Original Article

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Development, characterization and pharmacological evaluation of amino acid prodrugs of (+)-Ibuprofen

Nija B[*1], Arun Rasheed[1], Kottaimuthu A[2]

[1]Department of Pharmaceutical Chemistry, Al Shifa College of Pharmacy, Perinthalmanna, Malappuram District, Kerala, India
[2]Department of Pharmacy, Annamalai University, Annamalai Nagar - 608 002, Tamilnadu, India

Abstract

The current research work investigate the neuronal pathway associated with NSAIDs that lead to the reduction in the initiation and progression of neurodegenerative diseases such as Alzheimer's disease, Parkinsonism etc. This research was developed amino acid prodrugs of the active enantiomer of ibuprofen, (+)-IBN and checks the pharmacological effects, neuroprotective effect, anti inflammatory effect and anti-ulcerogenic effect. The treatment using (+)-IBN reduced the action of micoglia and the release of cytokine especially TNFα that are mainly involved in the neurodegenerative process. (+)-IBN reduced the deposition of soluble beta amyloid plaque by inhibiting the amyloid precursor protein, beta-secretase 1 and also enhancing the degradation process of beta amyloid via induction of insulin degrading enzyme. (+)-IBN also showed the property that the reduction in the TAU misfolding process. Therefore, the synthesized (+)-IBN amino acid prodrugs treatment effectively produce neuroprotective action, both restoring memory realed risk factors and reversing multiple brain neuropathological hallmarks. The current research study developed the three amino acid prodrugs of (+)-ibuprofen by conjugating with L-phenyl alanine, L-tryptophan and L-tyrosine also the physico-chemical characterization and spectral characterization was done. This study modify the carboxylic acid functional group present in the NSAIDs that lead to the formation of prodrugs with enhanced anti-inflammatory activity, reduced side effects and protective effect against neurodegenerative processes.

*Corresponding Author

Name: Nija B
Phone: +91-9446016773
Email: nija.b90@gmail.com

INTRODUCTION

The general pharmacological activities of Non-steroidal anti-inflammatory drugs (NSAIDs) are analgesic activity, antipyretic activity and anti inflammatory activity. The general mechanism of NSAIDs is explained through the inhibition of prostaglandins(PGs) by the action of two cyclooxygenase enzymes.

PGs having significant role in various cellular activities like gastrointestinal protection, inflammation, angiogenesis, hemostasis etc. (Gunaydin and Bilge, 2018). Many studies have been conducted for investigating the different pharmacological and therapeutical activities of NSAIDs.

Table 1: Physicochemical characterization of drug and synthesized prodrugs

Pro drug	Molecular weight	Colour	LogP	Protein binding value (%)	Melting point (^{0}C)	Yield (%)	Rf^a value	Elemental analysis		
									Percentage calculated	Percentage found
(+)-IBN 1	406	Off white	1.23	74	68 - 69	74	0.56	C	73.86	73.55
								H	7.44	7.48
								N	6.89	6.91
(+)-IBN 2	367	Off white	1.17	68	58 - 59	78	0.64	C	75.15	75.23
								H	7.95	7.96
								N	3.81	3.73
(+)-IBN 3	383	Yellowish white	1.16	58	56 - 58	70	0.67	C	72.04	71.90
								H	7.62	7.75
								N	3.65	3.33

^{a}ethyl acetate : n-hexane 1:2

Table 2: ADME prediction of synthesized prodrugs

Compound ID	QPlogPo/w ^{a}	QPPCaco^b	QPlogS^c	QPPMDCK ^{d}	% Absorption^e
(+)-IBN 1	5.21	1358.18	-5.95	950.47	100
(+)-IBN 2	4.681	1591.082	5.014	1105.55	100
(+)-IBN 3	4.67	609.32	-6.488	367.94	100
(+)-IBN	3.49	337.89	-3.755	194.74	92.67

Table 3: Brain targeting efficiency of (+)-IBN and its prodrugs

Drug	C_{Plasma} (μg/ml)	C_{brain} (μg/ml)	BTE
(+)-IBN	19.72±0.016	1.12±0.018	0.057±0.016
(+)-IBN 1	18.02±0.021^c	7.91±0.025^c	0.439±0.023^c
(+)-IBN 2	16.15±0.029^c	8.64±0.032^c	0.535±0.030^c
(+)-IBN 3	15.48±.019^c	4.32±0.021^c	0.279±0.020^c

Value expressed as mean±SEM of five experiments. Statistical significance by ANOVA followed by Dunnet's test, ^{c}p<0.01 vs (+)-IBN.

Table 4: Time – Concentration profile and BTE of (+)-IBN and (+)-IBN 2

Time (min)	Concentration (μg/mL) in Plasma		Concentration (μg/mL) in Brain		Brain targeting efficiency	
	(+)-IBN	(+)-IBN 2	(+)-IBN	(+)-IBN 2	(+)-IBN	(+)-IBN 2
10	19.72±0.016	16.15±0.029^b	1.12±0.018	8.64±0.032^b	0.0568±0.023	0.535±0.031^b
20	17.22±0.021	12.19±0.040^b	0.882±.024	6.23±0.038^b	0.0512±0.019	0.436±0.022^b
30	12.19±0.013	9.32±0.032^c	0.474±0.016	3.46±0.037^c	0.0443±0.033	0.367±0.036^b
60	7.82±0.031	5.18±0.028^c	0.223±0.036	1.14±0.029^c	0.0285±0.028	0.227±0.024^b
120	5.12±0.026	3.66±0.027^b	0.117±.028	0.815±0.031^b	0.0229±0.018	0.211±0.024^c
240	2.28±0.029	2.16±0.019^b	0.04±0.033	0.383±0.022^b	0.01750.030	0.1800.034^c

Value expressed as mean±SEM of five experiments. Statistical significance by ANOVA followed by Dunnet's test, ^{b}p<0.001 ^{c}p<0.01 vs (+)-IBN

Table 5: Brain targeting efficiencey parameters

Parameters	Brain
Relative Uptake Efficiency (RE)	9.21
Concentration Efficiency (CE)	7.71
Drug Targeting Index (DTI)	17.93

Table 6: Anti Inflammatory Activity

Group	Prodrug	Anti-inflammatory activity				
		0.5 hour	1 hour	2 hour	4 hour	6 hour
I	Normal	-	-	-	-	-
II	(+)-IBN	48±1.1	62.0 ± 1.2	60.6 ± 2.1	56.1 ± 1.2	43.3 ± 1.5
III	(+)-IBN 1	42.0 ± 1.2^d	50.1 ± 1.0^d	59.0 ± 1.3^d	71.7 ± 1.1^d	73.4 ± 1.2^d
IV	(+)-IBN 2	45 ± 1.1^d	62.5 ± 1.7^d	67.4 ± 1.1^d	71.7 ± 1.1^d	77.3 ± 2.2^d
V	(+)-IBN 3	42.0 ± 1.5^d	50.1 ± 1.8^d	54.4 ± 1.7^d	58.3 ± 1.0^d	64.5 ± 1.3^d

Values were the mean ± SD of six observations. $^d P < 0.05$, done by ANOVA followed by Dunnett's test. Comparison between group II vs III, IV and V.
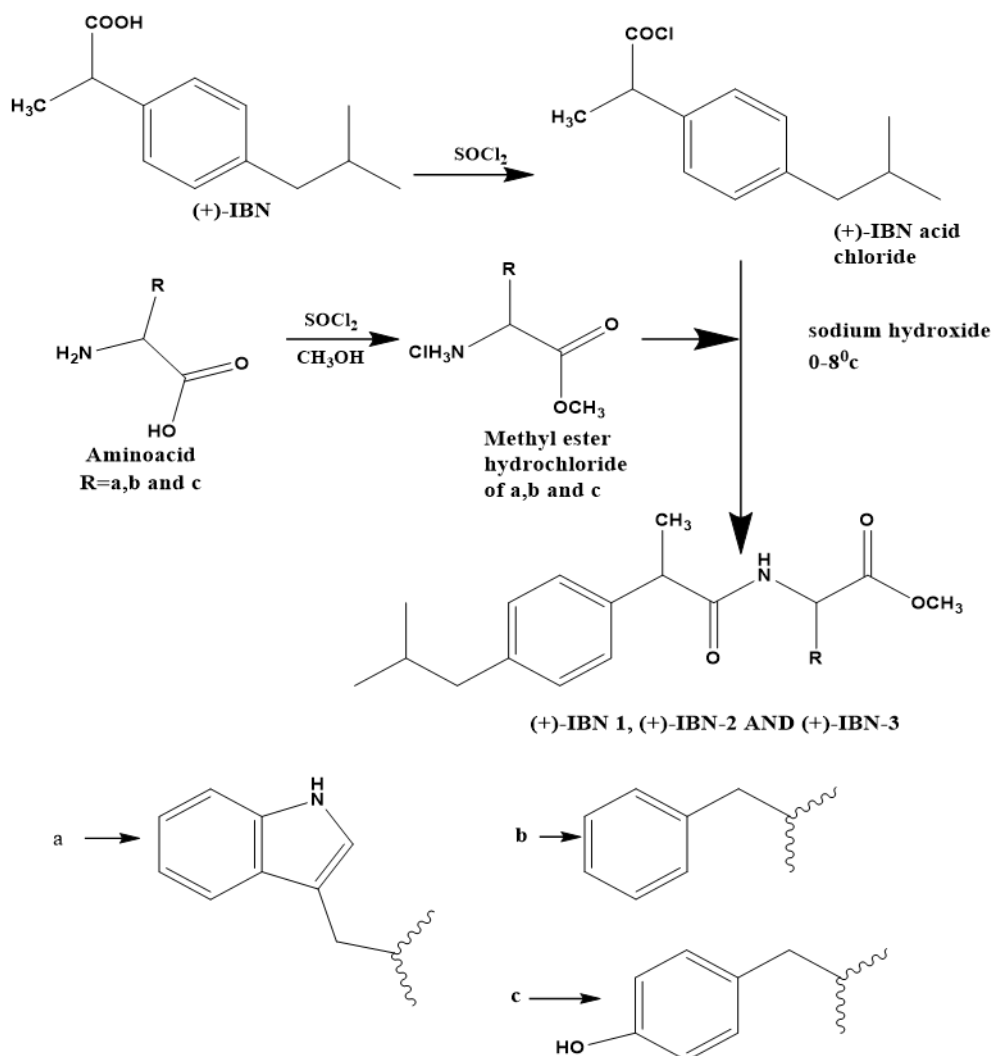
Figure 1: Scheme for the synthesis of (+)-IBN 1, (+)-IBN 2 and (+)-IBN 3

Studies revealed the significance of NSAIDs in various activities and treatment strategies like anticancer activity, anti-Alzheimer's activity, anti-parkinsonian activity etc. (Osafo and Agyare, 2017; Rebecca and Wong, 2019; Ettcheto *et al.*, 2017; Ajmone-Cat *et al.*, 2010).

The current research aims to develop the novel amino acid prodrugs of NSAID, (+)-IBN and investigate the effect of NSAIDs and their conjugates in different areas. The use of prodrugs based approach modify the carboxylic acid functional group of NSAIDs that produced the compounds with better physical-chemical properties, pharmacokinetic–pharmacodynamic properties, pharmacological activities and reduced toxicity profile (Rasheed *et al.*, 2011; Preethi *et al.*, 2018).

Literature survey showed that the long term use of NSAIDs reduce the risk and progression of neuro-degeneration by the significant effect on the microglial activity, reduction in beta amyloid production, facilitate the $A\beta$ degradation, reduction in the process of TAU protein hyperphosphorylation and reduced the problems like spatial learning and memory impairment (Khansari and Halliwell, 2019; Španić *et al.*, 2019).

So this study synthesize the three amino acid prodrugs and expect to give improved properties (Rasheed and Kumar, 2008).

MATERIALS AND METHODS

Reagent and chemicals

The amino acids were obtained from Hi-media chemicals, India, (+)-IBN was obtained from Alkem laboratories, Mumbai and all other reagents are analytical grade. The melting points were monitored by the Melting point apparatus and the elemental analysis was done in CDRI, Lucknow.

The infra red spectra were recorded by IR spectrometer, Al Shifa College of pharmacy. ^{1}H NMR, ^{13}C NMR and Mass spectra were recorded in IICB, Kolkata. The homogenate was prepared by using the homogeniser, Remi instruments division. The histopathological studies were carried out in Department of Pathology, KIMS Al-shifa hospital Kerala. The cell line studies of neurodegenerative disease were carried out in Biogenix, Trivandrum.

Experimental procedures

Synthesis

This research study synthesize the three novel prodrugs of (+)-IBN by Schotten-Baumann method in which the drug was conjugated with aminoacids like L-tryptophan(a), L-phenyl alanine(b) and
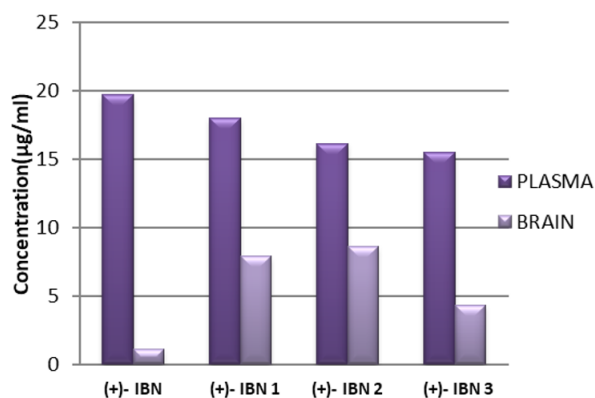
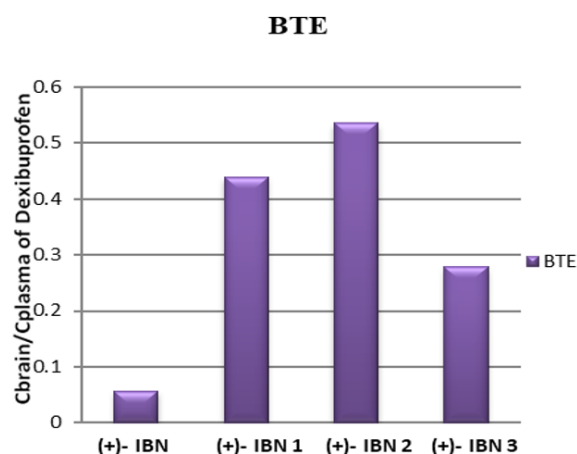**Figure 2: (+)-Ibuprofen concentration in brain and plasma**
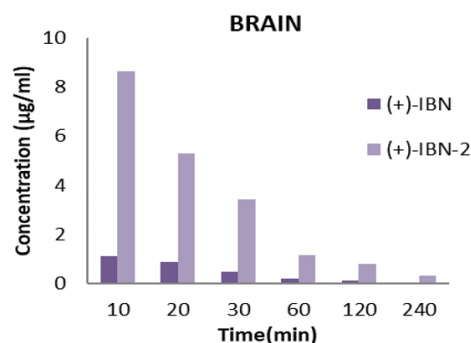
Figure 3: Brain targeting efficiency

Figure 4: Concentration – time plot of (+)-IBN and (+-IBN 2 in brain

L-tyrosine(c) produced (+)-IBN-1, (+)-IBN-2 and (+)-IBN-3 shown in Figure 1 as per the literature (Rasheed *et al.*, 2011).

(+)-IBN1(S(+)-methyl-3-(1H-indol-3-yl)-2-(2-(4-isobutyl phenyl)propanamido)propanoate)

FTIR(cm^{-1})3400(NH-amide), 3007(aromatic-CH)1581, 1660(CO-ester)1453(CN)1228, 1350(CO of ester); **[1] HNMR(δ, ppm)(D$_2$O):** 1.37(J=7.1Hz, 3H), 1.79(J=6.8Hz, 1H), 3.58(J=7.1Hz, 2H),
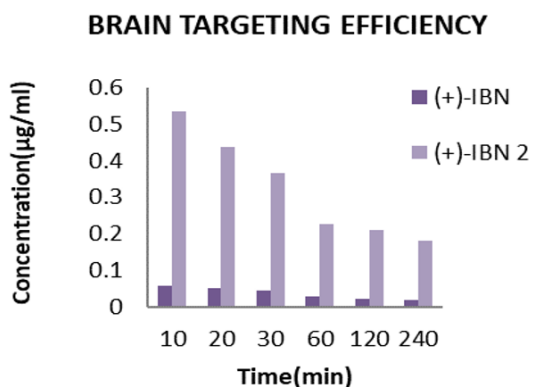
Figure 5: BTE of the (+)-IBN and (+)-IBN 2

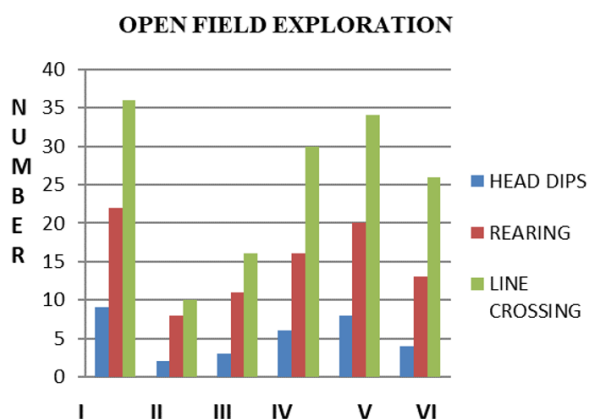**Figure 8: Water maze test**

Figure 6: Open field exploration

Figure 9: Antioxidant activity

7.19(5H, indole ring), 8.28(1H,NH), 8.22 (1H,NH), 7.2(4H,benzene ring),^{13}C NMR$(\delta,$ **ppm) (D$_2$O)**: 18.40, 21.56, 29.68, 44.15, 48.14, 112.71, 115.88, 116.98, 128.28, 129.21, 129.56, 133.68, 134.46, 134.68, 147.02, 171.87, 184;Mass (m/z):406(M$^+$).

(+)-IBN2(S(+)methyl2-(2-(4-isobutylphenyl)propanamido)-3-phenylpropanoate)

FTIR(cm^{-1})3346(NH-amide),2955,2921(aromatic-CH)1547(COester)1406(CN)1288(COester);**^{1}HNMR(δ, ppm)(D$_2$O) :**1.37(J=7.1Hz,3H), 3.61(J=7.2Hz,1H), 7.26(4H, benzenering), 8.52(1H,NH), 3.60(J=6.8Hz,1H), 7.25(4H, benzenering),^{13}**CNMR(δ,ppm)(D$_2$O)**: 18.41, 21.47, 29.68, 44.15, 48.14, 120.88, 122.02, 122.64, 123.95, 134.46, 127.28, 129.91, 130.62, 131.17, 133.16, 138.08, 147.42, 147.64, 157.02, 171.07, 184;Mass (m/z): 367(M$^+$).

Figure 7: Marble burying test

(+)-IBN3(S(+)methyl3-(4-hydroxyphenyl)2-(2-(4-isobutylphenyl)propanamido)-3 phenyl propanoate); FTIR(cm^{-1})3340 (NH-amide), 2954(aromatic-CH)1545(CO-ester)1406(CN)1291(CO of ester); **^{1} HNMR (δ, ppm)(D$_2$O):** 1.37(J=6.7Hz, 6H), 3.58(J=7.1Hz, 1H), 7.23 (J=8Hz, 4H, benzene ring), 7.11(J=8Hz, 4H, benzene ring), 8.28(1H,NH) ^{13}C NMR **(δ, ppm)(D$_2$O):** 13.54, 20.43, 39.10, 39.37, 39.51, 39.65, 39.79, 39.93, 40.05, 120.94, 122.07, 122.68, 123.98, 127.33, 129.92, 130.69, 131.25, 133.65, 138.13, 139.62, 147.69, 163.24, 175.05 ; **Mass(m/z):** 383(M$^+$)

Physical- chemical characterization methods

The progression of the reaction and the purity of the compounds confirmed by thin layer chromatography on pre-coated silica-G plates and the detection method is UV chamber visualization (Rao and Shil, 2019; Kemp, 1991). The melting point of the drug and prodrugs were done for confirming the product formation and it was done by using melting point apparatus (John and Young, 2013) . Solubility of the drug and amide prodrugs were done in different solvents to identify the nature of the compounds (Qiu et al., 2009).

The quantification of the elements present in the synthesized compounds and it was compared with that of the calculate values are significant method to confirm the successful synthesis (Margui and Van Grieken, 2014). The partition coefficient of the podrugs were done by shake falsk method explained by Paschke et al. (2004).

Protein binding of the drugs and prodrugs were to confirm that the drug is available for the pharmacological activity and it was done by equilibrium dialysis method explained by (Pinger et al., 2017; Barton et al., 2007).

Spectral characterization was very much important to confirm the desired structures and done by IR, ^{1}HNMR, ^{13}CNMR and Mass spectroscopy (Silverstein and Webster, 1998; Jurgen, 2004).

In silico Studies

ADME Optimization is significant in the drug development process that was done by QikProp according to the Schrödinger [Internet], USA (Schrödinger, 2015).

Biodistribution and pharmacokinetic studies

The best distributed prodrug in the brain can be found out by bio-distribution study and that can be monitored by the parameter brain targeting efficiency. The procedure was according to the (Zhang et al., 2012).

Pharmacological evaluation

The activties tested were anti-inflammatory activity,ulcerogenecity and protective effect in brain.

In brain, (+)-IBN and its synthesized prodrugs were evaluated for behavioral parameters, antioxidant parameter, histopathology of the brain cortex and the results were compared with that of the parent drug. Neurotoxicity was induced by aluminium induced model by the oral administration of aluminium chloride [100mg/kg/day] for three months (Shati et al., 2011). The animals were grouped in to six groups and each group contained six animals. The first group acts as control that receives normal saline. The second group received aluminium chloride only. The third, fourth, fifth, sixth group received (+)-IBN [1.39 mg], (+)-IBN 1 [2.75 mg], (+)-IBN 2 [1.6 mg] and (+)-IBN 3 [2.0 mg] respectively by oral administration. The animal experiment was 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.

Behavioral studies

Open f ield tests

The open field exploration study was carry out according to the procedure explained by (Taïr et al., 2016)

Marble burying test

The marble burying test was done by (Angoa-Pérez et al., 2013; Wolmarans et al., 2016).

Water maze task

This test access the spatial learning and memory conducted as per the procedure followed by (Vorhees and Williams, 2006; Nunez, 2008).

Biochemical estimations

Antioxidant parameters were estimated by testing the activity of the enzyme catalase and superoxide dismutase (SOD) (Pham-Huy et al., 2008). SOD activity was done by the procedure explained by (Weydert and Cullen, 2010) and the catalase activity was done as per the procedure (Goth, 1991; Hadwan and Abed, 2016).

Histopathological Studies

The cortex of the brain was collected and histopathologically evaluated by using haematoxylin-eosin dye in Department of pathology, KIMS-Al shifa hospital,kerala and The sections were examined microscopically for histopathological changes observed and compare with that of parent NSAID (Nobakht et al., 2011).
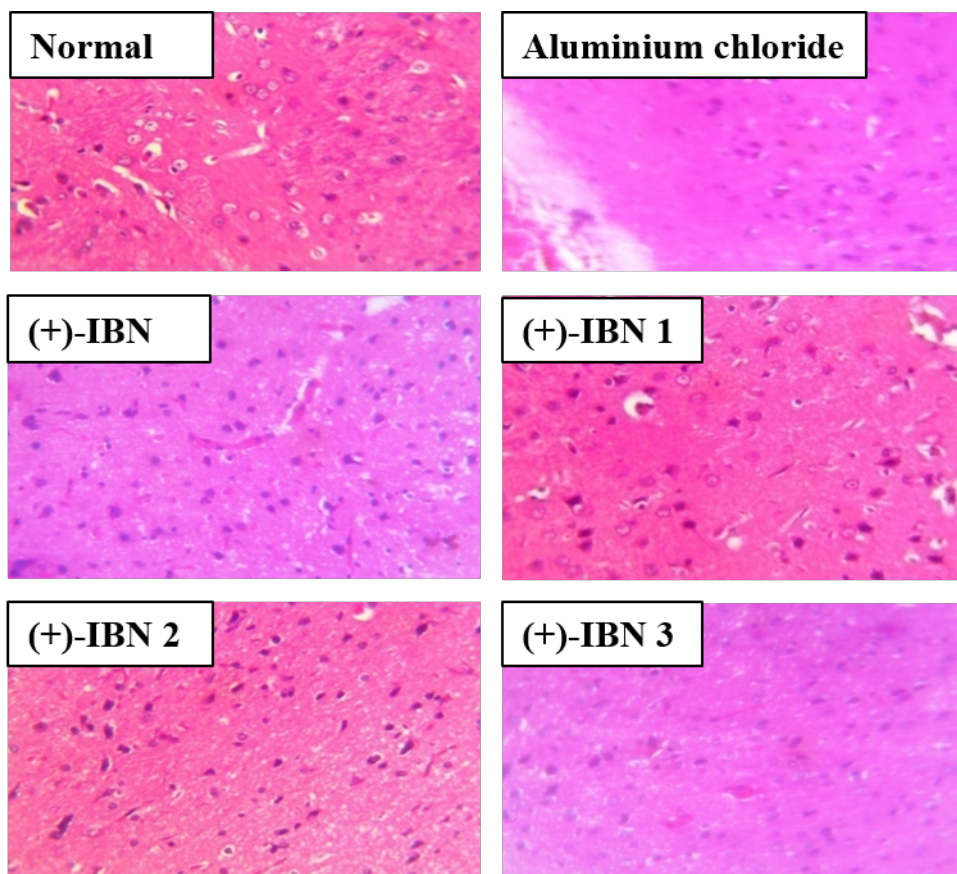
Figure 10: Histopathology of the brain cortex

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. (Martinez et al., 2020)

Anti –inflamamtory activity and ulcerogenecity study

Anti inflammatory activity was performed by carrageenan induced paw edema method explained by (Khedir *et al.*, 2016). The main side effect of the NSAIDs is the production of ulcers that were studied and monitored as per the procedure by (Rasheed *et al.*, 2016).

Statistical analysis

Statistical significance was done by ANOVA and the values were expressed as mean $\pm$ SD.

Figure 11: The % viability of prodrugs in different concentrations

RESULTS AND DISCUSSION

Physical and chemical characterization methods

The nature of the prodrugs was evaluated by various methods. Different methods adopted and their results were given in the Table 1. The data in the table revealed that the formation of pure amide prodrugs. Th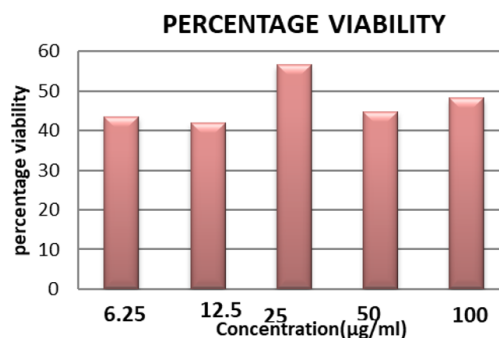e melting point determination and thin layer chromatography results showed the formation of the products. The solubility studies proved the organic solubility of the prodrugs that showed its lipophilic nature. Also the Log P value calculated from the partition coefficient method revealed the enhancement of lipophilic profile of the amide prodrugs. The lower protein binding values indicate the availability of the drug in various biological fluids. Elemental analysis data provided the comparable values with the theoretical values spectral data confirm the structure of the compounds with amide and ester linkages.
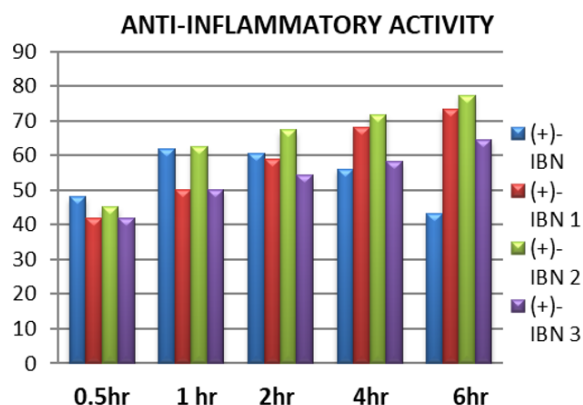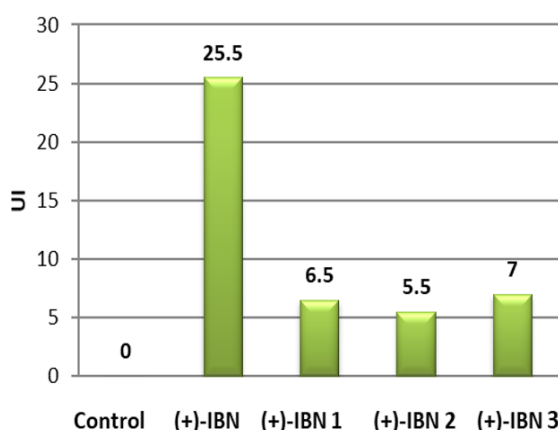
Figure 12: Anti-inflammatory activity

Figure 13: Comparative ulcerogenecity of (+)-IBN and its prodrugs

Insilico studies

The ADME optimization is very much significant and that was done by insilico tool. Data was given in Table **??**.

In Table **??** 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).

Biodistribution and Pharmcokine tics

The drug with more distribution in the brain and Brain targeting efficiency (BTE) can be found out from the biodistribution study and the data was presented in Table 3 and graphically represented in Figures 2 and 3. (+)-IBN 2 showed higher BTE value and brain distribution shown in Figure 3. So Pharmacokinetic studies were carried out using (+)-IBN

and (+)-IBN 2 .The concentration- time profile of plasma and brain and BTE was given in Table 4 graphically represented in Figures 4 and 5 respectively. The targeting efficiency parameters are presented in Table 5. The study revealed that the concentration of the prodrug after 10 minutes of administration is very high in brain compared to that of (+)-IBN. The brain targeting efficiency of the prodrugs were high compared to that of (+)-IBN. Also, the RE,CE and DTI showed the remarkable high distribution of (+)-IBN 2 compared to that of (+)-IBN.

Pharmacological Evaluation

Behavioral parameters were monitored by Open field test [Figure 6], Marble burying test [Figure 7], and Water maze test [Figure 8]. In open field test, head dipping, rearing and line crossing were considered for monitoring the cognitive activity. Behavioral data obtained from the marble burying test is a parameter to test the locomotion. The behavioral studies proved that the enhancement in the locomotion, memory, spatial learning etc shown by the prodrugs compared to that of the parent NSAID that indicates that the potential activity of (+)-IBN is nil due to the inability to cross BBB.

Biochemical Estimation

The evaluation of antioxidant parameters indicated the protective effect of the synthesized amide prodrugs against neurodegeneration.SOD and Catalase activity were graphically represented in Figure 9.

Histopathological analysis

The histopathology study of the brain cortex was taken and concluded that the prodrug treated group, all the three different aminoacid prodrugs shown the normal architecture of the cells without any spongiform cells. The study proved the protective nature of the NSAIDs shown in Figure 10.

Invitro neuroprotective effect determination by MTT assay

From the cell line studies, the effects of the samples (+)-IBN-2 on H_2O_2 induced cell death was examined in SH-SY5Y cells. As illustrated in the Figure 11 at a concentration of 25 μg.mL^{-1} all two samples are having a neuroprotective activity by measuring the percentage viability.

Anti Inflammatory Activity

The percentage anti inflammatory activity of (+)-IBN and its prodrugs were determined and are given in Table 6. A graphical representation was shown in Figure 12. From the study concluded that the synergistic activity profile of the synthesized amide prodrugs with the significance of < 0.05. After the six hours observations the amide prodrugs showed
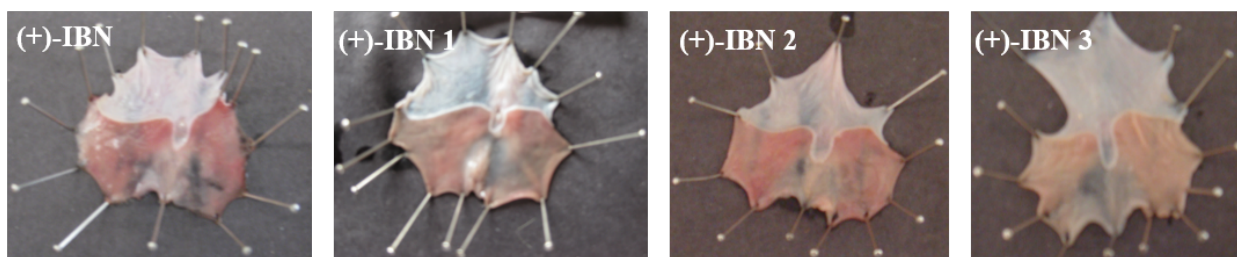
Figure 14: Rat stomach showing ulcers with (+)-IBN and its prodrug

about 70 percentage of anti inflammatory activity that is very high compared to that of the parent (+)-IBN.

Ulcerogenic Activity

The ulcer formation was monitored and quantitatively represented in ulcer index shown in Figures 13 and 14. The ulcer index of standard drug (+)-IBN was found as 25.3 while (+)-IBN 1, (+)-IBN 2, and (+)-IBN 3 showed very less values of 6.5, 7.2 and 8.9 respectively.

CONCLUSIONS

This research study proved that the new amide prodrugs showed better physico chemical properties, transport properties, bio-distribution and pharmacological activities. On the basis of the results, it can be concluded that prodrug approach could successfully attain the goal of improving the transport properties through the BBB and protective effect against the degeneration in brain. The prodrug approach on the NSAIDs provides the improvement in the anti inflammatory activity, reduction in the gastric toxicity and behavioral test, antioxidant parameters revealed the protective effect in the brain. The study revealed that the new synthesized prodrugs showed better lipophilicity, brain targeting efficiency, drug targeting index, relative efficiency and concentration efficiency, anti inflammatory activity and anti ulcerogenecity than the parent drug. On the basis of the results, concluded that the prodrug based drug design lead to the formation of the products with the enhancement of the properties.

Funding Support

None.

Conflict of Interest

None.

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