targets for future drug development.

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Statement of Sources

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institutions of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

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Statement of Contribution of Others

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Declaration of Ethics

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *James Cook University Policy on Experimentation Ethics, Standard Practices and Guidelines (2001)* and *James Cook University Statement and Guidelines on Research Practice (2001).*

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Introduction

The life expectancy in western society continues to increase, as medical breakthroughs decrease the prevalence of fatal conditions such as cancer and heart disease. The drawback of increased life expectancy is the concurrent increase in age-related dementia. In Australia, an estimated 245,000 people suffered from dementia in 2009, approximately 50% of whom suffered from Alzheimer's disease [2]. The progressive memory loss and inability to live independently witnessed in Alzheimer's is not only difficult for families and carers, but results in a significant burden on the Australian economy. As the average age of western countries increases, so will the number of patients suffering from this debilitating disease. Moreover, Australia will see the prevalence of dementia accelerate further, due to the large number of baby boomers in the demographic, who are now passing 65 years of age [2]. It is therefore imperative that preventative treatments are discovered.

Alzheimer's disease was first identified in 1907 [3], with the primary hallmarks being neurofibrillary tangles, senile plaques and significant neurodegeneration. Neurofibrillary tangles are intracellular aggregations of hyper-phosphorylated microtubule-associated protein Tau, whilst senile plaques are extracellular deposits primarily composed of aggregated β-amyloid protein. These abnormalities had been observed for some time before being linked to the corresponding neurodegeneration. It has only been in the last 25 years, that these protein abnormalities have been found to induce inflammation that exacerbates inflammation. This inflammation involves an array of inflammatory substances, including complement factors, acute-phase proteins and pro-inflammatory cytokines [4].

It is evident that senile plaques play a central role in the inflammatory cascade [5]. β-amyloid is able to elicit inflammatory responses from microglia and astrocytes, the resident macrophages and house-keepers of the central nervous system respectively, which are found in increased numbers surrounding senile plaques [6, 7]. This protein is also a target for alteration by 'advanced glycation endproducts,' which are known to act via the same receptor and are able to amplify inflammatory pathways [8]. Through activation by β-amyloid and advanced glycation endproducts, microglia and astrocytes play an integral role in the progression of AD, with the production of the cytokines Interleukin-1, Interleukin-6, Macrophage-colony stimulating factor and Tumour necrosis factor-α, accompanied by prostaglandins and free radicals [9-13].

The excessive inflammation in AD results in neurodegeneration, which causes the dementia observed in AD patients. Although there is evidence of neuronal apoptosis *in vivo*, it is a common misconception that apoptosis is the primary cause of cognitive decline [14]. Synaptic loss, via neurite retraction, correlates more closely to the degree of dementia witnessed in AD patients, indicating neurite retraction as the major symptomatic cause of AD [15].

Current treatment strategies for AD are not targeted at providing neuroprotection and therefore fail to address the underlying cause of the disease. The most recent addition to therapeutic regimes for AD, the N-methyl-D-aspartate receptor antagonist, Memantine, has demonstrated some neuroprotective properties, however Acetyl cholinesterase inhibitors continue to be the primary therapeutic agent of choice. Acetyl cholinesterase inhibitors have been used in the treatment of AD since the FDA approval of Aricept in 1996, and although an initial improvement in cognition is observed, they provide, at best, modest outcomes for patients. Acetyl cholinesterase inhibitors are only used following diagnosis of AD and can not be administered as a preventative treatment, which is a major downfall of this approach. Thus, it is essential that preventative therapies are identified in the near future.

Pathology of Alzheimer's Disease

Microglia

Microglia constitute 5-20% of the neuroglial population and under normal conditions are tightly regulated to remain in a resting state [16]. When activated, they perform tasks that assist in the growth and survival of both neurons and astroglia [17]. As the central nervous system equivalent of macrophages, a primary function of microglia is the phagocytosis of cellular debris [18], which may occur as a result of aging and normal cellular death, or due to head trauma or disease. Microglia do not normally possess a phagocytic phenotype, but acute stress results in microglia undertaking a more protective role [18]. It is possible that microglia become activated early in the onset of Alzheimer's disease (AD), where they are found in increased numbers surrounding senile plaques [19], presumably attempting to phagocytose and remove neurotoxic substances in the vicinity. The inability to remove these substances may cause detrimental chronic activation of the cells, resulting in phagocytic differentiation that amplifies neuronal stress and degeneration.

Under normal conditions microglia constitutively express cytokines and chemokines, but activation significantly up-regulates an array of pro-inflammatory substances, including Interleukin (IL) -1β, IL-6, IL-8, IL-10, IL-12, IL-15, Tumour necrosis factor-alpha (TNF-α), Macrophage inflammatory protein-1α (MIP-1α), MIP-1β, and Monocyte chemoattractant protein-1 (MCP-1) [20]. At present, the inflammatory factors believed to be of importance in relation to AD are reactive oxygen species (ROS), reactive nitrogen species (RNS), proteases and the cytokines IL-1β, IL-6, Macrophage-colony stimulating factor (M-CSF) and TNFα [9-13]. Evaluation of cytokine arrays in AD susceptible transgenic mice has confirmed the involvement of these cytokines, with TNFα in particular being significantly up-regulated [21].

The inflammatory factors present in AD are considered to be 'pro-inflammatory,' and are typical of an innate immune response, which, via MHC-I-dependant mechanisms, normally provide a rapid response in the case of acute neuronal injury. The innate inflammatory response in AD, however, is uncontrolled and consequently is a major contributing factor to neuronal degeneration. This point is emphasised by administration of IL-4, an adaptive immunity-associated cytokine, causing microglia to switch to an 'anti-inflammatory' phenotype that significantly enhances neuron survival in mouse models of the disease [22]. Although the exact mechanism is unclear, it has been demonstrated to involve an increase in both MHC-II expression and insulin-like growth factor-I (IGF-I) production, and a decrease in TNFα production. Moreover, this phenotypic switch has also been linked to increased microglial uptake of glutamate, an excitatory neurotransmitter that exacerbates neurodegeneration in AD [23].

Interestingly, Jimenez *et al.* found that microglia can undergo an age-dependent phenotypic switch [24]. Early in the disease process of PS1xAPP transgenics, microglia possessed an Aβ phagocytic phenotype, which was altered to a classical cytokine-expressing inflammatory phenotype as the disease progressed. Thus, microglial-specific immune modulation or prevention of the phenotypic switch may be a viable method of treating AD in the future.

Astroglia

Astrocytes are the most abundant cells in the brain, occupying over 50% of the total brain volume [25] and assist in the normal functioning of neurons by optimising the local environment. Some of the numerous tasks that these cells perform include local ion and pH control and importantly, the delivery of metabolic substrates, particularly glucose, as

astrocytes are the primary stores for glycogen in the brain [26, 27]. An especially significant function in terms of disease, is the clearance of neuronal waste products, particularly the excitatory neurotransmitter glutamate, in a bid to maintain sub-neurotoxic levels in the extracellular space [28]. Astrocytes also regulate neurogenesis [29] and influence the structural development of the brain, via promotion of neuronal connections. This has been demonstrated *in vitro*, where neurons co-cultured with astrocytes exhibit a seven-fold increase in synapses [30].

As research in this field progresses, astrocytes are being implicated as more significant contributors in the progression of AD than previously believed. When activated, astrocytes are capable of producing a similar profile of cytokines to microglia. More importantly however, astrocytes are more susceptible to activation by the AD-related cytokines TNFα and IL-1β than to Interferon(IFN)-γ [31]. It is possible that like microglia, astrocytes become overstimulated in AD, causing a disturbance in their housekeeping functions, placing increased stress on local neurons. Two mechanisms that are likely to be affected in this process are glutamate uptake and Nitric oxide (NO) production, as *in vitro* studies indicate decreased glutamate uptake and increased NO production by activated astrocytes [32-35].

The brains of AD patients display increased levels of glutamate and an over stimulation of N-methyl-D-aspartate (NMDA) receptors [36]. This is due, in part, to the inhibition of glutamate uptake by astrocytes, as demonstrated by knockdown of astroglial glutamate transporters, resulting in increased neuron death. In contrast, knockdown of a neuronal glutamate transporter does not dramatically alter neuron survival [37]. Astrocytic glutamate scavenging primarily occurs via the Na^+/K^+ ATPase-dependant GLAST and GLT-1 transporters [38]. These transporters not only display decreased expression in AD [39], but transportation activity can also be inhibited during oxidative stress [40], a persistent condition in AD [41, 42]

The second mechanism of interest in astrocytes, the production of NO, is partially linked to the presence of excessive extracellular glutamate. Glutamate stimulation of neurons and, to a lesser degree, astrocytes, results in increased intracellular Ca^{2+} (Calcium) levels [43]. Along with other numerous downstream effects of intracellular Ca^{2+}, a Ca^{2+} -calmodulin-dependant activation of nitric oxide synthase (NOS) is observed [44]. Combined with the expression of the inducible form of NOS (iNOS), which occurs in response to cytokines [45], significant amounts of NO can be produced by astrocytes.

High levels of lipid peroxidation [46, 47] suggest that antioxidant mechanisms are unable to manage the increase in reactive species such as NO, which causes continual stress to surrounding neurons. Surprisingly however, antioxidant capacity of cells does not appear to be significantly reduced in AD [48] and has actually been shown to be increased in affected brain regions [49]. Under physiological conditions, astrocytes release glutathione (GSH), which acts as a scavenger of oxidising species, thus relaying protection to neurons against oxidant damage [50]. Under physiological conditions, astrocytic expression and activity of the GSH-producing enzyme glutamate-cysteine ligase, together with trafficking of GSH into the extracellular space are increased in the presence of NO [51]. Extended exposure to NO during pathological conditions however, as is the case in AD, eventually causes a failure of this antioxidant mechanism and neuroprotection is not conveyed [52].

The mechanisms of astrocytic neuroprotection and neurodegeneration and the fine line that separates the two, are complex. Irrespective, it is clear that astrocytes play more than a simple by-stander role in the progression of AD. Support for a major role, particularly in the later stages of the disease, comes from a finding that the degree of dementia correlates more closely with astrocyte numbers than that of microglia [53]. Thus, it is possible that astrocytes may be a target for preventative treatments for patients already in the latter stages of the disease, while microglia are targeted before disease onset in patients at high risk of developing AD.

β-amyloid

The amyloid family of proteins are so named due to their ability to form β-sheet (amyloid) structures by cross-linking with similar proteins. Several diseases involve proteins of amyloidogenic nature, including systemic amyloidosis (lysozyme), diabetes mellitus type 2 (amylin), AD (β-amyloid), Huntington's disease (huntingtin) and Parkinson's disease (α-synuclein). Of these, systemic amyloidosis is the only disease that involves an amyloid protein as the primary effector, with most amyloid proteins merely contributing as a secondary factor. In the case of AD, however, this is debatable, as the fundamental consequence of genetic mutations in inherited forms of AD, including the Presenilin-1 (PS-1) and Apolioprotein-ε4 (APO-E) alleles, is a significant increase in β-amyloid (Aβ) [54-56].

Senile plaques, the primary hallmark of AD in the brains of patients, are at the centre of localised inflammation, due largely to the presence of Aβ. As a member of the amyloid family of proteins, Aβ has the ability to bind to other Aβ peptides and form aggregates. The first

stage of aggregation is a dimerization between two Aβ peptides, giving rise to a β-stranded secondary structure [57]. These dimers are then further aggregated, via side chain interactions with other dimers, in a quaternary β-sheet structure to form long twisting fibrils. These aggregates, known as fibrillar Aβ, are the predominant form associated with senile plaques, possessing greater toxicity than non-fibrillar forms [7, 58] and are thought to be responsible for continuous glial activation in AD. Activation by oligomeric forms of the peptide quickly declines, while fibrillar forms induce a sustained response, consistent with the chronic inflammation in AD [59].

Amyloid precursor protein (APP), a 770 amino acid transmembrane protein, is believed to function as a receptor and growth factor [60] that conveys neuron survival and neurite outgrowth during neural development [61]. This protein is the parent protein of Aβ, with proteolytic cleavage of the N- and C- termini by β- and γ- secretases [62] and further processing by α- secretase giving rise to the family of Aβ peptides. The combination of secretase actions results in a number of isoforms, including Aβ1-16, Aβ1-28, Aβ17-42, Aβ1-40 and Aβ1-42 [62].

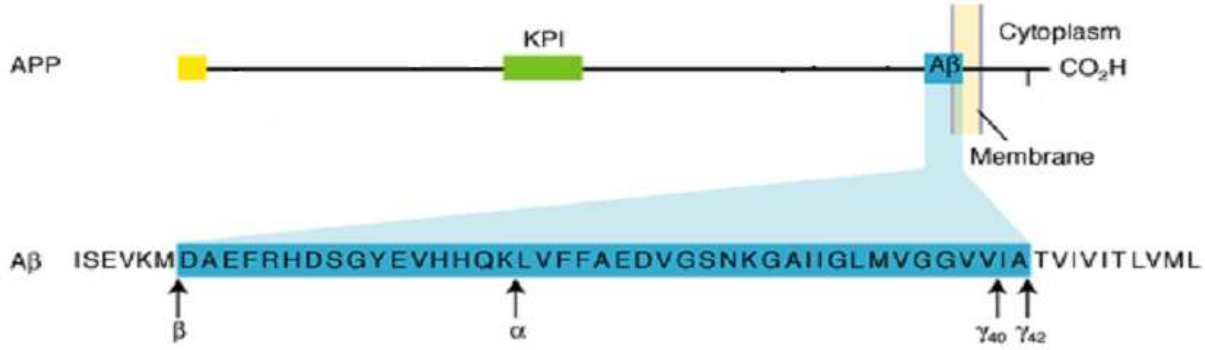

Figure A. Processing of APP to form β-amyloid. APP is cleaved by three secreatses, which gves rise to the various isoforms of the β-amyloid peptide. (Adapted from Expert Reviews in Molecular Medicine, 2002)

Increased accumulation of Aβ is an inevitable process of aging [63], but it is the over-production of the Aβ1-40 and Aβ1-42 isoforms that is correlated to AD pathology [64]. Aβ1-42 is believed to play a greater role throughout the progression of AD, as it has been closely correlated to AD-specific brain morphology and is found at higher concentrations at an earlier stage of disease than Aβ1-40 [55, 65]. Both of these peptides however, contain the Aβ1-16 portion of the Aβ peptide, which has a high affinity for the C1q receptor [66], which is involved in activation of the complement pathway and leads to the activation of astrocytes and microglia. The involvement of astrocytes and microglia in inflammation and the

localisation of C1q to Aβ plaques indicates the complement pathway, via the C1q receptor, is a possible source of early inflammatory activation in AD [67]. The role of the C1q receptor is indicated in studies with $APPQ^{-/-}$ transgenic mice (APP over-expressing Tg2576 mice crossed to C1q null mice), which display similar Aβ plaque burdens as their APP over-expressing parent strand, but significantly less activated microglia [68]. The 'amyloid cascade hypothesis' suggests a pathway that involves the deposition of an Aβ1-42 seed. This deposition prompts the formation of diffuse plaques, which are initially amorphous and non-fibrillar [69, 70], but compact over time, becoming fibrillar and therefore neurotoxic [71]. Further evidence to support this theory arises from studies that have compared the senile plaques in the brains of AD patients, to those of non-AD affected brains of the same age. AD brains display significant amounts of compact, fibrillar plaques. Interestingly, despite the absence of dementia, non-AD brains also display evidence of plaque formation, although of a non-fibrillar nature. In contrast to AD affected individuals, the predominant Aβ peptide present in non-demented individuals, is Aβ17-42, created by further cleavage of Aβ1-42 with α-secretase. Aβ17-42, known as the P3 fragment, is suggested to be relatively harmless, due to the absence of inflammation and degenerative neurites that usually accompanies senile plaque formation [58, 62, 72]. The poor reactivity of the P3 fragment again supports the role of the C1q receptor in initial activation, as P3 does not possess the peptide sequence responsible for C1q activation. The difference in Aβ isoforms between AD and non-affected individuals indicates that the onset of AD may be a result of incorrect processing of APP, although this theory is yet to be confirmed.

Aβ is known to exacerbate inflammation and cause neurodegeneration via numerous mechanisms, including increased permeability of the blood-brain barrier [73], increased vasoconstriction of vascular smooth muscle cells [74, 75], inhibition of proteases and trypsin [76] and inhibition of acetylcholine release and choline re-uptake in neurons [77]. The most notable of these effects, however, is the induction of pro-inflammatory stimuli by microglia and astrocytes including cytokines, chemokines [11, 78], NO [79, 80] and ROS [81],. Although fibrillar Aβ of senile plaques is accepted as more toxic, fibrillar peptide is not required for the activation of glial cells, which can therefore occur prior to plaque formation [82].

Cytokine and chemokine up-regulation in AD is a double-edged sword, as it is intended to induce neuroprotection and removal of Aβ by glial cells, but may in reality have the opposite effect. Transforming growth factor-β (TGF-β), produced by astrocytes and found

at high concentrations in AD brains [83], typifies this situation. TGF-β is able to decrease plaque burden in the brain by promoting Aβ clearance by microglia [84], but negates this positive action by increasing Aβ production via a direct elevation and stabilisation of APP mRNA [85].

Despite the obvious pro-inflammatory activity of Aβ, the perpetual state of inflammation in AD may not be as severe in the absence of co-stimulatory molecules. Although a response can be elucidated *in vitro* from microglia by Aβ alone [86, 87], a substantially greater response is usually observed by co-stimulation with TNF-α, IL-6, IL-1β or advanced glycation endproducts [7, 11, 88, 89], all of which are present in AD. Aβ is undoubtedly central in the progression of AD and therefore the most obvious target for therapeutic intervention. However, given its reliance on co-stimulation, the inhibition of pro-inflammatory substances that act as co-stimulators is also likely to provide parallel neuroprotection.

Advanced Glycation Endproducts

Glycation is one of the most common non-enzymatic modifications of proteins [90]. Advanced glycation endproducts (AGEs), also known as Maillard products, are sugar-derived oxidation products that covalently attach to long-lived proteins. AGE formation is an inevitable part of aging, with proteins including collagen, eye lens crystalline and neuron-associated proteins displaying significant glycation levels in aged individuals [91, 92]. Moreover, studies have demonstrated that the correlation is so close that AGE accumulation could be used as a determinant of age [93].

In AGE formation, the addition of the sugar generally takes place on an arginine or lysine residue and results in the formation of a chemically reversible Schiff base, with further stabilisation to an Amadori product. Although the formation of these initial products is reversible, subsequent alterations, including rearrangements, dehydrations and oxidations gives rise to the complex structures known as advanced glycation end products (AGEs), which are chemically irreversible [94, 95]. Once a protein has been modified by the addition of AGEs, it becomes cytotoxic, with the number or degree of modifications determining the level of toxicity [96].

AGEs have a broad range of effects, however the ability to cross-link proteins and to activate cellular pathways via cell surface receptors are the two of primary concern [97]. The

nature of these effects has linked AGEs to several diseases, including increased cartilage stiffness in osteoarthritis [98], β_2-microglobulin deposits in haemodialysis [99, 100] and diabetes mellitus, where high glucose levels induce chronic production of AGEs that leads to vascular complications and inflammation [97, 101, 102]. These diseases share common factors with AD, since AGEs are also increased in the brains of AD patients, and likely contribute to neurodegeneration.

Known to be present in considerably greater concentrations in the cortex and hippocampus, AGEs have a range of detrimental effects of interest in AD [103]. It has been recognised for some time that, given its longevity, Aβ is a candidate protein for glycation in AD [104]. Several other proteins, α-tubulin, ubiquinol-cytochrome C reductase complex protein I, the β chain of ATP synthase and importantly, Tau, have also been found to be highly glycated in AD patients [103, 105]. The cross linking of proteins by AGEs impairs normal function, which in the cases of ubiquinol-cytochrome C reductase and ATP synthase, may account for some of the depleted neuronal metabolism witnessed in AD [106]. A more problematic effect of glycation in AD, particularly for Aβ and Tau, however, is the enhanced resistance of proteins to breakdown by protease and macrophage attack [107], which may result in a more rapid accumulation of these proteins and augmentation of microglia activation.

While protease resistance and functional impairment may indirectly exacerbate the progression of AD, AGEs, in a similar manner to Aβ, are able to directly activate inflammatory pathways of astrocytes and microglia. *In vitro* studies have demonstrated up-regulation of the cytokines IL-1β, IL-6, TNF-α and macrophage-colony stimulating factor (M-CSF) in microglia [7, 108] and IL-1β, TNF-α in astrocytes [109, 110]. These responses are at levels comparable to those obtained from fibrillar Aβ [7]. Moreover, glycated proteins also contribute considerably to oxidative stress, as they not only induce a 50-fold increase in ROS and RNS compared to non-glycated proteins [111], but inhibit astrocytic GSH and superoxide dismutase (SOD) antioxidant mechanisms [110].

Receptor for AGEs (RAGE)

It is clear that, despite differences in size, origin and physiological function, Aβ and AGEs share many similar pro-inflammatory properties. One of the reasons for this is the activity of the receptor for advanced glycation endproducts (RAGE). So named because it was the first receptor identified in the binding of AGEs, RAGE is a member of the

immunoglobulin superfamily [112] that, despite its name, has several ligands other than AGEs, including Aβ, S100B and amphoterin.

The binding motifs of the various RAGE ligands have only recently been elucidated. Initially, it appeared that there was little similarity between the ligands, more recent investigations have since demonstrated potential commonalities in relation to RAGE binding. Amphoterin has been found to have a C-terminal binding motif similar to that of S100B [113], while another research group have found some homology between amphoterin and Aβ [114]. Sequence homology however, fails to account for the ability of AGEs to activate RAGE. It has been proposed that the β-sheet structure of a protein, a property common to all of the RAGE ligands including AGEs, is responsible for RAGE affinity and activation [114]. RAGE mediates a variety of cellular responses that depend not only on the identity of the ligand, but often on the concentration of that ligand [115].

It has been accepted for some time that the primary physiological role of RAGE is the induction of neurite outgrowth during embryonic development. Evidence supporting this function is the co-localisation of RAGE and amphoterin in the developing CNS [116, 117], together with *in vitro* studies that have displayed the outgrowth promoting properties of amphoterin [117]. If amphoterin is the physiological ligand for RAGE, it remains to be seen whether AGEs, Aβ and S100B coincidentally bind to RAGE, with aberrant results.

Previously, it was believed that RAGE possessed beneficial functions only during CNS development, as (after completion of development) it is overwhelmingly involved in the exacerbation of inflammation in pathological conditions. More recent data however, suggests that RAGE has a beneficial function in the promotion of neurite regeneration following peripheral nerve injury [118]. This function, combined with the knowledge that RAGE expression on neurons is highly up-regulated in AD [119], indicates that such up-regulation may be an attempt by neurons to induce regeneration of neurites following damage. It is possible however, that this outcome is not achieved and RAGE up-regulation in this environment actually causes exacerbation of cellular stress, which results in further neurite retraction and degeneration [reviewed by 120, 121].

The presence of immunoreactive RAGE, particularly in neuronal supporting cells, is significantly correlated ($R^2>0.6$, $p<0.005$) to the severity of AD, implicating it in the disease pathogenesis [122]. The intimate involvement of RAGE in Aβ-induced neurodegeneration is

further supported by studies with both over-expressing and signal-deficient transgenic mouse models. Respectively, these transgenics exacerbate and attenuate microglia-dependent neurodegeneration [123]. Interestingly, RAGE has recently been found to also induce expression of β-secretase, thereby increasing Aβ generation, further exacerbating plaque burden and inflammatory stimulation [124].

The outcome of RAGE activation in neurite repair or degeneration is likely to be dependant on the ligand that binds to the receptor, as several cellular pathways have been identified in RAGE signalling. Neurite repair occurs via amphoterin or S100B, which induce neurite outgrowth in a Rho GTPase (Rac and Cdc42)-dependant mechanism [125] that involves the phosphorylation of Cyclic AMP-Response Binding protein (CREB) [126]. In AD, the concentration of amphoterin and S100B is far outweighed by Aβ and AGEs. Both of these ligands result in the production of pro-oxidant species and activation of Ras GTPase, instead of Rho GTPases [125], effectors of major importance throughout the course of AD.

Active Ras has numerous downstream effects, many of which have been observed in microglia and astrocytes that, like neurons, display high RAGE expression in AD [109, 119]. The primary effect is a dramatic increase in activity of the transcription factor nuclear factor-κ B (NF-κB), via mitogen-activated protein kinase (MAPK) signalling. NFκ-B, as discussed in the following section, is a transcription factor that causes increased expression of several pro-inflammatory cytokines, enzymes and receptors. One enzyme under the regulation of NFκ-B is NADPH oxidase, which is partially responsible for RAGE-induced oxidant production [127]. The cytoplasmic domain of RAGE is required for signal transduction [125] and thus NADPH oxidase activity, but cells deficient in the cytoplasmic domain of the receptor continue to display oxidant production [115]. This suggests a mechanism of oxidant production independent of RAGE signalling that is yet to be elucidated.

RAGE possesses both intracellular signal-dependant and independent mechanisms of cellular activation. The up-regulation of inflammatory stimuli and production of ROS caused by binding of the various RAGE ligands induces further neurite retraction and degeneration, which is likely to contribute to the progression of AD. Thus, RAGE may ordinarily play a role in the regeneration of nervous tissue following injury, but has quite the opposite effect in AD. Inhibition of RAGE, either directly or indirectly via increased expression of the competitive, soluble, extracellular form, is therefore a possibility in the future treatment of AD.

NFκ-B

Nuclear factor-κ B is upregulated in AD [128] and is a primary link between RAGE and the production of pro-inflammatory signals in microglia and astrocytes. Signalling pathways including mitogen activated protein kinase (MAPK) and Protein Kinase C (PKC) are able to cause the nuclear translocation of NF-κB and subsequent transcription of target genes [125]. This occurs by phosphorylation of Inhibitor kappa B (IκB), the inhibitory protein of NF-κB, causing its dissociation, which exposes the nuclear translocation sequence of NF-κB, allowing its transport into the nucleus [129].

Once nuclear translocation of the p50/p65 NF-κB complex has occurred, the p65 subunit is phosphorylated, which allows binding of the NF-κB complex to consensus sequences. Numerous DNA motifs have been identified as NF-κB binding elements, but the sequence with the greatest affinity for NF-κB is GGGACTTTCC [130]. Moreover, this sequence is the most common within the promoter regions of the more than 50 inflammation-related targets of NF-κB [131].

Of the currently known NF-κB inflammatory targets, those of importance in AD are the cytokines TNF-α, IL-1β, and IL-6, which become involved in a positive-feedback loop, as all three cytokines utilise NF-κB in their respective signalling pathways. The nuclear translocation inhibitor of NF-κB, SN50 and various MAPK inhibitors are able to significantly decrease production of these pro-inflammatory signals in response to RAGE ligands [92], emphasising the importance of RAGE, RAS and NF-κB in microglial activation. This transcription factor and its signalling pathways therefore represent possible therapeutic targets in AD treatment.

Nitric oxide

NO has a variety of known cellular functions, particularly as a second messenger [132, 133] in the regulation of immune responses, neuronal signalling and synaptic plasticity [134], but also as a first messenger [135]. The exact role of NO in the progression of AD remains a topic of debate, however, with conflicting evidence demonstrating both neuroprotective [136] and neurodegenerative [137, 138] properties. Given that high NOS expression correlates with areas of substantial neurodegeneration in post-mortem brains of AD patients [137], it is plausible that NO is initially induced to play a role in neuroprotection, but ongoing over-expression renders it neurotoxic.

Evidence that NO indirectly activates ATP-sensitive K^+ (Potassium) channels via the small G-protein RAS and the mitogen-activated protein kinase (MAPK) pathway provides a theoretical mechanism for neuroprotection [139]. By activating K^+ channels, neuroprotection may be conferred by decreasing membrane potential, thus preventing hyperpolarisation and over-stimulation of neurons. This may be a function of NO early in AD, but there are several neurotoxic mechanisms that are likely to overwhelm any neuroprotective properties. The most notable of these is the induction of oxidant stress, with a concomitant decrease in cellular antioxidant capacity. NO has a short half-life, as it is highly reactive with other molecules. The most prominent being superoxide radicals ($O_2\cdot^-$), a major by-product of the mitochondrial electron transport chain [140]. The combination of these two radicals results in the formation of the powerful oxidant peroxynitrite ($ONOO^-$), which can cause lipid peroxidation, DNA damage and impair mitochondrial activity [141].

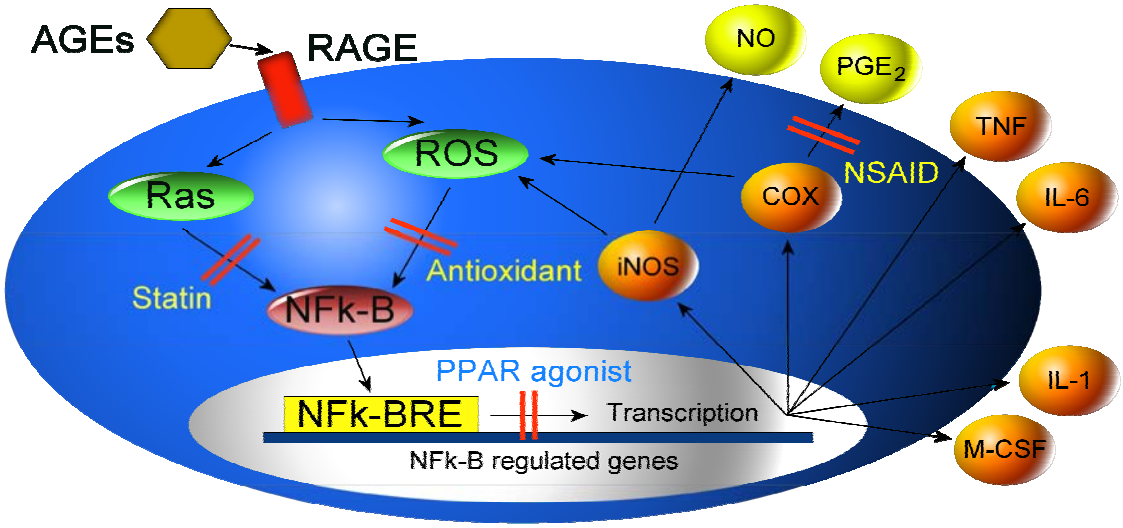

Figure B. Microglial inflammatory pathways in Alzheimer's disease. Microglia are activated by β-amyloid, AGEs or cytokines, causing the production of a variety of pro-inflammatory stimuli. The known targets of NSAIDs, statins, antioxidants and PPAR agonists are also included.

Current Therapy for Alzheimer's Disease

Presently no cure for AD exists, and although significant research is directed at the identification of targets for preventative treatments, existing ones are largely symptomatic and fail to address the underlying pathology of AD. The prime example of this situation is the

acetylcholinesterase (AChE) inhibitor class of drug, which has been used as the standard treatment for AD for several years. An alternative drug which has recently entered the market, an NMDA receptor antagonist, is the first to provide some neuroprotection, and is now becoming widely used in AD.

Acetylcholinesterase Inhibitors

Several transmitter pathways are involved in AD, but it is the cholinergic signalling pathway that is consistently the first to be affected by the loss of synaptic connections, as a result of neurite retraction. Not only are there fewer synaptic connections, but levels of acetylcholine (ACh) are lower in AD brains, making it more difficult for the remaining connections to elicit a response from neighbouring neurons. This reduction in ACh and cholinergic signalling is the basis for current AD therapy.

As acetylcholinesterase (AChE) continues to function at normal rates, the depleted ACh in AD brains may result in a failure of post-synaptic receptor activation. Following its release into neuronal synapses and subsequent signal transduction, ACh is degraded by AChE to its constituents, acetate and choline, which are recycled for further ACh synthesis. Under normal physiological conditions, this enzymatic reaction occurs rapidly so that low levels of ACh can be obtained and synaptic signalling terminated. By inhibiting AChE in AD patients, the concentration of ACh in neuronal synapses increases, thereby increasing the likelihood of activating post-synaptic receptors.

Acetylcholinesterase inhibitors have been used to treat AD since the release of Tacrine in the mid 1990's. This type of therapy provides modest outcomes for patients, with a slight improvement in cognition that is stable for approximately one to two years, depending on the severity of AD at time of administration. Tacrine has since been removed from use due to hepatotoxicity and superseded by Galantamine and Rivastigmine, which have displayed greater efficacy, for up to 5 years for the latter [142].

Both Rivastigmine and Galantamine are known to possess properties other than the inhibition of AChE. Concentrations of AChE can decrease by as much as 45% in AD brains, whereas butyrylcholinesterase (BuChE) concentrations increase by as much as 90%, particularly in close proximity to senile plaques [143-145]. Thus, for Rivastigmine, it is likely that increased efficacy is because of selective inhibition of BuChE. Alternatively, Galantamine is able to allosterically modulate nicotinic acetylcholine receptors (nAChRs). It

is believed that this modulation enhances the ability of ACh to bind to and elicit a response from nAChRs, thus further assisting in cholinergic signalling. Despite improvements in efficacy, AChE inhibitors do not provide neuroprotection and an eventual loss of cognitive ability is inevitable.

NMDA Receptor Antagonists

Excitotoxicity in AD is a result of N-methyl-D-aspartate (NMDA) receptors responding to the excessive levels of the excitatory neurotransmitter glutamate. This leads to over-stimulation of neurons, resulting in uncontrolled signalling and eventually neurodegeneration. The most recent drug class to be used in the battle against AD are antagonists of NMDA receptors that attempt to prevent over-stimulation of neurons and therefore decrease cellular stress and degeneration.

Under physiological conditions, magnesium (Mg^{2+}) functions as an endogenous, voltage-dependant blocker of NMDA receptors. High trans-membrane voltage-dependency and low affinity for the receptor means that in AD, continuous stimulation of NMDA receptors causes a failure in the Mg^{2+} block [For review see 146]. Dizocilpine is one of several high-affinity, non-competitive NMDA receptor antagonists that is able to replace Mg^{2+} as the ion channel inhibitor. The high affinity of such compounds, however, causes significant side effects, including hallucinations, paranoia and motor retardation, which contradicts their clinical use [147].

It has been known since the late 1980's that Memantine is an antagonist of NMDA receptors [148], but it took until 1993 to demonstrate that, unlike high affinity NMDA antagonists, Memantine replaces Mg^{2+} with similar voltage-dependency [149]. This enables the receptor to fulfil physiological functions, whilst filtering out 'synaptic noise' associated with excessive pathological stimulation. It also results in fewer side effects and can be used in conjunction with AChE inhibitors, rendering it more useful in the clinical setting, where it is currently used in patients with moderate to severe AD.

There exists vast *in vitro* evidence to support the neuro-protective effect of Memantine in the presence of Aβ, excessive NMDA receptors and energy depletion, which all occur in AD [150-152]. *In vivo* efficacy was demonstrated in pre-clinical trials, with modest benefit in moderate to severe AD patients in relation to cognition and tolerability [153, 154]. More recently it has also been demonstrated to decrease hippocampal glutamate levels via brain

imaging *in vivo* [155], which subsequently delayed neuro-degeneration. Memantine was approved in the U.S.A. for the treatment of AD in October 2003 and is becoming a frequent treatment option for moderate to severe cases, despite questions regarding its efficacy [156].

Future Targets for Alzheimer's Disease

Current treatment for AD begins at the time of diagnosis and temporarily increases cognitive functions, but does not target the pathological cause of the disease and therefore fails to slow its progression. The future of AD therapy lies in remedies that prevent the progression of AD via neuroprotective mechanisms. The numerous factors that contribute to the complex pathology of AD provide many targets for such neuroprotective treatments, as discussed in the following section.

β-amyloid

As the pathological protein common to all AD cases and with no apparent physiological functions in later life, Aβ is an ideal target for therapeutic intervention. There are several approaches that could theoretically result in lower levels of Aβ, including antibody targeting, altered processing and β-sheet breakage.

Targeting Aβ with antibodies, by either direct injection of specific antibodies or synthetic Aβ peptides, was seen as a likely therapy in the mid 1990's. By removing the primary inducer of inflammation, neurodegeneration in patients diagnosed with AD was expected to cease and in some cases, neurite outgrowth be promoted. Such a response would either slow the progression of the disease, or possibly reverse previous cognitive deficits.

This approach was expected to not only increase the immune response to the protein, but to switch the response from innate, cell-mediated to humoral, antibody-mediated. As previously mentioned, the antibody-associated cytokine IL-4 has displayed neuroprotective properties *in vivo*, suggesting the switch to a humoral response is a favourable one. Results from animal studies demonstrated promising results, with the administration of anti-Aβ antibodies in PDAPP mice leading to a rapid reduction in cerebral amyloid plaques and an equally rapid improvement in cognition [157, 158]. A clinical study of anti-Aβ antibody therapy also displayed benefit for AD patients, but was quickly withdrawn, due to dramatic side-effects in a small number of subjects, specifically vascular meningeal inflammation in 18 of the 300 subjects [159]. Despite this setback, studies into vaccination have continued in mouse models of AD. A recent report found that both Aβ and MAP-Tau protein levels are

decreased in well-progressed disease subjects and memory deficits could be reversed, although microhaemorrhage remained a significant issue [160].

Subsequent research has found that antibodies directed at the amino-terminal sequence of Aβ have a 1000-fold higher affinity for fibrillar Aβ than monomeric peptides, but carboxy-terminal antibodies favour monomeric peptides, possibly due to the C-terminus of the peptide being buried in the interior of amyloid fibrils [161]. It is possible that such a preference may lead to binding of antibodies to plaques that can not be removed, leading to excessive antibody-mediated inflammation. Aβ targeting with monoclonal antibodies with preference for monomeric peptides is still possible and research into this type of therapy is ongoing, with promising results [for review see 162].

A second mechanism that may be employed to decrease the amount of Aβ in the brain is the alteration of APP processing. By altering secretase activity, non-toxic and non-fibrillar forms of Aβ can be produced, instead of Aβ1-40 or Aβ1-42. Although yet uncharacterised, α and γ-secretases may be important, particularly in the generation of soluble APP fragments, which may have a physiological role [163]. Of the three secretases involved in APP processing, β-secretase (BACE1) appears to be the most acceptable as a therapeutic target and has not been indicated in any significant physiological functions, with BACE1 knockout mice demonstrating normal development and behaviour [164, 165]. A recent study however, indicates that caution is warranted. Double transgenic BACE knockout mice on an AD prone background displayed dramatic memory and sensory deficits, combined with seizures, indicating that BACE may also be required for normal APP processing [166].

To date, several inhibitors of BACE1 have been developed, but are not applicable to the clinical setting. These inhibitors are peptidomimetic sequences that act as transition state analogues, preventing APP from binding to the active site of the enzyme [167]. The problem with such inhibitors is that they are expensive to produce, have low stability, poor oral bioavailability and are unlikely to cross the blood-brain-barrier [168]. These problems have recently been overcome and have displayed promise in animal models [169, 170].

As previously mentioned, fibrillar Aβ is generally more neurotoxic than monomeric forms of the peptide and is highly resistant to macrophage and protease attacks. By preventing β-sheet formation, or breaking previously formed fibrillar-Aβ structures, neurotoxicity may

decrease and clearance of Aβ by microglia may increase. Thus, β-sheet or crosslink breakers are being assessed for their efficacy in AD.

β-sheet breakers are short peptides corresponding to the 'core' of the Aβ sequence, which block Aβ cross-linking and therefore prevent plaque formation [171]. These peptides are either modified to contain alternating *N*-methyl residues that eliminates the hydrogen bonding essential for cross-linking, or do not contain residues 25-35, which are believed to be the most toxic [172, 173]. Until recently however, these peptides had difficulty crossing the blood-brain-barrier. By substituting amino acid side chains with the naturally occurring polyamines, the ability of peptides to cross the blood-brain-barrier increases dramatically and has been used successfully in inhibiting Aβ plaque formation in the brain [174-177]. Unfortunately, little information on the progression of trials with this type of therapy has been available in recent years.

Another possible AD-preventative therapy related to Aβ is alteration of soluble Aβ. The soluble form of Aβ is non-toxic, crosses the blood brain barrier and acts as a 'sink' for Aβ in the periphery. Studies have found that by increasing the size of the 'sink', via antibody-mediated targeting or Low-density lipoprotein receptor related protein-1-mediated inhibition, Aβ load in the brain is reduced [178, 179]. It has been hypothesized that by decreasing soluble Aβ levels in the periphery, more Aβ crosses the blood brain barrier to maintain equilibrium, thereby decreasing fibril and plaque formation in the brain. This therapy, whilst in early stages of research, is especially promising, due to peripheral, as opposed to brain-specific treatment. Potentially, this could reduce severe side-effects, such as haemorrhages observed in other brain-specific antibody treatments.

Advanced Glycation Endproducts

The inflammatory capacity and contribution of AGEs in the progression of AD is yet to be fully understood. There is no doubt however, that decreasing the degree of glycation in the brain will reduce Aβ cross-linking and weaken the cellular response, thereby assisting in the survival of neurons. Several classes of AGE-inhibitors have been identified, with the main inhibitors of interest being aminoguanidine, carnosine, pyridoxamine and tenilsetam [180-182].

Reactive dicarbonyls, such as glyoxal and methylglyoxal, are common substrates for the production of AGEs. Aminoguanidine and carnosine are both believed to, at least partly,

prevent AGE formation by scavenging free dicarbonyls before they are able to attach to proteins. A large body of evidence supports the anti-crosslinking and neuroprotective abilities of these compounds, but the exact mechanisms are poorly understood. Studies with various truncated and modified forms of carnosine has demonstrated that the α-amino group of histidine is the most significant in crosslink inhibition, with imidazolium groups providing stabilisation of complexes [183]. This knowledge may assist in the refinement of AGE breaking compounds.

Scavenging of dicarbonyls, although effective at preventing the formation of AGEs, does not have an effect on AGEs already present. A new class of 'AGE-breaker' was discovered, in the thiazolium compounds, which destroy the chemical crosslink structure of AGEs [184]. Although the mechanism of crosslink breakage is not fully understood, these compounds also decrease AGE production through chelating and antioxidant activities [185]. Alteration of the structure has increased the stability, bioavailability and efficacy of thiazolium compounds such as ALT-711, which has shown greater promise in cardiac and diabetic clinical trials than dicarbonyl scavengers [186-189].

Transcription Factors

Transcription factors involved in the inflammatory process are candidate therapeutic targets that may be particularly useful after diagnosis, when inflammatory factors are already present. By regulating the transcriptional activity of NFκ-B or peroxisome proliferator-activated receptors (PPARs), it may be possible to reduce microglial and astrocytic activity and subsequent production of inflammatory factors.

Despite indications from initial research, NFκ-B has been found to have several physiological functions in cell proliferation, differentiation, oncogenesis, synaptic signalling, learning and memory [190, 191]. The primary function of this transcription factor however, is related to inflammatory responses, providing an ideal therapeutic target. Such intervention may provide significant benefit in AD, given that neurological damage can be correlated to NFκ-B activity [192]. There is also increasing evidence however, that inhibition of NFκ-B, through either direct or indirect mechanisms, confers neuroprotection, particularly when neurons are under excitotoxic stress [193-195].

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily, transcription factors that are upregulated in AD [196]. Once activated,

PPARγ and PPARα act via transcriptional repression of pro-inflammatory genes, including STAT1, AP-1 and NF-κB [197, 198]. Thus, agonising these receptors is an indirect method of down-regulating NFκ-B and related factors, with several added advantages, including the alteration of APP processing, by undefined mechanisms [199]. Studies in transgenic mouse models have demonstrated that PPARs are intimately involved in the progression of AD. Injection of a PPAR antagonist into the cerebellum, a brain region not normally affected by Aβ accumulation and neurodegeneration, significantly increased Aβ burden and decreased motor function [200].

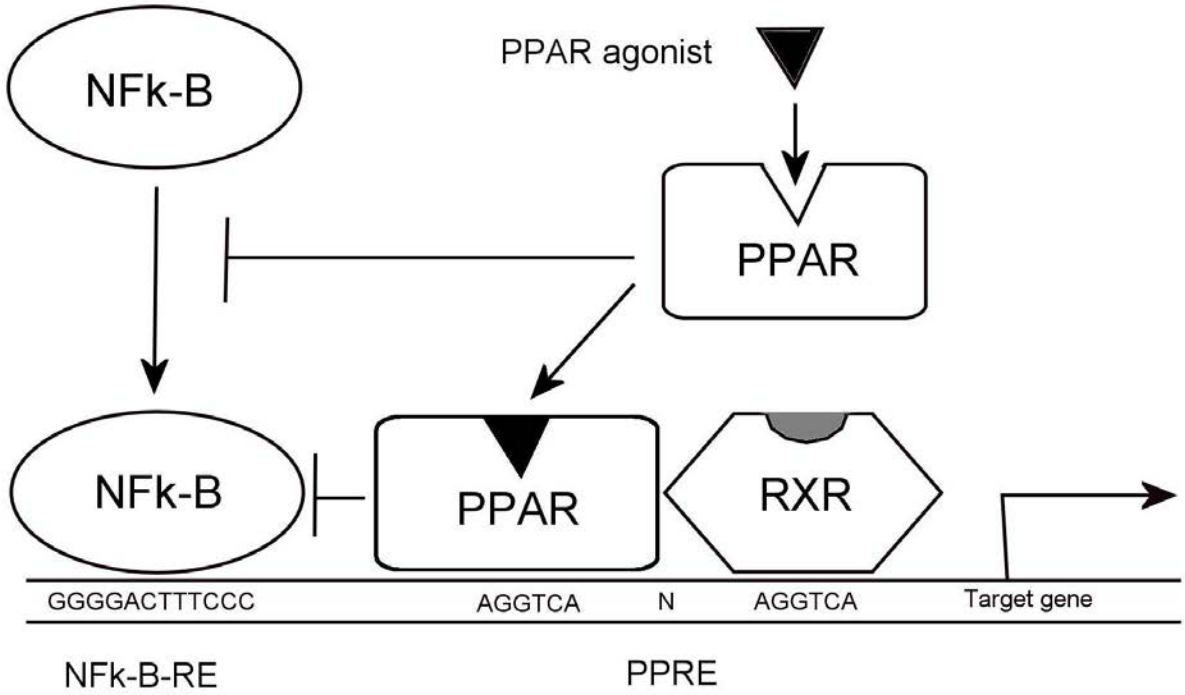

Figure C. Inflammatory inhibition by peroxisome proliferator-activated receptors. Following PPAR agonist binding, PPARs dimerize with Retinoid X Recetors and bind to DNA concensus sequence, thereby inhibiting transcription of inflammatory sequences.

Thiazolidinediones or glitazones, agonists of PPARs, were initially developed for the treatment of type 2 diabetes as they were identified as insulin-sensitising compounds [201]. They are now commonly used as therapeutic agents and have demonstrated efficacy and safety, even in elderly patients [202]. Moreover, PPAR agonists are often used safely in combination with other therapeutic agents, which is a further benefit in relation to the treatment of the multifactorial AD. Alone, agonists of PPARs, both synthetic and naturally occurring, have demonstrated neuroprotection *in vitro* and in animal models [203-206], and

more recently has displayed cognitive improvements and stabilisation of plasma Aβ40:42 ratio in sufferers of mild AD in a 6 month clinical trial [207].

G-protein Signalling

The Ras family of small G-proteins are often associated with human tumours and although involved with numerous physiological functions, appear to be non-essential or functionally redundant, as knockout mice are viable [208]. The inhibition of Ras in AD may therefore provide beneficial outcomes. Ras is activated by several mechanisms, namely RAGE, oxidant stress and via an NMDA receptor-dependant mechanism [209, 210] and is responsible for a significant portion of NFκ-B activation in AD [210]. Thus, the effect of Ras down-regulation on microglial and astrocytic activation will likely be similar to that achieved by NFκ-B and PPAR targeting. Inhibition of Ras also provides direct neuroprotection during excitotoxicity via a mechanism that is not fully understood [209].

The Rho family of G-proteins are not involved in RAGE signalling or the activation of NFκ-B, but inhibition may also provide beneficial effects in AD. Rho is known to function in actin remodelling, specifically, the inhibition of neurite formation [211, 212]. Since neurite retraction in AD is a major factor in cognitive decline, the inhibition of Rho may improve the regeneration of dendrites and synaptic connections. More importantly, research indicates that Rho activation induces APP processing and Aβ production via Rho-associated kinase (ROCK) pathway [213, 214]. *In vivo*, Rho inhibition may therefore provide neuroprotective benefits via a reduction in levels of Aβ production, resulting in fewer plaques and diminished cellular stress.

Anti-inflammatory Drugs in the Prevention of Alzheimer's Disease

The term 'anti-inflammatory' covers a wide range of drug classes and is not restricted to compounds that specifically target inflammatory processes. Drug classes of interest include Non-Steroidal Anti-Inflammatory Drugs (NSAIDs), statins and antioxidants, which may have different primary targets, but possess common anti-inflammatory properties.

Several studies, particularly the epidemiological Rotterdam Study that focussed on the use of anti-inflammatory medications in approximately 7,000 subjects, have demonstrated that anti-inflammatory medications decrease the prevalence of AD [215-218]. Such an effect is not surprising, given that the mechanisms of inflammation in AD are similar to those present in several other chronic inflammatory conditions such as arthritis and artheriosclerosis [219, 220]. Medications that appear to be likely candidates for the prevention of AD belong to the drug classes of Non-steroidal anti-inflammatory drugs (NSAIDS), statins and antioxidants.

A more in-depth epidemiological study by Vlad and colleagues found that only selected compounds from the NSAID class provided modest protection against the onset of AD [221]. This finding provided new insights into both the pathology of AD and specific cellular mechanisms of significant disease relevance. The outcomes indicate that the drug targets of importance in relation to prevention are not the primary therapeutic mechanism of these drugs.

Non-steroidal anti-inflammatory drugs

As one of the most commonly used classes of therapeutic drugs in the world, Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used as treatments for a variety of inflammatory conditions, particularly arthritis [222]. NSAIDs, in the form of natural remedies, have been used for centuries as therapeutic agents, with Bayer marketing Aspirin as the first commercially available drug in 1899. The anti-inflammatory properties of NSAIDs stem from the inhibition of cylooxygenase (COX) enzymes, which normally catalyse the production of prostaglandins [223].

By inhibiting the activity of COX enzymes, NSAIDs can significantly decrease the level of prostaglandin synthesis, leading to a general decrease in inflammation, as prostaglandins are then unable to potentiate any inflammatory responses [224]. Over time however, prolonged use of traditional NSAIDs commonly leads to gastrointestinal disruptions including ulcers and bleeding. This side-effect occurs through inhibition of COX-1 that normally provides mucosal protection in the intestines [225, 226]. It was discovered that the COX-2 isoform was not involved in gastric homeostasis, but is commonly associated with inflammation, which lead to the development of COX-2 specific inhibitors, such as celecoxib and rofecoxib [227]. These COX-2-selective compounds however, have since been removed from the market due to safety concerns.

NSAIDs have demonstrated an ability to provide a protective effect against the onset and development of AD that increases with the duration of therapeutic NSAID use for alternate conditions [216, 228]. Significant epidemiological evidence, including that obtained in the Rotterdam [215] and Rochester [229] studies supports the general neuroprotective nature of NSAIDs, however clinical trials with several drugs from this class have provided conflicting results. Complete reviews of epidemiological, clinical and transgenic studies with NSAIDs in AD have been published by Imbimbo and McGeer and McGeer [230, 231].

For some time, significant evidence, mainly the high expression observed in neurons, pointed towards the involvement of COX-2 in the progression of AD [232, 233]. This theory has been somewhat quashed, with clinical trials of the COX-2 inhibitors celecoxib and rofecoxib, failing to demonstrate therapeutic benefit for AD patients [234-236]. These two drugs merely add to the long list of traditional NSAIDs that have been trialled and failed in AD treatment, including naproxen and diclofenac [236, 237]. These results suggest that COX inhibition does not account for any neuroprotection conveyed by NSAIDs and that other mechanisms must be responsible.

It is now recognised that many of the anti-inflammatory properties of NSAIDs, not only in AD, are due to novel side effects, which target cellular pathways other than the inhibition of COX [238, 239]. Neuroprotection appears to be consistently conveyed by three NSAIDs; indomethacin, sulindac sulphide and ibuprofen [215, 240]. A focus of much research is therefore directed at the identification of cellular targets unique to these three NSAIDs. Although there are numerous possibilities, two mechanisms have been proposed as

likely candidates in neuroprotection by these NSAIDs; the alteration of APP processing and activation of PPARs.

Secretion of Aβ1-42 is increased in neuronal cells exposed to COX-2 selective NSAIDs, while non-selective forms decrease the secretion of both Aβ1-42 and Aβ1-40 in a concentration-dependant manner [241]. More interesting however, is that of the non-selective NSAIDs, ibuprofen, indomethacin and sulindac sulphide down-regulate Rho-dependent Aβ1-42 production by 50-65%, while other non-selective drugs show no inhibition [213]. This down-regulation of Aβ production is also correlated to the inhibition of Rho activity, providing another target of interest for NSAIDs in the prevention of AD [213].

It is possible that, as well as the inhibition of Rho, decreasing Aβ production is due to the PPAR agonist activity of certain NSAIDs. PPARs are able to increase APP degradation by ubiquitylation of APP and inhibition of secretase activity, with both mechanisms resulting in lower Aβ production [199, 242]. Interestingly, ibuprofen, indomethacin and sulindac sulphide, the NSAIDs that decrease Aβ production, are known ligands of PPARs [198, 243, 244]. As previously mentioned, PPARs are involved in numerous anti-inflammatory pathways and it is for this reason that it has been argued that the activation of PPARs is not merely an additional action of NSAIDs, but the primary anti-inflammatory mechanism of these drugs [245].

The evidence supporting the use of NSAIDs in AD suggests that the inhibition of COX is not an important factor in neuroprotection. The complex pathways involved in the progression of AD and the multiple cellular targets of NSAIDs has made it difficult to pinpoint the mechanisms that provide neuroprotection. Research into the neuroprotective ability of NSAIDs is of great importance. Not only will it assist in the development of better treatments for AD, but NSAIDs already in use have established therapeutic data and can be used immediately for AD treatment, although precautions must be taken to avoid gastric toxicity following long-term use.

Statins

The statin class of drugs are used predominantly in the treatment of hypercholesterolemia [246]. Their primary action is the inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the enzyme that regulates the synthesis of cholesterol [247]. Hypercholesterolemia has been found to increase glial activity and

neurodegeneration in transgenic mice [248], so it is not surprising that statins have been earmarked as preventative against AD in epidemiological studies, as reviewed by Ekert and colleagues [249]. Evidence continues to mount however, that although the inhibition of HMG-CoA reductase is likely to assist, the side effects of statins are likely to play a substantial role in neuron survival [217].

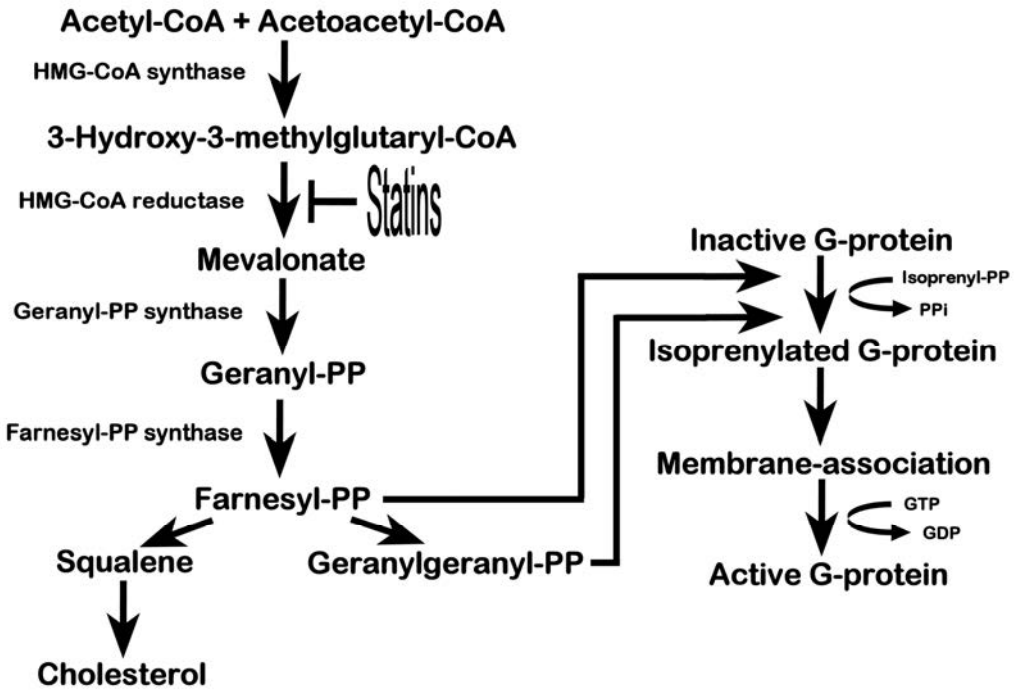

Figure D. Isoprenoid synthesis inhibition by statins. Statins inhibit the cholesterol pathway, which provides precursors for the isoprenoid intermediates involved in membrane targeting of proteins.

As a consequence of decreasing cholesterol via HMG-CoA reductase inhibition, statins may cause the previously discussed inhibition of Ras and Rho small G-protein signalling. Mevalonate is an intermediate of the cholesterol biosynthetic pathway and is a precursor for the isoprenoid lipids. Isoprenoid lipids are involved in the post-translational isoprenylation of several proteins, including the Ras and Rho family of proteins [250]. Isoprenylation usually occurs on a cysteine residue at the C-terminal end of the protein and is required for proper G-protein membrane localization and protein-protein interactions [251, 252]. In the absence of isoprenoid lipids, Ras and Rho function may be depleted dramatically, as isoprenylation can not occur, rendering them unable to associate with the cell membrane and become activated by RAGE or other inflammatory receptors. Again, the inhibition of Ras and Rho leads to decreased NFκ-B activity and lower Aβ production.

There is data that suggests the inhibition of isoprenylation is the primary factor in reducing microglial activation [253]. An overall decrease in cholesterol however, is likely to have a number of beneficial effects aside from microglial down-regulation. Although there is currently no clinical evidence using high cholesterol diets, this is supported by an increase in AD-like pathologies and memory deficits in rabbits and mice fed high cholesterol diets [248, 254-256]. In particular, the distribution of lipid rafts in neuronal membranes decreases under low cholesterol conditions, as is the case with statin therapy. It is apparent that lipids rafts are intimately involved in APP processing and Aβ accumulation and oligomerisation [257, 258]. Not only are high levels of Aβ multimers found in lipid rafts, but β-secretase is enriched, which preferentially cleaves proximal APP over α-secretase [259, 260]. Thus, a decrease in cholesterol and therefore lipid rafts may cause a reduction in both toxic Aβ and aggregated Aβ [259].

It is plausible that statins possess numerous side-effects that are yet to be isolated, such as that which directly protects neurons against NMDA-dependant excitotoxicity [261]. One side-effect that has already been well documented and which some anti-inflammatory activity can be attributed to, is the ability to activate PPARs [262, 263]. This knowledge provides a common factor between statins and the NSAIDs that are effective in AD prevention. This connection has been a major factor influencing the investigation of PPARs in neuroprotection. Research that investigates the neuroprotective effects of statins and NSAIDs, with comparisons of known side-effects profiles may provide important links to neuroprotective mechanisms.

Antioxidants

The term ‘antioxidant’ covers a broad range of substances. The common action of these substances is the scavenging of extracellular and, depending on their membrane-permeability, intracellular free radicals including reactive oxygen (ROS) and reactive nitrogen species (RNS) [264]. There are numerous naturally occurring antioxidants, with a majority being consumed through diet, but many are available as complementary medicines, with examples being fruit-derived flavenoids, vitamins C and E, *Ginkgo biloba*, α-lipoic acid and β-carotene. The brain is under significant oxidative stress in AD, so it is not surprising that antioxidants such as these are a possible treatment option [265]. Recent clinical studies with the antioxidants *Gingko biloba* and curcumin contradict this data and found no benefit to AD patients [266, 267]. Several aspects of the methodologies employed in these trials however,

which are a common issue in clinical AD studies and will be discussed later in this report, mean that antioxidants remain a possible preventative therapy for AD.

Several steps in the AD inflammatory pathway involve the production of free radicals such as superoxide and nitric oxide [268, 269]. The production of these radicals may play a more dominant role in the progression of AD than previously conceived [210]. ROS and RNS have several detrimental effects, including DNA and cell membrane damage, mitochondrial inhibition and the activation of redox-sensitive transcription factors such as NFκ-B, effects that all exacerbate the excessive inflammation in AD [8].

Oxidant damage alone is able to account for some neurodegeneration that occurs in AD [270]. The neuroprotective properties of several antioxidants that displayed prevention of AD, including vitamins E and C [271, 272], α-lipoic acid [218] and *Gingko biloba* [273] were therefore perceived to be a result of free radical scavenging. Results from alternative studies contradict this data, suggesting that although radical scavenging may provide some neuroprotection, effective prevention of AD requires secondary mechanisms of antioxidants [274].

Some antioxidants are known to possess side-effects, supporting the involvement of secondary factors in AD prevention. α-Lipoic acid, one of the effective antioxidants, is known to inhibit degradation of the inhibitor of NFκ-B ($I_\kappa B$) and also directly inhibit NFκ-B in a DNA-binding-dependant manner [275, 276]. The most notable antioxidant with anti-inflammatory capabilities is curcumin, a tumeric-derived curry spice that is believed to be responsible for the low prevalence of AD in India [277]. Curcumin shares some traits with α-Lipoic acid, including the inhibition of NFκ-B in DNA binding and IκB-dependant mechanisms, but has several other beneficial characteristics, as it increases production of the antioxidant glutathione [278] and directly inhibits Aβ aggregation and plaque formation [279]. The most intriguing characteristic of curcumin however, is that it is an agonist of PPARs [280-282]. Given that curcumin is proposed to possess remarkable neuroprotective properties, its ability to activate PPARs considerably strengthens the likelihood that PPAR activation conveys neuroprotection.

Article 1

Optimizing the Generation of Stable Neuronal Cell Lines via Pre Transfection Restriction Enzyme Digestion of Plasmid DNA

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Abstract

Transfection of mammalian cell lines is a widely used technique that requires significant optimization, including transfection method or product used, DNA vector, cell density, media composition and incubation time. Generation and isolation of stable transfectants from the large pool of untransfected or only transiently transfected cells can be laborious and time-consuming. Transfection of DNA is usually performed with a non-linearized plasmid, since it is assumed that cutting the plasmid beforehand leads to a lower efficiency of transfection or the degradation of linearized DNA by cytosolic nucleases. However, the transfected circular plasmid will be linearized by a random cut within the cell and it might be possible that sensitive parts of the plasmid such as the resistance gene or the gene of interest are destroyed upon linearization. On the other hand, linearizing a plasmid before transfection by a single, defined cut with a selected restriction enzyme in a non-coding area of the gene has the advantage of ensuring the integrity of all necessary gene elements of the plasmid. In this study, we transiently transfected and subsequently performed stable clone selection using uncut vector and vector digested with two distinct restriction enzymes in the Neuro2a neuronal cell line. We report that linearization of plasmid DNA prior to transfection can increase the efficiency of stable clone generation by approximately double and can also increase expression of the target gene. This activity however, is dependant on the site of linearization within the vector, with the optimum restriction enzyme for pEGFP-N1 stable clone formation found to be BsaI.

Introduction

Transfection, the introduction of foreign DNA into mammalian cells, is a widely used technique in molecular and cellular biology. Several methods, including calcium phosphate precipitation/transfection, electroporation and liposome-mediated transfection, allow foreign DNA to pass through the lipid bilayer membrane of mammalian cells. Any cell that harbors foreign DNA not incorporated into the chromosomes is transiently transfected, whereby the DNA is able to be transcribed, but cannot be not copied and therefore will be degraded over time and diluted during mitosis. Transient transfection is a useful tool, primarily used in short-term reporter assays. It is often a necessity however, to obtain a cell line that continually expresses the foreign gene of interest, known as stable transfection, which requires the integration of the foreign DNA into the chromosomal DNA of the host cell. It is well documented that the efficiency of transient transfection, particularly for liposome-mediated transfection, is cell-specific and affected by several factors. Transfection efficiencies of approximately 20-30% are generally observed, but can be optimized to achieve efficiency rates of 70-98% [1-5]. Stable transfection however, relies on insertion of the foreign DNA into the genome, a process which occurs infrequently, thus resulting in low numbers of stable clones.

The topology of DNA is known to affect transfection efficiency, as supercoiled or open-circular DNA provides greater efficiency than linear DNA [6]. Greater transfection efficiency of circular DNA potentially increases the chance of stable integration, but through a random cut in the vector, stable clones that do not express the gene of interest could be generated. Green Fluorescent Protein (GFP) has previously been shown to be an effective method for quantifying transient efficiency [7]. Thus, in this study, we used the enhanced-GFP-expressing vector pEGFP-N1 to measure both transient and stable transfection efficiency of Neuro2a and HT22 cells with circular/supercoiled and linearized vector by microscopic analysis. We demonstrate that although linearized DNA may result in similar transient transfection efficiency, it gives rise to a greater number of stable transfected cells.

Methods

Cell Culture

Murine Neuro2a and murine HT22 cells (both obtained from the University of Leipzig, Germany) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% foetal calf serum (FCS), supplemented with penicillin (200U/ml), streptomycin (200µg/ml) and fungizone (2.6µg/ml) (all GIBCO). Cells were maintained at 37^{o}C, containing 5% CO_2, in a humid environment. Cells were removed from flasks using rubber scrapers (Sarstedt) and counted in a counting chamber (Neubauer), then dispensed at a density of 1.2 x 10^6cells/well in 6-well plates (Sarstedt). The cells were then grown for 24 hrs in antibiotic-free DMEM containing 5% FCS prior to transfection.

Vector preparation

The 4.7kb vector, pEGFP-N1 (Clontech) was purified using the Pureyield Plasmid Midiprep System (Promega). DNA purity was >1.6, as determined by the spectrophotometric 260:280nm ratio. In duplicate, 2.5µg of pEGFP-N1 was cut with BsaI or SspI (New England Biolabs) and the appropriate buffer for 2 hours at 37^{o}C. A pseudo-digestion, containing buffer only, was also performed as uncut control. Restriction enzymes were then inactivated at 65^{o}C for 15 minutes and a sample run on a 1% agarose gel with untreated DNA as control, to confirm vector digestion. Three independent experiments in duplicate were performed (n=6).

Transfection

Transfections were performed using Lipofectamine-LTX (Invitrogen). Each 2.5µg of linearized and enzyme inactivated DNA was diluted to a volume of 500µl with FCS and antibiotic-free Opti-mem (Invitrogen), then combined with 2.5µl of Plus Reagent at room temperature for 10 minutes. 6.25µl of Lipofectamine-LTX was then added and allowed to complex at room temperature for 90 minutes. This extended incubation time was employed to ensure complete complexation of all fragments in the presence of added protein contamination post-digestion. Antibiotic-free DMEM containing 5% FCS was then added to a final volume of 2ml, before addition to the cells in 6-well plates. Cells were transfected at 37^{o}C for 24 hours before the Lipofectamine-containing media was replaced with fresh DMEM containing 5% FCS.

Analysis of transient transfection

24 hours after removal of the Lipofectamine-containing media, cell number and eGFP expression were measured using an FL MZIII Stereomicroscope (Leica). Images of three

randomly selected fields in each well were captured at 10x magnification, under both bright and UV light with an eGFP filter. Images were analyzed using Cell Profiler software and the number of eGFP-positive and negative cells were then counted. Fluorescent intensity of transfected cells was determined using Image J software V1.41g (NIH).

Analysis of stable transfection

Following transient transfection analysis, cells were exposed to 600 μg/ml Geneticin (GIBCO) in the original 6-well plate for 3 days. Cells of each well were then removed by scraping and transferred to a 10cm cell culture dish containing 900μg/ml of Geneticin for 10 days, with media changed every 3 days. The number of eGFP-positive and negative colonies was then counted using the MZIII Stereomicroscope (Leica).

Statistical analysis

Results were graphed and analyzed using Prism 4 (Graphpad Software). A one-way ANOVA and Dunnett's post test were performed using transfections with uncut vector as controls. Independent experiments were performed three times in duplicate (n=3) and errors bars represent the Standard error of the mean (SEM).

Results

Transient transfection efficiency

In this experiment, we wanted to determine whether transient transfection efficiency was affected by previous linearization of the pEGFP-N1 vector. 2.5μg of vector was either left uncut or was linearized with BsaI or SspI. These two enzymes were selected because neither of them would cut essential components of the vector, necessary for transcription in mammalian cells and would also result in blunt ended fragments. BsaI cuts at position 3746bp and SspI at 1665bp and 2218bp (Figure 1). Linearization was confirmed by electrophoresis (data not shown), following heat inactivation of restriction enzymes. The plasmids were then transfected into 1.2 x 10^6 Neuro2a or HT22 cells for 24 hours using Lipofectamine-LTX in 6-well plates. After a further 24 hours with media change, three random fields of each well were imaged at 10x magnification using a fluorescent stereomicroscope and eGFP-expressing cells counted using Cell Profiler software.

There was a marked difference between the two cell lines. Transfection efficiency was significantly higher in HT22 cells than Neuro2a cells, with 15.5% and 4.5% average efficiency for uncut vector (Fig. 2). There were also differences in efficiency between uncut and cut vectors. However, there was no clear transfection advantage for the circular plasmid compared with the linearized plasmid. Whereas the uncut plasmid provided the greatest efficiency for Neuro2a cells, digestion with BsaI resulted in the greatest number of transiently transfected HT22 cells. It remained consistent between the two cell lines however, that digestion with SspI decreased transfection efficiency (Fig. 2). Examination of the fluorescent images also demonstrates that cutting with BsaI, but not SspI, results in brighter fluorescence than undigested vector, as depicted in Figure 3.

Stable transfection efficiency

In this experiment, we aimed to determine whether the generation of stable clones was affected by previous linearization of the pEGFP-N1 vector. Following determination of transient transfection efficiency, Neuro2a and HT22 cells were treated with 600μg/ml of Geneticin for 3 days, then 900μg/ml for 10 day. eGFP-expressing colonies were then counted using a stereomicroscope with and without a GFP filter. No colonies were observed for any of the transfections performed using Neuro2a cells. The HT22 cells, however, yielded a significant number of stable colonies expressing eGFP. The highest number of eGFP-expressing HT22 colonies observed, from the initial 1.2 x 10^6 cells transfected, was 30, arising from cells transfected with BsaI-digested vector in a single well of a 6-well plate. This

was significantly higher than stable colonies resulting from either uncut vector or vector linearized with SspI, which obtained a maximum of 11 and 3 colonies per well respectively, in a single experiment. After transient transfection efficiency is taken into account, Fig. 4 demonstrates that stable integration of BsaI digested vector was significantly greater than and approximately twice as efficient as undigested vector and was nearly four times more efficient than SspI digested vector. Despite the differences in eGFP-expressing colonies between the three digestion treatments, the number of non-fluorescent stable colonies was similar. The overall efficiency of each transfection treatment is depicted in Fig. 5, where it is evident that given the same number of cells and transfection conditions, digestion of pEGFP-N1 with BsaI prior to transfection of HT22 cells, is three times more efficient than transfection with undigested vector and twelve times more efficient than prior digestion with SspI.

Discussion

The generation of clonal cell lines is a vital technique that can often be laborious and time consuming. Several factors, including cell type, transfection technique, carrier vector and the desired sequence can all dramatically affect both transient and stable transfection efficiency. Previous studies have found that little or no transfection was obtained with linear DNA [8]. During the development of neuronal high throughput screening assays, we have found that efficient transient transfection of a neuronal cell line can be achieved with linear DNA and also increases the number of stable integrations. Both of these events however, are dependant on the site of vector digestion. BsaI digests pEGFP-N1 immediately prior to the mammalian eGFP promoter, in the pUC bacterial origin, while SspI linearizes the vector by cutting at two sites between eGFP and the antibiotic resistance gene, in the f1 bacterial origin, giving rise to a small 550bp secondary fragment. This fragment will likely compete for liposomal complexation and transfection with the larger eGFP encoding fragment, which may have caused the observed decrease in transient transfection efficiency with SspI. Moreover, digestion immediately following the encoded eGFP protein may inhibit expression and therefore transfection detection. Such an effect may, in part, explain why von Groll and colleagues (2006) observed no efficiency with linearized vector, as their chosen enzyme (BamHI) also digested immediately after the encoded detection protein. Although comparative efficiencies between digestion sites in Neuro2a cells were similar to HT22, no stable Neuro2a colonies were obtained due to low transient efficiency, a feature of Neuro2a cells that has been previously reported [9].

Incorporation of the foreign DNA into the chromosome of the host cell is required for the generation of a clonal cell line. This process is most likely to occur during nuclear replication, as fragments of linear DNA can be randomly copied into new chromosomes. It is therefore not surprising that restriction digestion of DNA prior to transfection greatly effects the integration into host chromosomes. By taking into account the number of cells initially transiently transfected, thereby removing the effect of variation in transfection efficiency, the integration efficiency of restriction enzyme treatment can be observed in Fig. 4. This difference in chromosomal integration and eGFP expression is not due to a difference in single stranded overhangs of the vector, as both restriction enzymes are blunt end cutters. The site of digestion however, may alter expression of the eGFP and antibiotic resistence genes, thereby effecting clonal selection. This is supported by the observation that transient transfections with BsaI resulted in greater eGFP expression, as displayed by measurably

higher fluorescence intensity than other treatments, and also resulted in more numerous stable colonies (Fig. 3).

Interestingly, despite the variation in the number of eGFP-expressing colonies, all three transfection treatments resulted in a similar number of stable colonies that did not express eGFP (Fig. 4), indicating a consistent cellular digestion event and chromosomal insertion that conferred antibiotic resistance, but not eGFP expression.

Linearization of a vector is commonly used in transfections that have previously been difficult to isolate stable clones with uncut vectors. We have confirmed that linearization, via restriction enzyme digestion, of a vector prior to transfection affects the transfection and stable integration efficiency. In this report we have demonstrated that it is possible to increase the number of stable colonies by up to three-fold through vector linearization. It was also found however, that the affect of linearization is highly dependant on the restriction region chosen in the vector. To increase the likelihood of obtaining stably transfected cells, we suggest that transient transfection be optimized and vector linearization occur immediately prior to the gene of interest rather than between the gene of interest and selection cassette.

Acknowlegements

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Figures

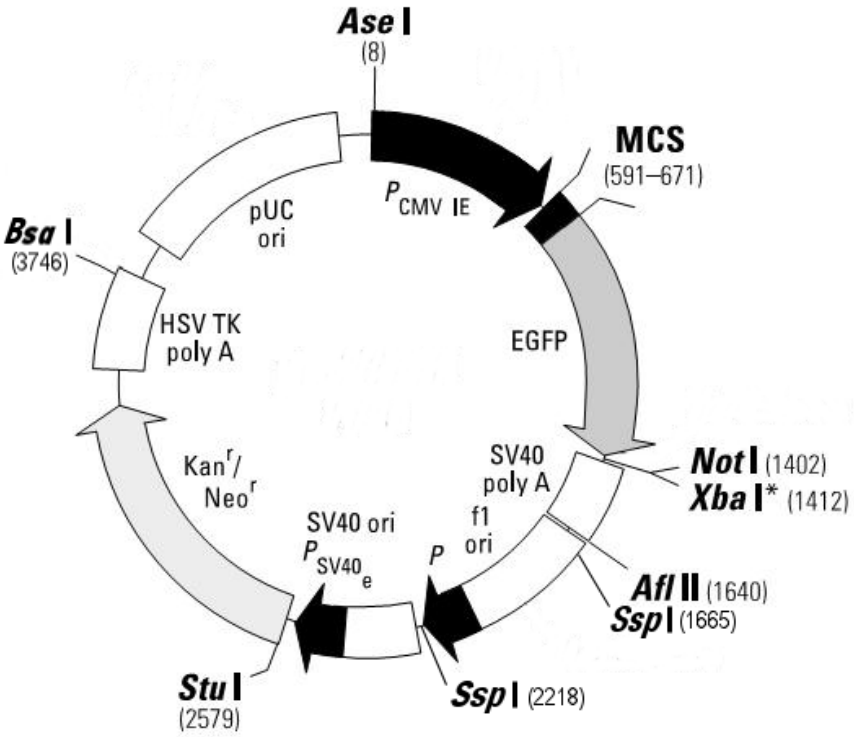

Figure 1. Restriction enzyme digestion sites of pEGFP-N1. *Bsa*I linearizes pEGFP-N1 by a single digestion at 3746bp at the terminal end of the poly A-tail of the Neomycin resistance gene. *Ssp*I linearizes by removal of a small fragment from 1665 to 2218bp, in the bacterial promoter region of the Neomycin resistance gene.

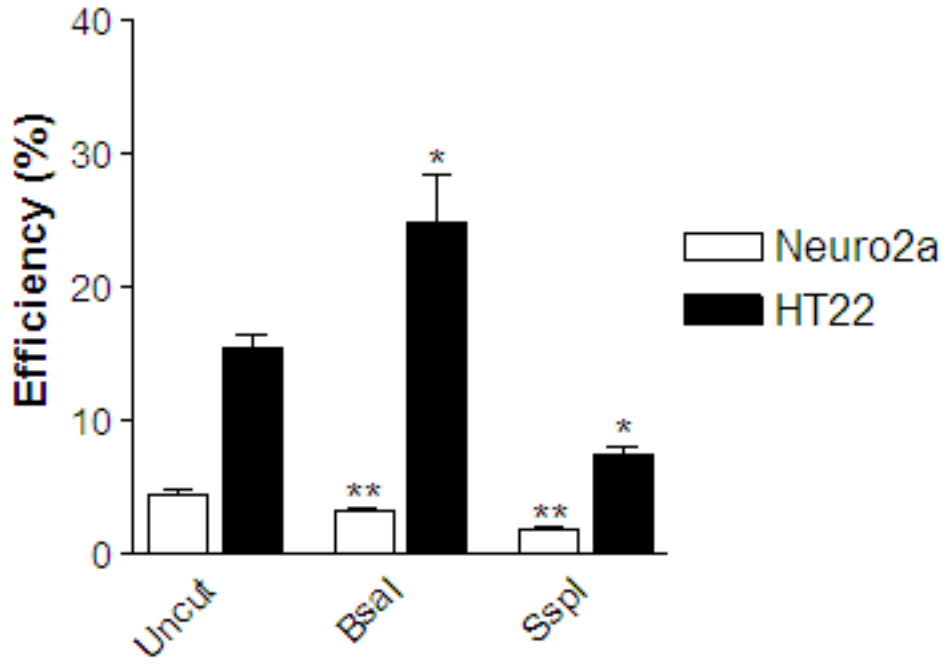

Figure 2. Transient transfection efficiency of Neuro2a and HT22 neuronal cells. 24 hours after transfection with pEGFP-N1, using Lipofectamine-LTX, cells were visualized and GFP-positive cells identified. (* $P<0.05$, ** $P<0.01$ compared to uncut controls. Error bars represent SEM).

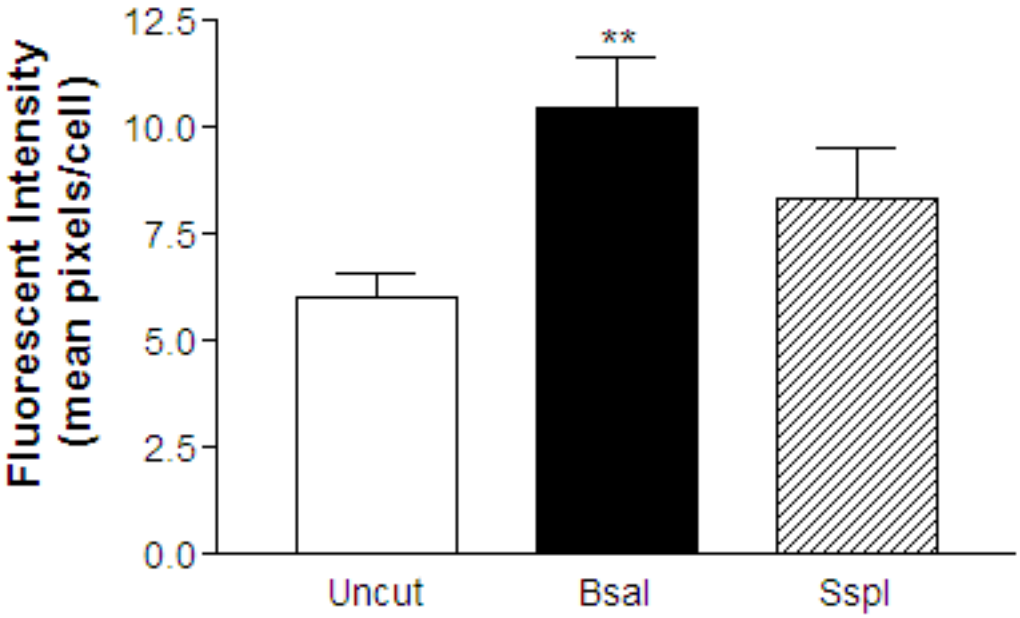

Figure 3. Fluorescent intensity of transiently transfected HT22 neurons. pEGP-N1 vector was left untreated, or digested with *Bsa*I or *Ssp*I prior to a 24 hour transfection with Lipofectamine-LTX. Twenty-four hours post-transfection, cells were imaged with a fluorescent microscope and analyzed with Image J software. Data is represented as mean pixel intensity per cell. (* $P<0.05$, ** $P<0.01$ compared to uncut controls. Error bars represent SEM).

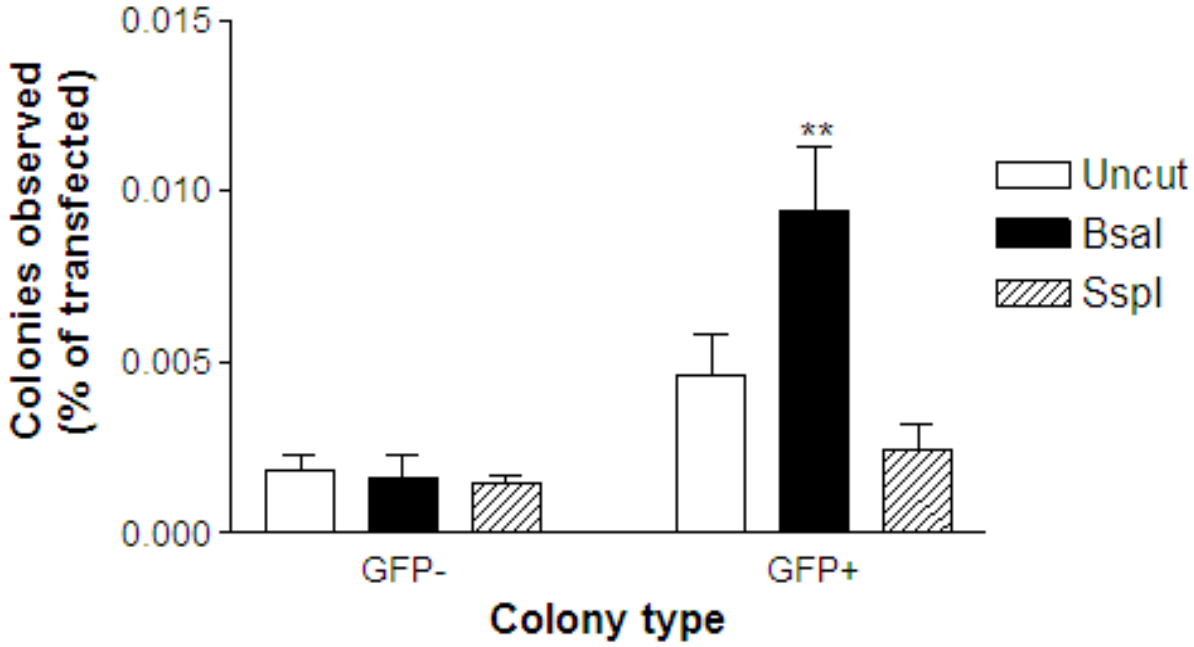

Figure 4. Stable integration efficiency of uncut and digested pEGFP-N1. $1.2x10^6$ HT22 cells were transfected using Lipofectamine-LTX . Stable colonies were counted after 13 days of selection with G418 antibiotic. Values are represented in relation to positive transient transfectants. (* P<0.05, ** P<0.01 compared to uncut controls. Error bars represent SEM).

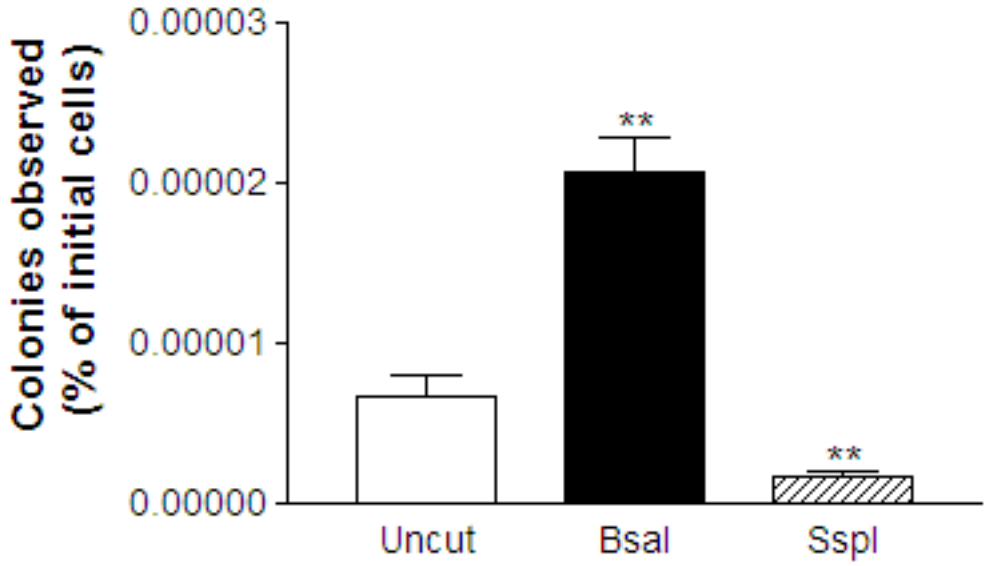

Figure 5. Stable transfection efficiency of pEGFP-N1 in HT22 neurons. $1.2x10^6$ HT22 cells were transfected using Lipofectamine-LTX . Stable colonies were counted after 13 days of selection with G418 antibiotic (* $P<0.05$, ** $P<0.01$ compared to uncut controls. Error bars represent SEM).

Article 2

HMG-CoA reductase-independent anti-inflammatory activity of statins in microglia and macrophages

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Abstract

The statin family of drugs inhibit HMG-CoA reductase, an enzyme that produces mevalonate, an early intermediate in the synthesis of cholesterol. The inhibition of HMG-CoA reductase alone was proposed to provide a certain degree of anti-inflammatory effects, as cholesterol pathway intermediates are required for the proper anchoring and thus functioning of small G-proteins, such as those involved in several inflammatory pathways. We determined the anti-inflammatory properties of the four statins, atorvastatin, simvastatin, fluvastatin and lovastatin and established whether the activity observed was linked to their inhibition of HMG-CoA reductase. Activation of N-11 microglia and RAW 264 macrophages with lipopolysaccharide or interferon-γ was assessed using TNF or nitric oxide as the readouts. The four statins displayed dramatically different anti-inflammatory potential, with atorvastatin being the most potent with an IC_{50} in the low micromolar range. Upon further investigation of the pathways, the HMG-CoA reductase-independent activity was confirmed, since exogenous mevalonate, the product of HMG-CoA reductase, did not modify the inhibitory actions of the statins.

Furthermore, statins displayed additional anti-inflammatory properties when farnesylation and subsequent G-protein signalling was inhibited by pamidronate, in combination with statins. This suggests that, currently unidentified, HMG-CoA reductase-independent properties of statins are most likely responsible for their potential anti-inflammatory activity.

Introduction

Statins inhibit the activity of HMG-CoA reductase, which catalyses the conversion of 3-hydroxy-3-methylglutaryl-CoA to mevalonate, an essential precursor for the production of cholesterol. Thus, this class of drug, which includes Atorvastatin, Simvastatin, Fluvastatin and Lovastatin, is widely used in the treatment of hypercholesterolemia, which increases the risk factor for the development of several cardiovascular diseases [1, 2]. Through the vast and ever-increasing number of patients using statins, significant evidence indicates that this class of drug is beneficial for conditions other than hypercholesterolemia.

Atherosclerosis, and a subsequent increase in cardiovascular disease, is a complication that is often associated with diabetes, but the incidence of this condition in diabetic patients taking statin medication is decreased [1-3]. This is supported by peritoneal dialysis patients undergoing statin therapy, who, in comparison to patients not using statins, also display a decrease in C-reactive protein, a marker for both inflammation and cardiovascular disease [4]. These conditions however, are known to be related to hypercholesterolemia, but statins have also demonstrated benefits for several conditions unrelated to hypercholesterolemia.

Epidemiological and observational studies initially displayed a promising link between statin use and a reduction in Alzheimer's disease (AD) [5]. Initial results of clinical trials in AD patients were also promising, although a recent meta-analysis has found that statins provide no benefit to AD patients [6, 7]. As a long-term preventative therapy for AD however, statins continue to be a treatment of interest, based on epidemiological data. In addition to AD studies, a mouse model has also found that the brain can be protected by statins against traumatic brain injury [8]. Although these conditions may appear to be unrelated to each other, as with atherosclerosis, inflammation is known to play a part in both conditions, indicating that statins possess anti-inflammatory effects. This theory is further supported by the ability of statins to dramatically decrease the chance of severe sepsis [9, 10].

The inhibition of HMG-CoA reductase alone can result in a decrease in the activity of several pro-inflammatory signals such as p38 Mitogen–activated protein kinase (MAPK), Nuclear Factor κ-B (NFκ-B) and phosphoinositide-3-kinase [11-14]. This is due to the requirement of small G-proteins, including Ras and Rho, to be anchored to the cell membrane before they can be activated to induce downstream cytosolic signalling. Proper anchoring requires the addition of small isoprenoids, either farnesylpyrophosphate or

geranylgeranylpyrophosphate, which are intermediates in the cholesterol pathway and are therefore absent when HMG-CoA reductase is inhibited (Figure 1) [15, 16].

The numerous conditions that statins appear to be beneficial for, however, do not all involve cytokines and inflammatory pathways that rely on G-protein dependent signalling. In the cases of sepsis and Alzheimer's disease, where lipopolysaccharide (LPS) and β-amyloid (Aβ) are the main stimuli respectively, MAPK cascades are known to play a major role in inflammation. These MAPK cascades can be initiated by receptors that are G-protein dependent, including Toll-like receptor 4 (TLR4), Receptor for Advanced Glycation Endproducts (RAGE), Tumour Necrosis Factor (TNF) receptor and Interleukin 1 receptor [17-21]. In contrast, IFNγ signalling is not G-protein-dependent, unlike LPS and Aβ, which rely on small G-proteins and isoprenylation for cellular signalling. Interferon-γ (IFNγ) however, is the predominant pro-inflammatory mediator in atherosclerosis [22] and is known to stimulate microglia via the dimerization of the Interferon-gamma receptor, leading to activation of JAK-STAT tyrosine kinases, with minimal involvement of MAPKs, including ERK, MEK or PKC (Figure 2) [23-25]. This difference suggests that that the beneficial effects observed for statins in inflammatory conditions might be not solely attributable to a decrease in isoprenylation and G-protein signalling, due to HMG-CoA reductase inhibition. Given the differing pathways of activation by these ligands, it would be expected that statins would induce a greater anti-inflammatory effect in response to G-protein-dependent ligands. Moreover, these effects should be abolished by addition of exogenous mevalonate, the product of HMG-CoA reductase, which is required for isoprenylation of the small G-proteins. Previous studies found that exogenous mevalonate did abolish the effect of statins, when cells were activated with stimuli such as Aβ, interleukin-1β (IL-1β) and LPS in microglia and macrophages [26-28].

In this study, we compared the anti-inflammatory efficacy of four statins (Atorvastatin, Fluvastatin, Lovastatin and Simvastatin) in microglia and macrophages by activating with LPS or IFNγ for 24 hours, using nitric oxide (NO) and TNF as determinants of inflammatory activity. The contribution of HMG-CoA reductase inhibition to these anti-inflammatory effects was also investigated, via the use of mevalonate (cholesterol and isoprenylation precursor) and pamidronate (farnesylation inhibitor). The addition of mevalonic acid allows pathways downstream of HMG-CoA reductase, especially isoprenylation, to occur, while alternative HMG-CoA reductase-independent actions of statins remain unchanged.

Pamidronate is an aminobisphosphonate that inhibits farnesyl-pyrophosphate synthase, a key enzyme in the HMG-CoA reductase pathway. Farnesyl-pyrophosphate is required for the isoprenylation of intracellular proteins which are targeted to the membrane. The consequent failure of isoprenyl attachment blocks G-protein tethering to the cell membrane and inhibits biological function, ultimately resulting in impaired signalling. Addition of pamidronate completely inhibits small G-protein-dependent signalling. Thus, any additional anti-inflammatory effects of statins when used in combination with pamidronate, must be mediated by non HMG-CoA related properties of the drugs.

The major outcomes of this research were that the four statins assayed displayed dramatically different anti-inflammatory properties, which were not related to their IC_{50} values for HMG-CoA inhibition. In addition, since this activity was not significantly altered by addition of mevalonate or pamidronate, we propose that – at least in our cell culture model – the anti-inflammatory activity of statins is not exclusively HMG-CoA reductase-dependent.

Materials and Methods

Cell maintenance

N-11 murine microglial cells (obtained from the University of Tüblingen, Germany) and J774 macrophages (supplied by Dr. J. Smith, James Cook University, Australia) were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% foetal calf serum (FCS), supplemented with penicillin (200U/ml), streptomycin (200μg/ml) and fungizone (2.6μg/ml). Cells were maintained at 37^{oC}, containing 5% CO_2, in a humid environment.

Activation and treatment of N-11 and J774 cells

Once grown to confluence in culture flasks, cells were removed using a rubber cell scraper, then counted using a Neubauer counting chamber. They were then dispensed into 96-well plates at a density of $5x10^4$/well and grown for 24 hours in serum-free DMEM.

Cells were then incubated with statins (concentrations ranging from 0.05μM to 100μM), with or without 500μM mevalonate or 200μM pamidronate for 90 minutes prior to activation with either 10μg/mL or 10U/mL IFN-γ for 24 hours at 37^{oC}. Mevalonate and pamidronate were both diluted in DMEM. Statins were initially dissolved in DMSO at a concentration of 10mM, and were further diluted in DMEM. The final concentration of DMSO applied to the cells did not exceed 0.5% and did not affect cell viability.

Nitric oxide determination

Nitric oxide production was monitored by measuring the concentration of nitrite in the media using the 'Griess reagent'. Conditioned media (75μL) from each well was transferred to a fresh 96-well plate and 75μL of Griess reagent (1%w/v sulfanilamide and 0.1%w/v naphthyethylene-diamine in 2.5% HCl) was added and the absorbance at 540nm measured using a plate reader (Multiskan *Ascent* with Ascent software v2.4, Labsystems, Helsinki, Finland). Concentrations were then determined by comparison to a standard curve prepared with sodium nitrite.

Determination of TNF in cell culture supernatant by ELISA

Following the 24 hour incubation with LPS and statins, the concentration of TNF was determined by a Sandwich Enzyme-Linked Immunosorbent Assay (ELISA), according to the manufacturer's manual (Peprotech, Rocky Hill, New Jersey). Briefly, capture antibody was used at a concentration of 1μg/ml in PBS (1.9mM NaH_2PO_4, 8.1mM Na_2HPO_4, 154mM NaCl) (pH 7.4). Serial dilutions of TNF standard from 0 to 2000pg/ml in diluent (0.05% Tween-20, 0.1% BSA in PBS) were used as internal standard. TNF was detected with a

biotinylated secondary antibody and an Avidin peroxidase conjugate, with TMB as the detection reagent. Absorbance was determined at 355nm in a 96 well plate reader.

Cell viability assays

Cell viability was determined by the reduction of resazurin to the fluorescent product resorufin, catalysed by mitochondria of viable cells. After the removal of conditioned media for measurement of nitric oxide and TNF concentration, the remaining media was aspirated for determination of cell viability. 100μL of serum-free DMEM containing 0.125mg/L resazurin (Alamar Blue) was added to each well and incubated for 2 hours at 37^{oC}. The fluorescence was then measured with excitation/ emission wavelengths of 545/ 595nm using a Wallac Victor V Fluorometer (Perkin Elmer, Massachusetts).

Statistical analysis

Results were graphed and analyzed using Prism 4 (Graphpad Software). A Dunnett's test was performed, using activated culture as controls for nitric oxide and TNF inhibition (* $P<0.05$). Error bars represent the standard error of the mean (SEM).

Results

In this study we investigated whether four commonly used statins possess the ability to down-regulate inflammatory responses in murine microglia and macrophages. N-11 murine microglia and J774 murine macrophages were activated for 24 hours with LPS or IFN-γ in the presence of Atorvastatin, Fluvastatin, Lovastatin or Simvastatin. Each statin was also assayed in the presence of mevalonate, the production of which is normally inhibited by statins, and pamidronate, a bisphosphonate that directly inhibits farnesylation (Figure 1). Activation of inflammatory pathways was determined by the measurement of both nitric oxide and TNF in the supernatant, known products of microglia in response to IFN-γ and LPS [23, 29].

Inflammatory inhibition in N-11 microglia by statins

NO and TNF displayed similar dose-dependent increases in both cell lines upon stimulation with LPS, indicating that they are both useful indicators of inflammation. Following activation of murine N-11 microglia with LPS, the four statins assayed caused a reduction in inflammation, as measured by NO and TNF, but to varying degrees (Figure 3). Atorvastatin and Simvastatin both caused a significant decrease in both NO and TNF production, with IC_{50} values in the range of 3μM and 30μM respectively. Lovastatin however, did not display any decrease in NO or TNF, while Fluvastatin demonstrated some anti-inflammatory activity, but failed to reach 50% inhibition at the highest concentration tested.

Inflammatory inhibition in J774 macrophages by statins

Experimental data with LPS-activated J774 murine macrophages supported the greater anti-inflammatory activity displayed by Atorvastatin and Simvastatin in microglia (Figure 3). Not only were IC_{50} values in the same range as those in microglia, but Atorvastatin again began decreasing NO and TNF production at concentrations much lower than any of the other statins tested, although Simvastatin caused a higher maximal NO inhibition. The most obvious difference in results between macrophages and microglia however, is that Fluvastatin and Lovastatin displayed a greater inhibition in macrophages. Fluvastatin decreased both NO and TNF at high concentrations (>50μM) in microglia, but was more effective at lower concentrations in macrophages, with an IC_{50} of approximately 15μM. Lovastatin also showed greater inflammatory inhibition in macrophages, but still failed to reach 50% inhibition at 100μM.

Mevalonate does not abolish anti-inflammatory activity of statins

Both microglia and macrophages were activated in the presence of statins in combination with exogenous mevalonate, the product of HMG-CoA reductase, to determine the anti-inflammatory involvement of HMG-CoA reductase. Anti-inflammatory activity by statins was still observed in the presence of mevalonate (Figure 4). The pattern of NO and TNF reduction did not markedly change when mevalonate was supplied to either N-11 or J774 cells. Although mevalonate attenuated some of the effect of statins, the IC_{50} and maximum inhibition values remained close to those observed for statins alone (Table 1). Importantly, complete rescue of statin-dependent NO and TNF reduction was not observed in the presence of mevalonate.

Anti-inflammatory activity of statins is G-protein independent

By activating microglia with IFN-γ, which signals primarily via the JAK-STAT pathway, small G-protein dependent signalling was minimal, and any residual G-protein activity was inhibited by co-administration of pamidronate, a known farnesylation inhibitor. Statins demonstrated similar anti-inflammatory activity when N-11 cells were activated with IFNγ (Figure 5), compared to the NO decrease observed following activation with LPS (Figure 3). The addition of pamidronate did not significantly alter the degree of NO inhibition for statins overall and IC_{50} values remained similar (Table 1), although the degree of maximal inhibition is marginally less for Atorvastatin, Fluvastatin and Lovastatin.

Discussion

The statin family of drugs, commonly used to treat hypercholesterolemia, are known to possess anti-inflammatory properties. This study presents evidence that the anti-inflammatory effects of statins, specifically associated with microglia and macrophages, is independent of cholesterol synthesis inhibition and therefore , independent of isoprenylation inhibition.

From the numerous studies that have found mevalonate to attenuate the anti-inflammatory effects of statins, it is clear that the inhibition of isoprenylation provides beneficial anti-inflammatory effects. Several facets of this study however, emphasize the importance of novel actions of statins in the down-regulation of microglia. If the inhibition of HMG-CoA reductase and subsequent decrease in isoprenylation was the only factor playing a role in inflammation inhibition, it would be expected that all statins would demonstrate similar anti-inflammatory profiles. This is not the case however, as only Atorvastatin and Simvastatin resulted in dramatic decreases in NO and TNF, while mild inhibition was observed for Fluvastatin and minimal inhibition for Lovastatin. Moreover, statins have IC_{50} values for HMG-CoA reductase in the low nanomolar range for cell culture systems [30]. The decreases observed in TNF and NO however, did not occur until the low micromolar range, concentrations that far exceed the IC_{50} for HMG-CoA reductase, indicating that the inhibition of this enzyme plays a minor role in their anti-inflammatory activity. Although the concentrations displaying anti-inflammatory properties far exceed cerebrospinal fluid levels observed in patients, they do correlate with the findings of numerous studies on the anti-inflammatory effects of statins *in vitro* [26, 31-33].

The negligible anti-inflammatory role of HMG-CoA reductase is further supported by the results obtained with statins in the presence of mevalonate and pamidronate. By adding mevalonate, the inhibition of HMG-CoA reductase is negated and cholesterol synthesis, farnesylation and small G-protein signalling can occur (Figure 1). The concentration of mevalonate used *in vitro* is generally between 50µM and 500µM [34-36], with 500µM previously shown to be suitable for up to 6 days of use. This concentration was therefore utilized to ensure mevalonate stores were not depleted during incubation and displayed no difference in toxicity or inflammatory response to mevalonate-free controls (data not shown). It would be expected that if the anti-inflammatory properties of statins were solely attributable to HMG-CoA reductase, addition of mevalonate would completely abolish any anti-inflammatory properties observed. This was not the case, as the addition of mevalonate failed

to abolish the observed effects of statins alone (Figure 4). This was not cell specific, as the same patterns of inhibition were observed in both the microglia and macrophage cell line. The only noticeable difference between the two cell types was that Fluvastatin and Lovastatin caused greater inhibition in macrophages, which may be due to the higher activation and levels of NO and TNF produced, giving more scope for down regulation.

200μM pamidronate was used in this assay as it has previously been shown to almost completely abolish TNF production in response to LPS, via the inhibition of farnesylation [37]. In macrophages, IFNγ signals primarily via the JAK-STAT pathway, with minimal small G-protein involvement [38, 39], supported by the lack of inhibition with pamidronate alone following activation with IFNγ (Figure 5). Assaying statins in the presence of pamidronate, with IFNγ as an activator, means that any minimal isoprenylation required for IFNγ-dependent signalling is constantly inhibited. Thus, statins are unable to inhibit inflammation via a reduction in isoprenyl-dependent G-protein signalling. The results indicate that statins decrease IFNγ-dependent inflammation at the same rate, regardless of the presence of pamidronate (Figure 5). This result, taken together with the inability of mevalonate to reverse the statins activity supports the minimal effect of isoprenylation and small G-protein signalling in the anti-inflammatory properties of statins. Some of the likely anti-inflammatory mechanisms previously identified include Peroxisome Proliferator-Activated Receptor (PPAR)-α and PPAR-γ agonism, Inhibitory κ-B up-regulation and stabilisation, up-regulation of antioxidant enzymes such as Heme Oxygenase, and direct antioxidant effects [40-45].

Given the link between the ApoE4 allele, high cholesterol and increased incidence of AD [46, 47], it is not surprising that cholesterol lowering drugs may have beneficial effects in relation to AD. These findings however, suggest that the pleiotropic anti-inflammatory properties of statins, rather than the inhibition of HMG-CoA reductase, are primarily responsible for dramatically inhibiting microglial inflammation. Thus, it is likely that it is the pleiotropic effects that provide neuroprotection against the onset of Alzheimer's disease. Moreover, the difference in novel side effects explains why some statins have previously not demonstrated efficacy in trials.

Acknowledgements

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Figures

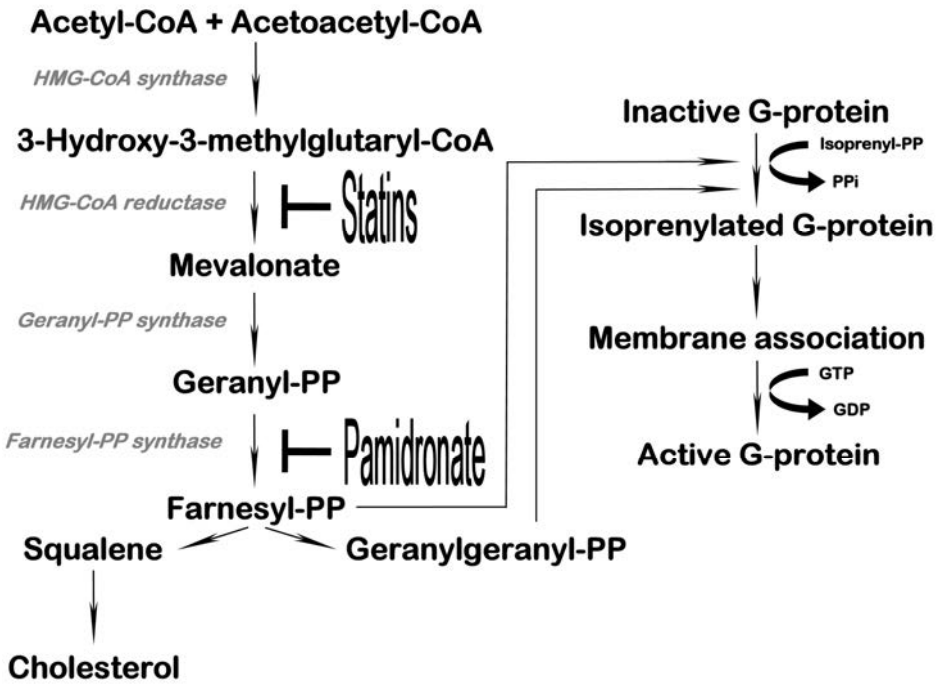

Figure 1. HMG-CoA reductase is a rate-limiting step in the synthesis of cholesterol. The cholesterol intermediate Farnesyl-pyrophosphate is required for isoprenylation of small G-proteins for proper membrane association. HMG-CoA reductase inhibition by statins effectively reduces isoprenylation and small G-protein signalling, while pamidronate directly blocks isoprenylation, but allows cholesterol synthesis to occur.

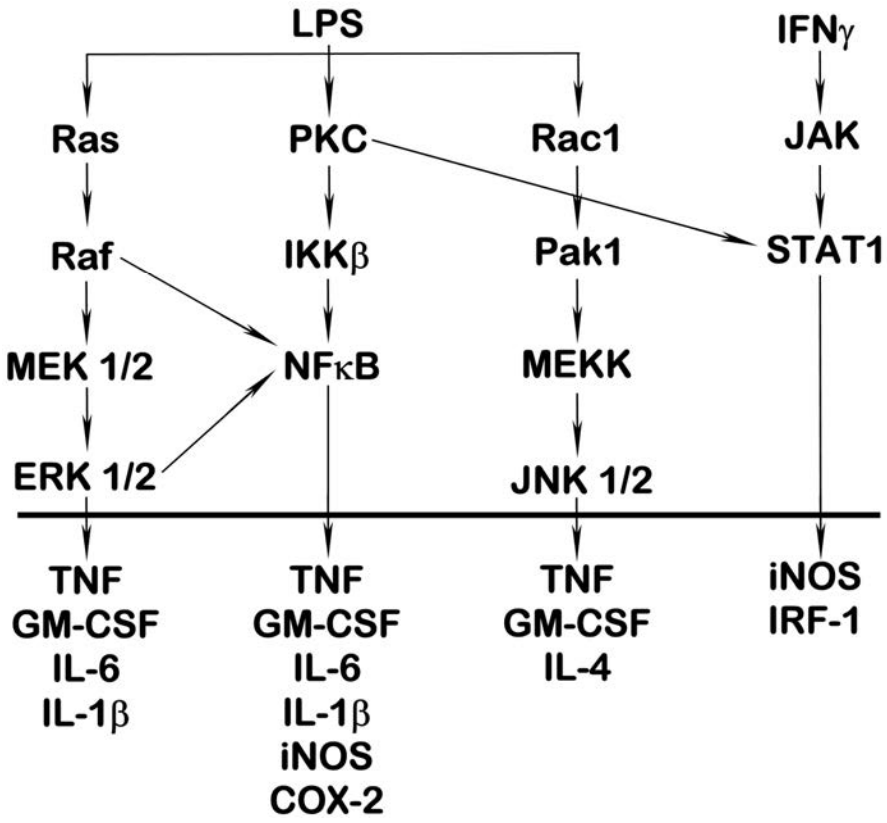

Figure 2. Signalling pathways involved in LPS and IFNγ stimulation. Isoprenyl-dependent small G-proteins, including Ras and Rac are activated following LPS stimulation of macrophages and microglia. Ras and Rac activate cytosolic MAPK cascades that result in production of pro-inflammatory mediators, primarily TNF, IL-6 and iNOS. IFNγ activation relies heavily on the JAK-STAT pathway, with minimal small G-protein involvement. iNOS and IRF-1 are pro-inflammatory mediators that are directly up-regulated by IFNγ-dependent STAT1.

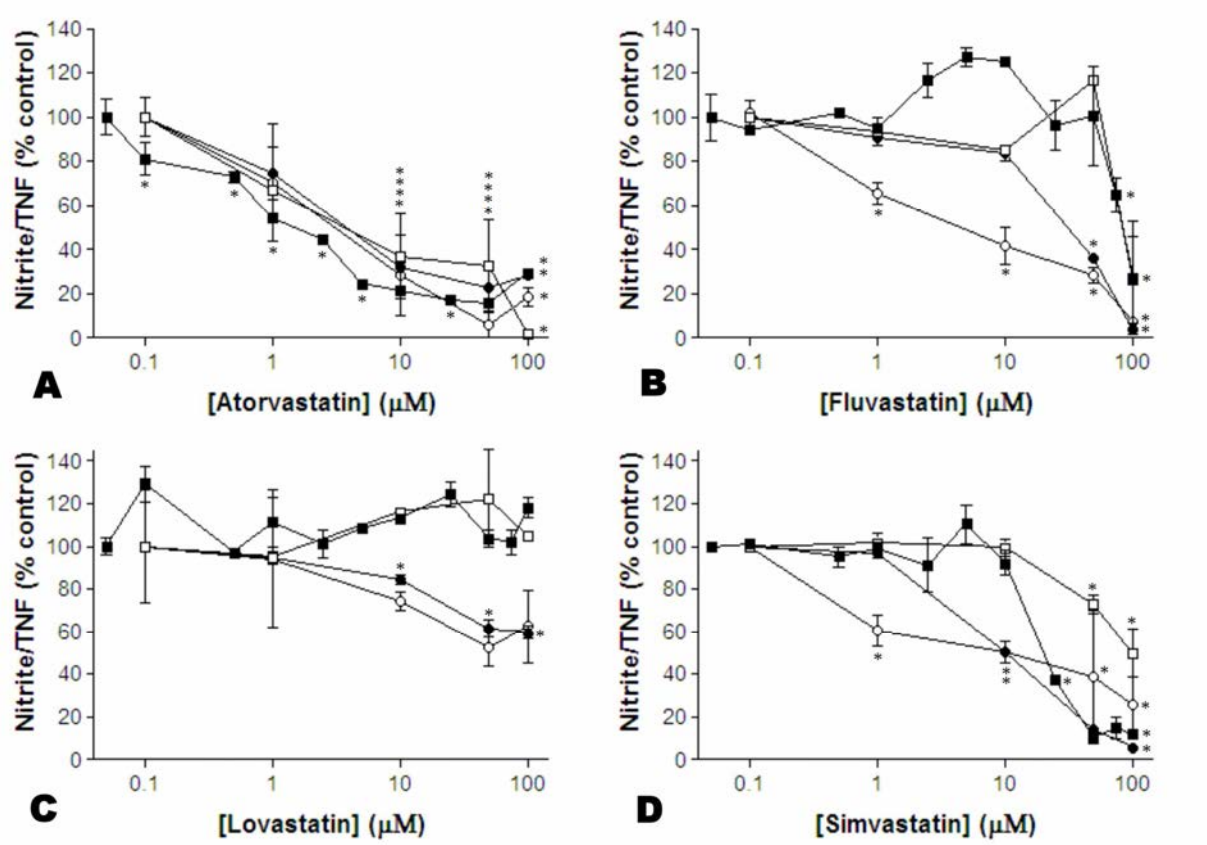

Figure 3. Anti-inflammatory effects of statins in LPS stimulated microglia and macrophages. N-11 microglia (squares) and J774 macrophages (circles) were incubated with statins for 90 minutes prior to activation with 10μg/ml of LPS. After 24 hours, nitric oxide (closed symbols) and TNF (open symbols) were determined by Griess reagent and ELISA respectively. Cell viability (data not shown) was measured by the Alamar Blue assay and did not fall below 90%.

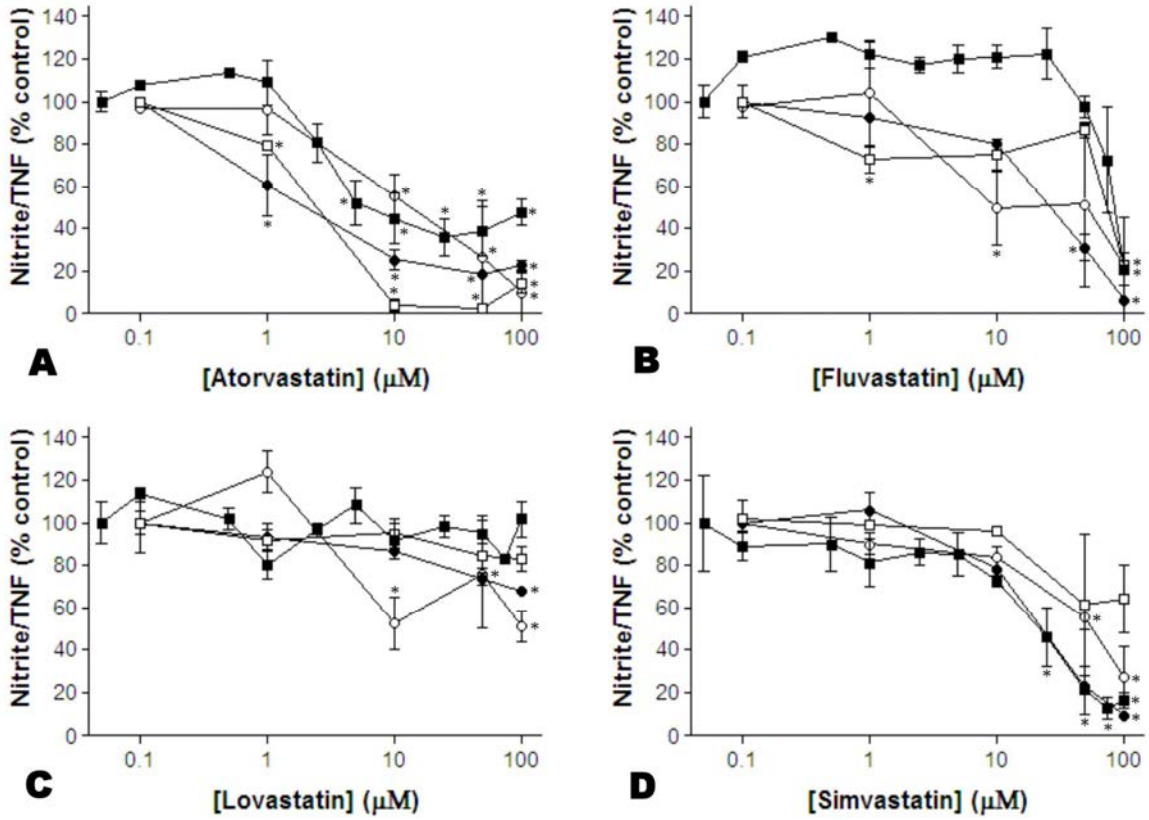

Figure 4. Mevalonate-independent anti-inflammatory effects of statins in LPS stimulated microglia and macrophages. N-11 microglia (squares) and J774 macrophages (circles) were incubated with statins and 500μM mevalonic acid for 90 minutes prior to activation with 10μg/ml of LPS. After 24 hours, nitric oxide (closed symbols) and TNF (open symbols) were determined by Griess reagent and ELISA respectively. Cell viability (data not shown) was measured by the Alamar Blue assay and did not fall below 90%.

	N-11 microglia				J774 macrophages			
	NO IC_{50} (μM)	NO Max Inhib (%)	TNF IC_{50} (μM)	TNF Max Inhib (%)	NO IC_{50} (μM)	NO Max Inhib (%)	TNF IC_{50} (μM)	TNF Max Inhib (%)
Atorvastatin	2	85	3	100	4	75	4	92
Atorvastatin + mevalonate	6	62	6	100	2	78	15	93
Fluvastatin	75	72	75	72	35	100	4	92
Fluvastatin + mevalonate	78	79	74	76	20	92	7	78
Lovastatin	-	0	-	0	-	40	-	45
Lovastatin + mevalonate	-	18	-	18	-	32	-	48
Simvastatin	20	90	-	44	11	95	13	67
Simvastatin + mevalonate	18	88	-	39	19	92	58	77

Table 1. Anti-inflammatory effects of statins in LPS stimulated microglia and macrophages.

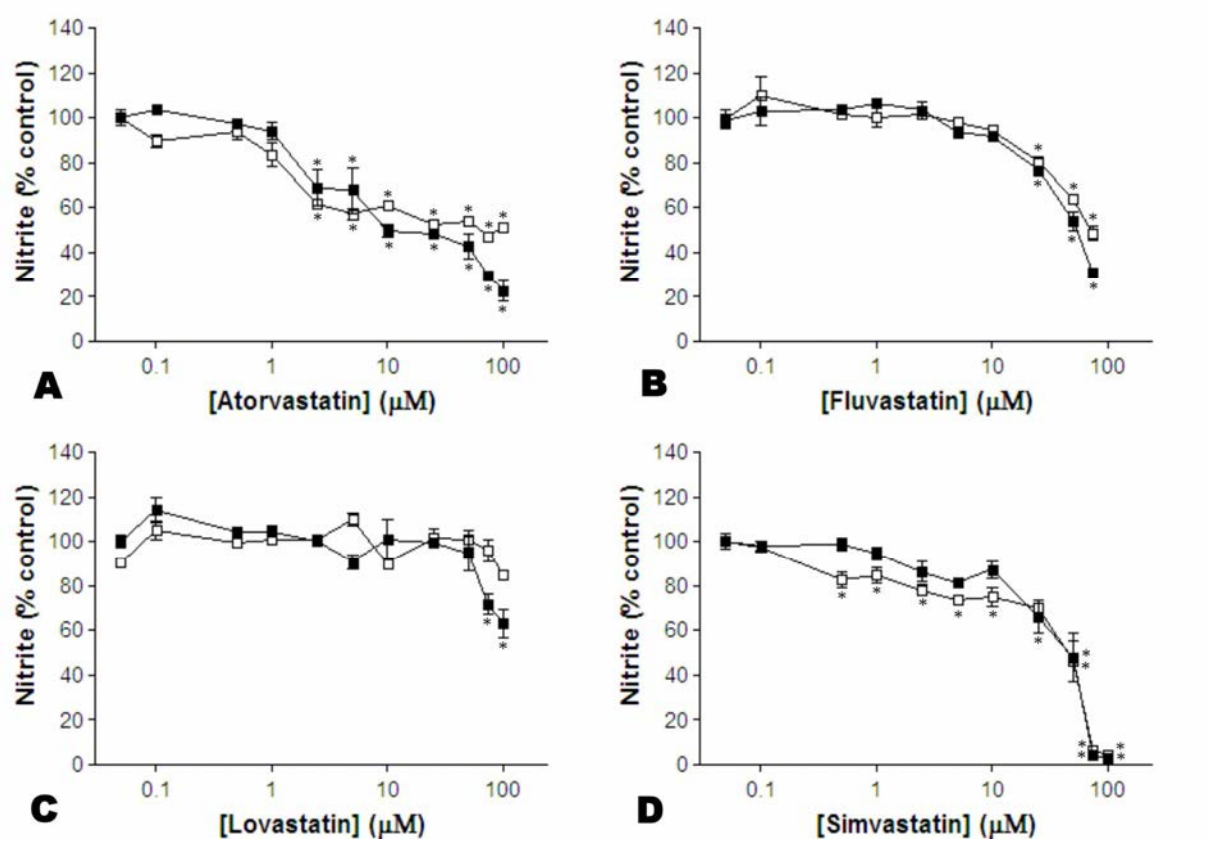

Figure 5. Small G-protein and isoprenylation-independent anti-inflammatory effects of statins in microglia. N-11 microglia were incubated with statins, with (□) or without (■) 200μM of pamidronate for 90 minutes prior to activation with 10U/ml of IFNγ. After 24 hours nitric oxide (■,□) was determined by Griess reagent. Cell viability (data not shown) was measured by Alamar Blue fluorescence and did not fall below 90%.

Article 3

Neurodegeneration is dependant on microglial proximity and nitric oxide production

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Abstract

Several neurodegenerative diseases, including Alzheimer's and Parkinson's disease can be attributed to excessive or ongoing micro-inflammation in the brain. The glial cells of the brain are responsible for production of pro-inflammatory cytokines, enzymes and radicals that eventually cause neurodegeneration. In this study, we aimed to develop an increased-throughput assay for the screening of neuroprotective compounds that more closely models the *in vivo* situation than current neuroprotective assays. Three separate co-culture methods were assayed for their ability to induce neuron death, including the transfer of activated microglial media to neurons, activation of microglia in co-culture with neurons separated by a membrane and a direct co-culture of microglia and neurons with no separation. The transfer of activated media and membrane co-culturing failed to induce neuron death, indicating that glial proximity to neurons is an important factor in microglial-induced neuron death. Furthermore, we demonstrate that inhibitors of Nitric oxide synthase are able to provide neuroprotection, suggesting that the free radical Nitric oxide plays a significant role in neuron death *in vitro*.

Introduction

Neurodegenerative diseases and pathologies can be accompanied by, or directly due to, inflammation. The presence of the inflammatory cytokines Tumour Necrosis Factor-α (TNF), Macrophage-colony stimulating factor, Interleukin-1β (IL-1β), Interleukin-6 , as well as nitric oxide, a small molecule produced by nitric oxide synthase, is well documented in a variety of neurodegenerative disease, including in Alzheimer's and Parkinson's diseases [1-8]. The up-regulation of these pro-inflammatory products is both a direct result, and the cause, of microglial migration and proliferation. Although the level of inflammation during these disease states may be low, particularly in comparison to that observed outside the brain, the net result is a continuous state of stress for local neurons. Initially, this may cause a retraction of dendrites and a decrease in synaptic signaling, but may eventually lead to neuronal death.

In the case of Alzheimer's disease, although many aspects of the disease process have been elucidated, a singular cause of neuronal loss has yet to be identified. As well as the pro-inflammatory β-amyloid (Aβ) present in senile plaques, affected areas of the brain are known to contain high levels of TNF, IL-1β, IL-6 and various reactive oxygen species, many of which have individually demonstrated neurotoxic potential *in vitro* [9-12]. Of these pro-inflammatory products, reactive oxygen species are likely to play a primary role in neuronal degeneration, given their ability to induce cellular damage via multiple pathways and to do so at low concentrations.

Although one pro-inflammatory product may play a significant role, a singular cause of neuron degeneration or death is unlikely to be identified, as the pathways leading to neuron degeneration *in vivo* are potentially multi-factorial, due to the presence of pro-inflammatory microglia. Previous studies have relied on a direct neurotoxic insult, such as addition of hydrogen peroxide, Aβ or glutamate to neurons alone [13-19]. Whilst this data provides neuroprotective information in regards to a single mechanism of neuron death, effective compounds may not translate well to an *in vivo* situation. Ideally, a neuroprotective assay that more closely models the situation *in vivo* would therefore include activated microglia, in co-culture with neurons.

Initially, this study aimed to develop three assays for determination of neuroprotective properties of compounds. These included a model that relied on the activation of microglia alone, then transferral of this conditioned media to neurons, a model that contained microglia and neurons in the same well, separated by a membrane and finally a model that contained

both microglia and neurons in the same well, without any separation. The third of these culture models is unique, as commonly used viability assays prevent the differentiation between cell types in co-culture. We therefore employed stably transfected neurons, expressing Green Fluorescent Protein (GFP) as a marker for neuron viability in the presence of microglia [20].

Consequently, a novel *in vitro* co-culture that enables high throughput testing of compounds for neuroprotective properties was developed. During the development of the three neuroprotection assays, we found that neuron death could not be induced in all three co-culture systems. Moreover, when neuron death was observed, neuroprotection was conferred by specific inhibitors of Nitric oxide synthase, indicating a role for nitric oxide in neurodegeneration.

Methods

Materials

Interleukin-1β, Interferon$_\gamma$ and Tumour Necrosis Factor-α were purchased from Peprotech (Rocky Hill, NJ, USA). β-amyloid was synthesized by Keck Laboratories (Yale University, USA). Rezazurin (Alamar blue), Lipopolysaccharide and Dimethylsulfoxide were purchased from Sigma-Aldrich (St Louis, MO, USA).

Cell Culture

Murine HT22-GFP and Neuro2a-GFP neurons, N-11 microglia and J774 macrophages were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO) containing 5% foetal calf serum (FCS), supplemented with penicillin (200U/ml), streptomycin (200μg/ml) and fungizone (2.6μg/ml) (GIBCO). HT22-GFP and Neuro2a-GFP were also supplemented with 0.25mg/mL G418 to maintain stable selection. Cells were maintained at 37^{oC}, containing 5% CO_2, in a humid environment. Cells were removed from flasks using rubber scrapers (Sarstedt) and counted in a counting chamber (Neubauer) before dispensing into assay plates. Following dispensing into plates, serum-free media was used for each of the three models of neurodegeneration.

Determination of cellular fluorescence

Neuro2a-GFP cells were plated at varying densities ($1x10^4$ – $1.5x10^5$) into a 96 well plate. Two hours after plating, cells either remained in plating medium only, or had Alamar blue added to a final concentration of 12.5μg/mL and incubated for a further 2 hours. The total medium was then measured at 545/595nm for Alamar blue fluorescence or media removed and cellular EGFP measured at 490/530nm using a Victor V fluorimeter (Perkin Elmer, Massachusetts).

Transfer of conditioned media

N-11 and J774 cells were dispensed into 24 well plates at a density of $2x10^5$ cells/well and incubated at 37^{o}C, containing 5% CO_2, in a humid environment for 24 hours. Cells were then activated with a combination of either 5U/mL IFNγ and 5μg/mL LPS or 10U/mL TNF, 10U/mL IL-1β and 500ng/mL β-amyloid for 48 hours. Twenty-four hours after activation of inflammatory cells, $2x10^5$ HT22-GFP and Neuro2a-GFP neurons/well were dispensed separately into fresh 24 well plates and incubated with standard media for 24 hours. At the end of 48 hours of activation, media was removed from the inflammatory cells and transferred

to plates containing neurons for a further 48 hours. Nitric oxide content and neuron viability were then determined.

Membrane co-culture

HT22-GFP and Neuro2a-GFP neurons were dispensed into 24 well plates at a density of $2x10^5$ cells/well in a volume of 70μL and allowed to adhere for 2 hours at 37°C, containing 5% CO_2, in a humid environment. A further 430μL was then added to complete the initial 24 hour incubation. N-11 and J774 cells were also dispensed at $2x10^5$ cells/well, into 24 well plate 0.2μm Anapore membrane inserts (Nunc) and allowed to adhere for 24 hours. Membrane inserts were then transferred into wells containing neurons, making sure the insert was centered over the neuron-containing area of the well. The co-culture of neurons and inflammatory cells were then activated for 48 hours with the same activation combinations as described above for transfer of conditioned media. Nitric oxide content and neuron viability were then determined.

Direct co-culture

HT22-GFP and N-11 cells were cultured simultaneously at a density of $2x10^5$ each/well in 24 well plates, as were Neuro2a-GFP and J774 cells. After an initial 24 hour incubation period, co-cultures were activated for a further 48 hours using the same combination of inflammatory activators as described above for transfer of conditioned media. Nitric oxide content and neuron viability were then determined.

Inhibition of iNOS in direct co-culture

Neuro2a neurons ($4x10^4$/well) and J774 macrophages ($1x10^4$/well) were dispensed together into 96 well plates and incubated for 20 hours at 37°C, containing 5% CO_2, in a humid environment. Various concentrations (1-25μM) of iNOS inhibitors S-(2-Aminoethyl)Isothiourea and S-Methylisothiourea (Sigma, St Louis, MO, USA) were then added. After 4 hours, cells were activated with 1U/mL IFNγ and 1μg/mL LPS for 48 hours. Nitric oxide content and neuron viability were then determined.

Nitric oxide determination

Nitric oxide production was monitored by measuring the concentration of nitrite in the media using the 'Griess reagent'. Conditioned media (75μL) from each well was transferred to a fresh 96-well plate and 75μL of Griess reagent (1%w/v sulfanilamide and 0.1%w/v naphthyethylene-diamine in 2.5% HCl) was added and the absorbance at 545nm measured

using a Victor V spectrometer. Concentrations were then determined by comparison to a standard curve prepared with sodium nitrite.

Neuron viability determination

Viability of HT22 and Neuro2a neurons, in the conditioned media transfer, membrane co-culture and direct culture systems, was determined by measurement of total EGFP fluorescence. After media was removed for nitric oxide determination, the remaining media was aspirated and the fluorescence of each well measured at 490/530nm using a Victor V plate spectrometer. Viability was determined as a percentage of positive and negative control wells present on each plate.

Statistical analysis

Results were graphed and analyzed using Graphpad Prism 4. A Dunnett's test was performed, using unactivated co-culture as controls for each co-culture system, and activated co-culture as controls for nitric oxide inhibitor assays (* $P<0.05$, ** $P<0.01$). Each data point represents three independent experiments performed in triplicate (n=3), error bars represent the standard error of the mean (SEM).

Results

Fluorescence of EGFP expressing neurons

To investigate whether neuronal GFP expression was suitable for cell viability determination, the fluorescent content of Neuro2a-GFP cells was measured against Alamar blue reduction. The fluorescent intensity of reduced Alamar blue measured was approximately 4 times greater than that for cellular EGFP across all cell densities measured. The relative fluorescence detected for Alamar blue and EGFP was found to be directly proportional to the number of cells present (Figure 1). Analysis of the data determined that although the gradients differ significantly ($P<0.0001$), both assays closely correlate fluorescence to cell number ($R^2>0.9$), making either assay suitable for cell viability determination.

Neuronal cell death after transfer of glia conditioned media

Inflammatory cells (N-11 microglia and J774 macrophages) were cultured individually and activated with known pro-inflammatory agents (IFNγ and LPS or TNF, IL-1β and β-amyloid). After 48 hours, the activated media (conditioned media) from these cells was transferred to neurons (HT22 or Neuro2a, respectively) for a further 48 hours. Controls were also performed with activators added directly to neurons, which did not effect viability compared to unactivated glial or fresh media controls. Transfer of IFNγ/LPS stimulated media was not found to significantly affect the viability of HT22 neurons (Figure 2). Transfer of media from J774 cells, with these activators, decreased the viability of Neuro2a neurons marginally (approximately 10%), but not statistically different from unactivated controls. This effect is most likely due to depletion of the media, as prior to transfer, media underwent a dramatic colour change in the presence of phenol red. The neurons may therefore have undergone glucose deprivation in the following 48 hour incubation, resulting in minor neuron death. In contrast to IFNγ and LPS activation, transfer of media conditioned with TNF, IL-1β and Aβ induced a significant proliferation of both HT22 and Neuro2a neurons. Nitric oxide was produced by both inflammatory cell lines in response to IFNγ and LPS (N-11>25μM and J774 >40μM), but not in the presence of TNF, IL-1β and Aβ.

Neuronal cell death in co-culture of cells separated by a membrane

Inflammatory cells were cultured in the same wells as neurons, separated by a membrane that is permeable to small molecules, including cytokines and free radicals. No decrease in viability was observed for HT22 or Neuro2a neurons under these conditions in the presence of IFNγ and LPS, despite nitric oxide production being comparable to other culture systems (N-11>18μM and J774>40μM) (Figure 3). Both neuronal lines however, proliferated when

activation of cells occurred with TNF, IL-1β and Aβ. Under these conditions, no nitric oxide was detected after 48 hours.

Direct co-culture

Neurons and microglia/macrophages were cultured under the same conditions as described for membrane co-culture, without membrane separation for 48 hours. While addition of activators to neurons alone had no effect, both HT22 and Neuro2a neurons were found to degenerate when activated in co-culture with IFNγ and LPS, with viability decreasing by 67% and 70% respectively (Figure 2). Nitric oxide production under these conditions (>25μM for HT22/ N-11 and >40μM) for Neuro2a/ J774, is comparable to that produced during both transfer of conditioned media and membrane co-culture (Figure 3). Unlike previous culture systems, activation with TNF, IL-1β and Aβ failed to induce proliferation, but importantly, did not decrease neuron viability below controls. Again, negligible concentrations of nitric oxide were detected with this combination of activators.

Role of iNOS in co-culture neuron degeneration

The direct co-culture system was utilized to determine the toxic species causing neuron death. Neuro2a neurons and J774 macrophages were incubated for 4 hours with an inhibitor of iNOS (Isothiourea or S-MT), prior to 48 hours of activation with IFNγ and LPS. At 1μM, neither inhibitor had an effect on nitric oxide production or neuron viability (Figure 4). Both inhibitors displayed inhibition of nitric oxide production at a concentration of 5μM, reducing nitrite content from an initial 45μM to approximately 30μM. At this concentration however, neither inhibitor significantly altered neuron viability. At 10μM, Isothiourea completely inhibits nitric oxide production, while S-MT inhibits approximately 75%. It is at this concentration that both iNOS inhibitors dramatically increase neuron viability to a maximum of 85% of controls. This dramatic increase in viability indicates that the presence of nitric oxide plays a pivotal role in the neurodegenerative process in this co-culture system. No further increase in neuron survival was observed, as concentrations of iNOS inhibitors above 10μM resulted in cellular toxicity.

Discussion

In this study we demonstrate a simple, yet effective method for determining neuron viability in co-culture with inflammatory microglia or macrophages, which can be used for rapid screening of neuroprotective compounds. Methods previously employed to separate cell viability in a co-culture system often involve fixing of the cells, staining with fluorescently conjugated cell-type-specific antibodies or stains, ELISA, confocal microscopic image analysis or FACS analysis [21-25]. These techniques are generally labour intensive, time consuming and expensive, which makes them unsuitable for analyzing the neuroprotective potential of numerous compounds in a medium or high throughput manner in most laboratories. We therefore employed stably-transfected green fluorescent neurons to allow differentiation between neuronal and microglial viability in an increased-throughput co-culture system. The suitability of these neurons is further supported by FACs analysis of neurons co-stained with Propidium iodide, demonstrating that dead neurons lose their green fluorescent potential [20].

During the development of three *in vitro* culture systems in this study, several points of interest regarding the interaction between neurons and inflammatory cells have been elucidated. The three culture systems indicate that proximity of inflammatory cells and neurons is paramount, as only a co-culture that allowed close proximity and direct cell contact resulted in a significant amount of neuron death. The exception to this finding was the result obtained following transferral of conditioned media from J774 macrophages to Neuro2a neurons, which caused a minor decrease in neuron viability due to depletion of the media, rather than inflammatory insult. Although this outcome was not further assessed, it is possible that this effect may be diminished by use of a media with higher glucose content, or by combining activated media with fresh media immediately before transfer to neurons.

The combination of IFNγ and LPS caused dramatic neuron death in a direct co-culture system, which correlates to previous findings [26]. In contrast, the combination of TNF, IL-1β and β-amyloid, which was chosen as a model for the neurodegenerative, inflammatory conditions surrounding senile plaques in Alzheimer's disease, did not induce neurodegeneration in any of the three co-culture models. Although TNF, IL-1β and oligomeric β-amyloid have all previously been found to be neurotoxic, either directly, or via the activation of glial cells [27-34], there are several notable differences in methods between this study and previous studies that may account for the lack of toxicity with this pro-inflammatory combination. Firstly, the β-amyloid used in this study was oligomeric, which is

able to induce inflammation, but is not as pro-inflammatory or neurotoxic as fibrillar β-amyloid [34]. The concentration of β-amyloid used here is also approximately 10 times lower than that used in other studies, despite TNF and IL-1β being used at concentrations previously demonstrated to induce inflammation or neurodegeneration [35-38]. Furthermore, the study by Xie and colleagues also demonstrates that β-amyloid-induced neurotoxicity is dependant on the presence of nitric oxide, the production of which was not observed in combination with TNF and IL-1β in this study. The most significant aspect however, may be that all studies previously demonstrating β-amyloid-induced neurodegeneration in a glial co-culture have utilized primary cultures, while this model of neurodegeneration is based on the use of neuroblastoma lines, for increased throughput. These cells are likely to be more resistant to β-amyloid and cytokine-induced degeneration than primary neurons and may explain why no decrease in neuron viability was observed in response to TNF, IL-1β and β-amyloid.

The use of cultured cell lines however, fails to account for the significant proliferation of neurons in the conditioned media and membrane culture systems in response to these stimuli. In agreement with data that undifferentiated neuroblastoma cells are resistant to TNF toxicity [31], the combination of TNF, IL-1β and β-amyloid had no effect on viability or proliferation when applied directly to neurons (data not shown), indicating that a microglial-derived factor is responsible for inducing neuron proliferation. Although unexpected, this phenomenon has been previously observed, whereby conditioned media from microglia induced proliferation of primary neurons [39]. The factors involved in this pathway require further investigation, but one of the mediators identified in primary neurons is mitogen-activated protein kinase, a pathway which is likely to be activated by factors derived from microglia in the presence of β-amyloid, TNF and IL-1β. Moreover, it is possible that the neuroproliferative effects of cytokines and other long-lived factors produced by microglia are negated by the activity of neurotoxic free radicals and reactive oxygen species. This action accounts for the lack of proliferation observed in the direct co-culture model, where short-lived factors are more likely to act on neurons, compared to the significant proliferation observed in transfer and membrane models where there is unlikely to be a significant effect of short-lived factors on neurons.

There is an ever-growing amount of evidence that supports the role of nitric oxide in neuron degeneration [26, 35, 40, 41]. In particular, Hemmer *et al.* used a similar co-culture method and demonstrated the importance of nitric oxide in neurodegeneration in comparison to TNF, cyclooxygenase or superoxide [25]. We found that in response to IFNγ and LPS,

nitric oxide was consistently produced in all three culture systems, despite only direct culture resulting in neuron death. The half-life of nitric oxide is only a few seconds, before conversion to the stable nitrite or nitrate [42]. It is therefore likely that all nitric oxide produced by inflammatory cells was degraded prior to transfer of conditioned media and that the distance separating inflammatory cells and neurons in membrane culture is enough to cause degradation before the radical can elicit an effect on neurons. Further evidence that nitric oxide plays a significant role in neuron death is the result obtained with iNOS inhibitors. Two inhibitors, S-(2-Aminoethyl)Isothiourea and S-Methylisothiourea, were found to protect neurons against microglial-induced death in a nitric oxide-dependant manner (Figure 4) and concentrations similar to, or lower than previously reported results [26, 43]. Both inhibitors increased viability of neurons to a maximum of approximately 85%, never reaching complete neuron rescue, a result supported by previous data that also resulted in incomplete rescue with iNOS inhibitors [26]. The inability to completely rescue neuronal viability indicates that nitric oxide is not the sole, but a primary mechanism of neuron death in a co-culture system.

This data highlights several points in regard to neuron degeneration: 1) Proximity of inflammatory cells to neurons is crucial in inducing neuron death. By separating neurons from inflammatory cells by as little as 1mm (the distance between cells using membrane co-culture), neuron viability remains unchanged during pro-inflammatory activation. This was demonstrated by the inability of both the conditioned media and membrane systems to induce neuron death. 2) Long-lived factors such as cytokines do not directly cause neuron degeneration. These factors will be present and able to elicit a response in all culture systems utilized, yet both transferral of media and membrane co-culture failed to cause a decrease in neuron viability. 3) Nitric oxide plays a significant role in neuron degeneration in vitro. Activation with a combination of TNF, IL-1β and β-amyloid did not decrease neuron viability and failed to induce nitric oxide production. Conversely, IFNγ and LPS induced the production of significant amounts of nitric oxide and caused dramatic neuron death. Moreover, neuron viability could be rescued using specific inhibitors of nitric oxide synthase. These factors may assist in the further elucidation of neurodegenerative mechanisms and the co-culture system we have described provides a new method for the screening of potential neuroprotective compounds.

Acknowledgements

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Figures

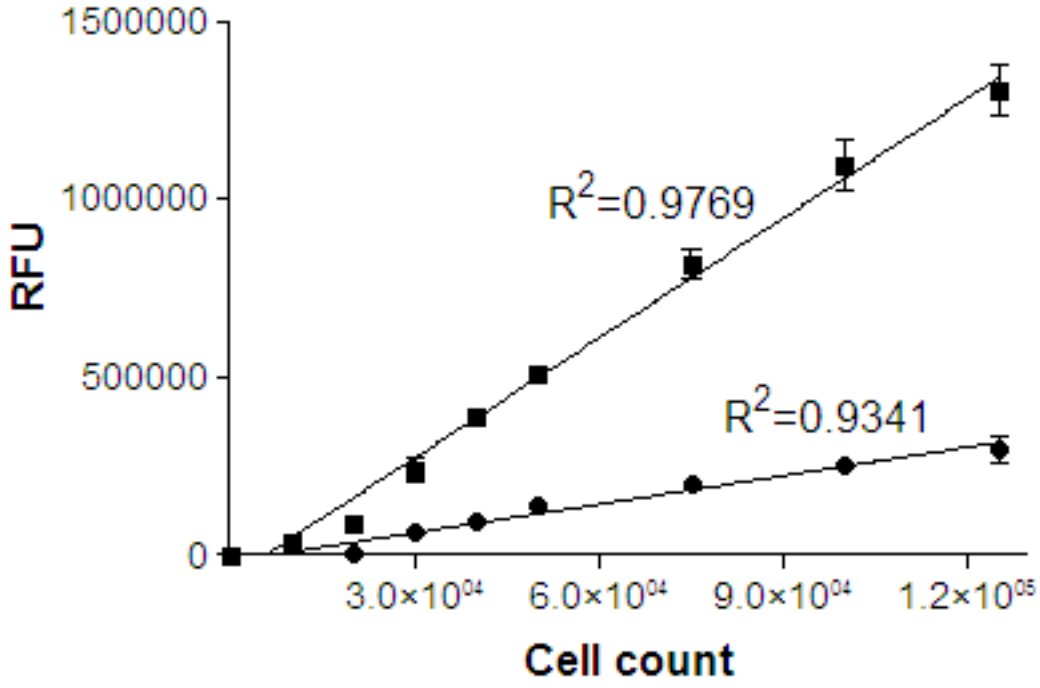

Figure 1. EGFP expression and Alamar Blue fluorescence are dependant on cell number. Neuro2a-GFP cells were seeded at various cell densities in 96 well plates for 2 hours, then left untreated, or 12.5µg/mL of Alamar blue was applied for a further 2 hours. EGFP (●) was measured at 490/530nm and Alamar blue (■) at 545/595nm.

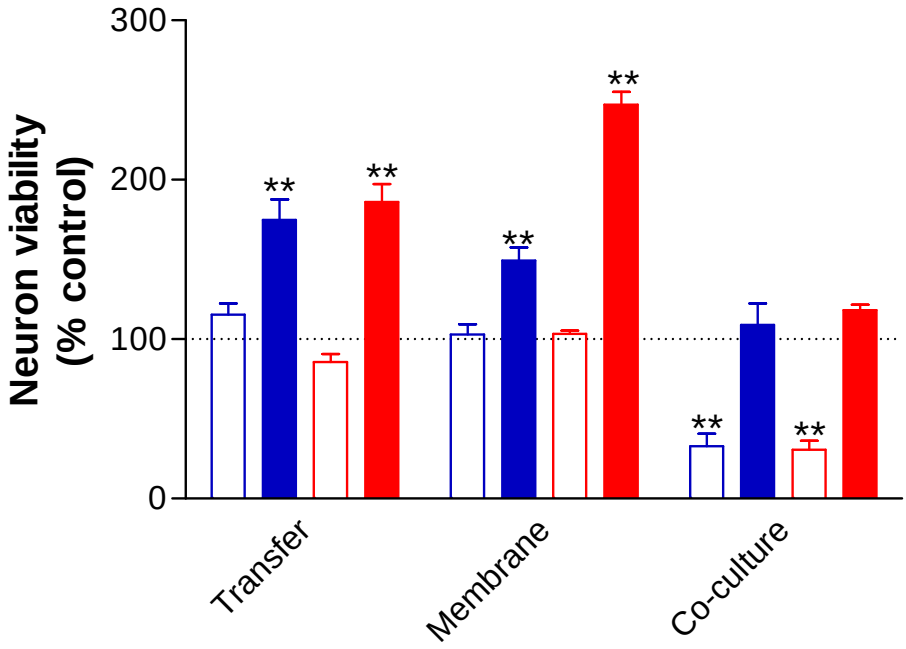

Figure 2. Neuron death in co-culture is dependant on inflammatory cell proximity. HT22 neurons and N-11 microglia (□,■) and Neuro2a neurons and J774 macrophages (□,■) were activated using three co-culture systems. Transfer= activation of microglia/macrophages alone for 48 hours, then conditioned media transferred to neurons alone for a further 48 hours. Membrane= microglia/macrophages cultured in the same well, separated by a membrane for 48 hours. Co-culture= microglia/macrophages cultured with direct contact to neurons for 48 hours. A combination of either 5U/mL IFNγ and 5μg/mL LPS (□,□) or 10U/mL TNF, 10U/mL IL-1β and 500ng/mL β-amyloid (■,■) was used to activate the co-culture systems. Neuron viability was measured by GFP expression at 490/530nm. (* $P<0.05$, ** $P<0.01$ compared to unactivated controls).

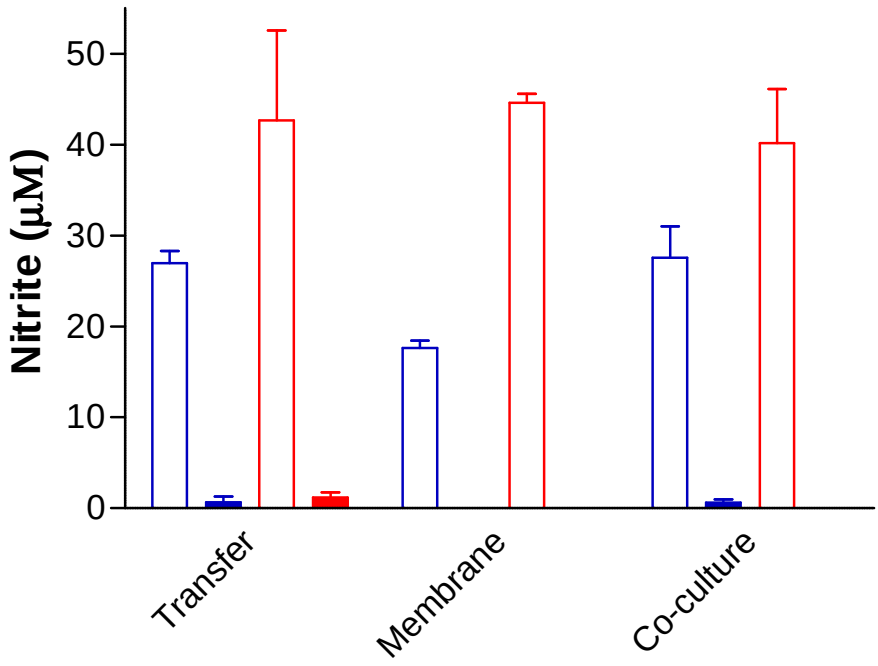

Figure 3. Neuron death in co-culture is dependant on microglia-derived nitric oxide production. HT22 neurons and N-11 microglia (□,■) and Neuro2a neurons and J774 macrophages (□,■) were activated using three co-culture systems. Transfer= activation of microglia/macrophages alone for 48 hours, then conditioned media transferred to neurons alone for a further 48 hours. Membrane= microglia/macrophages cultured in the same well, separated by a membrane for 48 hours. Co-culture= microglia/macrophages cultured with direct contact to neurons for 48 hours. A combination of either 5U/mL IFNγ and 5µg/mL LPS (□,□) or 10U/mL TNF, 10U/mL IL-1β and 500ng/mL β-amyloid (■,■) was used to activate the co-culture systems. Nitric oxide production was determined by measurement of nitrite with Greiss reagent at 545nm.

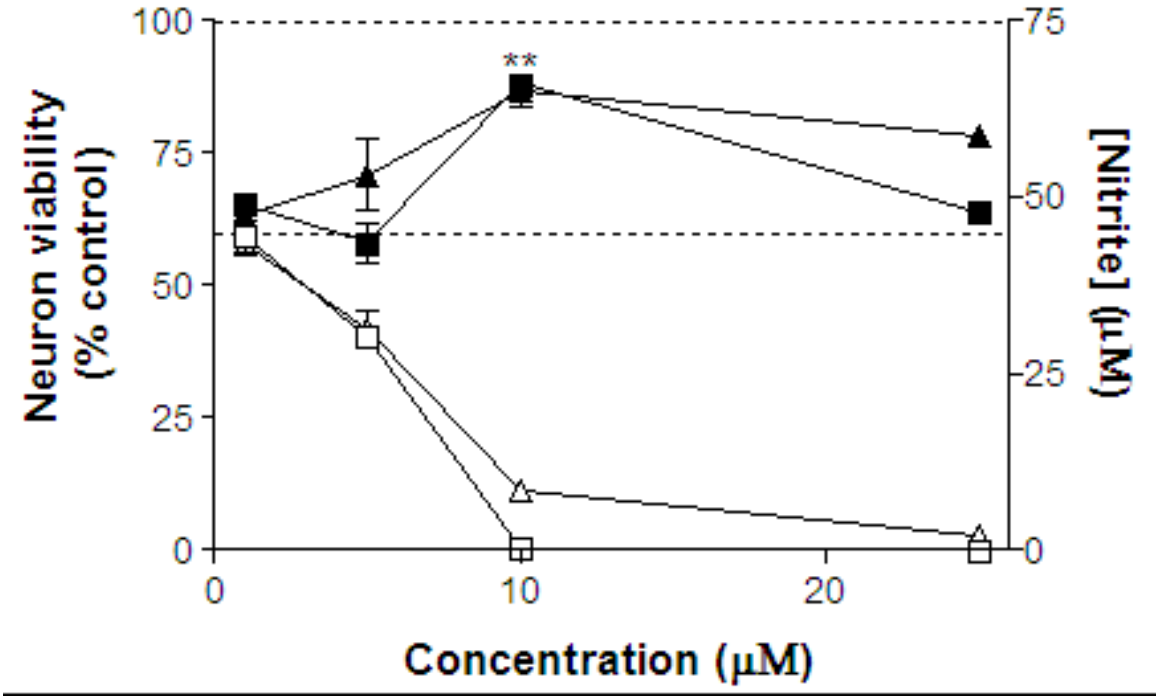

Figure 4. Inhibition of nitric oxide production increases neuron viability following inflammatory insult in co-culture. HT22 neurons and N-11 microglia were activated with 1U/mL IFNγ and 1μg/mL LPS for 48 hours in the presence of iNOS inhibitors Isothiourea (■) and S-MT (▲). Neuron viability (closed symbols) was measured by GFP expression at 490/530nm and nitric oxide (open symbols) was determined by Griess reagent at 545nm. (* P<0.05, ** P<0.01).

Article 4

Selective neuroprotective effects of anti-inflammatory drugs and statins *in vitro*

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Abstract

Following data from epidemiological studies, significant research has been focused on the use of Non-steroidal anti-inflammatory and statin drugs as potential preventative treatments for Alzheimer's disease. The promise of generally well tolerated and currently available therapeutics for the long-term treatment of Alzheimer's disease is what sparked significant interest amongst the research community. In this study, we utilized a co-culture model of neurodegeneration, whereby neuron death is induced by culturing with microglia and activating with pro-inflammatory mediators Interferon-γ and Lipopolysaccharide. Thirteen drugs were assayed in this system, with ibuprofen, indomethcain, sulindac sulphide, lovastatin and fluvastatin displaying neuroprotection. In agreement with previous *in vivo* data, this result indicates that not all anti-inflammatory and statin drugs protect neurons against degeneration *in vitro*. Additionally, isoform-selective anti-inflammatory drugs rofecoxib and celecoxib conferred no neuroprotection, while neuroprotection by statins was observed in the presence of exogenous mevalonate, their primary target in the cholesterol pathway. Neuroprotection *in vitro* is thus not dependant on the inhibition of cycloxygenase or HMG-CoA reductase, the primary targets of anti-inflammatories and statins, respectively. We propose that currently used anti-inflammatory drugs may be of benefit in the prevention of neurodegeneration, through the activity of unidentified targets and not documented primary targets, as initially hypothesized.

Introduction

The search for preventative treatments for Alzheimer's disease is becoming increasingly important, as the worlds' population continues to age and the prevalence of the debilitating disease increases. While the commercial sector focuses on new compounds aimed at the specific causes of Alzheimer's disease, such as β-amyloid (Aβ), β-secretase and Microtubule-Associated Protein-Tau (MAP-Tau), epidemiological evidence of possible neuroprotective anti-inflammatory drugs has recently become a source of relevant information for target discovery.

Initially, pharmaco-epidemiological evidence indicated that elderly patients using non-steroidal anti-inflammatory drugs (NSAIDs) for the treatment of arthritis, may have a decreased risk of Alzheimer's disease [1, 2]. The promise that readily available well established therapeutics, with minimal side effects, had potential as preventative treatments for Alzheimer's sparked great interest in the research community. A plethora of publications was the result of extensive *in vitro* and *in vivo* testing, including clinical trials. The findings of the clinical trials in particular, have since lead to a dramatic decrease in research regarding the efficacy of NSAIDs in the prevention of Alzheimer's disease. Several trials found that the administration of a NSAID caused no change in patient outcomes relative to controls [3-9], while only a few reported an improvement in measured outcomes [10, 11]. Negative results are not surprising, given the array of compounds within the NSAID drug class, the relatively short trial lengths (in comparison to normal disease progression) and the use of current disease sufferers in trials.

More recent epidemiological data provides optimistic outcomes, indicating that only selected drugs from the NSAID class, including ibuprofen and indomethacin, may be neuroprotective against the development of Alzheimer's disease [12]. Although the mechanisms of action have not been identified, this theory is supported by numerous studies that have demonstrated dramatic differences in the ability of NSAIDs to elicit effects on cellular targets other than their primary target, Cycloxygenase (COX). Some of the identified off-target effects of NSAIDs include the inhibition of Nuclear Factor κ-B (NFκ-B) and the activation of Peroxisome-proliferator activated receptors (PPAR), which are known to reduce inflammation and may play a part in neuroprotection [13-15]. This scenario is shared with drugs of the statin class, where epidemiology indicated a possible neuroprotective effect for patients undergoing treatment with statins, but further studies indicated otherwise (reviewed by Rockwood *et al.*, 2006) [16]. Like NSAIDs, statin compounds, which primarily inhibit the

action of Hydroxy-methyl glutaryl-Coenzyme A (HMG-CoA) reductase, are also known to differ greatly in their ability to activate or inhibit alternative cellular targets. It is possible that via the inhibition of HMG-CoA reductase some beneficial effects are observed in Alzheimer's disease, such as a decrease in β-secretase activity or a reduction in Ras-dependant inflammatory signaling. There is however, mounting evidence that side effects such as activation of PPAR [17, 18], as observed with NSAIDs, are the likely candidates that result in neuroprotection by statins and the reason for varied clinical outcomes.

An *in vitro* assay that allows rapid screening of neuroprotective compounds has been developed within our laboratory. This will allow the evaluation of NSAIDs and statins used in the prevention of Alzheimer's disease in an effort to address the conflicting data previously observed [19]. The co-culture system relies on the activation of microglia and release of pro-inflammatory species that, in close proximity, cause a dramatic degradation of neurons, beginning with neurite retraction and eventually cell death. The use of stably transfected, GFP-expressing neurons allows for the determination of neuron viability following this inflammatory insult. This method was used to screen 9 commonly used NSAIDs and 4 statins, to determine whether neuroprotective potential *in vitro* is similar within a drug class.

We demonstrate that despite compounds within a drug class sharing a common primary target, compounds within the NSAID or statin drug families possess significantly different neuroprotective properties. We found that three NSAIDS, Ibuprofen, Indomethacin and Sulindac sulfide, protected neurons against microglial-induced death at concentrations above 10μM, while a further six NSAIDS, including COX-2 selective compounds, demonstrated no protection of neurons against microglial insult. Drugs from the statin class also display significantly different neuroprotective profiles that are independent of HMG-CoA reductase inhibition, with Lovastatin and Fluvastatin demonstrating greatest neuroprotection, with EC_{50} values between 15μM and 20μM. Additionally, we found that neuroprotection is not strictly dependant on the inhibition of inducible Nitric Oxide Synthase (iNOS), despite our previous findings that neuronal death in this culture system is highly dependant on nitric oxide (NO) production.

Methods

Cell Culture

Murine HT22-GFP and N-11 microglia were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO) containing 5% foetal calf serum (FCS), supplemented with penicillin (200U/ml), streptomycin (200μg/ml) and fungizone (2.6μg/ml) (GIBCO). HT22-GFP were also supplemented with 0.25mg/mL G418 to maintain stable selection. Cells were maintained at 37^{oC}, containing 5% CO_2, in a humid environment. Cells were removed from flasks using rubber scrapers (Sarstedt) and counted in a counting chamber (Neubauer) before dispensing into assay plates in serum-free media.

Drug dilutions

Celecoxib (Sigms-Aldrich), diclofenac (Sigms-Aldrich), diflunisal (Sigms-Aldrich), ibuprofen (Sigms-Aldrich), indomethacin (Sigms-Aldrich), naproxen (Sigms-Aldrich), piroxicam (Sigms-Aldrich), rofecoxib (Merck), sulindac sulphide (Sigms-Aldrich), atorvastatin (Pfizer), fluvastatin (Novartis), lovastatin (Merck) and simvastatin (Merck) were initially diluted to 10mM in DMSO. Further dilutions were performed with serum-free media prior to cellular addition, with the final concentration of DMSO not exceeding 0.5% and had no effect on cell viability.

Neuroprotection in co-culture

HT22 neurons ($4x10^4$/well) and N-11 microglia ($1x10^4$/well) were dispensed together into 96 well plates and incubated for 20 hours. Various concentrations (1-50μM) of anti-inflammatory compounds were then added for 4 hours prior to activation. These concentrations were chosen based on a previous assay where no anti-inflammatory effects were observed at concentrations below 1μM and toxicity began above 50μM. For assays with statin compounds, 500μM Mevalonate was also added simultaneously with the test compound, prior to activation. Cells were activated with 1U/mL IFNγ and 1μg/mL LPS for 48 hours. Nitric oxide content and neuron viability were then determined.

Nitric oxide determination

NO production was monitored by measuring the concentration of nitrite in the media using the 'Griess reagent'. Conditioned media (75μL) from each well was transferred to a fresh 96-well plate and 75μL of Griess reagent (1%w/v sulfanilamide and 0.1%w/v naphthyethylene-diamine in 2.5% HCl) was added and the absorbance at 545nm measured using a Victor V

spectrometer. Concentrations were then determined by comparison to a standard curve prepared with sodium nitrite.

Neuron viability determination

Viability of HT22 was determined by measurement of total EGFP fluorescence. After media was removed for NO determination, the remaining media was aspirated and the fluorescence of each well was immediately measured at 490/530nm using a Victor V plate fluorimeter. Viability was determined as a percentage of positive and negative control wells present on each plate.

Statistical analysis

Results were graphed using Graphpad Prism 4 and are representative of three independent experiments, performed in triplicate (n=3). Statistical analysis was performed using a one-way ANOVA, with p=0.05 (*), comparing each data point to activated controls. Error bars represent the standard error of the mean (SEM).

Results

Neuroprotective effect of Non-steroidal anti-inflammatory drugs

Nine NSAIDs were assayed for neuroprotective potential using a co-culture system of HT22 neurons and N-11 microglia. Following a four hour incubation with various concentrations of each compound (1-50μM), microglia were activated for 48 hours with a combination of 1U/mL IFNγ and 1μg/mL LPS. These amounts of activators were chosen from a viability curve (data not shown) that resulted in a reduction of 50%, to reduce the possibility of therapeutics failing to protect neurons, due to overstimulation of microglia. The viability of neurons was then determined by measurement of the GFP fluorescence from the stably expressing neurons and compared to unactivated controls. Of the 9 compounds tested, only 3, Ibuprofen, Indomethacin and Sulindac sulfide, rescued neuronal viability (Figure 1A). At concentrations below 10μM, none of the 3 effective compounds demonstrated any increase in neuron viability, and only Indomethacin increased viability at 10μM. Both Ibuprofen and Indomethacin displayed a steady increase in viability at 25-50μM, while Sulindac only prevented neuron death at 50μM, the highest concentration tested. Although these concentration are 10-50 times greater than observed plasma concentrations [20, 21], the high concentrations of IFNγ and LPS as activators must also be noted. The maximum viability observed for the 3 compounds (all at 50μM) was remarkably similar, at approximately 50% of controls. The highest total increase however, was due to Ibuprofen which increased neuron viability by 19%. All other NSAIDs tested either displayed no change, or a decrease in neuron viability (Figure 1B). Of the non-selective NSAIDs, only Naproxen resulted in no viability change across the concentrations tested, while Diclofenac, Diflunisal and Piroxicam displayed toxicity and subsequently decreased viability by 16, 19 and 5% respectively, at 50μM. Two COX-2 specific inhibitors, Celecoxib and Rofecoxib, were also assayed for neuroprotective properties and demonstrated vastly different profiles. Rofecoxib had no effect on neuron viability, except at 50μM, where a slight increase, of less than 4%, was observed. Celecoxib however, cause dramatic neuron death at concentrations of 25μM and above, resulting in the greatest toxicity caused by any of the NSAIDs tested, with a decrease of 40% at 50μM. Direct toxicity of compounds to neurons and microglia individually was not measured, so these decreases in neuron survival may not be due to increased activation of microglia, but toxicity. Thus, no assumptions in regards to increasing inflammation and neurodegeneration in the disease state can be made.

Neuroprotective effect of statin drugs

Four compounds from the statin class of drug were assayed under the same conditions as described above, but at time of drug addition, mevalonate was also added, to a final concentration of 500μM. Mevalonate is the product of HMG-CoA reductase and serves as a precursor for cholesterol synthesis. The intermediates between mevalonate and cholesterol are involved in small G-protein anchoring, the inhibition of which has been suggested to be a primary anti-inflammatory mechanism of statins (Figure 3). Atorvastatin caused a sharp decrease in neuron viability as low as 5μM, with complete neuron death observed at concentrations of 10μM and greater. We have previously found that atorvastatin inhibited NO and TNF production to a greater extent than any of the statins assayed (unpublished). This data however, was after 24 hours with only microglia, where no toxicity was observed. Atorvastatin may be more toxic to neurons than microglia, particularly for the extended incubation period used in this assay, thereby conferring toxicity as opposed to neuroprotection.Simvastatin also caused neurotoxicity at 25 and 50μM, following a slight increase (9%) in viability at 5 and 10μM. The two statins that led to an increase in neuron viability were Fluvastatin and Lovastatin, the latter demonstrating greater efficacy. Neither drug significantly altered viability below 10μM, but both increased viability at 25μM and Lovastatin continued to exert positive effects at 50μM. The total increase in viability at this concentration of Lovastatin was 19%, which corresponds to the highest total viability increase observed in the presence of NSAIDs.

Nitric oxide production in relation to neuroprotective compounds

Following the 48 hours incubation with NSAIDs and statins, media was transferred to fresh 96 well plates and combined with the Griess reagent for the detection of nitrite (the stable product of NO) at 545nm. Nitrite was measured as we have previously found NO production to be correlated with neuron viability, by use of specific NOS inhibitors (unpublished). All compounds were therefore assayed for their effect on NO production and several compounds demonstrated a nitrite decrease, but were directly proportional to the toxicity observed (Figure 1). Therefore, only the nitrite content in the presence of neuroprotective compounds is displayed. Of the three NSAIDs that displayed neuroprotective effects (Ibuprofen, Indomethacin and Sulindac sulfide), only Indomethacin resulted in a significant decrease in NO production. This reduction was concentration-dependant across the concentrations assayed, with the highest NO reduction of 8μM (equivalent to 24% inhibition) observed at 50μM Indomethacin. Unlike NSAIDs, both of the neuroprotective statins decreased NO production. Fluvastatin displayed an almost linear pattern of concentration-dependant NO

inhibition across the concentrations assayed, with a maximum decrease of 5μM, equivalent to 12%. The inhibition observed for Lovastatin was far greater, with a maximum of 23μM (68%) observed at 50μM Lovastatin. There was no effect on NO production observed below 10μM, which coincides with the lack of neuron viability modification at the same concentrations. The inhibition of NO at 25μM (24%) and 50μM (68%) Lovastatin is also similar to the pattern of neuroprotection observed, although neuroprotection values were not as great, at 14% and 19% respectively.

Discussion

The elucidation of the disease process in Alzheimer's disease has led to the identification of several potential drug targets that may assist in delaying or even halting the progression of neuronal degeneration. A major drawback of investigating new targets is the unknown adverse effects that may occur through their inhibition or alteration, as was observed during immunization studies with synthetic β-amyloid. Although the treatment appears to reduce cognitive decline, via stimulation of an antibody-mediated immune response, encephalitis was observed in several trial subjects and the trial was discontinued [22]. Although this is an extreme case, adverse effects of any magnitude are of great concern in Alzheimer's disease treatment, which may last for several years. For this reason, the use of current drugs with known adverse effects is a promising prospect and one of the reasons that has lead to numerous studies with non-steroidal anti-inflammatory and statin drugs.

The use of an *in vitro* model of neuron degeneration, has allowed us to demonstrate that, like previous *in vivo* results, non-steroidal anti-inflammatory and statin drugs do not all share equivalent neuroprotective properties. Of the nine NSAIDs assayed, only three, ibuprofen, indomethacin and sulindac sulfide, protected neurons from microglia-mediated degeneration. Although no increase in neuron viability was observed for any NSAIDs at concentrations below 10μM, maximum neuroprotection was in the presence of ibuprofen, which at a concentration of 50μM, increased neuron viability from 32% to 51% of unactivated controls. Although this increase in viability is not as impressive as a complete rescue of neurons, when translated to the *in vivo* situation, this small amount of neuroprotection may delay the onset of Alzheimer's disease by several years. The IC_{50} for NSAIDs in relation to their primary target COX-2 is generally in the sub- to low micromolar range [23], significantly lower than the concentrations required to protect neurons against degeneration in this study. Therefore, given that not all NSAIDs provided neuroprotection and those that did, did so at concentrations far exceeding those required to inhibit COX-2, it is plausible that the difference in side effects of the NSAIDs are responsible for any neuroprotection conferred by this drug class. Interestingly, not only have the three effective NSAIDs previously been documented to have numerous side effects, particularly alteration of Peroxisome-proliferator activated receptors and Nuclear factor κ-B activity [13-15], but they have also been identified as more likely to prevent Alzheimer's disease through more recent observational studies [12]. Moreover, these results correlate with our *in vitro* data, indicating that ibuprofen is the most neuroprotective NSAID [12].

In the same manner as NSAIDs, there has been much debate regarding the role of the primary target HMG-CoA reductase, and its inhibition by statins, in the prevention of Alzheimer's disease. Simvastatin and pravastatin did not display efficacy in clinical trials, which is the opposite of the expected outcome if HMG-CoA reductase inhibition and subsequent isoprenylation and small G-protein signaling decreases were solely responsible [24]. It is therefore likely that the primary mechanism of neuroprotection is not related to HMG-CoA reductase inhibition, but alternate properties of selected statins. Our results support this hypothesis, as only lovastatin and fluvastatin increased neuron viability following microglial insult. Not only was this neuroprotection observed at concentrations far exceeding that required to inhibit their primary target, but this protection was afforded in the presence of mevalonate, which negates any effect of HMG-CoA reductase inhibition. All statins were also tested alone, without mevalonate, but all concentrations lead to a substantial decrease in neuron viability (data not shown). This effect was likely due to the length of the assay, with cells unable to survive for 48 hours in the absence of cholesterol and its intermediates, including Co-enzyme Q10, as this effect was not observed in the presence of mevalonate.

As previously mentioned, there are inherent issues with clinical trials for Alzheimer's disease, particularly when assessing preventative therapies as opposed to treating current dementia patients. The use of Alzheimer's disease patients in trials, the duration of treatment and the cognitive or physical outcomes used to measure success all contribute to the complexity of clinical trials and may account for some of the unfavorable results. It is for this reason that direct comparisons between *in vitro* and current clinical results are difficult. Long-term observational studies, such as that conducted by Vlad and colleagues [12] may provide a more accurate depiction of the neuroprotective effects of therapies, which as observed here, may correlate more closely to *in vitro* data.

Further supporting the unsuitability of current clinical methodology are outcomes with statins that have thus far contradicted epidemiological studies. A factor often suggested as a primary cause of these failures in clinical trials is the inability of the compounds to cross the blood-brain barrier. The two statins previously mentioned as ineffective in clinical trials, simvastatin and pravastatin, however, are lipohilic and hydrophilic respectively, suggesting that lipophilicity may not be related to neuroprotective potential. Our data also supports this outcome, as the four statins assayed are all considered lipophilic [25], yet demonstrated vastly different neuroprotective profiles in an *in vitro* system that is independent of lipophilicity and the blood-brain barrier. Again, it is possible that studies of a longer duration, in subjects not

currently diagnosed with Alzheimer's disease, using disease prognosis as an outcome may give a clearer indication regarding the efficacy of various statins as preventative treatments.

Our group and others, have previously found that neuron degeneration *in vitro* is highly dependant on microglia-derived NO production [26]. The inhibition of NO production by NSAIDs and statins in this co-culture system was therefore investigated. Unexpectedly, the correlation between NO inhibition and neuroprotection was not as clearly defined as previously observed. Of the five compounds that demonstrated neuroprotection, only indomethacin and lovastatin significantly inhibited NO production, but did not correlate closely with neuron viability (Figure 2).

In this report, we have demonstrated that compounds from the non-steroidal anti-inflammatory NSAID drug class do not exert equal neuroprotective effects *in vitro*. Although compounds are grouped into the NSAID drug class based on their ability to inhibit the cyclooxygenase enzymes, there are clearly other factors that result in vastly different neuroprotective potential by NSAIDs. This scenario is also apparent for drugs of the statin class, which not only display varying neuroprotective properties, but do so while the inhibition of their primary target, HMG-CoA reductase, is negated. Moreover, the NSAIDs and statins that demonstrated neuroprotection using this *in vitro* method (ibuprofen, indomethacin, sulindac sulfide, fluvastatin and lovastatin) primarily coincide with those previously earmarked as compounds more likely to prevent the development of Alzheimer's disease *in vivo*. Through further clinical trials, utilizing *in vitro* data such as this study to better select trial compounds, it is possible that currently used anti-inflammatory drugs may yet provide more favorable clinical outcomes in the prevention of Alzheimer's disease.

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Figures

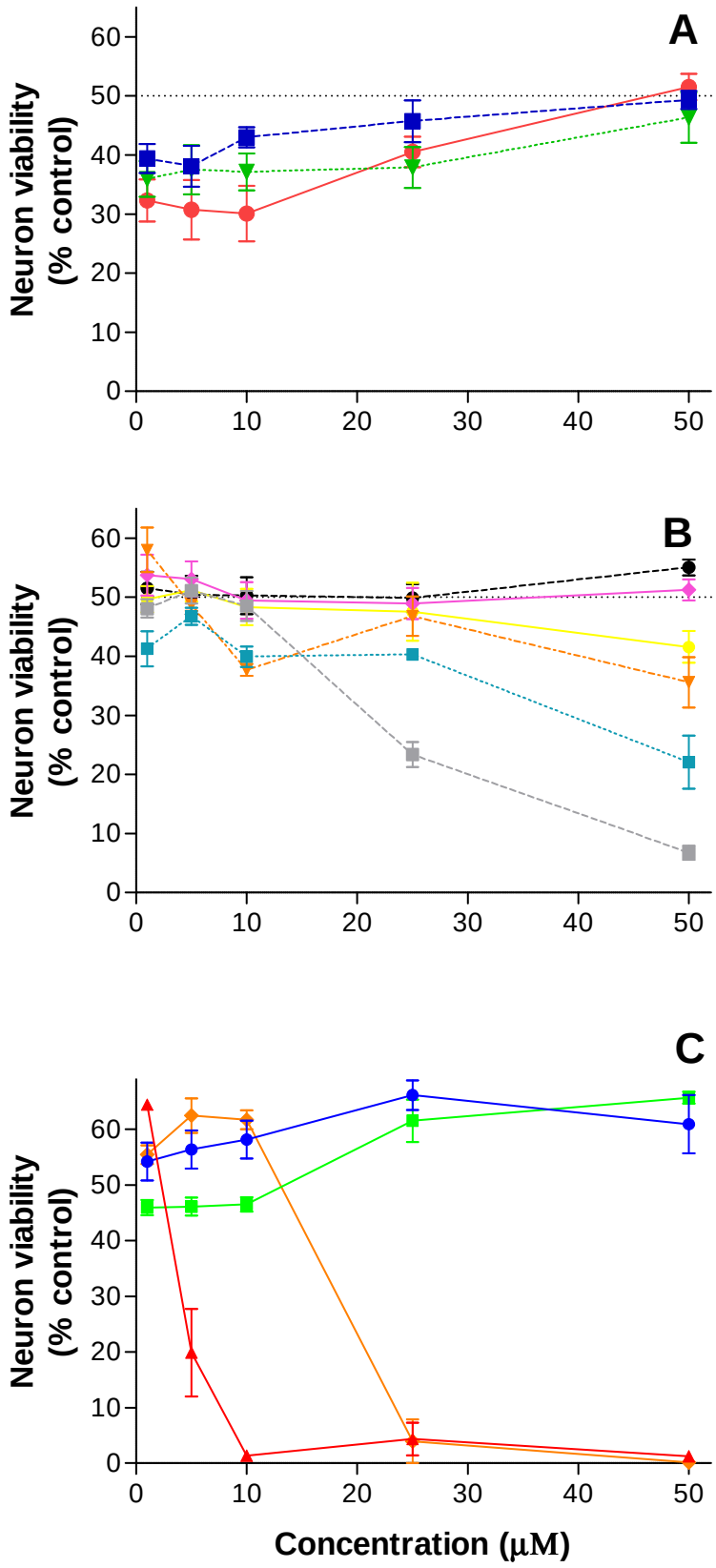

Figure 1. Effect of selected Non-steroidal anti-inflammatory and statin drugs on neuron viability following microglial insult. HT22 neurons and N-11 microglia were pre-incubated

with anti-inflammatory drug for 4 hours prior to activation with 1U/mL IFNγ and 1μg/mL LPS for 48 hours. Neuron viability was determined by neuronal GFP fluorescence. A) Non-selective NSAIDs demonstrating a neuroprotective effect: ibuprofen (●), indomethacin (■) and sulindac sulfide (▼). B) Non-selective: diclofenac (■), diflunisal (▼), naproxen (♦) and piroxicam (●) and COX-2 selective: celecoxib (□) and rofecoxib (○) NSAIDs with no neuroprotective effect. C) Effect of statins: atorvastatin (▲), fluvastatin (●), lovastatin (■) and simvastatin (♦) on neuron viability in the presence of mevalonate.

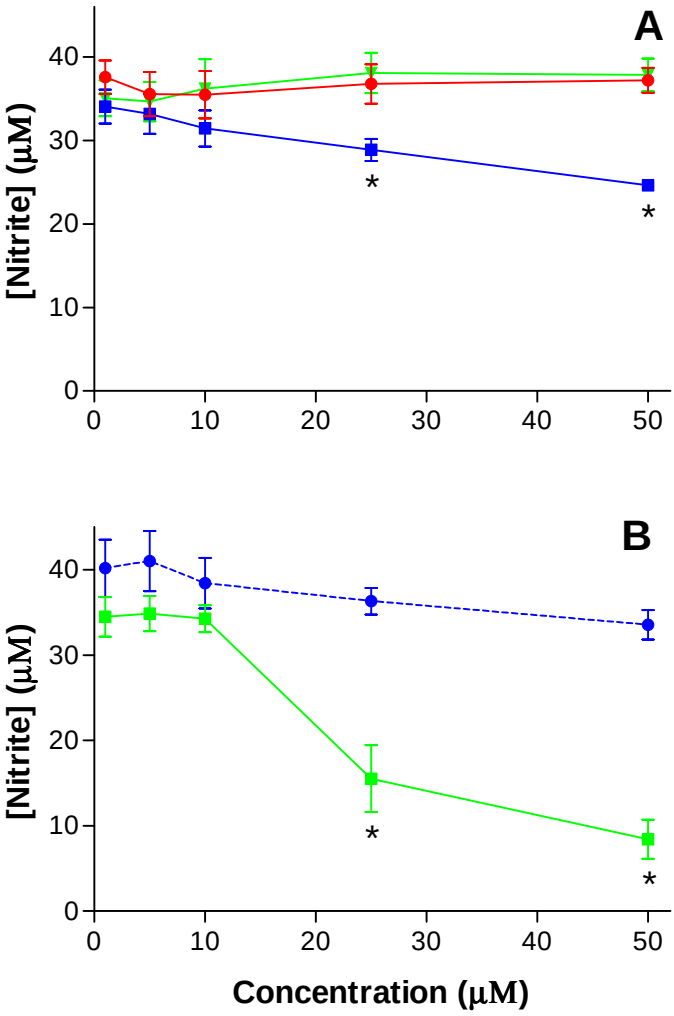

Figure 2. Effect of neuroprotective compounds on nitric oxide production. HT22 neurons and N-11 microglia were pre-incubated with anti-inflammatory drug for 4 hours prior to activation with 1U/mL IFNγ and 1μg/mL LPS for 48 hours. Nitric oxide was determined by measurement of nitrite with Griess reagent at 545nm. A) Nitrite production in the presence of neuroprotective NSAIDs: ibuprofen (●), indomethacin (■) and sulindac sulfide (▼). B) Nitrite production in the presence of neuroprotective statins and mevalonate: fluvastatin (●) and lovastatin (■).

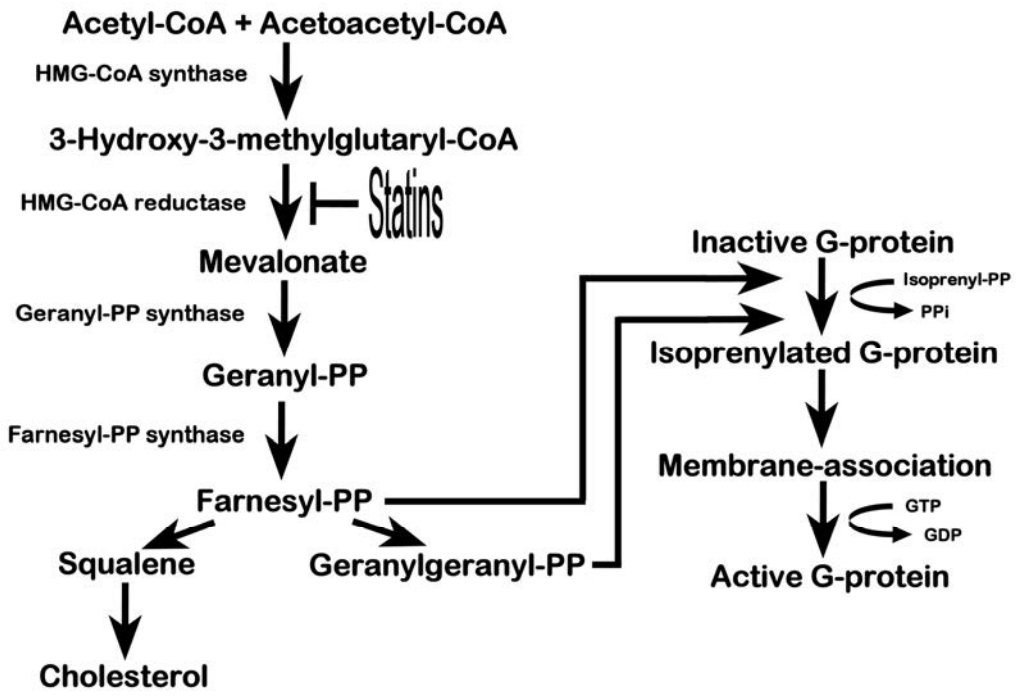

Figure 3. Relationship between cholesterol synthesis and small G-protein signaling. HMG-CoA reductase is known as the rate-limiting step in cholesterol synthesis and is inhibited by drugs from the statin class. The normal product of this enzyme is mevalonate that is converted to several isoprenyl intermediates, prior to cholesterol synthesis. These isoprenyl intermediates are also used as markers for localization of specific membrane-bound proteins, including small G-proteins such and Ras and Rho.

Article 5

Neuroprotective effects of natural product-derived compounds *in vitro*

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Abstract

The extended duration of Alzheimer's disease progression calls for low risk therapeutics that are well tolerated and therefore suitable for several years of therapy. The low toxicity and general safety associated with currently available nutraceuticals means that this treatment class may be of significance. Low cost and the ease with which patients can obtain these treatments are further benefits for the long term prevention of Alzheimer's disease. With these points in mind, we have used two cellular models of neurodegeneration to demonstrate the neuroprotective potential of several natural compounds. The models include a co-culture of microglia and neurons, activated by Interferon-γ and Lipopolysaccharide to simulate inflammation-dependant neurodegeneration and hydrogen peroxide addition to neurons to simulate oxidant-induced neuron death. Incubation with Co-enzyme Q10, Apigenin and Diosmetin significantly increased neuron viability following microglial inflammatory insult in the co-culture system. This neuroprotection was partially attributable to an inhibition of nitric oxide production, a key regulator of neuron death in this co-culture model. Of these nutraceuticals, Co-enzyme Q10 also displayed rescue of neurons against direct hydrogen peroxide attack, indicating it may provide neuroprotection via multiple anti-inflammatory and anti-oxidant pathways. The *in vitro* evidence we present suggests that selected nutraceuticals are able to rescue neurons *in vitro*, warranting further investigation in regards to their neuroprotective potential.

Introduction

The prevalence of Alzheimer's disease (AD) is steadily increasing, as the worlds' population continues to age. Globally, there will be an estimated 4.6 million newly diagnosed patients every year, leading to an estimated 42 million patients with this debilitating disease by 2020 [1].This emphasizes the need for more effective preventative treatments to replace the current regimes that primarily focus on symptomatic treatment, but do little to delay the progression of the disease. The commercial sector is primarily focused on new compounds aimed at the specific causes of Alzheimer's disease, such as β-amyloid, β-secretase and Microtubule-Associated Protein-Tau. The acute inflammatory nature of the disease however, means that therapies that result in a general decrease in brain inflammation, via non-specific targets, may also provide benefit for patients.

Non-steroidal anti-inflammatory drugs (NSAIDs) are amongst the most commonly used medications worldwide and epidemiological studies have indicated a potential role for selected drugs of this class in the prevention of Alzheimer's disease, leading to numerous *in vitro* and clinical studies [2-4]. These drugs however, are known to have side effects including gastric ulcers, decreased renal function and congestive heart failure, particularly following long-term use [5-7]. They are therefore not ideal for the ongoing treatment of Alzheimer's disease. There are however, numerous natural compounds with known anti-inflammatory and antioxidant abilities that are likely to be safer for long-term therapy [8-10].

Natural remedies are largely overlooked in favor of traditional commercial drugs, but are becoming more common place and have been shown to be beneficial for several diseases, including psoriasis, conjunctivitis, osteoarthritis and breast cancer [11-14]. Moreover, numerous compounds derived from natural product sources have been proposed as likely candidates in the prevention of AD, Lipoic acid, Curcumin and Ginkgo biloba extract being some of the front-runners [15-18]. As is the case with many Alzheimer's disease trials, the success of extracts in clinical studies has been less than impressive, with results varying from no benefit to minimal benefit, depending on the method of analysis [19, 20]. Trials in Alzheimer's disease have generally been conducted using patients already diagnosed, rather than assessing the ability of agents to delay the onset of the disease prior to diagnosis. These are two very different scenarios and it is possible that diagnosed patients are at an advanced stage of neuronal damage that can not benefit from anti-inflammatory or antioxidant therapy [21]. It is therefore not surprising that potential preventative therapies have not performed well in clinical trials. Given the unique differences between prevention and treatment in

Alzheimer's disease, long-term observational studies may be a more attractive approach as opposed to traditional clinical trials [22]. Such trials are not only lengthy and costly, but the number of antioxidant and anti-inflammatory extracts available is substantial. We therefore developed an *in vitro* assay that allows rapid screening for the identification of neuroprotective agents that are likely to provide more positive outcomes in future trials [23].

Here we use a co-culture system that relies on the activation of microglia and release of pro-inflammatory species that, in close proximity, cause dramatic neuron death. The use of stably transfected, GFP-expressing neurons allows for the determination of neuron viability following this inflammatory insult. As an initial study, this method was used to screen 6 natural compounds for neuroprotective potential. Three of these compounds, Estradiol, curcumin and Coenzyme Q10, have been suggested as neuroprotective compounds, while the remaining compounds share similar structures with minimal research on their cellular effects [24-28]. The same compounds were always assayed for their ability to directly protect neurons against toxic insult by hydrogen peroxide, in a neuron-only environment.

We demonstrate that Coenzyme Q10 is neuroprotective against both microglial-derived and hydrogen peroxide-induced neuronal death, at concentrations above 25µM. Apigenin and Diosmetin, the structures of which are closely related, displayed neuroprotection against microglial-induced death at concentrations greater than 5µM, but were not effective at direct protection against hydrogen peroxide. We have previously observed that neuronal death in the co-culture system is highly dependant on nitric oxide production. In this study however, although nitric oxide was partially inhibited by all three of the neuroprotective extracts, there was no direct correlation between nitric oxide inhibition and neuron survival.

Methods

Cell Culture

Murine HT22-GFP (James Cook University, Australia) and N-11 microglia (obtained from the University of Tüblingen, Germany) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO) containing 5% foetal calf serum (FCS), supplemented with penicillin (200U/ml), streptomycin (200μg/ml) and fungizone (2.6μg/ml) (GIBCO). HT22-GFP were also supplemented with 250μg/mL G418 to maintain stable selection. Cells were maintained at 37^{oC}, containing 5% CO_2, in a humid environment. Cells were removed from flasks using rubber scrapers (Sarstedt) and counted in a counting chamber (Neubauer) before dispensing into assay plates.

Compound dilutions

Apigenin (90%, Nutrafur), Diosmetin (90%, Nutrafur), Diosmin (90%, Nutrafur), Curcumin (95%, Jarrows Formulas), Coenzyme Q10 (Sigma-Aldrich) and 17β-estradiol (Sigma-Aldrich) were initially diluted to 10mM in DMSO. Further dilutions were performed with serum-free media prior to cellular addition, with the final concentration of DMSO not exceeding 0.5% and had no effect on cell viability.

Neuroprotection in co-culture

HT22 neurons ($4x10^4$/well) and N-11 microglia ($1x10^4$/well) were dispensed together into 96 well plates and incubated for 20 hours in serum-free media. Various concentrations (1-50μM) of compounds were then added for 90 minutes prior to activation. Cells were activated with 1U/mL IFNγ and 1μg/mL LPS for 48 hours. Nitric oxide content and neuron viability were then determined.

Neuron viability determination in co-culture

Viability of HT22 was determined by measurement of total EGFP fluorescence. After media was removed for nitric oxide determination, the remaining media was aspirated and the fluorescence of each well was immediately measured at 490/530nm using a Victor V plate fluorimeter. Viability was determined as a percentage of positive and negative control wells present on each plate.

Nitric oxide determination

Nitric oxide production was monitored by measuring the concentration of nitrite in the media using the 'Griess reagent'. Conditioned media (75μL) from each well was transferred to a

fresh 96-well plate and 75μL of Griess reagent (1%w/v sulfanilamide and 0.1%w/v naphthyethylene-diamine in 2.5% HCl) was added and the absorbance at 545nm measured using a Victor V spectrometer. Concentrations were then determined by comparison to a standard curve prepared with sodium nitrite.

Neuroprotection against Hydrogen peroxide-induced death

HT22 neurons ($5x10^4$/well) were dispensed into 96 well plates and incubated for 24 hours in serum-free media. Various concentrations (1-50μM) of natural extracts were then added simultaneously with Hydrogen peroxide (75μM final concentration) and incubated for 6 hours. Media was then aspirated and neuron viability was then determined via the reduction of Alamar Blue to a fluorescent product at 545/595nm in fresh media.

Statistical analysis

Results were graphed using Graphpad Prism 4 and are representative of three independent experiments, performed in triplicate (n=3). Statistical analysis was also performed using a one-way ANOVA, with $P<0.05$ (*), comparing each data point to activated controls. Error bars represent the standard error of the mean (SEM).

Results

Neuroprotective effect of natural compounds in co-culture

Six natural extracts were assayed for neuroprotective potential using a co-culture system of HT22 neurons and N-11 microglia. Following a 90 minute incubation with various concentrations of each compound (1-50μM), microglia were activated for 48 hours with a combination of 1U/mL IFNγ and 1μg/mL LPS. The viability of neurons was then determined by measurement of eGFP fluorescence from the stably expressing neurons. Viability is expressed as a percentage of unactivated co-culture controls.

Figure 2 demonstrates that three of the extracts provided neuroprotection against microglial insult. Of these, Apigenin displayed a positive effect at the lowest concentration of 5μM, with an increase in neuron viability of approximately 15%, which was also observed at 10μM. Higher concentrations of Apigenin not only failed to further increase neuron viability, but displayed toxicity. Diosmetin however, increased neuron viability to the same extent as Apigenin (approximately 15%), but did so at higher concentrations of 25μM and 50μM and did not display toxicity. The third of the extracts that demonstrated neuroprotection was Co-enzyme Q10. The mitochondrial-related co-factor caused a dramatic decrease in neuron viability below 10μM, but increased neuron viability above 25μM, leading to the highest neuron viability increase observed for any extract (22%), at 50μM. Diosmin and Estradiol did not significantly alter neuron viability in the concentration range assayed, while Curcumin caused significant toxicity to neurons at concentrations above 10μM.

Nitric oxide inhibition by natural extracts in co-culture

Following the 48 hour co-culture incubation with extracts, media was transferred to fresh 96 well plates and combined with the Griess reagent for the detection of nitrite (the stable product of nitric oxide) at 545nm. Nitrite was measured as we have previously found nitric oxide production to be correlated with neuron viability, by use of specific nitric oxide synthase inhibitors (unpublished). All compounds were therefore assayed for their effect on nitric oxide production.

Apigenin and Diosmetin, the two extracts that displayed neuroprotection at the lowest concentrations, also decreased nitric oxide production (Figure 3). This reduction however, is not directly related to the increase in neuron viability observed (Figure 2). At 10μM, Apigenin increased neuron viability by 15%, but the decrease in nitric oxide was much greater at approximately 27%. Likewise with Diosmetin, which at 25μm decreased nitric oxide

production by 38%, while neuron viability again increased by 15%. The highest concentration of Diosmetin (50μM) inhibited nitric oxide by a further 10%, but no further increase in neuron viability was observed. Coenzyme Q10, which conferred the greatest neuroprotection of all extracts at 50μM, marginally decreased nitric oxide production at 25μM ($P<0.05$), but at 50μM, nitric oxide production was not significantly different from controls, suggesting a highly effective neuroprotective mechanism independent of nitric oxide. Of the extracts that failed to protect neurons against degradation, only Curcumin significantly reduced nitric oxide content, but this was at concentrations (>10μM) that also resulted in cellular toxicity.

Neuroprotection against Hydrogen peroxide-induced death by natural extracts

The same extracts assayed for neuroprotective properties using the co-culture system were also assayed for their ability to protect neurons alone, against direct toxic insult by Hydrogen peroxide. Extracts were simultaneously added with 75μM Hydrogen peroxide to HT22 neurons for 6 hours, before neuron viability was determined via the reduction of Alamar blue. This concentration, which is comparable to, or less than used in previous studies, resulted in an approximate 60% reduction in neuron viability [29-31].

Diosmetin and Coenzyme Q10 were both significantly increased neuron viability following Hydrogen peroxide insult. At 5μM, these extracts increased viability by 8% and 11% respectively. Diosmetin maintained the same level of neuroprotection at all concentrations assayed. Coenzyme Q10 however, demonstrated increased neuroprotection at higher concentrations, with 25μM increasing neuron viability by a maximum of 22%. Estradiol and Diosmin had no significant effect on neuron viability, but Apigenin and Curcumin caused toxicity at concentrations above 10μM, in the same manner that was observed in co-culture assays.

Discussion

The inflammation that causes neuron degeneration in Alzheimer's disease is of an acute, but chronic nature, resulting in a gradual decline in neuron functionality over several years. For this reason, the use of therapeutics that have negligible adverse effects and are suitable for long-term therapy is of great importance. Natural extracts and compounds generally possess low toxicity, with the added benefit of low cost when compared to traditional drug therapies. With these factors in mind, we have utilized an *in vitro* model of neuron degeneration to demonstrate that several natural extracts, including Coenzyme Q10, Apigenin and Diosmetin, possess neuroprotective properties.

Coenzyme Q10, in particular, demonstrated dramatic protection of neurons against both microglia-derived inflammatory insult and direct attack by hydrogen peroxide. Essential in the mitochondrial electron transport chain, Coenzyme Q10 acts as both an electron acceptor and as a coenzyme in the production of adenosine triphosphate (ATP) [32-34]. It is therefore plausible that an increase in endogenous Coenzyme Q10 would increase available ATP, resulting in greater survival during neuronal stress. In addition to this neuroprotective mechanism, Coenzyme Q10 is known to possess several beneficial effects that may also promote neuron survival. It has been found to be antioxidant; scavenging free radicals in its reduced state, anti-inflammatory; inhibiting pro-inflammatory cytokines via both gene and protein inhibition, and anti-apoptotic; inducing expression of mitochondrial uncoupling proteins [35-38]. Of these, the most important mechanism of neuroprotective action of Coenzyme Q10 in this research is likely to be antioxidant activity, given the increase in neuron viability when challenged directly with hydrogen peroxide and the lack of inflammatory inhibition, as measured by nitric oxide production, in microglia-neuron co-culture.

Conversely, apigenin and diosmetin appear to impart neuroprotection by reducing inflammation, with less antioxidant involvement. This can be surmised from the results in co-culture, where nitric oxide production decreased as neuron viability increased, while minimal antioxidant action caused hydrogen peroxide-induced neuron death to remain relatively unchanged. These extracts, Diosmetin in particular, have not been as extensively studied compared to Coenzyme Q10, but have been found to inhibit p65 phosphorylation and Nuclear Factor κ-B activation, p38 Mitogen-activated protein kinase, c-Jun N-terminal kinase, STAT-1, Interleukin 1-β, Tumour Necrosis Factor, nitric oxide and prostaglandins, macrophage proliferation and reduce lipid peroxidation [39-45]. The result obtained with these extracts is

of interest, as they share remarkably similar structures, indicating that a shared portion of the structure conveys neuroprotection (Figure 1). The extract (-)-epigallocatechin-3-gallate (EGCG) also shares structural features and has been more extensively studied and has been shown to completely rescue neurons from hydrogen peroxide-induced death [46]. This is in stark contrast to the minimal increase in neuron viability we observed during this study, which can be attributed to methodological differences. The concentrations required to observe dramatic rescue with EGCG were in excess of 100µM and over a period of twenty-four hours, as opposed to the six hour incubation used in this assay. The increased protective effect of EGCG over the extended time period is due to the alteration of antioxidant enzyme expression, as opposed to direct oxidant scavenging effects [46]. Similar studies have also demonstrated that increased pre-incubation times with compounds can further increase protective effects [29, 47, 48], although a shorter incubation period was used in this assay, to display direct antioxidant effects, as opposed to secondary effects, such as those observed for EGCG. Given their structural similarities, it is plausible that, in the same manner as EGCG, increased incubation times and concentrations with apigenin and diosmetin would further protect neurons against hydrogen peroxide insult via secondary mechanisms. Interestingly, Diosmetin is a metabolite of diosmin, which failed to display any neuroprotective or anti-inflammatory properties in this study, highlighting the importance of the shared features between diosmetin, apigenin and EGCG. Despite their similarity, apigenin and Diosmetin displayed a disparity at concentrations above 25µM, where apigenin decreases neuron viability. This may be due to the induction of apoptosis, which has previously been demonstrated to occur at concentrations of apigenin above 25µM [49].

Our group and others have previously found that neuron degeneration *in vitro* is highly dependant on microglia-derived nitric oxide production [50]. In this study however, we have shown that inhibition of nitric oxide is not essential in providing neuroprotection. Apigenin and diosmetin, via their anti-inflammatory effects, decreased nitric oxide production, which lead to modest neuroprotection. The increase in neuron viability observed with Coenzyme Q10 however, was greater, despite less inhibition of nitric oxide (Figure 3). This may, in part, be due to Coenzyme Q10's ability to prevent the formation of the highly toxic peroxynitrite from nitric oxide, which would convey neuroprotection, while measurable nitric oxide (as nitrite) remains unchanged [51].

The use of an *in vitro* co-culture model of neurodegeneration has confirmed that several natural compounds possess neuroprotective properties, namely Coenzyme Q10,

apigenin and diosmetin. Moreover, these compounds impart neuroprotection via two alternate mechanisms; anti-inflammatory effects on microglia, thereby inhibiting production of nitric oxide and toxic radicals, or by antioxidant degradation of nitric oxide to non-toxic species. Coenzyme Q10 has previously been the subject of numerous neuroprotective studies *in vitro* and *in vivo*, but there is little knowledge of the role of apigenin and diosmetin in neuroprotection. Although information is scarce, these compounds appear to be well tolerated in subjects and further *in vivo* studies are therefore warranted for the long-term treatment of Alzheimer's disease.

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Figures

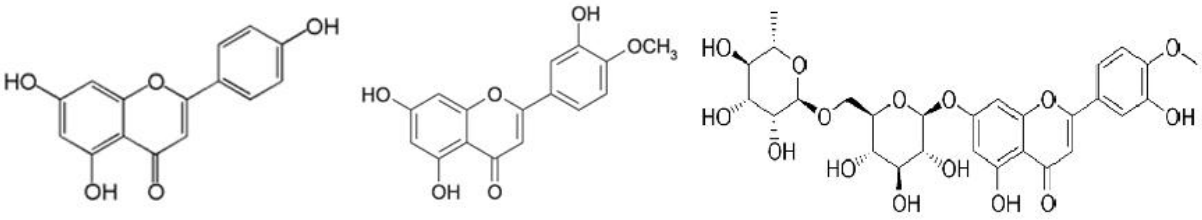

Figure 1. Apigenin, Disometin and Diosmin structures. These natural extracts share a similar three ring structure. Diosmetin (centre) is a metabolite of Diosmin (right) and possesses additional methyl and hydroxyl side chains in comparison to Apigenin (left).

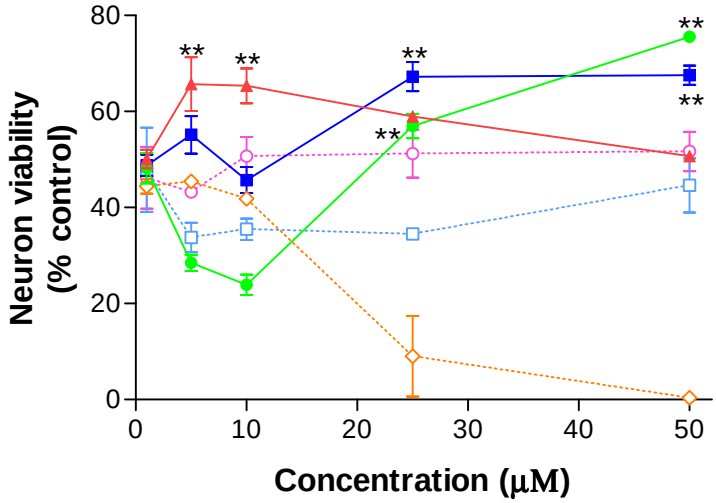

Figure 2. Effect of selected natural compounds on neuron viability following microglial insult. HT22 neurons and N-11 microglia were pre-incubated with extract for 90 minutes prior to activation with 1U/mL IFNγ and 1µg/mL LPS for 48 hours. Neuron viability was determined by neuronal GFP fluorescence. Apigenin (▲), Co-enzyme Q10 (●), Diosmetin (■), Curcumin (◊), Diosmin (□), and Estradiol (○). Solid symbols and lines represent extracts displaying a neuroprotective response, open symbols and broken lines represent ineffective extracts. (** = P<0.01 for neuroprotection).

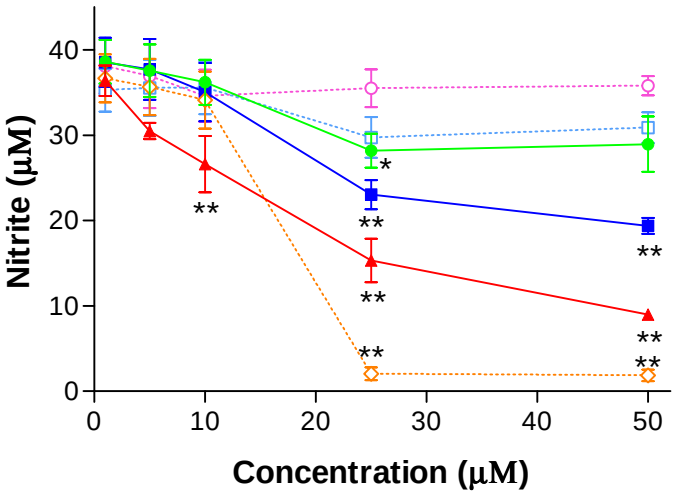

Figure 3. Effect of selected natural compounds on nitric oxide production in a neuron-microglia co-culture. HT22 neurons and N-11 microglia were pre-incubated with extract for 90 minutes prior to activation with 1U/mL IFNγ and 1µg/mL LPS for 48 hours. Nitrite was determined by addition of Griess reagent and absorbance measured at 545nm. Apigenin (▲), Co-enzyme Q10 (●), Diosmetin (■), Curcumin (◊), Diosmin (□), and Estradiol (○). Solid symbols and lines represent neuroprotective extracts, open symbols and broken lines represent ineffective extracts. (* = P<0.05, ** = P<0.01).

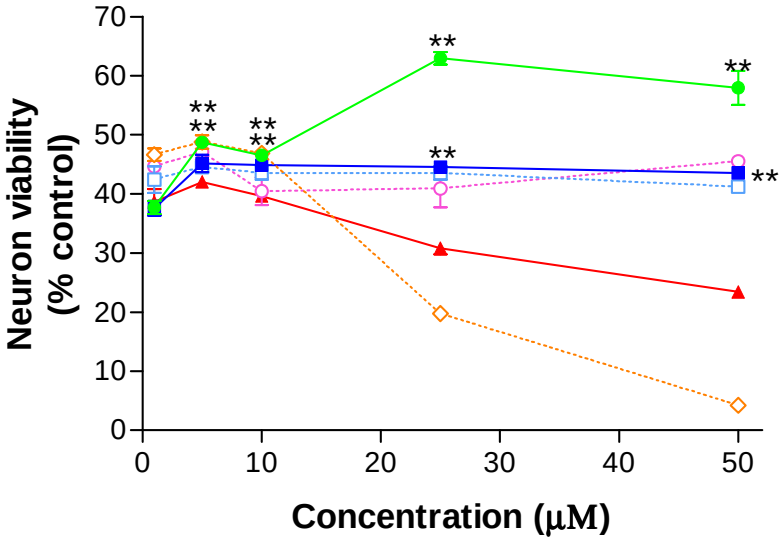

Figure 4. Effect of selected natural compounds on neuron viability following hydrogen peroxide insult. HT22 neuron death was induced with 75µM Hydrogen peroxide, with simultaneous addition of natural extracts at various concentrations (1-50µM). Neurons were treated for 6 hours before determination of cell viability, which was measured via reduction of Alamar Blue and fluorescence at 545/595nm. Apigenin (▲), Co-enzyme Q10 (●), Diosmetin (■), Curcumin (◊), Diosmin (□), and Estradiol (○). Solid symbols and lines represent neuroprotective extracts in co-culture, open symbols and broken lines represent ineffective extracts. (* = P<0.05, ** = P<0.01).

Summary

This summary will focus on four areas of importance in relation to the findings, namely nitric oxide, NSAIDs, statins and natural compounds.

Nitric oxide

Nitric oxide is increasingly indicated as a major causative agent in AD-related neurodegeneration [283-288]. The results presented in Article 3 of this report support this theory, demonstrating that NO plays a significant role in the induction of neuron death *in vitro*. Two specific inhibitors of iNOS, S-(2-Aminoethyl)isothiourea and S-Methylisothiourea, were both found to decrease NO production and increase neuron viability (Figure 3). The neuroprotective effects of NO inhibitors have been demonstrated by several groups [289-291].

Further evidence that supports the role of NO in neurodegeneration is the inability of TNF, IL-1β and Aβ to induce neuron death in this study (Article 3, Figure 2). Given that these pro-inflammatory mediators are present surrounding senile plaques in the brains of AD patients, and that TNF and IL-1β have also been found to amplify the direct toxic effects of Aβ on neurons, it would be expected that when used in combination in co-culture, significant neurodegeneration would be observed [9-13, 292]. This report demonstrates that this is not the case, even at high concentrations of the three activators. More importantly however, is the absence of TNF/IL-1β/Aβ-induced NO production, when compared to the LPS/IFNγ combination that induced significant NO production and neuron death. This factor further supports the major role of NO in the induction of neurodegeneration *in vitro*.

There is evidence to suggest species specificity of neuroglia, as human microglia do not produce NO, while rodent microglia are able to produce it in abundance [293]. A transgenic study further illustrated this by creating a model that expresses the entire human iNOS sequence, including all promoters, introns and exons [294]. When crossed to a iNOS null mice, NO produced by microglia was in the nanomolar range, while wildtype iNOS mice produce micromolar levels of NO. These observations may lead one to question the relevance of the use of murine-derived cells and the ability of microglia to induce neuron death in an NO-dependent manner in this and other AD research. Although murine microglia produce significant amounts of NO in response to pro-inflammatory stimuli, this function of microglia is redundant in humans, as astrocytes are responsible for the production of inflammation-

induced NO. This is demonstrated in the vicinity of senile plaques, where iNOS gene expression can be detected in astrocytes, but not in microglia [35, 295]. Moreover, astrocytic-derived NO is TNF- and IL-1β-dependent and occurs through NFκ-B, the same activators and pathway that have been shown to produce NO in microglia [269, 296]. Thus, regardless of the origin of NO *in vitro*, the pathways involved and the neurodegenerative outcome are the same. Consequently, rodent microglia provide an acceptable model for investigating neurodegeneration and the effect of anti-inflammatory compounds. Moreover, results *in vitro* need to be confirmed *in vivo*, which generally involves transgenic murine models of AD. The use of murine-derived cells *in vitro* simplifies translation to, and correlation with these *in vivo* models.

NO is believed to play a neuroprotective role at low concentrations under normal physiological conditions. It may even afford some neuroprotection during acute inflammation, as it decreases susceptibility to hydrogen peroxide induced death *in vitro* [297]. This effect however, may be time and concentration-dependant, as there is also data that demonstrates the opposite effect, where NO increases neuron susceptibility to hydrogen peroxide-induced death [298]. This research adds to the mounting evidence that suggests NO plays a dominant role in neurodegeneration during the progression of AD. The mechanisms that NO acts through to increase neuronal stress are extensive and well-documented, including direct mitochondrial disruption and protein nitrosylation, or via protein nitration and DNA damage following conversion to the powerful oxidant peroxynitrite [299, 300]. Given the extensive list of neurodegenerative-inducing effects of NO, it is surprising that research into the use of NO mimetics is underway [301]. Trials with mimetics began some years ago and although results are yet to be published, the outcomes will be interesting. They may further elucidate whether NO is able to play a neuroprotective role, or if the addition of endogenous NO to the already excessive concentration merely exacerbates the progression of AD.

Based on the NO-derived neurodegeneration observed in this study, NO may play a greater role in AD-related neurodegeneration than it is currently accredited for. Therefore, specific inhibitors of iNOS may be of value in the long-term prevention of AD. Although the inhibition of iNOS may not target Aβ deposition, the primary cause of the disease, it may delay neurodegeneration via decreasing glial cell recruitment and activation around Aβ plaques and by reducing oxidant stress on neurons. Furthermore, inhibitors with a greater affinity for the inducible isoform of NOS, as opposed to the neuronal NOS isoform, may be of

even greater benefit, given the possible neuroprotective effect of the neuronal isoform [302-305].

NSAIDs

The efficacy of NSAIDs in the prevention of AD continues to intrigue researchers, with results that contradict epidemiological evidence continually emerging. The numerous clinical trials with NSAIDs, including naproxen, diclofenac, celecoxib and more recently, indomethacin and ibuprofen are not only discouraging, but surprising [234-237, 306, 307]. The result of the latter two studies are particularly unexpected, given that re-analysis of early epidemiological studies and more recent observational studies indicate that use of these two NSAIDs, as well as sulindac sulfide, correlates to prevention of AD [221, 308]. Existing literature reviews highlight the numerous studies that currently provide conflicting data in this intensely investigated field [230, 231].

Clinical trials are the accepted standard for determining drug efficacy. The inability of clinical trials to replicate epidemiological evidence suggests that perhaps epidemiological data is not a fair predictor of AD in regards to NSAID usage. A more in-depth assessment however, indicates that it may be the clinical trials to date that are insufficient in determining drug efficacy for the prevention of AD. The operative word in this instance is prevention, as epidemiological studies have assessed the ability of NSAIDs to prevent the onset of AD in a random population, while clinical trials generally assess the ability of drugs to delay progression in patients already diagnosed with AD. A meta-analysis of AD trials confirms this, as no benefit was seen in any trial that used cognitive decline as an end point [309]. A further problem with clinical trials is the duration of treatment, which is generally six to twelve months for AD trials conducted to date. For the assessment of drugs in delaying symptomatic progression in diagnosed patients, this period of time may be acceptable, but for investigating the prevention of the initial disease onset, longer periods are required. It may be for this reason that epidemiological studies have provided more compelling evidence in this field. This is emphasized by a follow-up study of naproxen, which after an initial two year investigation showed no protective effect over control subjects, but after a further four years, demonstrated some protective benefit [310].

The translation of epidemiological and *in vitro* neuroprotective data to the clinical setting in AD is likely to be an ongoing issue for some time. Preventative clinical studies in large random populations over extended periods are likely to give results closer to those

observed in epidemiological studies, but are not feasible and therefore unlikely to take place. This raises the questions of how can the efficacy of therapies be effectively measured in the prevention of AD? The first and simplest method is the use of transgenic models of AD. There are numerous murine models, which through over-expression of APP and Presenilin-1, or mutations in APP, Tau, Presenilin-1, Apo-E and β-secretase, mimic the senile plaques, inflammation and neurodegeneration observed in AD patients (See [311, 312] for reviews). Although these animal models do not exactly represent the disease state, primarily due to the previously mentioned species-specificity of neuron-associated cells, they are continually improving and provide a rapid means of assessing therapeutic potential in the prevention of AD-associated pathology. Human trials however, still need to be performed to confirm transgenic outcomes. There are alleles known to be associated with an increased risk of AD, such as the ApoE-ε4 allele [313-315]. Subjects carrying such risk-related alleles may be preferential candidates for human trials, as the increased propensity of subjects developing AD may allow the sampling population and trial duration to be dramatically reduced. Furthermore, if used in conjunction with the recently advanced PET brain imaging and cerebrospinal fluid biomarkers for clinical outcomes, as opposed to traditional mental state examinations only, more accurate clinical results may be obtainable [316-318].

The issues with clinical trials to date renders the translation of *in vitro* to *in vivo* results difficult. Despite this, the general trend observed is that within the NSAID class, three drugs consistently demonstrate neuroprotective effects, including some minor benefits in patients [307]. These compounds are ibuprofen, indomethacin and sulindac sulfide. Given that these compounds appear to provide neuroprotection, activity that is not shared amongst all NSAIDs, combined with the fact that COX-2 specific inhibitors have continually failed to provide protection *in vitro* and *in vivo*, it is widely accepted that these NSAIDs possess a common target other than COX, which conveys neuroprotection.

Although over one thousand times less potent than traditional γ-secretase inhibitors in cell-based assays, the ability of some NSAIDs to reduce or alter Aβ production to a less toxic species, has lead numerous researchers to conclude that this activity is the basis of neuroprotection in AD [239, 319]. There are facets to this assumption however, that fail to address the results of clinical and transgenic trials. The three most promising NSAIDs, ibuprofen, indomethacin and sulindac sulfide, decrease Aβ production *in vitro* and *in vivo* by up to 80% via alteration of γ-secretase activity [239, 320]. The downfall of this however, is that diclofenac and diflunisal also decreased Aβ production, but failed to demonstrate

neuroprotection in the same study. Moreover, naproxen, despite a lack of neuroprotective potential in several assays, is also able to inhibit Aβ production, albeit at concentrations ten-fold greater than sulindac sulfide [241]. Furthermore, the Aβ-reducing ability of other NSAIDs, including the COX-2 selective celecoxib, has been assessed in transgenic mice, with many causing a decrease in Aβ load, whilst no changes in inflammatory markers or microglial activation were observed [321, 322]. Moreover, the beneficial action of ibuprofen, one of the three NSAIDs earmarked as neuroprotective, has been found to be independent of Aβ alteration in mice [323]. During this research, the design of the co-culture system meant that neurodegeneration was not dependent on Aβ production or addition. *In vivo*, Aβ is processed and deposited over time, causing microglial activation and neurodegeneration, but in this *in vitro* model, IFNγ and LPS were used as the primary microglial activators, thereby removing Aβ as a factor in neurodegeneration. Therefore, the observation of NSAID-derived neuroprotection in this study does not support the alteration of Aβ processing or production as the primary neuroprotective mechanism. It must be noted however, that these results do not refute the potential role of secretase inhibition by NSAIDs, a mechanism that may contribute to neuroprotection *in vivo*.

Although evidence is presented here to suggest that Aβ alteration is not the primary mechanism of neuroprotection by NSAIDs, this by no means implies that Aβ alteration does not assist in delaying the onset of AD. On the contrary, an NSAID-dependent reduction in Aβ would indeed assist in delaying AD and a study by Sastre and colleagues provides an important link between the alternative neuroprotective mechanisms of NSAIDs and Aβ alteration [242]. In agreement with several other studies, ibuprofen and indomethacin were found to decrease Aβ production *in vitro*. The study went further however, demonstrating that PPAR agonists are also able to decrease Aβ production and most importantly, the NSAIDs reduced production of Aβ in a PPAR-dependent manner, as PPAR antagonists blocked this ability. Thus, it is possible that previous reports have inadvertently correlated neuroprotection to Aβ reduction, were the actual mechanism responsible was PPAR agonism, which by proxy caused a reduction in Aβ.

In this report, the ability of ibuprofen, indomethacin and sulindac sulfide to agonize PPARs provides an explanation for their observed *in vitro* neuroprotection in comparison to other NSAIDs assayed [324, 325]. In the absence of Aβ-dependent neurodegeneration, the activation of PPARs may forward neuroprotection by inhibiting the JAK-STAT, NFκ-B, p38 MAPK and Akt pathways and subsequent cytokine production, thereby inhibiting microglial

activation [326-328]. Simultaneously, the resistance of neurons to degeneration could also be increased via an increase the antioxidant Superoxide dismutase and Glutathione systems and a reduction in the activity and expression of NADPH oxidase, all of which PPAR agonism can induce [329-332]. Neuroprotection via these mechanisms, particularly NADPH oxidase inhibition, correlates well to the results in this study, as minimal inhibition of NO was observed in the presence of neuroprotective NSAIDs, despite the importance of NO in induction of neurodegeneration in this culture model. Other groups found that PPAR agonists conferred neuroprotection in an iNOS-independent manner and that NO-induced degeneration occurred following conversion to peroxynitrite and only when NAPDH oxidase is simultaneously expressed [328, 333]. Thus, PPAR-dependent NADPH oxidase inhibition and a subsequent decrease in peroxynitrite may have forwarded neuroprotection in this study, whilst the change in NO produced and detected in the presence of NSAIDs remained insignificant. This mechanism may also account for the COX-independent decrease in NADPH oxidase and superoxide production by ibuprofen in transgenic mice [334].

The beneficial effects of PPAR agonists across are broad range of diseases are well documented. Relative to AD, numerous transgenic models have demonstrated the efficacy of PPAR agonists in preventing AD pathology [335-339], while antagonists exacerbate Aβ deposition and inflammation [200]. Derivatives of sulindac sulfide with increased PPARγ affinity have been developed [340]. A comparative study between the parent compound and those with increased PPAR agonist activity in transgenic mice would be an ideal method of determining the value of PPAR activity in neuroprotection.
Interestingly, a recent pilot trial with Pioglitazone displayed cognitive improvements and stabilization of Aβ production in AD patients [207]. PPAR agonism may therefore prove effective at both preventing disease onset as well as slowing disease progression in patients.

Since the initial epidemiological indication that NSAIDs may play a role in the prevention of AD, there has been significant research interest in this area. The overall outcome however, has not progressed dramatically since early research and the general consensus appears to be that only selected NSAIDs possess neuroprotective properties, but the target responsible is yet to be identified. This notion is supported by the observations during this study, indicating that numerous NSAIDs fail to protect, while ibuprofen, indomethacin and sulindac sulfide protect neurons from microglial insult in an *in vitro* model of AD. Utilizing these three NSAIDs in comparative studies with other members of this drug class provides a means to identify the alternative anti-inflammatory target that conveys

neuroprotection. Current evidence indicates that PPAR agonist activity is shared between the effective NSAIDs and is likely to play a role in their neuroprotective action. Regardless of the mechanism however, until a specific target is identified and a drug designed for the prevention of AD, the use of ibuprofen, indomethacin or sulindac sulfide is a favorable alternative for delaying AD. Given the current accessibility, low cost and relative safety of these compounds, they should be considered as long-term preventative measures for AD.

Statins

The efficacy of statins in preventing AD continues to be a topic of debate and will continue to be for some time. As is the case with NSAIDs, the inconsistencies between epidemiological and clinical data have not provided definitive evidence of their beneficial effect. As discussed for NSAIDs, these discrepancies may be due to the numerous problems faced when conducting clinical trials in AD, such as long trial periods, using clinically diagnosed or random cohorts, investigating symptomatic or preventative studies and the physiological or psychological endpoints used as measures. A recent Cochrane review of two randomized controlled trials addressed some of these issues, with longer follow-up periods, some exclusion of dementia patients and multiple cognitive measures [341]. Although the review concluded that statins given later in life do not prevent dementia or AD, the authors note that these trial conditions are still not ideal and larger trials over many years, comparing statin users to non-users are required for a definitive answer. The outcome of this review emphasizes the need to confirm neuroprotective drugs across multiple *in vitro* and animal models prior to administration in clinical trials, as the evidence presented in this study clearly demonstrates diversity in neuroprotective abilities of drugs within the same class. It was previously believed that the lipophilicity and ability to cross the blood-brain barrier may account for some of this diversity and observed differences in clinical results. This idea has since been repealed however, with the protective effect of statins being independent of lipophilicity, an outcome supported by the Cochrane review [341, 342]. The largest and longest clinical trial of a statin to date also supports this, as the lipophilic atorvastatin was not beneficial over 18 months in AD patients [343]. As suggested previously as a methodology for clinical trials of NSAIDs, statin use has been investigated in groups that are known to be more susceptible to developing AD, as opposed to random cohorts, with more promising results. Sufferers of Down's syndrome are at a higher risk of developing AD and the use of statins in this group shows a decrease in AD prevalence [344]. Another short-term study observed patients with a family history of AD and found that simvastatin also improved memory and cognition [345].

There is a correlation between cholesterol and Aβ content in the brain and high cholesterol is known to potentiate Aβ processing and Aβ-induced neruotoxicity, neuroinflammation and memory loss [248, 346, 347]. Furthermore, statin treatment of neurons decreases β-secretase activity, while simultaneously increasing α-secretase activity and promoting sAPP production as opposed to toxic Aβ species [348, 349]. For these reasons, any neuroprotective ability displayed by statins was initially attributed to the lowering of cholesterol levels by inhibition of HMG-CoA reductase. The study of Down's syndrome patients and statin use supports this hypothesis, as a protective effect against dementia was observed in patients with high baseline total cholesterol, but not in those with desirable cholesterol levels [344]. As discussed earlier, a reduction in total cholesterol is likely to aid in reducing the progression of AD via several mechanisms, primarily a reduction in membrane lipid rafts and concomitant decrease in processing APP to toxic Aβ species [257-259]. There is however, mounting evidence that a decrease in cholesterol and subsequent alteration of APP processing and Aβ production is not the critical factor in the efficacy of statins [350]. In support of this, the aforementioned study that established simvastatin to be effective in patients demonstrates that after treatment, no change was elicited in cerebrospinal fluid biomarkers for AD. This result has been replicated in other patient studies [351, 352] and also in transgenic mice, where simvastatin treatment did not decrease Aβ plaque burden, but improved neuron survival in aged mice and improved memory and learning in another study [353, 354].

It is becoming clear that the pleiotropic actions, as opposed to the primary cholesterol reducing activity, of statins convey neuroprotection. Numerous investigations have focused on the side effects of HMG-CoA reductase inhibition, namely the concomitant reduction in isoprenoid intermediates, which are responsible for isoprenylation and proper intercellular localization of proteins. Isoprenoids are significantly elevated in the brain of AD patients and their involvement in membrane localization of several inflammatory cascade initiators is what made them a mechanism of interest in preventing neurodegeneration [355]. Despite this interest however, several lines of evidence indicate that although likely to be beneficial in neuroprotection, inhibition of isoprenoid synthesis alone does not account for observed neuroprotection. The ability of statins to protect neurons against excitotoxic insult and reduce Aβ production independent of mevalonate and isoprenoid production are testament to this [356, 357]. The neuroprotection observed in this report is further evidence supporting this theory, as only two of the four statins assayed protected neurons against microglial-induced

degeneration. Additionally, this neuroprotection occurred in the presence of high concentrations of mevalonate, thereby negating the effect of HMG-CoA reductase inhibition and allowing isoprenoid-dependent pathways to function normally.

Figure 2 of Article 4 in this report demonstrates that both of the neuroprotective statins decreased NO production to some degree. Lovastatin was the most effective, inhibiting NO production by over 60%, resulting in a modest increase in neuron viability, which is not surprising given the correlation between NO production and neuron death in this co-culture model. Several studies have demonstrated the anti-inflammatory effect and NO inhibiting ability of statins, but have found these effects to be dependent on the inhibition of isoprenoid synthesis [358, 359]. Due to the addition of mevalonate to co-cultures, the anti-inflammatory decrease in NO observed here however, is independent of isoprenoids. This is highlighted in Article 1 of this report, where again, the addition of mevalonate did not change statin-dependent NO production in activated microglia or macrophages. Based on this information, the question remains: if not isoprenoid inhibition, which pleiotropic effect of statins is responsible for neuroprotection?

During this study, numerous attempts were made to address this problem, via development of reporter assays for specific cellular targets. Based on previous reports, NFκ-B and PPARγ were hypothesized to be the best candidate targets for these assays and reporter vectors were subsequently constructed with the intention of obtaining the first direct comparison between *in vitro* neuroprotective ability and reporter assay activity. Given that the cells of interest in regard to these targets in AD are inflammatory microglia, two microglial (N-11 and CHME-5) lines were transfected with PPARγ-Luciferase and NFκ-B-GFP reporter vectors. The aim was to observe drug-dependent changes in target activity during inflammatory stimulation, in the presence and absence of mevalonate. Initial experiments using Prostaglandin-J2 and LPS as control substances for PPARγ and NFκ-B respectively, failed to elicit a measurable response in the system used. Transfection with a control GFP-expressing vector confirmed the reason for the poor signal to be low transfection efficiency. Thus, two alternative inflammatory cell lines, RAW264.7 and J774 macrophages, were trialed for transfection ability but also demonstrated poor uptake and expression of the control vector. Reporter vectors were also modified to include gene cassettes for stable selection, to overcome the issue of transient transfection, but again, low transfection efficiency hindered creation of stable reporter clones. Therefore, no reporter assays were fully developed or performed for any compounds during this study.

Despite the inability to confirm its involvement during this study, PPARγ in particular remains a target of importance in regard to neuroprotection. A report by Seo and colleagues provides data to suggest a link between PPAR activity and the neuroprotection observed during this study [360]. Numerous reports have found statins to possess PPAR agonist activity, with simvastatin generally displaying the highest activity [361]. The Seo group however, via several reporter and expression assays, demonstrated that only fluvastatin consistently activated PPARα at concentrations below 20μM. Atorvastatin and simvastatin however, only activate PPARs at higher concentrations, which correspond to toxicity in co-culture, thus no neuroprotection via PPAR could be observed. Unfortunately, there is no PPAR data available for lovastatin, which would clarify this theory.

The exact mechanism by which statins regulate PPAR-mediated neuroprotection can only be speculated at this stage. It is known however, that statins activate PPARγ via an increase in Prostaglandin-J2 (PG-J2) in a COX-2-dependent manner [361, 362]. This is unusual, as COX-2, the target of NSAIDs is generally associated with inflammatory actions. The increase in COX-2 expression and activity in response to statins however, drives production towards the anti-inflammatory PPARγ ligand PG-J2, as opposed to the normal production of inflammatory PGE2. It is possible that neuroprotection is then initiated by PG-J2, rather than direct PPAR activation by statins, which satisfies previous findings that selected statins, including fluvastatin, do not directly bind to PPARs [363].

Although PPARs have been focused on during this discussion, there are other pathways that statins affect that are likely to provide some form of neuroprotection. The inhibition of NADPH oxidase by NSAIDs has been proposed in this report as a possible neuroprotective mechanism. Lovastatin and fluvastatin are potent inhibitors of NADPH oxidase, which potentially provides a link to the results obtained with these vastly different drug types. The inhibition of NADPH oxidase however, is dependent on mevalonate inhibition [359, 364] and neuroprotection in this study was afforded in the presence of endogenous mevalonate. Thus, NADPH oxidase is not a mechanism that conveys neuroprotection for statins in this assay, although it is likely to provide benefits *in vivo*. Two isoprenoid-independent actions of statins that could possibly have played a role in neuroprotection in this assay however, are the ability to increase neuronal energy production and antioxidant systems via inhibition of Akt signaling [365, 366] and upregulation of the anti-apoptotic Bcl-2 protein [367]. An increase in the energy available to neurons combined

with a decrease in oxidant stress will increase the resistance of neurons to degeneration, while an increase in anti-apoptotic signals will provide a further decrease in neuron death. An additional effect of statins is the ability to blocks cell cycle progression [368]. Although not quantified during this research, stimulation of microglia, both *in vitro* and *in vivo*, causes proliferation as well as activation. By inhibiting cell cycle progression, it is possible that microglial proliferation in co-culture was inhibited, thereby decreasing the number of microglia producing pro-inflammatory products such as NO and thus forwarding neuroprotection. This activity of statins *in vivo* may assist greatly in inhibiting progression of AD, as the proliferation of microglia surrounding senile plaques is crucial to the progression of the disease.

The pleiotropic actions of statin are vast, given that they are able to elicit effects both dependently and independently of HMG-CoA reductase inhibition. The identification of the neuroprotective target will assist greatly in understanding the pathology of AD and the design of specific therapeutics for AD treatment. This report has not assisted in elucidating the cellular target of importance in regards to neuroprotection, but has provided evidence that neuroprotection can be conveyed in the presence of mevalonate and is therefore not solely attributable to the inhibition of HMG-CoA reductase. Although *in vivo* data has been less than promising, this *in vitro* data suggests that statin therapy should still be investigated for the treatment of AD. In particular, lovastatin treatment should be pursued, as it displayed the greatest neuroprotection and is also the most lipophilic statin, meaning it can cross the blood-brain-barrier to exert neuroprotective effects more readily than other hydrophilic statins. Despite the positive effect on neurons in this study, the use of statins as temporary preventative treatments for AD is more complicated than NSAIDs. Although statins are generally well tolerated for several years in hyperlipidemic patients, cholesterol inhibition and subsequent side effects in normal subjects would require frequent monitoring. Further more, the requirement for a clinician visit and prescription, combined with the high cost of statins makes them unattractive as a long term preventative measure. This class of drug should therefore be utilized primarily in the research setting, to allow elucidation of the pathway, or pathways, that assist in preventing AD.

Natural compounds

The use of natural remedies is becoming more common for numerous diseases and has been suggested to be of benefit for AD treatment for some time. This is not surprising, given that antioxidant levels in plasma are lower in both early and late stage AD patients than in

controls [369]. Results in the clinical setting however, have not supported the use of natural compounds in the treatment of AD, including trials with Vitamin E and the highly regarded curcumin [266, 370]. The reasons for this are likely to be carried over from previous discussions in this text about the use of inappropriate cohorts, trial periods and outcome measures. These issues may be overcome in the future however, as a trial has been initiated with the *Ginkgo biloba* extract EGb761 that is assessing the ability of this compound to prevent the transition of memory complaints to AD diagnosis over a five year period, rather than the short term treatment of current AD patients [371]. Unfortunately, the methodology employed in this trial may lead to an outcome similar to a Cochrane review that reports Egb761 to be ineffective at inhibiting cognitive impairment [372]. Subjects in the current trial are elderly patients, who although classed as non-demented, have previously complained of memory deficits. With advanced age and memory deficit part of the inclusion criteria, it is plausible that these subjects are already in the early stages of AD progression, when Aβ plaque deposition has already occurred and neurodegeneration has been initiated. In the same manner as numerous previous trials, this study may therefore be accessing prevention of cognitive decline with EGb761, rather than prevention of AD. This conundrum is universal in clinical trials in AD and although difficult, needs to be addressed. Even under ideal clinical trial settings, treatment with natural compounds is further complicated compared to NSAIDs and statins, as there is a vastly greater number of compounds available for use and a distinct lack of interest from pharmaceutical companies for trial funding. Thus, the importance of effective *in vitro* models for identification of neuroprotective natural compounds for further, more targeted *in vivo* testing is amplified.

This study provides a simple *in vitro* model of neurodegeneration and clearly demonstrates that not all compounds that are considered to be ‘antioxidant’ are either anti-inflammatory or neuroprotective. Of the six compounds tested, three, apigenin, diosmetin and Coenzyme Q10 (Q10), protected neurons against microglial insult. An unexpected result, which cannot be ignored is not only the lack of neuroprotection, but the toxicity displayed by curcumin. Curcumin is known to have a wide variety of anti-inflammatory and antioxidant effects and is one of the more promising natural compounds, with positive outcomes *in vitro* and *in vivo* [279, 373-376]. Other *in vitro* studies have found curcumin to be neuroprotective at similar concentrations to those found to be toxic in this study [377, 378]. Possible causes for this discrepancy include the length of incubation; some studies were conducted for six to twenty four hours, as opposed to the 48 hours employed in this co-culture model, media pH instability due to compound; concentrations above 10μM caused an immediate yellowing of

the media, assumed to be due to compound colour, but may have been pH indicator related, compound solubility and the concentration of solvent present in each assay; the concentrations of dimethylsulphoxide used in this assay may not have been high enough to maintain solubility at high concentrations, and finally, the formulation used for testing; this study utilized commercially available 95% pure curcumin, it is possible the 5% unknown impurities vary between supplier and contain toxic compounds. Regardless of the reasons, curcumin failed to provide neuroprotection in this co-culture model and therefore cannot be compared to other neuroprotective compounds for analysis of neuroprotective mechanisms.

The detection of compounds that provide neuroprotection via inhibition of microglial-derived pro-inflammatory species was the primary objective of the *in vitro* co-culture system utilized in this study. Apigenin and diosmetin appear to protect neurons in such a manner, primarily via anti-inflammatory inhibition of NO, as the observed reduction in NO correlates to the increase in neuron viability. This is not surprising, given that of several natural compound species, flavones demonstrate the greatest inhibition of iNOS, with apigenin and diosmetin the two most effective [379]. Unexpectedly however, Q10 provides greater neuroprotection than is conveyed by the inhibition of NO alone. This demonstrates that, not surprisingly, natural compounds and those classed as antioxidants are able to provide neuroprotection by multiple pathways.

The anti-inflammatory activity displayed by apigenin and diosmetin correspond to previous findings in macrophages, where TNFα, iNOS and NO levels were inhibited at similar concentrations [380]. Moreover, apigenin has also been confirmed to inhibit NO and protect neurons by another group [381] and that the inhibition of NO production extends to astrocytes, indicating that treatment may also translate to the human disease state where, as previously mentioned, astrocytes, rather than microglia, are responsible for NO production [382]. Also, diosmetin has previously been found to be active in cellular protection against oxidant insult, while it's parent compound diosmin displayed no activity [383]. This evidence agrees with the neuroprotective results in this study, indicating that the small three ring structure shared between apigenin and diosmetin possesses neuroprotective properties.

The structural similarity between apigenin and diosmetin indicates that the mechanism, or mechanisms, of action are likely to be the same. The antioxidant, free radical scavenging potential of these compounds is negligible, particularly compared to compounds with related structures such as quercetin that have high antioxidant potential [384]. This

supports the previous statement that neuroprotection is likely to be a product of anti-inflammatory action as opposed to direct free radical scavenging. Although the mechanisms were not studied during this research, some of the plausible pathways responsible can be surmised from previous publications. NFκ-B, one of the targets highlighted as a possible neuroprotective mechanism prior to beginning this research is effectively blocked by apigenin at both the level of expression and activation [385, 386]. Unfortunately, no data is available for diosmetin to confirm NFκ-B as a shared target, but the ability of 17β-estradiol to inhibit NFκ-B activation in the absence of neuroprotection suggests that this is not the primary mechanism [387-389]. The other target of interest, which has been discussed at length in regards to NSAIDs and statins, is PPARs. As observed for these drug types, the natural compounds found to provide neuroprotection, apigenin and diosmetin, are able to activate PPARs [390-392]. In contrast, although there is evidence that 17β-estradiol can upregulate PPAR expression, there is no data to indicate it is able to activate PPARs [393, 394]. It is therefore plausible that apigenin and diosmetin convey neuroprotection, at least in part, by agonism of PPARs *in vitro*.

In vivo, apigenin and diosmetin possess further effects that are likely to provide additional benefits in the prevention of AD. Apigenin is able to prevent excitotoxicity in human neurons [395] and stimulate neurogenesis in adult rats [396]. Furthermore, it directly inhibits β-secretase at low levels, which over a period of years may further delay progression of AD [397]. Apigenin has been more widely studied, so more data is available, although the structural similarities suggest that diosmetin would also possess such activity, particularly given that it is generally more potent in previous comparative studies [384]. As discussed in Article 5 of this report, apigenin and diosmetin share structural similarities with the antioxidant (-)-epigallocatechin-3-gallate (EGCG). The anti-inflammatory and anti-oxidant efficacy of EGCG and quercetin is well documented across numerous assays [398-408], emphasising the likely importance of the shared structural features, indicating that apigenin and diosmetin may be of value in future studies.

Q10 is a widely used and studied compound, recognized to have substantial free radical scavenging ability. Although no data exists for its ability to directly scavenge NO, it is able to slow the conversion of NO to peroxynitrite, which would undoubtedly decrease oxidant stress on neurons [409], particularly when neurons are more susceptible to peroxynitrite-induced mitochondrial disruption than glial cells [410]. In addition to direct scavenging of free radicals, Q10 may further protect neurons by overcoming an oxidant-

induced decrease in mitochondrial respiration. In competition with oxygen, NO is also able to induce neurodegeneration and apoptosis via inhibition of Cytochrome c oxidase [411-413]. As the substrate of Complex III in the electron transport chain, Q10 may negate mitochondrial-driven neurodegeneration by a direct increase in Complex III activity. This would subsequently provide more electrons to Cytochrome c oxidase, thereby increasing its activity and resulting in increased energy production, overcoming NO-dependant, mitochondrial-induced neurodegeneration. This activity potentially explains why during this study at 50μM, greater neuroprotection was observed than at 25μM, while NO inhibition remained the same between the two concentrations. In addition to protection against microglial insult, Q10 was the only natural compound tested that also reduced hydrogen peroxide-induced neuron death. This result supports the free radical scavenging, antioxidant abilities of Q10 and further highlights the therapeutic potential of this compound. The efficacy of Q10 displayed in this *in vitro* model also translates to *in vivo* studies. Q10's lipophilicity means that therapeutic treatment increases mitochondrial concentrations and provides neuroprotection [414]. Furthermore, it protects neurons against Aβ-induced death and decreases brain carbonyl levels in mice, which may provide increased resistance to AD in the clinical setting [415, 416].

This study demonstrates that, as observed *in vivo*, *in vitro* neuroprotective potential is not common to all natural compounds, including those previously believed to be protective against the development of AD, namely 17β-estradiol. Although modest, the level of neuroprotection conveyed by selected natural compounds however, is equivalent to that imparted by commercial NSAIDs and statins. Unlike commercial compounds however, natural compounds were able to rescue neurons via both anti-inflammatory and antioxidant mechanisms. Although the mechanisms of anti-inflammatory neuroprotection were not studied during this research, previously defined targets of the compounds assayed suggests than again, there may be a link between neuroprotection and PPAR activity. In summary, the use of natural extracts and compounds in the prevention of AD is promising, especially when considering the relative safety, low cost and ease of access for treatment in comparison to commercial drugs. It must be noted however that, as with any treatment regime, patients need to be aware that even though natural compounds appear to be safe and may, in some instances, be more tolerable than commercial drugs [266, 417, 418], undesirable side-effects may occur and should be monitored. Additionally, the ability of natural compounds to provide neuroprotection via separate anti-inflammatory and antioxidant pathways may allow a safe, multi-targeted therapeutic neuroprotective approach to the prevention of AD.

Future Directions

The fluorescent assay developed during this study demonstrates that a co-culture of neurons and microglia can effectively be used to identify neuroprotective compounds. The primary issue with the assay however, is the origin of the cell lines utilized, given the species specificity as previously discussed. All of the cell types in this study were of murine origin, which potentially poses issues in translating to the *in vivo* human disease state. The simplest and most effective method of overcoming this issue is to repeat the study using cells of human origin. Neuronal lines that may be suitable include CHP-212, IMR-32 or SH-SY5Y, while microglial lines such as CHME-5 would be ideal. The latter two cell lines became available during this study. No co-culture could be performed with them however, as poor transfection efficiencies of the SH-SY5Y neuroblastoma cells resulted in no stable GFP-expressing colonies being obtained.

The outcome of human microglial and neuronal cell lines in co-culture may also further elucidate the role of NO in neuron death. This study found that microglia-derived NO played a significant role in murine neuronal death, but as discussed previously, human microglia do not produce NO [293]. If NO production is a primary cause of neuron degeneration in co-culture, it would therefore be expected that minimal degeneration would be observed when human microglia and neurons are co-cultured. A further extension of this human-derived cell study should include a co-culture of neurons with astrocytes, in place of microglia. Given that in the Alzheimer's brain, astrocytes are known to produce NO surrounding senile plaques, it would be expected that their inflammatory response in culture would include NO, which may induce neuron death in the same manner as microglia-derived NO [35, 295].

The method of detecting neuron viability in this study was a simple whole-well measurement of cytosolic, fluorescent protein remaining in the neurons. By using FACS, this method was demonstrated to effectively determine neuron viability [1], but a downfall of this method is the use of neuron death as an end-point. Given that the majority of neurodegeneration observed in AD is synaptic retraction and not neuron death, a more ideal method of investigating neurodegeneration and neuroprotective compounds would therefore be the measurement of dendritic retraction. Further advantages of an image-based assay would be the lower concentrations of inflammatory activators required to induce neurite retraction as opposed to neuron death and the ability to investigate multiple factors simultaneously. Such an assay could have been performed during this study, but not in an

increased-throughput manner. Although culture wells could have been analyzed individually under a stereo microscope, it is a time-consuming and labor-intensive process that is not an option when assaying multiple compounds. Recent advances in high content imaging and high throughput screening however, provide an ideal platform to extend the capability and accuracy of this research.

There is currently limited information available on high content imaging and high throughput screening in relation to AD. From the publications available however, the majority of research in this area is focused on screening for compounds that inhibit either Tau hyperphosphorylation [419] or Aβ formation and aggregation [420-423]. These processes make ideal targets for drug screening, given that Aβ lies at the centre of AD initiation and progression, while Tau is a major cause of neurite disruption. But this screening fails to utilize the power of high content imaging and does not take into account cell-cell interactions or identify compounds that may assist in delaying progression of neurodegeneration once it has begun. Hu and colleagues however, have demonstrated an effective method of investigating neurite retraction and neuroprotection [424]. In summary, primary cortical cells were cultured in the presence of Aβ and selected compounds, then cell-specific antibodies were used to identify glia and neurons, whilst simultaneously allowing tracking of neurite formation. Although primary cortical cells more closely represent the *in vivo* situation when investigating neuroprotective compounds, their availability and preparation make them unsuitable for high throughput screening. A similar experimental approach could be employed with cultured cell lines however, including a co-culture of neurons, microglia and astrocytes. The use of fluorescently labeled neurons, as used during this report, would allow for tracking of neurite outgrowth and retraction, whilst avoiding the need for costly and time-consuming antibody staining. Retinoic acid has traditionally been used to induce neuron differentiation and neurite outgrowth and may be an effective method prior to inflammatory activation, when screening for inhibitors of neurite retraction. The use of Retinoic acid in neuroprotective assays has recently come under question however, due to alteration of tolerance to neurotoxins, so care should be taken in experimental design and controls [425].

An experiment that would assist in identifying the origin of neuroprotective potential in anti-inflammatory drugs would be the development of several reporter assays. As previously discussed, there is evidence, including the results of this report, that indicates alternative targets and mechanisms of action of both NSAIDs and statins are responsible for conveying neuroprotection. By correlating results of reporter gene assays with the

neuroprotective potential observed *in vitro*, particularly the results in this report, it may be possible to identify the target, or targets, that are shared between compounds and convey neuroprotection. Although the list of possible targets suitable for reporter gene assays is extensive, several reporter assays have previously been conducted on NSAIDs and statins, with candidate targets. No report however, has been comprehensive enough to include more than a few drugs within a drug class. Differences in methodologies between articles, combined with the fact that not all drugs from the NSAID and statin classes have been assayed, makes the interpretation of reporter assays and their correlation to neuroprotective potential difficult. The solution to this problem is the simultaneous screening of all compounds with reporter assays for candidate targets. During this research, the development of reporter gene assays for NFκ-B and PPARγ were attempted, but not successful. This was primarily due to the low transfection efficiencies achieved with microglial (N-11 and CHME-5) and macrophage (RAW264.7 and J774) cell lines. With the low transfection rates observed, a suitable assay window could not be achieved using transient reporter assays and no stable clones could be isolated using G418 selection. These reporter assays however, provide an ideal starting point for assessing the alternative anti-inflammatory properties of neuroprotective compounds in a comparative manner.

Finally, the neuroprotective compounds identified in this study require confirmation of their efficacy *in vivo*. Although some of them have displayed positive outcomes in murine models of AD, not all of them have been analyzed. A direct comparison between the neuroprotective potential observed *in vitro* with outcomes in mice, including both behavioral and physical measures such as Aβ plaque and Tau loads, microglial proliferation, levels of NO and other pro-inflammatory markers, neurite retraction and mitochondrial respiration of neurons would further distinguish effective compounds for human trials.

Conclusions

The ability of anti-inflammatory compounds to delay the onset or progression of AD has been a topic of great interest and debate for an extended period. The primary reason for the continued debate is the discrepancy between *in vitro* and epidemiological data and clinical trials, where compounds earmarked as potential neuroprotective compounds have failed in clinical trials. As previously discussed, this inconsistency is likely to be due to the conditions and subjects used during trials, as neuroprotective compounds are more likely to prevent or delay AD prior to diagnosis, yet trials tend to assess delaying of progression once AD has been diagnosed. To overcome this issue, future clinical trials should follow the example described by Andrieu and collegues, where patients with memory complaints are enrolled prior to AD diagnosis, then followed for several years, with AD diagnosis as the end point [371]. Prior to clinical trials however, compounds need to be further investigated for neuroprotective potential in cellular models.

Previous clinical trials have somewhat arbitrarily selected compounds for testing based on their drug class, possibly on the assumption that all compounds within a class share the same neuroprotective properties. The neuroprotective inequality between compounds of the NSAID, statin and antioxidant classes has been highlighted by this study. The simple co-culture model employed emphasizes the differences in anti-inflammatory and neuroprotective potential of drugs within a class. It is for this reason that previous trials with compounds such as naproxen, diclofenac, rofecoxib and atorvastatin are likely to have provided unfavourable results, as none of these compounds were found to be neuroprotective using this *in vitro* model [236, 237, 342, 426]. Thus, compound selection for future clinical trials should be more closely based on *in vitro* data, rather than selection based on drug class. The results of this study indicate that ibuprofen, indomethacin, sulindac sulfide, lovastatin, fluvastatin, apigenin, diosmetin and Coenzyme Q10 provide neuroprotection *in vitro* and may therefore provide more positive outcomes in the clinical setting than previous trials.

This study also provides several lines of evidence that highlight the involvement of nitric oxide in neurodegeneration *in vitro*. The production of NO is correlated to the induction of neuron death in a proximity-dependent manner, which agrees with the findings of other groups [427, 428]. Several compounds tested were able to rescue neurons by an indirect NO decrease via anti-inflammatory action on microglia, but interestingly, selective iNOS inhibitors provided equivalent neuroprotection without effecting other microglial pathways. It

is therefore proposed that NO plays a greater role in the induction of neurodegeneration and progression of AD than is currently attributed to it. This argument is in agreement with *in vivo* data, where genetic ablation of iNOS alone dramatically reduces AD pathology in transgenic mice [288]. Based on this theory, selective inhibitors of iNOS, particularly those specific for neuronal iNOS, present themselves as possible treatments for AD and should be pursued further, beginning with transgenic studies.

As a known activator of inflammatory pathways involved in AD, NFκ-B has been a focal point of neuroprotective studies, which is not surprising, given that several NSAIDs are known to inhibit NFκ-B [128, 429, 430]. This study included NSAIDs with such activity, but no neuroprotection was observed. It can therefore be surmised that, at least *in vitro*, NFκ-B is not the primary mechanism that anti-inflammatory compounds impart neuroprotection. Conversely, a majority of effective compounds in this *in vitro* assay have been previously shown to possess PPAR agonist activity. It is for this reason that PPAR is proposed as a dominant factor in conferring neuroprotection by NSAIDs, statins and natural compound therapeutics. The data regarding PPARs in the treatment of neurodegenerative disorders is increasing rapidly. Recently, the PPAR agonist pioglitazone inhibited microglial and astrocytic activation, COX and iNOS expression and Aβ production to the same extent as ibuprofen in a murine AD model [337]. This is a promising result and further investigation into the direct use of PPAR agonists in AD, as well as direct comparative studies of anti-inflammatory drug-derived PPAR activity is warranted.

Alzheimer's disease is a complex and multifactorial condition. Due to the multitude of targets and pathways involved, treatment for AD is likely to be a multi-drug approach. Moreover, the therapeutic regime is likely to change throughout the initiation and progression of AD. For example, prior to diagnosis, those categorised as at-risk of developing AD may take preventative measures for several years, aimed at inhibiting β-amyloid production and the microglial inflammatory response. With advances in *in vivo* imaging and diagnostic tests, early detection of AD pathology is sure to improve in the future, which will provide for a second stage of intervention. β-amyloid deposition and senile plaque formation will have occurred by this stage, so the focus may shift from inhibiting β-amyloid production to assisting in its removal. Agents such as β-sheet breakers may be employed for this purpose, but cytokine therapy to induce microglial phagocytosis of β-amyloid is also a potential therapy. Patients suffering dementia in the later stages of AD are likely to continue treatment with current therapies aimed at improving cognitive function, but in combination with

therapies that provide neuroprotection, thereby prolonging neuron survival and function simultaneously. A recent clinical trial with a combination of antioxidants administered to AD patients emphasized the beneficial effects of a combinatorial approach, as significant cognitive improvements were observed, even when the antioxidants were administered in addition to the traditional AChE inhibitor donepezil [431]. While compounds in this study displayed neuroprotection alone, it is plausible that in some combinations the effects will be additive. A combination of particular interest is that of lovastatin and Q10. Statins are known to reduce Q10 levels as a side effect of cholesterol inhibition [432]. Although this factor is yet to be investigated, statins may be a double-edged sword in AD prevention, providing anti-inflammatory neuroprotection, whilst simultaneously increasing mitochondrial stress on neurons via Q10 inhibition. In light of the substantial neuroprotection Q10 demonstrates here, it is expected that co-administration of Q10 would not only negate the statin-dependent decrease of Q10, but provide additional neuroprotection [433]. It may therefore be advisable that patients considered 'at risk' for AD, who are treated with statins for hypercholesterolemia should also be administered Q10 to offset the Q10-lowering effect of statins. With beneficial effects such as those described in this report, supplementary nutraceutical therapies such as Q10 are likely to be employed for AD therapy in addition to targeted AD drugs in the future.

The evidence presented in this report supports the use of NSAIDs, statins and naturally occurring antioxidant compounds as neuroprotective agents. Selected compounds from these classes, via pleiotropic mechanisms, are able to prevent microglial-induced neuron death *in vitro* and may therefore provide protection against the inflammatory processes present in AD. Of the compounds investigated here, the NSAIDs and antioxidants have a relatively safe profile, are suitable for long-term therapy, easily accessible and inexpensive. Thus, although the efficacy of these compounds is yet to be confirmed in the clinical setting, it is suggested that the neuroprotective compounds in this study be considered as temporary preventative measures for AD, particularly in subjects in the high risk category. Based on this study, an ideal combinatorial therapy would include diosmetin or ibuprofen with Coenzyme Q10.

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