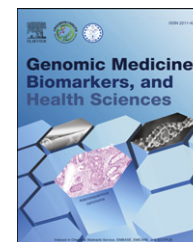


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## ORIGINAL ARTICLE

# Biochemical effects of chronic administration of efavirenz on the intracranial auditory relay centers of adult Wistar rats

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Received 2 March 2012; received in revised form 31 August 2012; accepted 1 October 2012

Available online 5 November 2012

## KEYWORDS

biochemical effects;  
efavirenz;  
inferior colliculus;  
medial geniculate  
body;  
Wistar rats

**Abstract** The biochemical effects of chronic administration of efavirenz, which is commonly used as part of highly active antiretroviral therapy for treatment of human immunodeficiency virus (HIV) type 1, on the intracranial auditory relay centers [i.e., the inferior colliculus (IC) and medial geniculate body (MGB)] of adult Wistar rats were carefully studied. Rats of both sexes ( $n = 30$ ), with an average weight of 200 g, were randomly assigned into treatment ( $n = 15$ ) and control ( $n = 15$ ) groups. Rats in the treatment group each received the recommended daily dose of 600 mg/70 kg (1.71 mg/200 g) of body weight of efavirenz daily for 30 days, while the control group rats received an equal volume of distilled water alone daily for 30 days. The rats were sacrificed after 30 days, and the IC and MGB were carefully removed for further biochemical assays. These analyses indicated that levels of malonyldialdehyde (MDA), a non-enzyme biomarker of oxidative stress, were significantly higher ( $p < 0.05$ ) whereas levels of superoxide dismutase (SOD) were significantly lower ( $p < 0.05$ ) in treated tissues than in control tissues. Chronic administration of efavirenz may therefore have an adverse effect on the auditory sensibilities by affecting the IC and MGB of adult Wistar rats. Copyright © 2012, Taiwan Genomic Medicine and Biomarker Society. Published by Elsevier Taiwan LLC. All rights reserved.

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## Introduction

Efavirenz is an antiretroviral drug that belongs to the class of drugs called non-nucleoside reverse transcriptase inhibitors used as part of highly active antiretroviral therapy to treat human immunodeficiency virus (HIV) type 1.<sup>1</sup> Efavirenz has been found to be effective in many combination regimens for HIV treatment. It has been combined successfully with nucleoside consisting of lamivudine or emtricitabine plus abacavir, didanosine, stavudine, tenofovir, or zidovudine to achieve virologic suppression in a high percentage of recipients.<sup>2,3</sup> Most antiviral agents do not efficiently cross the blood–brain barrier or are actively transported out of the central nervous system (CNS).<sup>4</sup> Even after antiviral treatment that successfully controls the virus in the treatment compartments, the CNS may suffer continuing damage induced by HIV infection.<sup>5</sup> Efavirenz, however, can cross into the CNS and spinal fluids and can be taken once a day without regard to mealtimes.<sup>6,7</sup>

Some adverse effects in the CNS are commonly associated with efavirenz.<sup>8</sup> Efavirenz intake has been reported to be associated with several health problems, including confusion, insomnia, abnormal and vivid dreams, dizziness, and headaches.<sup>9–12</sup> Nonetheless, efavirenz has emerged as a cornerstone of highly active antiretroviral therapy regimens. The side effect profile of the drug is generally regarded as satisfactory. However, there are conflicting study results in the medical literature as well as conflicting reports from patients and physicians regarding neuropsychiatry problems associated with efavirenz.<sup>13</sup> Lipodystrophy, moderate or severe pain, abnormal vision, arthralgia, asthenia, dyspnea, gynecomastia, myalgia, myopathy, and tinnitus have been reported to be linked with efavirenz.<sup>1</sup>

The inferior colliculus (IC) and medial geniculate body (MGB) constitute the intracranial auditory relay centers. The MGB is the target of ascending projections from the IC and descending input from the auditory cortex; this is the obligatory synaptic target in the thalamus for hearing.<sup>14</sup> The cerebral cortex strongly affects the MGB through descending projections. These projections are thought to consist primarily of small areas with slow conduction velocities.<sup>15</sup> It has been demonstrated that auditory cortex neurons have great physiological plasticity when rats are exposed to specific stimuli coupled with concurrent stimulation of a forebrain subcortical structure in the nucleus basalis. Observed changes have included massive expansion of frequency-specific representation.<sup>16</sup> The IC is essential for normal hearing and for the startle reflex.

Cortical structures such as the medial and lateral geniculate bodies and the inferior and superior colliculi have higher glucose utilization than other structures.<sup>17</sup> There is a correlation between functional activity and metabolic rate much as in the visual and auditory systems.<sup>17</sup> Because efavirenz crosses the blood–brain barrier, it is relevant to investigate the possible role of oxidative stress in efavirenz neurotoxicity in the intracranial auditory relay centers of adult Wistar rats.

## Materials and methods

### Animals

The School of Basic Medical Sciences at the University of Benin in Benin City, Edo state, Nigeria, granted approval for the care and use of laboratory animals before the work began. Thirty adult Wistar rats of both sexes with an average weight of 200 g were randomly assigned into control ( $n = 15$ ) and treatment ( $n = 15$ ) groups. The rats were obtained and maintained in the Animal Holdings of the Department of Anatomy, School of Basic Medical Sciences, University of Benin, Benin City, Edo State, Nigeria. They were fed with grower's mash obtained from Edo Feeds and Flour Mill Limited, Ewu, Edo State, Nigeria, and given water *ad libitum*. Efavirenz was obtained from the President's Emergency Plan for AIDS Relief Unit, University of Benin Teaching Hospital, Benin City, Edo State, Nigeria.

### Drug administration

The rats in the treatment group received 1.71 mg of efavirenz per 200 g body weight, which is equivalent to the recommended daily dose of 600 mg/70 kg. Efavirenz was dissolved in distilled water and orally administered to the treatment group rats with orogastric tube for 30 days, while control rats received an equal volume of distilled water for the same period. The body weights of both groups were measured using a Mettler Toledo weighing balance before, during, and after the period of treatment before being sacrificed.

### Dissection of the brain, IC, and MGB

The rats in both groups were sacrificed by cervical dislocation, and the skull was quickly opened with the aid of a pair of bone forceps to expose the brain. The IC and MGB were carefully removed, weighed, and further subjected to various biochemical assay techniques.

### Preparation of samples

The samples (of IC and MGB) from the experimental animals were removed and homogenized in a mortar and pestle with a pinch of acid washed sand and a total of 5 mL normal saline (0.9%) added sequentially during the homogenization process. The homogenates were centrifuged at 3500 rpm for 5 minutes with the aid of a centrifuge. The clear supernatants were then collected using a micropipette, transferred into an empty specimen container, and refrigerated until needed for the assays.

### Superoxide dismutase assay

The superoxide dismutase (SOD) activities in these tissues were determined using the method described by Misra and Fridovich.<sup>18</sup> An aliquot (0.4 mL) of the supernatant was added to 5 mL of 0.05M carbonate buffer (pH 10.2) equilibrated in the spectrophotometer for 2–3 minutes. The reaction was then initiated by the addition of 0.6 mL of

freshly prepared 0.3 mM adrenaline as a substrate to the buffered supernatant mixture, which was quickly mixed by inversion and the absorbance taken. The reference cuvette contained 5 mL carbonate buffer, 0.6 mL substrate, and 0.4 mL distilled water. The increase in absorbance at 420 nm due to the adrenochrome formed was monitored every 30 seconds for 120 seconds. One unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the auto-oxidation of adrenaline to adrenochrome during 120 seconds. It was allowed to equilibrate before the addition of adrenaline solution and then read immediately. The absorbance was measured at 420 nm.

### Catalase assay

The method of Cohen et al.<sup>19</sup> was adopted for the catalase assay. Aliquots of the homogenate supernatant (0.5 mL) were added into ice cold test tubes while the blank contained 0.5 mL distilled water. The reaction was initiated by adding sequentially, at fixed intervals, 5 mL of cold 30 mM hydrogen peroxide followed by thorough mixing by inversion. The test samples and the blank were taken one at a time, and 7 mL of 0.01M potassium permanganate was added and then mixed twice by inversion and absorbance at 480 nm. Each sample was read within 30–60 seconds. The spectrophotometer standard was prepared by adding 7 mL of 0.01M potassium permanganate to a mixture of 5.5 mL of 0.05M phosphate buffer with pH 7.0 and 1 mL of 6M tetraoxosulfate VI acid solution. The spectrophotometer was zeroed with distilled water and the activity of the enzyme was then estimated.

### Peroxidase assay

The peroxidase assay was based on the method of Chance and Maehly.<sup>20</sup> First, 0.4 mL of the sample homogenate was added to clean test tubes, followed by the addition of 5 mL phosphate buffer and 5 mL hydrogen peroxide, then by 3 mL distilled water. Finally, 5 mL pyrogallol was added, and the absorbance was measured at 430 nm. The blank was prepared by the addition of 0.45 mL phosphate buffer, followed by 5 mL hydrogen peroxide. Then, 3 mL distilled water was added, and finally, pyrogallol was added to zero the spectrophotometer before taking the absorbance of the test.

### Malonyldialdehyde assay

Lipid peroxidation was estimated in terms of thiobarbituric acid reactive species (TBARS) using malonyldialdehyde (MDA) as the standard according to the method of Beuge and Aust.<sup>21</sup> First, 1.0 mL of the sample extract was added to 2.0 mL TCA–TBA–HCL reagent [15% (w/v) TCA, 0.375% (w/v) TBA and 0.25N HCL]. The contents were boiled for 15 minutes, cooled, and centrifuged at 10,000×g to remove precipitate. The absorbance was read at 535 nm, and the malonyldialdehyde concentration of the sample was calculated using the extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1} \text{ Cm}^{-1}$ .

### Glucose-6-phosphate dehydrogenase assay

This assay was based on the method of Hess et al.<sup>22</sup> Glucose-6-phosphate dehydrogenase (G-6-PDH) activity was determined by the measurement of the rate of absorbance change at 340 nm due to the reduction of  $\text{NAP}^+$ . Aliquots of the homogenate supernatant (15  $\mu\text{L}$ ) were added into test tubes while the blank contained 15  $\mu\text{L}$  distilled water. The reaction was initiated by adding sequentially, at fixed intervals, 1000  $\mu\text{L}$  of reagent R1 (31.7 mmol/l triethanolamine buffer, pH 7.6, and 3.2 mmol/L EDTA) followed by the addition of 30  $\mu\text{L}$  of reagent R2 (0.34 mmol/L NADP), and then followed by thorough mixing and incubation for 5 minutes at 37 °C. The test samples and the blank were taken one at a time, and 15  $\mu\text{L}$  of reagent R3 (0.58 mmol/L glucose-6-phosphate) and 15  $\mu\text{L}$  of reagent R4 (digitonin) were added and mixed twice by inversion, and the absorbance was read at 340 nm within 0–3 minutes. The spectrophotometer was zeroed with distilled water, and the activity of the enzyme was estimated.

### Lactate dehydrogenase assay

This assay was based on the method of Weisshaar et al.<sup>23</sup> Lactate dehydrogenase (LDH) enzyme activity was determined by the measurement of the rate of absorbance change at 340 nm due to the reduction of  $\text{NADH}^+$ . Aliquots of the homogenate supernatant (0.02 mL) were added into test tubes while the blank contained 0.02 mL distilled water. The reaction was initiated by adding sequentially, at fixed intervals, 1.0 mL of the reagent, and the absorbance was read at 340 nm within 0–3 minutes. The spectrophotometer was zeroed with distilled water, and the activity of the enzyme was estimated.

### Statistical analysis

The results were calculated for mean and standard error of means. The results from the various assays were analyzed using one-way analysis of variance for a significance level of  $p > 0.05$ .

### Results

The results of the biochemical assays were calculated; the results are shown in Table 1, and presented as bar charts in IC (Fig. 1) and MGB (Fig. 2), respectively. The activities of SOD and LDH in the efavirenz-treated MGBs and ICs decreased significantly ( $p < 0.05$ ) compared to those of the control group. The activities of catalase were found to decrease in the efavirenz-treated IC and increase in the efavirenz-treated MGB, but these differences were not statistically significant ( $p < 0.05$ ) in comparison with the IC and MGB samples of the control group. The glutathione peroxidase activities, MDA, and G-6-PDH levels increased significantly ( $p < 0.05$ ) in efavirenz-treated ICs and MGBs as compared to ICs and MGBs of the control group.

**Table 1** Biochemical assay results for the IC and MGB of the animals.

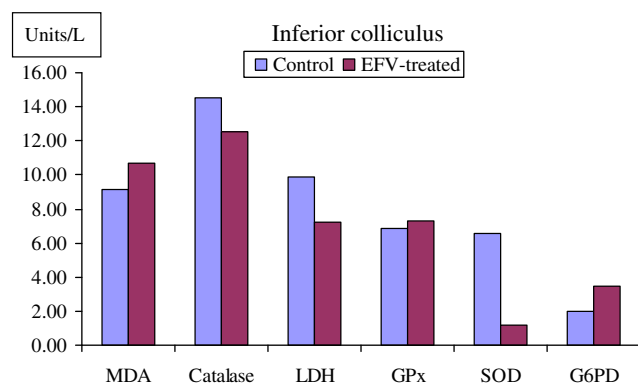
Biochemical effect	Brain tissue	
	IC	MGB
SOD (units/L)		
Control	5.99 ± 0.06*	6.53 ± 0.03*
EFV	1.22 ± 0.02*	1.15 ± 0.02*
Catalase (units/L)		
Control	0.0145 ± 0.0005	0.0120 ± 0.001
EFV	0.0125 ± 0.0005	0.0135 ± 0.0005
GPx (units/L)		
Control	0.685 ± 0.005*	0.665 ± 0.015*
EFV	0.730 ± 0.010*	0.795 ± 0.005*
MDA (units/L)		
Control	0.913 ± 0.001*	0.935 ± 0.006*
EFV	1.070 ± 0.001*	1.077 ± 0.002*
G-6-PDH (U/L)		
Control	0.0020 ± 0.001*	0.0015 ± 0.001
EFV	0.0035 ± 0.005*	0.003 ± 0.000
LDH (Units/L)		
Control	0.99 ± 0.06*	0.53 ± 0.03*
EFV	0.72 ± 0.02*	0.15 ± 0.02*

\*Significant ( $p < 0.05$ ).

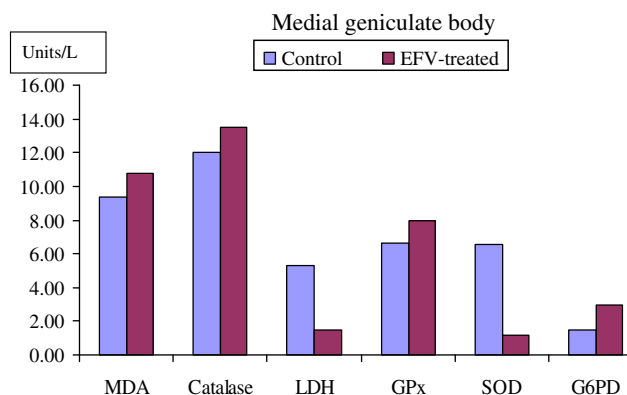
EFV = efavirenz; G-6-PDH = glucose- 6-phosphate dehydrogenase; GPx = glutathione peroxidase; IC = inferior colliculus; LDH = lactate dehydrogenase; MDA = malonyldialdehyde; MGB = medial geniculate body; SOD = superoxide dismutase.

## Discussion

Efavirenz has been reported to be associated with a variety of health problems, including problems related to the CNS. Common among such problems are confusion, insomnia, abnormal and vivid dreams, dizziness, and headaches.<sup>9–12</sup> It has been suggested that chronic administration of efavirenz may also affect the weight and microanatomy of the brain and colliculi in adult Wistar rats.<sup>24–26</sup> However, the underlying mechanism involved in this toxicity is not well



**Figure 1** Bar chart showing biochemical assay results for the inferior colliculus (IC) of the animals. G6PD = glucose 6-phosphate dehydrogenase; GPx = peroxidase; LDH = lactate dehydrogenase; MDA = malonyldialdehyde; SOD = superoxide dismutase.



**Figure 2** Bar chart showing biochemical assay results for the medial geniculate body (MGB) of the animals. G6PD = glucose 6-phosphate dehydrogenase; GPx = peroxidase; LDH = lactate dehydrogenase; MDA = malonyldialdehyde; SOD = superoxide dismutase.

understood. This study was aimed at investigating the possible role of oxidative stress in efavirenz neurotoxicity in the intracranial auditory relay centers of adult Wistar rats. It was hypothesized that oxidative stress may be a biochemical mechanism for the toxicity. Thus, oxidant biomarkers (MDA and SOD) and antioxidant enzymes have been implicated in this investigation.

The results of this experiment showed that oxidative stress measured in terms of MDA was found to increase significantly ( $p < 0.05$ ) in all treated tissues under investigation as compared with their parallel controls. Concomitantly, the activity of SOD was found to decrease significantly ( $p < 0.05$ ) in the treated tissues compared with their controls. These results demonstrate that efavirenz exerts its toxic effects by promoting oxidative stress in the IC and MGB of adult Wistar rats. The observation reported in this experiment is consistent with the studies carried out by Otitoju et al.,<sup>27</sup> who investigated the possibility of an oxidative mechanism in insecticide toxicity and reported that the oxidative stress increased in all experimental groups.<sup>27</sup>

Antioxidant levels are lower whereas pro-oxidant polyunsaturated fatty acid levels are higher in the CNS relative to other tissues. Therefore, the CNS is exceptionally at risk of oxidative damage. Antioxidant enzymes such as catalase, glutathione peroxidase, G-6-PDH, LDH, and SOD are responsible for the brain's basic functions, both physical and cognitive.<sup>28–30</sup> It is known that the activities of antioxidant enzymes are significantly modified in the CNS during intoxication, when a decrease in activity may indicate oxidative modification of the enzymatic proteins. Other viable explanations associated with the decreased activities of antioxidant enzymes may include a decrease in the synthesis rate. In this study, catalase, G-6-PDH, glutathione peroxidase, and LDH demonstrated inconsistent levels across the ICs and MGBs of adult Wistar rats. An increase in the activity of these antioxidant enzymes may often be due to an adaptive response to excess free radicals. Furthermore, catalase may be unreliable in this respect because the decrease in its activity may be due to an enhancement of protein synthesis as a confounding factor.<sup>28</sup> There was also a confounding factor for G-6-PDH

that is capable of inactivating the enzyme.<sup>29</sup> These confounding factors may explain the inconsistency in the relative levels of these enzymes observed in this study. It is known that the metabolic products of efavirenz include hydroxylated products and their *O*-glucuronide, *N*-glucuronide, and sulfate conjugates among others.<sup>31</sup> It is recommended that further studies review the metabolic products of efavirenz to determine which of them have pro-oxidant properties, with a view to determining how to manage the oxidative damage resulting from efavirenz therapy.

## Acknowledgments

We thank the medical team of the President's Emergency Plan for AIDS Relief Unit, University of Benin Teaching Hospital, Benin City, Edo State, Nigeria, for their understanding and assistance in obtaining efavirenz. I offer my sincere gratitude to Dr V. Makelele, Dr H. Egbo, Dr N. Mokogwu, Dr Ezekiel Nwose, and Mr Bobby N. Aguebor-Ogie for their assistance in making this work a reality.

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