Possible carcinogenic potential of dimethyl dimethoxy biphenyl dicarboxylate in experimental animals

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J. Adv. Pharm. Technol. Res.

ABSTRACT

Dimethyl dimethoxy biphenyl dicarboxylate (DDB) has been extensively used in the treatment of liver diseases accounting for 1–6% of the global disease burden. Cell replication, DNA synthesis, and proliferation, providing significant information about behavior of cells were examined in mice exposed to subchronic administration with DDB. Conventional liver functions specifically gamma-glutamyltransferase (γ-GT), a marker expressing liver canceration was also investigated. Normal mice were allocated into two groups each of 10 mice. The 1st and 2nd groups were treated with DDB in a dose of 50 mg/kg/day, 5 days/week for 1 month and 3 months, respectively. Comparable groups of normal mice were left without treatment as controls. Compared to normal control group, animals receiving DDB for 3 months showed marked elevations of both alanine aminotransferase and γ-GT, significant inhibition in cytochrome P450, a significant increase in the mean ploidy and 4C with moderate to marked increase in S-phase populations and the number of proliferating cell nuclear antigen-positive cells. In conclusion, this is the first report on the potential relationship between the subchronic administration of DDB and the increase in the hepatocyte proliferation, cell replication and DNA synthesis that may raise an alarm regarding possible DDB insult on the biological behavior of cells.

Key words: Cytochrome P450, dimethyl dimethoxy biphenyl dicarboxylate, DNA pattern, gamma-glutamyltransferase, mice, proliferating cell nuclear antigen

INTRODUCTION

Dimethyl dimethoxy biphenyl dicarboxylate (DDB), which is a traditional Chinese medicine, has been registered as liver support medication in China, and is currently used for the treatment of chronic viral hepatitis B (HBV) and hepatitis C (HCV) in Asia, for example, China, Vietnam, Indonesia, Pakistan, and Egypt. At a national level, HCV prevalence in Egypt ranges from 11% to 14%. Although directly acting antiviral drugs have been successfully introduced with cure rates of >95%, DDB has been given (10 pilules 3 times daily) for years to HBV and HCV patients as supplement and/or alternative treatment to the conventional old treatment regimen (interferon and rebavirin).

In Egypt and according to the National Control Strategy 2008 on viral hepatitis, liver mortality is over 40,000/year and is increasing annually. One model predicts 700,000 cases of cirrhosis and over 140,000 cases of hepatocellular carcinoma (HCC) within the next 20–30 years. Based on the GLOBOCAN 2008 estimates, HCC has been doubling in incidence over a decade and ranks the 2nd and 6th most...
common cancer among men and women, respectively, in Egypt.\textsuperscript{6} Although HCC is known to develop on top of chronic viral hepatitis B, C or co-infections, cofactors may be playing a role in the higher incidence of HCC in Egyptian patients, including cigarette smoking, occupational exposure to chemicals such as pesticides, aflatoxins, and endemic infections such as schistosomiasis or fascioliasis.\textsuperscript{7}

This work investigates the effect of subchronic administration of DDB on the hepatocytes proliferative status including inflammation and neoplasia using proliferating cell nuclear antigen (PCNA), a main component of eukaryotic DNA replication machinery controlling several metabolic pathways.\textsuperscript{8} Conventional liver functions involving alanine aminotransferase (ALT), total protein, albumin and γ-GT as an alarming marker expressing liver canceration when its activities are gradually increasing\textsuperscript{9} were also investigated.

MATERIALS AND METHODS

Animals

Male Swiss albino mice (CD-1 strain) weighing 20–25 g, obtained from the Schistosome Biological Supply Center at the Theodor Bilharz Research Institute (TBRI), were fed a standard rodent pellet chow and water ad libitum and housed under standard laboratory conditions. All the animal experiments were conducted in accordance with the international guidelines and were approved by the Institutional Review Board of TBRI.

Experimental design

Normal mice were allocated into two groups each of 10. The 1\textsuperscript{st} and 2\textsuperscript{nd} groups were treated with DDB (Beijing Union Pharmaceutical Factory, P.R. China) in a dose of 50 mg/kg/day,\textsuperscript{10} 5 days/week for 1 and 3 months, respectively. Comparable groups were left without treatment as controls. After 1 and 3 months of DDB treatments, blood samples were collected; sera were separated and stored at −70°C pending assay.

Assessment of biochemical parameters

Concentrations of serum ALT, γ-GT, total protein, albumin, total bilirubin and urea were estimated by the methods of Reitman and Frankel,\textsuperscript{11} Persijn and van der Slik,\textsuperscript{12} Weichselbaum,\textsuperscript{13} Doumas et al.,\textsuperscript{14} Weigl et al.,\textsuperscript{15} and Patton and Crouch,\textsuperscript{16} respectively.

Evaluation of DNA content, proliferating cell nuclear antigen and cytochrome antigens

Feulgen stain specifically and quantitatively stains the nuclear DNA blue that can be quantified as integrated optical density,\textsuperscript{17} using the computerized image analysis system (Zeiss, Germany) with software program “AxioVision 4.8 (Carl Zeiss Microscopy GmbH 07745 Jena, Germany).” A number of 150–200 cells with single monolayer nuclei were subjected to DNA analysis, identified, and classified according to Eskelino et al.\textsuperscript{18} Immunohistochemical reaction was performed using the avidin-biotin complex immunoperoxidase technique according to Hsu et al.,\textsuperscript{19} using anti-mouse antibodies against PCNA and cytochrome P450 (CYP450) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The number of PCNA positive nuclei was assessed in 1000 hepatic nuclei in 10–15 high power fields (×400)/section. PCNA proliferation index was expressed as percentage of positive epithelial nuclei to total nuclei.\textsuperscript{20} The number of CYP450 positive cells as cytoplasmic stain\textsuperscript{21} in five random fields (approximately 1000 hepatocytes)/section was assessed under ×10 objective lens of a light microscope.

Statistical analysis

Results were expressed as mean ± standard error. A two-tailed Student’s t-test was used to detect the significance of difference between the means of different groups. Results were considered statistically significant if $P \leq 0.05$.

RESULTS

Biochemical parameters

Treatment with DDB for 3 months resulted in significant elevation in γ-GT level ($P < 0.05$) when compared to the corresponding control. Compared with the DDB-treated group for 1 month, there were significant increases in both ALT ($P < 0.05$) and γ-GT ($P < 0.001$) levels in the DDB-treated group for 3 months [Table 1].

Table 1: Biochemical changes in sera of mice treated with dimethyl dimethoxy biphenyl dicarboxylate for 1 and 3 months (n=10 per group)

| Animal groups | ALT (U/L) | γ-GT (U/L) | Total protein (g/dL) | Albumin (g/dL) | Globulins (g/dL) | A/G ratio (g/dL) | Blood urea (mg/dL) |
|---------------|-----------|------------|----------------------|----------------|-----------------|----------------|-------------------|
| 1 month       |           |            |                      |                |                 |                |                   |
| Untreated     | 12.60±1.51 | 1.12±0.12  | 5.66±0.23            | 3.05±0.12      | 2.61±0.11       | 1.21±0.13      | 50.5±3.80         |
| DDB-treated   | 14.31±1.31 | 1.23±0.14  | 5.47±0.32            | 2.84±0.22      | 2.64±0.13       | 1.16±0.14      | 55.3±3.90         |
| 3 months      |           |            |                      |                |                 |                |                   |
| Untreated     | 15.62±1.32 | 2.48±0.86  | 6.62±0.23            | 3.38±0.19      | 3.14±0.17       | 1.08±0.07      | 52.5±4.21         |
| DDB-treated   | 19.33±1.73 | 6.86±1.3** | 6.56±0.43            | 3.18±0.39      | 3.38±0.22       | 0.96±0.13      | 57.94±3.69        |

Values are mean±SE. **$P<0.01$ versus the corresponding untreated group, $^aP<0.05$. **$P<0.001$ versus DDB-treated for 1 month. SE: Standard error; ALT: Alanine aminotransferase; γ-GT: Gamma-glutamyltransferase; DDB: Dimethyl dimethoxy biphenyl dicarboxylate.
Cytochrome P450 expression

Compared to untreated controls, expression of CYP450 in the cytoplasm of hepatocytes was numerically decreased in the DDB-treated group for 1 month [Table 2, Figure 1a and b] and significantly increased (P < 0.01) in the treated group for 3 months [Table 2, Figure 1c and d].

DNA content pattern

Treatment with DDB for 1 month showed significant increase (P < 0.01) in the percentage of hepatocytes occupying the S-phase fraction on the expense of cells at 2C (P < 0.01) when compared to parallel untreated control. Meanwhile, treatment for 3 months showed significant increases (P < 0.01) in the mean ploidy, 4C (P < 0.05) and in the percentage of hepatocytes occupying the S-phase fraction on the expense of cells at 2C (P < 0.01). Compared with the DDB-treated for 1 month, a significant increase (P < 0.05) in S-phase on the expense of 2C was recorded in the treated group for 3 months [Table 2]. Image analyzer automatically expresses the DNA content of each individual cell. The percentage of each cell out of the total number of cells examined are then classified into four categories, namely, diploid (2C), proliferation index (S-phase cells) (3C), tetraploid (4C), and cells with more than 4–5C DNA content indicating aneuploidy. The DNA content in hepatocytes of control and DDB-treated groups for 1 and 3 months is shown in Figures 2 and 3.

Immunohistochemical expression of proliferating cell nuclear antigen (positive indices)

Liver of treated mice for 1 and 3 months [Figure 4b and d] showed scattered hepatocytes with binucleated nuclei and a significant increase in the number of inflammatory cells (P < 0.05, P < 0.01) compared with their corresponding untreated controls, respectively [Figure 4a and c]. Regarding PCNA, liver sections of untreated control groups left for 1 and 3 months showed few nuclei with positive reaction [Figure 5a and c]. Table 2 shows an increase in the number of liver PCNA positive nuclei both in hepatocytes (P < 0.05) and in the epithelial cells lining the sinusoids (P < 0.05, P < 0.01) of treated mice for 1 [Figure 5b] and 3 months [Figure 5d], respectively, compared to the respective untreated control groups [Table 2].

Table 2: Nuclear DNA pattern, cytochrome P450 expression and the proliferating index (proliferating cell nuclear antigen; positive indices) in hepatocytes of mice treated with dimethyl dimethoxy biphenyl dicarboxylate for 1 and 3 months (n=10 per group)

| Animal groups | DNA ploidy | 2C | 4C | S-phase % | CYP450 | PCNA (PI) |
|---------------|------------|----|----|-----------|--------|-----------|
|               |            |    |    |           |        |           |
| 1 month       |            |    |    |           |        |           |
| Untreated     | 1.9±0.42   | 94.6±1.22 | 1.0±0.78 | 4.4±0.84 | 93.0±7.88 | 12.2±3.35 | 17.4±2.36 |
| DDB-treated   | 2.4±0.51   | 85.8±4.82* | 2.4±0.53 | 11.8±5.00** | 73.0±11.35 | 24.6±4.29* | 29.0±4.42* |
| 3 months      |            |    |    |           |        |           |
| Untreated     | 2.0±0.02   | 93.6±1.42 | 1.2±0.13 | 5.2±1.39 | 84.0±5.16 | 15.2±3.48 | 18.0±2.00 |
| DDB-treated   | 3.2±0.42** | 67.2±5.59** | 2.8±0.29* | 30.0±5.57** | 53.0±9.35** | 38.6±6.97* | 45.0±10.19** |

Values are mean±SE. *P<0.05, **P<0.01 versus the corresponding untreated groups, 1P<0.05 versus DDB-treated for 1 month. SE: Standard error, DDB: Dimethyl dimethoxy biphenyl dicarboxylate, PCNA: Proliferating cell nuclear antigen, CYP450: Cytochrome P450, PI: Positive indices
DISCUSSION

This study investigated the effect of DDB on hepatocyte proliferation and nuclear pattern of DNA in healthy mice, hand in hand with some of the liver enzymes expressing hepatic function and tendency to canceration. Treatment for 3 months resulted in significant elevation in $\gamma$-GT when compared to control group. Liu et al.\textsuperscript{[9]} reported an extremely low $\gamma$-GT in the adult liver with gradually increasing pattern during the courses of liver canceration revealing the use of $\gamma$-GT as a hepatocarcinoma marker. Hanigan\textsuperscript{[22]} considered it a factor influencing tumor growth and conferring survival advantages to rapidly dividing neoplastic cells through the enhancement of the intracellular recovery of cysteine; crucial for protein synthesis. The presence of elevated $\gamma$-GT levels seems to reflect a state of persistent oxidative stress as part of the biological pathway related to cancer development.\textsuperscript{[23]}

In this study, the expression of CYP450 in the cytoplasm of hepatocytes, a key player in the metabolism and activation/inactivation of various toxicants and carcinogens was significantly decreased 3 months post-DDB treatment. Kim et al.\textsuperscript{[24]} demonstrated inhibition of CYP450 isoform “CYP3A4” activity when DDB was preincubated with NADPH in microsomes. This inhibition may modulate the elimination of other co-administered drugs metabolized by CYP3A4. It can also alter susceptibility to toxins and carcinogens, which are either activated or detoxified by P450 enzymes.\textsuperscript{[25]} Nii\textsuperscript{[26]} hypothesized that the orally ingested CYP inhibitors limit the CYP metabolism in all tissues, and the remaining proximate or ultimate carcinogens in liver migrate to extrahepatic tissues where they induce tumors.

DNA quantitation revealed moderate to marked increase in S-phase populations in 60% and 80%, in conjunction with significant increase in PCNA-positive cells, in DDB-treated mice for 1 and 3 months, respectively. In the hepatocytes, clonal perpetuation of accumulated DNA aneuploid affecting growth regulatory genes, which eventually leads to a continuation of severe progressive abnormalities characterized by dysplasia, formation of adenoma and finally cancer.\textsuperscript{[27]} The elevated levels of PCNA expression appear in the nucleus during the late G1 phase with maximum expression during S-phase and decline during G2 and M phase. Therefore, the accumulation of PCNA gene products in cycling cells is considered an index of the degree of cellular proliferation and DNA synthesis.\textsuperscript{[28]} Therefore, detection of significant amount of this protein marker is a reliable indicator of cell replication. Interestingly, following exposure of cells to DDB for 3 months, a tightly bound form of PCNA was detected in nuclei in all phases of the cell cycle that refer to the increase in the proliferative ability of liver cells with continuation of DDB treatment.
CONCLUSION

Continuous administration of DDB may be an irritating material and could lead to the development of alterations in DNA replication and division in the hepatocytes and the sinusoids. This gives us an alarm that DDB may have insulted the biological behavior of cells, specifically cell proliferation a precursor to tumorigenesis. Until a long-term randomized control trial is accomplished addressing the histological impacts of DDB on hepatocytes, the use of DDB should not be encouraged.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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