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Synthesis, characterization and pharmacological investigation of lysine and glycine conjugated amide prodrugs of (+)-ibuprofen

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ABSTRACT

(+)-ibuprofen [(+)-IBN] is a Non-steroidal anti inflammatory drug (NSAIDs) that is pharmacologically active stereoisomer of racemic form of ibuprofen. Literature showed that Non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, naproxen and flubiprofen, are widely provide the protective effect against the neurodegenerative conditions. But the therapeutic application of NSAIDs in the CNS disorders were limited due to their limited brain distribution across the physiological barrier, blood brain barrier (BBB). BBB is composed of tightly connected endothelial cells of brain capillaries and the surrounding astrocytes and pericytes. The compounds which are able to cross the BBB by passive diffusion are small, lipophilic and uncharged at physiological P^H. This study make an attempt to synthesize the derivatives (+)-IBN by conjugating with amino acids such as glycine and lysine that produced the two amide prodrugs, (+)-IBN-G and (+)-IBN-L. The objectives of this study are to synthesize the amide prodrugs of (+)-IBN with glycine and lysine produced (+)-IBN-G, (+)-IBN-L respectively and to perform detailed study on their physical and chemical properties, distribution profile in the brain, brain targeting efficiency parameters, pharmacological activities. The compounds synthesized will act as prodrugs of (+)-IBN and after the administration the (+)-IBN was released at the desired site by enzymatic or non-enzymatic hydrolysis and synthesized prodrugs showed the enhanced brain distribution, protective against neurodegeneration, enhanced anti inflammatory activity, reduction in the gastric side effect such as ulcer formation in the stomach.

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INTRODUCTION

Neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, Huntington's disease, prion diseases and amyotrophic lateral sclerosis are an important threat to the human population and that are presently untreatable pathologies. Neurodegeneration is the common condition of the diseases even though the clinical and neuropathological hallmarks 0f the disorders are different (Aaron and D, 2017; Babitha and Vazhayil, 2014). The mechanisms of neurodegenerative diseases are still unclear and effective treatment methods are not available. Many hypotheses were developed to explain the mechanisms behind Neurodegenera-

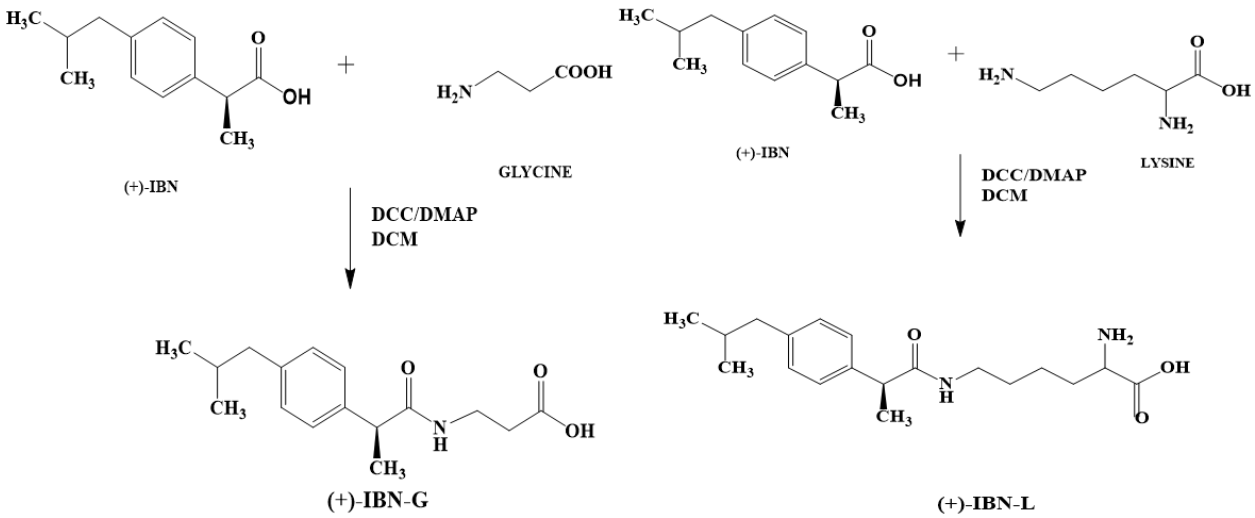

Figure 1: Schematic representation of synthesis of (+)-IBN-G and (+)-IBN-L

tion (Tutar and Tutar, 2010).

Neuroinflammation is one of the emerging hypotheses to explain the mechanisms leads for the different neurodegenerative disorders. Many studies suggest that the role of inflammation in the initiation of the neurological disorders and in brain glial cells regulate the inflammation. Glial cells in the brain made up of atrocytes that is the most abundant cells as well as important in the brain functioning and maintaining homeostasis. The activation of microglia results the production of inflammatory mediators thus enhance the inflammatory process. The chronic inflammation is one of the key pathologic factor in most of the neurodegenerative diseases like AD, Huntington's disease etc (Seibenhener and Wooten, 2015; Lau *et al.*, 2007).

Non steroidal anti-inflammatory drugs (NSAIDs) showed not only the pharmacological activities like anti-inflammatory, analgesic, and antipyretic activities but also the activities like anticancer activity, anti-Alzheimer's activity, anti-parkinsonian activity etc. Many studies have been conducted to establish the application of NSAIDs in different areas (Osafo *et al.*, 2017; Rebecca and Wong, 2019; Ettcheto *et al.*, 2017). Based on the different studies, the NSAIDs showed the mechanisms like inhibitory mechanism on nitric oxide synthesis, important role as agonists for peroxisome proliferator-activated receptor gamma, inhibit amyloid beta-protein-induced neurotoxicity and some unknown pharmacological effects. The different mechanisms of NSAIDs affect the various hypotheses of neurodegenerative diseases thus this can be used for the treatment of neurodegenerative disorders (Khansari and Coyne, 2012; Asanuma *et al.*, 2004). The penetration abililty of the NSAIDs across the BBB are limited due to their hydroliphilic nature. Therefore the prodrug based approach enhances the lipophilicity of the NSAIDs and produce the desired pharmacological activity in brain by brain targeted drug delivery systems (Biswas *et al.*, 2017; Mannila *et al.*, 2005).

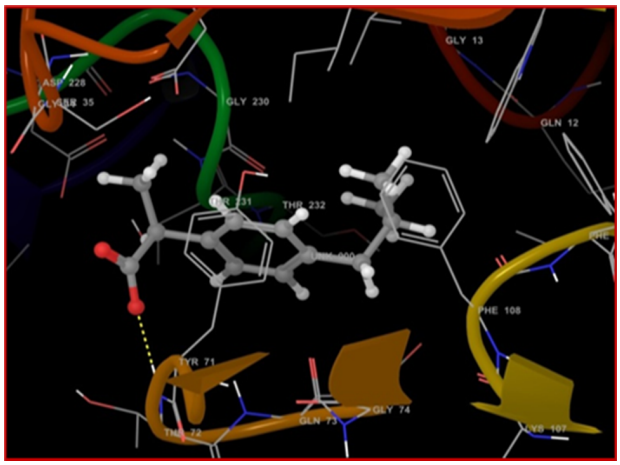

Figure 2: Ligand interaction diagram for (+)-IBN

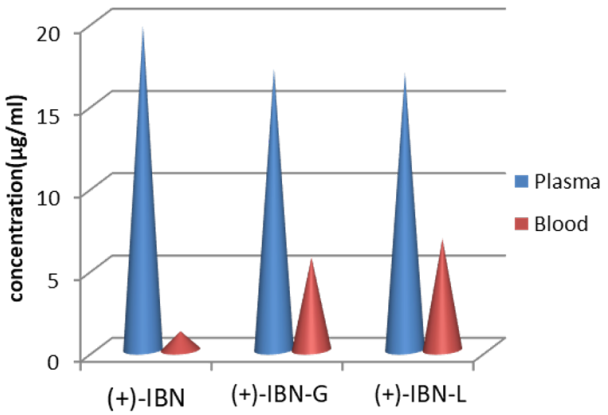

Figure 3: Concentration of drug in the plasma and blood

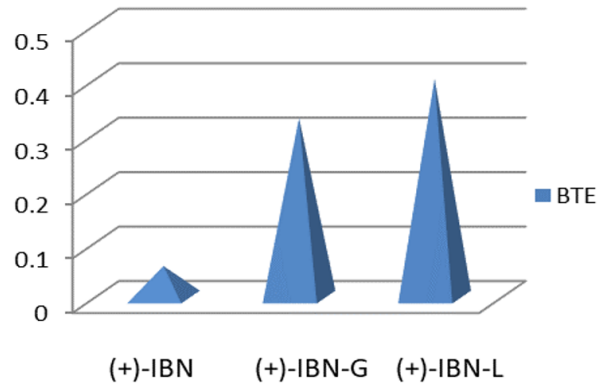

Figure 4: Brain targeting efficiency of drug and prodrugs

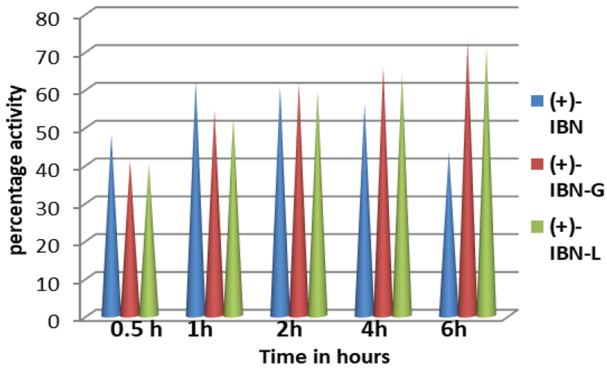

Figure 5: Anti inflammatory activity

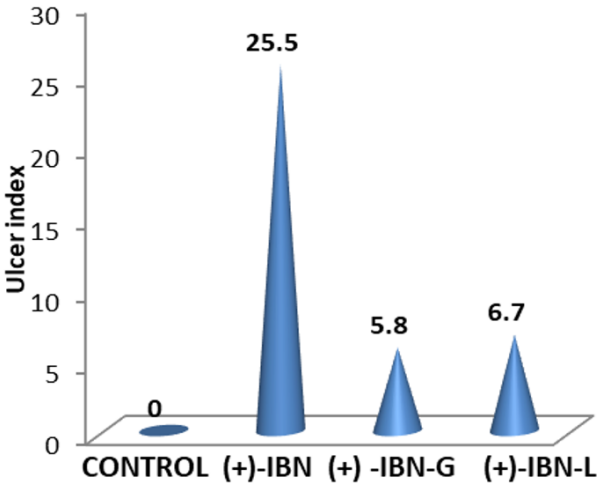

Figure 6: Comparative ulcerogenecity

MATERIALS AND METHODS

The drug (+)-IBN was obtained from Shasun Pharmaceuticals Ltd, Puducherry, India. The amino acids were obtained from Nice Chemicals and Ron Lab Chemicals, Vennala, Cochin. All other reagents and solvents were used here as analytical grade. The Infra red spectra were recorded on IR spectrophotometer (Shimadzo 8201 PC), Al Shifa College of Pharmacy. The Elemental Analysis was done in CDRI, Lucknow .^{1}H NMR and ^{13}C NMR spectra were performed in SAIF, Panjab University, Chandigarh. The Mass spectra of the synthesized prodrugs were recorded using Mass spectrophotometer in CSIF, IIISM, SRM University, Chennai. The melting points of the prodrugs were recorded using melting point apparatus (Sigma instruments, Mumbai). The homogenate was prepared by using the homogenizer, Remi Instruments division. The supernatant was prepared by using the Centrifuge, Kemi. The histopathological studies were carried out in Pathology department, KIMS Al Shifa hospital, Kerala. The cell line studies of neurodegenerative disease were carried out in Biogenix, Trivandrum.

Experimental procedures

The amide prodrugs of (+)-ibuprofen with glycine and lysine were synthesized by coupling with dicyclohexylcarbodimide (DCC) and DMAP. 10 mmol of (+)-IBN was treated with 30 ml dichloromethane and to this add 0.01M DCC and 110 mg DMAP. The mixture was stirred in a magnetic stirrer half an hour at room temperature. Then the reaction was conducted in ice bath for two hour and continued overnight at room temperature. The progression of reaction was monitored by thin layer chromatography using ethyl acetate-hexane (1:2) as mobile phase. After, completion of reaction the dicyclohexyl urea was removed. Then the filtrate was evaporated and to the residual mass 10 ml ethyl acetate was added. Then it was washed with saturated sodium bicarbonate solution. Organic layer was removed and dried using magnesium sulphate. The crude product obtained was recrystallised from ethanol. The schematic representation of the synthesis of (+)- IBN conjugates with glycine and lysine are shown in the Figure 1, (Makhija, 2013; Valeur and Bradley, 2009).

(+)-IBN-G: IR spectra (KBr, cm^{-1}): 3283 (NH stretching of amide), 2918 and 2851 (aromatic CH stretching), 1535 (C=O stretching of ester), 1449 (C=N), 1279 (CO of ester);^{1}**H NMR(δ, ppm) (D_2O):** 2.04, 2.05(J = 7.4 Hz), 0.84(J = 4.1 Hz), 1.1(J = 4.1Hz), 1.14, 1.00(J = 3.9 Hz), 2.02, 1.24(J = 2 Hz), 3.85,5.59, 3.12, 3.34, (J = 1-2Hz)^{13}**C NMR (δ, ppm) (D_2O):** 179.79, 174.83, 157.79, 154.11,

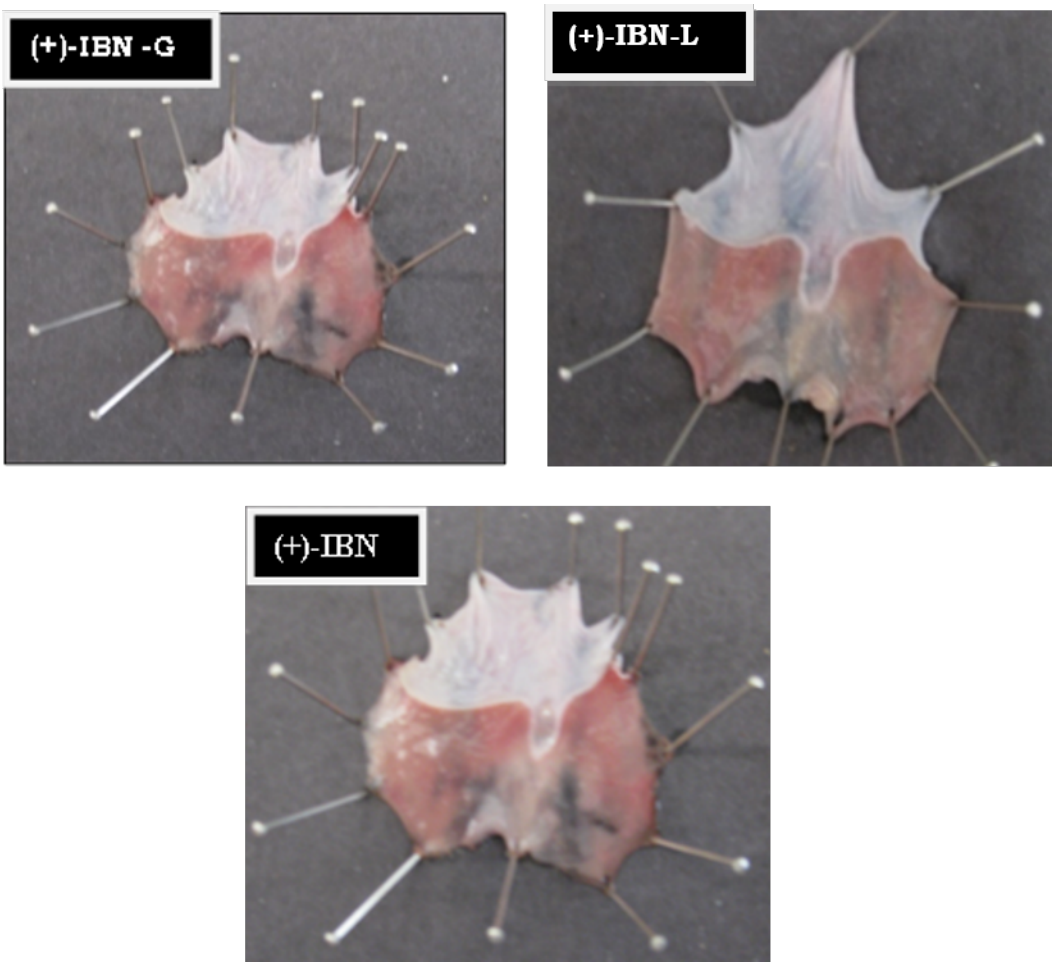

Figure 7: Ulcer production with (+)-IBN and its prodrugs

140.44, 140.11, 138.80, 129.29, 127.29, 126.93, 77.51, 76.88, 60.65, 55.90, and 49.83. **Mass (m/z):** 285(M+)**UV Absorbance(nm):** 226 in methanol

(+)-IBN-L: IR spectra (KBr, cm^{-1}): 3319(NH of amide), 2921 (aromatic CH stretching), 1561 (CO of ester), 1438 (C=N), 1305, 1268 (CO of ester); **^{1}H NMR (δ, ppm) (D_2O):**0.42, 0.12, 1.02, 1.08 (J = 7-8Hz), 1.29(J = 5.2 Hz), 0.59, 0.75, 1.76(J = 3.2, 4 Hz) 1.10, 3.56(J = 2 Hz), 4.00, 2.12, 1.63, 3.83, 5.04(J =1-2 Hz), 3.33; **^{13} C NMR (δ, ppm) (D_2O):** 45.90, 77.42, 126.92, 127.31, 129.29, 130.41, 140.50, 154.13, 157.74, 174.88, and 179.97 ;**Mass (m/z):** 336(M+), **UV Absorbance (nm):**226nm in methanol

Biodistribution and brain targeting efficiency

Biodistribution of the synthesized compounds can be monitored by using the in vivo studies. The distribution of drugs and prodrugs in plasma and brain was done by using the methodology described by (Zhang *et al.*, 2012) and the brain targeting efficiency of the prodrugs were calculated.

Pharmacological evaluation

The pharmacological activities such as anti-inflammatory activity, anti-ulcerogenecity and the effect in the brain were tested by in vivo methods. The results of the each study were compared with that of the parent NSAID, (+)-IBN.

Neuroprotective activity the synthesized prodrugs

Aluminium chloride induced neurotoxicity model

Neurotoxicity was produced by chronic aluminum chloride induced mice model. The animals were grouped in to six and each group contained six animals. The first group acts as control that receives normal saline. The second group received aluminum chloride only.

The third, fourth, fifth group received (+)-IBN [2.50 mg], (+)-IBN-G [2.00 mg], and (+)-IBN-L [2.00 mg] respectively by oral administration (Singh and Goel, 2015; Shati *et al.*, 2011).

The animal experiment were performed as per the guide lines of the animal ethical committee (Reg. No: 1195/PO/Re/S/08/CPCEA) Al Shifa College of Pharmacy, Kerala, India.

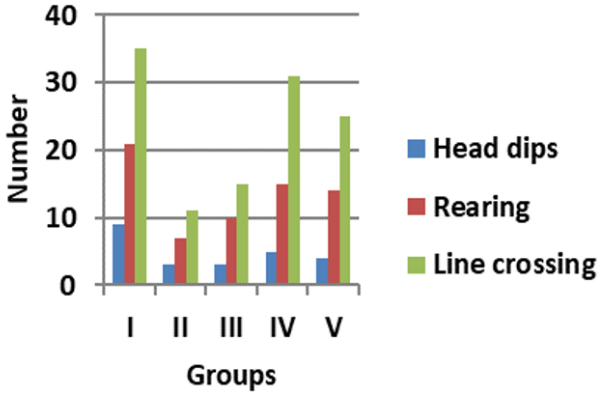

Figure 8: open field test

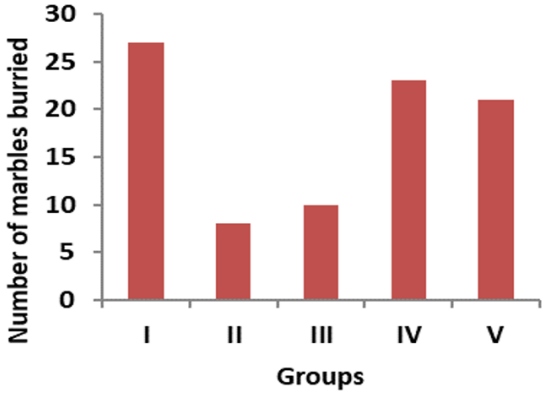

Figure 9: Marble burying test

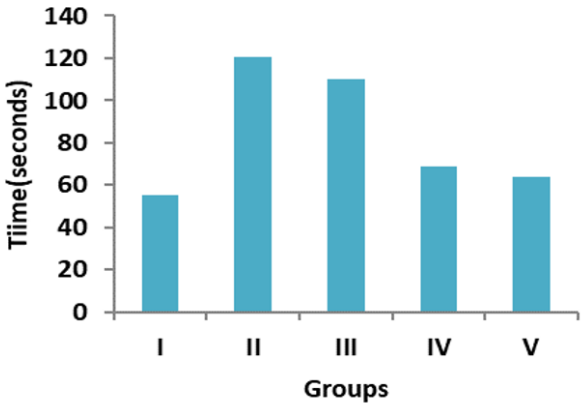

Figure 10: Water maze test

Behavioral studies

Behavioral studies provide simultaneous measures of locomotion, exploration and anxiety. The open field test, marble burying test and water maze test were used here.

Open field habituation

The Open Field behavioral test is one of the most known primary behavioral tests to monitor the locomotion and exploration. This was done according to the procedure explained by (Seibenhener and Wooten, 2015; Carner and Shieh, 2015).

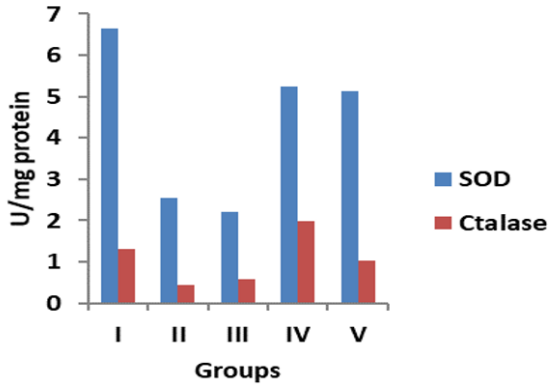

Figure 11: Antioxidant activity

Marble burying test

Marble burying test was according to the procedure explained by (Gallo *et al.*, 2014; Aliskan *et al.*, 2017).

Water maze test

Water maze test was done procedure explained by (Nunez, 2008).

Biochemical estimations

Oxidative stress is one of the main reason for neurodegenerative conditions so the estimation of the antioxidant are significant. Antioxidant enzyme were significant role against reactive oxygen species leads to cell destruction (Kurhaluk, 2019). In this the SOD and CAT activity was measured by the procedure by (Weydert and Cullen, 2010; Khan *et al.*, 2012).

Histopathological Studies

Histopathology of brain cortex was done by haematoxylin-eosin dye method. histopathological changes of the brain cortex were monitored microscopically (Nobakht *et al.*, 2011).

Cell Line study- *invitro* neuroprotective effect determination by MTT assay

Cell line was conducted by the MTT assay and percentage viability was found out at different concentrations.

Anti inflammatory activity

Anti inflammatory activity was performed by carrageenan induced paw edema method explained by (Rasheed *et al.*, 2011).

Ulcerogenicity

The main side effect of the NSAIDs is the production of ulcers that were studied and monitored as per the procedure by (Rasheed and Kumar, 2010).

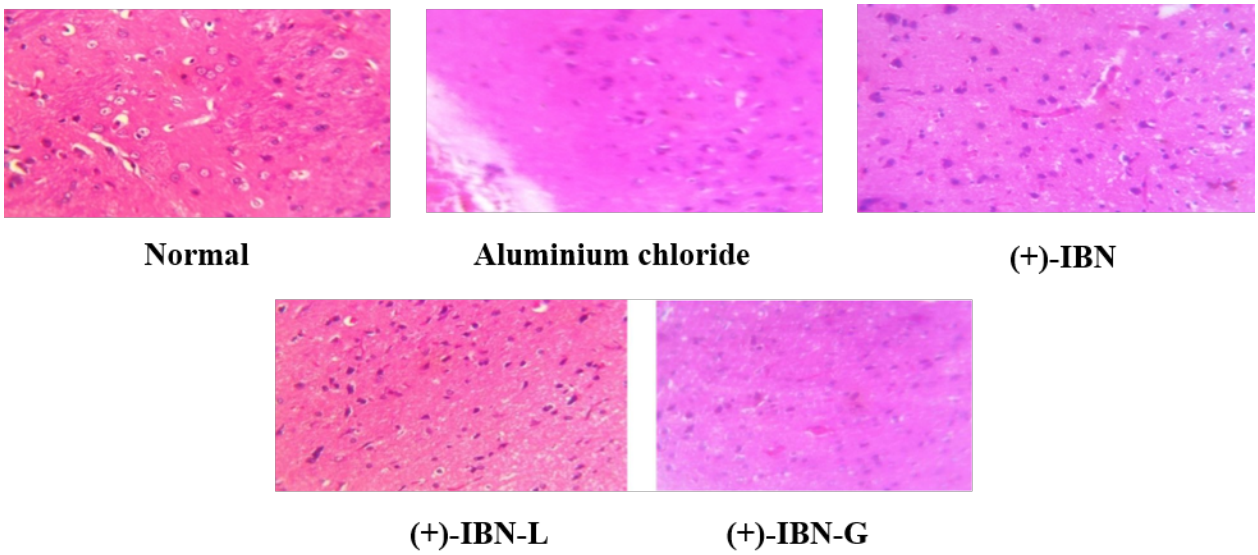

Figure 12: Histopathology of brain cortex

Statistical analysis

Statistical significance was done by ANOVA and the values were expressed as mean ± SD.

RESULTS AND DISCUSSION

Physico-chemical characterization

The physico-chemical characterization was done and the data was given in the Table 1. The remarkable difference in the melting point, Log P value, R_f value Molecular weight from mass spectroscopy and other spectral characterizations confirmed the formation of a pure product with amide linkage. The increased Log P value indicates the enhanced lipophilic profile of the prodrugs. The elemental analysis was performed and find out the percentage of C, H and N in the compounds that was comparable with that of theoretical values.

Molecular modeling study

The molecular modeling study revealed that (+)-IBN is capable to interact with the active site of the Beta-Secretase enzyme, with a docking score of -5.68 that was given in the Figure 2. The pharmacokinetic profile of the prodrugs were done by insilico tool proved the enhancement of the properties of the prodrugs that was given in the Table 2.

In Table 2 shows, [a]Predicted octanol/water partition co-efficient log p (acceptable range: −2.0 to 6.5). [b]Predicted Caco-2 cell permeability in nm/s (acceptable range, <25 is poor and >500 is great). [c]Predicted aqueous solubility; S in mol/L (acceptable range: −6.5 to 0.5). [d]Predicted apparent MDCK cell permeability for the blood-brain barrier, (acceptable range, < 25 is poor and >500 is great), [e]Percentage of human oral absorption (< 25% is poor and >80% is high).

In vivo biodistribution study

The distribution of the drug and prodrugs can be evaluated by bio-distribution conducted in rats. The parameter used for evaluating the brain distribution of the drug and prodrugs is brain targeting efficiency(BTE) and that can be calculated by $(C_{brain}/C_{plasma})_{(+)-IBN.}$ The $(C_{brain}/C_{plasma})_{(+)-IBN}$ ratios of the 10 min after administration of (+)-IBN, (+)-IBN-G and (+)-IBN-L prodrugs were 0.057±0.016, 0.329±0.023 and 0.401±0.020 respectively. The data was given in the Table 3 and Figure 3. Brain targeting efficiency of the drug and prodrugs were graphically represented in the Figure 4.

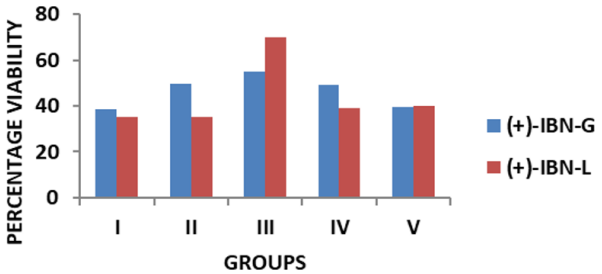

Figure 13: The percentage viability of prodrugs in different concentrations

Pharmacological evaluation

Anti inflammatory activity

From the anti inflammatory test the pharmacological activity of the (+)-IBN-G and (+)-IBN-L was found to increase from 41 to 78 and 40 to 70.8 respectively that was shown in the Table 3 and Figure 5. This

Table 1: Physico-chemical characterization of the synthesized prodrugs

Prodru	Molecula weight	Colou	MP(^{0}C)	Percentage yield (%)	Protein binding (%)	R_f Value	Elemental data			
							Percentage calculated		Percentage found	
(+)-IBN-G	291	Off white	175-180	82	59.88	0.58	C H N	68.42 8.04 5.32	C H N	68.40 8.02 5.28
(+)-IBN-L	334	Off white	205-210	80	65.25	0.62	C H N	68.23 9.04 8.38	C H N	68.26 9.02 8.29

Table 2: pharmacokinetic parameters using Qikprop

Compound	QPlogPo/w [a]	QPPCac[b]	QPlogS[c]	QPPMDCK [d]	% Absorption
(+)-IBN-G	3.947	342.15	-3.755	197.39	44.52
(+)-IBN-L	2.738	61.755	-3.549	52.212	92.75
(+)-IBN	0.490	6.639	-3.611	5.235	75.16

Table 3: Anti inflammatory activity of the (+)-IBN and its prodrugs

Group	Prodrug	Dose (mg p.o.)	Anti Inflammatory activity (%)				
			0.5hr	1 hr	2 hr	4 hr	6 hr
I	Control[a]	1% CMC	-	-	-	-	-
II	(+)-IBN	3.6	48.0 ± 1.1	62.0 ± 1.2	60.6 ± 2.1	56.1 ± 1.2	43.3 ± 1.5
III	(+)-IBN-G	5.3	41.0 ± 1.0[c]	54.3 ± 1.3[c]	61.4 ± 1.4[c]	65.9 ± 1.4[c]	72.8 ± 1.3[c]
IV	(+)-IBN-L	5.5	40.0 ± 1.0[c]	52.3 ± 1.3[c]	59.4 ± 1.4[c]	63.9 ± 1.4[c]	70.8 ± 1.3[c]

Values were the mean ± SD of six observations. [C] P <0.05,done by ANOVA followed by Dunnett's test. Comparison between group II vs III and IV.

study proved that prodrug approach applied on the NSAIDs effectively attain the goal to enhance the anti inflammatory activity.

Ulcerogenicity

Ulcerogenic study revealed the reduction in the toxicity by prodrug based approach used in NSAIDs. (+)-IBN produced the mean ulcer index of 25.5 but the synthesized amino acid conjugates of drug produced very less toxicity profile that can be understood by mean ulcer index that was given in the Figures 6 and 7.

Activity in the brain

Pharmacological activity of the synthesized amide compounds in brain was done by monitoring the behavioral tests, antioxidant parameters and histopathology of the brain cortex. The graphical representations of the results were given in the Figures 8, 9, 10 and 11. The results of the behavioral studies showed that the increased locomotor activity, memory and spatial learning capacity of the synthesized prodrugs compared with that of (+)-IBN. The results of SOD and catalase activity provide the information about the protective effect of the prodrugs in neurotoxicity condition compared to that of (+)-IBN. The histopathology analysis on the brain cortex also proved the protective nature of prodrugs compared with that of (+)-IBN that was shown in the Figure 12.

In vitro cell line study

The results suggest that (+)-IBN-G and (+)-IBN-L were involved in protection of SH-SY5Y cells. The neuroprotective effects of samples were confirmed using the percentage viability data that is shown in

theFigure 13.

CONCLUSIONS

The aim and objectives of the study were successfully completed and the main aim of the study was the synthesis of the glycine and lysine conjugated (+)-IBN, characterization and determination of its pharmacological activities. The physico-chemical and spectral characterization of the synthesized amide prodrugs confirmed the structure and purity. Prodrug based approach on the NSAIDs successfully attain the goal for the improvement in the transport property across the BBB and better bio distribution in brain compared with that of the parent NSAID, (+)-IBN. The pharmacological studies proved that the enhanced anti-inflammatory activity, reduction in the gastro-intestinal toxicity and protective action against neurodegeneration. The modification of the free carboxylic acid group in the NSAID by conjugating with amino acid produced the amide prodrugs that overcome the side effects of the NSAIDs and also improve the pharmaco-kinetic and pharmaco-dynamic profile.

Conflict of Interest

None.

Funding Support

None.

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