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REVIEW ARTICLE

Neuroprotective effects of multifaceted hybrid agents targeting MAO, cholinesterase, iron and β-amyloid in ageing and Alzheimer's disease

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Alzheimer's disease (AD) is accepted nowadays as a complex neurodegenerative disorder with multifaceted cerebral pathologies, including extracellular deposition of amyloid β peptide-containing plaques, intracellular neurofibrillary tangles, progressive loss of cholinergic neurons, metal dyshomeostasis, mitochondrial dysfunction, neuroinflammation, glutamate excitoxicity, oxidative stress and increased MAO enzyme activity. This may explain why it is currently widely accepted that a more effective therapy for AD would result from the use of multifunctional drugs, which may affect more than one brain target involved in the disease pathology. The current review will discuss the potential benefits of novel multimodal neuroprotective, brain permeable drugs, recently developed by Youdim and collaborators, as a valuable therapeutic approach for AD treatment. The pharmacological and neuroprotective properties of these multitarget-directed ligands, which target MAO enzymes, the cholinergic system, iron accumulation and amyloid β peptide generation/aggregation are described, with a special emphasis on their potential therapeutic value for ageing and AD-associated cognitive functions. This review is conceived as a tribute to the broad neuropharmacology work of Professor Moussa Youdim, Professor Emeritus in the Faculty of Medicine and Director of Eve Topf Center of Excellence in Technion-Israel Institute of Technology, and Chief Scientific Officer of ABITAL Pharma Pipeline Ltd., at the occasion of his 75th birthday.

LINKED ARTICLES

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Abbreviations

Aβ, amyloid β peptide; AChEIs, AChE inhibitors; AD, Alzheimer's disease; APP, amyloid precursor proteins; BACE 1, β-site amyloid precursor protein cleaving enzyme 1; EPO, erythropoietin; GAP-43, growth-associated protein-43; GPx, glutathione peroxidase; HFD, high-fat diet; NFT, neurofibrillary tangles; PHDs, prolyl-4-hydroxylases; PS1, presenilin 1; RNS, nitrogen species; SIN-1, 3-morpholinosydnonimine; STZ, streptozotocin; TfR, transferrin receptor; T2DM, diabetes mellitus; VAR, VAR10303; 5′UTR, 5′ untranslated region

Tables of Links

TARGETS	
Nuclear hormone receptors[a]	**Enzymes**[d]
PPAR-γ	AChE
Catalytic receptors[b]	BChE
InsR	Casein kinase 1δ (ck1δ)
TrkB	CDK-5
Transporters[c]	GSK-3β
GLUT-1	HIF-1α
	IRE
	iNOS
	MAO-A
	MAO-B

LIGANDS	
5-HT	IL-6
Amyloid β (Aβ)	L-DOPA
ASS234	NGF
ATP	Noradrenaline
Bcl-2	Presenilin-1 (PS1)
BDNF	Rasagiline
Donepezil	Rivastigmine
Dopamine	Scopolamine
Erythropoietin (EPO)	Selegiline
GDNF	Tacrine
H_2O_2	TNF-α
Insulin	VEGF
IL-1β	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c,d]Alexander *et al.*, 2013a,b,c,d).

Introduction

Alzheimer's disease (AD) is the most prevalent neurodegenerative disease in the elderly population (Bullock, 2004). Its predominant clinical manifestation is progressive memory deterioration and other cognitive functions, including disordered behaviour and impairment in language, comprehension and visual-spatial skills (Tsolaki *et al.*, 2001; Weintraub *et al.*, 2012). The neuropathology of AD is characterized by several features, including extracellular deposition of amyloid β peptide (Aβ)-containing plaques in the cerebral cortical regions, accompanied by the presence of intracellular neurofibrillary tangles (NFT, intracellular lesions consisting of paired helical filaments formed of hyperphosphorylated tau) and a progressive loss of basal forebrain cholinergic neurons, leading to reduction in cholinergic parameters, such as ACh and choline acetyltransferase (ChAT) levels and muscarinic and nicotinic ACh receptor binding (Selkoe and Schenk, 2003; Schliebs, 2005). Additionally, there is accumulating evidence indicating that elevated oxidative stress, increased MAO-B enzyme activity, metal accumulation, ion dyshomeostasis, mitochondrial dysfunction, neuroinflammation, glutamate excitoxicity, gene mutations and an impaired ubiquitin–proteasome system are all involved in the dysfunctional brain network associated with AD (Figure 1) (Smith *et al.*, 2000; Rogers and Lahiri, 2004; Joseph *et al.*, 2005). A significant reduction in 5-hydroxytryptaminergic and noradrenergic transmission also occurs, which might explain the relatively high incidence of depression found in AD patients (Palmer *et al.*, 1988; Newman, 1999).

Due to the multifactorial aetiology of AD, it is currently widely accepted that a more effective therapy for AD would result from multifunctional drugs, able to simultaneously target the multiple processes involved in the disease pathology. Thus, based on a multimodal drug design paradigm, Youdim and collaborators have developed a series of non-toxic brain permeable multifunctional compounds, capable of targeting

Major mechanisms involved in pathogenesis of AD

* Oxidative stress (increase in reactive oxygen and nitrogen species)
* Iron accumulation
* Mitochondrial dysfunction
* Abnormal protein folding and aggregation (e.g. Aβ, Tau)
* Aβ production
* Tau hyperphosphorylation
* Glutamatergic excitotoxicity
* Inflammation
* Ubiquitin-proteasome system dysfunction
* Ion dyshomeostasis
* Metabolic impairment
* Decline in growth factors' levels
* Neurotransmitter imbalance
* Synaptic dysfunction
* Neuronal death

Figure 1
Major mechanisms involved in the pathogenesis of AD. Full explanation is discussed in the text.

the numerous mechanisms that underlie AD, including MAO enzymes, the cholinergic system, iron accumulation and Aβ generation/aggregation. In this review, we discuss the molecular mechanisms involved and the beneficial effects of these novel multitarget compounds in ageing and AD.

Pathophysiological targets in ageing and AD

The exact biochemical mechanism of the pathogenesis of AD is still unknown, but a marked loss of cholinergic cells and ACh, necessary for cognition and memory, and oxidative stress-related pathways have been suggested to play an important role in the neurodegenerative processes associated with AD (Maccioni *et al.*, 2001).

The cholinergic system is the primary neurotransmitter system responsible for the cognitive dysfunction in normal ageing and AD (Davies and Maloney, 1976; Perry *et al.*, 1981; Bartus *et al.*, 1982) and thus, restoration of cholinergic function may improve cognitive functions. There are several AChE inhibitors (AChEIs) [rivastigmine (Jann, 2000; Grossberg and Desai, 2001; Machado, 2009), donepezil (Dooley and Lamb, 2000; Doody *et al.*, 2012) and galantamine (Prvulovic *et al.*, 2010)] for the treatment of AD, but despite their mild effectiveness as cognitive enhancers, these drugs have not been shown to significantly affect the neurodegenerative process (Francis *et al.*, 1999; Giacobini, 2002; Lopez *et al.*, 2002; Racchi *et al.*, 2004). Thus, it is widely accepted nowadays that an effective therapeutic strategy in AD is likely to be more complex than simply replacement of lost ACh.

Many lines of evidence suggest that mitochondrial dysfunction and oxidative stress play an important role in the development of neurodegenerative processes in ageing and AD (Schulz *et al.*, 2000; Fukui and Moraes, 2008; Bar-Am *et al.*, 2015). The apparent imbalance between the generation and removal of reactive oxygen species (ROS) and/or nitrogen species (RNS) potentially leads to free radical-mediated processes, that target cellular proteins, DNA, lipids and polysaccharides (Andreyev *et al.*, 2005; Zorov *et al.*, 2014). The most consistent defects in mitochondria in ageing and AD are functional deficits in several key mitochondrial respiratory enzymes, mainly mediated by a functional decline in complexes I and IV, and reduced mitochondrial membrane potential and ATP levels (Keil *et al.*, 2006; Su *et al.*, 2010). Iron-induced lipid peroxidation may mediate alterations in mitochondrial function, as a result of damage to the mitochondrial membrane, resulting in the generation/accumulation of ROS in synaptic mitochondria and an impaired synaptic function (Stauch *et al.*, 2014).

Dysregulation of brain iron homeostasis and enhanced iron accumulation along with reduced antioxidant defence mechanism [e.g. catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx)] and increased basal lipid peroxidation have been reported in patients with AD and in ageing (Reiter, 1995; Arlt *et al.*, 2002; Montine *et al.*, 2002; Zecca *et al.*, 2004; Raven *et al.*, 2013). It was suggested that increased concentrations of iron might be caused by several factors, including increased blood brain barrier (BBB) permeability, inflammation, redistribution of iron within the brain and modifications in iron homeostasis (Mesquita *et al.*, 2012; Ward *et al.*, 2014). A defective iron homeostasis has been implicated in the misfolding process associated with the two major pathologies in AD: Aβ generation and tau hyperphosphorylation, which may generate oxidative stress by inducing the production of ROS and RNS (Castellani *et al.*, 2004; Moreira *et al.*, 2005; Ward *et al.*, 2014). Indeed, it was found that senile plaques and NFTs contain redox-active transition metals and may exert pro-oxidant/antioxidant activities, depending on the balance between neuronal antioxidants and reductants (Sayre *et al.*, 2000).

A direct link between iron metabolism and Aβ pathologies was previously provided by Rogers and collaborators (2002) who demonstrated the presence of an iron-responsive element (IRE) in the 5′ untranslated region (5′UTR) of the amyloid precursor proteins (APP) transcript. Consequently, APP 5′UTR is selectively responsive to intracellular iron levels in a pattern that reflects iron-dependent regulation of intracellular APP synthesis. Indeed, iron levels were shown to regulate mRNA translation of APP in neuronal cells, similarly to the mRNAs of ferritin-L and ferritin-H (Rogers *et al.*, 2002; Rogers *et al.*, 2008). Overall, these findings led to the hypothesis that chelation therapy should be considered as a valuable therapeutic strategy for AD, to prevent iron-induced ROS, oxidative stress and Aβ generation/aggregation in specific brain regions (Youdim, 2006).

Furthermore, it was suggested that various redox-sensitive transcription factors, regulated by oxygen accessibility might bind specific DNA consensus sequences and activate the expression of various genes, particularly those controlling adaptive cellular homeostasis and known to compensate for oxidative stress in the brain (Bergeron *et al.*, 1999; Semenza, 2001). Among such factors, the hypoxia-inducible factor (HIF)-1, whose activation state is differentially regulated by the levels of oxygen and iron within its vicinity, is particularly important (Wang and Semenza, 1995; Bergeron *et al.*, 1999; Zaman *et al.*, 1999; Semenza, 2001), since its activation and nuclear translocation may enhance the transcription of specific neuroprotective-target genes that encode proteins involved in various adaptive/survival cascades (Hewitson and Schofield, 2004; Siddiq *et al.*, 2008). During ageing, HIF-1α accumulation and signalling activation in the brain are reduced in response to hypoxia and ischaemia (Frenkel-Denkberg *et al.*, 1999; Chavez and LaManna, 2003). Decreased HIF-1 was also demonstrated in AD brain (Liu *et al.*, 2008).

An additional contribution to oxidative stress in the brain may arise from the increased activity of the mitochondrial enzymes MAO-A and MAO-B, which catalyse the oxidative deamination of various biogenic and xenobiotic amines, including monoamine neurotransmitters, such as 5-HT, noradrenaline and dopamine (Youdim and Riederer, 2004; Finberg, 2014), with concomitant generation of aldehydes and H_2O_2 (Halliwell and Gutteridge, 1986). Previously, it was reported that MAO-B activity is significantly increased in specific brain regions in ageing (Fowler *et al.*, 1980; Strolin Benedetti and Dostert, 1989; Cohen, 2000) and AD (Hirvonen *et al.*, 2009) and may contribute to neurodegenerative processes, secondary to ROS production. Furthermore, MAO-A activity and gene expression have been found to be up-regulated in different brain areas of AD patients (Burke

et al., 1999; Emilsson et al., 2002; Naoi et al., 2015). It was suggested that decreased levels of the CNS monoamines, such as dopamine, noradrenaline and 5-HT (Adolfsson et al., 1979) and an altered monoaminergic neurotransmitter system (e.g. the 5-hydroxytryptaminergic and dopaminergic), demonstrated in the ageing brain (Carlsson, 1987), and particularly in depressed and aggressive patients with AD (Vermeiren et al., 2014), may play an important role in the deterioration of memory and other cognitive functions in ageing and AD (Palmer and DeKosky, 1993; Engelborghs and De Deyn, 1997). This led to the therapeutic strategy of developing dual MAO-A and MAO-B inhibitors for the treatment of AD (Youdim and Buccafusco, 2005a; Youdim et al., 2005b), as MAO inhibition might increase amine neurotransmitters, reduce the generation of ROS and exert beneficial neuroprotective effects (Riederer et al., 2004; Finberg, 2014).

Multitarget-directed compounds

Rasagiline (*N*-propargyl-1-R-aminoindan) (Azilect®, Teva pharmaceutical Co. Ltd, Netanya, Israel) is a new generation, highly potent irreversible MAO-B inhibitor, anti-Parkinsonian drug (Youdim, 2003). Rasagiline is effective as monotherapy or adjunct therapy to L-DOPA for early and late PD patients (Parkinson Study Group, 2002; Parkinson Study Group, 2004; Parkinson Study Group, 2005; Olanow et al., 2008; Olanow et al., 2009). Previously, structure-activity studies have demonstrated that the propargyl moiety confers not only MAO inhibitory activity but also a wide range of neuroprotective/neurorestorative activities, exerted by rasagiline (Weinreb et al., 2005; Weinreb et al., 2010a). Employing the propargyl moiety, we have designed and synthesized a series of multifunctional, non-toxic, lipophilic brain permeable drugs, including (i) M30 (5-[*N*-methyl-*N*-propargylaminomethyl]-8-hydroxyquinoline) and VAR10303 (VAR) (5-[2-(methyl-prop-2-ynyl-amino)-ethyl]-quinolin-8-ol dihydrochloride) with iron chelating, brain-selective MAO-A and MAO-B inhibitory and neuroprotective activities and HLA20 (5-[4-propargylpiperazin-1-ylmethyl]-8-hydroxyquinoline) with iron chelating, weak MAO inhibitory and neuroprotective activities (Figure 2A). (ii) Ladostigil [(*N*-propargyl-(3R) aminoindan-5yl)-ethyl methyl carbamate], which was designed to combine the AChE and butyrylcholinesterase (BChE) inhibitory activities of the anti-AD drug rivastigmine with the neuroprotective and MAO inhibitory activities of rasagiline (Figure 2B).

Iron-chelating and MAO inhibitory compounds

The multimodal iron-chelating compounds, M30, VAR and HLA20 were designed from the prototype brain-permeable iron chelator, VK28 (5-[4-(2-hydroxyethyl) piperazine-1-ylmethyl]-quinoline-8-ol) (Varinel Inc., West Chester, PA, USA) and enriched with the propargyl moiety of rasagiline and thus, inherit some of their neuroprotective/neurorestorative properties (Ben-Shachar et al., 2004; Gal et al., 2005; Zheng et al., 2005a; Avramovich-Tirosh et al., 2007b; Avramovich-Tirosh et al., 2007a; Kupershmidt et al., 2009; Avramovich-Tirosh et al., 2010; Gal et al., 2010).

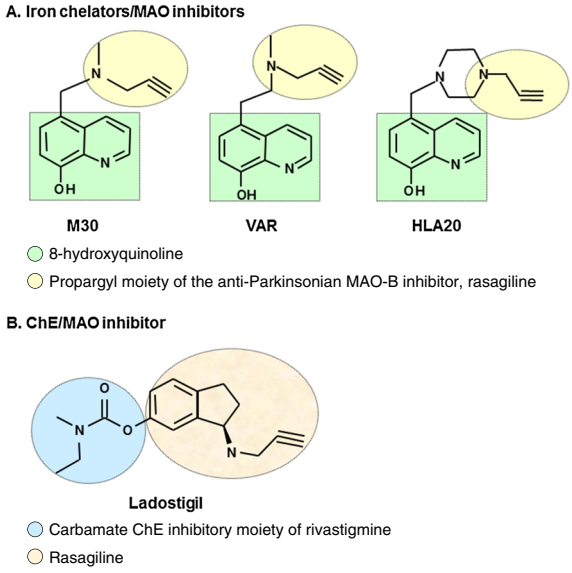

Figure 2
The structures of multifunctional, non-toxic, lipophilic brain permeable drugs, including (A) M30 (5-[*N*-methyl-*N*-propargylaminomethyl]-8-hydroxyquinoline) and VAR10303 (VAR) (5-[2-(methyl-prop-2-ynyl-amino)-ethyl]-quinolin-8-ol dihydrochloride) with iron chelating, brain selective MAO-A and MAO-B inhibitory and neuroprotective activities and HLA20 (5-[4-propargylpiperazin-1-ylmethyl]-8-hydroxyquinoline) with iron chelating, weak MAO inhibitory and neuroprotective activities. The multimodal iron-chelating compounds, M30, VAR and HLA20 were designed from the prototype brain-permeable iron chelator, VK28 (5-[4-(2-hydroxyethyl) piperazine-1-ylmethyl]-quinoline-8-ol) and enriched with the propargyl moiety of the anti-Parkinsonian MAO-B inhibitor, rasagiline. (B) Ladostigil [(*N*-propargyl-(3R) aminoindan-5yl)-ethyl methyl carbamate] with neuroprotective, AChE and BChE and brain selective MAO-A and MAO-B inhibitory activities. The underlying principle of the design of ladostigil was to amalgamate the carbamate ChE inhibitory moiety of rivastigmine into the 6 position of the pharmacophore of rasagiline.

M30, HLA20 and VAR demonstrated high antioxidant activity against iron-induced lipid peroxidation with IC_{50} value comparable with that of the prototype iron chelator, deferoxamine (Zheng et al., 2005a; Bar-Am et al., 2014). It is well established that strong iron chelators could form inactive complexes with iron and interfere with the Fenton reaction, leading to a decrease in hydroxyl free radical production, and thus block lipid peroxidation. M30, HLA20 and VAR that are shown to possess high iron binding capacity (Zheng et al., 2005b; Bar-Am et al., 2014) may also be active through this mechanism to inhibit free radical formation. In addition, electron parametric resonance spin-trapping studies suggest that M30 and HLA20 can behave as radical scavengers to directly scavenge hydroxyl radicals (Zheng et al., 2005c).

Regarding inhibition of MAO activity, M30 and VAR were found to be highly potent brain selective (striatum, hippocampus and cerebellum) inhibitors of both MAO-A and MAO-B activities *in vivo* [IC_{50} values (μM) for M30: MAO-A,

0.037 ± 0.02; MAO-B, 0.057 ± 0.01], with little effect on peripheral MAO activities (liver and small intestine), thus limiting the adverse, potentiating effect of catecholamine releasing agents, such as tyramine, on the cardiovascular system ('cheese reaction') (Gal *et al.*, 2005; Zheng *et al.*, 2005a; Bar-Am *et al.*, 2014). The compound HLA20 was shown to be a weak MAO-A and MAO-B inhibitor [IC_{50} values (μM) for HLA20: MAO-A, >200; MAO-B, >50] (Zheng *et al.*, 2005a).

Molecular mechanism of action of neuroprotective effects

Antioxidant/iron-chelating activities. Our previous study demonstrated that M30 and HLA20 significantly reduced cell mortality induced by H_2O_2 and the peroxynitrite ion generator, 3-morpholinosydnonimine (SIN-1) in mouse motor neuron-neuroblastoma fusion line, NSC-34 cells (Kupershmidt *et al.*, 2009). In addition, M30 and HLA20 induced a dose-dependent increase in transferrin receptor (TfR) levels, further indicating the iron-chelating effect of these drugs (Kupershmidt *et al.*, 2009). Similarly, recent *in vitro* studies demonstrated that VAR elicited a significant neuroprotective effect against oxidative stress-induced damage by H_2O_2 in human neuroblastoma SH-SY5Y cells (Bar-Am *et al.*, 2014). Thus, the antioxidant activity may act synergistically with the direct iron-chelating effects of these multifunctional iron-chelating drugs, modulating the toxicity of H_2O_2-Fe^{2+} reactivity. In support of this hypothesis, it was shown that three antioxidant enzymes (catalase, SOD-1 and GPx) were up-regulated in a brain region-dependent manner, by chronic treatment of mice with M30 (Kupershmidt *et al.*, 2011).

Regulation of HIF-1α cascade. Previous studies have described several iron chelators and molecule inhibitors of prolyl-4-hydroxylases (PHDs) as a route to HIF-1 activation and neuroprotection (see reviews Lee and Andersen, 2006; Harten *et al.*, 2010; Nagel *et al.*, 2010; Weinreb et al., 2010b). Indeed, M30 and HLA20 were found to regulate the HIF-PHD system, up-regulate HIF-1α and significantly increase the levels of HIF-1-dependent neuroprotective genes, enolase-1, vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF) in NSC-34 cells (Kupershmidt *et al.*, 2009). In addition, in rat primary embryonic cortical neurons, these multitarget iron chelators up-regulated HIF-1α and its dependent protective genes [e.g. TfR, VEGF, BDNF, the glycolytic enzyme enolase 1, the cyclin-dependent kinase (CDK) inhibitor p21 and erythropoietin (EPO)] (Avramovich-Tirosh *et al.*, 2010; Maoz *et al.*, 2012). Supporting these findings, it was demonstrated that chronic administration of M30 to adult mice resulted in up-regulation of HIF-1α protein levels and HIF-1 target genes, VEGF, EPO, enolase-1, TfR, haem oxygenase 1(HO-1), inducible nitric oxide synthase (iNOS) and glucose transporter (GLUT)-1 in the brain (Kupershmidt *et al.*, 2011). Given the wide spectrum of cellular functions regulated by HIF-1-target genes, it is suggested that this compensatory neuroprotective pathway may be involved in many of the neuroprotective effects of M30.

APP regulation and Aβ reduction. Previous studies demonstrated that M30 reduced APP protein expression in SH-SY5Y neuroblastoma cells (Avramovich-Tirosh *et al.*, 2007a) and mice hippocampus (Avramovich-Tirosh *et al.*, 2007b). It was suggested that this effect is mediated by chelating the intracellular iron pool, resulting in an effect on APP translation via the IRE in 5′UTR of the APP transcript (Rogers *et al.*, 2002; Rogers and Lahiri, 2004). Indeed, M30 was found to suppress the translation of a luciferase receptor mRNA via the 5′UTR sequence that includes the APP IRE (Avramovich-Tirosh *et al.*, 2007b). Moreover, it was shown that M30 reduced the levels of β-C-terminal fragment and Aβ in the medium of Chinese hamster ovary cells, stably transfected with the APP 'Swedish' mutation (CHO/ΔNL) (Avramovich-Tirosh *et al.*, 2007a), and exerted protection against $Aβ_{25-35}$-induced toxicity in primary cultured neurons (Avramovich-Tirosh *et al.*, 2010). M30 was also found to activate the non-amyloidogenic pathway of APP processing, thus increasing the levels of secreted sAPPα and α-C-terminal fragment in CHO/ΔNL cells (Avramovich-Tirosh *et al.*, 2007a). Hence, the reduction in Aβ levels, demonstrated in response to M30, may result from both mRNA translational regulation of APP levels, as well as from activation of the non-amyloidogenic pathway by α-secretase cleavage. The latter will result in an increased secretion of the neuroprotective sAPPα, at the expense of the production of the neurotoxic Aβ.

Neurite outgrowth and anti-apoptotic activity. An additional aspect of the mechanism of action of the multitarget iron chelating compounds is cell differentiation and anti-apoptotic effects. All three compounds, M30, HLA20 and VAR, were demonstrated to stimulate neurite extension and induce neuronal differentiation, accompanied by a significant increase in the expression of the neuronal specific axonal marker of differentiation, growth-associated protein-43 (GAP-43) in SH-SY5Y and PC12 cells (Avramovich-Tirosh *et al.*, 2007a; Bar-Am *et al.*, 2014). Moreover, M30 and HLA20 were shown to increase the number of PC12 cells in G_0/G_1 and decrease the number in S phase and their proportional number in the G_2 phase, indicating that these multitarget compounds can inhibit cell progression beyond G_0/G_1 phase (Avramovich-Tirosh *et al.*, 2007a).

With regard to the anti-apoptotic activity, M30 caused a marked decrease in the level of apoptotic cells, down-regulated the expression of phosphorylated histone H2A.X (a marker of apoptosis) and cleaved caspase-3, increased the levels of Bcl-2 and conversely decreased Bax expression in SH-SY5Y cells exposed to long-term serum deprivation (Avramovich-Tirosh *et al.*, 2007a).

In addition, in recent studies it was demonstrated that HLA20 has neuroprotective potential as it reduced NMDA and non-NMDA glutamatergic-mediated excitoxicity in rat primary hippocampal and cortical cultures (Maoz *et al.*, 2012). It was suggested that the neuroprotective effect of HLA20 may be associated, at least in part, with its down-regulating effect on caspase-3 and annexin V, and inactivation of the apoptotic pathway involving nuclear translocation of GAPDH and consequent activation of MAO-B gene transcription. Furthermore, it was shown that HLA20 activated the HIF-1-related neuroprotective deacetylase, sirtuin and increased the nuclear content of its downstream effector,

the mitochondrial biogenesis-related PPAR-γ coactivator-1α (Maoz *et al.*, 2012).

Neuroprotective/neurorestorative effects in ageing and AD

As regards the ageing process, chronic treatment of aged mice with M30 exerted significant positive effects on neuropsychiatry functions and cognitive age-related deficits: thus the drug reduced levels of anxiety and aggression, induced locomotor activity and improved memory and nest behaviour (Kupershmidt *et al.*, 2012a). The observed improvement in behavioural deficits was accompanied by a significant reduction in the cerebral accumulation of iron and Aβ plaques (Kupershmidt *et al.*, 2012a) (Table 1). Furthermore, M30 caused a significant irreversible inhibition of both MAO-A and MAO-B activities in aged mice brain (Kupershmidt *et al.*, 2012a). The other multifunctional compound in the M30 series, VAR, was also shown to induce neuroprotective effects on age-related changes in neurobehavioural functions; treatment with VAR in aged rats exerted a significant beneficial effect on depressive-like behaviour, induced locomotor activity and improved cognitive deficits (Bar-Am *et al.*, 2014). In addition, assessment of the neuroprotective effects of VAR revealed that the drug enhanced mRNA expression levels of the growth factors, glial cell line-derived neurotrophic factor (GDNF), nerve growth factor (NGF), BDNF and its receptor Trk B; synaptic plasticity markers (e.g. synapsin-1 and GAP-43) and the anti-apoptotic, Bcl-2 in the brain of aged rats (Bar-Am *et al.*, 2014) (Table 1). These findings in aged animals indicate that the novel MAO inhibitor/iron-chelating drugs, M30 and VAR, acting against multiple brain targets and age-associated memory impairments, may provide a potential strategic treatment against the progression of age-related neurodegeneration.

Further studies in AD animal model (Table 2) demonstrated that M30 markedly attenuated various cognitive deficits, including spatial learning and memory retention, working memory, learning abilities, anxiety levels and memory for novel food recognition and nesting behaviour in APP/presenilin-1 (PS1) transgenic mice (Kupershmidt *et al.*, 2012b). In addition, M30 was found to reduce brain iron accumulation and induce a significant reduction in a number of cerebral AD-like phenotypes, such as APP, phospho-APP, Aβ levels, Aβ plaques formation and aggregation, phospho-tau and CDK-5, as well as elevation of the levels of phospho-PKB and phospho-glycogen synthase kinase (GSK-3β) (Kupershmidt *et al.*, 2012b). Chronic treatment with M30 significantly elevated cortical insulin (Ins) and insulin receptor (InsR) mRNA and protein expressions, and increased the levels of cerebral HIF-1α and the expression of HIF-1-target genes involved in glycolysis, including aldolase A, enolase-1 and GLUT-1 in APP/PS1 mice (Mechlovich *et al.*, 2014a). Treatment with M30 also increased the hepatic protein expression levels of InsR and GLUT-1 and attenuated the increase in blood glucose levels following glucose tolerance test in APP/PS1 mice (Mechlovich *et al.*, 2014a). These findings indicate that iron chelating drugs, such as M30 may also affect impaired neuronal Ins signalling and GLUT-1 expression, implicated in AD (Solano *et al.*, 2000; Salkovic-Petrisic *et al.*, 2006), presumably through its regulation of glucose metabolism.

Considering the close correlation between AD and type 2 diabetes mellitus (T2DM) (Roriz-Filho *et al.*, 2009; Cholerton *et al.*, 2011), additional studies have recently demonstrated that M30 exerted neuroprotective regulatory effects in high-fat diet (HFD) and ob/ob transgenic mouse models of T2D (Mechlovich *et al.*, 2014b) (Table 2). Indeed, M30 treatment increased cerebral levels of Ins/InsR and p-GSK-3β, produced a significant up-regulation of cerebral HIF-1α protein levels and induced the expression of several HIF-1-target genes involved in neuroprotection, glycolysis, neurogenesis, oxidative stress and anti-inflammation in these T2DM mice models (Mechlovich *et al.*, 2014b). In addition, M30 caused a significant inhibition of brain MAO-A and MAO-B activities, reduced brain levels of the metabolites of dopamine and increased the levels of 5-HT and noradrenaline (Mechlovich *et al.*, 2014b). Glucose tolerance was also improved after M30 treatment in both models of T2DM (Mechlovich *et al.*, 2014b). These findings suggest that M30 exerts various beneficial neuroprotective regulatory effects that may act synergistically and delay or prevent AD-related neurodegenerative processes associated with T2DM.

In the rat model of a sporadic form of AD, developed by i.c.v. administration of streptozotocin (STZ) (Knezovic *et al.*, 2015), M30 was found to exert neuroprotective activity by completely preventing the development of, or ameliorating already developed, cognitive deficits in the preventive or neurorestoration paradigms respectively (Knezovic *et al.*, 2012) (Table 2). M30 was shown to prevent the STZ (administered i.c.v.)-induced decrease in catalase activity, in a region-dependent manner (Sofic *et al.*, 2014). Further experiments showed that the multifunctional iron chelator HLA20 also exerted neuroprotective effects in STZ (i.c.v.)-induced memory impairments (Šalković-Petrišić *et al.*, 2015) (Table 2).

The ChE and MAO inhibitory compound, ladostigil

The underlying principle of the design of ladostigil was to amalgamate the carbamate ChE inhibitory moiety of the anti-AD drug, rivastigmine ((S)-3-[1-(dimethylamino)-ethyl]phenyl *N*-ethyl-*N*-methylcarbamate) into the 6 position of the pharmacophore of rasagiline (Weinstock *et al.*, 2000b; Weinstock *et al.*, 2000a; Weinstock *et al.*, 2001; Sterling *et al.*, 2002). The resulting molecule, ladostigil is a dual AChE-BChE inhibitor (the inhibitory effect is ~100 times more potent against AChE than BChE) and brain-selective MAO-A and MAO-B inhibitor, with little or no MAO inhibitory effects in the liver and small intestine; an important property that enables this drug to exert only limited potentiation of blood pressure in response to oral tyramine (Weinstock *et al.*, 2001; Youdim and Weinstock, 2001; Weinstock *et al.*, 2002; Weinstock *et al.*, 2006) (presently in stages of completion of Phase IIb clinical study). Previous *in vivo* studies have shown that administration of ladostigil ($26\,mg{\cdot}kg^{-1}$) to rats for 2 weeks, inhibited brain MAO-A and MAO-B activity by ~70%, with very little or no effect in the intestine (Weinstock *et al.*, 2000a; Weinstock *et al.*, 2002).

Table 1

In vivo pharmacological profiles of multitarget-directed hybrid drugs in ageing

Multitarget compounds	Species and strain	Dose/experimental design	Behavioural alterations	Molecular alterations	Ref.
Iron chelators/MAO inhibitors					
M30	C57BL mice; male (15 months-old)	1 and 5 mg·kg^{-1}, oral gavage 4 times a week for 6 months	Reversed age-associated memory impairments	Reduced cerebral accumulation of iron and Aβ plaques	(Kupershmidt *et al.*, 2012a)
VAR	Sprague-Dawley rats; male (18 months-old)	1 mg·kg^{-1}, s.c. 4 times a week for 6 months	Improved cognitive behavioural deficits	Increased expression levels of GDNF, BDNF, NGF and Bcl-2; decreased expression levels of Bax	(Bar-Am *et al.*, 2014)
ChE/MAO inhibitor					
Ladostigil	Wistar rats; male	1 mg·kg^{-1}·day^{-1} in drinking water for 4 months	Prevented the loss of novel object discrimination; improved reference memory	Reduced CD11b expression and microglial neuroinflammation in the fornix and parietal cortex	(Weinstock *et al.*, 2013)
	Rhesus aged macaques; male and female	Chronic escalating regimen, 1–7 mg·kg^{-1} p.o.	Improved working memory; enhanced motivational aspects		(Buccafusco *et al.*, 2003)
	Wistar rats; male (27 months-old)	1 mg·kg^{-1}·day^{-1}, gavage for 30 days		Induced significant changes in hippocampal expression of various mitochondrial and key regulator genes involved in neurodegeneration, cell survival synaptogenesis and oxidation	(Weinreb *et al.*, 2007b)
Ladostigil	Wistar rats; male (16 months-old)	1 mg·kg^{-1}·day^{-1} in drinking water for 6 months	Prevented the development of spatial memory deficits	Decreased gene expression of IL-1β, IL-6, TNF-α and iNOS in the parietal cortex	(Panarsky *et al.*, 2012)
	Wistar rats; male (16 months-old)	1 mg·kg^{-1}·day^{-1} in drinking water for 6 months	Improved spatial memory	Prevented increase in activated astrocytes and microglia in hippocampus and white matter areas; increased pro-NGF in hippocampus; prevented reduction in cortical AChE activity and increase in hippocampal BChE activity; reduced gliosis	(Weinstock *et al.*, 2011)

Table 2

In vivo pharmacological profiles of multitarget-directed hybrid drugs in AD

Multitarget compounds	Species and strain	Dose/experimental design	Behavioural alterations	Molecular alterations	Ref.
Iron chelators/MAO inhibitors					
M30	APP/PS1 mice; male	1 and 5 mg·kg^{-1}, oral gavage 4 times a week for 9 months	Improved cognitive behaviour deficits; improved memory loss	Reduced APP levels and Aβ levels/plaques aggregation; reduced cerebral iron; modulated cerebral HIF-1α-related glycolytic genes and insulin signalling	(Kupershmidt *et al.*, 2012b); (Mechlovich *et al.*, 2014a)
	STZ-i.c.v. Wistar rats; male	10 mg·kg^{-1}·day^{-1}, oral gavage for 5 days	Neuroprotection: prevented development of spatial memory impairment	Decreased catalase activity	(Knezovic *et al.*, 2012); (Sofic *et al.*, 2014)
		2 and 10 mg·kg^{-1}, oral gavage 3 times a week for 11 weeks	Neurorescue: ameliorated already developed cognitive deficits		
	HFD C57BL mice; male	5 mg·kg^{-1}, oral gavage 3 times a week for 5 months		Modulated cerebral HIF-1α-related glucolytic Modulated cerebral HIF-1a-related glycolytic-neuroprotective-neurogenesis and oxidative stress genes	(Mechlovich *et al.*, 2014b)
	Ob/ob mice; male	1 mg·kg^{-1} oral gavage 3 times a week for 5 months			
HLA20	STZ-i.c.v. Wistar rats; male	5 mg·kg^{-1}·day^{-1} oral gavage for 5 days	Neuroprotection: prevented development of spatial memory impairment		(Šalković-Petrišić *et al.*, 2015)
ChE/MAO inhibitor					
Ladostigil	Adult rats	25–100 μmol·kg^{-1}; oral gavage (90 min before scopolamine)	Antagonized the impairment of spatial memory	Increased brain ACh	(Weinstock *et al.*, 2000a)
	STZ-i.c.v. Sprague-Dawley rats; male	1 mg·kg^{-1}·day^{-1}; oral gavage 1 week before until 8 weeks after STZ	Prevented memory deficits	Prevented glial changes and the increase in nitrotyrosine immunoreactivity	(Shoham *et al.*, 2007)
	NaN_3-induced cytochrome oxidase inhibition in Sprague-Dawley rats; male	17 mg·kg^{-1}·day^{-1}; oral gavage for 5 weeks	Prevented spatial memory deficits	Prevented the decrease in ChAT and the compensatory increase in synaptic plasticity in the subgranular layer of the dentate gyrus and the increase in TfR expression in interneurons in the hilus	(Luques *et al.*, 2007)

Chronic treatment of rats with ladostigil for 3 weeks (52 mg·kg^{-1}) was shown to inhibit cerebral ChE activity by ~50% (Sagi *et al.*, 2005).

Molecular mechanism of action of neuroprotective effects

Regulation of cell survival signalling pathways and neurotrophic factors. Previous studies have demonstrated that in apoptotic cells, AChE aggregates in the nucleus, suggesting that AChE might play an important role in cell apoptosis and favour the association between AChE and neuronal apoptosis in AD (Yang *et al.*, 2002). Thus, it is suggested that cholinergic neurons expressing AChE are considerably more vulnerable to apoptosis (Toiber and Soreq, 2005). In agreement with this, it was reported that various AChEIs exhibited neuroprotective effects by increasing the expression levels of anti-apoptotic/pro-apoptotic genes and proteins (Villarroya *et al.*, 2004; Wang and Tang, 2005). Indeed, in the neurotoxic high-density cultured SK-N-SH cells, ladostigil was shown to have significant neuroprotective activity, including inhibition of caspase-3 activation, induction of Bcl-2 and reduction of Bad and Bax genes and protein expression (Yogev-Falach *et al.*, 2006). In addition, ladostigil was reported to prevent the fall in mitochondrial membrane potential and thus inhibit the initiation of the apoptotic cascade in SH-SY5Y cells exposed to SIN-1 (Maruyama *et al.*, 2003). These protective properties of ladostigil may be associated with its AChE inhibitory activity. Nonetheless, previous studies demonstrated that the propargyl moiety of rasagiline also promoted neuronal survival, mediated by PKC-dependent and MAPK-dependent activation, associated with Bcl-2 family members (Weinreb *et al.*, 2004) and mitochondrial membrane stabilization (Maruyama *et al.*, 2003; Yogev-Falach *et al.*, 2006), and thus this effect might have also a crucial role in the neuroprotective activity of ladostigil.

In addition, it was demonstrated that the neuroprotective effects of ladostigil involve or result from activation of BDNF/GDNF-MAPK pathways (Weinreb *et al.*, 2007a). Thus, the elevation of neurotrophic factors, induced by ladostigil, may possibly initiate respective cell signalling cascades, suggesting the involvement of neurotrophic factors may be involved in the molecular mechanism of action of ladostigil.

Antioxidant activity. Ladostigil was found to exert a significant neuroprotective effect against H_2O_2-induced damage in SH-SY5Y cells (Bar-Am *et al.*, 2009), demonstrating an antioxidant activity via direct scavenging effect on free radicals overproduced in H_2O_2-treated neuronal cells, and an indirect effect by stimulating the expression and activity of cellular antioxidant enzymes, such as catalase and glutathione reductase (Weinreb *et al.*, 2008). Similarly, results obtained from high density cytotoxic model of SK-N-SH cells, also revealed that ladostigil induced an increase in the mRNA expression levels of these antioxidant enzymes (Bar-Am *et al.*, 2009). In support of these findings, previous studies showed that ladostigil has neuroprotective effects against other *in vitro* insults associated with oxidative damage: SIN-1, serum withdrawal (Yogev-Falach *et al.*, 2003) and glucose-oxygen deprivation (Weinstock *et al.*, 2001).

Regulation of APP processing. An essential molecular mechanism of action of ladostigil may involve its ability to regulate the processing of APP by the non-amyloidogenic α-secretase pathway (Vetrivel and Thinakaran, 2006; Bar-Am *et al.*, 2010). In support of this, previous findings demonstrated that ladostigil markedly suppressed holo-APP protein levels and elevated sAPPα release (Yogev-Falach *et al.*, 2002; Bar-Am *et al.*, 2004; Yogev-Falach *et al.*, 2006), indicating that this drug can be of clinical value for accelerating the non-amyloidogenic APP processing, thereby reducing Aβ generation. The observation that ladostigil did not alter APP mRNA levels suggests that the decrease in APP protein and Aβ levels can be attributed to suppression of APP translation (Yogev-Falach *et al.*, 2006). Furthermore, the stimulation of sAPPα release induced by ladostigil was blocked by a hydroxamic acid-based metalloprotease inhibitor, indicating that α-secretase and metalloprotease are involved in this effect (Yogev-Falach *et al.*, 2002). Using several signalling inhibitors it was demonstrated that PKC and MAPK signalling pathways might be involved in the enhancement of sAPPα release by ladostigil (Yogev-Falach *et al.*, 2006). In accordance with inhibitory studies, it was shown that ladostigil stimulated ERK1-MAPK and ERK2-MAPK phosphorylation (Yogev-Falach *et al.*, 2002). *In vivo* studies revealed that ladostigil reduced the levels of holo-APP and up-regulated the levels of p-PKC, PKCα and PKCε in the mice hippocampus (Bar-Am *et al.*, 2004).

Neuroprotective/neurorestoration effects in ageing and AD

Regarding the effect of ladostigil on age-related alterations, previous *in vivo* studies demonstrated that the drug improved cognitive deficits in aged monkeys (Buccafusco *et al.*, 2003) and prevented the development of age-related memory deficits in aged rats (Weinstock *et al.*, 2011; Panarsky *et al.*, 2012; Weinstock *et al.*, 2013) (Table 1). These beneficial effects may be associated with immunomodulatory (microglial activation) and antioxidant effects of ladostigil (Weinreb *et al.*, 2008; Weinstock *et al.*, 2013). In agreement with this hypothesis, aged rats treated with ladostigil showed an increase in pro-NGF in various brain regions, presumably resulting from enhanced neuronal activity (Weinstock *et al.*, 2011). It was also shown that ladostigil can restore gene expression levels of iNOS, IL-1β, IL-6 and TNF-α in the brain of ageing rats to the respective levels of cognitively intact adult rats (Panarsky *et al.*, 2012).

Additional studies on the neuroprotective activity of ladostigil in aged rats identified significant changes in various hippocampal genes and proteins, related to the iron-mediated oxidative stress pathway, such as areduction in antioxidant enzymes and induction of ferritin (Weinreb *et al.*, 2007b). It was also found that ladostigil reversed the effect of ageing on mRNA expression levels of various genes associated with metabolism and oxidation processes in the hippocampus of old rats, including the GPx precursor, glutathione S-transferase, glutathione synthetase, thioredoxin

peroxidase and glucose-6-phosphate dehydrogenase (Weinreb *et al.*, 2008) (Table 1).

With regard to the potential beneficial effects of ladostigil in AD (Table 2), it was shown that the drug antagonized the spatial memory deficits induced by scopolamine in rats, indicating that the drug caused an increase in brain ACh, sufficient to compete with scopolamine for muscarinic receptors subserving memory (Weinstock *et al.*, 2000a; Weinstock *et al.*, 2001). In addition, ladostigil was shown to prevent gliosis, oxidative-nitrative stress and memory impairments in the STZ (i.c.v.) rat model of AD (Shoham *et al.*, 2007). Ladostigil also prevented the decrease in ChAT in the diagonal band, and the compensatory increase in synaptic plasticity and TfR and restored spatial memory deficits, induced in rats by the cytochrome oxidase inhibitor, sodium azide (a chronic AD animal model) (Luques *et al.*, 2007). Genomic studies demonstrated that ladostigil significantly down-regulated rat hippocampal levels of the familial AD-linked PS1 gene (Weinreb *et al.*, 2007b). This effect can be of value in reducing Aβ formation, because PS1 is a major component of the γ-secretase complex, which facilitates the generation of Aβ peptide via intramembranous proteolysis of APP (Kern *et al.*, 2006; Vetrivel and Thinakaran, 2006). Furthermore, ladostigil down-regulated mRNA expression of casein kinase 1δ, associated with pathological hallmarks in several neurodegenerative diseases (Weinreb *et al.*, 2007b).

Concluding remarks and future perspectives

The complex pathology of AD led to the development of single drug molecules with the ability to address multiple pathophysiological targets for the treatments of dementia and AD. This approach may also reduce the risk of drug–drug interactions in AD patients and enhance patient compliance. Accordingly, many research groups have recently designed and synthesized a variety of multifunctional molecules that can interact with multiple symptomatic or AD modifying targets by combining different pharmacophores, mainly derived from existing drugs (e.g. donepezil, tacrine, rivastigmine, rasagiline and selegiline).

An example of these is the series of multitarget MAO-A and MAO-B/AChE/BChE inhibitors, which have been designed as hybrids from the AChE inhibitor, donepezil and the selective potent MAO-B inhibitor, PF9601N (Samadi *et al.*, 2011; Samadi *et al.*, 2012). Among these multitarget hybrid compounds, ASS234 has been shown to retain the anti-apoptotic and antioxidant properties of PF9601N and to cross the BBB (Bolea *et al.*, 2011; Bolea *et al.*, 2013; Esteban *et al.*, 2014). *In vitro* results showed that further to its MAO/ChE inhibitory properties, ASS234 can inhibit Aβ1-42 self-aggregation and block AChE-mediated Aβ1-40/Aβ1-42 aggregation (Bolea *et al.*, 2013). Additionally, ASS234 reduced Aβ1-42-induced toxicity and apoptosis and prevented Aβ1-42-mediated depletion of the antioxidant enzymes catalase and SOD-1 (Bolea *et al.*, 2013). In an experimental model of vascular dementia, it was demonstrated that rats treated with ASS234 tended to perform various working and reference memories better than the untreated control rats (Stasiak *et al.*, 2014). Another multitarget-directed ligand of AChEI/β-secretase-1 (the amyloidogenic β-site amyloid precursor protein cleaving enzyme 1 (*BACE 1*) enzyme) inhibitor and anti-Aβ aggregation (Cavalli *et al.*, 2007; Bolognesi *et al.*, 2009) is the quinone-bearing polyamine, memoquin (2,5-bis-(6-,[ethyl-(2-methoxy-benzyl)-amino]-hexylamino)-[1,4] benzo-quinone). Memoquin was shown to restore cholinergic impairments and behavioural deficits linked to attention and memory and to reduce Aβ expression and accumulation and tau hyperphosphorylation and deposition in several AD animal models (Bolognesi *et al.*, 2009). It was also reported that memoquin was able to ameliorate various aspects of cognitive impairments (e.g. spatial, episodic and short- term and long-term memory) in scopolamine-induced and Aβ-induced amnesia mouse models (Capurro *et al.*, 2013). In addition, several hybrid multifunctional compounds have been recently developed, synthesized and evaluated as potential multitarget drug candidates for AD, including: a chimeric molecule of tacrine and the selective MAO-B inhibitor, selegiline (Lu *et al.*, 2013b); *o*-hydroxyl and *o*-amino benzylamine-tacrine hybrids (Mao *et al.*, 2012), a series of resveratrol derivatives (Lu *et al.*, 2013a) and tetrahydrobenzo[h][1,6]naphthyridine-6-chlorotacrine hybrids, possessing ChE inhibitory and Aβ and tau anti-aggregation activities (Di Pietro *et al.*, 2014).

The development of a multitarget-directed ligand that combines more than two pharmacophores would represent an advanced strategy for the design of a more effective drug candidate that can simultaneously modulate various pathological pathways of AD. Youdim, Zheng and other collaborators have recently described a structure-based strategy for the rational design of more advanced site-activated multitarget ligands derived from the compound M30, which possess ChE and MAO-A and MAO-B inhibitory, as well as site-activated chelating and neuroprotective activities (Zheng and Monnot, 2012). Preliminary studies have shown that these novel compounds act as pro-chelators, being metabolized to the active chelator M30 following pseudo-inhibition of AChE (Zheng *et al.*, 2014). In addition, the synthesis and biological evaluation of a new class of metal chelator-ChE and MAO inhibitor compounds, derived from donepezil, propargylamine and 8-hydroxyquinoline for the potential prevention and treatment of AD has been reported (Wang *et al.*, 2014).

For future therapeutic applications of multifunctional compounds that address the multifaceted nature of AD, such as MAO elevation and oxidative stress, Aβ and tau protein accumulation/aggregation, cholinergic hypofunction and iron dyshomeostasis, a detailed pharmacological evaluation should be conducted (e.g. cellular and BBB penetration, metabolite stability and undesired toxic side effects) and presumably, if needed, optimized by structural modifications.

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Author contributions

M. B. H. Y., T. A., O. B. and O. W. wrote the manuscript. All authors have revised and approved the manuscript.

Conflict of interest

M. B. H. Y. is CSO of ABITAL Pharma Pipeline Ltd. He is a part owner of the company and has shares.

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