Summary

"Unlocking the therapeutic potential of repaglinide for the management of metabolic syndrome linked Alzheimer's Disease"

Background

Metabolic syndrome (MetS) is a condition which includes clusters of metabolic disorders like glucose intolerance, hypertension, dyslipidemia, obesity, insulin resistance, and that together raise the risk of coronary heart disease, diabetes, and stroke. Among all, type 2 DM (T2DM) is the most common cause of mortality and morbidity caused by MetS. T2DM is prominently characterized by hyperglycemia due to insulin resistance (IR). IR is mediated by reduced insulin receptor expression and dysregulation of signaling cascades such as phosphoinositide 3-kinase (PI3K)/ Glycogen Synthase Kinase-3β (GSK-3β). In the recent past, MetS abnormalities linked to progressive brain insulin resistance (BIR) with consequent impairment of central insulin signaling processes, accumulation of neurotoxins, neuronal stress, and resulting in a progressive course of neurodegeneration is also termed Type 3 DM. The dysregulated PI3K/GSK-3β in the brain can increase the production and secretion of amyloid-beta (Aβ) and generates hyperphosphorylated tau which prompts neurodegenerative disorders such as Alzheimer's (AD).

Recently, comorbid conditions including DM linked with AD are mounting at an alarming rate and has become a major health issue in developing and developed countries. Although, several treatment options are available for T2DM management which can temporarily delay or reduce the symptoms of the disease including the classes of drugs- Dipeptidyl Peptidase-4 (DPP-4) inhibitors, Sodium-glucose Cotransporter-2 (SGLT2) inhibitors, etc. but they are effectively unable to control the diabetic complications and co-morbidities. However, recent pre-clinical and clinical studies have indicated the benefits of the meglitinides class of drugs not only in DM but also in comorbid conditions like AD and Huntington's disease (HD). Repaglinide (REP) a meglitinides class drug is believed to act by targeting ATP-binding cassettes and stimulating the release of insulin from the β-cells of the pancreas. REP exerted a neuroprotective effect against kainic acid-induced neuronal cell death in the CA3 region of hippocampal and rotenone-induced Parkinson's disease. Further, recent studies have reported that REP exerted a strong neuroprotective effect in HD which may possibly work via multiple intracellular channel modulators, maintaining calcium homeostasis, gene expression, enzymatic activities, and targets neuronal calcium receptors by specifically attaching to them in a calcium-dependent manner. Recent evidence also indicated that REP down-regulates the

expression of downstream regulatory element antagonist modulator (DREAM), (a calcium-binding protein) that regulates calcium homeostasis and is involved in the pathogenesis of HD and AD. It has been reported that REP increases neuronal survival via upregulating activating transcription factors-6 (ATF6) gene (endoplasmic stress sensor) that may possibly work via activating autophagy and inhibiting endoplasmic stress-induced apoptosis along with inhibition of the DREAM. Thus, there is a huge possibility that REP regulates neurodegeneration via modulating the necrotic and apoptotic cell death protein expression of pro-apoptotic protein, anti-apoptotic protein, calcium homeostasis, and may eventually reduce neuronal cell death. Moreover, clinical reports suggest that repeated doses of REP need to be administered to exhibit pharmacological action as it has a poor pharmacokinetic profile, low solubility, low absorptivity, high protein binding, and substantial first-pass metabolism. This evidence clearly indicated the possible benefit of REP in DM and co-morbid conditions but the lack of strong evidence of its efficacy in AD and pharmacokinetic challenges are emergent needs to be circumvented along with its efficacy to be ascertain.

Methodology and Results

In vitro and *in vivo* studies were conducted to understand the neuroprotective potential, molecular targets, identifying proteins, and activating cellular signaling pathways of the REP. In the cellular *in vitro* studies, primarily cytotoxicity assay was performed on SHSY-5Y neuroblastoma cells. It was observed that the REP shows cellular viability of ~80-85% at 1mM concentration and no morphological changes in the cells were found. Additionally, to mimic the neurodegenerative condition similar to AD, the SHSY-5Y cells were treated with streptozotocin (STZ, 2.5mM) as it accelerates neuronal aging, Aβ aggregation and depolarized the mitochondrial membrane, increases apoptosis, and decrease glucose uptake. The results have shown that REP increased the cell viability of STZ-treated SHSY-5Y cells by ~1.2-fold and significantly ($p<0.01$) decreased the oxidative stress in H_2O_2-treated cells, indicating the possible neuroprotective potential of REP. Further, to check the efficacy of REP in AD, *in vivo* studies were performed using a high-fat diet (HFD) fed STZ-induced murine model. The HFD was fed for 16 weeks followed by administration of STZ (30mg/kg; *i.p*) in wistar rats for induction of DM-linked AD. The significant ($p<0.001$) increase in the level of AD pathogenesis modulators i.e; Aβ, hyperphosphorylated tau proteins, and pro-inflammatory cytokines (TNF-α, IL-6), oxidative marker (Malondialdehyde (MDA), Nitrite (NO)) and decreased brain-derived neurotrophic factor (BDNF), antioxidants (Glutathione (GSH), Superoxide dismutase (SOD)) enzymes level was observed in brain homogenate of HFD fed STZ rats (disease rats) as compared to normal rats. After treatment of REP (4 mg/kg,*p.o*) for 4

weeks in DM-linked AD wistar rats, a significant ($p<0.01$) decrement in Aβ, tau proteins, TNF-α, IL-6, MDA, NO, and increment in BDNF, SOD, GSH level was observed compared to disease control rats. Further, behavioral studies (passive avoidance task (PAT), and Morri's water maze (MWZ)) were conducted in HFD-fed STZ rats to understand the effect of REP on memory cognitive dysfunctions in AD. A significant improvement ($p<0.01$) in the retention memory and spatial memory was observed after the REP treatment compared to HFD-fed STZ. Additionally, the apoptosis marker proteins (Bcl-2, Bax, Caspaase-3) and ATF6 gene expression in brain homogenate were measured to understand the mechanistic approach of REP. The decreased intensity of Bcl-2, ATF-6 and increase in the intensity of Bax, Caspase-3 represents activation of apoptosis in HFD+STZ induced rats compared to normal control but after treatment with REP a significant ($p<0.05$) increase in Bcl-2, ATF-6 expression, and decrement in Bax, Caspase-3 expression indicates a reduction in neuronal cell death then the disease rats. Moreover, the pharmacokinetic (PK) studies confirmed the short half-life ($t_{(1/2)}$ 2.65 ± 1.54 h), high clearance rate (163.84 ± 17.39 mL/h/kg), and low volume of distribution (2209.63 ± 603.29 mL/kg) of REP after performing the study on wistar rats. Therefore, to circumvent the observed problems *i.e.,* low absorptivity, high protein binding, first-pass metabolism, and poor pharmacokinetic and pharmacodynamic of REP, the targeted nano drug delivery systems were developed and biologically evaluated.

Primarily, an amphiphilic di-block co-polymer (mPEG-PCL) was synthesized using a ring-opening polymerization reaction and was characterized thoroughly using Nuclear Magnetic Resonance (NMR), Gel permeation chromatography (GPC). Further REP was loaded into the polymer using nanoprecipitation method and formulated polymeric nanoparticles (PNPs). In developed PNPs, the role of various process parameters such as drug: polymer, sonication time, and stabilizer amount on response factors like particle size (PS), polydispersity index (PDI), and entrapment efficiency (EE) were studied to optimize the PNPs using Quality by design (QbD) approach response surface (Box Behnken) methodology. Further, the REP-loaded PNPs showed a high redispersibility index with trehalose and organic residual content in PNPs was estimated using headspace gas chromatography (GC-HS). The morphological characterization of PNPs was characterized by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) and showed that REP-loaded PNPs have isometric shape with the regular surface. Moreover, *in vitro* release study indicated that REP-loaded PNPs (~70% in 48h) followed the Korsmeyer-Peppas model with a Fickian diffusion release pattern. Additionally, REP-loaded PNPs enhance intestinal absorption by ~ 1.3 folds and improve brain permeation by ~ 1.2 folds compared to free REP. Furthermore, cellular studies confirmed that REP-loaded

PNPs significantly enhance cell viability, and cell uptake, decrease STZ induce neurodegeneration and oxidative stress in SHSY-5Y cells compared to free REP. Further, in *in vivo* pharmacokinetic study the PNPs loaded with REP showed significant increments in t_{max} (2.4-fold), C_{max} (1.2-fold), $t_{1/2}$ (5.9-fold) and decrement in cL (1.2-fold) as compared to REP. In the biodistribution study, REP-loaded PNPs improve the mean resistance time in the brain more than free REP. Likewise, in pharmacodynamic studies, the REP-loaded PNPs significantly ($p<0.01$) attenuated the Aβ, tau proteins, TNF-α, IL-6, MDA, NO levels and improves the BDNF, SOD, GSH levels compared to REP. Furthermore, the behavioral studies (PAT, MWZ, Novel object recognition (NOR)) also showed a significant ($p<0.01$) improvement in the retention, spatial, and recognition memory after being treated with REP-loaded PNPs compared to free REP. Also, improvement in health neuronal count was observed in the REP-loaded PNPs compared to REP after performing hematoxylin and eosin (H&E) staining in the cornu ammonis (CA) and dentate gyrus (DG) regions of hippocampal.

Additionally, polymer lipid hybrid nanoparticles (PLHNPs) were formulated with soy phosphatidylcholine (SPC) as a phospholipid, poly (lactic-co-glycolic acid) (PLGA) as a biodegradable polymer, 1,2 distearoyl-sn-glucero-3-phospoethanolamine-N- amino (polyethylene glycol)-2000] (DSPE-PEG 2000) a lipid polymer and Pluronic F127 as a stabilizer. The PLHNPs were prepared using a nanoprecipitation self-assemble process and to optimize the formulation, the effect of various process parameters (Drug: polymer; amount of lipid and sonication time) on response factors (PS, PDI, and EE) were studied using the QbD approach response surface methodology (Box Behnken). The morphological characterization of REP-loaded PLHNPs was carried out by SEM and TEM and showed a core-shell structure with a spherical shape and smooth surface. The REP-loaded PLHNPs showed the release of REP in a controlled manner (~58% in 48h) followed by the Korsmeyer-Peppas model with a Fickian diffusion release pattern. Further REP loaded PLHNPs showed stability in the gastrointestinal environment, also enhance the intestinal permeation rate (~ 3.3 folds), and improved the brain permeation (~1.6 folds) compared to REP. Moreover, in *in vitro* cellular studies, REP-loaded PLHNPs exhibit significant improvement in cellular uptake (~21%), enhance cell viability, reduce STZ-induced neurodegeneration, and decrease the oxidative stress in neuroblastoma SHSY-5Y cells compared to free REP. Moreover, *in vivo* pharmacokinetic studies indicated significant ($p<0.05$) improvement in t_{max} (4.4-fold), C_{max} (1.9-fold), $t_{1/2}$ (9.1-fold), and decrease in the cL rate (1.8-fold) compared to free REP in wistar rats. Further, in biodistribution studies, it decreases the brain clearance rate (~ 5.6-fold) and increases the area under the curve (~10.1 fold) when compared with the free REP. Likewise,

in pharmacodynamic studies, the REP-loaded PLHNPs significantly (*p<0.01*) attenuated the Aβ, tau proteins, TNF-α, IL-6, MDA, NO levels and improves the BDNF, SOD, GSH levels compared to REP. Furthermore, the behavioral studies (PAT, MWZ, NOR) also showed a significant (*p<0.01*) improvement in the retention, spatial, and recognition memory after treated with REP-loaded PLHNPs compared to REP. Also, improvement in the neuronal count was observed in the REP-loaded PLHNPs compared to REP after performing H&E staining in the CA and DG regions of the hippocampal.

Furthermore, a comparative evaluation was conducted between PNPs and PLHNPs to examine the best nanocarrier system for targeting REP to the brain by oral route. It was observed that both the nanocarriers were efficient to deliver REP in the brain but PLHNPs exhibits significant changes like delaying the release of REP more efficiently and showed good stability in the simulated gastrointestinal fluids compared to PNPs. Additionally, PLHNPs showed significant enhancement in effective intestinal permeability by ~3.13-fold, absorption rate (K_a) by ~1.69-fold, and improved brain permeability compared to PNPs. The *in vitro* cellular uptake study of PLHNPs showed ~ 1.2 % more uptake than PNPs. Furthermore, PLHNPs formulation significantly prevents the neuronal cell death induced by STZ and H_2O_2 in SHSY-5Y cells as evidenced by increased cell viability by 0.97-fold than the PNPs. Moreover, pharmacokinetic studies also indicate the significant improvement of t_{max} by 1.8-fold, C_{max} by 1.4-fold, $t_{1/2}$ by 1.5-fold, and CL 1.4-fold by PLHNPs when compared to PNPs. Likewise, in pharmacodynamic studies, PLHNPs strongly attenuated the level of Aβ, tau-protein, pro-inflammatory cytokines (IL-6, TNF-α), MDA, NO and increased the antioxidant enzyme level (GSH), BDNF when compared to PNPs. Furthermore, behavioural studies (PA, MWM, and NOR test) also indicated highly significant improvement in the cognitive dysfunctions by PLHNPs when compared to PNPs, and H&E staining also confirmed, a healthier neuronal count in PLHNPs when compared with PNPs. Hence, the comparative study confirmed that oral administration of PLHNPs efficiently promotes the brain delivery of REP more than the PNPs, due to its core-shell nanoparticulate structure which imparts GI stability, enhanced permeation, and reduced immunogenicity. The polymeric core in PLHNPs encapsulates the REP and SPC monolayer surrounding the core which reduces the outward diffusion and enhances the stability of REP. The outer layer of DSPE-PEG 2000 prolongs the circulation time of nanocarriers by avoiding reticuloendothelial system (RES) uptake, which is indispensable for increasing the brain's uptake of nanocarriers. PEGylation facilitates the ligand–receptor interactions at the brain endothelium to ease the entry of REP into the brain.

Additionally, the additive and/or synergic neuroprotective potential of REP in combination with MEM was also examined using the HFD+STZ model. The mechanistic co-delivery approach of REP will be a more rational approach for providing novel and better therapeutic solutions for effective AD management. The low dose of MEM (5mg/kg; *p.o*) and REP (4mg/kg; *p.o*) were administered in HFD+STZ neurodegenerative rats and measured the level of neurochemicals (Aβ, tau protein, BDNF), proinflammatory cytokines (TNF-α, IL-6) and oxidative stress biomarkers (GSH, SOD, NO, MDA). After 3 weeks of treatment with REP+MEM, a significant (*P<0.01*) amelioration in the level of Aβ, tau protein, TNF-α, IL-6, and an increase in the level of BDNF has been observed when compared to free REP. The results confirmed an additive effect when REP was combined with MEM. Strikingly, when the combination of REP and MEM was compared with the PLHNPs -REP loaded formulation group, no additional significant differences in attenuation of Oxidative, neuroinflammatory, and AD markers were observed. Therefore, these results clearly indicated that the REP-loaded PLHNPs might exert a neuroprotective effect similar to the combination of REP and MEM group and proved an attractive translational possibility of REP-loaded PLHNPs formulation for clinical benefits in AD patients comorbid with BIR.

Conclusion

- In overall conclusion, for the first time, our research work confirmed the neuroprotective potential of REP in MetS-induced AD. Therefore, REP pave the way and suggested a therapeutic clinical repurposed drug for treating AD, co-morbid in MetS patients.
- Furthermore, targeted drug delivery systems like PNPs and PLHNPs -REP loaded formulations have been developed that improved the various pharmacokinetic problems i.e., short half-life, high clearance rate, high protein binding, low absorptivity, and first-pass metabolism as well as improved the efficacy of REP. Moreover, the comparative studies between developed and optimized PNPs and PLHNPs loaded REP nanocarriers, concluded that the PLHNPs exerted more significantly targeted brain delivery than the PNPs due to its core-shell structure and PEGylation on the outer surface which facilitates the ligand–receptor interactions at the brain endothelium to ease the entry of REP into the brain.
- In line with these, we also observed that REP-loaded PLHNPs have a potential neuroprotective effect similar to that exerted by the combination of low-dose MEM with REP.

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