The Effect of Dimethyl Dimethoxy Biphenyl Dicarboxylate (DDB) against Tamoxifen-induced Liver Injury in Rats: DDB Use Is Curative or Protective

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Tamoxifen citrate is an anti-estrogenic drug used for the treatment of breast cancer. It showed a degree of hepatic carcinogenesis, when it used for long term as it can decrease the hexose monophosphate shunt and thereby increasing the incidence of oxidative stress in liver rat cells leading to liver injury. In this study, a model of liver injury in female rats was done by intraperitoneal injection of tamoxifen in a dose of 45 mg/kg body weight for 7 successive days. This model produced a state of oxidative stress accompanied with liver injury as noticed by significant declines in the antioxidant enzymes (glutathione-S-transferase, glutathione peroxidase and catalase) and reduced glutathione concomitant with significant elevations in TBARS (thiobarbituric acid reactive substance) and liver transaminases; sGPT (serum glutamate pyruvate transaminase) and sGOT (serum glutamate oxaloacetate transaminase) levels. The oral administration of dimethyl dimethoxy biphenyl dicarboxylate (DDB) in a dose of 200 mg/kg body weight daily for 10 successive days, resulted in alleviation of the oxidative stress status of tamoxifen-intoxicated liver injury in rats as observed by significant increments in the antioxidant enzymes (glutathione-S-transferase, glutathione peroxidase and catalase) and reduced glutathione concomitant with significant decrements in TBARS and liver transaminases; sGPT and sGOT levels. The administration of DDB before tamoxifen intoxication (as protection) is more little effective than its curative effect against tamoxifen-induced liver injury. The data obtained from this study speculated that DDB can mediate its biochemical effects through the enhancement of the antioxidant enzyme activities and reduced glutathione level as well as decreasing lipid peroxides.

Keywords: Dimethyl dimethoxy biphenyl dicarboxylate, Glutathione peroxidase, Glutathione-S-transferase, Reduced glutathione, Tamoxifen, Thiobarbituric acid reactive substance

Introduction

Tamoxifen citrate, 1-[4-(2-dimethyl-aminoethoxy)phenyl]-1,2-diphenyl-1-butene), is a nonsteroidal antiestrogen drug that is used in the treatment and prevention of all stages of hormone-dependent breast cancer (Desai et al., 2002; Jordan, 2003). Tamoxifen reduce the level of estrogen and estrogen receptor with no change in progesterone contents (Liu et al., 2004). This effect is mediated by the inhibition of the action of both 17β estradiol and esterone (Yu and Bender, 2002; Stefano et al., 2003) and competition with estrogen for binding to its receptor (Ali and Coombes, 2002). Also, tamoxifen is able dramatically to inhibit the formation of 17β estradiol and esterone epoxides as measured by both the loss of their ability to inhibit DNA-dependent RNA synthesis and to bind to nuclear DNA (Yu and Bender, 2002).

Tamoxifen is metabolized by human cytochrome P450s 3A4, 2C9, 2B6, 2C8, 2C19, 2D6, 1A1, 1A2, and 2A6 (Dehal and Kupfer, 1997, 1999) and is also an effective inducer of P450s 2B2, 2B1, and 3A in rat liver at doses comparable with the therapeutic doses used in humans (Emile et al., 1995). Kupfer et al., 1994 and Sridar et al., 2002, reported that, tamoxifen has been found to be metabolized by liver primarily into three metabolites, tamoxifen-N-oxide, formed by flavin-containing monooxygenase, and N-desmethyl and 4-hydroxytamoxifen, formed by cytochrome P450. The N-demethylation was demonstrated to be catalyzed by P450A3 in rat and human liver; however, the P450s catalyzing the 4-hydroxylation have not been identified.

It was obvious that, certain toxicants (may be drug
overdose or harmful substances) have been characterized by their ability to induce liver injury following the cleavage by cytochrome P450 to form free radicals (Rosen et al., 1999). Lipid peroxidation via substitution of hydrogen from unsaturated fatty acids forms carbon-centered lipid radicals (McCay et al., 1984). This molecule adds molecular oxygen to form lipid peroxyl radicals, thereby initiating the process of lipid peroxidation (Letteron et al., 1990). It was revealed that, tamoxifen in high dose is a known liver carcinogen in rats (Ahotupa et al., 1994; Calballero et al., 2001) which is due to oxygen radical overproduction which occurs during tamoxifen metabolism. Oxidative DNA and lipid damage was detected by the measurement of DNA adduct and lipid peroxides, respectively (Pagano et al., 2001). Tamoxifen-DNA adducts have been detected in rats administered tamoxifen (Han and Liehr, 1992). On the other hand, tamoxifen has been shown to potentiate the nitric oxide production in breast cancer patients through the enhancement of the nitric oxide synthase II expression (Simeone et al., 2002). This strong hepatocarcinogenic effect of tamoxifen in rats raises issues bearing on the prophylactic chronic administration to healthy women (Williams et al., 1993). Also, tamoxifen can exert some side effects such as hot flushes, nausea, hypertriglyceridemia and decreased HDL cholesterol (Saarto et al., 1996; Atalay et al., 2004).

Dimethyl dimethoxy biphenyl dicarboxylate (DDB) is a synthetic analogue of Schizandrin C, one of compounds isolated from *Fructus schizandrae*, which is a traditional Chinese medicine (Kim et al., 1999). Although, Schizandrae have a long history of medicinal use within the traditional Chinese system, the world begun recently to understand its pharmacological possibilities and clinical applications. Experimental evidence suggests that DDB has hepatoprotective abilities and functions as a potent antioxidant and have proven clinically in the treatment of chronic viral and chemically-induced hepatitis (Liu, 1989; Fu and Liu, 1992; Ip et al., 2000). Decocts of DDB were found to possess strong *in vitro* inhibitory action on *Bacillus subtilis*, *Bacillus dysenteriae*, *Bacillus typhi* and *Staphylococcus aureus* (Hong, 1986). So that, it is indicated in cases of chronic cough and dyspnea, diarrhea, night sweats, wasting disorders, irritability, palpitations, dream-disturbed sleep and insomnia (Sinclair, 1998). The major chemical constituents of DDB are schizandrins, deoxyxyschizandrin, schisanhenol, schizandrol, sesquicarene, β-chamigrene, citral, stigmasterol and vitamins C and E (Hong, 1986; Bensky and Gamble 1993). It was reported that, DDB protects against carbon tetrachloride, D-galactosamine, and thioacetamide-induced hepatic injury (Yu et al., 1987). It lowers sGPT level in patients with chronic viral hepatitis and decrease the hepatotoxicity in animals with few side effects (Liu et al., 1978). A pharmacological study conducted by Li, 1991, showed that DDB increased liver protein and glycogen synthesis and had an inducing effect on the cytochrome P-450 enzyme system. Also, DDB was found to inhibit vitamin C/NADPH induced lipid peroxidation in rat liver microsomes and appear to be more effective than vitamin E at the same concentration. DDB formed a metabolite-P450 complex with P450 upon incubation with human liver microsomes in the presence of NADPH (Kim et al., 2001). P450s were demonstrated to be the primary enzymes responsible for the metabolism of DDB in human liver microsomes *via* demethylation and by generating secondary metabolites (Baek et al., 2001). The aim of this work is to study and gain insight the biochemical effects exerted upon DDB treatment of tamoxifen-induced liver injury and lipid peroxidation.

Materials and Methods

**Chemicals** Dimethyl Dimethoxy Biphenyl Dicarboxylate (DDB) was received as a gift from Arab company for pharmaceuticals and medicinal plants (Mepaco), Egypt. Tamoxifen citrate was a kind gift obtained from medical union pharmaceutical drug company (MUP), Egypt. All the other chemicals were of analytical grade and purchased from Sigma (St. Louis, USA) and Fluka (Buchs, Switzerland).

**Animals** Fifty adult female albino rats weighed 120-170 gm were used as experimental animals in this study. The animals were housed in the animal care centre of faculty of Pharmacy, Al-Azhar University. They were kept under identical conditions with free access to standard laboratory food (Purina Chow) and water.

**Experimental Protocol** The rats were randomly divided into 5 groups of 10 animals each as follows:-

- **Group 1**: Normal control untreated rats.
- **Group 2**: Tamoxifen-intoxicated rats: Rats were treated with tamoxifen citrate i.p. in a dose of 45 mg/Kg body weight in 0.1 ml dimethylsulfoxide (DMSO)/Kg body weight for 7 successive days (Hard et al., 1993).
- **Group 3**: DDB-treated rats: Rats were orally administered 200 mg DDB in 1 ml tween/Kg body weight daily for 10 successive days (Zohny et al., 2001).
- **Group 4**: DDB (P) rats: Rats were orally administered DDB (in a dose similar to group 3) prior tamoxifen intoxication as protection (P) against liver injury induced by tamoxifen.
- **Group 5**: DDB (C) rats: Rats were orally administered DDB (in a dose similar to group 3) after tamoxifen intoxication as a curative (C) against liver injury induced by tamoxifen.

At the end of the experiment, animals were subjected to light ether anaesthesia and killed by cervical dislocation. The blood sample were collected in heparinised centrifuge tube and centrifuged to obtain serum. The abdomen was excised and the liver was removed immediately by dissection, washed in ice-cold isotonic saline and blotted between two filter papers. The liver was wrapped in aluminum foil and stored at 80°C. A 10% (w/v) liver homogenates was prepared in ice-cold 0.1 M potassium phosphate buffer, PH 7.5 using Branson sonifier (250, VWR Scientific, USA).

**Biochemical parameters** Fresh aliquot from liver homogenate was used to estimate glutathione-S-transferase (Habig et al., 1974), glutathione peroxidase (Qi-chang et al., 1987), catalase (Clairborne, 1987), catalase (Clairborne, 1987), catalase (Clairborne,
1985), reduced glutathione (Ellman, 1959) and thiobarbituric acid reactive substance (Ohkawa et al., 1979). However, serum was used to measure the level of sGPT and sGOT liver transaminases (White et al., 1970). The determination of sGOT and sGPT transaminases based on the fact that phenylhydrazone that produced after incubation the substrate with the enzyme, was measured spectrophotometrically. The amount of phenylhydrazine formed was directly proportional to the enzyme quantity.

The glutathione-S-transferase was determined spectrophotometrically using aromatic substrate (1-chloro-2,4 dinitrobenzene) and monitored the change in absorbance due to thioester formation. One unit of the enzyme is defined as the amount of glutathione-S-transferase which needed to catalyze the formation of 1 µmol of thioester per minute and the specific activity is expressed as nmol/min/mg protein. The glutathione peroxidase was determined by reversed phase HPLC using LDC analytical system equipped with C18 econosil 5 µµODS 250×4.6 mm column packed with 5 µµ particle size at flow rate 1 ml/min. This procedure based on the fact that glutathione peroxidase converts the GSH (reduced glutathione) into GSSG (oxidized glutathione). The increase in GSSG amount is directly proportional to glutathione peroxidase activity and the specific activity is expressed as U/mg protein. The catalase activity was measured using H2O2 as substrate that can be decomposed by catalase enzyme. One unit of catalase is defined as the amount needed to decompose 1 nmol H2O2 of per minute and the specific activity is expressed as U/mg protein.

The reduced glutathione was measured spectrophotometrically using 5,5′dithiobis(2-nitrobenzoic acid), that was converted to 2-nitro-S-mercaptobenzoic acid per one mole of glutathione. The amount of reduced glutathione is expressed as nmol/mg protein. The lipid peroxides expressed as nmol of thiobarbituric acid reactive substance (TBARS) per mg protein was measured spectrophotometrically using 1,1,3,3-tetraethoxypropane.

Statistical analysis All data were expressed as mean ± SEM. The standard error was calculated by dividing the standard deviation by the square root of the number of observations. Paired t-test was carried out to compare populations using GraphPad Prism software (San Diego, CA). Statistical significance was defined as a p value < 0.005 and 0.01.

Results

The administration of high dose tamoxifen citrate elicited dramatic increase in the activity of sGPT and sGOT liver transaminases. The Liver damage was evaluated by the measurement of serum level of GPT and GOT transaminases. Rats that orally administered DDB for 10 days, showed no significant change with the normal control group.

The pretreatment (protection) and treatment (curative) of tamoxifen-intoxicated rats with DDB significantly decreased sGPT and sGOT levels in comparing to the tamoxifen-induced liver injury group. The sGPT was significantly decreased by ↓61.0% and ↓50.0% for protected and curative group, respectively. While, the sGOT was significantly decreased by ↓50.0% and ↓35.0% for protected and curative group, respectively (Table 1).

The influence of tamoxifen citrate intoxication and DDB on the antioxidant enzyme activities (glutathione-S-transferase, GST; glutathione peroxidase, GPX and catalase, CAT), reduced glutathione (GSH) and thiobarbituric acid reactive substance (TBARS) of rats liver are showed in Table 2. The level of thiobarbituric acid reactive substance (TBARS) is an indicator for lipid peroxidation of hepatic cells. The tamoxifen-intoxication resulted in liver injury in rats as manifested by significant decline in GST, GPX, CAT and GSH enzyme levels by ↓26%, ↓35.0%, ↓41.0% and ↓27.0%, respectively compared to the normal control. However, TBARS level showed a huge significant increase in tamoxifen-intoxicated rats by ↑2220% in comparing to the normal control group. The administration of DDB to normal rats resulted in significant increase of GST and GSH enzymes levels by ↑10.4% and ↑13.3%, respectively. However the enzyme levels of GPX, CAT and TBARS showed non significant changes compared to the normal rats. On the other hand, the administration of DDB to normal rats resulted in non significant decrease in TBARS compared to normal control group (Table 2).

### Table 1. The influence of oral administration of dimethyl dimethoxy biphenyl dicarboxylate (DDB) to normal rats and before (protective) and after (curative) tamoxifen citrate intoxication on serum GPT and serum GOT liver transaminases

<table>
<thead>
<tr>
<th>Animal Groups</th>
<th>sGPT (U/L)</th>
<th>sGOT (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Control</td>
<td>M ± S.E.</td>
<td>60 ±2.0</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>50-66</td>
</tr>
<tr>
<td>TAM-intoxicated</td>
<td>M ± S.E.</td>
<td>181 ±4.0*</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>160-209</td>
</tr>
<tr>
<td>% Change</td>
<td>↑+ 300%</td>
<td>↑+ 132 %</td>
</tr>
<tr>
<td>DDB-treated</td>
<td>M ± S.E.</td>
<td>59 ±2.0</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>51-69</td>
</tr>
<tr>
<td>% Change</td>
<td>↑+ 2.0%</td>
<td>↓− 0.30%</td>
</tr>
<tr>
<td>DDB (P)</td>
<td>M ± S.E.</td>
<td>70 ±1.0*</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>63-80</td>
</tr>
<tr>
<td>% Change</td>
<td>↓− 61.0%</td>
<td>↓− 50.0%</td>
</tr>
<tr>
<td>DDB (C)</td>
<td>M ± S.E.</td>
<td>90 ± 2.0*</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>78-94</td>
</tr>
<tr>
<td>% Change</td>
<td>↓− 50.0%</td>
<td>↓− 35.0%</td>
</tr>
</tbody>
</table>

DDB(P): protection group; DDB(C): curative group; TAM: tamoxifen citrate.
Values are expressed as mean ± S.E.
Number of rats per group n=10.
*Significance difference from TAM-intoxicated group at p<0.005.
#Significance difference from normal control group at p<0.005.
Moreover, the pre-treatment or treatment with DDB (Table 2), improved the activities of antioxidant enzymes such as glutathione-S-transferase, glutathione peroxidase and catalase as well as the level of reduced glutathione and thiobarbituric acid reactive substance, in comparing to tamoxifen-intoxicated liver injured rats group.

The pretreatment of tamoxifen-intoxicated rats with DDB (Table 2) elicited the strongest effect in inducing a highly significant decline in TBARS and a highly significant increase in the activities of antioxidant enzymes and the level of reduced glutathione in comparison to the tamoxifen-intoxicated rats. The results showed a significant increment in the levels of GST, GPX, CAT and GSH enzymes by ↑+20%, ↑+18.0%, ↑+19% and ↑+5.0%, respectively compared to the normal control. The administration of DDB after tamoxifen-intoxication resulted in significant decrease of TBARS level by ↓-43.0% (Table 2).

**Discussion**

Tamoxifen citrate is an anti-estrogenic drug widely used for the treatment of breast cancer (Desai et al., 2002). It was obvious that tamoxifen in toxic doses lead to oxidative liver damage (Hard et al., 1993) as it have been elucidated to be a hepatocarcinogen in rats (Ahotupa et al., 1993; Karki, 2000). This study was conducted in order to investigate the role of DDB in alleviating the oxidative stress status obtained due to tamoxifen intoxication in rats. It was reported that, DDB have a strong scavenging effect against active oxygen radicals (Li et al., 1990). Tamoxifen may be more toxic to the liver because it

<table>
<thead>
<tr>
<th>Animal Groups</th>
<th>GST nmol/min/mg protein</th>
<th>GPX U/mg protein</th>
<th>CAT U/mg protein</th>
<th>GSH nmol/mg protein</th>
<th>TBARS nmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normal Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M± S.E.</td>
<td>270 ±7</td>
<td>26 ± 1.5</td>
<td>36 ± 2.0</td>
<td>30 ±2.0</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Range</td>
<td>220-300</td>
<td>22-29</td>
<td>32-43</td>
<td>25.9-33</td>
<td>0.6 1.2</td>
</tr>
<tr>
<td><strong>TAM-intoxicated rats</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M±S.E.</td>
<td>200±4.0*</td>
<td>17± 1.0*</td>
<td>21 ± 1.0*</td>
<td>22± 1.0*</td>
<td>21 ± 0.6*</td>
</tr>
<tr>
<td>% Change</td>
<td>↓- 26.0*%</td>
<td>↓-35.0*%</td>
<td>↓-41.0*%</td>
<td>↓-27.0*%</td>
<td>+2220*%</td>
</tr>
<tr>
<td><strong>DDB- treated normal rats</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M±S.E.</td>
<td>298±5.0*</td>
<td>27±1.0</td>
<td>39 ± 1.0</td>
<td>34 ±0.4*</td>
<td>0.8 ± 0.01</td>
</tr>
<tr>
<td>Range</td>
<td>260-320</td>
<td>20-31</td>
<td>33-45</td>
<td>30-37</td>
<td>0.5-0.9</td>
</tr>
<tr>
<td>% Change</td>
<td>↑+10.0*%</td>
<td>↑+ 4.0%</td>
<td>↑+ 8.0%</td>
<td>↑+13.3%</td>
<td>↓- 11.00%</td>
</tr>
<tr>
<td><strong>DDB (P)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M±S.E.</td>
<td>258±5.0*</td>
<td>23±1.0*</td>
<td>31 ± 2.0*</td>
<td>25 ±0.6*</td>
<td>8.1 ± 0.1*</td>
</tr>
<tr>
<td>Range</td>
<td>240-270</td>
<td>17-26</td>
<td>28-33</td>
<td>21-27</td>
<td>7.3-9.8</td>
</tr>
<tr>
<td>% Change</td>
<td>↑+29.0*%</td>
<td>↑+35.0*%</td>
<td>↑+48.0*%</td>
<td>↑+14.0*%</td>
<td>↓- 61.0%</td>
</tr>
<tr>
<td><strong>DDB (C)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M±S.E.</td>
<td>240±5.0*</td>
<td>20± 0.9*</td>
<td>25 ± 0.8*</td>
<td>23 ±0.7</td>
<td>12 ± 0.5*</td>
</tr>
<tr>
<td>Range</td>
<td>230-252</td>
<td>16-3-22.8</td>
<td>21-4-28</td>
<td>20-25</td>
<td>9.8-14</td>
</tr>
<tr>
<td>% Change</td>
<td>↑+20.0*%</td>
<td>↑+18.0*%</td>
<td>↑+19.0*%</td>
<td>↑+ 5.0%</td>
<td>↓- 43.0%</td>
</tr>
</tbody>
</table>

**DDB(P):** protection group; **DDB(C):** curative group; **TAM:** tamoxifen citrate.

Values are expressed as mean ± S.E.

Number of rats per group n=10.

*Significance difference from TAM-intoxicated group at p<0.01.

*Significance difference from normal control group at p<0.005.
has much higher affinity for hepatic tissue than for any other tissues (Desai et al., 2002). It results in a shift toward the growth of diploid hepatocytes in rat liver cells (Dragon et al., 1998). Tamoxifen was found to produce five DNA adducts in rat liver that appeared to be responsible for carcinogenesis (Hemminki et al., 1995; Hellmann-Blumberg et al., 2000). In accordance with the data obtained from this study, Stanley et al., 2001, reported that tamoxifen administration resulted in significant increase in thiobarbituric acid reactive substance (TBARS) production. The lipid peroxidation may be attributed to the fact that hexose monophosphate shunt (HMP) in rat liver is strongly inhibited by high dose of tamoxifen, so that the NADPH levels inside cells is decreased. The state of oxidative stress observed during tamoxifen administration in high dose was accompanied by decreased hepatic glutathione content and increased peroxidation (Ahotupa et al., 1994). The activities of some intracellular antioxidant enzymes decreased with the increase of lipid peroxidation levels (Diplock et al., 1994) which was concomittent with the results achieved from this study.

The pre-treatment as well as treatment of tamoxifen intoxicated rats with DDB, inhibited the tamoxifen-induced hepatic injury and thereby the level of oxidative stress as it can decrease lipid peroxidation and enhance the antioxidant enzyme activities. The decrease in antioxidant defence systems in animals render them more susceptible to the hepatotoxicity (Palomero et al., 2001). The liver damage was determined by the measurement of sGOT and sGPT while the level of TBARS in liver was used as an indicative of lipid peroxidation. The levels of antioxidant enzyme activities in liver homogenates (glutathione-S-transferase, glutathione peroxidase and catalase) and reduced glutathione were significantly increased whereas the level of TBARS were significantly decreased comparable to tamoxifen-intoxicated group (Table 2). On the contrary, Oge et al., 2003, showed that catalase did not show any significant difference after tamoxifen intoxication.

In agreement with the results obtained in this study, Lu and Liu, 1991, reported that when DDB given orally to mice, there was significant reduction in TBARS formation with increased superoxide dismutase and catalase activity. Also, it was obvious that, DDB increased the concentration of nitric oxide and cortisol in blood plasma and saliva (Panossian et al., 1999) and improved the liver functions as the elevated serum GPT and GOT in liver hepatitis patients have been decreased (Liu, 1989). The pretreatment with DDB exerted a little more beneficiary improvements than its use as a treatment (curative) against tamoxifen-intoxication.

In conclusion, the present data indicate that the oral administration of DDB, have a beneficial effects on damaged liver cells to prevent lipid peroxidation and improve antioxidant enzyme activities of tamoxifen-intoxicated rats. Moreover, the use of DDB before tamoxifen treatment (as protection) is more effective than its curative effect (DDB administered after tamoxifen treatment) against tamoxifen-induced liver injury. The data obtained from this study revealed that DDB can mediate its biochemical effects through the enhancement of the antioxidant enzyme activities and reduced glutathione level as well as decreasing the level of lipid peroxides.

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Antioxidant Potentials of Dimethyl Dimethoxy Biphenyl Dicarboxylate (DDB) against Tamoxifen-induced Liver Injury in Rats


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