ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 7, Issue2, 2014

ISSN - 0974-2441

Research Article

BIOLOGICAL EVALUATION OF SCHIFF BASES OF NEW ISATIN DERIVATIVES FOR ANTI ALZHEIMER'S ACTIVITY

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Received: 26 January 2014, Revised and Accepted: 22 February 2014

ABSTRACT

Isatin can be used for synthesis of many heterocyclic compounds having diverse pharmacological activities. Several Isatin derivatives are being developed as promising drugs in the area of central nervous system diseases. In the present study, six novel Isatin derivatives, especially Schiff bases were screened for possible Anti-Alzheimer's activity using the scopolamine model with. Acute oral toxicity, along with in vivo and invitro tests like Radial arm maze (RAM), Morris water maze (MWM), Jumping box test along with assessment of Lipid peroxidation (LPO), Super oxide dismutase (SOD), Catalase and Acetyl choline esterase levels followed by histopathological study of rat brain tissues. Various Schiff bases of Isatin derivatives showed significant anti-amnesic activity as assessed by behavioural test using RAM, MWM and Jumping box test. There was a significant increase ($p < 0.05$) in brain LPO levels in animals treated with scopolamine when compared to scopolamine and Isatin group. There was a significant decrease ($p < 0.05$) in brain SOD, catalase activity in animals treated with scopolamine when compared to scopolamine and Isatin group. Also, the animals treated with Isatin and scopolamine showed a significantly decreased acetyl cholinesterase activity when compared to scopolamine treated groups. The histopathological study also revealed less amyloid formation in groups treated with Isatin derivatives when compared to control group. The anti amnesic effects of Isatin derivatives demonstrated in the present study may be due to anti-oxidant action. Considering the results of behavioural, biochemical and histopathological studies, we hypothesize that Schiff bases of Isatin derivatives may act directly as a free radical scavenger or regulator to inhibit AChE, oxidative activity and ionic homeostasis imbalance in neurons induced by scopolamine.

Keywords: Anti-Alzheimer's activity, Schiff bases, Isatin

INTRODUCTION

Indole and its derivatives have occupied a unique place in the chemistry of nitrogen heterocyclic compounds, because of their varied biological properties. One of the important derivatives of this group is Isatin and its derivatives which are versatile substrates. Isatin is chemically known as 1H- indole-2, 3 dione. Isatin has been known for about 150 years and has been recently found like oxindole and endogenous polyfunctional heterocyclic compounds, to exhibit biological activity in mammals.[1] Isatin is an endogenous indole present in mammalian tissues and fluids. It has distinct and discontinuous distribution in brain and other tissues; highest concentrations in the brain have been shown in the hippocampus. [2]

Isatin can be used for synthesis of many heterocyclic compounds having diverse pharmacological activities. In the past few years, Isatin and its derivatives have received much attention due to their chemotherapeutic values. Studies on several Isatin derivatives reported show considerable pharmacological actions such as antimicrobial, antiviral, anticonvulsant, anti oxidant, anticancer, antibacterial[3-9]. From these results, ideas for future molecular modifications leading to compounds with greater favorable pharmacological properties may be derived.

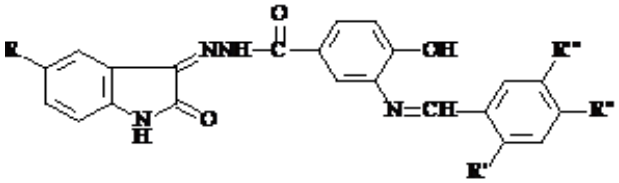
Alzheimer's disease (AD) was first described by the German neurologist Alois Alzheimer in 1906, which is a physical disease affecting the elderly. AD is a progressive dementia affecting cognition, behavior, and functional status with no known cause or cure.[10] AD is associated with a shrinkage of brain tissue, with localized loss of neurons mainly in the hippocampus and basal forebrain. The exact pathophysiological mechanism underlying are not clearly known. [11] Several genetic and environmental factors have been associated in the pathogenesis of AD. Few majorly accepted hypotheses are the Amyloid cascade hypothesis, cholinergic hypothesis and Oxidative stress. AD is characterized by the deposition of extracellular β-amyloid plaques and intracellular neurofibrillary tangles (composed of paired helical filaments) in the brain of AD patients. The loss of cholinergic neurons in the hippocampus and frontal cortex is a feature of the disease. [11, 12] The reduction in cholinergic activity is correlated with the degree of cognitive impairment. Several drugs have been designed to enhance cognitive function in AD patients by targeting acetylcholinesterase (AChE), in an attempt to maximize the effect of ACh by increasing its permanence in the synaptic cleft. Acetylcholinesterase inhibitors (AchEIs) are the best developed therapy and are used for mild to moderate disease. The mechanism by which AchEIs slow progression of disease is thought to be by decreasing levels of amyloid-β protein precursor (AβPP) and production of Aβ and amyloidogenic compounds. Tacrine was the first widely used AchEI. Second generation drugs like donepezil, galantamine and rivastigmine have been developed, These drugs have fewer side effects, longer half-lives, and greater efficacy.[13,14] Among oxidative stress, reactive oxygen species (ROS) and reactive nitrogen species (RNS), including superoxide anion, hydrogen peroxide, hydroxyl radicals, singlet oxygen, alkoxyl radicals, peroxyl radicals, and peroxynitrites, are thought to be associated in etiology of numerous diseases. In particular, peroxynitrites formed by the *in vivo* reaction of nitric oxide with superoxide anions has been implicated in Aβ formation and accumulation, with high levels of Aβ also augmenting peroxynitrites generation in the brain of AD patients. [15, 16] Although present treatment helps to improve AD symptoms, they do not delay disease progression. Therefore, new therapeutic agents that obstruct the disease progression are essential. Several Isatin derivatives are being developed as promising drugs in the area of central nervous system diseases. Presently we have targeted synthesis and screening of novel derivatives of Isatins, especially Schiff bases for possible activity against AD.

MATERIALS AND METHODS

Synthesis of Schiff Bases of Isatin Derivatives

(Isatin-3-[N²-(3-benzalamino-4-hydroxybenzoyl)]hydrazones)

Isatin was prepared by the method developed by Sandmeyer which furnished Isatin in >75% overall yield. [17] Synthetic Schiff bases of Isatin derivatives were prepared using suitable procedures in laboratories of Talla Padmavathi College of Pharmacy. Derivatives were purified chromatographically and authenticated by spectral analysis. More than 20 compounds were synthesized, among them 6 derivatives were screened for anti-Alzheimer's activity.



Compound	R	R'	R''	R'''	IUPAC name of test compounds
TEST-1	H	H	Cl	H	Isatin-3[N"-(3-(4-CI-benzalamino)-4-hydroxybenzoyl)hydrazone]
TEST-2	H	H	$N(CH_3)_2$	H	Isatin-3[N"-(3-(4-dimethylamino)-4-hydroxybenzoyl)hydrazone]
TEST-3	Cl	H	Cl	H	7-ChloroIsatin-3[N"-(3-(4-CI-benzalamino))-4-hydroxybenzoyl)hydrazone]
TEST-4	Cl	H	$N(CH_3)_2$	H	7-ChloroIsatin-3[N"-(3-(4-dimethylamino)-4-hydroxybenzoyl)hydrazone]
TEST-5	CH_3	H	Cl	H	7-methylIsatin-3[N"-(3-(4-CI-benzalamino)-4-hydroxybenzoyl)hydrazone]
TEST-6	CH_3	H	$N(CH_3)_2$	H	7-methylIsatin-3[N"-(3-(4-dimethylamino)-4-hydroxybenzoyl)hydrazone]

Ethical clearance

The care and maintenance of the animals were carried out as per the approved guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi. The research protocol was approved by the Institutional Animal Ethical Committee (IAEC).

Drugs and chemicals

Scopolamine hydrobromide (Buscopan, Boehringer Ingelheim), acetylcholine chloride, 5, 5-dithio-bis2–nitrobenzoic acid, (Ellman's reagent), acetylthiocholine iodide, trichloroacetic acid, thiobarbituric acid (TBA) all were purchased from Sigma-Aldrich (Bangalore, India), Piracetam (Nootropil®)

Acute oral toxicity study

The procedure was followed by using OECD guidelines (Organization of Economic co-operation and Development) 423 (Acute Toxic Class Method).[18] Twelve female mice weighing 20-30g were used for study. The starting dose level of Isatin derivatives was 300mg/kg body weight p.o. As most of the Isatin derivatives posses LD value more than 300mg/kg body weight p.o. so the strating dose was used is 300mg/kg bodyweight p.o. Dose volume was administered 0.1ml/10gm body weight to the mouse which were fasted over night with water ad libitium. Food was withheld for a further 3-4 hours after administration of drug. Body weight of the mice before and after termination were noted and any changes in skin and fur, eyes, mucous membranes, respiratory, circulatory, autonomic and central nervous system, somatomotor activity and behavior pattern were observed. Also, the mice were checked for signs of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma were noted.

Anti-Alzheimer's activity and Histopathology

For evaluation of anti Amnesic activity animals were assigned into nine groups of six animals each. The drug treatment protocol is shown in **(Table-1)**. Animals were trained for maze task performance by conducting one daily training trial. It took 7 days to get the animal completely trained, during which they did not receive any drug. Completely trained animals were chosen for the study. These animals were dosed once in a day with the respective drugs for 8 days along with daily training trial. Scopolamine was given on 8th day 45 min prior to the treatment. After one hour all animals were tested on Radial arm maze and Morris water maze task performance and passive conditioned avoidance test.

Biochemical Estimation of Markers of Oxidative Stress

Biochemical tests were conducted 24 h after last behavioral test (Morris water maze). The animals were sacrificed by decapitation. Brains were removed and rinsed with ice-cold isotonic saline. Brains were then homogenized with ice-cold 0.1 mmol/l phosphate buffer (pH 7.4). The homogenates (10% w/v) were then centrifuged at 10,000 *rpm* for 15 min and the supernatant so formed was used for the biochemical estimations. The amount of lipid peroxidation products present in the homogenate samples of brain was estimated by the thiobarbituric acid reactive substances (TBARS) method.[19] Superoxide dismutase (SOD) activity was assayed spectrophotometerically, as the inhibition of photochemical reduction of nitro-blue tetrazolium (NBT) at 560 nm.[20] Catalase measurement was done based on the ability of catalase to oxidize hydrogen peroxide following the method of Luck.[21] AChE is a marker of loss of cholinergic neurons in the forebrain. The AChE activity was assessed by Ellman method.[22]

Histopathological studies

After draining the blood, brain samples were excised, washed with normal saline and processed separately for histological observations. Initially, the materials were fixed in 10% buffered neutral formalin for 48 h and then with alcohol and xylene solution for 6 h. Paraffin sections were taken at 5 mm thickness, processed in alcohol–xylene series and were stained with hematoxylin and eosin. The sections were examined microscopically for histopathological changes.

Statistical analysis

The results are presented as the mean ± SEM. For evaluation of antiamnestic activity statistical analysis was done by one way ANOVA followed by Dunnet's t-test.

RESULTS

For the acute oral toxicity, the starting dose of 300mg/kg B.W/P.O of Schiff bases of Isatin compounds were administered. There was no significant changes in body weight before and after termination of the experiment and no signs of toxicity was observed. The experiment was terminated on 14th day. The experiments were repeated again with the same dose level, 300mg/kg b.w/p.o of Schiff bases of Isatin derivatives for 3 days more. No significant changes were observed from the first set of experiment. LD cut off mg/kg body weight was observed as X (unclassified) and Globally Harmonized System (GHS) classes also comes under X (Unclassified).

Various Schiff bases of Isatin derivatives showed significant antiamnesic activity as assessed by behavioral test using Radial arm Maze (RAM) and Morris water Maze (MWM). In Radial arm Maze, there was significant reduction of pretreatment with Schiff bases of Isatin derivatives (30mg/kg,P.O) and Piracetam (200 mg/kg, I.P) in time taken for successful trail on 1st day (acquisition) and 8th day (retention) when compared to Positive control group, while scopolamine 1 mg/kg,I.P. significantly increased the time taken for successful trial compared to control group. In Morris water Maze Schiff bases of Isatin derivatives (30mg/kg,P.O)and piracetam (200mg/kg,I.P) significantly decreased latency when compared to positive control, indicating their effect on improvement in learning

and memory in retention trial. However, scopolamine increased the latency significantly .In Jumping box test, i.e., active avoidance

paradigm there was a significant decrease in avoidance response on 5th day as compared to 1st day in the control group**.** Significant increase in avoidance response was observed in scopolamine treated group compared to control group. However standard drug (Piracetam) treatment and Schiff bases of Isatin derivatives (3omg/kg (p.o) treatment significantly decreased the avoidance response compared to scopolamine treated group. This reflects the effectiveness of Piracetam as well Isatin derivatives during memory loss. Piracetam group showed decreased avoidance response compared to Isatin derivatives (30 mg/KG(P.O) treated group at the end of 5th day. All the observations are noted in **Table-2.**

Scopolamine treatment significantly increased the brain LPO levels, decreased the SOD and Catalase activity when compared to control group. Piracetam and Schiff bases of Isatin derivatives (30 mg/kg, P.O) treatment significantly decreased ($p < 0.05$) brain LPO levels, increased the SOD and Catalase activity to their corresponding scopolamine treated group **(Table-3)**. Also, Scopolamine treatment significantly increased acetyl cholinesterase activity in brain as compared to control. However, Piracetam and Isatin derivatives (30mg/kg, P.O) treatment significantly decreased acetyl cholinesterase activity as compared to positive control **(Table-3)**. The Histopathology of brain samples were studied by Professional Medical Pathologist. The Hippocampal lesions were assessed microscopically at 40 magnifications. The observations are given in **Table-4 & Figure-1.**

DISCUSSION

Formation of memory is the most complex process and involves multiple neuronal pathways and neurotransmitters. It is well known that the cholinergic neuronal system plays an important role in learning and memory in humans and animals.[23] Cholinergic deficits are neuropathological occurrences that are consistently associated with memory loss and are correlated with the severity of Alzheimer's disease [24] and it mimics the cognitive symptomology of AD.[25] Based on a cholinergic hypothesis, many attempts have been made to reverse cognitive deficits by increasing brain cholinergic activity via acetylcholinesterase (AChE) inhibitors. Administration of AchE inhibitors that increase the availability of Acetylcholine (Ach) at cholinergic synapses are rationale target for developmental programs targeting treatment of Alzheimer's symptoms. Learning and memory can be conceived as both a psychological process, as well as a change in synaptic neural connectivity. [26]

Age, stress and emotion are conditions that may lead to memory loss, amnesia, anxiety, high blood pressure, dementia, to more ominous threat like schizophrenia and AD. The degeneration of some cholinergic brain nuclei as those located in basal forebrain, particularly within the septohippocampal acetylcholinergic systems involved in learning and memory Processes is a main change affecting a specific neural system in the brain of patients with AD.[27]

The present study demonstrates that beneficial effect of Isatin derivatives on scopolamine induced amnesia. These derivatives significantly ameliorated the cognitive deficit. Isatin derivatives showed significant antiamnesic activity as assessed by behavioral test using Morris water Maze (MWM) and Radial arm Maze (RAM). Series of paradigms for evaluation of memory performance is carried out that work upon different mechanisms.[28] Various mazes are used conventionally to assess the learning and memory paradigms in animals.[29]

Scopolamine-induced impairment of spatial working memory in rats has been reported in previous studies and a dose of 0.4 mg/kg, i.p was used for the induction of amnesia and memory dysfunction.[30] Thus, the scopolamine-induced amnesic murine model is useful for investigating age-related senile CNS dysfunction. Hippocampal cholinergic neurotransmission plays a critical role in the process of underlying learning and memory.[31,32] Scopolamine, a nonselective muscarinic antagonist, blocks cholinergic signaling without changing the acetylcholine concentration, and produces memory deficits that are similar to those found in age-related senile CNS dysfunction.[33] It has been demonstrated by previous animal and human studies that learning and memory can be modified by drugs affecting the central cholinergic system.[34] Acetyl cholinesterase is the enzyme responsible for acetylcholine hydrolysis which terminates the cholinergic transmission. It is currently believed that the action of this enzyme could affect the underlying processes in AD. [35]

RAM performance is an appetitive motivated task and is also useful to assess the spatial reference as well as spatial working memory performance and agents that affect these processes.[28] Results of this study showed that oral administration of Schiff bases of Isatin derivatives significantly decreased the time taken for successful trial that had received the scopolamine, suggesting that these derivatives ameliorated the memory impairment. The Morris water maze learning task is used to assess hippocampal-dependent spatial learning ability. [36] The paradigm we used in the- Morris water maze test enabled the simultaneous analysis of distinctions between working and reference memory processes.[36,37] Escape latency reductions from day to day reflect learning with respect to reference or long-term memory.[36] Impairment in long term memory was observed in the scopolamine treated group. Pretreatment with Isatin derivatives (30mg/kg, P.O) significantly shortened the escape latency time that had received the scopolamine, suggesting that Isatin derivatives ameliorated the memory impairment. From the behavioral test i.e. jumping box active avoidance test, it is clearly seen that there was general decrease in the performance in the active avoidance in scopolamine treated groups. The Schiff bases of Isatin derivatives (30mg/kg, P.O) increase the active avoidance performance which indicates therapeutic efficacy of derivatives against memory loss. Other important activity has been shown by these derivatives is that it has acetylcholinesterase (AchE) inhibiting activity. This activity tends to allow the more retention of acetylcholine in the brain, which is important for the cognitive function, learning and memory. The Histopathological study also showed that the beneficial effects of Isatin derivatives in reducing the amyloid formation when compared to scopolamine group.

Many clinical studies have reported strong evidence that oxidative stress is involved in the pathogenesis of Alzheimer's disease.[38] Oxidative stress is critical detriment factor in the stimulation of neuronal cell death, and Aβ toxicity results in an increase in the Reactive oxygen species (ROS) and superoxide radicals, which result in oxidative damage within the cell. The toxicity of Aβ is attenuated by treatment with antioxidants such as vitamin E, as well as the agents that decrease intracellular superoxide levels.[39] In our experimental conditions, scopolamine administration resulted in a significant increase in TBARS, an important marker for lipid peroxidation, and in a reduction in both SOD and Catalase activities in amnesic rats.[40]

Pretreatment with Schiff bases of Isatin derivatives (30mg/kg,P.O) produced a significant decrease in TBARS and SOD and Catalase activities are restored. Our results suggest that the anti amnesic effects of Isatin derivatives demonstrated in the present study may be due to anti-oxidant action. Based on the results of behavioral, biochemical and histopathological studies, we hypothesize that Schiff bases of Isatin derivatives may act directly as a free radical scavenger or regulator to inhibit AChE, oxidative activity and ionic homeostasis imbalance in neurons induced by scopolamine. In addition these derivatives, an antioxidant medication may also be prescribed to a patient with elevated brain oxidative stress parameters.

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