Non-steroidal anti-inflammatory agents, tolmetin and sulindac, inhibit liver tryptophan 2,3-dioxygenase activity and alter brain neurotransmitter levels

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Abstract

Hepatic tryptophan 2,3-dioxygenase (TDO) is one of the rate-limiting enzymes in tryptophan catabolism and plays an important role in regulating the physiological flux of tryptophan into relevant metabolic pathways. In this study, we determined the effect of the non-steroidal anti-inflammatory agents, tolmetin and sulindac, on rat liver TDO activity and the subsequent changes in the hippocampal and striatal neurotransmitter levels. The amount of melatonin produced by the pineal gland was also measured using high performance liquid chromatography (HPLC). Treatment of rats with tolmetin or sulindac (5 mg/kg/bd for 5 days) significantly inhibited liver TDO activity. The results show that whilst tolmetin and sulindac increase serotonin levels in the hippocampus, these agents also significantly reduce dopamine levels in the striatum. Tolmetin, but not sulindac, increased the amount of melatonin produced by the pineal gland. The results of this study suggest that whilst tolmetin and sulindac may be beneficial for patients suffering from depression, these agents also have the potential to induce adverse effects in patients suffering with neurological disorders such as Parkinson's disease.

Introduction

Hepatic tryptophan 2,3-dioxygenase (TDO) is the rate-limiting enzyme in the oxidative breakdown of tryptophan to kynurenine in the body. Its activity determines the relative tryptophan flux into the serotonergic and kynurenine pathways. Hepatic TDO is specific for tryptophan as the substrate (Hayaishi, 1980).

Tryptophan is hydroxylated to 5-hydroxytryptophan by tryptophan hydroxylase, which is present in high concentrations in the pineal gland, and is the rate-limiting step in the synthesis of 5-hydroxytryptamine (5-HT) (Lovenberg et al., 1968). It is the amount of tryptophan available and not the enzyme activity that influences the rate of the reaction (Deguchi and Barchas, 1972 and Bensinger et al., 1974). In the pineal gland there is sufficient aromatic amino acid decarboxylase, which decarboxylates 5-hydroxytryptophan to produce 5-HT.

Serotonin is an important regulator in a wide range of physiological and biochemical processes in vertebrates. Serotonin is involved in the maintenance of circadian rhythmic functions, blood pressure regulation, acts as a neurotransmitter at central and peripheral regions (Kema et al., 2000), and is known to act as its own trophic factor (Mohanakumar et al., 1995). Alterations in brain serotonin levels are known to result in mood disorders, particularly depression (van Praag, 1982). Serotonin can be *N*-acetylated to form *N*-acetyl serotonin, in a reaction catalyzed by the enzyme *N*-acetyltransferase. In most circumstances this is the rate-limiting step in melatonin synthesis (Klein et al., 1997). The 5-hydroxy group of *N*-acetylserotonin is *O*-methylated in the presence of hydroxyl-*O*-methyltransferase to form melatonin.

In the last several years, melatonin, the chief secretory product of the pineal gland (Reiter, 1991), has been found to be both a direct free radical scavenger and an indirect antioxidant (Hardeland et al., 1995, Hardeland, 1997, Reiter et al., 1995 and Reiter et al., 1997), in addition to its function as a neurohormone. Because of these actions, melatonin has been pharmacologically tested for its ability to reduce oxidative damage in a variety of experimental neurological processes and has been found to be highly effective in this respect (Reiter et al., 1997 and Reiter, 1998). Melatonin is both lipid (Costa et al., 1995) and water soluble (Shida et al., 1994), although its lipid solubility is greater and has the ability to traverse almost every organ in the body (Reiter, 1995). It has been demonstrated to be a powerful antioxidant and free radical scavenger (Tan et al., 1993) and reduce oxidative damage in the central nervous system (Reiter, 1998).

Neuroinflammation has been implicated in the pathogenesis of neurological disorders such as Alzheimer's and Parkinson's disease (McGeer, 1988, Castano et al., 1998, Langston et al., 1999 and Cicchetti et al., 2002). Although non-steroidal anti-inflammatory drugs (NSAIDS) may offer neuroprotection via anti-inflammatory action, other mechanisms may also be involved at the neurotransmitter level (Mohanakumar et al., 2000 and Sairam et al., 2003). Any drug used for the treatment of whichever disease should not aggravate the underlying pathology. In this study, we determined the effects of tolmetin and sulindac, commonly used NSAIDS for pain, inflammation, arthritis and rheumatoid arthritis, on brain neurotransmitter levels in the hippocampus and striatum. The effect of these NSAIDS on rat liver TDO activity and biogenic amines such as serotonin, dopamine and their metabolites as well as the amount of melatonin produced by the pineal gland were determined.

Materials and methods

Chemicals

Tolmetin, sulindac, melatonin, L-tryptophan, hematin, dopamine (DA), serotonin, 5-hydroxy-3-indoleacetic acid (5-HIAA) and dihydroxyphenyl acetic acid, (DOPAC), phosphoric acid (PA) and octane sulphonate (OSA) were purchased from Sigma Chemicals Company, St Louis, MO, U.S.A. Trichloroacetic acid (TCA), triethylamine (TEA) and perchloric acid ($HCLO_4$) were obtained from Saarchem, Johannesburg, ethylenediamine tetraacetic acid (EDTA) was purchased from HOLPRO Analytics, (PTY) LTD, Johannesburg, South Africa. HPLC grade, acetonitrile (ACN), methanol (MeOH) and chloroform were purchased from BDH Laboratory Supplies, Poole, England. Minimum Essential Medium (MEM) was purchased from Highveld Biologicals, South Africa. All other reagents were of the highest quality available.

Animals

Adult male rats of the Wistar strain, weighing between 250–300 g were purchased from South African Institute for Medical Research (Johannesburg, South Africa). The animals were housed in a controlled environment with a 12-h light: dark cycle, and were given access to food and water ad libitum. The Rhodes University animal ethics committee approved protocols for the experiments.

Methods

Treatment regimes

Animals were divided into three groups A, B and C. Group A served as the control while groups B and C received either tolmetin or sulindac at a dose of 5 mg/kg/bd for 5 days. On the 6th day animals were killed by neck fracture. The livers were removed, perfused with cold normal saline and stored at − 70 °C until use. The hippocamppi and striatum were dissected from the brain, frozen in liquid nitrogen and stored at − 70 °C until use. The pineal gland from each rat was removed and incubated for 24 h at 37 °C in 50 μl of MEM that was supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ml) in a sterile humidified incubator.

Liver tryptophan 2,3-dioxygenase activity

The activity of the enzyme was determined according to a modified method previously described by Badawy and Evans (1975). The livers were thawed, chopped into fine pieces and homogenised with 60 ml 140 mM KCl/2.5 mM NaOH using a glass–teflon hand held homogenizer. The homogenate was sonicated for a period of 2 min at 30 s intervals for complete release of enzymes from the cells. Finally 0.2 M sodium phosphate buffer pH 7.0 was used to make up the volume required to yield a 10% w/v homogenate. The entire procedure, where possible, was conducted on ice. An aliquot of 15 ml homogenate was added to a flask containing 12.5 ml water. An aliquot of haematin (100 μl) at a final concentration of 2 μM (Badawy and Evans, 1975) was added to samples that were used to determine the total activity of the enzyme. This was stirred for 1 min to allow for the activation of the enzyme. Finally, 2.5 ml of 0.03 M L-tryptophan was added to all flasks and gently stirred. The assay was conducted in triplicate. Aliquots of 3 ml of the assay mixture was transferred to test tubes, stoppered under carbogen and incubated for 1 h at 37 °C in an oscillating water bath. The enzyme activity was determined in the absence and presence of haematin in order to determine the activity of the holo- and apoenzymes of TDO. The

apoenzyme in isolation is inactive but in the presence of haematin becomes fully active. The holoenzyme activity was measured in the absence of haematin while the total activity was measured in the presence of added haematin. The reaction was terminated with the addition of 2 ml of 0.9 M TCA to the reaction mixture and incubated for 2–4 min. The mixture was filtered through a Whatman no. 1 filter paper. Filtrate (2.5 ml) was added to 1.5 ml of 0.6 M NaOH and vortexed. The kynurenine present in the solution was measured at 365 nm spectrophotometrically using the molar extinction coefficient of kynurenine: $\varepsilon = 4540\ M^{-1}.cm^{-1}$. The blank consisted of 2 ml TCA and 1.5 ml NaOH. The TDO activity was expressed as nmol/mg protein/h. Protein estimation was performed using the method described by Lowry et al. (1951).

High performance liquid chromatography (HPLC)–electrochemical evaluation of biogenic amines

Instrumentation and chromatographic conditions

Samples were analyzed on an isocratic HPLC coupled to an electrochemical detector. The chromatographic system consisted of a Waters Millipore Model 510 pump, a Waters electrochemical detector and a Rikadenki chart recorder. Samples were introduced into the system using a Rheodyne Model 7725i fixed loop injector, fitted with a 20 µl loop. Separation was achieved using a C_{18} (Waters Spherisorb©, 5 µm, 250 × 4.6 mm o.d.s. 2 column). The mobile phase consisted of 13% ACN, 8.32 mM OSA, 0.27 mM, EDTA, 0.4–0.45% TEA and 0.2–0.25% PA (v/v) and made to 1000 ml using Milli-Q water. Mobile phase was degassed twice using a 0.45 µM membrane filter prior to use. The flow rate was set at 0.8 ml/min and the electrochemical detector was set at a potential of + 0.74 V.

Biochemical analyses of biogenic amines

5-HT, DA, 5-HIAA and DOPAC were measured according to a similar method described by Muralikrishnan and Mohanakumar (1998). The frozen hippocamppi and striatum were thawed and weighed, deproteinised in 10% w/v of ice-cold $HCLO_4$ (0.1 M) containing 0.01% EDTA. These samples were thereafter sonicated at 50 Hz for 30 s using an ultrasonic cell disruptor. The homogenates were kept at 4 °C on ice for approximately 20 min prior to centrifugation at 10,000 ×*g* for 10 min. The supernatant (10 µl) was directly injected into the HPLC–electrochemical detector system for analyses. Results are expressed as pmol/mg tissue.

HPLC–UV determination of the amount of melatonin produced by the pineal gland

Instrumentation and chromatographic conditions

Instrumentation used was as described above; however a Waters 2487 dual absorbance UV–VIS detector was used instead of an electrochemical detector. The mobile phase consisted of ACN:MeOH:H_20 (4:1:5) at a flow rate of 1 ml/min. The detector wavelength was set at 304 nm and the injection volume was 20 µl. The Breeze software was used to control the HPLC system and to acquire and analyze data.

Method validation

A stock solution of melatonin (0.1 mg/ml) was freshly prepared for each test by weighing melatonin and dissolving it in mobile phase. Dilutions of this stock solution were made appropriately for the concentration range to be studied. Samples for the assessment of linearity and precision were prepared by serial dilution of the stock solution to yield concentrations over the range 100–500 ng/ml. In addition, two concentrations within the calibration range were prepared independently for use as accuracy standards. The limits of quantification and detection were also determined. Specificity was determined by mass spectrometry. Quantitative determination of melatonin was made by comparison of peak heights using known concentrations of melatonin.

Extraction efficiency

To 40 μl of MEM, 10 μl of mobile phase containing known amounts of melatonin was added. The final concentrations of melatonin were 200, 300 and 400 ng/ml. To this, 1 ml of chloroform was added and vortexed. An aliquot of 0.5 ml of the chloroform was removed and evaporated to dryness on a hot water bath. The residue (melatonin) was dissolved in 50 μl of mobile phase and 20 μl was injected onto the column for analyses.

Amount of melatonin produced by the pineal gland

After the pineal glands had been incubated in the 50 μl of MEM at 37 °C for 24 h, the glands were removed and 1 ml of chloroform was added and vortexed. The rest of the experiment was conducted as described above. Results are represented as amount of melatonin produced per pineal in 24 h and are shown as uncorrected values.

Mass spectrometry

The HPLC system consisted of a spectrasystem AS300 autosampler, a spectrasystem UV200 detector and a spectrasystem P2000 pump. The mobile phase consisted of A: ammonium acetate made to pH 3 using formic acid, and B: methanol. A linear gradient from 15% to 90% MeOH was run over 17 min. Separation was achieved on a C_{18} (Waters Spherisorb©, 5 μm, 250 × 4.6 mm o.d.s. 2 column). The LC instrument was interfaced with a Finnigan MAT LCQ mass spectrometer equipped with an electrospray ionization (ESI) source. The mass spectrometer was configured in the positive ion mode and to use selective ion monitoring (SIM) for the melatonin parent ion in the *m/z* range 60–240. The mass spectrometer parameters were as follows: the nebulizer nitrogen gas flow rate was set at 80, discharge voltage of 4.5 kV, the capillary temperature was set at 220 °C, the capillary voltage was 20 V and the discharge current was 5 μA. The LC–MS workstation Xcalibur, version 1.3, was used for system control and data acquisition.

Statistical analyses

Results were analyzed using a one-way analysis of the variance (ANOVA) followed by the Student–Newman–Keuls Multiple Range Test. The level of significance was accepted at $p < 0.05$ (Zar, 1974).

Results

Fig. 1 shows that both the NSAIDS, tolmetin and sulindac, inhibit the holoenzyme but have no effect on the apoenzyme, and both agents significantly reduced the total TDO activity.

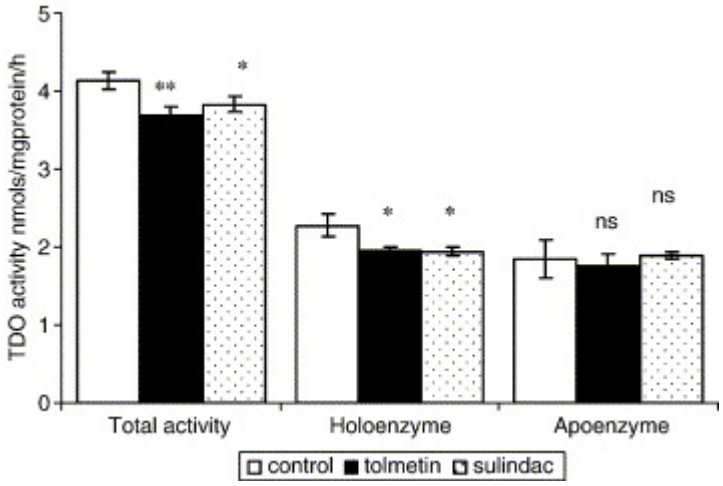

Fig. 1. Effect of tolmetin and sulindac (5 mg/kg/bd for 5 days) on liver TDO activity. Each bar represents the mean ± SD ($n = 6$). $^{ns}(p > 0.05)$, $*(p < 0.05)$ and $**(p < 0.01)$ as compared to controls.

Tolmetin and sulindac treated animals (5 mg/kg/bd for 5 days) show an increase in the levels of serotonin (6.12 ± 0.25 and 5.84 ± 0.20 pmol/mg tissue respectively) in the hippocampus compared to controls (5.19 ± 0.24 pmol/mg tissue) (see Fig. 2). Tolmetin treated animals also show an increase in the 5-HIAA levels in the hippocampus. DA and DOPAC levels in the hippocampus demonstrated no significant changes after treatment. As shown in Fig. 3, in the striatum, tolmetin and sulindac significantly reduces the DA levels (60.85 ± 3.18 and 68.87 ± 5.19 pmol/mg tissue respectively) when compared to controls (72.70 ± 2.94 pmol/mg tissue). DOPAC levels were significantly higher in tolmetin treated animals but not the sulindac treated animals.

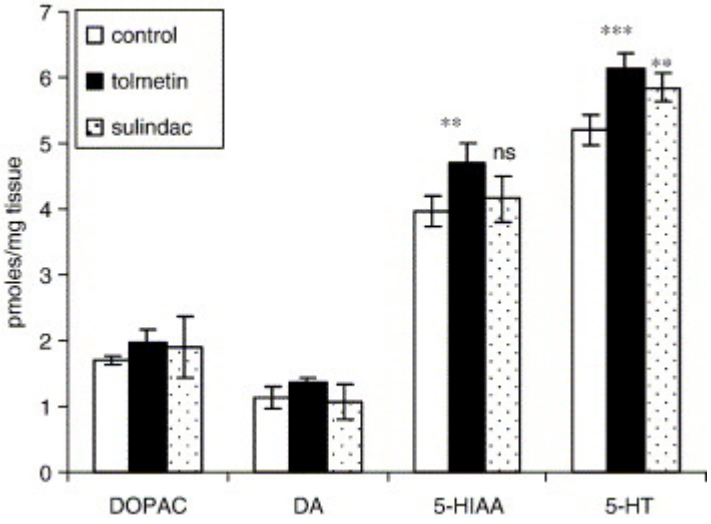

Fig. 2. Effect of tolmetin and sulindac (5 mg/kg/bd for 5 days) on rat hippocampal biogenic amine levels. Each bar represents the mean ± SD ($n = 5$). $^{ns}(p > 0.05)$, $**(p < 0.01)$ and $***(p < 0.001)$ as compared to controls.

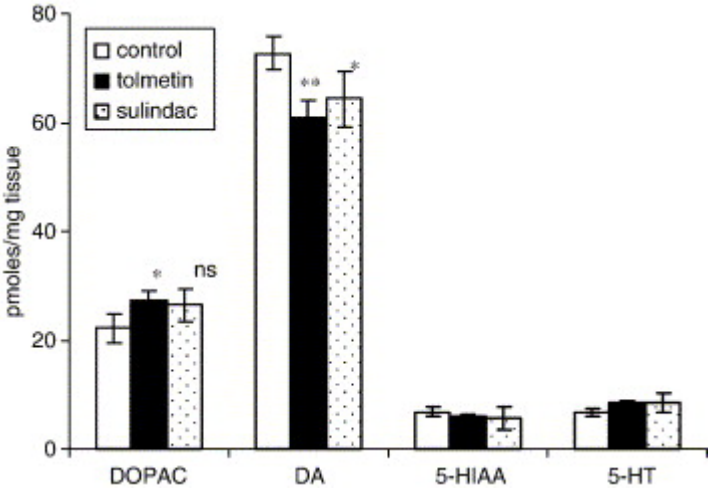

Fig. 3. Effect of tolmetin and sulindac (5 mg/kg/bd for 5 days) on rat striatum biogenic amine levels. Each bar represents the mean ± SD ($n = 5$). $^{ns}(p > 0.05)$, $*(p < 0.05)$ and $**(p < 0.01)$ as compared to controls.

Chromatograms of melatonin obtained show symmetrical peaks which were resolved from the solvent front with a retention time of 4.1 min. Regression analysis shows that the concentration versus peak height is linear over the range studied i.e.100–500 ng/ml ($r^2 = 0.9998$). The limits of detection (LOD) and limits of quantification (LOQ) were 5 ng/ml and 15 ng/ml respectively. Inter- and intra-assay precision was determined by calculating the relative standard deviation (RSD) of peak heights of the calibration standards ($n = 5$) and is reported as RSD %. The intra-assay precision show RSD % values of 0.86%–2.28% and the inter-assay precision show RSD % values of 0.58%–2.11%. Approximately 80% of the melatonin was extracted from 50 μl of MEM (containing 200, 300 or 400 ng/ml melatonin) using 1 ml of chloroform. The standard sample of melatonin and the extract from the pineal glands showed base peaks at *m/z* 174 (mass spectral data not shown). It has been suggested (Xie et al., 1998), that this molecular ion is formed by the loss of the HN═COH–CH3 fragment from the protonated parent molecule with the formation of a four-membered ring from the side chain.

As shown in Fig. 4, tolmetin but not sulindac, significantly ($p < 0.05$) increases melatonin synthesis. Controls release 11.96 ± 0.13 ng of melatonin in 24 h while tolmetin and sulindac treated animals release 12.33 ± 0.14 and 11.99 ± 0.17 ng of melatonin in 24 h respectively.

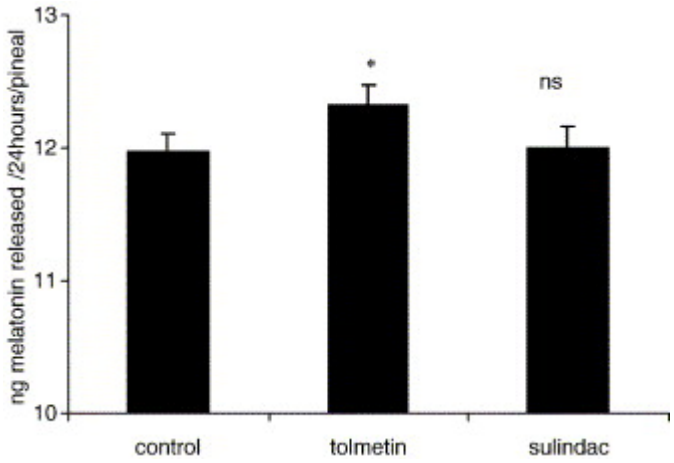

Fig. 4. Effect of tolmetin and sulindac treatment on melatonin synthesis in the rat pineal gland. Each bar represents the mean ± SD ($n = 5$). $^{ns}(p > 0.05)$ and $*(p < 0.05)$ when compared to controls.

Discussion

Tryptophan is an essential amino acid having various important biological functions. One of its biochemical pathways is known as the tryptophan–niacin or kynurenine pathway (Musajo and Benassi, 1964, Price et al., 1965 and Wolf, 1974). In adults about 90% of dietary tryptophan is metabolized along this pathway (Wolf, 1974). It has been shown that the administration of antidepressant drugs, including tricyclic antidepressants, results in the inhibition of liver TDO resulting in the elevation of brain tryptophan and serotonin concentrations (Hardeland and Rensing, 1968 and Walsh and Daya, 1998). In the present study, tolmetin and sulindac both inhibit the total liver TDO enzyme activity. Both agents inhibit the holoenzyme, resulting in the inhibition observed in total enzyme activity. The reason why these agents do not alter apoenzyme activity needs further investigation. One possibility is that these agents do not alter the ability of heme to bind to the active sites. This was accompanied by a significant increase in 5-HT in the hippocampus. Despite the differences, inhibition of TDO could be one possible mechanism via which both agents increased serotonin levels in the hippocampus.

Depression is a complex condition involving abnormalities in the sympathetic nervous system as well as the endocrine and immune systems (Szelenyi and Selmeczy, 2002). In the present study, much attention was focused on the hippocampus and striatum, where monoamines are present, since these regions play crucial roles in behavioral functions such as learning, memory and emotion, all of which may be affected in depression (Butterweck et al., 2002). The results show that both tolmetin and sulindac increase 5-HT levels in the hippocampus but not in the striatum implying that these agents may have potential use in patients suffering from depression. Inhibition of liver TDO is known to result in the increased circulating levels of tryptophan, making more of this amino acid available for uptake into the brain where it is converted to 5-HT (Curzon, 1969 and Curzon and Bridges, 1970).

Dopamine (DA) has received a great deal of attention in the mammalian CNS (Albin et al., 1989, Williams and Goldman-Rakic, 1995 and Greengard et al., 1999). This neurotransmitter plays an important role in multiple functions such as movement, memory, mental and psychological activities (Albin et al., 1989, Kalivas and Stewart, 1991, Williams and Goldman-Rakic, 1995 and Knable and Weinberger, 1997). Abnormalities of DA signaling may result in several neurological disorders such as Parkinson's disease, epilepsy, schizophrenia and drug abuse (Kalivas and Stewart, 1991, Koob, 1992, Koob and Le Moal, 1997 and Nestler and Aghajanian, 1997). Parkinson's disease is caused by a loss of dopaminergic neurons in the substantia nigra pars compacta, leading to a reduction in DA levels in the striatum (Deumens et al., 2002). This study shows that both tolmetin

and sulindac reduce DA levels in the striatum, suggesting that these NSAIDs may have the potential to exacerbate or induce DA-deficient neurological disorders.

NSAIDs are thought to exert most of their effects via prostaglandin synthesis inhibition (Vane, 1971 and Flower and Vane, 1972) and it is known that prostaglandins markedly enhance melatonin synthesis at night (Cardinali et al., 1979, Cardinali et al., 1982 and Voisin et al., 1993). Thus, NSAIDS have the ability to reduce melatonin synthesis. For example, it has been shown that NSAIDs such as aspirin (Murphy et al., 1986) and ibuprofen (Surrall et al., 1987 and Murphy et al., 1986) reduce melatonin synthesis in humans. However, in the present study, tolmetin, but not sulindac, increases the amount of melatonin produced by the rat pineal gland. It is unlikely that the rise in melatonin induced by tolmetin is a consequence of inhibition of TDO in the liver as it has been shown (Daya et al., 1989), that such inhibition does not alter melatonin levels. A possible reason for the tolmetin-induced increase in melatonin synthesis could be a direct effect of this drug on the melatonin synthesis pathway in the pineal gland.

Conclusion

Tolmetin and sulindac inhibit TDO with a concomitant increase in 5-HT levels in the hippocampus, suggesting that theses agents may be beneficial for patients suffering from depression. However, these agents simultaneously reduce DA levels in the striatum and thus do have implications in Parkinson's patients.

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