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(54) **Title:** ANIMAL MODEL

(57) **Abstract:** There is disclosed a method of generating an animal model exhibiting an age-related pathological condition of the brain, the method comprising orally administering to the animal galactose and sodium benzoate. Also described is an animal model produced by this method. Further, a method of identifying a compound for the treatment of age-related pathological conditions of the brain is disclosed, the method comprising administering a candidate compound to a test animal model and detecting a change in an age-related pathological condition of the brain in the test animal model relative to a control.



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Animal Model

Field of the Invention

- 5 The invention relates to a method of generating an animal model that exhibits age-related pathologies of the brain, for example, Alzheimer's disease-like pathologies.

Background to the Invention

Sporadic Alzheimer's disease (sAD) is a growing pandemic and is fast becoming an economic
10 burden worldwide due to the ever increasing aged population. The disease is characterised by a progressive loss of cognitive function precipitating from multiple pathologies including protein aggregation, oxidative stress, mitochondrial dysfunction and neuroinflammation. The exact etiology of sAD is complex, remains elusive and is probably the result of complex interactions among multiple genetic, epigenetic and environmental factors. Contrary to this,
15 the etiology of familial Alzheimer's disease (fAD), which affects less than 1% of AD cases, have been linked to mutations in three genes: the amyloid β -protein precursor (APP) gene, the presenilin-1 (PS1) gene, and the presenilin-2 (PS2) gene. These mutations have been overexpressed in mice to produce transgenic (*Tg*) preclinical animal models in an attempt to better understand and study the disease. Although these models have proven to be invaluable
20 tools for investigating the pathological mechanisms of AD, they all share a common flaw in that their etiology is representative of fAD and not sAD and the pathology is driven by the overexpression of mutant proteins. Contrary to sAD, these *Tg* fAD models display a significant amount of pathology in adolescence which persists into adulthood, eventually leading to a behavioural phenotype. Although recent genome-wide association studies have
25 shown certain genes to be linked with sAD, these genes do not seem to be the sole factors contributing to the pathology. Rather, they represent a risk factor for disease progression or rate of onset which may precipitate under the right environmental conditions.

Success in the clinic is dependent on early pharmacological intervention during disease
30 progression. This presents a problem during the drug discovery process which utilises preclinical models for proof of principle. To date, the pharmaceutical industry have had to rely on *Tg* animals to model sAD, despite their significant differences in etiology. The lack of an accurate preclinical model may be one of the main underlying reasons as to why so many drugs have failed to translate into the clinic.

A mouse model which better represents sAD is desperately needed. It has been shown that D-Galactose administered chronically by injection can accelerate natural aging in mice (Xu, 1985; Zhang *et al.*, 1990). In addition to advanced senescence, these mice display cognitive
5 deficits and AD like pathologies such as neuroinflammation, oxidative stress, mitochondrial dysfunction and amyloid beta and hyperphosphorylated tau accumulation (Xiao *et al.*, 2011; Wang *et al.*, 2009; Shan *et al.*, 2009; Zhang *et al.*, 2010). This pathology is not the product of overexpression of mutant proteins and as such is not developmental. The disease progression is also more representative of what is predicted to happen in the clinic, although this is
10 speculative.

As age is the greatest risk factor for developing sAD, a preclinical model which presents a phenotype of advanced senescence accompanied by, non-genetic, AD like-pathologies and cognitive deficits would benefit the sAD field particularly when translating therapeutics from
15 the preclinical to the clinical phase.

To this end, the inventors have developed a more simple and efficient way to generate an animal model showing age-related pathologies of the brain than previous models.

20 Summary of the Invention

In a first aspect of the invention, there is provided a method of generating an animal model exhibiting an age-related pathological condition of the brain, comprising orally administering to the animal galactose and sodium benzoate.

25 The inventors have surprisingly found that sodium benzoate contributes to the pathology of the animal model. Administration of galactose and sodium benzoate causes the animal to develop an age-related pathological condition of the brain. In particular, the combination of galactose and sodium benzoate causes pathologies such as an increase in soluble amyloid beta and p-tau in the cortex of the animal.

30

An "animal model" refers to a non-human vertebrate, including but not limited to mammal, primate, and rodent. Non-limiting preferred model organisms are mouse, rat, guinea pig, dog,

cat, rabbit, pig, chimpanzee and monkey. Preferably, the animal model is a rodent model. Most preferably, the animal model is a mouse model.

The animal should preferably be an adult animal to which the galactose and sodium benzoate
5 are administered. An adult animal is one which is fully grown or developed. For example, when the animal is a mouse, the mouse is preferably at least 1 month old when first administered the galactose and sodium benzoate. More preferably, the mouse is at least 2 months old when first administered the galactose and sodium benzoate. In some
10 embodiments, the mouse is between 1 and 16 months in age when first administered the galactose and sodium benzoate. In other embodiments, the mouse is between 1 and 10 months in age when first administered the galactose and sodium benzoate. In various embodiments, the mouse is between 2 and 6 months in age when first administered the galactose and sodium benzoate. In further embodiments, the mouse is between 2 and 4 months when first administered the galactose and sodium benzoate. In particular
15 embodiments, the mouse is about 3 months when first administered the galactose and sodium benzoate.

The expression "an age-related pathological condition of the brain" means that the animal model exhibits one or more pathologies or symptoms which are associated with aging. After
20 a normal animal reaches adulthood, changes in the brain will occur as the animal becomes older, for example, as the animal approaches and reaches old age. It is these changes which cause the one or more pathologies or symptoms which are associated with an increased age or aging of the animal. Such pathologies or symptoms include protein aggregation, amyloid beta accumulation, phosphorylated tau accumulation, hyperphosphorylated tau accumulation,
25 oxidative stress, mitochondrial dysfunction, neuroinflammation, neurotoxicity, neuronal loss, advanced senescence, cognitive deficits, increased advanced glycation end products, decreased glutamate release, increased bace1 activity, synaptic dysfunction, endoplasmic reticulum stress, calcium regulation dysfunction, plaque formation, mononuclear phagocyte activation and neurofibrillary tangles formation. Preferably, the animal model exhibits at
30 least one of protein aggregation, amyloid beta accumulation, phosphorylated tau accumulation, hyperphosphorylated tau accumulation, neuroinflammation, neurotoxicity, neuronal loss, plaque formation and neurofibrillary tangles formation. Preferably, the animal model exhibits at least accumulation of amyloid beta and/or hyperphosphorylated tau. In

some embodiments, the animal model exhibits a plurality of the pathologies or symptoms identified above.

The one or more pathologies or symptoms referred to above may be exhibited in the cortex of the brain of the animal. Alternatively, they may be exhibited in the hippocampus of the brain of the animal. Preferably, accumulation of amyloid beta and/or hyperphosphorylated tau takes place in the hippocampus and cortex of the brain of the animal.

In some embodiments, the animal model exhibits a pathological condition of Alzheimer's disease meaning that the animal model exhibits one or more pathologies or symptoms which are associated with Alzheimer's disease. These pathologies or symptoms are as described above. Put another way, the animal model may exhibit one or more Alzheimer's disease-like pathologies or symptoms.

The expression "orally administering" means that the route of administration of the galactose and sodium benzoate is through the mouth of the animal. Oral administration also includes buccal administration (dissolved inside the cheek), sublabial administration (dissolved under the lip), and sublingual administration (dissolved under the tongue).

Galactose and sodium benzoate may be administered in the form of tablets to swallow, chew or dissolve in water or under the tongue, capsules or chewable capsules, time-release or sustained-release tablets and capsules, powders or granules, or in liquid form. Alternatively, the galactose and sodium benzoate can be added to food so that they can be administered to the animal in its food. Preferably, the galactose and sodium benzoate are dissolved in a solvent so that they can be administered to the animal in its drinking water. Preferably, the solvent is water, e.g. such that the galactose and sodium benzoate are dissolved in the drinking water of the animal.

The advantage of administering the galactose and sodium benzoate to the animal orally is that it is not necessary to inject the animals with the substances. This significantly reduces the labour involved in administering the substances and also reduces the risk of harm to the animal and/or the person administering the substances as needles are not involved.

The galactose and sodium benzoate should be administered at an effective amount so that the animal develops an age-related pathological condition of the brain.

Preferably, the galactose is D-galactose. Preferably, the galactose is administered at between
5 about 0.5 and about 7 grams of galactose per kilogram of animal, i.e. between about 0.5 and about 7 g/kg. In various embodiments, the galactose is administered at between about 1 and about 6 g/kg. In some embodiments, the galactose is administered at between about 2 and about 6 g/kg. In other embodiments, the galactose is administered at between about 2 and about 5 g/kg. In further embodiments, the galactose is administered at between about 3 and
10 about 5 g/kg. In particular embodiments, the galactose is administered at about 4 g/kg. Preferably, the animal receives this level of galactose daily.

When the animal model is a mouse and the galactose is in the drinking water of the mouse, the amount of galactose in the drinking water is as follows (assuming a mouse drinks about 2
15 ml of water per day). Preferably, the drinking water contains between about 0.25 and about 3.5 milligrams of galactose per gram of animal per millilitre of water, i.e. between about 0.25 and about 3.5 mg/g/ml. In various embodiments, the drinking water contains between about 0.5 and about 3 mg/g/ml. In some embodiments, the drinking water contains between about 1 and about 3 mg/g/ml. In other embodiments, the drinking water contains between about 1 and
20 about 2.5 mg/g/ml. In further embodiments, the drinking water contains between about 1.5 and about 2.5 mg/g/ml. In particular embodiments, the drinking water contains about 2 mg/g/ml.

Therefore, in view of the above, for a 30 gram mouse, the drinking water may contain about
25 7.5-105 mg/ml, 15-90 mg/ml, 30-90 mg/ml, 30-75 mg/ml, 45-75 mg/ml or 60 mg galactose/ml of drinking water.

Preferably, the sodium benzoate is administered at between about 1 and about 100 milligrams of sodium benzoate per kilogram of animal, i.e. between about 1 and about 100 mg/kg. In
30 some embodiments, the sodium benzoate is administered at between about 10 and about 100 mg/kg. In other embodiments, the sodium benzoate is administered at between about 10 and about 70 mg/kg. In more embodiments, the sodium benzoate is administered at between about 30 and about 70 mg/kg. In further embodiments, the sodium benzoate is administered

at between about 50 and about 70 mg/kg. In particular embodiments, the sodium benzoate is administered at about 66 mg/kg. Preferably, the animal receives this level of sodium benzoate daily.

- 5 The sodium benzoate may be used as an additive in food or water. In such cases, the sodium benzoate is preferably used as an additive at between about 0.001% and about 10% by weight. In various embodiments, the sodium benzoate is present at between about 0.001% and about 5% by weight. In some embodiments, the sodium benzoate is present at between about 0.001% and about 3% by weight. In other embodiments, the sodium benzoate is present at
10 between about 0.001% and about 2% by weight. In further embodiments, the sodium benzoate is present at between about 0.001% and about 1% by weight. In particular embodiments, the sodium benzoate is present at between about 0.001% and about 0.5% by weight. In various embodiments, the sodium benzoate is present at between about 0.001% and about 0.2% by weight. In some embodiments, the sodium benzoate is present at between
15 about 0.001% and about 0.15% by weight. In other embodiments, the sodium benzoate is present at between about 0.01% and about 0.15% by weight. In further embodiments, the sodium benzoate is present at between about 0.05% and about 0.15% by weight. In particular embodiments, the sodium benzoate is present at about 0.1% by weight.
- 20 Preferably, the galactose and sodium benzoate are administered together.

- The galactose and sodium benzoate are preferably administered continuously over time until the animal develops an age-related pathological condition of the brain. This means that there is no substantial break in which the animal is not subjected to the influence of the galactose
25 and sodium benzoate. This is easily achieved by having the galactose and sodium benzoate in the drinking water of the animal. It will be appreciated that whilst the galactose and sodium benzoate are preferably administered continuously over time such that there is no substantial break in which the animal is not subjected to the influence of the galactose and sodium benzoate, this continuous administration can still be fulfilled by periodic or intermittent
30 dosing. For example, when the galactose and sodium benzoate are in the drinking water, the animal will not be continuously drinking water. Instead the animal will be drinking water intermittently at intervals. However, even with this intermittent dosing, there will be no

substantial break in which the animal is not subjected to the influence of the galactose and sodium benzoate and the two agents will be present in the plasma and body of the animal.

The galactose and sodium benzoate should be administered until the animal develops an age-related pathological condition of the brain. Preferably, the galactose and sodium benzoate are administered for at least about 5 days. In various embodiments, the galactose and sodium benzoate are administered for at least about 10 days. In further embodiments, the galactose and sodium benzoate are administered for at least about 15 days. In some embodiments, the galactose and sodium benzoate are administered for at least about 20 days. In other embodiments, the galactose and sodium benzoate are administered for at least about 25 days. In further embodiments, the galactose and sodium benzoate are administered for at least about 30 days. In particular embodiments, the galactose and sodium benzoate are administered for at least about 35 days. In some embodiments, the galactose and sodium benzoate are administered for at least about 40 days. In other embodiments, the galactose and sodium benzoate are administered for at least about 45 days. In further embodiments, the galactose and sodium benzoate are administered for at least about 50 days. In particular embodiments, the galactose and sodium benzoate are administered for at least about 55 days. In additional embodiments, the galactose and sodium benzoate are administered for at least about 60 days.

The invention also provides an animal model produced by the method described above. In particular, the invention provides an animal model produced by the method described above, wherein the animal is a C57B16 mouse. More preferably, the animal is a male C57B16 mouse.

A second aspect of the invention provides a method of identifying a compound for the treatment of age-related pathological conditions of the brain, comprising administering a candidate compound to a test animal model generated by the method of the present invention and detecting a change in the pathological condition in the test animal model relative to a control.

30

In a particular embodiment, there is provided a method of identifying a compound for the treatment of Alzheimer's disease, comprising administering a candidate compound to a test

animal model generated by the method of the present invention and detecting a change in the pathological condition in the test animal model relative to a control.

A "control" is an alternative subject or sample used in an experiment for comparison purposes. For example, where the purpose of the experiment is to test whether a candidate compound is capable of ameliorating or advancing an age-related pathological condition of the brain (e.g. Alzheimer's disease) in a test animal model, it is generally preferable to use a control (an animal or a tissue or cell sample from the animal), which typically exhibits the same or similar pathological condition but is not exposed to the candidate compound or is exposed to a lower dose of the compound, or for a shorter period of time.

Preferably, the candidate compound ameliorates the age-related pathological condition of the brain (e.g. Alzheimer's disease). Preferably, the candidate compound ameliorates one or more symptoms or pathologies which are associated with aging. More preferably, the candidate compound ameliorates one or more symptoms or pathologies which are associated with Alzheimer's disease. These symptoms and pathologies include protein aggregation, amyloid beta accumulation, phosphorylated tau accumulation, hyperphosphorylated tau accumulation, oxidative stress, mitochondrial dysfunction, neuroinflammation, neurotoxicity, neuronal loss, advanced senescence, cognitive deficits, increased advanced glycation end products, decreased glutamate release, increased bace1 activity, synaptic dysfunction, endoplasmic reticulum stress, calcium regulation dysfunction, plaque formation, mononuclear phagocyte activation and neurofibrillary tangles formation. Preferably, the candidate compound ameliorates one or more symptoms or pathologies selected from protein aggregation, amyloid beta accumulation, phosphorylated tau accumulation, hyperphosphorylated tau accumulation, neuroinflammation, neurotoxicity, neuronal loss, plaque formation and neurofibrillary tangles formation. Preferably, the compound is capable of reducing or inhibiting the accumulation of amyloid beta peptide and/or hyperphosphorylated tau. The compound may act at any point of the signal transduction pathway leading to one or more of the aforementioned pathological conditions.

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Preferably, the compound is selected from the group consisting of an antisense oligonucleotide, a ribozyme, an antibody, a liposome, a small interfering RNA, a small molecule and an inorganic compound.

Candidate compounds of the present invention include a biological or chemical compound such as a simple or complex organic or inorganic molecule. Such compounds may include, but are not limited to, antisense oligonucleotide, small interfering RNA, a ribozyme, a
5 ribozyme derivative, liposomes, peptides such as, for example, soluble peptides, and combinatorial chemistry-derived molecular libraries made of D- and/or L- configuration amino acids, molecules from natural product libraries, antibodies (including, but not limited to, polyclonal, monoclonal, humanized, anti- idiotypic, chimeric or single chain antibodies, and FAb, F(ab')₂ and FAb expression library fragments, and epitope-binding fragments
10 thereof). In addition, a vast array of small organic or inorganic compounds from natural sources such as fungal, plant or animal extracts, and the like, can be employed in the screening assay. Preferably, the compound is selected from the group consisting of an antisense oligonucleotide, a ribozyme, an antibody, a liposome, a small interfering RNA, a small molecule and an inorganic compound.

15

In practicing this embodiment, candidate compounds can be administered into the animal model through a wide variety of routes of administration and dosage forms. Any suitable route of administration may be employed for providing a mammal, primate, rodent, and especially a mouse, an effective dosage of a candidate compound of the present invention.
20 For instance, the candidate compound can be administered intravenously, subcutaneously, intramuscularly, intraperitoneally, intradermally, orally, intranasally, or intrapulmonarily. Illustrative dosage forms include tablets, troches, dispersions, suspensions, solutions, capsules, creams, ointments, aerosols, and the like. The effective dosage of active ingredient employed may vary depending on the particular compounds employed, the mode of
25 administration, the condition being treated and the severity of the condition being treated. Such dosage may be ascertained readily by a person skilled in the art.

Detailed Description of the Invention

The invention will now be described in detail by way of example only with reference to the
30 figures in which:

Figure 1 shows the evaluation of amyloid beta 40 levels in the soluble fraction of the cortex, frontal cortex, hippocampus and cerebellum. One way ANOVA, n=10.

Figure 2 shows the evaluation of amyloid beta 42 levels in the soluble fraction of the cortex, frontal cortex, hippocampus and cerebellum. One way ANOVA, n=10.

- 5 Figure 3 shows the evaluation of amyloid beta 40 levels in the insoluble fraction of the cortex, frontal cortex, hippocampus and cerebellum. One way ANOVA, n=10.

Figure 4 shows the evaluation of amyloid beta 42 levels in the insoluble fraction of the cortex, frontal cortex, hippocampus and cerebellum. One way ANOVA, n=10.

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Figure 5 is a graph showing the standard deviation of error within each treatment group for insoluble amyloid beta 40.

- Figure 6 is a graph showing the standard deviation of error within each treatment group for
15 insoluble amyloid beta 42.

Figure 7 shows the evaluation of phosphorylated Tau levels in the soluble fraction of the cortex, frontal cortex, hippocampus and cerebellum. One way ANOVA, n=10.

20 Example 1

Materials and methods

Animals:

- Male C57Bl6 mice, three months of age, were group housed on a 12 hour:12 hour light–dark cycle; food and water were available *ad libitum*. All procedures described here were
25 approved by the Home Office, and were designed with commitment to reduce numbers and undue suffering in accordance to the Animals (Scientific Procedures) Act 1986.

Animal treatment:

- Forty animals were divided into four treatment groups, 10 animals per group. All animals
30 received compound/s supplemented in their drinking water for a period of 8 weeks.

Group 1 received 0.1% sodium benzoate (veh + Bz)

Group 2 received 4g/kg D-galactose with 0.1% sodium benzoate (Dgal + Bz)

Group 3 received no compounds (veh)

Group 4 received 4g/kg D-galactose (Dgal)

Dosing started when animals were three months of age. Animals received fresh drinking water every second day. A fresh stock of drinking water supplemented with compound/s was made up every time the water was changed in the cages. Animals could access their drinking
5 water *ad libitum* and this was their only water source throughout the experiment.

Compound formulation:

D-galactose test groups received 4mg/g D-galactose (Sigma, G0625) per ml of drinking water for eight weeks while the vehicle group received water. The veh + Bz and Dgal + Bz groups
10 received 0.1% sodium benzoate (Sigma, 71300) in their drinking water throughout the 8 week period.

A stock solution of D-galactose (Sigma, G0625) was made by making 150g up to 1L with mouse drinking water.

15

To make 4g/kg D-galactose:

$$\begin{aligned} &= 4\text{mg/g} \\ &= 4\text{mg/g} \times \text{mouse weight (30g)} \\ &= 120\text{mg} \end{aligned}$$

20

Assuming a mouse drinks 2ml water a day therefore:

$$\begin{aligned} &= 2\text{mg/g/ml drinking water.} \\ &= 120\text{mg} / 2\text{ml} \\ &= \underline{\underline{60\text{mg /ml for a 30g mouse}}} \end{aligned}$$

25

$$\begin{aligned} &= 60\text{g} / \text{L} \\ &= 400\text{ml of 150g/L stock D-Gal make up to 1 L with mouse drinking water.} \end{aligned}$$

Tissue processing:

Animals were euthanized with sodium pentobarbital and brains carefully removed. The
30 hippocampus, frontal cortex, cerebellum and cortex were dissected and snap frozen in liquid nitrogen. Frozen sections were homogenized in PBS supplemented with complete protease inhibitors (Roche); 100µl for hippocampus and frontal cortex, 400µl for cortex and cerebellum; using the LT Tissue lyser (Qiagen) (20Hz for 2.5 minutes for each tissue). Protein

levels were quantified with a BCA assay (Pierce) according to manufacturer's instructions. Samples were normalized to 5.5mg/ml with PBS and 1% of a 10% lauryl chloride solution was added to each homogenate. Samples were briefly mixed and left on ice for 30 minutes. Samples were then centrifuge at 16000xg for 20minutes at 4°C. Supernatants (soluble
5 fraction) were removed and the remaining pellets were solubilized in Guanadine buffer (6M guanidine-HCL, 1% sarkosyl, 50mM Tris pH8). Guanadine samples were placed on a rotator overnight at room temperature. Guanadine samples were diluted 40x in PBS prior to use (guanadine fraction).

10 MSD ELISA:

Amyloid beta was quantified using the abeta MSD Vplex kit (K15199E-2), 25µl of soluble or guanidine fractions were loaded for each brain region. Total and phosphorylated Tau was quantified using a Phospho (Thr231)/Total Tau Kit (K15121D). Soluble samples were diluted 80x in PBS and the previously 40x diluted guanidine samples were used.

15

Results:

Amyloid beta quantification:

The soluble and the guanidine fractions from the cortex, frontal cortex, hippocampus and cerebellum were used to quantify amyloid beta 40 (Aβ40) and amyloid beta 42 (Aβ42) levels.
20 Guanadine is a highly caotrophic agent able to solubilize dense protein aggregates. An accumulation of insoluble protein aggregates is a common feature of neurodegenerative diseases such as AD. These insoluble proteins begin by forming small oligomers which grow into large aggregated protein plaques which ultimately affect normal cellular processing. Amyloid beta is a peptide which has a high propensity to aggregate and aggregated amyloid
25 beta is one of the hallmarks of AD pathology. Amyloid beta is not toxic in its monomeric form but has been shown to be highly toxic upon the formation of dimers, trimers and oligomers. Aβ42 is thought to be more toxic than Aβ40 because it has a higher propensity to form aggregates. It is thought that Aβ42 forms the seed for aggregation. These aggregates can then attract various species of amyloid beta, initiating a feedforward loop.

30

Fig 1 depicts the Aβ40 levels over the four brain regions analysed for the four treatment groups. The circles indicate individual animals' Aβ40 levels within each group. It is evident from fig 1 that there is a significant increase in Aβ40 levels for the Dgal+Bz group vs the

veh+Bz group in the cortex ($p=0.011$), frontal cortex ($p=0.0007$) and hippocampus ($p=0.025$). There is however no change in A β 40 levels between the veh and Dgal groups in the frontal cortex, hippocampus or cerebellum. The Dgal group did show a significant decrease in A β 40 levels compared to the veh group in the cortex fraction ($p=0.017$). This data suggests that the

5 D-galactose is only effective at elevating A β 40 in the brain if co-dosed with sodium benzoate.

Fig 2 shows soluble A β 42 levels in the four brain regions for the four treatment groups. There is a significant increase in A β 42 levels for the Dgal+Bz group compared to the veh+Bz group in the cortex fraction ($p=0.018$). There is no change in A β 42 levels for the Dgal group relative

10 to the veh group in any of the brain fractions analysed.

Fig 3 depicts the A β 40 levels in the insoluble brain fractions. As with figure 1 there is a significant increase in A β 40 levels for the Dgal+Bz group compared to the veh+Bz group in the cortex ($p=0.0005$), frontal cortex ($p=0.047$) and hippocampus ($p=0.013$). There is no

15 change in A β 40 levels for the Dgal group in comparison to the veh group for any of the brain fractions analysed.

In fig 4 there is a significant increase in insoluble A β 42 levels for the Dgal+Bz group in the cortex ($p=0.026$) and hippocampus ($p=0.008$) fractions compared to the veh+Bz group. Once

20 again there is no change in insoluble A β 42 levels for the Dgal group compared to the veh group in any of the brain fractions analysed.

It is apparent from figures 3 and 4 that there is more variation of A β 40 or A β 42 levels for animals within the veh and Dgal group compared to animals within the veh+Bz or the

25 Dgal+Bz groups. This variation is more obvious in figures 5 and 6 which plots the standard deviation for each group from figures 3 and 4. It is clear from figures 5 and 6 that there is a lot more variation within the groups not treated with sodium benzoate. It seems that sodium benzoate reduces in group variation allowing for a more stable phenotype to precipitate.

30 **Phosphorylated tau quantification:**

Hyperphosphorylated tau accumulation is another hallmark of AD type pathology. Tau promotes assembly and stability of microtubules and vesicle transport. However in disease state tau becomes hyperphosphorylated and in so doing becomes insoluble, lacks affinity for

microtubules, and self-associates into paired helical filament structures (Querfurth and La Ferla, 2010). The MSD ELISA kit used to detect phosphorylated tau (pTau) uses the AT180 antibody which is specific for tau which is phosphorylated on a threonine amino acid at position 231 (pThr231) within the protein sequence (Amniai *et al.*, 2011). Phosphorylation of tau at Thr231 appears early in mild cognitive impairment (MCI) neurons and proceeds to accumulates in only degenerating neurons as AD progresses, localizing to dystrophic neurites, which are known to correlate well with memory loss (Nakamura *et al.*, 2012).

Figure 7 shows a significant increase in pTau for the Dgal+Bz group compared to the Veh+Bz group in the cortex ($p=0.0001$), frontal cortex ($p=0.0074$) and cerebellum ($p=0.038$) fractions. A significant decrease of pTau was observed for the Dgal group compared to the Veh group in the cortex ($p=0.0001$) and cerebellum ($p=0.0001$) fractions, suggesting a decrease in pathology. No significant changes were observed in the other brain fractions.

15 **Conclusions:**

Amyloid beta 40 and 42 accumulation and elevated hyperphosphorylated tau levels are common hallmarks of AD pathology. The amyloid hypothesis put forward by John Hardy and Dennis Selkoe proposed the sequence of pathological events leading to AD is dependent on an accumulation of amyloid beta. This suggests that increased amyloid beta production leads to the formation of amyloid aggregates which affect neuronal synaptic health. The aggregates can promote neuroinflammation and oxidative stress, causing further damage to neurons and synapses. These multiple events could eventually lead to tau hyperphosphorylation which ultimately leads to neuronal death (Hardy and Selkoe, 2002). The etiology of sporadic AD (sAD) is still highly debated among the scientific community and many do not believe the amyloid hypothesis in its entirety. However the scientific community cannot dispute the fact that amyloid beta and hyperphosphorylated tau are in some way involved in the disease, however whether it be a cause or consequence is still a matter of debate. Despite these caveats it has been demonstrated by various groups, in preclinical models, that amyloid beta aggregates and hyperphosphorylated tau are toxic to neurons and promote memory impairment and cognitive decline. Developing a mouse model of neurodegeneration we would expect to see an elevation of amyloid beta and hyperphosphorylated tau in the brain.

- Two separate models of neurodegeneration were run side by side in this study to compare the degree of pathology. The first model received D-galactose along with 0.1% sodium benzoate in their drinking water for 8 weeks. The second group received D-galactose without sodium benzoate in their drinking water for 8 weeks. It is clear from the data that there was no amyloid beta 40 or 42 or hyperphosphorylated tau pathology in the Dgal group compared to the Dgal+Bz group. It is also apparent that the insoluble fractions from the Dgal and veh groups had a high degree of variation of amyloid beta levels between animals compared to the two groups treated with sodium benzoate.
- 10 It is clear from the data that the group treated with 4g/kg D-galactose and 0.1% sodium benzoate produce a significant amount of AD type pathology in the form of amyloid beta and hyperphosphorylated tau (figures 1-4 and 7). It is also evident that the inclusion of sodium benzoate reduces animal variation within the group (Figures 5 and 6) allowing for a more stable and robust phenotype.

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Claims

1. A method of generating an animal model exhibiting an age-related pathological condition of the brain, comprising orally administering to the animal galactose and sodium
5 benzoate.
2. The method of claim 1, wherein the galactose and sodium benzoate are administered to the animal in its food or drinking water.
- 10 3. The method of claim 2, wherein the galactose and sodium benzoate are dissolved in the drinking water of the animal.
4. The method of any preceding claim, wherein the galactose is administered at between about 1 and about 6 grams of galactose per kilogram of animal.
- 15 5. The method of any preceding claim, wherein the galactose is administered at about 4 g/kg.
6. The method of any preceding claim, wherein the galactose is administered in the
20 drinking water of the animal and wherein the drinking water contains between about 0.5 and about 3 milligrams of galactose per gram of animal per millilitre of water.
7. The method of any preceding claim, wherein the galactose is administered in the drinking water of the animal and wherein the drinking water contains about 2 milligrams of
25 galactose per gram of animal per millilitre of water.
8. The method of any preceding claim, wherein the sodium benzoate is administered at between about 1 and about 100 milligrams of sodium benzoate per kilogram of animal.
- 30 9. The method of any preceding claim, wherein the sodium benzoate is administered at between about 50 and about 70 mg/kg.

10. The method of any preceding claim, wherein the sodium benzoate is administered in the drinking water of the animal and wherein the drinking water contains between about 0.001% and about 10% sodium benzoate by weight.
- 5 11. The method of any preceding claim, wherein the sodium benzoate is administered in the drinking water of the animal and wherein the drinking water contains between about 0.001% and about 0.2% sodium benzoate by weight.
12. The method of any preceding claim, wherein the sodium benzoate is administered in
10 the drinking water of the animal and wherein the drinking water contains about 0.1% sodium benzoate by weight.
13. The method of any preceding claim, wherein the galactose and sodium benzoate are administered for at least about 15 days.
- 15 14. The method of any preceding claim, wherein the galactose and sodium benzoate are administered for at least about 50 days.
15. The method of any preceding claim, wherein the animal is a rodent.
- 20 16. The method of any preceding claim, wherein the animal is a mouse.
17. The method of any preceding claim, wherein the animal model exhibits a pathological condition of Alzheimer's disease.
- 25 18. The method of any preceding claim, wherein the pathological condition is accumulation of amyloid beta and/or hyperphosphorylated tau.
19. The method of claim 1, wherein the animal is a rodent, wherein the galactose and
30 sodium benzoate are dissolved in the drinking water of the animal, wherein the drinking water contains between about 1 and about 2.5 milligrams of galactose per gram of animal per millilitre of water and between about 0.05% and about 0.15% sodium benzoate by weight, and wherein the galactose and sodium benzoate are administered for at least about 25 days.

20. An animal model produced by the method of any preceding claim.

21. The animal model of claim 20, wherein the animal is a C57B16 mouse.

5

22. A method of identifying a compound for the treatment of age-related pathological conditions of the brain, comprising administering a candidate compound to a test animal model according to claim 20 or 21 and detecting a change in an age-related pathological condition of the brain in the test animal model relative to a control.

10

23. The method of claim 22, wherein the compound ameliorates the age-related pathological condition of the brain.

24. The method of claim 22 or claim 23, wherein the compound ameliorates the
15 pathological condition of Alzheimer's disease.

Figure 1

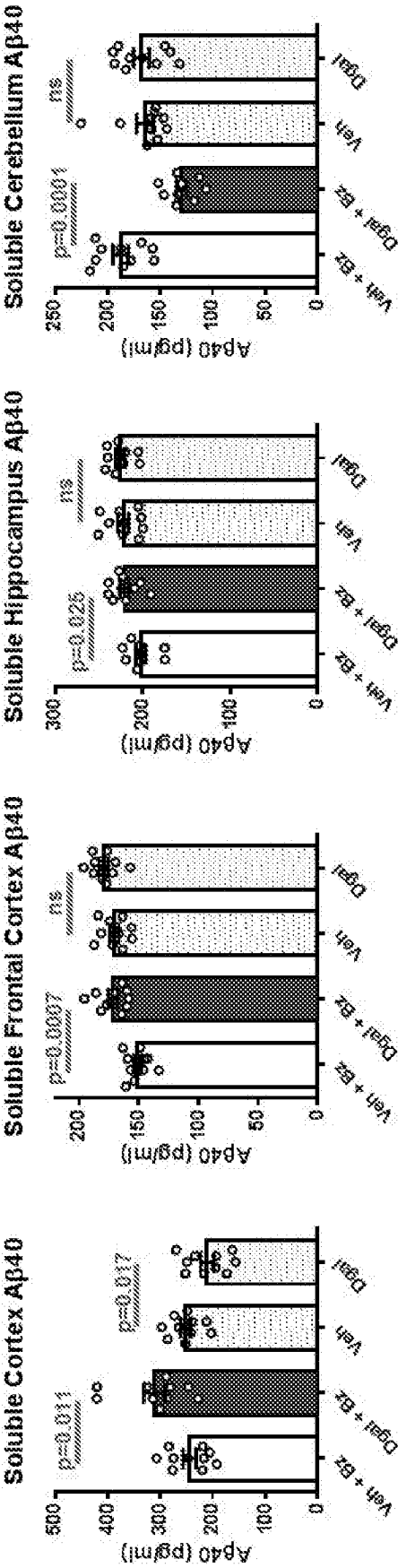


Figure 2

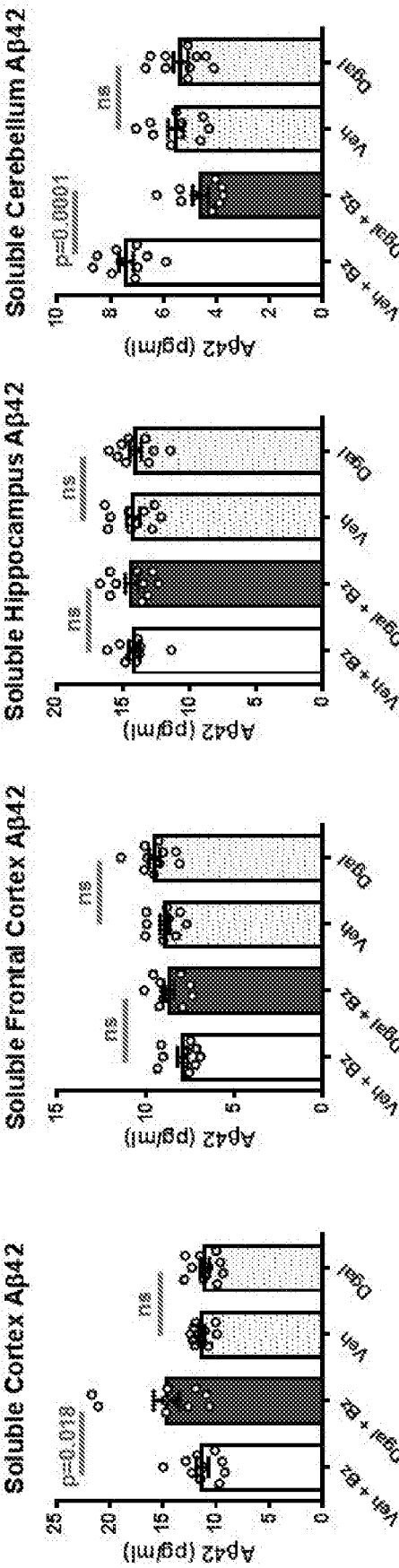


Figure 3

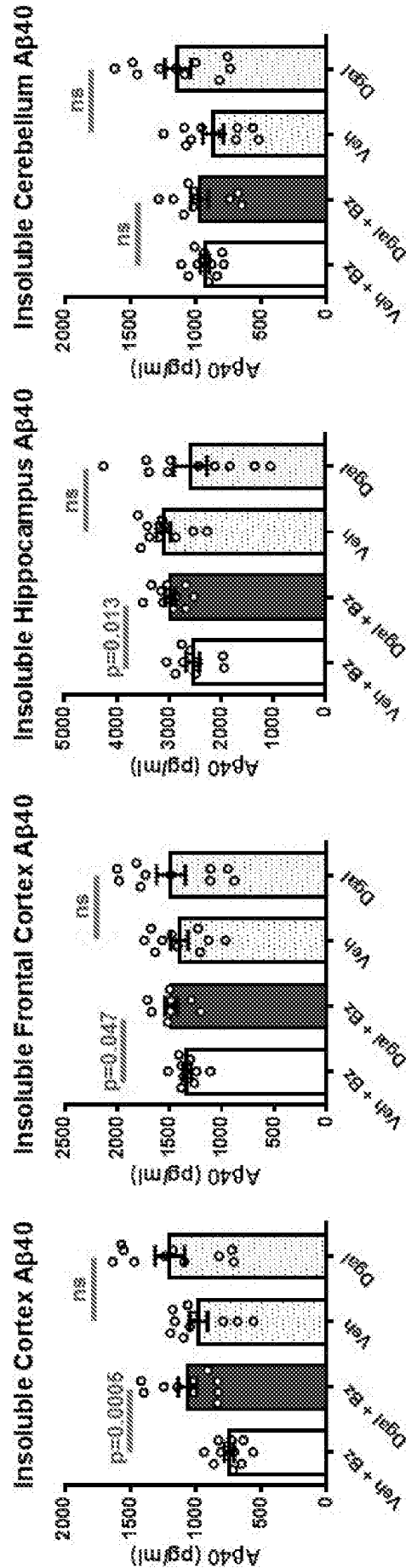


Figure 4

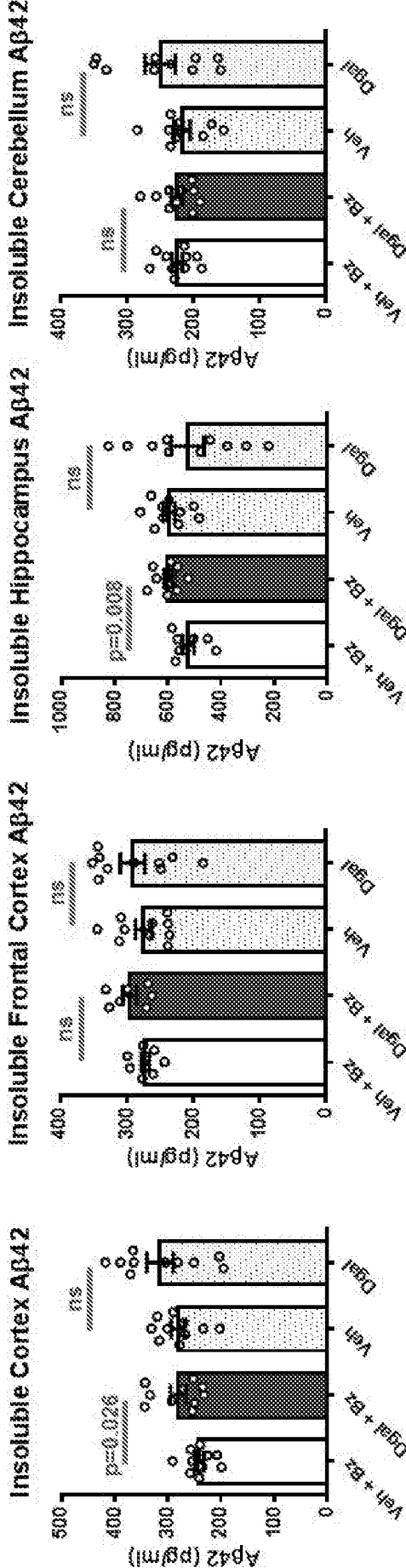


Figure 5

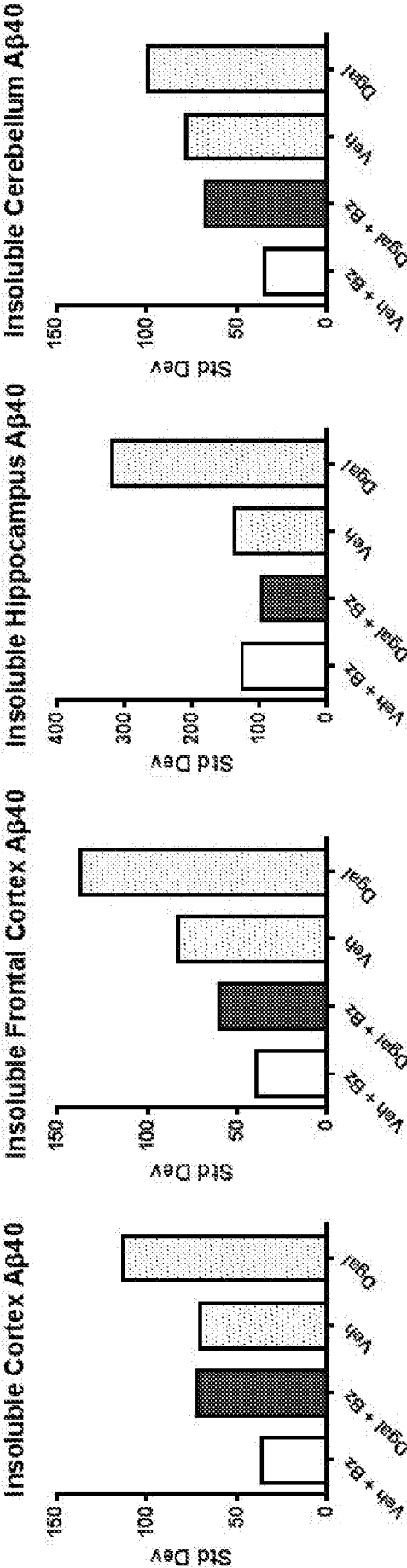


Figure 6

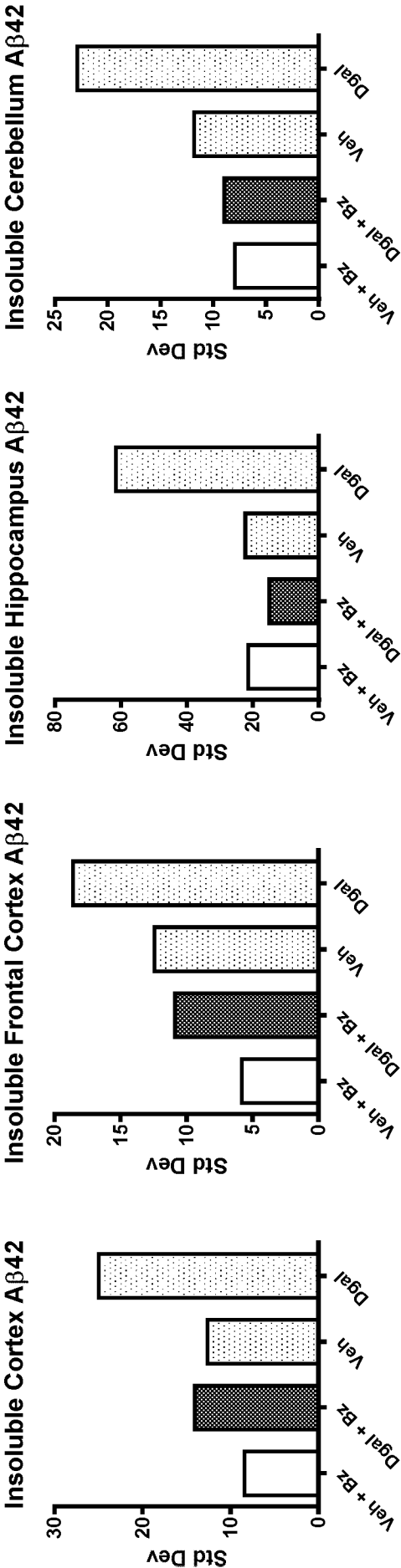
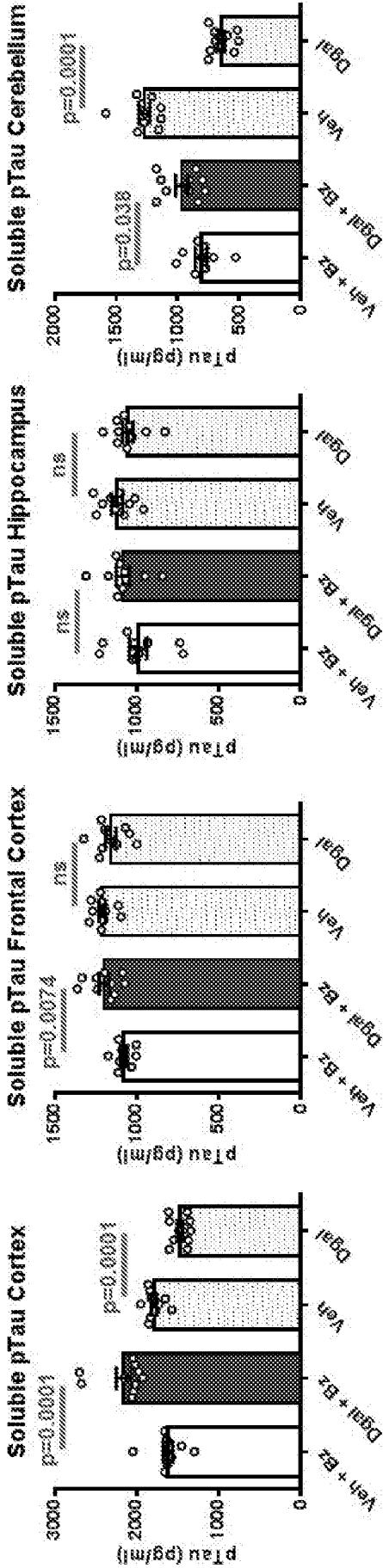


Figure 7



INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2016/051716A. CLASSIFICATION OF SUBJECT MATTER
INV. A01K67/027 G01N33/50
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A01K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, CHEM ABS Data, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HSIEH H M ET AL: "Soy isoflavones attenuate oxidative stress and improve parameters related to aging and Alzheimer's disease in C57BL/6J mice treated with d-galactose", FOOD AND CHEMICAL TOXICOLOGY, PERGAMON, GB, vol. 47, no. 3, March 2009 (2009-03), pages 625-632, XP025916536, ISSN: 0278-6915, DOI: 10.1016/J.FCT.2008.12.026 [retrieved on 2009-02-07]	20-24
Y	page 626 - page 631 ----- -/--	1-19



Further documents are listed in the continuation of Box C.



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Date of the actual completion of the international search

19 July 2016

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Deleu, Laurent

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2016/051716

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SAMANE GHANBARI ET AL: "Effects of IMOD(TM) and Angipars(TM) on mouse D-galactose-induced model", DARU JOURNAL OF PHARMACEUTICAL SCIENCES, BIOMED CENTRAL LTD, LONDON, UK, vol. 20, no. 1, 27 October 2012 (2012-10-27), page 68, XP021123018, ISSN: 2008-2231, DOI: 10.1186/2008-2231-20-68	20,22,23
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Y	----- CN 1 545 868 A (UNIV JINAN [CN]) 17 November 2004 (2004-11-17) claims 1-3	1-19
Y	----- ALI NOORAFSHAN ET AL: "Stereological studies of the effects of sodium benzoate or ascorbic acid on rats` cerebellum", SAUDI MEDICAL JOURNAL, vol. 35, no. 12, December 2014 (2014-12), pages 1494-1500, XP055289614, SA ISSN: 0379-5284 page 1498 - page 1499	1-19
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2016/051716

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CN 1545868	A	17-11-2004	NONE	

CN 104610384	A	13-05-2015	NONE	
