REVIEW

Highlights of ASS234: a novel and promising therapeutic agent for Alzheimer's disease therapy

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Abstract
There is no effective treatment to face Alzheimer's disease complexity. Multitarget molecules are a good approach against the multiple physiopathological events associated with its development and progression. In this context, N-((5-(3-(1-benzylpiperidin-4-yl) propoxy)-1- methyl-1H-indol-2-yl)methyl)-N-methylprop-2-yn-1-amine (ASS234) has been tested achieving promising results. ASS234 has demonstrated to cross the blood-brain barrier *in vivo*, and a good *in silico* safety profile being less toxic than donepezil. Besides, ASS234 reversibly inhibits human acetyl- and butyryl-cholinesterase, and irreversibly inhibits human monoamine oxidase A and B. Moreover, this multitarget molecule has antioxidant and neuroprotective properties, and inhibits $A\beta_{1-42}$ and $A\beta_{1-40}$ self-aggregation. Inquiring about the mechanism of action, several signaling pathways related to Alzheimer's disease had been explored showing that ASS234 induces the wingless-type MMTV integration site (Wnt) family and several members of the heat shock proteins family and moreover counteracts neuroinflammatory and oxidative stress-related genes promoting the induction of several key antioxidant genes. Finally, *in vivo* experiments with ASS234 in C57BL/6J mice displayed its ability to reduce amyloid plaque burden and gliosis in the cortex and hippocampus, ameliorating scopolamine-induced learning deficits. Here we gather the information regarding ASS234 evaluated so far, showing its ability to face different targets, necessary to counteract a neurodegenerative disease as complex as the Alzheimer's disease.

Key Words: *AChE; BuChE; gene expression; heat shock proteins; inflammation; in silico toxicology; MAO A/B; neuroprotection; oxidative stress; Wnt signaling*

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Introduction
Alzheimer's disease (AD) involves progressive memory loss and dementia, which is increasingly affecting the elderly in developed countries around the world. Indeed, AD is the most common neurodegenerative disorder (Fiest et al., 2016). The etiology of AD has been studied, finding an increasingly complex scenario. Nowadays, no available theories for curing AD. The involvement of extracellular deposits of β-amyloid (Aβ) and the accumulation of hyperphosphorylated tau (León et al., 2013; Spillantini and Goedert, 2013; Lue et al., 2017) seems clear. Multiple factors are related to AD, however, there is a consensus that AD is a complex disease in which both genetic and environmental factors can influence (Scheltens et al., 2016). Furthermore, several studies have related to AD; chronic oxidative stress (OS) (Liu et al., 2017), neuroinflammation (Bolos et al., 2017), mitochondria structure and function disturbances (Yan et al., 2013), metal ion metabolism disorders (Brewer, 2017), lipid metabolism abnormalities (Wong et al., 2017) and/or deregulation of the Cyclin-dependent kinase 5 (Liu et al., 2016).

This multifactorial nature frames AD in a complex scenario, difficult to approach in a simple way. Unfortunately, any of the five molecules approved for this pathology reached expectations. These five molecules are tacrine, donepezil, rivastigmine, galantamine and memantine, four acetylcholinesterase inhibitors (AChEI) and one N-methyl-D-aspartate receptor antagonist, respectively. Based on the fact that these therapeutic strategies were directed to only one biological target, in order to reach higher efficacy, the design and synthesis of new molecules try to tackle AD complex pathophysiology through multiple pathways and at several steps.

The multitarget-directed ligands (MTDLs) theory has attracted a great interest as a design and synthetic strategy. Obviously, a molecule able to interact with two or more biological targets may confer a high efficacy to treat a multifactorial disease such as AD (de Oliveira Pedrosa et al., 2017; Ramos et al., 2017).

In this review, our aim is to describe ASS234 properties, making a summary of the results obtained from the multiple studies conducted with this multipotent molecule so far (Bolea et al., 2011, 2013; Del Pino et al., 2014, 2018; Esteban et al 2014, 2017; Ramos et al., 2017, 2018a, b; Serrano 2017) (retrieved from PubMed database).

Why and How ASS234 Was Designed and Synthesized?
After more than a century after the first case of AD was diagnosed, there is still no effective drug available in the market, most likely due to the multifactorial nature mentioned above. To find this desired therapeutic agent, the MTDL approach, based on "one molecule, multiple targets" (Cavalli et al., 2008) has reached a great acceptance in the scientific community (León et al., 2013). Currently, a considerable number of MTDL with several biological targets had been

reported (Rosini et al., 2003; Rodríguez-Franco et al., 2005; Marco-Contelles et al., 2006; Elsinghorst et al., 2007; Fang et al., 2008; Ramos et al., 2017). The strategy to treat AD, hence, was to design new hybrids able to inhibit both monoamine oxidase (MAO) and cholinesterase (ChE), based on previous developments of MAO inhibitors (MAOIs) to treat neurodegenerative diseases (Schafer et al., 2000).

Consequently, the novel hybrids were designed by combining selected pharmacophoric motifs from donepezil, and a previously investigated MAOIs (N-[(5-benzyloxy-1-methyl-1H-indol-2-yl)methyl]-N-methylprop-2-yn-1-amine) at the IQOG (CSIC, Madrid, Spain) (**Figure 1**). The underlying strategy was to retain the key responsible motif for the MAO inhibition. The new compounds were expected to behave as dual binding site AChEIs, resulting in a significant increase of the AChE inhibition. Which is of a great interest managing AD symptomatology, enhancing the cholinergic deficit. Furthermore, it also blockades the peripheral site, which appears to mediate the Aβ-proagreggating action of AChE (Inestrosa et al., 1996).

ASS234 was then identified as the most potent MAOI, showing, moreover, the most potent activity towards AChE inhibition, being the most promising hit-compound among a new series of new hybrid derivatives. These derivatives contained the N-benzylpiperidine moiety of donepezil and the indolyl propargylamine moiety of PF9601N (**Figure 1**).

In Silico Safety Profile

For the selection and optimization of new compounds with therapeutic properties, alternative methods such as computational predictive tools are very useful, which aims to reduce the number of synthesized and tested ligands, and consequently, the animals used for the *in vivo* tests. Nonetheless, sometimes, it is a difficult mission to identify these risks previous to clinical and post-marketing experiences. For this reason, predictive tools that allow toxicity identification risks are strongly emerging and developed.

To assess the metabolism and carcinogenic risk of ASS234, an *in silico* study was carried out, obtaining the first approach about its safety profile (Ramos et al., 2018a). A phase I metabolism prediction was developed with Lhasa ltd. Meteor Nexus version 3.1.0. (KB 2018 1.0.0), obtaining 24 likely metabolites. The evaluation of its mutagenic potential, under the ICH M7 guideline, was performed using two complementary (Q)SAR methodologies from Lhasa ltd. (Leeds, UK), Derek Nexus V3.2.0 (expert rule-based methodology) and Sarah Nexus V3.0.0 (statistical-based methodology). Predicting an absence of structural alerts for 22 of the 24 metabolites, concluding that there is no mutagenic concern for them. While the presence and quantity of the other two, should be confirmed with analytical techniques.

Activity and Neuroprotective Profile of ASS234

Up to now, ASS234 has been reported as an inhibitor of AChE, butyrylcholinesterase (BuChE), and MAO-A/B. It is able to cross the blood-brain barrier (BBB), to inhibit Aβ aggregation and to modulate apoptosis. Moreover, it has been described as a more potent than donepezil inhibitor of MAO-A and Aβ aggregation (Bolea et al., 2011, 2013).

The enzymatic activity of BuChE increases while AChE decreases in AD patients brain (Greig et al., 2001). Therefore, a compound able to inhibit both AChE and BuChE would be very relevant in this therapeutic field. It is interesting to mention the ability of ASS234 to inhibit BuChE, in the submicromolar range. Moreover, the reported potential to inhibit of MAO-B is essential in order to modulate the cholinergic and the serotoninergic neurotransmission (Bolea et al., 2011).

It is critical to verify whether the compound crosses the BBB, since its effects are sought to be effective in the brain. To define this ability, the parallel artificial membrane permeation assay for the blood-brain barrier was used (Di et al., 2003). Obtained results suggest that ASS234 efficiently penetrates into central nervous system (CNS) tissues (Bolea et al., 2013).

In SH-SY5Y cells, ASS234 inhibits Aβ peptide fragments self-aggregation and AChE-dependent aggregation ($A\beta_{1-40}$ and $A\beta_{1-42}$). Moreover, in a micromolar range, ASS234 significantly reduced $A\beta_{1-42}$ mediated toxicity in a dose-depen-

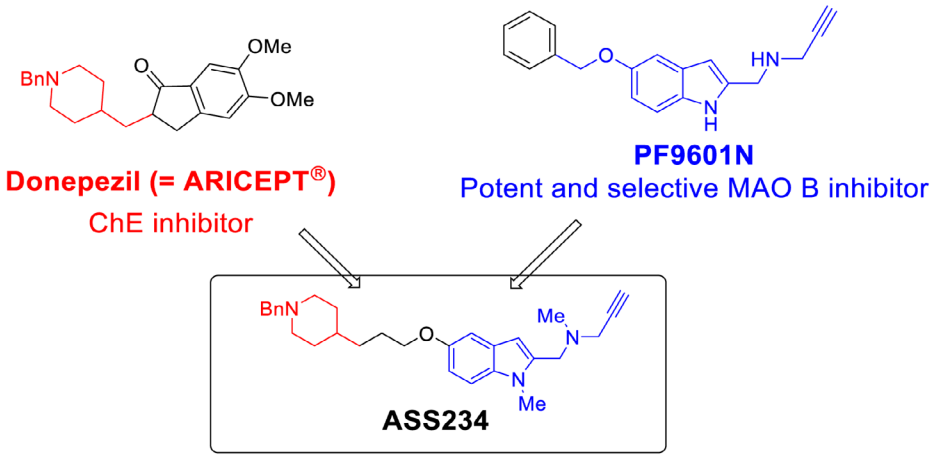

Figure 1 ASS234 design from donepezil and PF9601.
CHE: Cholinesterase; MAO: monoamine oxidase.

dent manner (Bolea et al., 2013).

Aβ has an extensive role in the pathogenesis of AD. Specifically, $A\beta_{1-42}$ induces the depletion of antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD-1) (Bolea et al., 2013). Herein, ASS234 showed an antioxidant profile through two different ways. Firstly, after testing the molecule with the oxygen radical absorbance capacity by fluorescence method (Ou et al., 2001), ASS234 was able to act as a moderate free radical species scavenger *in vitro*. The antioxidant effect observed was a half oxygen radical absorbance capacity value than the reference compound Trolox. Lastly, a pre-incubation in SH-SY5Y with ASS234, dose-dependently counteracted the $A\beta_{1-42}$-induced reduction of CAT and SOD-1 expression (Bolea et al., 2013).

Moreover, $A\beta_{1-42}$ induces apoptotic features that, presumably, based on previous results and ASS234 chemical characteristics, they could also be counteracted. Therefore, this group studied *in vitro* the activation of three initiator caspases (caspase-8, caspase-9, and caspase-12) and one executioner caspase (caspase-3) after Aβ-induced apoptosis. ASS234 decreased caspase-3 activation, and, among the three initiator caspases, only caspase-9 was involved in the anti-apoptotic effect observed. Thereby, suggesting that ASS234 may, directly or indirectly, antagonize the mitochondrial pathway of apoptosis (Bolea et al., 2013). These findings point to ASS234 as a possible modulator of the intrinsic pathway of apoptosis, avoiding AD-related neuronal cell death.

In AD brain, there is an increased activity of the mitochondrial enzymes MAO-A and MAO-B. They catalyze the oxidative deamination of biogenic and xenobiotic amines. The increase of serotonin (5-HT), noradrenaline (NA), and dopamine (DA) deamination enhances OS, aggravates brain homeostasis and contributes to depression and aggression in AD (Finberg, 2014; Vermeiren et al., 2014).

Next, it was carried out a biochemical and structural analysis to confirm the activity of ASS234 on human enzymes. This assay was able to confirm that this multitarget molecule reversibly inhibits AChE and BuChE and irreversibly inhibits MAO-A (Esteban et al., 2014).

Later, the predictable modulation of ASS234 on the monoaminergic metabolism and remaining activity of MAO was assessed *in vitro*, in PC12 and SH-SY5Y cells. In the same study, obtained results were confirmed *in vivo*, measuring 5-HT, DA, and NA levels by a microdialysis experimental model (Esteban et al., 2017). In both SH-SY5Y and PC12 cell lines, ASS234 fully inhibited MAO-A. In SH-SY5Y cells, ASS234 increased 5-HT levels, which is associated with a reduction in its metabolite, 5-hydroxyindoleacetic acid levels (Esteban et al., 2017). This effect is known to be a result of the prolonged irreversible inhibition of MAO-A, these results were in accordance with the previously reported ones (Bolea et al., 2013). Additionally, ASS234 incubation in PC12 cells decreased DA content significantly after 24 hours, which confirms its beneficial effects on MAO inhibition (Esteban et al., 2017).

Up to this moment, there was no *in vivo* confirmation as a MAO-A inhibitor. For this purpose, they used three doses (5, 15 and 30 mg/kg) for the *in vivo* microdialysis study in Wistar rats. Results revealed that ASS234 alters the extracellular levels of monoamines in the two main cerebral regions affected in AD (hippocampus and prefrontal cortex) (Esteban et al., 2017). ASS234 could serve as an enhancer of the monoaminergic system, and this brain region-specific increased monoamines may improve the brain environment in AD patients.

The great variety of properties therapeutically useful of ASS234 observed lead researchers to test this molecule in other models. The permanent bilateral occlusion of the common carotid arteries (BCCAO) rat model is useful to test pharmacological properties for impaired cognitive functions. ASS234 treatment in BCCAO rats improved the cognitive parameters (Stasiak et al., 2014).

Effects of ASS234 on Gene Expression and Neuroprotective Significance

The complexity of AD pathophysiological events displays a difficult scenario to evaluate molecular changes. In this line, understanding the possible modulation of molecular pathways by ASS234 may shed light to develop new therapeutic approaches. There are several signaling pathways that arouse great interest towards age-related diseases. These pathways or genes may act as therapeutic targets either increasing or decreasing their activity to improve AD development. Up to now, several molecular mechanisms induced by ASS234 had been pointed out. Herein, we make a brief review of the influence of ASS234 on Wnt, antioxidant, neuroinflammation and HSPs related genes.

The Wnt plays an important role in the CNS throughout life. It has emerged as a target to improve brain functioning against AD (Riise et al., 2015). In 2014, we investigated whether ASS234 acts on the expression of some members of the Wnt family (Wnt1, Wnt2b, Wnt3a, Wnt6 and Wnt5a). After ASS234 incubation with SH-SY5Y cells for a 24 hour-period, the expression of Wnt2 and Wnt5a were significantly increased (del Pino et al., 2014). Wnt2 expression level decreased in the aged brain, which is a problem since it has a critical participation in the decay of neurogenesis and synapse maintenance. Furthermore, Wnt5 has also been related to the promotion of synaptic assembly on both excitatory and inhibitory hippocampal neurons (Cuitino et al., 2010). The modulation of Wnt, by ASS234, in the CNS may open a new therapeutic approach.

Neuronal apoptosis and OS are important concerns in AD (Rojas-Gutierrez et al., 2017). Indeed, in the AD brain, an alteration of antioxidant enzymes expression and an extensive OS can be observed. Recently, ASS234 has been tested in this context, after an *in vitro* 24-hour period incubation in SH-SY5Y cells, ASS234 induced the gene expression of antioxidant enzymes; CAT, SOD-1, glutamate-cysteine ligase catalytic subunit, gamma-glutamylcysteine synthetase, glutathione reductase, sequestosome 1, thioredoxin reductase 1 and quinone oxidoreductase 1 (Ramos et al., 2016). We hypothesize that this ASS234 induced overexpression of antioxidant enzymes, may be mediated through the nuclear

factor (erythroid-derived 2)-like 2 (NRF2). NRF2 is the master regulator of the NRF2 pathway. And indeed, an ASS234 treatment in SH-SY5Y cells caused the overexpression of NRF2 with respect to the controls (Ramos et al., 2018b). The increase in the expression of these genes entails the mitigation of free radical generation and a reduction in neuronal tissue damage caused by the oxidative nature of this tissue in AD patients.

In the search for more possible mechanisms of action of the molecule, our group set out to investigate several of the main members of the HSPs family (Ramos et al., 2018b). And also, the heat shock transcription factor 1, that has been pointed as a master regulator of HSPs expression. This family is related to the life cycle of proteins, processes of pathological inflammation, OS and cell death (Gorenberg and Chandra, 2017).

The results we obtained were as expected, the expression of the HSPs related genes was significantly induced by ASS234 in SH-SY5Y cells (heat shock transcription factor 1, HSP105, HSP90AB1, HSPA1A, HSPA1B, HSPA5, HSPA8, HSPA9, HSP60, DNAJA1, DNAJB1, DNAJB6, DNAJC3, DNAJC5, DNAJC6, HSPB1, HSPB2, HSPB5, HSPB6, HSPB8, and HSP10) (Ramos et al., 2018b). Reportedly, the overexpression of HSPs modulates inflammation, reactive oxygen species formation and apoptosis induction (Ikwegbue et al., 2017). These results suggest that ASS234 may be a potent HSP inductor, which might prevent protein misfolding aggregation and cell death in AD.

Inflammation plays an important role in AD, therefore, modulating neuroinflammation is considered as a powerful therapeutic tool. The presence of a common N-propargylamine moiety in the ASS234 molecule may confer an anti-inflammatory activity (Bolea et al., 2013; Weinreb et al., 2016). Consequently, our group focused on this area and tried to describe the possible anti-inflammatory effects of ASS234. We observed that ASS234 was able to counteract induced inflammatory effects in RAW 264.7 cells (del Pino et al., 2018). We also evaluate seven neuroinflammation related genes expression profiling after ASS234 (5 μM) treatment in SH-SY5Y cells. Proinflammatory genes TNF-α, TNFR1, IL-6, IL-1β, and NF-κB and anti-inflammatory genes IL-10 and TGF-β were evaluated. Proinflammatory related genes appeared downregulated, and anti-inflammatory related genes appeared upregulated. As described above, upregulation of Wnt and NRF-2 signaling pathways appear to be ASS234 neuroinflammation protective mechanisms. These data, together with the neuroinflammation related gene expression fold changes, lead us to conclude that ASS234 shows a beneficial profile, that may face AD inflammatory environment.

Analysis in Animal Experimental Model

Taking together all this information, ASS234 arises as a multipotent AChE/BuChE/MAO A-B inhibitor with a potent inhibitory effect on Aβ aggregation, as well as antioxidant and antiapoptotic properties. It appears as a promising alternative drug to treat cognitive decay and neurodegeneration underlying in AD.

At this point, there was a need to reliably correlate all this information. Thereby, we examined the effect of ASS234 on the cognition of healthy adult C57BL/6J mice in a scopolamine model. The *in vivo* cholinergic properties of ASS234 were confirmed with this study. It is noteworthy, that it was at least as efficacious as donepezil improving cognition. The previous results obtained for ASS234 that revealed its potent inhibitory AChE and MAO profile (Bolea et al., 2013) were also confirmed *in vivo* in our laboratory. When ASS234 was peripherally administered to a transgenic mouse model (APPswe/PS1ΔE9) of AD, this multipotent molecule enters to the CNS and reduces pathology (Serrano et al., 2017).

Moreover, we studied the effect of ASS234 on neuroinflammation evaluating the immunohistochemical distribution of the astrocyte marker protein galial fibrillary acidic protein and of the microglia/macrophage-specific protein Iba-1. The precursor protein APP induce Aβ deposits that lead to OS, promoting in turn, neuronal injury loss, inflammation and characteristic activation of microglia and astrocytic cells, which plays a critical role in the pathogenesis and development of AD (Niranjan, 2013; Perez-Nievas et al., 2013; Orre et al., 2014; Trujillo-Estrada et al., 2014). The well characterized APPswe/PS1ΔE9 mouse model of AD, displays microgliosis and astrocytosis which is an indicative of neuroinflammation (Hong et al., 2010; Kamphuis et al., 2012; Kook et al., 2014; Wang et al., 2015). In this model, after an ASS234 treatment our group observed a reduction of amyloid plaque burden and gliosis in the cortex and hippocampus. A significant reduction of GFAP and iba-1 immunostainings were observed in the cortex of the ASS234 treated transgenic mice (Serrano et al., 2017). *In vivo* results support the previously observed beneficial effects of ASS234 on neuroinflammation.

Conclusions

The multifactorial heterogeneity of AD opens many possible therapeutic targets that require multitarget compounds, such as ASS234. This multitarget directed propargylamine appears to act in several points of the pathophysiological cascade (**Tables 1** and **2**).

To sum up, ASS234 has a potential disease-modifying profile, displaying a good preclinical safety profile, has a good MAO/AChE inhibitory potency profile, a relevant neuroprotective and antiapoptotic activity, and moreover, it is able to regulate several signaling pathways involved in the brain activity. Besides, to manage one single chemical entity simplifies dosing regimens, improves patient compliance, obviating thus the challenge of administering multiple single-drug entities, which could have different bioavailability, pharmacokinetics, and metabolic profiles. All together, these investigations and results suggest that MTDL ASS234 is a promising agent to fight AD. Nevertheless, determination of effective doses and the optimal timing of administration should be more deeply investigated before extrapolating the obtained data to preclinical studies.

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Table 1 Summary of ASS234 reported properties in several models

System	Model	Properties/characteristics	Reference
In silico	Lhasa Ltd.	Absence of structural toxicity alerts	Ramos et al., 2018a
In vitro	HepG2	Lower toxicity than donepezil and tacrine.	Serrano et al., 2017
In vitro/ In silico	Biological AChE and BuChE/ Docking (RiboDock)	AChE and BuChE inhibitor (submicromolar range)	Bolea et al., 2011
In vitro/ In silico	MAO Rat liver/Docking (RiboDock)	MAO (A and B) inhibitor	Bolea et al., 2011
In vitro	PAMPA assay	Crosses the BBB	Bolea et al., 2013
In vitro	$A\beta_{1-40}$ and $A\beta_{1-42}$ peptides incubation	Ab aggregation inhibitor	Bolea et al., 2013
In vitro	SH-SY5Y	Antiapoptotic	Bolea et al., 2013
In vitro	ORAC	Antioxidant effect	Bolea et al., 2013
In vitro	Spectroscopy and crystallographic studies	Confirmation: AChE and BuChE inhibitor MAO (A and B) inhibitor	Esteban et al., 2014
In vitro	SH-SY5Y and PC12 cells	Full MAO-A inhibition	Esteban et al., 2017
In vitro	SH-SY5Y and PC12 cells	Increases 5-HT levels	Esteban et al., 2017
In vivo	Wistar rats	MAO-A inhibitor	Esteban et al., 2017
In vivo	BCCAO rats	Cognitive improvement	Stasiak et al., 2014
In vivo	C57BL/6J mice	Cognitive improvement	Serrano et al., 2017
In vivo	APPswe/PS1ΔE9 mice	Reduction of Ab plaque load and gliosis; beneficial against neuroinflammation	Serrano et al., 2017

5-HT: Serotonin; AChE: acetylcholinesterase; BBB: blood-brain barrier; BCCAO: permanent bilateral occlusion of the common carotid arteries; BuChE; butyrylcholinesterase; MAO: monoamine oxidase; ORAC: oxygen radical absorbance capacity; PAMPA: parallel artificial membrane permeation.

Table 2 *In vitro* gene expression modulation by ASS234

Cell line	Target	Neuroprotective significance	Reference
SH-SY5Y	Wnt family	Improves brain functioning against AD	del Pino et al., 2014
SH-SY5Y	CAT, SOD-1, GCLC, GCLM, GSR, SQSTM1, TXNRD1 NQO1 and NRF2 pathway	Antioxidant pathway	Ramos et al., 2016, 2018b
SH-SY5Y	HSPs family and HSF1	Death-life cycle Anti-inflammatory Antioxidant	Ramos et al., 2018b
RAW 264.7	Proinflammatory genes TNF-α, TNFR1, IL-6, IL1β, and NF-κB and anti-inflammatory genes IL-10 and TGF-β	Neuro-inflammation modulation	del Pino et al., 2018

CAT: Catalase; GCLC: glutamate-cysteine ligase catalytic subunit; GCLM: gamma-glutamylcysteine synthetase; GSR: glutathione reductase; HSF1: heat shock transcription factor 1; HSPs: heat shock proteins; IL-1β: interleukin 1β; IL-6: interleukin 6; IL-10: interleukin 10; NF-κB: nuclear factor κB; NQO1: quinone oxidoreductase 1; NRF2: nuclear factor (erythroid-derived 2)-like 2; SOD-1: superoxide dismutase; SQSTM1: sequestosome 1; TGF-β: transforming growth factor beta; TNF-α: tumor necrosis factor α; TNFR1: tumor necrosis factor receptor 1; TXNRD1: thioredoxin reductase 1; Wnt: wingless-type MMTV integration site.

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