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REVIEW

A Novel Neuroregenerative Approach Using ET_B Receptor Agonist, IRL-1620, to Treat CNS Disorders

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Summary

Endothelin B (ET_B) receptors present in abundance the central nervous system (CNS) have been shown to have significant implications in its development and neurogenesis. We have targeted ET_B receptors stimulation using a highly specific agonist, IRL-1620, to treat CNS disorders. In a rat model of cerebral ischemia intravenous administration IRL-1620 significantly reduced infarct volume and improved neurological and motor functions compared to control. This improvement, in part, is due to an increase in neuroregeneration. We also investigated the role of IRL-1620 in animal models of Alzheimer's disease (AD). IRL-1620 improved learning and memory, reduced oxidative stress and increased VEGF and NGF in Aβ treated rats. IRL-1620 also improved learning and memory in an aged APP/PS1 transgenic mouse model of AD. These promising findings prompted us to initiate human studies. Successful chemistry, manufacturing and control along with mice, rat and dog toxicological studies led to completion of a human Phase I study in healthy volunteers. We found that a dose of 0.6 µg/kg of IRL-1620 can be safely administered, three times every four hours, without any adverse effect. A Phase II clinical study with IRL-1620 has been initiated in patients with cerebral ischemia and mild to moderate AD.

Key words

Endothelin • ET_B receptors • Neuroregeneration • Alzheimer's disease • Ischemic stroke • Amyotrophic lateral sclerosis

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Introduction

Endothelin (ET), an endogenous 21 amino acid peptide, was first isolated from porcine aortic endothelial cells nearly 3 decades ago (Yanagisawa *et al.* 1988). There are 3 distinct isopeptides: ET-1, ET-2, and ET-3 which are present in various mammalian tissues performing a myriad of physiological and pathological roles such as regulation of blood pressure and perfusion, apoptosis and cellular proliferation and migration (Ehrenreich *et al.* 2000, Inoue *et al.* 1989, Vidovic *et al.* 2008, Yanagisawa *et al.* 1988). The ET peptides produce their biological effects through activation of G-protein-coupled receptors: ET_A and ET_B (Arai *et al.* 1990). Initial studies suggested two subtypes of ET_B receptors in the brain; ET_{B1} receptors with super high affinity and ET_{B2} receptors with high affinity binding to ET ligands (Sokolovsky *et al.* 1992). Subsequently, it was suggested that there are two subtypes of ET_B receptors; ET_{B1} which are IRL-1620 sensitive and ET_{B2} which are IRL-1620 insensitive receptors (Brooks *et al.* 1995). While assessing the role of endogenous ET it was reported that there are two subtypes of ET_B receptors; RES-701 sensitive mediating vasodilation ET_{B1} receptors and RES-701 insensitive mediating vasoconstriction ET_{B2} receptors (Gellai *et al.* 1996, Miasiro *et al.* 1998). However, ET_B receptor subtypes have never been cloned and are not recognized as receptors (Davenport 2002) and no further Family A GPCRs have been identified that might bind ET peptides (Davenport *et al.* 2016).

However, ET-1 and its receptors are not limited to the vascular system. Indeed, high concentrations of ET-1

are made by neurons, astrocytes and glial cells in the central nervous system (CNS) (MacCumber *et al.* 1990). ET_A and ET_B receptors in the CNS are important regulators of homeostatic conditions – regulating the sympathetic nervous system and cerebral blood flow (CBF) as well as neuronal migration, proliferation and apoptosis (Ehrenreich *et al.* 2000, Gulati *et al.* 1992, Gulati and Srimal 1993, Vidovic *et al.* 2008). The development and use of selective and non-selective agonists and antagonists for the ET_A and/or ET_B receptor has allowed researchers to delineate the actions of these receptors with regards to CNS development, pathogenesis and repair.

IRL-1620 [N-Succinyl-[Glu9, Ala11,15] endothelin 1] is a synthetic analog of ET-1 which was synthesized in 1992 (Takai *et al.* 1992). The names PMZ-1620, SPI-1620 and IRL-1620 are synonyms with amino acid sequence Suc-Asp-Glu-Glu-Ala-Val-Tyr-Phe-Ala-His-Leu-Asp-Ile-Ile-Trp, molecular weight of 1821.9 and molecular formula $C_{86}H_{117}N_{17}O_{27}$; CAS # 142569-99-1. Different companies have code named the same compound, SPI-1620 by Spectrum Pharmaceuticals, Inc. and PMZ-1620 by Pharmazz, Inc.; they both are IRL-1620 (originally made by Ciba, Japan). IRL-1620 is a potent and specific agonist for the ET_B receptors, with K_i values for ET_A and ET_B receptors of 1.9 μM and 16 pM, respectively, making it ~120,000 times more selective for ET_B over ET_A receptors. Since its synthesis, IRL-1620 has been used in numerous studies to determine the biological actions of ET_B receptors in the pulmonary, hepatic, renal, gastrointestinal, dermatological and endocrine systems (Bauer *et al.* 2000, Fellner and Arendshorst 2007, Khan *et al.* 2006, Lawrence *et al.* 1995, Mathison and Israel 1998, Mazzoni *et al.* 1999). We and others have used IRL-1620 to determine the role of ET_B receptors in the CNS (Briyal *et al.* 2015, Briyal *et al.* 2014, Gulati *et al.* 1997, Gulati *et al.* 1996, Gulati *et al.* 1995, Gulati *et al.* 2017b, Kaundal *et al.* 2012, Leonard *et al.* 2011, Leonard *et al.* 2012, Leonard and Gulati 2013, Leonard *et al.* 2015) which are present in high concentrations (Druckenbrod *et al.* 2008, Hostenbach *et al.* 2016, MacCumber *et al.* 1990, Schinelli 2006). The human brain contains a high density of ET receptors (Schinelli 2006), with ET_B accounting for 90 % of total ET receptors in the cerebral cortex (Harland *et al.* 1995), localized to neuronal regions. The relative expression of ET_B receptors was found to be highest in the human cerebellum, brainstem, hypothalamus, cerebral cortex, hippocampus, striatum, olfactory bulb and lungs (Davenport *et al.* 2016). ET_B receptors were not detected in the vascular structures or leptomeninges (Davenport *et al.* 2016). We have found that IRL-1620 promotes neuronal cell proliferation (Gulati 2016) and the location of neuronal stem cells are predominantly in the subventricular zone (SVZ), lining the wall of the lateral ventricles, hippocampal dentate gyrus (Eriksson *et al.* 1998, Kuhn *et al.* 1996) and spinal cord (Barnabe-Heider *et al.* 2010) of the adult CNS. In addition, a direct contact between endothelial cells and neuronal stem cells lining the ventricles is critical for maintaining the stemness (Ottone *et al.* 2014). Considering these factors, it appears that the main site of action of IRL-1620 for neurogenesis will be the lining of the walls of the cerebral ventricles.

While numerous studies have been performed to determine the role of central ET_A receptors by pharmacologically stimulating and blocking these receptors, however, the role of central ET_B receptors has largely been ignored and only few studies have been conducted to explore the function of these receptors. The present review will focus on studies that bring insight into the functionality of ET_B receptors in the CNS. Studies have been carried out to determine the role of ET_B receptors in the development of CNS and effect of stimulating ET_B receptors *via* selective agonist, IRL-1620, in animal models of cerebral ischemic stroke, Alzheimer's disease (AD) and hypoxic ischemic encephalopathy. This review highlights the potential for utilizing central ET_B receptors as a target for the treatment of CNS disorders.

Endothelin B receptors in the developing CNS

ET_B receptors are known to be an essential component of the developing nervous system. During both pre- and post-natal development, ET_B receptors help to regulate the differentiation, proliferation and migration of neurons, melanocytes and glia of both the enteric and central nervous systems (Druckenbrod *et al.* 2008). Serious or even fatal birth defects have been associated with pre-natal disturbances in ET_B receptor expression or function. The rodent ET_B receptor knockout model, which leads to mortality within 4 weeks of birth, is characterized by craniofacial malformation and congenital aganglionosis in the gastrointestinal tract as well as alterations in neuronal and glial cells (Dembowski *et al.* 2000). Within the CNS, these ET_B deficient rats show high levels of ET-1 with the cerebrovasculature demonstrating an enhanced constrictor response, along with an increase in apoptosis and a distinct decrease in the number of neural progenitor cells (Ehrenreich *et al.* 2000, Ehrenreich *et al.* 1999, Vidovic *et al.* 2008). In the

early human embryo, ET_B mRNA expression is limited to the neural tube, sensory and sympathetic ganglia and endothelium (Brand *et al.* 1998). While these studies demonstrate the actions and importance of ET_B receptors in the pre-natal CNS, little was previously known about the role of ET_B in post-natal CNS development.

Post-natal rat brain ET_B receptor expression studies conducted in our laboratory have shown that ET_B expression decreases by 72 % in the normal rat brain from post-natal day 1 to 28 (Puppala *et al.* 2015). Specifically, there was a significant decrease in ET_B expression in the cerebral cortex and SVZ by the 7^{th} and 14^{th} days of life, while expression within the cerebrovasculature increased. These results suggest that ET_B receptors are involved in the structural maturity and development of the CNS during post-natal period, after which the requirement of ET_B receptors diminishes leading to decreased expression in the CNS. Indeed, a further study found that the decrease in ET_B expression in the neuronal tissue coincided with a similar decrease in nerve growth factor (NGF), while administration of IRL-1620 on post-natal day 21 resulted in a significant increase in both ET_B and vascular endothelial growth factor (VEGF) in the cerebrovasculature (Leonard *et al.* 2015).

The findings that ET_B receptor stimulation is necessary for pre- and post-natal development and can influence growth factors like VEGF and NGF indicated that these receptors could serve as a potential target for neurovascular remodeling in the adult CNS as well. The endogenous neurorestorative processes within the adult brain attempt to repair damage due to disease, trauma or hypoxia by initiating neurogenesis, angiogenesis and oligodendrogenesis. It is possible that pharmacological interventions such as IRL-1620-induced stimulation of ET_B receptors can enhance these innate processes to improve neurovascular repair and remodeling or neuroregeneration.

Targeting ET_B receptors for cerebral ischemia

Just as neurogenesis occurs throughout brain development, it has come to light in recent years that neurogenic niches are present in the adult brain. These areas of neuronal progenitor cells in the adult brain, notably the dentate gyrus and SVZ, continue to form new neurons throughout life, often helping to repair and restore function in the case of CNS disease or trauma (Eriksson *et al.* 1998, Spalding *et al.* 2013). The National Institute of Neurological Disorders and Stroke as well as the Stroke Therapy Academic Industry Roundtable have identified neuroplasticity and neuronal repair as targets for novel therapies to treat ischemic stroke and other neurodegenerative diseases (Albers *et al.* 2011, Fisher *et al.* 2009). Stroke, with ~800,000 every year in the U.S., 3 out of 4 of which are first-time infarcts, is the fifth leading cause of death along with being one of the most prevalent causes of long-term disability worldwide. Despite the fact that over 85 % of strokes are of the ischemic classification, there exists only one FDA-approved pharmacological treatment for the disease, rtPA, which is limited by a short therapeutic time window and a risk of hemorrhagic transformation (Benjamin *et al.* 2017). As a result of the severity and complexity of the disease, a large number of clinical trials are currently underway for the treatment of ischemic stroke (Table 1) focusing on a range of mechanisms from restoration of blood flow to neuroprotection to neuroregeneration.

Shortly after the discovery of ET and its cardiovascular properties, the potential implications of this endogenous system in ischemic stroke were noted and identified as possible targets for novel therapeutic interventions. Levels of ET and ET immunoreactivity were found to be elevated in the CNS and blood following both ischemic and hemorrhagic stroke (Viossat *et al.* 1993). Due to ET's constriction of cerebral arteries, the elevated levels of circulating ET-1 adversely restrict regional CBF further exacerbating neuronal injury and other ischemic damage. Indeed, due to its potent vasoconstrictor properties, high concentrations of ET-1 have been historically utilized as a model for inducing ischemic stroke in animals (Reid *et al.* 1995). It should be noted that while the concentrations of ET-1 used to induce ischemia in this model are much higher than either physiological or pathological concentrations found within the body, high levels of ET in the damaged brain have been implicated widely ranging from delayed hypoperfusion to excitotoxicity, blood brain barrier (BBB) disruption and edema to inflammation (Kaundal *et al.* 2012). Initial studies targeting this pathologic elevation in ET following stroke focused on antagonizing the ET_A receptors. While selective antagonism of ET_A receptors showed promise pre-clinically in reducing infarct volume and neurological deficit (Barone *et al.* 2000, Briyal and Gulati 2010), this target proved unsuccessful in clinical trials (Kohan *et al.* 2012). Conversely, deficiency or antagonism of ET_B receptors exacerbates ischemic injury, leading to poorer outcomes (Chuquet *et al.* 2002, Ehrenreich *et al.* 1999), indicating that functional ET_B receptors may play a critical role in

recovery from cerebral ischemia. Given the importance of functional ET_B receptors in proper CNS development as well as the fact that stimulation of these receptors appears to enhance neuroregenerative growth factors, it was of interest to examine the effects of selective ET_B receptor stimulation in an adult rat model of cerebral ischemia.

Table 1. Current clinical trials for the treatment of cerebral ischemia (as of 11/17/2017 according to clinicaltrials.gov) arranged according their mechanism of action.

Agent	Mechanism of Action	Clinical Trial Identifier	Sponsor
Clopidogrel	Anticoagulant	NCT02776540	Ain Shams University
		NCT00991029	University of California, San Francisco
Cilostazol	Anticoagulant	NCT01013532	Asan Medical Center
		NCT02483169	
Tenecteplase	Thrombolysis	NCT03181360	University Hospital of North Norway
		NCT02388061	Neuroscience Trials Australia
		NCT02101606	University of Alberta
TF0023	Thrombolysis	NCT02785120	Techfields Inc.
DLBS1033	Thrombolysis	NCT02133521	Dexa Medica Group
DS-1040b	Thrombolysis	NCT02586233	Daiichi Sankyo, Inc.
3K3A-APC	Neuroprotection Anticoagulant	NCT02222714	ZZ Biotech, LLC
Allopurinol	Neuroprotection	NCT02122718	NHS Greater Glasgow and Clyde
Tocotrienol	Neuroprotection	NCT02263924	Seberang Jaya Clinical Research Center
SP-8203	Neuroprotection	NCT02787278	Shin Poong Pharmaceutical Co. Ltd.
Natalizumab	Neuroprotection	NCT02730455	Biogen
JPI-289	Neuroprotection	NCT03062397	Jeil Pharmaceutical Co., Ltd.
Minocycline	Neuroprotection	NCT03320018	Stony Brook University
HT-3951	Neuroprotection Neuroregeneration	NCT02530307	Dart NeuroScience, LLC
Atorvastatin	Neuroprotection	NCT02452502	Zhejiang University
	Neuroregeneration	NCT02458755	Samsung Medical Center
Autologous bone marrow mononuclear cells	Neuroregeneration	NCT02178657	Andalusian Initiative for Advanced Therapies
HT047	Neuroregeneration	NCT02828540	Hocheol Kim, Kyunghee University
Allogenic mesenchymal stem cells from adipose tissue	Neuroregeneration	NCT01678534	Instituto de Investigacion Hospital Universitario La Paz
SB623 (modified stem cells)	Neuroregeneration	NCT02448641	SanBio, Inc.
Fluoxetine	Neuroregeneration	NCT02767999	University Hospital, Toulouse
		NCT02737930	Bogachan Sahin, University of Rochester
Allogenic umbilical cord blood	Neuroregeneration	NCT03004976	Joanne Kurtzberg, MD

In adult rats subjected to permanent middle cerebral artery occlusion (pMCAO), intravenous treatment with 5 µg/kg IRL-1620 at 2, 4 and 6 h post-insult resulted in a significant recovery in neurological and motor function. Coinciding with this functional improvement, infarct volumes in IRL-1620 treated animals were significantly reduced (24.47±4.37 and 54.06±14.12 mm^3) for a 84.0 % and 69.5 % improvement over vehicle at 24 h and 7 days post-pMCAO, respectively (Leonard *et al.* 2011, Leonard *et al.* 2012). Similarly, in co-morbid Type II diabetic animals, IRL-1620 treatment lead to 69.4 % reduction in infarct volume as compared to diabetic

animals treated with vehicle. These improvements were blocked when animals were administered ET_B receptor antagonist, BQ788, thus confirming that the effects were due selective stimulation of the ET_B receptors by IRL-1620. Additionally, treatment with IRL-1620 led to a recovery of CBF back to pre-occlusion baseline by day 7 following pMCAO along with a significant reduction in pro-apoptotic protein Bax ($p<0.01$) and an increase in anti-apoptotic protein Bcl-2 ($p<0.01$) as compared to vehicle-treated animals (Gulati *et al.* 2017a). The anti-apoptotic effect was confirmed, with an 80.4 % decrease in TUNEL-positive cells within the ischemic hemisphere of IRL-1620-treated pMCAO rats when compared to vehicle (Gulati *et al.* 2017a).

Neuroregeneration following CNS injury has recently become a focal point for various interventions. Stem cell therapy, transcranial current stimulation, and a number of pharmacologic treatments including exogenous growth factors and hormones as well as anti-depressants have been investigated in experimental stroke models for their ability to stimulate the endogenous neurogenic niche (Abeysinghe *et al.* 2015, Braun *et al.* 2016, Guan *et al.* 2012, Khan *et al.* 2015, Kim *et al.* 2016, Shen *et al.* 2016). While neuroprotective effects such as reductions in oxidative stress and apoptosis were noted along with the decreased infarct volume (Leonard *et al.* 2011, Leonard *et al.* 2012) in pMCAO animals treated with IRL-1620, we determined to further investigate the impact of selective stimulation of ET_B receptors on neurogenic and angiogenic markers following cerebral ischemia in order to examine whether or not neuroregeneration was affected by this novel therapy.

IRL-1620 treatment post-pMCAO significantly increased expression of VEGF and NGF as determined by both Western blot and immunofluorescent techniques. VEGF-positive vessels per 30 µm brain slice increased 170 %, while NGF-positive cells increased 281 % in the SVZ in IRL-1620-treated animals as compared to vehicle. Proliferating cells, as determined by BrdU staining, increased 146 %, 229 % and 219 % in the cortex, striatum and SVZ, respectively, in IRL-1620-treated animals (Leonard and Gulati 2013). Selective stimulation of ET_B receptors *via* IRL-1620, therefore, appears to significantly enhance neurovascular repair following cerebral ischemia by enhancing the production of growth factors and increasing the number of proliferating cells within the CNS.

Preclinical studies using a rat model of pMCAO for cerebral ischemia indicate that selective stimulation of ET_B receptors *via* its agonist, IRL-1620, is highly beneficial. In addition to neuroprotection as evidenced by significantly decreased infarct volumes, reduced oxidative stress and apoptosis, IRL-1620 treatment appears to enhance neuroregeneration through increases in angiogenic and neurogenic growth factors (Fig. 1). Given these promising preclinical findings, it was important to establish the safety, tolerability and pharmacodynamics of IRL-1620 in healthy human volunteers. It was also of interest to determine whether or not such selective ET_B receptor stimulation could produce similar results in other neurodegenerative diseases such as Alzheimer's disease.

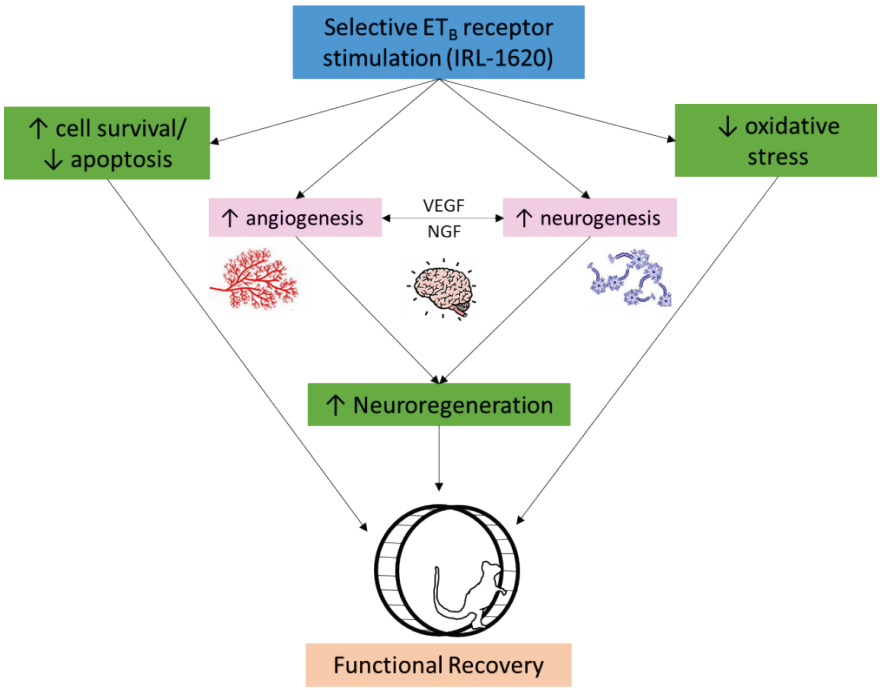

Fig. 1. Effect of ET_B receptor stimulation by agonist, IRL-1620, on neuroprotection and neuroregeneration in pre-clinical neurodegenerative models. IRL-1620 decreases oxidative stress and apoptosis leading to increased cell survival. IRL-1620 also increases vascular endothelial growth factor (VEGF) and nerve growth factor (NGF), which, in turn, enhance neuroregeneration and, ultimately, functional recovery.

Table 2. Current clinical trials for the treatment of Alzheimer's disease (as of 11/17/2017 according to clinicaltrials.gov) arranged according their mechanism of action.

Agent	Mechanism of Action	Clinical Trial Identifier	Sponsor
AD-SVF cells	Regenerative: AD-SVF cell infusion	NCT02912169	Ageless Regenerative Institute
hUCB-MSCs	Regenerative: Stem cell therapy	NCT02054208	Medipost
Allopregnanolone injection	Regenerative: GABA receptor modulator	NCT02221622	University of Southern California, NIA
hMSCs	Regenerative: Stem cell therapy	NCT02600130	Longeveron LLC
Atomoxetine	Anti-amyloid: Adrenergic uptake inhibitor SNRI	NCT01522404	Emory University, NIA
AZD0530 (saracatinib)	Anti-amyloid: Kinase inhibitor	NCT02167256	Yale University, ATRI, AstraZeneca
BAN2401	Anti-amyloid: Monoclonal antibody	NCT01767311	Eisai
Crenezumab	Anti-amyloid Monoclonal antibody	NCT01998841	Genentech, NIA, Banner Alzheimer's Institute
Crenezumab	Anti-amyloid: Monoclonal antibody	NCT02353598	Genentech
CT1812	Anti-amyloid: Sigma-2 receptor modulator	NCT02907567	Cognition Therapeutics
E2609	Anti-amyloid: BACE inhibitor	NCT02322021	Eisai, Biogen
LY3202626	Anti-amyloid: BACE Inhibitor	NCT02791191	Eli Lilly
PQ912	Anti-amyloid and anti-inflammatory: Glutaminyl-peptide cyclotransferase inhibitor	NCT02389413	Probiodrug AG, Julius Clinical, VU University Medical Center, Amsterdam
Sargramostim (GM-CSF)	Anti-amyloid: Granulocyte colony stimulator; amyloid removal	NCT01409915	University of Colorado, Denver, The Dana foundation
UB-311	Anti-amyloid: Monoclonal antibody	NCT02551809	United Neuroscience
Valacyclovir	Anti-amyloid: Antiviral agent	NCT02997982	Umea University
Lu AF20513	Anti-amyloid: Polyclonal antibody	NCT02388152	H. Lundbeck A/S
LY3002813	Anti-amyloid: Monoclonal antibody	NCT02624778	Eli Lilly and Company
LY3303560		NCT02754830	
MK-8931 (verubecestat)	Anti-amyloid: BACE Inhibitor	NCT02910739	Merck
NGP 555	Anti-amyloid: Gamma-secretase modulator	NCT02537938	NeuroGenetic Pharmaceuticals
PF-06751979	Anti-amyloid: Undisclosed mechanism	NCT02793232	Pfizer
BI409306	Neuroprotective: Phosphodiesterase 9A inhibitor	NCT02240693 NCT02337907	Boehringer Ingelheim
Cilostazol	Neuroprotective: Phosphodiesterase 3 antagonist	NCT02491268	National Cerebral and Cardiovascular Center, Japan
BI409306	Neurotransmitter based: Phosphodiesterase 9A inhibitor	NCT02392468	Boehringer Ingelheim
BPN14770	Neuroprotective: Negative allosteric modulator of phosphodiesterase 4D	NCT02840279 NCT02648672	Tetra Discovery Partners

ANAVEX 2-73	Neuroprotective: Sigma-1 receptor agonist	NCT02244541	Anavex Life Sciences
Candesartan blocker	Neuroprotective and anti-inflammatory: Angiotensin receptor	NCT02646982	Emory University
CPC-201	Neuroprotective: Cholinesterase inhibitor 1 peripheral cholinergic antagonist	NCT02549196	Chase Pharmaceuticals
Formoterol	Neuroprotective and anti-inflammatory: β-2 adrenergic receptor agonist	NCT02500784	Palo Alto Veterans Institute for Research, Mylan, Alzheimer's Association
Rasagiline	Neuroprotective: Monoamine oxidase B inhibitor	NCT02359552	The Cleveland Clinic
Riluzole	Neuroprotective: Glutamate receptor antagonist; glutamate release Inhibitor	NCT01703117	Rockefeller University
Simvastatin 1 L-Arginine 1 Tetrahydrobiopterin (SLAT)	Neuroprotective: HMG-CoA reductase inhibitor and antioxidant	NCT01439555	University of Massachusetts, Worcester
Telmisartan	Neuroprotective and anti-inflammatory:Angiotensin II receptor blocker, PPAR-gamma agonist	NCT02085265	Sunnybrook Health Sciences Centre, ADDF
Telmisartan	Neuroprotective and anti-inflammatory: Angiotensin II receptor blocker, PPAR-gamma agonist	NCT02471833	Emory University
DAOIB	Neurotransmitter based: NMDA enhancer	NCT02103673 NCT02239003	Chang Gung Memorial Hospital, Taiwan
Levetiracetam	Neurotransmitter: based Anticonvulsant	NCT02002819	University of California, San Francisco
Lithium	Neurotransmitter based: Ion channel modulator	NCT02129348	New York State Psychiatric Institute, NIA
Piromelatine	Neurotransmitter based: Melatonin receptor agonist; 5-HT 1A and 1D receptor agonist	NCT02615002	Neurim Pharmaceuticals
Pimavanserin	Neurotransmitter based: 5-HT2A inverse agonist	NCT02992132	Acadia
RVT-101	Neurotransmitter based: 5-HT6 antagonist	NCT02910102	Axovant Sciences
SUVN-502	Neurotransmitter based: 5-HT6 antagonist	NCT02580305	Suven Life Sciences
Bisnorcymserine (BNC)	Neurotransmitter based: Butyrylcholinesterase inhibitor	NCT01747213	NIA
HTL0009936	Neurotransmitter based: Muscarinic M1 receptor agonist	NCT02546310	Heptares Therapeutics
TAK-071	Neurotransmitter based: Muscarinic M1 receptor modulator	NCT02769065	Takeda

Targeting ET_B receptors for Alzheimer's disease

Alzheimer's disease is the sixth-leading cause of death in the United States. In the year 2017, an estimated 5.5 million Americans were diagnosed with AD. The numbers are projected to double by 2050. The cost in 2017 for all individuals with AD and other dementias is estimated at $259 billion, of which Medicare and Medicaid are expected to cover up to 67 percent. Presently, the only approved therapies for AD are the cholinesterase inhibitors and an N-methyl-D-aspartate receptor antagonist (Parsons *et al.* 2013). Existing drugs help mask the symptoms of AD, but do not treat the underlying disease or delay its progression. During the 2002 to 2012 observation period, 413 AD trials were performed with an overall success rate of 0.4 % (99.6 % failure) (Cummings *et al.* 2014). As it is now known that amyloid is deposited early during the course of the disease, even before clinical symptoms appear (Murphy and LeVine 2010), specifically targeting only amyloid in patients may not be enough to prevent neurodegeneration. As a result, current research is closely examining the repair process following biochemical cascades that promote cell survival and regeneration, re-networking of neuronal circuitry and functional recovery in hopes of developing new and more effective treatments. With the social, economic and human costs associated with AD on the rise, a large number of clinical trials are currently underway for the treatment and prevention of this disease, with 55 trials in Phase I, 95 trials in Phase II, and 48 trials in Phase III (Table 2). These clinical trials focus on a range of mechanisms such as anti-amyloid, anti-inflammatory, neuroprotective, and regenerative activity (Cummings *et al.* 2017).

Several studies have demonstrated the involvement of ET in AD (Haynes and Webb 1994, Palmer *et al.* 2012). Despite early reports of lower cerebrospinal fluid ET-1 concentrations in AD patients (Yoshizawa *et al.* 1992), follow-up studies indicate that ET-1 like immunoreactivity in the cortical region of AD patients' brains was significantly increased (Minami *et al.* 1995). ET-1 plays a central role in the regulation of cardiovascular functions and regional blood flow (Gulati *et al.* 1997, Gulati *et al.* 1996, Gulati *et al.* 1995) and may be part of the mechanism by which Aβ interferes with vascular function, as ET-1-induced vasoconstriction in middle cerebral and basilar arteries has been found to be enhanced following exposure to Aβ (Paris *et al.* 2003). Aβ exposure to SHSY5Y human neuroblastoma cells and human brain microvascular endothelial cells has been shown to upregulate ECE-1 and ECE-2, resulting in increased production and release of ET-1 (Palmer *et al.* 2009, Palmer *et al.* 2012, Palmer *et al.* 2013). ECE-1 has been found to assist in the clearance of Aβ by fragmentation of the peptide (Wang *et al.* 2006). ET-1 is rapidly cleared by ET_B receptors (Kelland *et al.* 2010), hence stimulation of these receptors by IRL-1620 could help to clear ET-1. Taken together, upregulation of ECE-1 due to Aβ and upregulation of ET_B receptors by IRL-1620 could help to clear Aβ and ET-1. We have previously demonstrated that selective ET_A receptor antagonists significantly reduced the Aβ-induced impairment in learning and memory as well as preventing oxidative stress (Briyal *et al.* 2011). Non-selective ET_A/ET_B antagonists, on the other hand, did not demonstrate any improvement in the Aβ-induced neurodegenerative changes. In light of this finding, it was of interest to investigate the specific role of ET_B receptors in Aβ-induced memory deficit. Our team found that intravenous administration of an ET_B receptor agonist, IRL-1620, increased CBF in normal rats (Leonard and Gulati 2009) and that the expression of anti-apoptotic marker, Bcl-2 was found to be increased, and pro-apoptotic marker, Bax was found to be decreased with liposomal IRL-1620 in neuronal PC-12 cells (Joshi *et al.* 2016), indicating that there may be a benefit to selectively stimulating ET_B receptors in an Aβ model. Moreover, functional ET_B receptors play a very important role in the CNS development, and stimulation of these receptors appears to enhance growth factors. Therefore, our aim was to investigate what effect ET_B receptor stimulation by IRL-1620 might have on cognitive impairment and oxidative stress in an animal model of AD.

Our findings suggested a beneficial effect of IRL-1620 on memory in Aβ-treated rats along with reduction in oxidative stress (Briyal *et al.* 2015, Briyal *et al.* 2014). We conducted behavioral studies using the Morris water maze to examine whether ET_B receptor agonist, IRL-1620, improves the memory deficit caused by Aβ. As expected, exposure to Aβ resulted in a significant impairment in spatial memory as evidenced by significantly longer escape latencies and no preference for the target quadrant. However, when the rats were administered specific ET_B receptor agonist, IRL-1620, the spatial memory deficit caused by Aβ treatment significantly improved (Briyal *et al.* 2014). Oxidative stress was significantly increased by Aβ exposure, while treatment with IRL-1620 significantly reduced this effect.

The positive results obtained with IRL-1620 were reversed by administering BQ788, an antagonist of the ET_B receptors.

Adult neurogenesis is one of the most important mechanisms contributing to brain development, learning, and memory. Alterations in neurogenesis underlie a wide spectrum of brain diseases (Pozhilenkova *et al.* 2017). In addition to reducing apoptosis and oxidative stress in neurodegenerative diseases like AD, the concept of enhancing innate neuroregeneration has recently become a target for developing new treatments. In order to determine the possible mechanism involved in IRL-1620 mediated neuroprotection following Aβ injection and to examine the possibility of a neuroregenerative effect, we further investigated the impact of ET_B receptor stimulation on VEGF, NGF and synapsin I. Our studies indicate that treatment with ET_B receptor agonist IRL-1620 produced an increase in NGF expression as well as NGF stained neurons in the rat brain suggesting that ET_B receptor stimulation might be augmenting neurogenesis *in vivo* in neurodegenerative diseases like AD (Fig. 1) (Briyal *et al.* 2015). Our preliminary data also indicate that treatment with ET_B receptor agonist IRL-1620 produced an increase in synapsin I expression in the rat brain, suggesting that the mechanism of functional recovery from memory deficit may also involve synaptogenesis (Ridgeway *et al.* 2017). Decreased expression of synapsin I protein has been characterized in Alzheimer's disease as part of the pathophysiology involved in disrupted cognition (Goetzl *et al.* 2016). There is evidence that phosphorylation of synapsin I functions in driving short-term neural plasticity (Giachello *et al.* 2010). These initial preclinical findings suggest that IRL-1620 may be a promising therapy for mild to moderate AD as it improves learning and memory while reducing oxidative stress and enhancing neuroregeneration. In addition to the experimental rat model, we also investigated the effect of IRL-1620 on memory deficit in APP/PS1 transgenic mice. This mouse model expresses mutant human amyloid precursor protein (APP) and presenilin protein 1 (PS1), causing AD-like amyloid plaque formation as early as 4 months of age while cognitive impairment becomes observable around 6 months of age (Faure *et al.* 2011, Kurt *et al.* 2001, Oakley *et al.* 2006). To 3 months old male APP/PS1 mice, we intravenously injected vehicle or IRL-1620 at a dose of 5 μg/kg, intravenously (IV) three times at 2 h intervals on days 1, 3 and 6 of every month until 6 months. As controls, we intravenously injected age-matched male wild-type (WT) mice with IRL-1620 or vehicle. We subjected these mice to the Morris water maze test 7 days after the last administration of IRL-1620 to assess alterations in learning and memory abilities. We found that APP/PS1 transgenic mice produced a significant impairment and IRL-1620 treatment significantly reduced (45 %) learning and memory deficit in 6 months aged transgenic mice (unpublished observation). These experiments are continuing, however, present results support the idea that IRL-1620 may be capable of significantly reducing the progressive neurodegeneration associated with AD.

Overall, our preclinical findings indicate that IRL-1620 administration results in a reduction of oxidative stress and an increase in growth factors suggesting enhanced neuroregeneration, ultimately leading to improved functional recovery in AD (Fig. 1). Combined with the results from our preclinical ischemic stroke studies, this data further encouraged us to initiate clinical safety and tolerability studies on IRL-1620 for the possible future use of this novel therapy in a variety of neurodegenerative disorders.

Targeting ET_B receptors for other CNS disorders

Traumatic brain injury

Traumatic brain injuries (TBIs), ranging from mild to severe, are defined as an impact or trauma to the head which results in disruption of normal brain function. TBI, which results in ~2.5 million emergency room visits per year in the United States, can lead to impaired memory, movement, sensation, and/or emotional functioning, lasting anywhere from a few days to a lifetime (Taylor *et al.* 2017). While diffuse axonal injury and direct vascular disruption occur immediately following TBI, many of the long-term neurodisabilities are a result of secondary injuries affecting metabolism, edema and dysfunctional autoregulation of the CBF (Graves and Kreipke 2015).

Disruption of CBF autoregulation may be explained, at least in part, by an increase in ET-1 concentration within the CSF following TBI, a phenomenon which has been noted in both human and animal models (Armstead and Kreipke 2011). An increase of ET-1, leading to vasoconstriction and decreased CBF, ultimately results in poor cognitive outcome for TBI patients. Interestingly, while ET_A receptors appear elevated as early as 4 h post TBI, ET_B receptors also increase, but at a later time period – 24 to 48 h after TBI, when CBF regulation is returning to

normal levels (Dore-Duffy *et al.* 2011). As in the case of ischemic stroke, preclinical studies of ET_A specific antagonists have yielded some promising results in the treatment of TBI and restoration of cerebral blood flow. Nevertheless, the impact of selectively activating ET_B receptors, which have been shown to both increase CBF and ET-1 clearance, has not been investigated. It would be interesting to see if selective ET_B receptor stimulation not only improves CBF, but also has neuroregenerative effects which could, in turn, result in an improved long-term functional outcome for patients with TBI.

Spinal cord injury

There are ~17,000 new cases of spinal cord injury (SCI) every year in the United States, a majority of which will require long-term treatment and rehabilitation (National Spinal Cord Injury Statistical Center 2016). Similar to TBI, there are two stages of injury in SCI – a primary injury consisting of fracture or distortion of the spinal cord, damage to axons, blood vessels and neurons, and a secondary injury consisting of a strong immune response, oxidative stress and the generation of proteolytic enzymes (Beck *et al.* 2010). Despite years of belief to the contrary, it has now been demonstrated that neural progenitor stem cells are found in both white and grey matter and neurogenesis does take place in the mature spinal cord, just as it does the mature brain (Fiorelli *et al.* 2013, Yamamoto *et al.* 2001).

Our findings showing neuroregeneration and functional recovery following selective ET_B receptor stimulation in animal models of cerebral ischemia and Alzheimer's disease and the existence of stem cell niche in the spinal cord, encouraged us to explore the efficacy of IRL-1620 in an experimental model of SCI. Adult male Sprague-Dawley rats were subjected to a moderate spinal contusion of 150 kdyn at the thoracic level (T10) and treated with either saline or IRL-1620 (1, 3, or 5 μg/kg) intravenously at 2 h intervals on days 1, 3, and 6 post injury. Motor functions, as determined by Basso, Beattie, Bresnahan scale, significantly improved in the hind limb of rats treated with IRL-1620 as compared to vehicle-treated animals (unpublished observation). IRL-1620 treatment increased the expression of ET_B receptors and synapsin in the spinal cords of rats following SCI. These results indicate that IRL-1620 significantly improves hind limb motor functions following SCI which may be attributed to synaptic remodeling and/or neuroregeneration. Studies are in progress to further investigate the mechanism of action of IRL-1620.

Neonatal hypoxic-ischemic encephalopathy

Hypoxic ischemic encephalopathy (HIE) is defined as a dysfunctional brain disorder that has devastating clinical outcomes in the neonate, including mortality, cerebral palsy, seizure disorders, and neurodevelopmental disorders. The incidence of HIE as estimated from population and hospital based studies ranges from 1 to 8 per 1000 live births, with a mortality between 15-20 % and occurrence of neurodevelopmental disorders of ~25 % (Graham *et al.* 2008, Kurinczuk *et al.* 2010, Vannucci and Perlman 1997). Presently, the only proven treatment for HIE is therapeutic hypothermia, which lowers the metabolic rate. Although therapeutic hypothermia is the current standard treatment, a significant number of infants still die or suffer from disabilities related to HIE whether they receive or do not receive hypothermia treatment (Gluckman *et al.* 2005, Gunn *et al.* 1998, Higgins *et al.* 2011, Thoresen and Whitelaw 2000). Several adjuvant therapies to hypothermia are undergoing experimentation to improve survival and neurodevelopmental outcomes of newborns that develop HIE.

One such potential adjuvant or alternative therapy for neonatal HIE could be a selective ET_B receptor agonist such as IRL-1620. As previously mentioned, IRL-1620 has been shown to be both neuroprotective and neuroregenerative in adult models of cerebral ischemia (Leonard *et al.* 2012, Leonard and Gulati 2013). Additionally, stimulation of ET_B receptors in young, healthy rats was found to promote angiogenesis (Leonard *et al.* 2015). It would therefore be of interest to determine the efficacy of IRL-1620 in an animal model of neonatal HIE.

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by progressive loss of motor neurons (MNs) particularly in the cerebral cortex, brainstem and spinal cord (Shaw and Ince 1997). Recent evidence suggests that factors secreted by activated astrocytes might contribute to degeneration of MNs. Endothelin-1 is a vasoactive peptide produced by activated astrocytes and microglia and is involved in initiating and supporting reactive gliosis in neurodegenerative disorders. Evidence suggest that ET-1 plays a role in the pathophysiology ALS. ET-1 has been reported to be abundantly expressed in reactive astrocytes of the spinal cord of SOD1-G93A mice and ALS patients and exerts a toxic effect on cultured MNs (Barbeito *et al.* 2004, Ranno *et al.* 2014). Studies have

shown that ET-1 toxicity is not directly caused by oxidative stress or activation of cyclooxygenase-2 but requires the synthesis of nitric oxide and is mediated by a reduced activation of the phosphoinositide 3-kinase pathway in an *in vitro* model of mixed spinal cord cultures enriched with reactive astrocytes (D'Antoni *et al.* 2017). ALS progression is associated with the dysfunction of astrocytes, and earlier studies have shown that ET-1 influences a number of cellular pathways implicated in ALS progression. Recently, gene expression studies have shown that levels of ET-1 and ET_B receptors are elevated in patients with ALS (Ostrow 2015). This further suggests that ET-1 may contribute to MN death and corroborates the view that the modulation of ET-1 signaling might be a potential therapeutic target to slow down MN degeneration in ALS.

Multiple sclerosis

Multiple sclerosis (MS) is a demyelinating autoimmune disease of the CNS with possible involvement of vascular dysregulation secondary to endothelial dysfunction caused by destruction of the vessel wall (Compston and Coles 2008). Vascular dysregulation leads to excessive vasoconstriction or insufficient vasodilatation, resulting in vasospasm mediated by ET-1, a potent and long-lasting mediator (Yanagisawa *et al.* 1988). A number of studies found that CBF is globally impaired in early diagnosed relapsing-remitting MS and primary progressive MS, indicating that it is an integral part of the disease that is already present at the time of diagnosis (Adhya *et al.* 2006, D'Haeseleer *et al.* 2011, Law *et al.* 2004). Animal studies have shown that chronic hypoperfusion of the brain can lead to neurodegenerative changes, including axonal degeneration (Wakita *et al.* 2002). However, the underlying mechanism of progressive degeneration of axons, which is a primary determinant of long-term disability in MS, is not clear and treatment is lacking. Plasma levels of the potent vasoconstrictor ET-1 were found to be elevated in patients with MS (Haufschild *et al.* 2001). These findings demonstrate that reduced CBF in MS may mediated by ET-1, which is likely released in the cerebral circulation from reactive astrocytes. Therefore, restoring CBF by targeting the ET-1 system warrants further investigation as a potential new therapeutic option. It may be interesting to explore whether reduced CBF in MS can be reversed with an ET_B receptor agonist along with potential for regeneration of neurons to counter the degenerative changes in MS.

Parkinson's disease

Parkinson's disease (PD), a neurodegenerative disorder involving primarily dopaminergic neurons in the substantia nigra, is yet another CNS disorder which has a severe medical and social impact. Stress to the endoplasmic reticulum (ER) caused by disrupted Ca^{2+} homeostasis, glucose starvation, hypoxia, and oxidative stress can lead to cell death (Jain *et al.* 2012, Schinelli 2006). Unfolded or misfolded proteins that accumulate during conditions of ER stress can form aggregates in the ER, as well as the cytosol, which are highly toxic and are a key pathological factor in Parkinson's disease (Hetz and Mollereau 2014, Takalo *et al.* 2013). In addition to modulating the vascular tone, ET-1 is implicated in a plethora of different biochemical pathways, which include induction of oxidative stress responses and inflammation, as well as ER stress. The link between ET-1 and the induction of ER stress has been reported and studies have shown increased levels of ET-1 in PD (Jain 2013, Jain *et al.* 2012). Given the wide range of pathological processes that involve ET-1, further research is warranted on ET-1 in PD. It is hoped that current progress on ET-1-induced pathology in different diseases can be exploited to advance knowledge on the precise role of ET in PD. The potential for selective stimulation of ET_B receptors to both assist in clearance of excess ET-1 as well as possible neuroregeneration may be worth exploring in PD.

Clinical development of IRL-1620

The pharmacokinetics of IRL-1620 in humans has been determined in previous studies in cancer patients (NCT00613691; NCT01741155; NCT01773785) with the mean C_{max} ranging from 0.45±0.09 ng/ml to 3.73± 1.66 ng/ml following single intravenous administrations of 0.05 μg/kg and 0.41 μg/kg, respectively. The half-life ($T_{1/2}$) of IRL-1620 ranged from 4.38 min to 8.29 min at doses of 0.11 μg/kg and 0.29 μg/kg, IV, respectively (Reddy *et al.* 2013, Tolcher *et al.* 2011). A longer duration of action with a short half-life of IRL-1620 is possible because it has been suggested that ET-1 and its receptors form complexes that are internalized and continue to signal (Archer *et al.* 2017). Internalization of ET-1 and its receptor complex into caveolin-containing vesicles occurs within 10 min of ET-1 application (Bremnes *et al.* 2000, Chun *et al.* 1995) but continues to signal and provide effects that last for days (Bremnes *et al.* 2000).

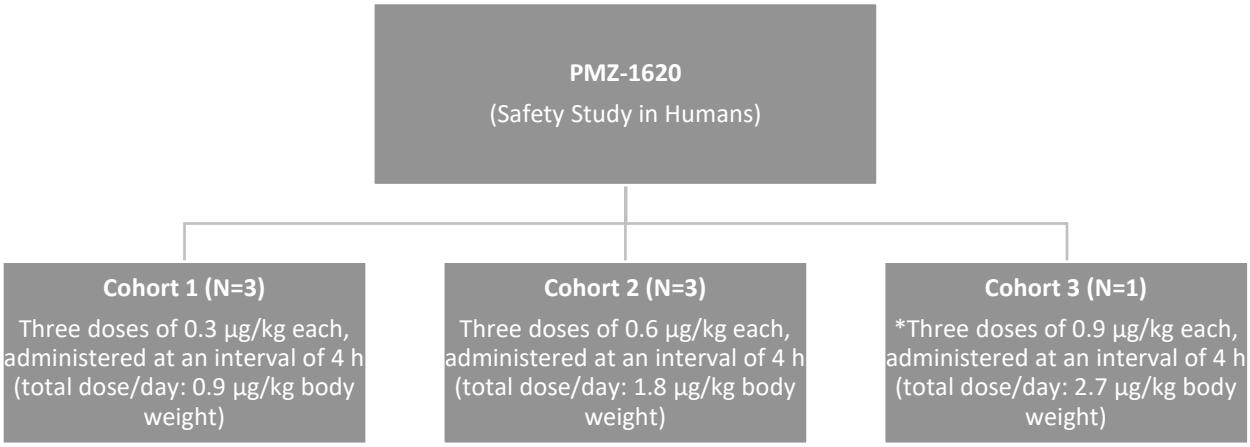

Fig. 2. Study design of Phase I clinical trial (CRTI/2016/11/007509) for the safety, tolerability and pharmacodynamics of multiple ascending doses of PMZ-1620 in healthy male volunteers. PMZ-1620 was administered as an intravenous bolus over 1 min. * The first subject of Cohort 3 experienced mild adverse events after administration of the first and second dose (0.9 µg/kg body weight). The events observed were mild, transient and resolved without any intervention. Further dosing in this cohort was stopped and the dose of 0.9 µg/kg was concluded as the Minimum Intolerable Dose (MID).

In view of our preclinical findings, we established the production of IRL-1620 (PMZ-1620) for human administration with successful chemistry, manufacturing and control studies. Following the completion of mice, rat and dog toxicological studies, we initiated an open label, Phase I study to determine the safety, tolerability and pharmacodynamics of multiple ascending doses of PMZ-1620 in healthy male volunteers (CTRI/2016/11/007509). The study was designed with 3 cohorts, each cohort having 3 subjects and each subject receiving 3 doses of either 0.3, 0.6 or 0.9 µg/kg administered at an interval of 4 h as an intravenous bolus over 1 min (Fig. 2). Therefore, in the first cohort each subject received a total dose of 0.9 µg/kg, in the second cohort each subject received a total dose of 1.8 µg/kg and in the third cohort each subject was to receive 2.7 µg/kg. However, a total of 4 non-serious adverse events (uneasiness, sweating, abdominal discomfort and vomiting) were reported by the first subject of cohort 3 after the first dose of 0.9 µg/kg of PMZ-1620. The adverse events resolved in about 10 min without any intervention. Four hours later a second dose of 0.9 µg/kg was given to the same subject and again uneasiness, sweating and abdominal discomfort were reported but there was no vomiting; all the events resolved within 10 min without any intervention. No further dosing was carried out and the study was concluded. The events observed at both doses were mild, transient and resolved without any sequelae. No subjects experienced serious adverse effects in any cohort. PMZ-1620 did not have any significant effect on vital signs, ECGs or laboratory parameters of the healthy male volunteers. PMZ-1620 was well tolerated and found safe when administered as multiple ascending doses in healthy subjects. The Minimum Intolerable Dose (MID) was established as 0.9 µg/kg and the Maximum Tolerated Dose (MTD) was 0.6 µg/kg. For Phase II, the proposed therapeutic dose of PMZ-1620 in patients with cerebral ischemia or Alzheimer's disease is 0.3 µg/kg, which is lower than the established MTD.

The convincing results of both preclinical efficacy studies and Phase I human safety and tolerability studies have encouraged us to further investigate the safety and efficacy of PMZ-1620 in two human Phase II studies, one in patients with cerebral ischemia, and the other in patients suffering from mild to moderate Alzheimer's disease.

Conflict of Interest

Dr. Gulati has a pending patent and Dr. Lavhale is presently employed by Pharmazz, Inc. having rights to pending patent.

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