Anticancer Effect of a Novel Palladium–Saccharinate Complex of Terpyridine by Inducing Apoptosis on Ehrlich Ascites Carcinoma (EAC) in Balb-C Mice

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Abstract. *Background/Aim: [Pd(sac)(terpy)](sac)•4H_2O (sac=saccharinate and terpy=2,2':6',2"-terpyridine) is newly-synthesized palladium(II) (Pd) complex. We investigated the antiproliferative and apoptotic effects of this complex on Ehrlich ascites carcinoma (EAC). Materials and Methods: EAC cells were administered to 33 Balb/c mice. Mice were divided randomly into four groups: control, cisplatin, Pd(II) complex and paclitaxel. Control group animals received 0.9% NaCl; other groups received treatments cisplatin, Pd(II) complex and paclitaxel on days 7 and 12. At day 14, animals were sacrificed. Expression of active caspase-3, p53 and proliferating cell nuclear antigen (PCNA) was investigated and apoptosis was evaluated by terminal deoxynucleotidyltransferase (TdT)-mediated nick-end labelling (TUNEL) technique. Results: Expression of p53 and PCNA were found to be decreased ($p<0.0001$), cells with active caspase-3 and TUNEL-positive cells were found to be increased ($p<0.0001$) in all treatment groups. Conclusion: Like cisplatin and paclitaxel, this Pd(II) complex has a strong anticancer activity against EAC by inducing apoptosis and suppressing proliferation in vivo.*

Breast cancer is one of the major causes of morbidity, mortality and therapy costs in women (1). Although there are many drugs on the market for the therapy of breast cancer, the response to such therapy is still very poor and therefore more efficient drugs should be developed (2). Recently, palladium (Pd), which is chemically similar to platinum, showed a significant antitumour effect on cancer cells, as well as relatively weak side-effects in comparison to platinum (3). Although synthesis of palladium complexes dates back 30 years because of their anti-fungal, anti-viral and anti-bacterial activities (4), research on anticancer activity of palladium complexes only progressively increased after the 1990s. Different methods in chemistry eased the synthesis of new stable Pd(II) complexes, thus eliminating the significant disadvantage of the lack of *in vivo* stability of these complexes (2). Some Pd(II) complexes with significant cytotoxic activity against cancer cell lines have been synthesized and reported in recent years (5, 6). Palladium complexes not only exhibit anti-tumoural activity against cancer cells, but they also have fewer side-effects than platinum-based compounds (3). As a major property of anticancer agents containing metals, it is expected that palladium complexes cause less kidney toxicity than cisplatin (7). It was reported that palladium complexes demonstrate significant anti-tumour activity when compared to cisplatin (8). In addition, novel palladium complexes with strong cytotoxicity to different cancer cell lines were reported (7, 9).

In our previous studies, we evaluated the anticancer properties of some platinum and palladium complexes and explored their mechanism of cell death induced in breast and lung cancer cell lines (10, 11, 12). In these preliminary studies, it was demonstrated that the anticancer activity against human breast and lung cancer cells of certain complexes occurs by inducing apoptosis through cell death receptors (12). One newly-synthesized palladium(II) complex is formulated as [Pd(sac)(terpy)](sac)•4H_2O, where sac is saccharinate, and terpy is 2,2':6',2"-terpyridine (2). Even though these studies examined the *in vitro* effects of this complex, there is no information regarding the mechanism of its effects, such as its apoptotic and antiproliferative activity *in vivo*.

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Therefore, in this study, we aimed to investigate the antiproliferative and apoptotic effects of this complex and compare its effects with clinically used drugs such as cisplatin and paclitaxel. For the elucidation of the mechanism, p53 and Proliferating Cell Nuclear Antigen (PCNA) were investigated with immunohistochemistry, while apoptosis was evaluated by terminal deoxynucleotidyltransferase (TdT)-mediated nick-end labelling (TUNEL) technique and the expression of active caspase-3 (AC3) in induced Ehrlich ascites carcinoma (EAC) *in vivo*.

Materials and Methods

Animals. The protocol used in this study was approved by the Istanbul University's Ethic Board (no: 36/30.03.2009). Female Balb/c mice weighing 22 g on average, bred at the Faculty of Veterinary Medicine, were used in this study. The animals were housed in polypropylene cages in a controlled environment (12 h dark/light cycles), fed with standard laboratory chow, and given up tap water *ad libitum*.

In vivo experiments. Thirty-three Balb-c female mice were subcutaneously injected with 2.5×10^6 EAC cells (Istanbul University, Department of Experimental Animal Biology and Biomedical Application Techniques, Istanbul, Turkey) to induce a solid carcinoma (13) (day 1) and then they were randomly divided into four groups: the control group (n=4); and three experimental groups (n=9-10). On days 7 and 12, the control group animals received 0.5 ml of 0.9% NaCl; the second group received 4 mg/kg cisplatin (Kocak Farma, İstanbul, Turkey) *i.p.*; the third group received 2 mg/kg of the Pd(II) complex (Uludag University, Science Faculty, Department of Chemistry, Bursa, Turkey, Patent Number: TR 2011 00198 B) *i.p.*; and the fourth group received 12.5 mg/kg paclitaxel(Sandoz, Novartis, İstanbul, Turkey) *i.p.* The dose and duration of cisplatin and paclitaxel treatment were slightly modified from a previously published study (14). On day 14, animals were sacrificed cervical dislocation and tumour tissues were extirpated measured with a caliper and, the tumor volume was calculated using the formula $V(mm^3)=a \times b^2/2$, where $V(mm^3)$ is the tumor volume in mm^3, a=length, b=width of the tumors. Then tumors were fixed and embedded in paraffin as routinely performed.

Immunohistochemical detection of AC3, p53 and PCNA. Tissue sections from paraffin blocks were collected into positively charged slides. They were put through de-paraffinization, antigen retrieval, endogen peroxidase and protein blocking procedures and incubated with antibody to AC3 (diluted 1:100, overnight at 4˚C, Millipore, USA, cat no: AB3623), p53 (diluted 1:750, 1 hour, room temperature, AbCam, Cat No:PAb240 (Ab26), Cambridge, UK) and PCNA (diIuted 1:20, 1 h, room temperature) then they were treated with a commercial secondary antibody kit (Invitrogen, Histostain-Plus IHC Kit, HRP, broad spectrum, Cat. No: 85-9043, Paisley, UK) and marked with 3,3'-Diaminobenzidine (DAB) chromogen (Invitrogen, DAB-Plus Substrate Kit, Cat. No: 00-2020, Paisley, UK). Finally, the sections were counterstained with Mayer's haematoxylin. Negative control sections were incubated with Phosphate Buffer Saline (PBS) instead of the primary antibody. Antigen retrieval was performed using citrate buffer solution.

Detection of DNA fragmentation in tumour tissues. TUNEL assay was performed by using a commercially available kit (ApopTag® Plus *in situ* Apoptosis Detection Kit, Catalogue Number: S7101, Millipore, USA). Tissue sections (5 μm) from paraffin blocks prepared from tumour tissues were collected onto positively charged slides and incubated overnight at 37˚C. Sections were deparaffinized with xylene, digested with proteinase K (20 μg/ml, 15 min), endogen peroxidase was quenched and slides were incubated with equilibration buffer. TdT enzyme was applied for 1 hour at 37˚C. Anti-digoxigenine-peroxidase was applied for 30 min at room temperature and for colour development sections were marked with 3,3'-Diaminobenzidine (DAB) chromogen. Methyl green was used for counterstaining. For negative controls, distilled water was used instead of TdT

Evaluation of p53 staining. Staining was determined according to the following categories: 0: no staining; 1+: weak but detectable; 2+: moderate or distinct staining; 3+: intense staining. For each tissue, the HSCORE was given by the following formula: $HSCORE=S\times Pi\times(i+1)$, where i is the intensity score and *Pi* the corresponding percentage of cells presenting a given staining intensity. For each slide, five randomly selected areas were evaluated under a light microscope, and the percentage of the cells at the various intensities within these areas was determined at different times by two researchers in a blind fashion (15, 16).

Proliferative index, apoptotic index and statistical analyses. Sections were examined under Image Pro-Plus (MediaCybernetics, USA). PCNA-positive cells were used to quantify the proliferative index (percentage of PCNA-positive cells in 800-1000 cells). Similarly, AC3 labelling and TUNEL-positive cells were used to quantify the apoptotic index (17).

Statistical analysis was performed using GraphPad Prism version 5.2 for Windows (GraphPad Software, San Diego, CA, USA). One-way variance analyses were used with Tukey *post-hoc* analyses when $p<0.05$. For all analyses, $p<0.05$ was considered significant.

Results

The complex was found to significantly reduce the growth (size) of tumors. Tumor sizes were; 14 690.9±9054.6 mm^3 Control, 4780.4±2562.9 mm^3 in Complex, 9343.9±3298.0 mm^3 in Cisplatin and 5498.7±2593.1 mm^3 in Paclitaxel groups. These results regarding to tumor sizes have been previously published (11).

When compared to the control, the expressions of p53 and PCNA were found to be decreased ($p<0.0001$) and the expression of AC3 and the percentage of TUNEL-positive cells were found to be increased ($p<0.0001$) in cisplatin-, Pd(II) complex- and paclitaxel-treated groups (Figure 1). The expression of p53 in the control group was dense and intense in the nuclei, but very light in the cytoplasm (Figure 2A). p53 expression by HSCORE decreased considerably in the group treated with Pd(II) complex (Figures A and 2B), and in the cisplatin-, and paclitaxel-treated groups in comparison to the control ($p<0.0001$). Moreover, the decline in p53 expression induced by the Pd(II) complex was similar to that of paclitaxel and slightly better than that of cisplatin (Figure 1A).

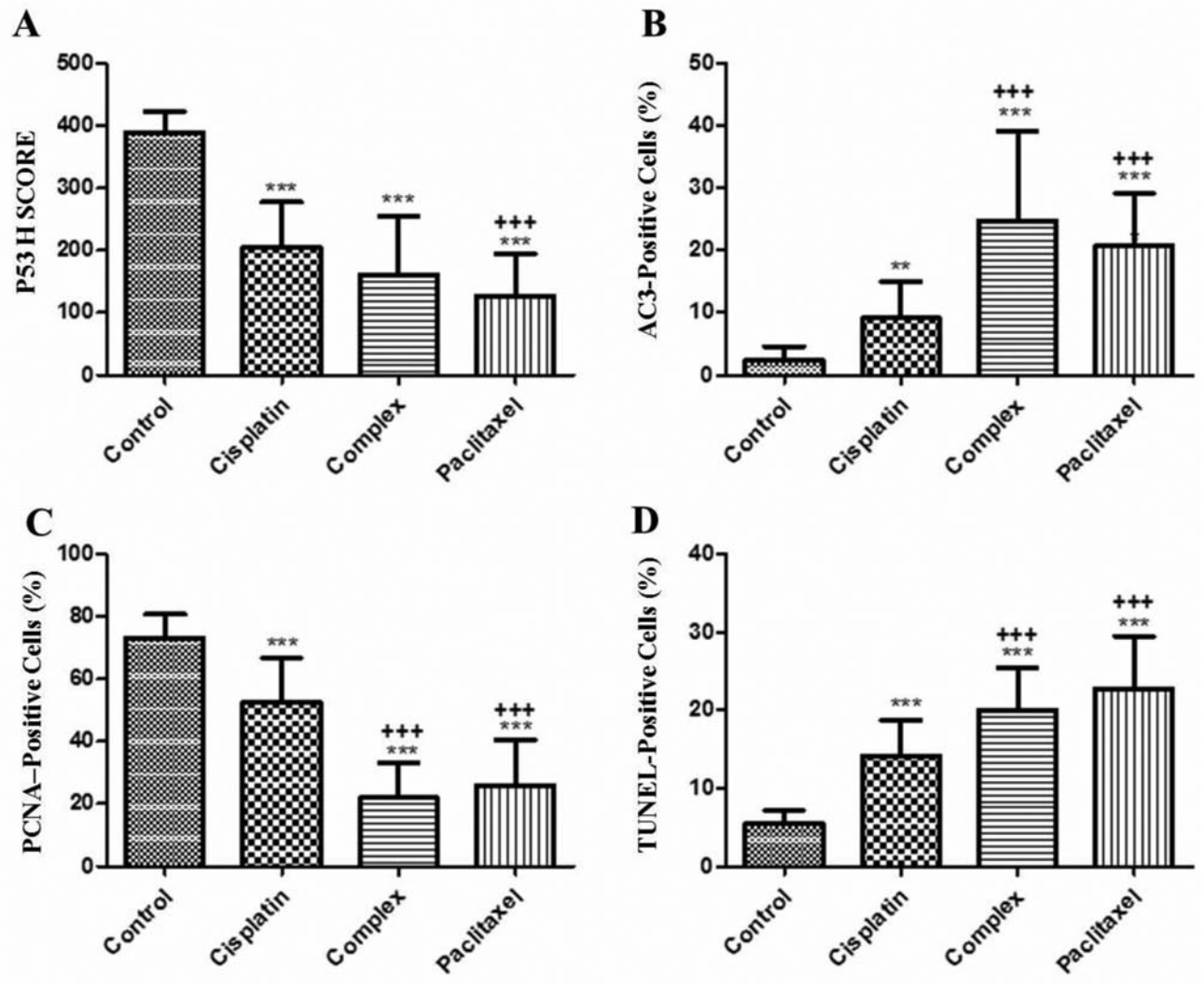

Figure 1. *Comparison of results between groups of treated mice. A: P53; B: active caspase-3 (AC3); C: proliferating cell nuclear antigen (PCNA); D: terminal deoxynucleotidyltransferase (TdT)-mediated nick-end labelling (TUNEL) **p<0.001 and ***p<0.0001 versus control; +++p<0.0001 versus cisplatin.*

The expression of AC3 in the controls was very low (Figure 2C). AC3 expression was increased in the groups treated with cisplatin, Pd(II) complex, and paclitaxel, but was the greatest in the Pd(II) complex- treated group (Figure 2D). The expression of AC3 in Pd(II) complex- and paclitaxel-treated groups was higher than that of cisplatin (Figure 1B) ($p<0.0001$). The increase in AC3 expression noticed by Pd(II) complex was nearly two-fold that by cisplatin and slightly higher than that by paclitaxel.

The intensity of PCNA expression was dense and nuclear (Figure 2E). The PCNA reaction decreased in groups treated with cisplatin, Pd(II) complex (Figure 2F) and paclitaxel in comparison to the control. The proliferative index by PCNA was significantly reduced by all treatments ($p<0.0001$), with Pd(II) complex and paclitaxel being more effective than cisplatin ($p<0.0001$), and the Pd(II) complex having the greatest effect (Figure 1C).

The reaction in the TUNEL assay was also evaluated as an index. In the control group, the apoptotic index was low (Figure 1D and 3A). The TUNEL reaction increased in all groups (Figure 1D; Pd(II) complex-treated group) (Figure 3B) in comparison to the control ($p<0.0001$), with paclitaxel having the greatest effect.

Discussion

In our previous study (11), we already showed that this Pd(II) complex had strong anticancer activity against EAC. In the evaluation of the tumor sizes in mice demonstrate that the regression in tumor size of the Pd(II) complex-treated

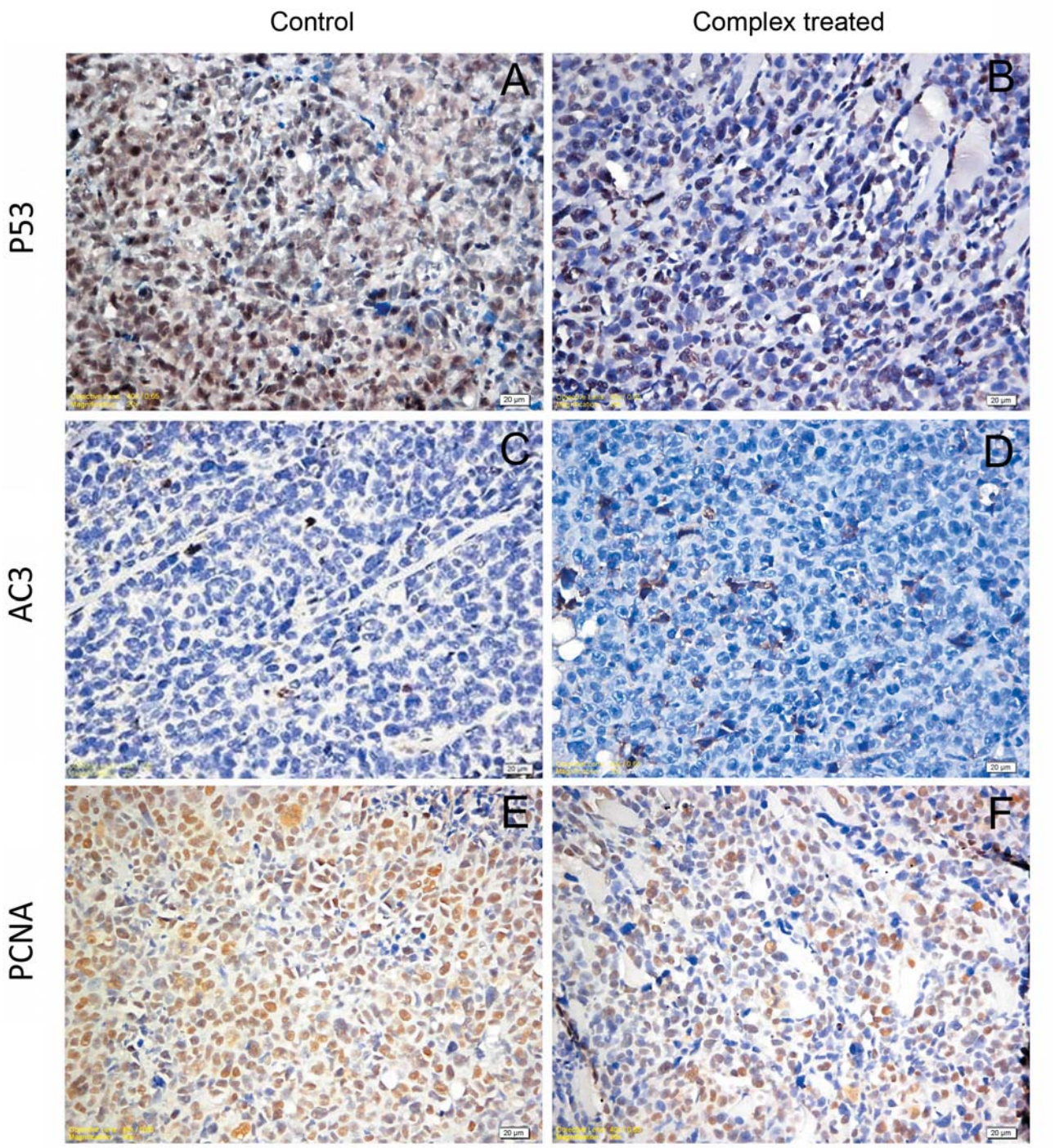

Figure 2. *Expression of P53, active caspase-3 (AC3) and proliferating cell nuclear antigen (PCNA) in control (A, C, E) and palladium (II) complex-treated (B, D, F) groups (Bar=20 µm).*

group was similar to that under paclitaxel treatment and even better than that under cisplatin. Moreover, the number of drug-related deaths in the Pd(II) complex- and paclitaxel-treated groups was one, while it was two in the cisplatin-treated group. Taking into account this promising finding, in the current study, we further explored the effect *in vivo* of the Pd(II) complex on some parameters related to proliferation and apoptosis.

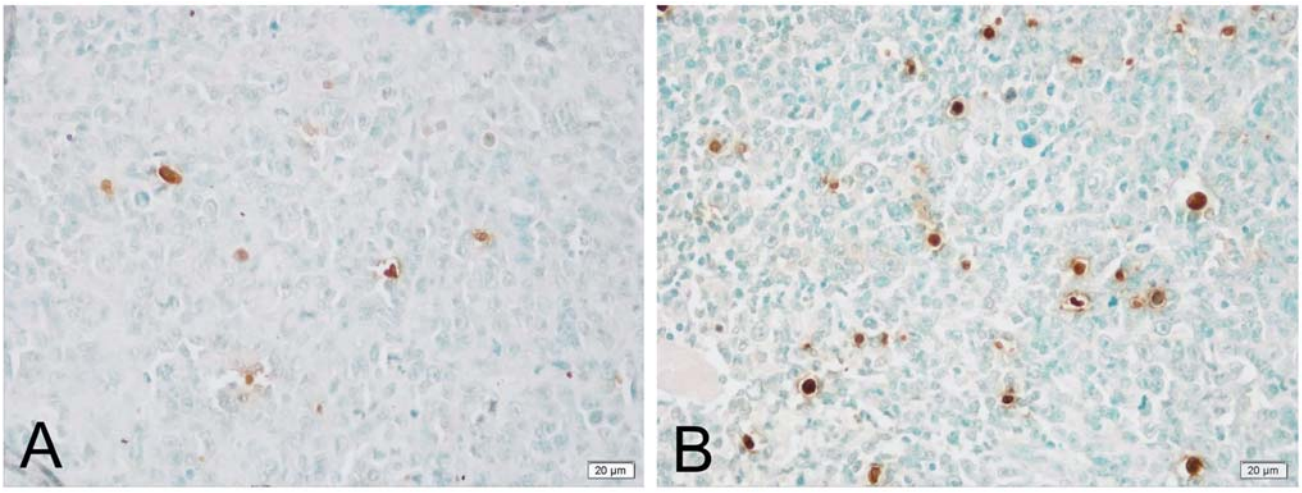

Figure 3. *Terminal deoxynucleotidyltransferase (TdT)-mediated nick-end labelling in control (A) and palladium (II) complex-treated. B) groups (Bar=20 µm).*

p53 is a protein that in humans is encoded by the *TP53* gene. The p53 protein is crucial in multicellular organisms, where it regulates the cell cycle and, thus, functions as a tumor suppressor, preventing cancer. As such, p53 has been described as 'the guardian of the genome' because of its role in conserving stability by preventing genome mutation (18). p53 activation regulates genes involved in many cellular functions, the most important being cell-cycle arrest and apoptosis. It is often remarked that 50% of carcinomas have mutated or inactivated p53; however, the true number is probably much higher when the involvement of the entire p53 pathway in tumorigenesis is examined (19). Tumor types with cytoplasmic confinement of p53 are less responsive to genotoxic stress induced by radiotherapy or chemotherapy; while cytoplasmic accumulation of p53 is an independent unfavourable prognostic factor in cancer. Relocation of p53 to the nucleus after cellular stress is desirable to inhibit the growth of malignant cells. Moreover, in a number of tumor types that retain wild-type p53, loss of p53 activity is associated with cytoplasmic localization (20). In such cases, p53 is excluded from the nucleus as a result of cytoplasmic sequestration, or hyperactive nuclear export (21-23). In the present study, we used antibody to p53 [PAb240 (ab26)] which recognizes both mutant and wild-type p53 under denaturing conditions, and according to the manufacturer's manual, cellular localization is predominantly nuclear but p53 is translocated to the cytoplasm following cell stress. Compared to the control group, the p53 expression decreased approximately 2.89-fold in paclitaxel- and 2.42-fold in Pd(II) complex-treated groups, which were quite similar to each other. In a normal cell, the p53 level is very low. Overexpression of p53 in the control group led us to believe that the majority of this population consisted of mutant type p53. According to Kacar *et al*., a different Pd (II) complex might represent a promising anticancer agent that exhibits Bcl-2 Associated X Protein (BAX)-mutant, and caspase 3-mutant cancer cells (24). The decrease in expression of p53 in the Pd(II) complex-treated group was similar to their finding. Thus we believe that the decrease in our study was because of cytotoxicity of Pd(II) in p53-mutant cells (24).

The anti-growth effect of Pd complex could be achieved by suppression of proliferation, activation of cell death, or both. Thus we first stained cells for PCNA to determine proliferation. PCNA is a protein expressed in the G_1/S phase of the cell cycle (25). In this study, the Pd(II) complex and other drugs reduced PCNA expression, which also shows the proliferation rate was reduced. This might explain the reduction of tumour diameters in treated groups observed in the previous study. Apoptosis was shown by AC3 staining and was confirmed by the TUNEL assay. Caspase-3 protein is a member of the cysteine-aspartic acid protease (caspase) family (26). Sequential activation of caspases plays a central role in the execution phase of cell apoptosis. Caspase-3 is activated in apoptotic cell both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways (27, 28). The zymogen feature of caspase-3 is necessary because if unregulated, caspase activity would kill cells indiscriminately (29). As an executioner caspase, the caspase-3 zymogen has virtually no activity until it is cleaved by an initiator caspase after apoptotic signalling events have occurred (30).

According to our results, expression of AC3 was very low in the control group. AC3 levels were highest in the Pd(II) complex-treated group and these results were approximately 2.7-fold higher then by cisplatin and slightly higher than that

of The paclitaxel-treated group. In accordance with this finding, TUNEL staining, which measures double-stranded breaks in DNA, was highest in the paclitaxel-treated group but the difference between the group treated with Pd(II) complex and that treated with paclitaxel was small. Caspase activation is a relatively early event and DNA strand breaks are late events in apoptosis (31). The results of these two assay methods were in concordance, which indicates that apoptosis was also another factor contributing to the decrease in the size of the tumor resulting from treatment with the Pd(II) complex.

In a similar study with a different Pd complex in murine cancer cell lines, it was reported that the complex had an activity equivalent to that of cisplatin (32). In this study, our results were compatible with these findings, the Pd(II) complex demonstrated even better results than cisplatin. The mechanisms of action of paclitaxel and cisplatin in cancer treatment differ. Cisplatin crosslinks DNA in several different ways, interfering with cell division by mitosis. The damaged DNA elicits DNA repair mechanisms, which in turn activate apoptosis when repair proves impossible (33). Microtubule-arresting drugs like paclitaxel inhibit mitotic progression leading to mitotic and post mitotic arrest and cell death (34). The Pd(II) complex led to similar and sometimes better antiproliferative effect than paclitaxel in this study. We observed that the greatest difference was observed between groups in terms of the PCNA and AC3 results.

Only a single drug-related death occurred in each of the groups treated with Pd(II) complex and paclitaxel, whereas two animals died in the cisplatin-treated group. This suggests that the Pd(II) complex had a less than moderate toxicity when compared to cisplatin. As no side effects like tremor, paraplegia and weight loss were observed in any of the groups (11). Parallel to these findings although cisplatin was applied two-fold higher than Pd(II) complex, the anti-proliferative and apoptotic effect of Pd(II) complex was higher than cisplatin and nearly similar to paclitaxel.

In conclusion, the Pd(II) complex used in this study had strong anticancer activity on EAC by inducing apoptosis as well as inhibiting proliferation *in vivo*. However, more *in vivo* studies with different types of cancer models need to be performed in order to demonstrate the detailed mechanism of action of this Pd(II) complex.

Conflicts of Interest

The Authors have no conflicts of interest to declare.

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