Isatin, an endogenous MAO inhibitor, and a rat model of Parkinson’s disease induced by the Japanese encephalitis virus

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Summary: A single dose of isatin (indole-2,3-dione)(i.p.), an endogenous MAO inhibitor, significantly increased norepinephrine and 5-hydroxytryptamine concentrations in the rat brain and also significantly increased acetylcholine and dopamine (DA) levels in the rat striatum. Urinary isatin concentrations in patients with Parkinson’s disease tend to increase according to the severity of disease. We have developed a rat model of Parkinson’s disease induced by the Japanese encephalitis virus (JEV). The distribution of the pathological lesions of JEV-rats resemble those found in Parkinson’s disease. Significant behavioral improvement was observed in JEV-rats after isatin, L-DOPA and selegiline administration using a pole test. Both isatin and selegiline prevented the decrease in striatum DA levels of JEV-rats. The increased turnover of DA (DOPAC=DA) induced by JEV was significantly inhibited by isatin, but not selegiline. These findings suggest that JEV-infected rats may serve as a model of Parkinson’s disease and that exogenously administered isatin and selegiline can improve JEV-induced parkinsonism by increasing DA concentrations in the striatum.

Endogenous monoamine oxidase (MAO) inhibitory component was first discovered in normal human urine by Glover et al. (1980) and the compound responsible for the MAO activity was subsequently given the name “tribulin” (Sandler, 1982). In 1988, isatin was identified as a major constituent of tribulin (Glover et al., 1988). We also identified isatin as one of the extracts in the urine of stroke-prone spontaneously hypertensive rats (SHRSP) (Hamaue et al., 1992) and in SHRSP brains (Hamaue et al., 1994) using gas chromatography-mass spectrometry (GC-MS). Tribulin may be responsible for the metabolites of isatin-related compounds (McIntyre and Norman, 1990). MAO inhibitory drugs such as selegiline have been widely used in clinical practice, originally for depressive illness, anxiety and for Parkinson’s disease. There is, however, virtually no information regarding the detailed central nervous system (CNS) function of isatin. Some experiments suggested that isatin can serve as a marker of stress and anxiety (Glover et al., 1991; Tozawa et al., 1998). We previously reported that exogenously administered isatin increased dopamine (DA) levels in the rat striatum (Hamaue et al., 1999a) and recently demonstrated that a significant increase in urinary isatin excretion was present in patients with Parkinson’s disease (Yahr’s classifications III, IV, V) (Hamaue et al., 2000). These results suggest that isatin may be associated with the pathogenic process in Parkinson’s disease. In this study, we analyzed neurochemical and pathological changes in our rat model of Parkinson’s disease induced by the Japanese encephalitis virus (JEV). By analyzing the DA levels together with movement problems, we evaluated the potential treatment of JEV-induced parkinsonism by endogenous MAO inhibitor isatin and synthesized MAO-B inhibitor selegiline. Furthermore, we analyzed and evaluated the MAO-inhibitor effects on neurochemical and pathological changes in our rat model of Parkinson’s disease induced by JEV.

Chemistry and MAO activity of isatin, an endogenous MAO inhibitor

Several previous reports have shown that stress can induce a decrease in MAO inhibitory activity (Clow et al., 1988). Isatin was identified as a major constituent of tribulin, a low-molecular-mass inhibitor of MAO type B (Glover et al., 1988). Tribulin output in human urine increases during various conditions of stress and anxiety (Clow et al., 1988). Cold immobilization stress has been shown to be associated...
with the serotonergic system (Oxenkrug and McIntyre, 1985). Cold-restraint stress increases the tribulin content in the rat heart and kidney (Armando et al., 1988).

Tribulin, which acts on central benzodiazepine receptors, has been proposed as an anxiety-promoting agent (Sandler, 1982). The MAO inhibitory and benzodiazepine receptor binding inhibitory components of tribulin are roughly equipotent (Clow et al., 1983). Tribulin differs from most other benzodiazepine receptor ligands in that it is not a peptide, because tribulin is extracted with ethyl acetate (Elsworth et al., 1986). Glover et al. (1988) reported that endogenous isatin has properties similar to tribulin. Isatin emerged as one of the major MAO inhibitory compounds to be isolated from human urine. Substantial concentrations of isatin are present in urine and tissues of both the rat and human.

Isatin is well known as a pharmacological agent and its effects have been studied in a variety of systems. It is a selective MAO-B inhibitor. At much higher concentrations it inhibits a variety of other enzymes, such as alkaline phosphatase (Bansal et al., 1988). There are also several reports of its in vivo effects. In rodents, isatin is anxiogenic and urinary excretion is increased after cold exposure (Tozawa et al., 1998). It remains unclear, however, whether all of the MAO inhibitory and benzodiazepine displacing activity attributed to tribulin is due to isatin (Glover et al., 1988). Jarman et al. (1990) have measured urinary tribulin and isatin in parallel and found a highly significant but not complete correlation between the two ($r = 0.68$, $n = 18$, $p < 0.001$ in migraine patients). Tribulin may be responsible for metabolites of isatin or related endogenous compounds (McIntyre and Norman, 1990). The physiological and pathological roles of isatin are not yet clear. Its molecular formula and weight are $C_8H_5NO_2$ and 147.14. It dissolves easily in water as well as in organic solvents such as ethyl acetate, although not in acetone (Glover et al., 1991).

In our in vitro study, isatin was found to be a potent MAO inhibitor that was more active against MAO-B than MAO-A. The $IC_{50}$ of the MAO total, the MAO-A and the MAO-B, was $1.5 \times 10^{-5}$, $5.8 \times 10^{-5}$ and $1.4 \times 10^{-5}$ M, respectively (Hamaue et al., 1992). Line Weaver-Burk plot and Dixon analysis indicated that isatin competitively inhibited the MAO activity of the rat liver homogenate in a concentration-dependent manner (Hamaue et al., 1992).

Tribulin has endogenous MAO and benzodiazepine binding inhibitory activity. It is extractable from biological tissues and body fluids into ethyl acetate. Research (Glover et al., 1988) on tribulin (low molecular weight endogenous inhibitory activity of MAO) has confirmed that its level is increased in both human urine and rat tissues by stress or anxiety, and by anxiogenic drugs. Isatin is thought to be a portion of tribulin that is a selective inhibitor of MAO-B. Other portions of tribulin, the ethyl and methyl esters of indoleacetic and 4-hydroxyphenylacetic acids, selectively inhibit MAO-A (Medvedev et al., 1996). Pathways for the synthesis and metabolism of isatin have not been established. One possible source would be via the action of the gut flora. It has been suggested that dietary tryptophan may be converted into indole by the gut flora and then transported to the liver where it is oxidized. Urinary isatin excretion is significantly reduced in germ free rat urine (0.22 μg/ml) compared with the urine of control rats (0.66 μg/ml) (Glover et al., 1991). This suggests that isatin is derived, at least in part, from the gut flora, which act perhaps on tryptophan containing food.

The stroke-prone spontaneously hypertensive rat (SHRSP)-stroke model in which stroke was clearly detected by pathological examination had significantly higher plasma norepinephrine (NE) levels than the controls. Plasma NE levels of the Wistar Kyoto rats (WKY), SHRSP-control and SHRSP-stroke groups were $248.8 \pm 23.2$, $346.9 \pm 35.2$ and $466.0 \pm 85.7$ pg/ml, respectively, while the mitochondrial MAO activity of the three groups was $146.4 \pm 13.3$, $87.1 \pm 11.2$ and $72.1 \pm 8.64$ (mean ± SE, $n = 12$) nmol/hr/mg protein, respectively. An inverse relationship was demonstrated between plasma NE levels and kidney MAO activity in SHRSP and WKY. Parvez and Parvez (1973) reported that catechol-o-methyltransferase (COMT) activity was related to the amount of substrate present in normotensive rats. It was assumed that the relative increase in red cell COMT activity was due to an increased level of circulating catecholamines. A compensatory increase in COMT and MAO activity may be an important factor in the control of plasma catecholamines in normotensive rats.

In brain and kidney extracts (Hamaue et al., 1992), tribulin-like activity was found to be significantly higher in SHRSP than in WKY. Tribulin activity was also significantly greater in the extract of SHRSP urine than in that of WKY (Hamaue et al., 1992). It has been suggested that impairment of the central adrenergic neurons is associated with blood pressure control and MAO activity in SHRSP (Minami et al., 1988). Thus, it is important in the regulation of monoamine concentrations (Blaschko, 1973). Although mechanisms other than enzyme concentration have been postulated for the regulation of MAO activity in vivo, evidence of short term regulation of the enzyme is scarce. Normal human urine inhibits MAO (Glover et al., 1988). This inhibitory activity is also present in SHRSP urine (Hamaue et al., 1992). Although the mechanism of decreased MAO activity induced by tribulin (or isatin) has not
yet been elucidated, it was postulated that increased blood pressure may be associated with increased monoamine concentrations induced by tribulin (or isatin) in SHRSP.

In line with the above, we isolated and identified isatin as one of the extracts in SHRSP urine (Hamaue et al., 1992) and in SHRSP brains (Hamaue et al., 1994) using GC-MS.

Characteristics of the biological properties of isatin

Isatin has a wide spectrum of biological properties against stress and certain infections (Glover et al., 1991). Isatin has been shown to inhibit a number of enzymes such as acid phosphatase (Singh et al., 1977), alkaline phosphatase (Bansal et al., 1988), hyaluronidase (Kumar et al., 1977), xanthine oxidase (Susheela et al., 1969), as well as MAO. Isatin has been found to act as an antiseizure agent in a variety of tests (Chocholova and Kolinova, 1979). It potentiates the antiseizure action of propranolol (Muller and Schramek, 1989). It also appears to increase vigilance and reduce slow wave sleep (Chocholova and Kolinova, 1989). It also appears to increase vigilance and reduce slow wave sleep (Chocholova and Kolinova, 1989), and Schramek, 1989). It also appears to increase vigilance and reduce slow wave sleep (Chocholova and Kolinova, 1989).

Acute effect of exogenously administered isatin on tissue monoamine concentrations in the rat

A single dose of isatin (50 or 200 mg/kg, i.p.) increased 5-HT concentrations measured 2 hours later in various brain regions of WKY and SHRSP (Hamaue et al., 1994). The magnitude of changes caused by isatin in SHRSP was lower than that observed in WKY. The ratio of 5-hydroxyindoleacetic acid (5-HIAA)/5-HT was significantly decreased by isatin in both WKY and SHRSP. In vitro, 5-HT is primarily metabolised by MAO-A (Yang and Neff, 1974). These data indicate that isatin significantly affects 5-HT activity which may, in turn, have an important physiological effect on CNS function. Several studies have shown that acute peripheral administration of isatin causes an increase of monoamines such as NE and 5-HT in the brain (Battacharya and Acharya, 1993; Hamaue et al., 1994; McIntyre and Norman, 1990; Yumier, 1990). Isatin passes into the brain from the periphery, but a peripheral dose of 50 or 100 mg/kg results in a brain concentration of about 9 mg/kg (Battacharya et al., 1993). The dose of isatin for these experiments was selected on the basis of previous reports that doses of higher than 40 mg/kg produced physiological changes (Chocholova et al., 1981; McIntyre and Norman, 1990). A single injection of isatin (50 or 200 mg/kg, i.p.) did not induce any significant cardiovascular or behavioral effects in either WKY or SHRSP.

Effects of isatin on acetylcholine and dopamine concentrations in the rat brain

Kumar et al. (1993) first reported that isatin inhibits acetylcholine esterase (AChE) activity in the rat brain and erythrocytes. We determined the levels of ACh, choline
From the striatal cholinergic interneuron is induced by D2 release (Nilsson et al., 1992), indicating that ACh release into the hippocampus. Apomorphine also increases ACh release. Many reports have demonstrated that perfusion with DA increased the release of ACh from the striatum (Gorell and Czarnecki, 1986; Imperato et al., 1993; Scatton, Hamaue et al., 1992). Ohue et al. (1992) reported that D2 dopaminergic receptors increase ACh release, therefore, may be increased by DA rise after isatin administration.

Table 1. Acetylcholine (ACh), choline (Ch), and dopamine (DA) concentration in the striatum of Wistar Kyoto rats after isatin administration

| Treatment | ACh  | Ch  | DA   |
|-----------|------|-----|------|
| Control   | 42.7 ± 2.8 | 14.7 ± 1.7 | 25.7 ± 4.1 |
| 50 mg/kg  | 38.5 ± 0.7  | 12.4 ± 0.3  | 39.8 ± 4.0* |
| 200 mg/kg | 53.1 ± 3.2* | 20.5 ± 1.5* | 53.6 ± 4.6** |

Mean ± SEM, n = 5–7; ACh, Ch, DA pmol/mg wet tissue; * p < 0.05; ** p < 0.01 vs control

(Ch) and DA in the rat brain 2 hrs after isatin administration (Hamaue et al., 1999a) using HPLC-ECD according to the method of Matsumoto et al. (1990). As shown in Table 1, ACh and Ch levels in the striatum of the group receiving isatin (50 or 200 mg/kg, i.p.) significantly increased. Striatal DA levels also increased after isatin treatment (Hamaue et al., 1999a). In other words, isatin simultaneously increased ACh and DA levels in the WKY striatum. In our in vitro study, 10^{-4} M of isatin induced an approximate 93% inhibition of MAO and a 5% inhibition of AChE in the rat brain. It is clear that isatin has a higher affinity to MAO than to AChE. Isatin administration also increased Ch, an AChE metabolite of ACh, in various brain regions. These results suggested that isatin increased ACh levels not by inhibiting AChE activity, but rather through another pathway.

In the microdialysis study, rats were placed in a stereotaxic apparatus and their right striatum were implanted with a guide cannula (0.8 mm outer diameter) under pentobarbital anesthesia (60 mg/kg, i.v.) according to the method of Paxinos and Watson (1980). Isatin administration (10^{-4} M) caused a significant rise in the extracellular levels of ACh and DA (Hamaue et al., 1999a). During microdialysis with isatin, the DA release from the WKY striatum was greater than the ACh release. Many reports have demonstrated that D2 dopaminergic receptors increase ACh release (Gorell and Czarnecki, 1986; Imperato et al., 1993; Scatton, 1992; Wedzong et al., 1988). Ohue et al. (1992) reported that perfusion with DA increased the release of ACh from the hippocampus. Apomorphine also increases ACh release (Nilsson et al., 1992), indicating that ACh release from the striatal cholinergic interneuron is induced by D2 receptor stimulation. The output of striatal ACh is inhibited by D2 receptors (Lehmann and Langer, 1983; Stoof et al., 1992). The pronounced stimulatory effects of DA antagonists on the release of striatal ACh and their opposite effect on tissue concentration have been reported (Sethy and van Woert, 1974; Stadler et al., 1973). ACh release, therefore, may be increased by DA rise after isatin administration.

Urinary isatin excretion in patients with Parkinson’s disease

We have reported that exogenously administered isatin significantly increased ACh and DA levels in the rat striatum (Hamaue et al., 1999b). In order to elucidate the relation between isatin and Parkinson’s disease, we measured the urinary isatin excretion of patients with Parkinson’s disease (Hamaue et al., 2000). We have developed a convenient, alternative method for the determination of isatin by high-performance liquid chromatography (HPLC) (Hamaue et al., 1998) to replace the GC-MS determination (Hamaue et al., 1994).

Urinary isatin concentration in Parkinson’s disease tended to increase in accordance to the degree of Hoehn and Yahr criteria (1967). A significant increase in urinary isatin excretion was observed in patients with Stage III (102.71 ± 28.29, p < 0.05, n = 13), VI (129.29 ± 65.92, p < 0.05, n = 6) and V (267.05 ± 154.48, p < 0.01, n = 8) Parkinson’s disease as compared with that of healthy control subjects (52.00 ± 24.29, n = 11). At these stages, Parkinson’s patients demonstrate severe clinical symptoms such as tremor, spastic gait, freezing and masked face. One of the reasons for increased urinary isatin in Parkinson’s disease might be due to the stress of this disease or to the compensatory increase response to a lower level of cerebral dopamine content. Patients taking drugs for the treatment of Parkinson’s disease were included in this study. Urinary isatin concentrations in drug-treated patients with Parkinson’s disease at Stages I (38.11 ± 25.22, n = 6) and II (62.97 ± 27.64, n = 15) tended to decrease compared with those of patients without medication. These results suggest that urinary isatin is an endogenous marker for the clinical severity of Parkinson’s disease.

A rat model of Parkinson’s disease induced by the Japanese encephalitis virus

Animals and virus

The virus strain used was the JaGA-r01 strain of JEV. The supernate from 10% homogenates of infected mouse brains (10^6 PFU/ml) were diluted with 20% Hemaccel (Hoechst) in Eagle’s minimum essential medium and stored at -70°C until use. The virus (0.03 ml containing 3 x 10^6 PFU) was inoculated intracerebrally with a specially designed two-step thin 27 gauge needle (Hoshimori Iryoki KK, Tokyo, Japan) with a stopper 3 mm from the tip. The site of inoculation was located at the midpoint of the line connecting the left eye to the midpoint between the right and left ears.
of Albino rats of the Fischer strain. We used animals older than 17 days because the mortality rate of animals infected when they were older than 12 days decreased with age, with 13-day-old rats having a 50% mortality rate and 14-day-old rats having 8.3%. Animals inoculated when they were older the 17 days showed 0% mortality.

**Neuropathologic study**

The topographical distribution of JEV antigen in the developing rat brain was determined 3 days after JEV inoculation (Ogata et al., 1991). The neuropathologic changes in rats infected with JEV on days 12, 13 and 14 after birth were examined. Animals were sacrificed 3 days, 10 days, 12 weeks or one year after inoculation under ether anesthesia by perfusion fixation via the aorta with 4% freshly prepared paraformaldehyde in 0.1 M phosphate buffer. Coronal brain sections were taken from the frontal tip to the medulla, embedded in paraffin and stained with hematoxylin-eosin, Luxol fast blue-cresyl violet (Klüver-Barrera method), anti-JEV antibody and anti-tyrosine hydroxylase (TH) monoclonal antibody (Chemicon). The avidin-biotin-peroxidase complex (ABC) method was used in this immunohistochemical study (Hsu et al., 1981). After deparaffinization, the specimens were treated with 0.3% H2O2-methanol to suppress endogenous peroxidase activity, incubated with 10% normal goat serum, and allowed to react with anti-JEV rabbit serum or anti-TH monoclonal antibody, diluted in 1% bovine serum albumin (BSA) at 4°C overnight. Incubation with 1% BSA was used as a negative control. The sections were reacted with anti-JEV antibody. Anti-TH monoclonal antibody were then reacted with biotinylated goat anti-rabbit IgG and biotinylated goat anti-mouse IgG (Vestain), respectively. ABC reaction products were visualized with 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and counterstained with hematoxylin.

**Assessment of motor function**

A pole test (Ogawa et al., 1985) was performed to evaluate bradykinesia in the rats. The time it took the rats to descend from the top of a rough-surfaced pole (2.5 cm in diameter and 100 cm in height) to the floor was recorded in JEV-infected adult rats and control adult rats. We measured the motor activity of the adult rats (12 weeks after infection) infected with JEV at the age of 13 days and age-matched control rats. The difference between the JEV-infected rats and the control rats was significant (p < 0.001). The pole test showed a marked bradykinesia in the JEV-infected rats. Masked faces or tremor could not be assessed in the rats.

**Characteristics of the model for Parkinson’s disease induced by the Japanese encephalitis virus**

The pathogenesis of Parkinson’s disease currently is thought to depend upon hereditary, aging and environmental factors (Burns et al., 1983; Calne and Langston, 1983; Ballard et al., 1985; Cohen, 1986; Nagatsu and Yoshida, 1988; Dexter et al., 1989; Riederer et al., 1989; Adams and Odunze, 1991). Among the toxic factors, exogenous toxins such as MPTP and endogenous toxins such as free radicals or tetrahydroisoquinoline (Yoshida et al., 1990) have been implicated. Viruses can also selectively attack the substantia nigra and induce parkinsonism (Duvoisin and Yahr, 1965; Kristensson, 1992). Post-encephalitic parkinsonism is well documented (von Economo, 1917; Yahr, 1978). Walters (1960) described a 54-year-old woman who manifested symptoms of parkinsonism while convalescing from meningococcal meningitis due to Coxsackie B virus. Influenza A virus (Hudson and Rice, 1990), poliovirus (Bojinov, 1971) and measles virus (Alves et al., 1992) have also been suspected from case reports as possible viral causes. Fishman et al. (1980) reported an experimental model with a selective attack on the substantia nigra and subthalamic nucleus induced by a strain of mouse hepatitis virus which is a coronavirus that causes persistent CNS infection. Recently similar features have been described in rats infected with influenza virus (Takahashi et al., 1995).

JEV is a positive-strand enveloped RNA virus that belongs to the family of the flaviviruses and is the most common cause of arthropod-borne human encephalitis worldwide. Goto (1962) detected parkinsonian sequelae in 11.6% of 143 unselected patients five years after they had Japanese encephalitis. The parkinsonian syndrome after Japanese encephalitis differs from that which follows encephalitis lethargica in several respects. In general, parkinsonism following Japanese encephalitis is mild, develops in the acute phase and occasionally improves slightly over a long period. Recently, it was reported that MRI abnormalities were seen mainly in the substantia nigra and putamen in a case of typical parkinsonism following Japanese encephalitis (Shoji et al., 1993). Patients without a clear history of encephalitis who follow the clinical course and have pathologic findings consistent with postencephalitic parkinsonism have been reported (Gibb and Lees, 1987; Geddes et al., 1993). Although there were no Lewy bodies found in the substantia nigra in the JEV-treated rats, the pathologic findings otherwise resembled those of idiopathic Parkinson’s...
The effects of isatin and selegiline on bradykinesia and dopamine levels in a rat model of Parkinson’s disease induced by the Japanese encephalitis virus

JEV is the most common cause of arthropod-borne human encephalitis in Asia (Johnson et al., 1985) and may also be a cause of post-encephalitis parkinsonism (Dickerson et al., 1952). Ogata et al. (1997, 1998) have reported pathological results that in adult Fisher rats sacrificed 12 weeks after infection with JEV at 13 days, the number of tyrosine hydroxylase (TH)-positive cells was decreased in the substantia nigra, suggesting post-encephalitis parkinsonism. Many of the existing therapies of Parkinson’s disease counteract the detection of DA levels in the striatum as a result of the disease. The JEV-infected rat model showed marked bradykinesia, with significant behavioral improvement being observed following administration of L-DOPA (Ogata et al., 1997). Next, we compared the effects of isatin, an endogenous MAO-inhibitor, on the motor function and DA levels of JEV-induced Parkinson’s model rats with those of selegiline, a selective MAO-B inhibitor (Hamaue et al., 2004). It is an important observation that exogenously administered isatin or selegiline ameliorated the bradykinesia observed in JEV-induced parkinsonism rats. Also, isatin or selegiline significantly increased striatum DA levels in the JEV-infected rats. Selegiline was found to be a potent inhibitor that is more active against MAO-B than MAO-A. Selegiline competitively inhibited the MAO-B activity of the rat brain in a dose-dependent manner (Tipton et al., 1976). This drug is frequently used as an adjunct therapy in the treatment of Parkinson’s disease (Berry et al., 1994; Gerlach et al., 1996). As a MAO-B inhibitor and a derivative of amphetamine, selegiline can alter catecholaminergic neurotransmission resulting in a neuroprotective effect (Bursey and Eichenbaum, 1996). The dose of selegiline used in the treatment of parkinsonism (5–10 mg daily in tablet) is considered to be low enough to block MAO-B selectively while leaving MAO-A activity unaffected (Knoll, 1978). We medicated the JEV rat with 0.2 mg/kg selegiline. Selegiline recovered movement function. The striatum DA concentrations in JEV-infected rats were increased by selegiline medication. These results suggest a high efficacy and a therapeutic role in Parkinson’s disease. In conclusion, both MAO-B inhibitor isatin and selegiline prevented the decrease in striatum DA levels in JEV-rats. The increased turnover of DA (DOPAC/DA) induced by JEV was significantly inhibited by isatin, but not by selegiline. The effect of isatin on DA turnover may be one of its main roles as an endogenous MAO-B inhibitor. Hence, isatin could be a new treatment for Parkinson’s disease as an endogenous MAO-B
inhibitor, despite the fact that isatin is unlikely to be directly related to the etiology of Parkinson’s disease (Ogata et al., 2002) by increasing DA levels in the striatum.

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