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Apoptotic effects of dipyrido [3,2-a:2′,3′-c] phenazine (dppz) Au(III) complex against diethylnitrosamine/phenobarbital induced experimental hepatocarcinogenesis in rats

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Abstract We evaluated the effects of dipyrido [3,2-a:2′,3′-c] phenazine (dppz) Au(III) complex ([Au(dppz)Cl_2]Cl) on apoptosis during chemically induced hepatocellular carcinoma. 48 male Spraque-Dawley rats were divided into six groups; group I (control), group II [Dimethyl sulfoxide (DMSO)], group III ([Au(dppz)Cl_2]Cl), group IV [diethylnitrosamine + Phenobabital (DEN + PB)], group V (DEN + PB + [Au(dppz)Cl_2]Cl (2nd week)), and group VI (DEN + PB + [Au(dppz)Cl_2]Cl (7th week). The rats in groups IV through VI were administrated with DEN in a single dose of intraperitoneal 175 mg/kg. After 2 weeks of DEN administration, these groups of rats were given daily PB in a dose of 500 ppm. In group V, after two weeks of DEN administration, [Au(dppz)Cl_2]Cl complex (2 mg/kg) was given once a week by intraperitoneal injection. In the group VI, the rats were given a dose of 2 mg/kg [Au(dppz)Cl_2]Cl complex once a week, 7 weeks after DEN administration. At the end of the study, blood and tissue samples were collected from the rats to determine levels of serum AST, ALT, and LDH, and caspase 3, p53, Bax, Bcl-2 and DNA fragmentation in liver. AST, ALT, LDH, and Bcl-2 levels were higher in group IV, compared to group I, but caspase 3 and p53 levels were lower. In group V, caspase 3, p53, Bax, and DNA fragmentation levels were higher than those of group IV. Caspase 3 and p53 levels increased in group VI compared with group IV. In conclusion, [Au(dppz)Cl_2]Cl complex induced apoptosis by elevating levels of caspase 3, p53, Bax, and DNA fragmentation.

Keywords Gold(III) complexes · Apoptosis · Bax · Bcl-2 · Caspase 3

Abbreviations

dppz	Dipyrido [3,2-a:2′,3′-c] phenazine
[Au(dppz)Cl_2]Cl	Dipyrido [3,2-a:2′,3′-c] phenazine (dppz) Au(III)
DMSO	Dimethyl sulfoxide
DEN	Diethylnitrosamine
PB	Phenobarbital
Cisplatin	Cis-diammine-dichloroplatinum (II)
HCC	Hepatocellular carcinoma
TUNEL	Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling
LD50	50 % Lethal dose
SPSS	Statistical package for the social sciences

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Introduction

For living organisms, metals are fundamental cellular components chosen by nature for the functioning of some indispensable biochemical processes. A large number of disorders have required many metal compounds for their treatment [1, 2]. Metal complexes are favored for their diagnostic and anticancer uses in medicinal chemistry [1, 3]. The discovery of cis-diammine-dichloroplatinum (II) (cisplatin) initiated studies on anticancer agents [1].

Cisplatin is a potent anti-cancer drug widely used to treat different human solid carcinomas. However, cisplatin-based chemotherapy response rate in hepatocellular carcinoma (HCC) patients were low, and therefore it failed to extended patient survival [4]. HCC is a major worldwide public health concern with increasing incidence [5]. Thus, new and nontoxic anticancer drugs are immediately required to treat HCC [4].

In the last two decades, a great diversity of gold(I) [6] and gold(III) [7] compounds have qualified as ideal nominees for further pharmacological assessment because they are stated to possess antiproliferative attributes in vitro against numerous human cancer cell lines. Gold(III) compounds show isoelectronic and isostructural properties with platinum (II) and have similar uptake and DNA interference activity. Gold(III), compared to platinum(II), is an additional superiority for linking with DNA [8]. Gold compounds have been found to display promising antitumor activities [4]. For cytotoxicity or antitumour activities, a wide variety of gold(III) complexes were studied [9, 10] and some of them even retain efficacy against the cisplatin-resistant cell lines [11, 12]. Several research groups reported the antitumor activity of gold compounds, which indicates the remarkable interaction of gold ions with biomolecules [13]. The cytotoxicity and antitumor attributes of these organogold(III) compounds are apparently the result of direct DNA damage [14]. However, mechanistic studies propose that DNA is not an initial destination for gold(III) complexes. Their cytotoxicity is rather arbitrated by their capability to change mitochondrial activity and block protein synthesis [15].

Recent studies have shown that gold(III) complexes induce apoptosis through both caspase-dependent and independent apoptotic pathways [16]. Caspases play an essential role in p53 mediated apoptosis [17]. The p53 gene is a tumor suppressor which exhibits a crucial role in the control of the normal cell cycle; hence, it is a key factor in apoptosis induction in response to chemotherapy [18]. Transcriptional up-regulation of Bax gene expression is modulated by the p53. Bax gene product binds with Bcl-2 and inhibits the ability of Bcl-2 from preventing apoptosis [19]. In an in vitro study, the gold complexes were reported to affect the levels of antiapoptotic Bcl-2 and proapoptotic Bax. Aldinucci et al. [10] recently stated that gold(III) compounds decreased Bcl-2 and induced Bax in cultured cells.

In the current investigation, the use of one of the dipyrido [3,2-a:2′,3′-c] phenazine (dppz) Au(III) complex ([Au(dppz)Cl_2]Cl) in treating HCC was clarified in an experimental rat model. A combination of DEN and PB was preferred to induce experimental hepatocarcinogenesis in Spraque-Dawley male rats. This model of chemical carcinogenesis was chosen because nitrosamine is a hepato-specific carcinogen predominantly affecting the liver and also has biochemical, morphological, and histological similarities to human HCC [20]. The aim of this study was to investigate the possible apoptotic effects of newly synthesized [Au(dppz)Cl_2]Cl complex in a rat model of HCC.

Materials and methods

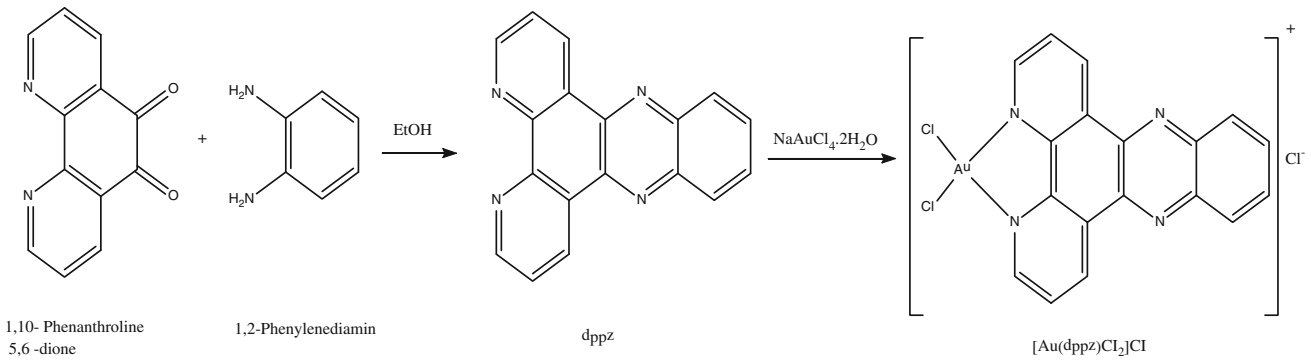
Synthesis of [Au(dppz)Cl_2]Cl

Dipyrido [3,2-a:2′,3′-c] phenazine (dppz) was prepared by the method of Dickeson and Summers [21]. 1,10-phenanthroline-5,6-dione, sodium tetrachloroaurate dihydrate ($NaAuCl_4 \cdot 2H_2O$), and potassium tetrachloroplatinat (K_2PtCl_4) were purchased. All other reagents were used as purchased without any further purification. Chemical analysis for C, H, and N were carried out on a Perkin-Elmer elemental analyzer. Infrared spectra in the region 4,000–200 cm^{-1} were recorded on Perkin-Elmer FT-IR. Spectra were measured using KBr pellets technique. ^{1}H-NMR spectra were acquired on Bruker DX400 instrument utilizing DMSO-d6 as solvent.

[Au(dppz)Cl_2]Cl was synthesised as reported (Scheme 1) [22, 23]. Ligands were dissolved in C_2H_5OH-H_2O including metal salts ($NaAuCl_4.2H_2O/K_2PtCl_4$) and then stirred for about 1 h at 30 °C. The precipitate was filtered and washed several times with water and then diethylether, and it was dried in vacuum dessicator. The filtrate was washed five times with water and air dried. The vapor diffusion technique with diethyl ether was used to crystalize the product from the solution of the compound in acetone. The compound was characterised by IR, ^{1}H-NMR, and MASS spectroscopic data.

Dipyrido [3,2-a:2′,3′-c] phenazine (dppz)

($C_{18}H_{10}N_4$; % C: 76.58; H: 3.57; N: 19.85). Selected IR (KBr) n_{max} (cm^{-1}): 3148, 3110, 3075 (Ar C–H), 1645, 1630, 1450, 1391 (C=N, C=C). ^{1}H-NMR(400 MHz) (DMSO-d_6) d(ppm) : 7.5–7.8 (6H, m), 8.3–8.8 (4H, m). MS(EI) (m/z): 282.3 (M^+).



Scheme 1 Synthesis of $[Au(dppz)Cl_2]Cl$ complex

[Au(dppz)Cl₂]Cl

($C_{18}H_{10}AuCl_3N_4$; % C: 36.92; H: 1.72; N: 9.57). Selected IR (KBr) n_{max} (cm^{-1}): 3175, 3110, 3081 (Ar C–H), 1650, 1624, 1450, 1382 (C=N, C=C), 327 (Au–Cl), 290 (M–N). ^{1}H-NMR(400 MHz) (DMSO-d_6) d(ppm) : 7.7–7.9 (6H, m), 8.5–8.9 (4H, m). MS(EI) (m/z): 585.6 (M^+).

The compounds provided satisfactory elemental analysis. The ^{1}H NMR spectral findings were also good agreement with the assigned structures, and all the proton peeks are determined to be at desired regions. The NMR of the complexes displayed remarkable downfield shift for the protons proximal to the polypyridyl donor nitrogen atoms suggesting ligand coordination, as in literature [24]. From the IR spectra results, the studied structure displays three characteristic absorption bands: the one is N–H at 3,175–3,075 cm^{-1}, second is C=N at 1,650 cm^{-1}, and the last one is C=C at 1,396 cm^{-1} with the stretching vibration. The Au–Cl bond in the title complex was provided by the presence of the band in the region 390–320 cm^{-1} [25]. All spectral data conform to literature.

Animal subjects

Two months old male Sprague–Dawley rats, 200–250 g in weight, were purchased from Eskisehir Osmangazi University Experimental Research Centre. The rats used in this investigation were kept in polycarbonate cages in a room, at constant temperature (21 ± 2 °C) and humidity (45–55 %). They had constant access to standard rat chow and permitted to drink water ad libitum. All protocols for this experiment were approved by Eskisehir Osmangazi University Institutional Local Animal Care committee (Permit number: 124).

Acute toxicity test

The 50 % lethal dose (LD50) of the $[Au(dppz)Cl_2]Cl$ was determined for male Sprague–Dawley rats. The rats were randomly divided into nine groups of five animals each. $[Au(dppz)Cl_2]Cl$ complex was dissolved in DMSO before use, and the final concentration of the solvent was 5 %.

Group-I (Control group)

The first group received a single intraperitoneal dose of DMSO.

Groups II–IX ([Au(dppz)Cl₂]Cl treated groups)

Eight groups were injected intraperitoneally with different doses (2, 4, 8, 16, 32, 64, 128, 256 mg/kg) of $[Au(dppz)Cl_2]Cl$ complex only once.

The latency of death and the number of deaths during each 24 h-period were determined during 3 days. The LD1, LD10, LD50, LD90, and LD99 were calculated by logarithmic regression analysis of the number of deaths according to the statistical Probit method [26]. We evaluated the intraperitoneal acute toxicity of $[Au(dppz)Cl_2]Cl$ complex on rats and demonstrated that $[Au(dppz)Cl_2]Cl$ complex presented no toxic effect if not more than 2 mg/kg.

Experimental design

The animals were divided into six groups of eight rats in each; group I (control), group II (Dimethyl sulfoxide (DMSO)), group III ($[Au(dppz)Cl_2]Cl$), group IV (DEN + FB), Group V (DEN + FB + $[Au(dppz)Cl_2]Cl$ (2nd week)), and group VI (DEN + FB + $[Au(dppz)Cl_2]Cl$ (7th week)).

Control group

The rats of control group no medication or treatment was given during the experimental period. The control rats were fed with standard diet.

Group II

Group II rats received 5 % DMSO intraperitoneally at 1 ml for the first day of experiment. Two weeks after this injection, the rats were given DMSO again once a week for 11 weeks.

Group III

Drug control animals were administered intraperitoneally once a week with 2 mg/kg body weight of $[Au(dppz)Cl_2]Cl$ complex dissolved in 1 ml of 5 % DMSO, for the entire experimental period between the 3rd week and the 13th week.

Group IV–VI

All rats were given a single intraperitoneal injection of DEN (175 mg/kg body weight) [27] dissolved in 1 ml of 5 % DMSO to initiate hepatocarcinogenesis. After 2 weeks on basal diet, they were administered drinking water containing PB at doses of 500 p.p.m. [28] for 11 weeks from weeks 3 to 13.

Group V

The rats were treated intraperitoneally with $[Au(dppz)Cl_2]Cl$ complex at the dose of 2 mg/kg body weight for two week after the administration of DEN and it continued till the end of the experiment. The $[Au(dppz)Cl_2]Cl$ complex was dissolved in 1 ml of 5 % DMSO.

Group VI

These rats were treated intraperitoneally with $[Au(dppz)Cl_2]Cl$ complex at the dose of 2 mg/kg body weight for seven weeks after the administration of DEN and it continued till the end of the experiment. The $[Au(dppz)Cl_2]Cl$ complex was dissolved in 1 ml of 5 % DMSO.

At the end of the experimental period, intracardiac blood samples were withdrawn under ether anesthesia, and the rats were sacrificed by cervical dislocation. To separate serum, blood samples were immediately centrifuged at $3500\times g$ for 10 min and then stored at -20 °C until analysis. Liver tissue samples were cleaned using an ice-cold solution of isotonic NaCl for the removal of blood spots, and they were then dried with blotting paper. Liver was divided into two pieces; one piece for biochemical studies and the other for histology and immunohistochemistry.

Enzyme assessments

The serum enzymes (AST, ALT, LDH) were analysed with a Roche Diagnostic Modular Analyser using Roche Hithachi kit.

p53

Levels of p53 protein were measured in liver homogenates. The p53 Pan ELISA kit was used for determine the levels of p53 protein (Roche Molecular Biochemicals, Germany, Cat. Nr. 11828789001). Weighed samples of liver tissue (1 g) was homogenized in ice cold RIPA buffer (low-salt) containing 20 mM Tris, 0.5 mM EDTA, 1 % Nonidet P40, 0.5 % sodium deoxycholate, 0.05 % sodium dodecyl sulfate, 1 mM phenylmethylsulphonylfluoride, 1 μg/ml aprotinin, and 2 μg/ml leupeptin. The homogenate was centrifuged at $10{,}000\times g$ for 10 min at 4 °C. The resulting supernatants were accumulated. The supernatants were transferred to a streptavidin-coated microtiter plate with an anti-human-p53 polyclonal antibody. They were processed according to the manufacturer's instructions. Absorbance was read at 450 nm with a microplate reader. Finally, tissue p53 concentrations were expressed as pg/g wet tissue weight.

Caspase 3

USCN Life Science Inc. caspase 3 ELISA kit (Cat. Nr. E90626Ra) was used to measure liver caspase 3 concentrations. The concentration of caspase 3 was determined according to caspase 3 ELISA kit. Briefly, liver tissues (1 g) were minced and then homogenized in ice cold PBS (0.02 mol/L, pH 7.0) and then centrifuged at $5{,}000\times g$ at 4 °C for 5 min. The supernatant was used to estimate the caspase 3 concentration. Levels were expressed as ng/g wet tissue weight.

Hepatic morphology and immunohistochemistry

The removed livers were fixed in neutral formalin and processed routinely for paraffin embedding followed by preparing liver slides in 4 mm thickness. Sections of rat liver tissue were stained by hematoxylin–eosin (H–E). All sections were scanned after an examination with Olympus BH-2 photomicroscope. During histological examination, hepatocyte degeneration, necrosis, and infiltration were evaluated by light microscopy. Severity of changes was assigned using scores of none (−), mild (+), moderate (++) and severe (+++) [29].

For immunohistochemical analysis, the deparaffinized and redehydrated tissue sections were quenched for endogenous peroxidase activity by incubating in 3 %

H_2O_2 for 10 min. To inhibit nonspecific binding, Ultra V Block (Lab Vision, USA) was applied for 20 min. Excess liquid was discarded and then sections were reacted with primary antibody Bcl-2 (ab7973 Abcam) and Bax (ab7977 Abcam) at room temperature for 1 h and washed in PBS. After each section was treated with streptavidin-peroxidase conjugate, colour was improved with amino-ethyl carbazole (AEC) chromate. The immunostained sections were counterstained for 1 min with hematoxylin and then dehydrated in sequential ethanol series for sealing and microscope observation. During immunohistochemical examination, Bax and Bcl-2 were evaluated by light microscopy. Severity of changes was assigned using scores of none (−), mild (+), moderate (++) and severe (+++) [29].

Induction of apoptosis was measured by quantitative terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) kit (Apoptag; Chemicon International, Inc) in liver sections (4–5 μm thick) from studied rats. A TUNEL-positive nucleus was stained brown.

Statistical analysis

The data were analyzed by statistical package for the social sciences (SPSS) 20.0 packet program. The Shapiro–Wilk test and the Kolmogorov–Smirnov test were used for verification of the data's normality. Continuous variables were demonstrated using n (sample size) and mean and SD. n (sample size) and median and 25th and 75th percentiles were used to demonstrate categorical variables. Continuous normally distributed measurements were compared across the groups using one-way analysis of variance. Non-parametric comparisons of samples were conducted by the Kruskal–Wallis test. Immunohistochemistry and histology results were analyzed with the Kruskal–Wallis test followed by Dunn's multiple comparison test. A value of $p < 0.05$ was considered to be significant.

Results

Table 1 shows the levels of serum hepatic marker enzymes in control and experimental rats. DEN + PB induced hepatocarcinogenesis caused abnormal liver function in all rats. The activities of all the marker enzymes (AST, ALT, LDH) showed a significant increase ($p < 0.001$, $p < 0.001$, $p < 0.05$ respectively) in DEN + PB animals compared to the control. On the other hand, there was no difference in marker enzymes between in both group II and group III compared to control group ($p > 0.05$). The values ALT decreased significantly in both group V ($p < 0.001$) and group VI ($p < 0.001$) as compared with group IV. AST and LDH amounts were not chanced in groups V ($p > 0.05$) and VI ($p > 0.05$) compared with group IV. Nevertheless, there was no significant difference in ALT, AST, and LDH levels between groups V and VI ($p > 0.05$).

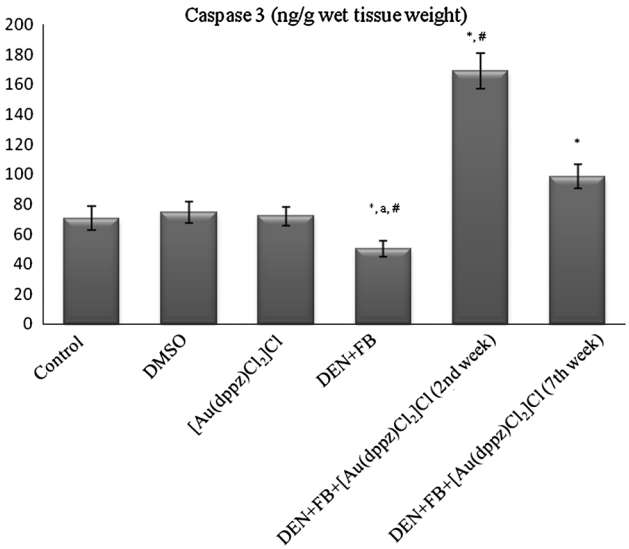


Fig. 1 Tissue levels of caspase-3 protein by ELISA analysis in control and experimental rat livers. Results are expressed as mean ± SE, and data was analyzed by one-way analysis of variance using SPSS 20.0 software. $^{*}p < 0.001$ versus groups I, II, and III, $^{a}p < 0.001$ versus group V, $^{\#}p < 0.001$ versus group VI

Table 1 Serum levels of ALT, AST, and LDH in control and experimental animals

Groups	ALT(U/L)	AST(U/L)	LDH (U/L)
Control ($n = 8$)	65.13 ± 6.40	125.25 ± 14.50	1,890.38 ± 138.35
DMSO ($n = 8$)	64.88 ± 7.26	127.63 ± 10.25	1,874.13 ± 226.21
[Au(dppz)Cl_2]Cl ($n = 8$)	65.00 ± 9.67	126.75 ± 12.76	1,928.00 ± 265.70
DEN + FB ($n = 8$)	128.50 ± 11.17*	168.13 ± 8.01*	2,326.38 ± 206.61**
DEN + FB + [Au(dppz)Cl_2]Cl (2nd week) ($n = 8$)	66.25 ± 5.80***	151.38 ± 22.13**	2,291.63 ± 409.31**
DEN + FB + [Au(dppz)Cl_2]Cl (7th week) ($n = 8$)	71.00 ± 4.54***	160.25 ± 21.57*	2,107.88 ± 125.92

Results are expressed as mean ± SE, and data was analyzed by one-way analysis of variance using SPSS 20.0 software

* $p < 0.001$ versus groups I, II, and III, ** $p < 0.05$ versus groups I, II, and III, *** $p < 0.001$ versus group IV

As depicted in Fig. 1, there were no significant differences in protein levels of caspase 3 in both group II and III cases compared to group I ($p > 0.05$), and there was a significant decrease in caspase 3 content in group IV compared to group I ($p < 0.001$). Caspase 3 protein levels in groups V and VI were significantly higher than group IV ($p < 0.001$). Caspase 3 was also higher in group V than in group VI ($p < 0.001$).

No difference was found between group I and group II for liver level of p53 ($p > 0.05$). p53 was significantly lower in group IV than in group I. p53 protein level of group III was higher than those of group II ($p < 0.001$) and group I ($p < 0.001$); however, it was significantly lower than group V and group VI ($p < 0.001$). p53 increased significantly in group III ($p < 0.001$), group V ($p < 0.001$), and group VI ($p < 0.001$) compared with group IV (Fig. 2).

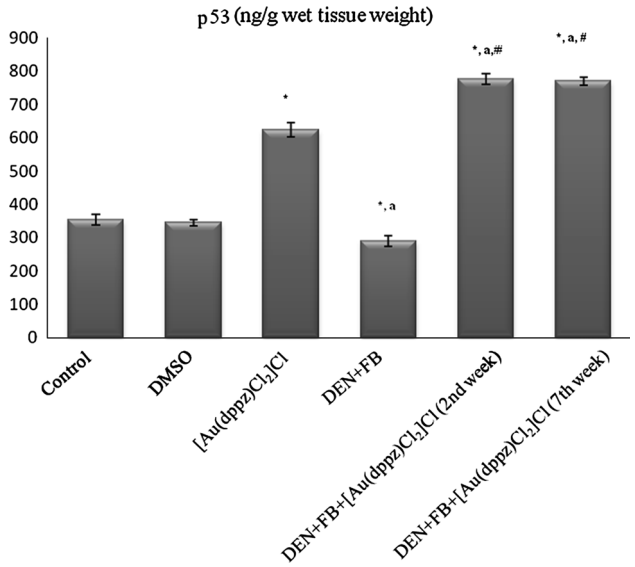


Fig. 2 Tissue levels of p53 protein by ELISA analysis in control and experimental rat livers. Results are expressed as mean ± SE, and data was analyzed by one-way analysis of variance using SPSS 20.0 software. *$p < 0.001$ versus groups I and II, $^{a}p < 0.001$ versus group III, $^{\#}p < 0.001$ versus group IV

Table 2 Tissue levels of Bax and Bcl-2 protein by immunohistochemical analysis in control and experimental rat livers

Groups	Bax	Bcl-2
Control ($n = 8$)	0 (0–0)	0 (0–0)
DMSO ($n = 8$)	0 (0–0)	0 (0–0.75)
[Au(dppz)Cl_2]Cl ($n = 8$)	0 (0–0)	0 (0–0)
DEN + FB ($n = 8$)	0 (0–0)	1 (1–1) ^{b,c,e}
DEN + FB + [Au(dppz)Cl_2]Cl (2nd week) ($n = 8$)	1 (0.25–1) ^{a,d,e,f}	0.50 (0–1)
DEN + FB + [Au(dppz)Cl_2]Cl (7th week) ($n = 8$)	1 (0–1)	0.50 (0–1)

Results are expressed as medians and range of extremes, and data was analyzed according to the Kruskal–Wallis test followed by the post-hoc Dunn test performed for multiple comparisons using SPSS 20.0 software

^{a} $p < 0.01$ versus groups I, $^{b}p < 0.001$ versus groups I, $^{c}p < 0.05$ versus group II, $^{d}p < 0.01$ versus group II, $^{e}p < 0.01$ versus group III, $^{f}p < 0.01$ versus group IV

Immunohistochemical studies indicated that there were no differences in Bax and Bcl-2 levels in both group II and III cases compared to group I ($p > 0.05$) (Table 2). Bax proteins were significantly higher in group V ($p < 0.01$) compared with others (groups I, II, III and IV). There was no significant difference in Bax values between group IV and the other groups ($p > 0.05$). The level of Bcl-2 in group IV significantly increased compared with groups I, II, and III ($p < 0.001$). Bcl-2 decreased in groups V and VI, but there was no statistically significant difference between each other and that in groups IV (Table 2).

No significant histological changes in the liver were seen in groups I (Fig. 3), II, and III. Severe histopathological changes were consistently observed in the livers of all the rats from group IV (Figs. 4, 5). The predominant lesions were extensive hepatocyte degeneration, necrosis, and infiltration of inflammatory cells (Table 3). Also, in group IV, the histology confirmed liver damage as evidenced by the presence of preneoplastic focus in the respective liver sections. The rats in group V and group VI exhibited significantly lesser pathology compared to the extensive liver damage found in the group IV. The administration of [Au(dppz)Cl_2]Cl in group V decreased the infiltration, hepatic necrosis, and degeneration.

TUNEL method was used to detect DNA fragmentation that results from apoptotic signalling cascades. As shown in Fig. 6, the section of groups I and II demonstrated TUNEL-negative staining. In groups III and IV, a few TUNEL positive cells were observed in the liver sections. Many apoptotic cells were positively stained in the groups V and VI.

Discussion

Programmed cell death is a highly preserved mechanism that plays a fundamental role in tissue homeostasis through targeted elimination of single cells without disrupting the biological functionality of the tissue [30]. Down regulation of the apoptosis pathways are implicated in the development of various cancers. Therefore, induction of apoptosis in cancer cells has significant importance for the discovery of anticancer agents [31].

Some recent studies have shown that novel gold(III) compounds can cause marked proliferative inhibition in human hepatic cell lines in vitro [4, 32, 33]. It has been

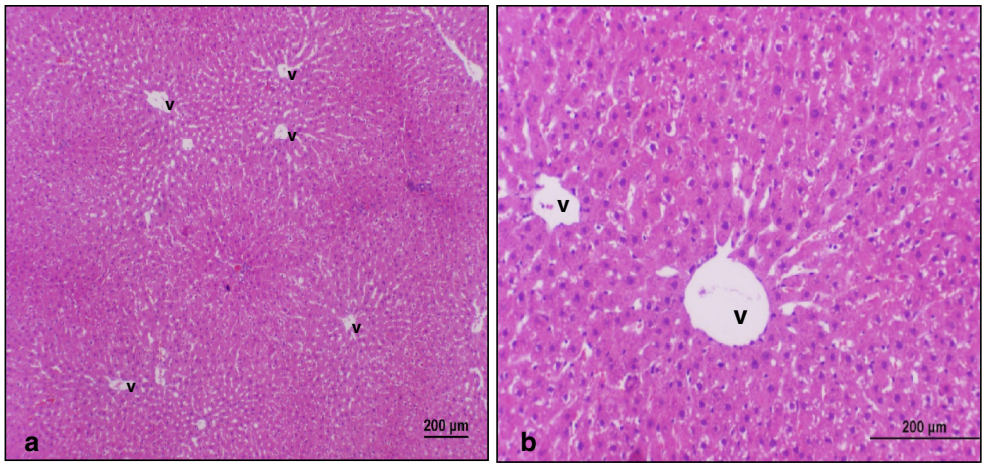


Fig. 3 **a**,**b** Light microscopy of liver section in control group. Histopathological studies in the liver of control group (a, b) revealed a normal architecture with the central vein (v). There was no observable necrosis, degeneration, and infiltration in the control group liver tissues (H & E, *bar*: 100 µm)

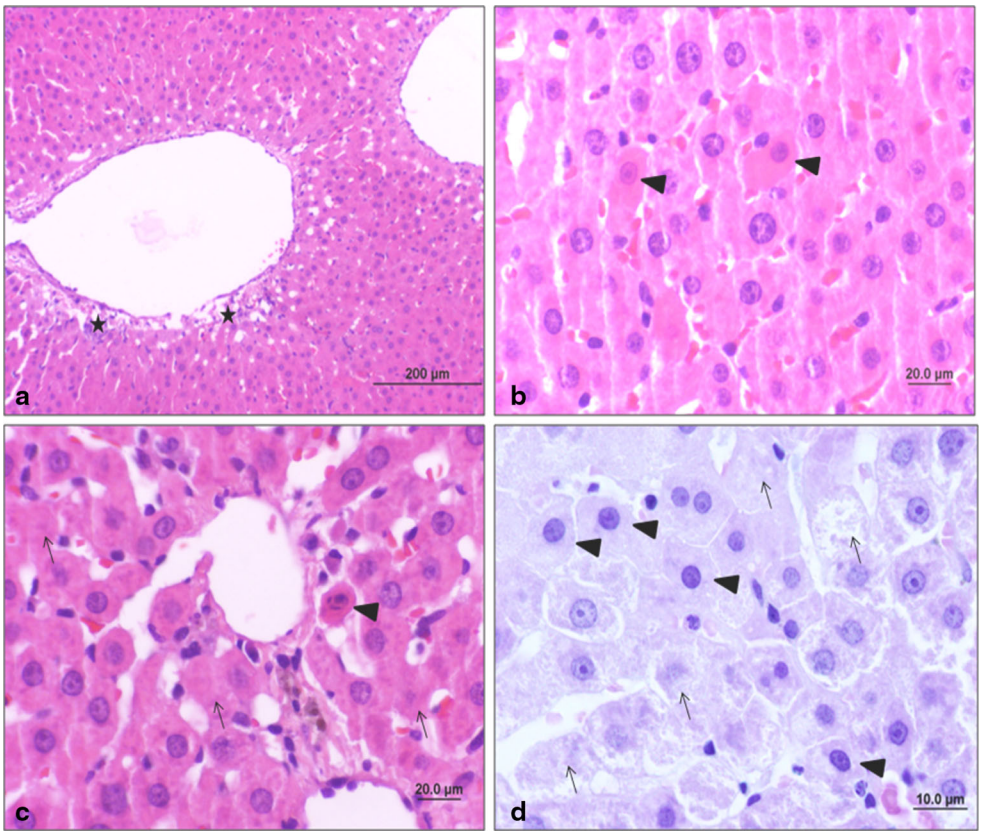


Fig. 4 **a**–**d** Light microscopy of liver section in DEN + PB group. Histopathological observation of DEN + PB group shows extensive cell damage (*asterisk*) (**a**), especially around the vena centralis (v). Hepatic tissue of DEN + PB group rats showed cells necrosis with eosinophilic cytoplasm, pyknotic nuclei (*black right pointing pointer*) (**b**, **c**, **d**), and karyolysis (*arrow*) (**c**,**d**) [H & E, *bar*: 200 µm (**a**), *bar*: 20.0 µm (**b**), *bar*: 20.0 µm (**c**), *bar*: 10.0 µm (**d**)]

proposed that induction of apoptosis is associated with cytotoxicity [31, 34]. The anticancer activities of gold(III) compounds were explored as a result of the fact that gold(III) is isoelectronic with platinum(II), and also tetra-coordinate gold(III) complexes are in the same square-planar geometries as cisplatin [35]. In this study, we

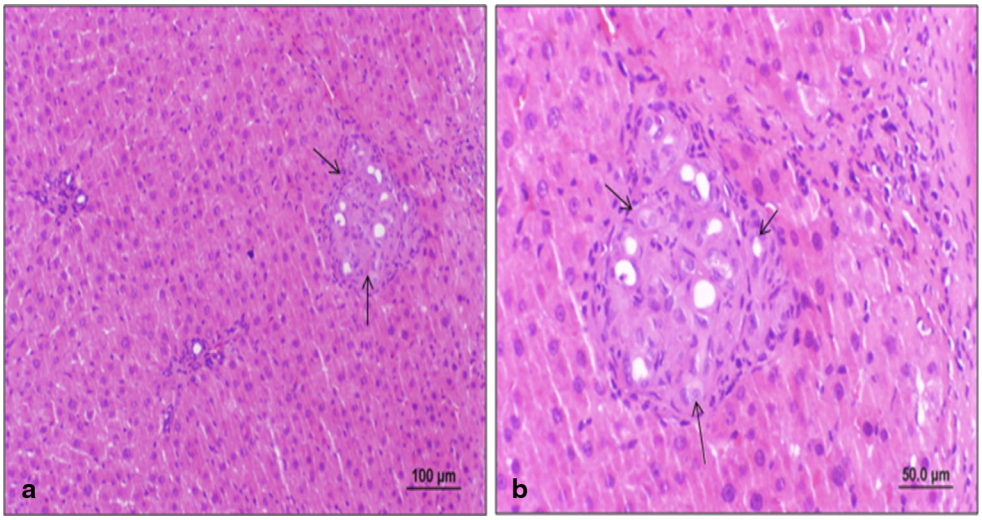


Fig. 5 (**a**,**b**): Light microscopy of liver section in DEN + PB group. The section of DEN + PB group, which increased preneoplastic focus (*arrow*), being found following hematoxylin and eosin staining [H & E, *bar*: 100 µm (**a**), *bar*: 50 µm (**b**)]

Table 3 Histopathological changes in the liver tissues of the studied groups

Groups	Necrosis	Degeneration	Infiltration
Control ($n = 8$)	0 (0–0)	0 (0–0)	0 (0–0)
DMSO ($n = 8$)	0 (0–0)	0 (0–0)	0(0–0.75)
[Au(dppz)Cl_2]Cl ($n = 8$)	0 (0–0)	0 (0–0)	0 (0–1)
DEN + FB ($n = 8$)	2 (2–3)$^{b, e, h}$	3(2.25–3)$^{b, e, h}$	3(2–3)$^{b, d, g}$
DEN + FB + [Au(dppz)Cl_2]Cl (2nd week) ($n = 8$)	0 (0–0.75)^{j}	1(0–1)^{i}	0(0–1)^{j}
DEN + FB + [Au(dppz)Cl_2]Cl (7th week) ($n = 8$)	1(0.25–1.75)	1(1–1.75)$^{a, c, f}$	1(0.25–2)

Results are expressed as medians and range of extremes, and data was analyzed according to the Kruskal–Wallis test followed by the posthoc Dunn test performed for multiple comparisons using SPSS 20.0 software

^{a} $p < 0.01$ versus group I, $^{b}p < 0.001$ versus group I, $^{c}p < 0.05$ versus group II, $^{d}p < 0.01$ versus group II, $^{e}p < 0.001$ versus group II, $^{f}p < 0.05$ versus group III, $^{g}p < 0.01$versus group III, $^{h}p < 0.001$ versus group III, $^{i}p < 0.05$ versus group IV, $^{j}p < 0.01$versus group IV

wanted to further investigate the effect of [Au(dppz)Cl_2]Cl on apoptosis in DEN/PB induced hepatocarcinogenesis in rats. With the application of [Au(dppz)Cl_2]Cl in this chemical carcinogenesis model, the outcomes specified here may be valuable in the discovery and development of tumor chemotherapy and other antitumor associated investigation.

Light microscopic investigation showed that the hepatic tissue of groups I, II, and III exhibited normal large polygonal cells with prominent round nuclei and eosinophilic cytoplasm, and few spaced hepatic sinusoids arranged in-between the hepatic cords with fine arrangement of kupffer cells. In contrast, infiltration, hepatic necrosis, degeneration, and preneoplastic focus were observed in the livers of group IV. Pyknotic nuclei with irregular nuclear membrane were also present in group IV. Our data are corroborated by previous studies reported by other investigators on DEN/PB induced hepatocarcinogenesis in animals [36, 37]. These observations suggest that the detected alterations may be associated to the neoplastic phenomenon and can be used as early indicators of tumourogenicity. However, [Au(dppz)Cl_2]Cl complex in group V reduced more pathological degenerations in the early stage of hepatocarcinogenesis of rats.

Increased levels of intracellular enzymes, such as AST, ALT, and LDH are regarded as sensitive indicators of liver cell damage. ALT is recognised to be a largely liver specific enzyme. Conversely, AST might be a non-specific index in the liver because AST is present in the heart, skeletal muscle, kidney, and brain [38]. LDH is a highly sensitive marker for solid neoplasm. LDH activity is elevated in various types of tumor [39]. Also, these enzymes themselves could be used as biomarkers of HCC response to therapy [40]. In this study, elevated levels of marker enzymes were observed in the serum of HCC-induced animals. The increase in serum ALT and AST indicated that DEN may induce liver injury. The increased levels of LDH may be due to its overproduction by tumor cells.

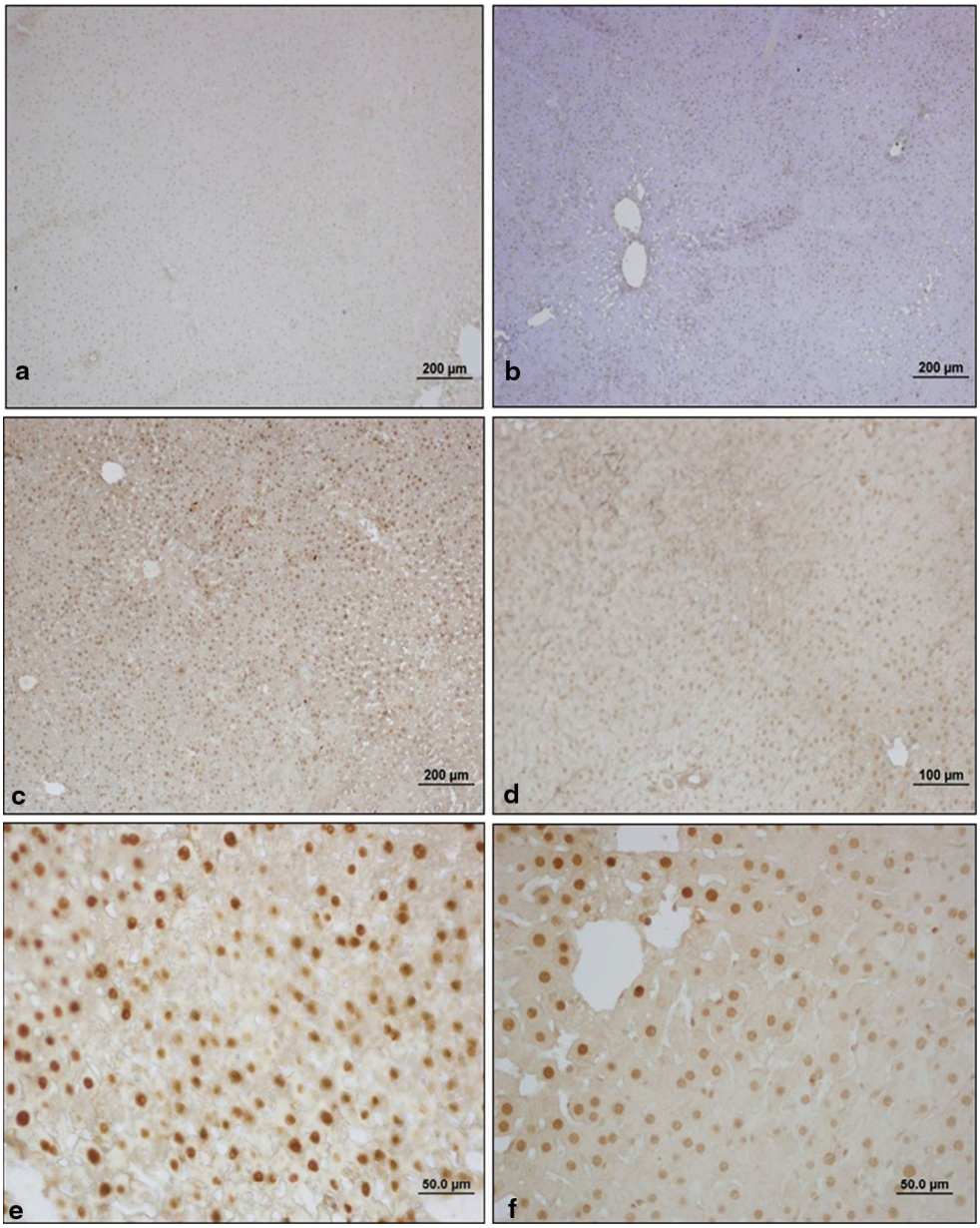


Fig. 6 **a**–**f** TUNEL staining in the liver tissues of the studied groups. The section of control and DMSO groups showed TUNEL-negative staining (**a**,**b**) (*bar*: 200 µm). In [Au(dppz)Cl_2]Cl and DEN + PB groups, a few TUNEL positive cells were observed in the liver section (**c**,**d**) (*bar*: 200 µm, *bar*: 100 µm). Many apoptotic cells were positively stained in the [DEN + FB + [Au(dppz)Cl_2]Cl (2nd week)] group and [DEN + FB + [Au(dppz)Cl_2]Cl (7th week)] group. Treatment of [Au(dppz)Cl_2]Cl markedly increased the number of TUNEL positive cells (**e**,**f**) (*bar*: 50 µm)

Proliferating malignant cells display very high proportion of glycolysis, which afterwards leads to elevated LDH activity [41]. However, these enzyme levels were not different in group III compared to group I, indicating that [Au(dppz)Cl_2]Cl complex did not cause damage to normal liver tissue. As seen in this study, application of [Au(dppz)Cl_2]Cl complex reduced serum ALT levels in groups V and VI compared to group IV. [Au(dppz)Cl_2]Cl complex treatment brought down the levels of ALT close to normal, suggesting its anti-cancer potential.

It is stated by some studies that for the induction of cell growth arrest and apoptosis in human cells following DNA damage or other kinds of cellular stress such as hypoxia, nucleotide depletion, activated oncogenes, tumor suppressor gene p53 is very significant. [42]. p53 is reported to induce apoptosis by activating numerous pro-apoptotic genes, leading to the activation of APAF-1 and caspase 9 through several pathways. p53 is estimated to organize excess of 100 genes, many of which can help population growth arrest or apoptosis [43]. An increased risk of tumor

formation and development is thought to be related to the loss of normal p53 function [42]. There was a significant reduction in the amounts of p53 protein in group IV rats compared to group I. This attenuation might be important for resistance to apoptotic cell death and may have an impact on DEN/PB induced carcinogenesis. In the previous studies, it was shown that a series of gold(III) complexes induced upregulation of p53 in a in vitro cancer study [44]. In accordance with these data, we also found that [Au(dppz)Cl_2]Cl substantially elevated the protein levels of p53 in group V and group VI. An increase of the p53 protein might be responsible for the greater cell damage induced by the [Au(dppz)Cl_2]Cl and, consequently, the increased cell death. One unanticipated finding was that a significant elevation in the p53 levels was determined in group III animals. Because of the absence of any changes in other parameters that were measured in group III, the increase was limited only to the amount, and it does not effect the function of healthy cells.

The apoptosis induction is related to the activation of cysteine aspartic acid-specific proteases (caspase 3, caspase 6, and caspase 8); a conserved family of enzymes that irreversibly lead a cell to death. The release of cytochrome c from mitochondria to cytosol after being induced by a variety of apoptosis-inducing agents causes the activation of procaspase 9 and then caspase 3 [45]. Caspase 3 is stated as a significant downstream effector cysteine protease in the apoptotic pathway [46]. Resistance of tumors to treatment and apoptosis are related to the loss of caspase 3 expression. Resistance to apoptosis by a wide variety of apoptotic stimuli including chemotherapeutic drugs due to defect of expression or activation of caspase 3 has been shown in a variety of cancer cell lines [47]. In this study, a significant decrease in caspase 3 levels was seen in DEN/PB-treated animals. The findings of the current study are consistent with those of Saraswati et al. [48]. Because caspase 3 is the effector caspase in the apoptosis pathways, we think that a reduced level of caspase 3 may play an important role in HCC carcinogenesis. Hence, loss of apoptosis can impact tumor initiation, progression, and metastasis. Palanichamy et al. [44] have reported that a series of gold(III) complexes induced upregulation of caspase 3 in an in vitro cancer study. Similarly, a significant increase in caspase 3 expression levels was observed on [Au(dppz)Cl_2]Cl complex treatment in both group V and group VI. A possible explanation for this might be that the release of cytochrome c from mitochondria as a result of permeability alterations to the mitochondrial membranes is a significant pathway of caspase 3 elevation. Therefore, the result suggested that an up-regulation caspase 3 proteins determined in this investigation may be one of the considerable mechanisms through which [Au(dppz)Cl_2]Cl complex induced apoptosis.

The Bcl-2 family, with its prosurvival proteins Bcl-2, Bcl-xl, Mcl-1, and A1 and death promoting proteins Bax, Bcl-xs, Bak, Bad, and Bik has a centralized role in the regulation of death of cells. The overexpression of anti-apoptotic proteins of this gene family, such as Bcl-2, brings to cancer pathogenesis, and it may be related to the resistance to cancer treatment. Bax plays a central role in regulation of apoptosis and also counteracts the antiapoptotic effect of Bcl-2, and it may directly induce apoptosis [49]. Bax and Bcl-2, which are central regulators of apoptotic cell death, also have the ion channel perforation activity and may control apoptosis by influencing the permeability of the membranes and cytochrome c release from mitochondria. Overexpression of Bcl-2 prevents cytochrome c release in response to a variety of apoptotic stimuli. Down-regulation of Bcl-2 thus causes to cytochrome c release from other membrane of mitochondria. Furthermore, Bcl-2 heterodimerization with Bax exerts dominant negative inhibition of proapoptotic Bax activity. Therefore, when the Bcl-2 expression level is low and the Bax expression level is maintained, homodimers of Bax will always be formed and apoptosis will be stimulated [50]. Recently, Aldinucci et al. have declared on the antiproliferative and apoptotic activities of two newly synthesized gold(III)-methylsarcosinedithiocarbamato derivatives against human acute myeloid leukemia cells. These new gold(III) compounds seem to stimulate a potent downregulation of the antiapoptotic Bcl-2 molecule and an upregulation of the proapoptotic Bax protein [10]. Similarly, Nandy et al. [51] have reported that gold(I) N-heterocyclic carbene complex induced downregulation of Bcl-2, upregulation of Bax. Our results show that no significant difference of Bax levels was found between group IV and group I. On the other hand, the level of Bcl-2 in group IV significantly increased compared with group I. In our opinion, the high Bcl-2 expression in group IV probably protects this group of rats from apoptosis and is essential for proper cell proliferation. A possible explanation for this might be that the effects of apoptosis induction are more dependent on the ratio between Bcl-2 and Bax than on the quantity of Bcl-2 alone [52]. The increased level of Bax in group V compared with group IV observed in our study indirectly indicate the alteration in the signaling phase of apoptosis in cancer cells. The increase in Bax is also consistent with the increase in p53 level observed in group V. The increased Bax expression following the induction of cell death has been concerned with the induction of the p53 expression since Bax is known to be a target for transcriptional activation by p53 in response to DNA damage [53]. It is important to note that [Au(dppz)Cl_2]Cl complex began to be used at different time in group V and VI; specifically, the application of [Au(dppz)Cl_2]Cl complex did not induce Bax level in group VI. The [Au(dppz)Cl_2]Cl complex was

able to up-regulate the proapoptotic molecule Bax and induce apoptosis. These findings suggest that [Au(dppz)Cl_2]Cl complex induce apoptosis via Bax pathway.

Apoptosis has been crucial for normal development of several systems [54]. Besides, as a result of a defense mechanism such as immune reactions or when cells are damaged by disease or noxious agents, apoptosis is seen to happen. DNA damage in some cells may result from irradiation or drugs used for cancer chemotherapy, leading to apoptotic death through a p53-dependent pathway [55]. Internucleosomal DNA fragmentation is considered to be a biochemical hallmark of apoptosis [54]. A primary physiological characteristic is the DNA fragmentation, which states an early event in apoptosis, and it marks a one way road to cell death. This happens as a result of no new cellular protein being synthesized for cell survival [56]. Various gold complexes with possible in vitro apoptotic activities were reported. In a more recent study, the gold(III) meso-tetraarylporphyrin 1a was stated to induce apoptosis of HCC cells both in vivo and in vitro [4]. We did not detect any DNA fragmentation with our TUNEL assay within groups I and II. TUNEL staining showed evidence of increased apoptosis in groups V and VI after application of [Au(dppz)Cl_2]Cl complex. [Au(dppz)Cl_2]Cl complex induced apoptosis in this rat model of hepatocarcinogenesis. It may be associated with the increased levels of the tumor-suppressor protein p53, Bax, and caspase 3. Transcriptional activation by p53 of pro-apoptotic Bax can increase the permeability of the mitochondrial membrane, as a result leading to the release of apoptogenic factors [57]. However, the study suggests that the increased levels of caspase 3 and p53 may relate to the induction of internucleosomal DNA fragmentation. All results indicated that [Au(dppz)Cl_2]Cl complex could increase apoptosis-related protein levels and result in apoptosis in the DEN/PB induced rats. It can thus be suggested that [Au(dppz)Cl_2]Cl complex induced cell apoptosis which might lead to inhibition of proliferation.

In conclusion, we have showed the novel observation that [Au(dppz)Cl_2]Cl complex induced apoptosis in DEN/PB induced rats involved with the modulation of p53, caspase 3 and Bax protein levels. To our knowledge, this is the first study to demonstrate the in vivo anticancer effect of [Au(dppz)Cl_2]Cl complex on DEN/PB induced rat model of hepatocarcinogenesis. This finding is a promising avenue in the application of gold compounds as novel anticancer drug candidates for the treatment of HCC.

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