Dual Effect of Alternariol on Acetyl Cholinesterase and Monoamineoxidase Extracted from Different Parts of Rat Brain

Hassan M.Y. Osman* Mohamed Y. Osman*

ABSTRACT Alternariol is a metabolite product of different strains of alternaria tenuis fungus. Its structure has a similarity to cannabinol derivatives and it has a chemical formula $3,4,5$ trihydroxy 6-methyl dibenzoxy-α-pyrone. The effect of the alternariol on both acetylcholinesterase (AChE) and monoamineoxidase (MAO-A) enzymes extracted from whole and five different parts of male albino rat brains; namely: frontal cortex, basal ganglia, cerebellum, pons, medulla oblongata was studied. Kinetic studies were done to determine the type of inhibition of AChE and MAO-A enzymes and the enzyme – inhibitor dissociation constants (Ki) by alternariol. The results indicated that alternariol inhibited both AChE and MAO-A enzymes of the cortex and medulla oblongata more than the extracts of the other parts of the brain. These parts are responsible for perception, motor, sensory, psychic activities and reflex centers of respiration. The inhibition of these enzymes increased with increasing the amount of alternariol added to the assay mixture, i.e., the inhibition was dose dependent and of the competitive type. The values of Ki for alternariol – AChE enzyme extracts varied from 12.0 to 15.0 mmol/L and for alternariol – MAO enzyme extracts varied from 28 to 30 mmol/L and were of the same order of magnitude. The difference in the degree of inhibition of the extracts of these brain parts could be attributed to the slight difference in the structure i.e; arrangement of their amino acids (isozyme phenomenon) and to their distinct gene loci. The inhibition of AChE and MAO-A by alternariol may save ACh and biogenic amines which are of great importance for the patients suffering from Alzheimers and dementia.

Keywords: Acetylcholine; 5 Hydroxytryptamine (Serotonin); Alternariol; Acetyl Cholinesterase; Monoamine Oxidase.

INTRODUCTION

Alternariol is a metabolite produced by different strains of alternaria tenuis. [1] Its structure was established as $3,4,5$ trihydroxy 6-methyl dibenzoxy -α-pyrone $C_{14}H_{10}O_5$ [I]. It has striking similarity to cannabinol derivatives [II].

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Several reports [2,3] have attempted to correlate the behavioral effects of tetrahydrocannabinol (Delta 8 THC) with changes in the level of 5-hydroxytryptamine (serotonin) or its metabolite. THC is an important psychoactive ingredient in marijuana [4], which is the most widely illegal recreational drug in USA.

Our preliminary experiments showed that alternariol manifested inhibitory effect on whole rat brain acetylcholinesterase (AChE) and monoamine oxidase (MAO) activities [5].

Cholinestrases are a group of enzymes that degraded the esters of choline and play a role in neurotransmission in the autonomic and somatic motor nervous system. Their inhibition results in accumulation of acetylcholine. AChE is used as a human biomarker of organophosphorus pesticides exposure [6]. Monoamine oxidase E.C. 1.4.3.4 (MAO) is an enzyme that oxidizes mono amine neurotransmitters and neuro- mediators as well as exogenous bioactive amines [7,8].

Inhibitors of MAO-A have shown to be effective antidepressents, and have been shown to be of value in the treatment of Parkinson’s disease [9].

The present study was conducted to investigate the inhibitory effect of alternariol on AChE and MAO-A isozymes, extracted from whole and five parts of rat brain, namely; frontal cortex, basal ganglia, cerebellum, pons and medulla oblongata.

Enzyme kinetic studies were done to determine the type of inhibition and enzyme inhibitor dissociation constant (Ki) of AChE and MAO-A by alternariol, and to know
which of these parts was inhibited by alternariol more than other parts.

**MATERIAL AND METHODS**

Alternariol was prepared by growing a strain of alternaria tenuis, Catalogue number S.M. 108, on Czepek-Dox medium [1] using either glucose or molasses, as a carbon source [1]. The metabolite was extracted from the dried defatted mycelian with ether, then purified by repeated crystallisation from dioxane giving colorless needles m.p. 350° (decomp).

**Chemicals:** were purchased as follows: acetylthiocholine iodide (AThChI) from BDH chemical, Ltd, Poole (England) Dithio-bis-nitro benzoic acid from Aldrich chemical Co.Ltd Gillingham, England. 5-hydroxytryptamine creatinine sulphate (contains 43.5% of 5HT, serotonin) from May and Baker LH, Dagenham (England)

**Animals:** Fifty male albino rats (weighing 100-150 g) aged 2 months were used in the experiments. Rats were supplied from the Medical Research Institute animal house, Alexandria University, Egypt. Rats were housed in group cages (5 in each cage) and allowed free access of food and tap water. The rats were killed by decapitation after subjected to an overnight fast, with free access of water. Each brain weighed about 1.6 g.

**Preparation of AChE from whole and different parts of rat brain:** Whole brain as well as the different parts obtained from 10 g brain tissues were isolated, weighed and homogenized in ice cold phosphate buffer (0.1 mol / L, pH 8.0) [10]. The resulting homogenates were centrifuged twice at 600×g for 10 min to remove cellular debris. The supernatant was centrifuged at 10,000 ×g for 20 min, and the resulting precipitate was suspended with the same buffer and used as a source of the enzyme in the assay.

**AChE assay:** The activity of AChE was measured by the method of Ellman et al [11]. The assay mixture contained: 2.0 ml phosphate buffer (0.1 M , pH 8.0) containing an amount of brain tissue equivalent to 0.1 unit of AChE, 100 ul dithio bis nitro benzoic acid (DTNB,0.1 mol/L) and 20 ul acetyl thiocholine iodide (AThChI, 21.67 mg/ml).
The type of inhibition and the enzyme – inhibitor dissociation constant (Ki) of AChE were measured at different concentrations of alternariol: 0 (control), 5.0, 10.0 and 15.0 mmol/L, while AThChI concentrations were varied, (2.5, 5.0, 7.5 and 10.0 mmol/L) for each concentration of alternariol. AThChI and alternariol were added simultaneously to the assay mixture.

**Preparation of MAO-A from whole and different parts of rat brain:** The MAO enzymes were prepared from whole and different parts of rat brain as described before [12,13,14]. Ten brains out of 40 were used (15g) as whole brain enzyme preparation. From the 30 other brains, the appropriate parts were isolated, washed with ice cold saline and weighed each separately. These parts were the frontal cortex (15g), basal ganglia (8g), pons (7g), medulla oblongata (10g) and cerebellum (15g). Whole brain as well as the different parts of the brain were homogenized in ice cold phosphate buffer Na₂HPO₄: NaH₂PO₄, PH 7.4, 0.1 mol/L containing 0.25 mol/L sucrose. The homogenates were centrifuged at 12000xg for 20 minutes. The resulting supernatants from the second centrifugation were discarded, and the mitochondrial precipitates were resuspended in the same phosphate buffer to give a final protein concentration of 10 mg/ml. The protein content was determined by Lowry’s method [15].

**MAO assay** The activity of MAO-A was measured by the method of Udenfreind et al [16,13,14]. The assay mixture contained: 1 ml 5HT (166 umol/ml) and 1 ml enzyme containing 10 mg protein in phosphate buffer. After incubation at 37°C for 1 hr, 1 ml of each of 1-nitroso, 2-naphthol (0.1% in 95% ethyl alcohol) and acid nitrite (freshly prepared), reagents were added to react with the unhydrolyzed 5 HT. Measurements were carried out at 540 nm. The type of inhibition and Ki of MAO-A were measured at different concentrations of alternariol: 0 (control), 5, 10 and 15 mmol/L while 5HT concentrations were varied, (111, 166, 222 and 333 umol/L) for each concentration of alternariol. 5HT and alternariol were added simultaneously to the assay.
mixture.

RESULTS

The activity of AChE in whole and different parts of rat brain was measured at constant substrate concentration (0.5 mmol/L AThChI). It has been found that the basal ganglia extract possessed the highest specific activity. The values of Km obtained from Lineweaver-Burk plot [17] of 1/V versus 1/S in the case of AChE extracts varied between 0.12 and 0.17. (table 1)

The activity of MAO in whole and different parts of rat brain was measured at constant substrate concentration (166 umol/L 5HT). It has been found that the basal ganglia and frontal cortex extracts possessed the highest activity of MAO. The values of Km in case of MAO-A extracts varied between 0.28 and 0.33 mmol/L (table 2).

A double reciprocal plot of velocity versus substrate concentrations in the absence and presence of alternariol gave curves of the competitive type for the inhibition of both AChE and MAO (fig. 1 and 2). This was confirmed by Cleland replot [18] of the slopes of the lines of (fig 1 and 2) versus inhibitor concentrations taking basal ganglia for AChE and MAO as examples (fig. 1 and 2).

The value of enzyme-inhibitor dissociation constant (Ki) of alternariol with whole brain AChE extract was 1.8mmol/L, and with different parts of the brain varied from 1.6 to 2.1mmol/L.

The values of Ki of alternariol with whole brain MAO-A extract was 2.4mmol/L and with the extracts of different parts varied from 2.4 to 3.8mmol/L.

The results also indicated that the highest affinity (Ki/Km) of alternariol was with the extracts of AChE obtained from frontal cortex and medulla oblongata (13.12 and 13.33mmol/L) and to MAO-A extracts of frontal cortex and medulla oblongata also (11.88 and 11.20).
**Table 1:** Rate of hydrolysis of acetyl thiocholine iodide by acetyl cholinesterase extracted from whole and different parts of rat brain under the effect of alternariol. Values represent mean±S.D. of 3 repeated experiments.

<table>
<thead>
<tr>
<th>Part of the brain</th>
<th>AChE activity umol/min/g wet wt</th>
<th>(a) Km mmol/L</th>
<th>(b) Ki mmol/L</th>
<th>(c) Ki/Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole brain</td>
<td>18.4±0.12</td>
<td>0.15</td>
<td>1.8</td>
<td>12.0</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>15.2±0.09</td>
<td>0.16</td>
<td>2.1</td>
<td>13.12</td>
</tr>
<tr>
<td>Basal ganglia</td>
<td>27.8±0.18</td>
<td>0.14</td>
<td>1.8</td>
<td>12.85</td>
</tr>
<tr>
<td>Pons</td>
<td>14.3±0.10</td>
<td>0.15</td>
<td>1.7</td>
<td>11.33</td>
</tr>
<tr>
<td>Medulla oblongata</td>
<td>16.5±0.12</td>
<td>0.12</td>
<td>1.6</td>
<td>13.33</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>5.5±0.07</td>
<td>0.17</td>
<td>2.0</td>
<td>11.76</td>
</tr>
</tbody>
</table>

a) Km, Michaelis constant.
b) Ki, enzyme inhibitor (alternariol) dissociation constant.
c) Ki/Km: affinity constant

**Table 2:** Rate of hydrolysis of 5-hydroxy tryptamine by monoamine oxidase extracted from whole and different parts of rat brain under the effect of alternariol. Values represent mean±SD of 3 repeated experiments.

<table>
<thead>
<tr>
<th>Part of the brain</th>
<th>MAO activity umol/L 5HT hydrolyzed/mg protein/hr</th>
<th>(a) Km mmol/L</th>
<th>(b) Ki mmol/L</th>
<th>(c) Ki/Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole brain</td>
<td>135±6.7</td>
<td>0.30</td>
<td>2.4</td>
<td>8.0</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>138±5.3</td>
<td>0.32</td>
<td>3.8</td>
<td>11.88</td>
</tr>
<tr>
<td>Basal ganglia</td>
<td>142±7.8</td>
<td>0.33</td>
<td>3.2</td>
<td>9.69</td>
</tr>
<tr>
<td>Pons</td>
<td>132±6.9</td>
<td>0.30</td>
<td>3.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Medulla oblongata</td>
<td>104±5.8</td>
<td>0.25</td>
<td>2.8</td>
<td>11.2</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>124±4.4</td>
<td>0.30</td>
<td>3.0</td>
<td>10.0</td>
</tr>
</tbody>
</table>

a) Km, Michaelis constant.
b) Ki, enzyme inhibitor (alternariol) dissociation constant.
c) Ki/Km: affinity constant
Inset: Cleland replot of the slopes obtained from fig. (1) against inhibitor concentration [I].

**Figure (1):** Competitive inhibition, obtained from Lineweaver-Burk plot of 1/V versus 1/S, under the effect of alternariol on the rate of hydrolysis of AThChl by frontal cortex (AChE) extract. (●) Control, (○) 5.0, (△) 10.0 & (o) 15mmol/L.

Inset: Cleland replot of the slopes obtained from fig. (2) against inhibitor concentration [I].

**Figure (2):** Competitive inhibition obtained from Lineweaver-Burk plot of 1/V versus 1/S, under the effect of alternariol on the rate of deamination of 5 HT by frontal cortex (MAO) extract (●) Control, (○) 5.0, (△) 10.0 & (o) 15mmol/L.
Discussion

The differences in the activity and enzyme-substrate dissociation constants (Km) values, between the extracts of the parts of the brain in case of both AChE and MAO-A extracts are attributed to isozyme phenomenon [19].

A-Effect of Alternariol on brain AChE:

The value of enzyme-inhibitor dissociation constant (K_i) of alternariol with whole brain AChE extract was 1.8 mmol/L, and with different parts of the brain varied from 1.6 to 2.1 mmol/L. When comparing the value of K_i obtained in the reaction of alternariol with brain AChE with those previously obtained in the reactions of some biogenic amines, namely; 5-HT (18 mmol/L), epinephrine (E) and norepinephrine (NE) (12.2 and 8.5 mmol/L), the present results indicate that alternariol is 10, 7.0 and 5.0 times more potent than 5-HT, E and NE, respectively - in its inhibitory effect on AChE. [20, 21]

Alternariol possessed higher affinity and binding to the enzyme extracts of medulla oblongata than with the extracts of the other parts. This means that it affects the areas of perception, psychic activities and the reflex centers for respiration.

Alternariol is more effective on AChE than cannabinoids (delta-8-THC and 11-hydroxy deltaTHC). These compounds showed only slight changes on AChE isolated from synaptic membranes [4]. In connection with the present results, Eubanks et al. [22] demonstrated that delta-9-THC, the active component of marijuana (which has structural similarity to alternariol) competitively inhibited AChE activity.

Effect of Alternariol on brain MAO-A

MAO-A inhibitors have shown to be effective antidepressants and of value in the treatment of Parkinson’s disease [9]. As MAO-A metabolizes 5-hydroxytryptamine (serotonin) and noradrenaline (nor epinephrine), and is a neural enzyme, it was only natural that most drug research emphasized on MAO-A inhibitors.
In the present work, the lowest Km values of MAO-A using 5HT as substrate was obtained with the extract of medulla oblongata 0.25mmol/L, showing that the substrate has more affinity to the enzyme extracted from M.O than to the extracts of other parts.

The lowest Ki value was obtained in case of the reaction of alternariol with MAO-A extract of medulla oblongata (2.8mmol/L) indicating that alternariol has more affinity to the active site of this extract than to the extracts of the other parts. When comparing the value of Ki obtained in the reaction of alternariol with MAO-A (2.4 mmol/L) with that previously obtained [14] with the reaction of acetylcholine with MAO-A (3.7 mmol/L), the results indicate that alternariol is 1.5 times more potent than acetylcholine in its inhibitory effect on MAO-A.

Previous reports (2) mentioned that delta-9 THC (which is one of the main psycho-active components of cannabis) has drastic effect on isolated rat liver mitochondria, detectable in the form of damage to cristae and outer membranes as well as changes in the respiration and ATPase activity. These findings coincide with the action of alternariol on MAO-A which is a mitochondrial enzyme (similar to ATPase).

The results showed that the degree of its inhibitory effect was varied between the extracts of the different parts of the brain. The lower Ki values of alternariol with MAO-A is markedly with the extract of medulla oblongata which is responsible for respiration. It is note-worthy to mention that the values of Ki obtained in cases of alternariol-AChE reactions were about half the values obtained in case of the reaction of alternariol with MAO-A extract. This indicates that the affinity of alternariol to AChE enzyme extracts was higher than its affinity towards MAO-A enzyme extracts. Moreover, both AChE and MAO extracts of the medulla oblongata were inhibited by
alternariol than the extracts of other brain parts. However, the difference in the values of Km and Ki suggesting the existences of different substrate binding site of both enzymes: AChE and MAO [14].

All these discrepancies indicate that one aminoacid can be responsible for binding of some (but not all) substrates and inhibitors [14,23], or due to the sequences of amino acids in the enzyme. The dual inhibition of AChE and MAO by alternariol may save AChE and the biogenic amines which are of great importance for the patients suffering from Alzheimers and dementia.

REFERENCES
5. Osman H M.Y., Osman M.Y. Dual effect of alternariol on acetyl cholinesterase and monoamine oxidase extracted from different parts of rat brain. FASEB (2008), Abstract#564.


