ORIGINAL ARTICLE

Possible carcinogenic potential of dimethyl dimethoxy biphenyl dicarboxylate in experimental animals

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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,[1] 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,[2] and Egypt.[3] At a national level, HCV prevalence in Egypt ranges from 11% to 14%.[4] 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.[5] Based on the GLOBOCAN 2008 estimates, HCC has been doubling in incidence over a decade and ranks the 2nd and 6th most

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common cancer among men and women, respectively, in Egypt.[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.[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.[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[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 1st and 2nd groups were treated with DDB (Beijing Union Pharmaceutical Factory, P.R. China) in a dose of 50 mg/kg/day,[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 proteins, albumin, total bilirubin and urea were estimated by the methods of Reitman and Frankel,[11] Persijn and van der Slik,[12] Weichselbaum,[13] Doumas *et al*.,[14] Weigl *et al*.,[15] and Patton and Crouch,[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,[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*.[18] Immunohistochemical reaction was performed using the avidin-biotin complex immunoperoxidase technique according to Hsu *et al*.,[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 optical fields (×400)/section. PCNA proliferation index was expressed as percentage of positive epithelial nuclei to total nuclei.[20] The number of CYP450 positive cells as cytoplasmic stain[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	52.58±4.21
DDB-treated	19.33±1.73$^{\$}$	6.86±1.13**$^{,\$\$\$}$	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, $^{\$}P<0.05$, $^{\$\$\$}P<0.001$ 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].

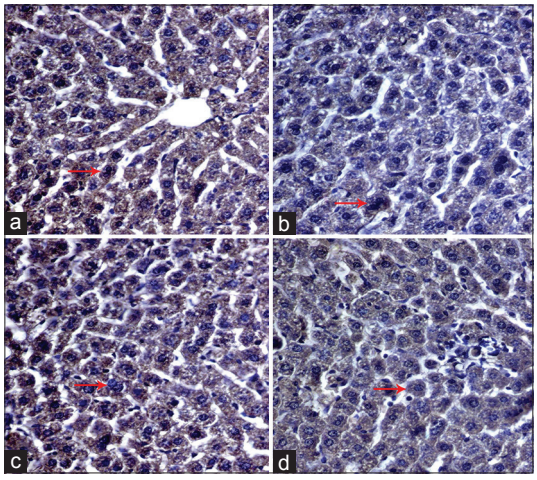

Figure 1: Hepatocytes of untreated (a and c) and dimethyl dimethoxy biphenyl dicarboxylate-treated (b and d) mice for 1 and 3 months, respectively (IHC, DAB, ×400), showing large number of positive cytochrome P450 (a and c) with marked intensity (brownish cytoplasmic stain) and binucleated nuclei (red arrows), and mild-moderate number of positive cytochrome P450 (b and d) with binucleated nuclei (red arrow)

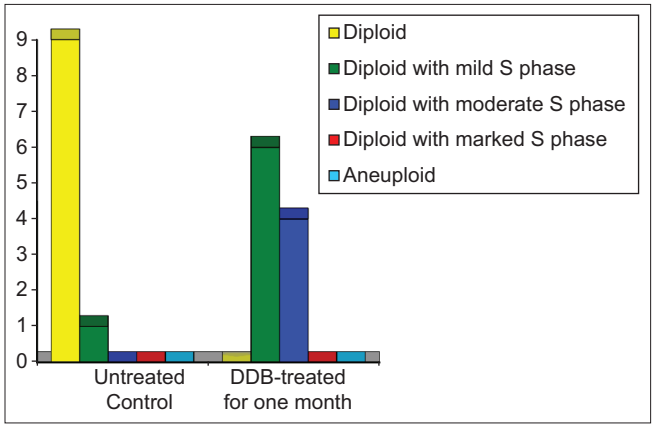

Figure 2: DNA pattern in dimethyl dimethoxy biphenyl dicarboxylate-treated group for 1 month versus untreated control

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)	
						Hepatocytes	Sinusoidal lining cells
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, $$P$<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

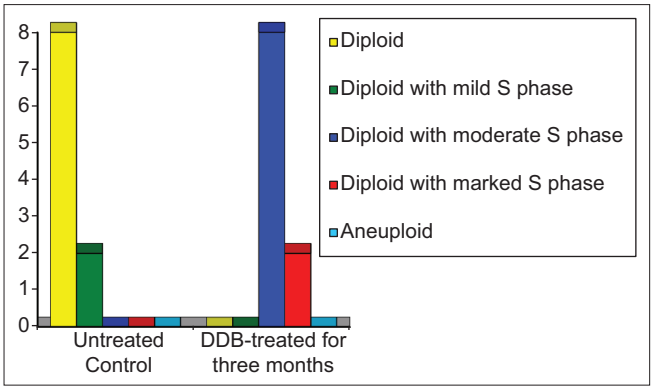

Figure 3: DNA pattern in dimethyl dimethoxy biphenyl dicarboxylate-treated group for 3 months versus untreated control

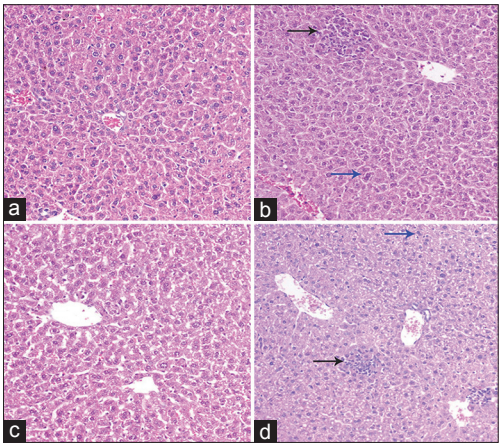

Figure 4: Hepatic tissues of untreated (a and c) and dimethyl dimethoxy biphenyl dicarboxylate-treated (b and d) mice for 1 and 3 months, respectively (H and E, ×200), showing normal architecture with regular arrangement of hepatocytes around central vein (a and c) and scattered hepatocytes with binucleated nuclei (blue arrow) and mild interlobular inflammation (black arrow) (b and d)

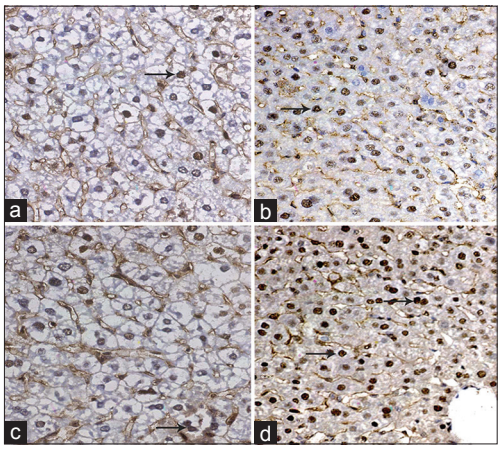

Figure 5: Hepatocytes of untreated (a and c) and dimethyl dimethoxy biphenyl dicarboxylate-treated (b and d) mice for 1 and 3 months, respectively (IHC, DAB, ×400), showing 10% and 15% of positive proliferating cell nuclear antigen (a and c) with strong intensity (brownish nuclear stain, black arrow) and 20–30% of positive proliferating cell nuclear antigen (b and d) with strong intensity (brownish nuclear stain, black arrow)

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 γ-GT when compared to control group. Liu *et al.*[9] reported an extremely low γ-GT in the adult liver with gradually increasing pattern during the courses of liver canceration revealing the use of γ-GT as a hepatocarcinoma marker. Hanigan[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 γ-GT levels seems to reflect a state of persistent oxidative stress as part of the biological pathway related to cancer development.[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.*[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.[25] Nii[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.[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.[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 of 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.

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Nil.

Conflicts of interest
There are no conflicts of interest.

REFERENCES

1. Lee KH. Research and future trends in the pharmaceutical development of medicinal herbs from Chinese medicine. Public Health Nutr 2000;3:515-22.
2. Sun H, Liu GT. Chemopreventive effect of dimethyl dicarboxylate biphenyl on malignant transformation of WB-F344 rat liver epithelial cells. Acta Pharmacol Sin 2005;26:1339-44.
3. Salama HM, Amer AR, Hammad OM, El-Sayed WF. Effect of DDB monotherapy and in combination with amantadine hydrochloride and ribavirin in patients with chronic hepatitis C virus infection. Sci Med J ESCME 2004;16:1.
4. Strickland GT. Liver disease in Egypt: Hepatitis C superseded schistosomiasis as a result of iatrogenic and biological factors. Hepatology 2006;43:915-22.
5. Mohamed MK. Epidemiology of HCV in Egypt 2004. Afro Arab Liver J 2004;3:41-52.
6. GLOBOCAN 2008. Cancer Incidence and Mortality Worldwide, Database (version 1.2). Available from: http://www.globocan.iarc.fr. [Last accessed 2015 Nov 15].
7. Anwar WA, Khaled HM, Amra HA, El-Nezami H, Loffredo CA. Changing pattern of hepatocellular carcinoma (HCC) and its risk factors in Egypt: Possibilities for prevention. Mutat Res 2008;659:176-84.
8. Moldovan GL, Pfander B, Jentsch S. PCNA, the maestro of the replication fork. Cell 2007;129:665-79.
9. Liu Z, Liu G, Zhang S. Reversing effect of dimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-dicarboxylate (DDB) on the phenotypes of human hepatocarcinoma cells line. Cancer Lett 1996;108:67-72.
10. Kim SN, Kim SY, Yim HK, Lee WY, Ham KS, Kim SK, *et al.* Effect of dimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-dicarboxylate (DDB) on chemical-induced liver injury. Biol Pharm Bull 1999;22:93-5.
11. Reitman S, Frankel S. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Am J Clin Pathol 1957;28:56-63.
12. Persijn JP, van der Slik W. A new method for the determination of gamma-glutamyltransferase in serum. J Clin Chem Clin Biochem 1976;14:421-7.
13. Weichselbaum TE. An accurate and rapid method for the determination of proteins in small amounts of blood serum and plasma. Am J Clin Pathol 1946;10:40-9.
14. Doumas BT, Watson WA, Biggs HG. Albumin standards and the measurement of serum albumin with bromcresol green. Clin Chim Acta 1971;31:87-96.
15. Weigl E, Bach H, Krieg D. Serum bilirubin in an obvious healthy population (author's transl). Med Klin 1975;70:664-9.
16. Patton C, Crouch S. Determination of serum urea. Anal Chem 1977;49:464-9.
17. Schulte E, Wittekind D. Standardization of the Feulgen-Schiff technique. Staining characteristics of pure fuchsin dyes; a cytophotometric investigation. Histochemistry 1989;91:321-31.
18. Eskelino R, Mokashy K, Yamaha R. Flow cytometric analysis of nuclear DNA content in endoscopic biopsy tissues of gastric cancer. Am J Clin Oncol 1995;18:325.
19. Hsu SM, Raine L, Fanger H. The use of antiavidin antibody and avidin-biotin-peroxidase complex in immunoperoxidase technics. Am J Clin Pathol 1981;75:816-21.
20. Akyol G, Dursun A, Poyraz A, Uluoglu O, Ataoglu O, Edalý N, *et al.* P53 and proliferating cell nuclear antigen (PCNA) expression in non-tumoral liver diseases. Pathol Int 1999;49:214-21.
21. Zhao X, Zhang JJ, Wang X, Bu XY, Lou YQ, Zhang GL. Effect of berberine on hepatocyte proliferation, inducible nitric oxide synthase expression, cytochrome P450 2E1 and 1A2 activities in diethylnitrosamine- and phenobarbital-treated rats. Biomed Pharmacother 2008;62:567-72.
22. Hanigan MH. Expression of gamma-glutamyl transpeptidase provides tumor cells with a selective growth advantage at physiologic concentrations of cyst(e)ine. Carcinogenesis 1995;16:181-5.
23. Pompella A, Corti A, Paolicchi A, Giommarelli C, Zunino F. Gamma-glutamyltransferase, redox regulation and cancer drug resistance. Curr Opin Pharmacol 2007;7:360-6.
24. Kim JY, Baek M, Lee S, Kim SO, Dong MS, Kim BR, *et al.* Characterization of the selectivity and mechanism of cytochrome P450 inhibition by dimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-dicarboxylate. Drug Metab Dispos 2001;29:1555-60.
25. Forrester LM, Henderson CJ, Glancey MJ, Back DJ, Park BK, Ball SE, *et al.* Relative expression of cytochrome P450 isoenzymes in human liver and association with the metabolism of drugs and xenobiotics. Biochem J 1992;281(Pt 2):359-68.
26. Nii H. Possibility of the involvement of 9H-pyrido[3,4-b] indole (norharman) in carcinogenesis via inhibition of cytochrome P450-related activities and intercalation to DNA. Mutat Res 2003;541:123-36.
27. Dragan YP, Pitot HC. The role of the stages of initiation and promotion in phenotypic diversity during hepatocarcinogenesis in the rat. Carcinogenesis 1992;13:739-50.
28. Bravo A, de Torrontegui G, Díaz R. Identification of components of a new stability system of plasmid R1, ParD, that is close to the origin of replication of this plasmid. Mol Gen Genet 1987;210:101-10.