ABSTRACT

Dementia is a multifactorial neurodegenerative disorder (NDD) that mainly affects the elderly and is characterized by the gradual loss of structure or function of neurons, leading to cognitive and motor impairments. There are about 69 million dementia patients worldwide and this number is expected to rise to 82 million by 2030 and 152 million by 2050. There are limited monotherapy drugs available as cholinesterase inhibitors and are used as combination therapy with NMDA antagonists and other drugs for the management of the disease. Out of many causes, hyperactivity of monoamine oxidase B (MAO-B) and acetylcholinesterase (AChE), impaired metal homeostasis are highly implicated in the pathophysiology of the multifactorial disease. MTDLs capable of inhibiting both brain MAO-B and AChE and chelating brain iron ions have significant advantages over other MTDLs in treating NDDs. Besides elevating the brain's dopamine and ACh levels, these MTDLs can reduce oxidative stress and protein misfolding and thereby minimize neuronal damage. Despite several multitarget-directed ligands (MTDLs) are identified as promising anti-neurodegenerative agents, there is a dearth of clinically approved MTDLs for NDDs. Thus, there is a need to design novel MTDLs possessing potential dual MAO-B/AChE inhibitory profiles including iron chelation and antioxidant properties. Ligand-guided pharmacophore-based design is one of the emerging approaches for the design of novel MTDLs against complex neurological disorders. In the current study, we employed pharmacophore-based virtual screening coupled with ligand-guided lead optimization techniques to identify and develop new MTDLs as anti-neurodegenerative agents.

Our initial objective was to identify a novel MAO-B inhibitory chemical scaffold by employing pharmacophore-based virtual screening (PBVS) and assess its multifunctional properties in *in vitro* biochemical and *in vivo* pharmacological models. Utilizing a pharmacophore developed from the known MAO-B inhibitor safinamide, we identified a novel benzothiazole-based thioacetamide. For further validation and SAR exploration, we rationally designed and synthesized a set of benzothiazole-derived thioacetamides (**ZBTI01**-**ZBTI14**) and investigated

their MAO and AChE inhibition properties. Among all screened molecules, compound **ZBTI13** emerged as the lead dual MAO-B (IC_{50} = 0.015 ± 0.007 µM) and AChE (IC_{50} = 0.114 ± 0.003 µM) inhibitor that blocked these enzymes in a competitive-reversible and mixed-reversible mode, respectively. Selected compounds have displayed iron-chelation and antioxidant properties. Further, computational assessment of ligand binding affinity and pharmacokinetic parameters of all new compounds and molecular dynamic simulation of compound **ZBTI13** with MAO-B and AChE were carried out to understand ligand efficiency, pharmacokinetic, and virtual molecular interaction profile, respectively. The cytotoxicity studies of the lead dual MAO-B/AChE inhibitor **ZBTI13** against fibroblast (L929) and neuroblastoma (SH-SY5Y) cells revealed that compound **ZBTI13** was non-toxic up to 10 µM and 50 µM against tested cells, respectively. Furthermore, compound **ZBTI13** displayed neuroprotection against dexamethasone-induced oxidative stress in neuroblastoma cells. Additionally, compound **ZBTI13** showed *in vivo* neuroprotection against scopolamine-induced amnesia and was non-toxic to adult female Albino mice with no histopathological toxicity up to a dose of 2000 mg/kg (as per OECD 423).

Inspired from the in-house MTDL (Compound **4i**, Kumar *et al.*, 2022), we attempted to exploit the sesamol scaffold for developing MTDLs possessing multifunctional properties including antioxidant and iron chelation through lead optimization. Thus, a set of O-acetamides and O-alkyl carbohydrazones was designed via linker modification and evaluated their multifunctional profile. The designed sesamol-derived O-acetamides (**SMA01-11**) and O-alkyl carbohydrazones (**SMH01-17**) were synthesized, and evaluated *in vitro* for their dual MAO and AChE inhibition properties. The 3,4-dichloro derivative (compound **SMA08**) was the most potent MAO-A inhibitor (IC_{50} = 0.053 ± 0.001 µM) while the 4-methyl analog (compound **SMA05**) emerged as a lead MAO-B inhibitor (IC_{50} = 0.019 ± 0.001 µM) compared to the reference inhibitors clorgyline (MAO-A IC_{50} = 0.096 ± 0.003 µM) and selegiline (MAO B IC_{50} = 0.021± 0.002 µM). For the SMH series compound, **SMH07** was identified as the most potent MAO-A inhibitor ((MAO-A IC_{50} = 0.076 ± 0.009 µM) while **SMH06** was a potent MAO-B inhibitor (MAO B IC_{50}

= 0.064 ± 0.003 µM). Further, the 2,4-dichloro analog (compound **SMA09**) emerged as a potent dual MAO (MAO-A IC_{50} = 0.160 ± 0.009 µM, MAO-B IC_{50} = 0.071 ± 0.002 µM) and ChE (AChE IC_{50} = 2.611 ± 0.086 µM and BChE IC_{50} = 4.22 ± 0.162 µM) inhibitor in competitive-irreversible mode. 2,6-dichloro analog Compound **SMH06** emerged as a dual MAO-B-AChE inhibitor (MAO-A IC_{50} = 0.076 ± 0.009 µM, and MAO B IC_{50} = 0.064 ± 0.003 µM) in a mixed-reversible mode. Selected MAO-AChE inhibitors of **SMA** and **SMH** series showed significant antioxidant and iron-chelation properties, suggesting their potential in treating neurological disorders characterized by impaired iron homeostasis. Further, cell-based toxicity against fibroblast (L929) and neuroblastoma cells revealed that the compound **SMA09** was non-toxic up to 50 µM against both cells while compound **SMH06** was non-toxic to fibroblast cells up to 50 µM and against neuroblastoma up to 10 µM. Compounds **SMA09** & **SMH06** both displayed neuroprotection against dexamethasone-induced oxidative stress in the neuroblastoma (SH-SY5Y) cell line. Moreover, compound **SMA09** showed *in vivo* neuroprotection against scopolamine-induced amnesia and was non-toxic to adult female Albino mice with no histopathological toxicity up to a dose of 2000 mg/kg (as per OECD 423).

Further, as a part of lead optimization, ring modification strategy was attempted wherein the sesamol moiety of the identified **SMH** lead (**SMH06**) is replaced with eugenol or isoeugenol which afforded another set of carbohydrazones (**SEH** & **SIEH** series). Accordingly, a library of 22 carbohydrazones derived from eugenol and isoeugenol was synthesized and evaluated for their dual MAO and AChE inhibition properties. The *in vitro* enzyme inhibition studies of compounds **SEH01**-**SEH11** & **SIEH01**-**SIEH11** resulted in the potency of compounds micromolar to nanomolar concentration against MAOs and ChEs. Compound **SEH06** was identified as the most potent MAO-A (IC_{50} = 0.053 ± 0.012 µM) inhibitor whereas compound **SIEH06** emerged as the most potent MAO-B (IC_{50} = 0.024 ± 0.011 µM) inhibitor among tested compounds. Compound **SIEH10** was identified as a potent and selective MAO-B inhibitor over MAO-A. Further, enzyme kinetics and time-dependent reversibility studies of compound **SIEH10** against MAO-B and AChE revealed competitive irreversible and mixed reversible

inhibition, respectively. Additionally, a free radical scavenging assay of selected compounds was carried out in which compound **SIEH10** emerged as a potent antioxidant with ~75 % free radical scavenging activity. Iron chelation study of compounds **SEH05**, **SIEH06** and **SIEH10** were found as iron chelator. Moreover, the cell-based toxicity studies of compound **SIEH10** against fibroblast (L929) and neuroblastoma (SH-SY5Y) cells revealed that compound **SIEH10** was non-toxic up to 10 μM and toxic against tested cells, respectively. Furthermore, compound **SIEH10** showed neuroprotection against dexamethasone-induced oxidative stress in neuroblastoma cells but unfortunately, it didn't show *in vivo* neuroprotection.

In the first series (ZBTI), SAR exploration of the identified virtual lead (**ZINC02181408**; 2,6-dimethyl analog) produced an isomeric 3,4-dimethyl analog (**ZBTI13**) as a dual MAO-B/AChE inhibitor with significant *in vitro* and *in vivo* neuroprotection and safely profile in animal model. Subsequently, in the second series **(SMA & SMH)** the attempted linker modification on known scaffold significantly enhanced the MAO-B inhibition activity, drastically decreased AChE potency, and produced highly CNS-penetrant MTDLs (**SMA09** & **SMH06**) with significant in vivo neuroprotection activity. Further, in the third series (**SEH & SIEH**) the attempted ring modification on lead (**SMH06**) obtained in previous series significantly enhanced the MAO-B-AChE inhibition activity, produced a lead brain penetrant MTDL (**SIEH10**) with strong antioxidant and *in cellulo* neuroprotective properties. In summary, we applied pharmacophore-based virtual screening followed by ligand-guided lead optimization techniques to identify and develop new MTDLs as multifunctional agents for treating NDDs. The study produced promising MTDLs possessing benzothiazole, sesamol and eugenol scaffolds (**ZBTI13**, **SMA09**/**SMH06** and **SIEH10**, respectively) as potential dual MAO-B/AChE inhibitors with significant *in vitro* neuroprotective efficacy and/or *in vivo* safety profile.