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Apoptosis-inducing Effect of a Palladium(II) Complex-[PdCl(terpy)](sac).2H2O] on Ehrlich Ascites Carcinoma (EAC) in Mice

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Abstract. *Background/Aim: New compounds for cancer treatment are needed due to persistenly unsatisfactory management of cancer. [PdCl(terpy)](sac)·2H2O] (sac=saccharinate, and terpy=2,2':6',2''-terpyridine) is a compound synthesized for this purpose. We investigated its anti-proliferative and pro-apoptotic effects on Ehrlich Ascites Carcinoma (EAC) in vivo. Materials and Methods: 42 Balb-c female mice were subcutaneously (s.c.) injected with EAC cells (1st day) and then randomly divided into 5 groups: control (0.9% NaCl), complex (2 mg/kg), complex (3 mg/kg) cisplatin (4 mg/kg) and paclitaxel (12.5 mg/kg). On the 5th and 12th day animals were drug administrated. At 14th day, animals were sacrificed. Expression of cell death and/or cell cycle-related markers (Bcl-2, Bax, active caspase-3, p53, PCNA) and apoptosis were investigated immunohisto-chemically. Survival-related markers (Akt, GSK-3β, IGF-1R, IR, IRS-1, p70S6K, PRAS40) were evaluated by luminex analysis. Results: Expression of p53, PCNA, Bcl-2 was found decreased ($p<0.001$) and that of active caspase-3, Bax, and apoptotic cells was found increased ($p<0.001$) in all groups. The survival-related markers did not show any statistical difference in complex groups. Conclusion: The Pd(II)-complex seems to have a strong anticancer activity on EAC by inducing apoptosis via both suppression of proliferation and activation of apoptosis in vivo, similar to the effects of cisplatin and paclitaxel.*

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Despite numerous drugs becoming available in cancer treatment during the last 50 years, full success has not been achieved on the fight for the disease. Thus, development of new compounds/modalities is of great importance. Anticancer drugs containing platin, carboplatin and oxaliplatin have been used in the clinical setting for different types of cancer. Cisplatin has become the most frequently used platinum-containing drug in chemotherapy in the last 30 years. Drugs containing platinum are used as combination or as single-treatment of diverse cancer types like ovarian, testicular, rectal, gastric, melanoma and bladder cancer (1). All platinum-containing agents cause cytotoxicity through combination with DNA, RNA and proteins after entering the cell. By causing single- or double-strand breaks in DNA, they result in apoptosis (2). Complexes containing platinum, such as cisplatin and carboplatin activate a variety of signaling pathways in cancer cells (3).

As a metal that is present in the same chemical group as platinum, palladium(II) has been demonstrated to be effective in cancer treatment (4). In a study by Keter *et al*. (2008) it was shown that complexes containing platinum(II) and palladium(II) cause apoptosis by increasing cell death in cancer cells (5, 6). Also, it was reported that different palladium complexes cause high DNA damage and induce apoptosis (7).

Quite similar to the palladium(II) [Pd(II)] complex of this study, another Pd(II) complex ([Pd(sac)(terpy)](sac)•4H2O (sac=saccharinate and terpy=2,2':6',2''-terpyridine), sythesized by our group was shown to suppress tumor cell growth and activate cell death *in vitro* and *in vivo* (8-10). The Pd(II) complex of the present study ([PdCl(terpy)](sac).2H2O]) was observed to suppress tumor cell growth in different lung cancer and mammary cell lines in tissue cultures (11, 12). However, there is no information on its *in vivo* activity as yet.

In this study, we aimed to investigate the effects of this complex on proliferation, apoptosis, and survival pathways. The effects of the complex were compared against those of clinically and commonly used drugs; cisplatin and paclitaxel. In EAC, we investigated its mechanism of action *in vivo* by analyzing the expression of p53, proliferating cell nuclear antigen (PCNA), Bax, Bcl-2, active caspase-3 (AC3) and apoptotic cell count with terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL). In addition, components of a well-known survival pathway (Akt, GSK-3β, IGF-1R, IR, IRS-1, p70S6K, PRAS40) have been investigated. We found that the Pd(II) complex had an anti-growth effect through inducing apoptosis.

Materials and Methods

Animals. The protocol used in this study was approved by the Istanbul University's Ethic Board (No:136/27.09.2012). 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), observed after injection applications, fed with standard laboratory chow, and given up tap water *ad libitum*.

Ehrlich Ascites Carcinoma (EAC) solid tumor model. An ethical board decision (No:136/27.09.2012) was obtained from the Istanbul University Animal Experiment Local Ethical Committee. In order to develop solid mammary tumors 42 young adult female Balb-c mice (22±2 g) were used. Animals and EAC tumor cells were obtained from Istanbul University Experimental Medical Research Institute (DETAE) Laboratory animal production unit. All animals received 2.5×10^6 EAC tumor cells subcutaneously (*s.c.*) (day 1) and then they were randomly divided into 5 groups: control group (n=6); and 4 experimental groups (n=9). On the 5th and 12th days control group animals received 0.5 ml 0.9% NaCl intraperitoneally (*i.p.*), 2nd group received 2 mg/kg complex *i.p.* (Uludag University, Faculty of Art and Science, Bursa, Turkey, Patent Number: TR 2011 00198 B), 3th group received 3 mg/kg complex *i.p.*, 4th group received 4 mg/kg cisplatin *i.p.*, and the 5th group received 12.5 mg/kg paclitaxel *s.c.* (Uludag University, Faculty of Medicine's Pharmacy, Bursa, Turkey). The dose and duration of cisplatin and paclitaxel treatment were administered according to a previously published study (10). At 14th day, animals were sacrificed with cervical dislocation. Tumor 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.

Immunohistochemical protocol for cell death/cell cycle markers. Tumor tissues were fixed and embedded in paraffin. Immunohistochemical expression of AC3, p53, Bax, Bcl-2 and PCNA levels were demonstrated. 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 PCNA (dilution 1:100, 1.5 h, room temperature), AC3 (dilution 1:100, overnight at 4˚C), p53 (dilution 1:750, 1.5 h, room temperature), Bax (dilution 1:100, overnight at 4˚C) and Bcl-2 (dilution 1:300, 2 h, room temperature) then they were treated with commercial secondary antibody kit (Invitrogen) and marked with 3,3'-diaminobenzidine (DAB) chromogen. Finally, the sections were counterstained with Mayer's hematoxylin. Negative control sections were incubated with phosphate buffer saline (PBS) instead of the primary antibody. Antigen retrieval was performed using citrate buffer solution.

TUNEL protocol for apoptosis. TUNEL assays were 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 tumor 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 h at 37˚C. Anti-digoxigenine-peroxidase was applied for 30 min at room temperature and for color development sections were marked with DAB chromogen. Methyl green was used for counterstaining. For negative controls, distilled water was used instead of TdT (13).

Luminex protocol for survival markers. Tumour tissues were stored in –80˚C till used. To prepare lysates, tissue samples were extracted in protein inhibitors (1 mM phenylmethylsulfonyl fluoride and phosphatase inhibitor 1 and 2 cocktail), and protein concentrations were determined using BCA Protein Assay Kit. With BioSource and Luminex Bio-Plex Systems (Invitrogen, Akt Phospho 7-Plex Panel, catalog no: LHO0001, CA, USA) levels of phosphorylated Akt, GSK-3β, IGF-1R, IR, IRS-1, p70S6K, PRAS40 were determined.

Proliferation and apoptotic index, Hscore and statistical analyzes. After IHC and TUNEL techniques slides were investigated under light microscopy (Leica DM4000 B Microscope, Leica). At 40× magnification, positively reactive cells were determined. For every slide areas (5-10) containing 800-1,000 cells were counted. For cells that reacted positively to antibody, Hscore, proliferation index and apoptotic index were performed (10, 13, 14). One-way ANOVA test were performed using the SPSS software to obtained results, values $p<0.05$ were accepted as statistically significant. Luminex data were analyzed according to Salphati *et al*. 2010 (15).

Results

When tumor volumes were evaluated complex-3 mg/kg group had the lowest values ($p<0.001$). Tumor volumes (mm^3) were 5340.56±1375.28 in control, 3587.65±758.62 in complex-2 mg/kg, 1,609.48±241.85 in complex-3 mg/kg,

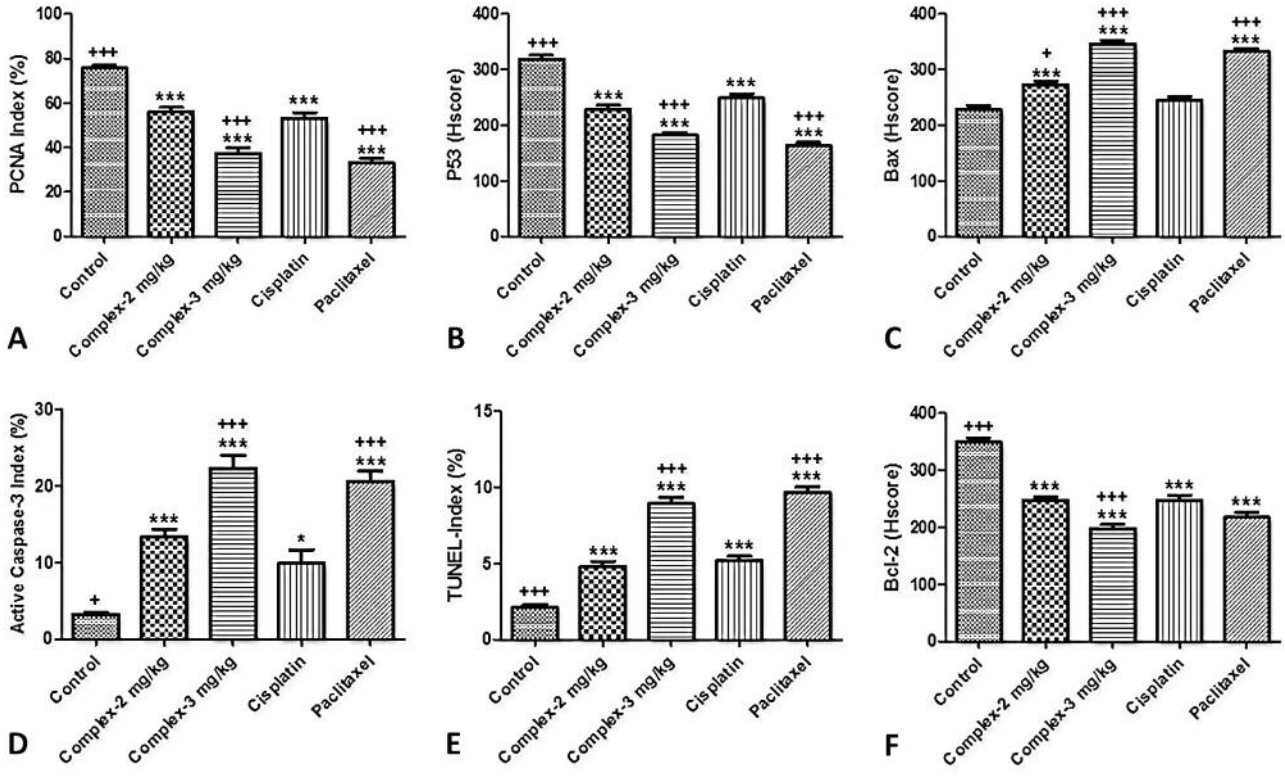

Figure 1. *Comparison of immunohistochemistry and TUNEL assay data. A: Proliferating cell nuclear antigen (PCNA), B: P53, C: Bax, D: active caspase 3 (AC3), E: TUNEL, F: Bcl-2. *p<0.05, ***p<0.001, versus control group. +p<0.05, +++p<0.001, versus cisplatin group.*

2,483.73±251.99 in cisplatin, 2,163.55±309.25 in paclitaxel group. Only an animal death was observed in the complex-3 mg/kg group while administering 2nd dose. No other deaths were recorded until the end of study. But in animals receiving 3 mg/kg complex after 2nd injection, temporary paraplegia, sensitivity to sound, trembles were observed, these findings disappeared in 12-14 h (12).

When compared to the control group, PCNA, p53, and Bcl-2 expressions statistically significantly decreased in all groups (p<0.001). While AC3 and TUNEL-positive cells increased in cisplatin, complex-2 mg/kg, complex-3 mg/kg and paclitaxel groups (p<0.001), Bax expression did not change only in cisplatin group (Figures 1 and 2). According to PCNA and p53 results, statistical significance was detected in paclitaxel and complex-3 mg/kg compared to control group and their results were similar to each other. When compared to control group, Bax expression were highest in complex-3 mg/kg group and there was statistical significance in cisplatin group. As expected; on contrary to Bax results the biggest decrease in Bcl-2 expressions was in complex-3 mg/kg group. This value was followed by paclitaxel, complex-2 mg/kg and cisplatin groups, respectively. Apoptotic cell number increment was highest in complex-3 mg/kg, according to the TUNEL assay (Figure 2). This increase was highest in paclitaxel group followed by complex-3 mg/kg.

GSK-3 Beta was highest in cisplatin (p<0.001) and paclitaxel (p<0.01) groups and IRS-1 was only highest in paclitaxel group (p<0.05) when compared to control group according to data from luminex analyses. No statistical significance was detected in complex-2 mg/kg and 3 mg/kg groups with respect to Akt, GSK-3β, IGF-1R, IR, IRS-1, p70S6K, PRAS40 proteins in Akt phospho 7-Plex Panel (Figure 3).

Discussion and Conclusion

In the present study, the effect of a Pd(II) complex (Pd(II)-saccharinate complex of terpyridine) on cell death, cell proliferation and cell survival was investigated *in vivo*. Several Pd(II)-based complexes have been studied and published so far and they seem to have a varying degree of cytotoxic potential on cell cultures of different tumor types.

One of such Pd(II) complexes was reported to have a strong anticancer activity against EAC (12). In the study presented, regarding tumor size in both complex doses, a significant decrease compared to the control group was

observed. The fold-decreases were 1.49-fold and 3.31-fold in complex-2 mg/kg and complex-3 mg/kg, respectively. In addition, it was 1.88-fold in cisplatin and 2.47-fold in the paclitaxel groups. In a study with a slightly different Pd(II) complex by Ulukaya *et al*. (2011a) with the dose of 2 mg/kg, there was a 3.07-fold decrease. In our study, 2-mg/kg dose of Pd(II) complex shrunk the tumor by 1.49-fold.

The complex used in this study shrunk the tumor 3.31-fold when administered in 3 mg/kg dose. This shows that the Pd(II) complex used in Ulukaya *et al*. (2011a) was more effective on tumor sizes when it was applied as a dose of 2 mg/kg (9). We presume that this difference can result from the structural differences of complexes. The other explanation might be the time point of the first application of the complex. In our study, it was the 5th day. One of the important indicators of cancer prognoses is evaluation of cell proliferation. In the clinical setting, the evaluation of cell proliferation can be performed partially by counting mitotic cells with histopathological grading or labelling of cells in S-phase. In addition to this, PCNA is another important parameter of proliferation. It has a critical role in the onset of cell cycle and increases in G1-S phases of the cell cycle (16-19). In this study PCNA values decreased especially in the complex-3 mg/kg group. In the group of 2 mg/kg complex, the results were similar to the cisplatin group. Results of 3 mg/kg-complex group were quite similar to the paclitaxel group. These results imply that dosing is a critical factor. There are two types of apoptotic pathways in mammalian cells; extrinsic and intrinsic pathways. While the extrinsic pathway is stimulated by death receptors, the intrinsic pathway is a pathway controlled by mitochondria participated Bcl-2 protein family (20, 21). Bcl-2 family composed of anti-apoptotic and pro-apoptotic two groups that have a role in cell death and DNA damage. The irregularity of anti-apoptotic Bcl-2 family members is one of the identifying indicators of cancer cells compared to normal cells. Bcl-2 proteins are important targets in terms of resistance of cancer cells to treatments, thus facilitating new treatment methods (22).

In parallel to PCNA result and tumor sizes, Bcl-2 decreased in all treatment groups compared to control group. The decrease in complex-3 mg/kg administered group was higher than the cisplatin group. Bax protein is a pro-apoptotic molecule (23). It has been reported that Bax expressions were induced by γ-radiation, chemotherapeutic drug and other genotoxic factors (24). Bcl-2 family members play key roles in many cellular activities by forming hetero or homo dimers. Bax that is a Bcl-2 homolog protein, promotes cell death by competing with Bcl-2. While increase of Bax-Bax homodimerization effects as apoptotic inductive, increase of Bcl-2-Bax heterodimerization generating a survival signal for cells (24). In previously conducted studies the decrease in Bax protein expression in mammary cancer was reported to be proportional to chemotherapy response and survival (25). In studies conducted in mammary cancer cell lines it was reported that the normality of Bax expression was found to be causing inhibition in tumorigenesis (26) and increased sensitivity in treatment

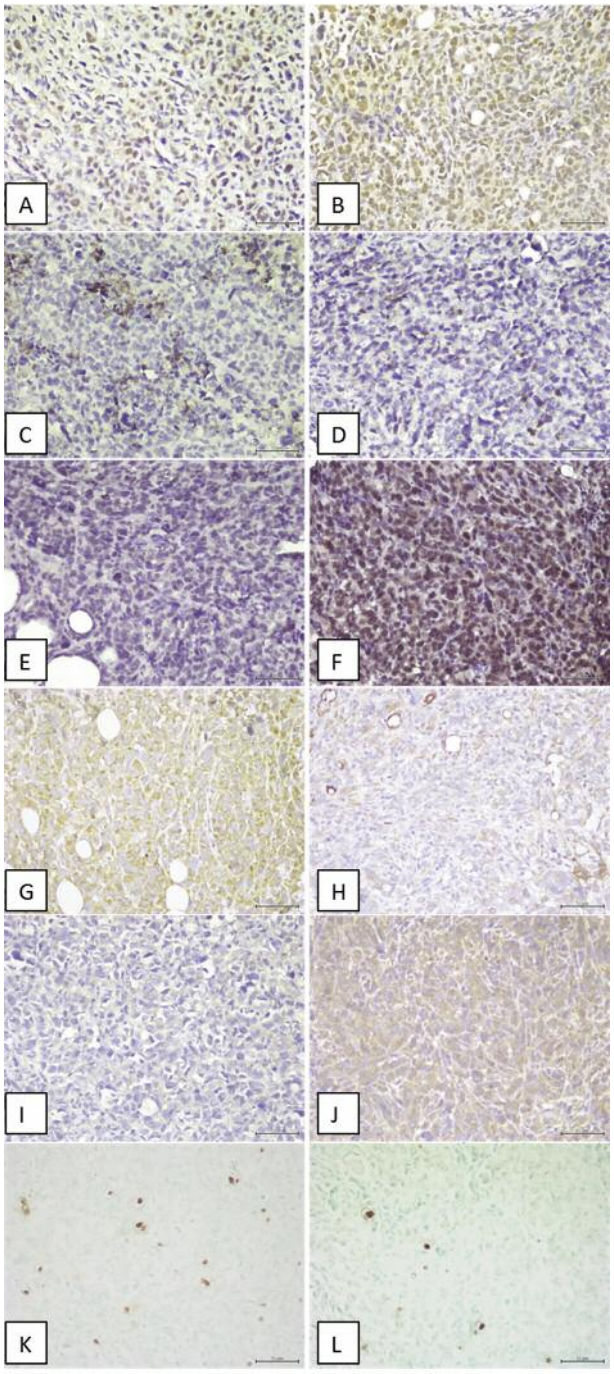

Figure 2. *Expression of proliferating cell nuclear antigen (PCNA), active caspase-3 (AC3), P53, Bax, and Bcl-2 in complex-3 mg/kg treated (A, C, E, G, I) and control (B, D, F, H, J) groups. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) in complex-3 mg/kg treated (K) and control (L) groups (Bar=50 μm).*

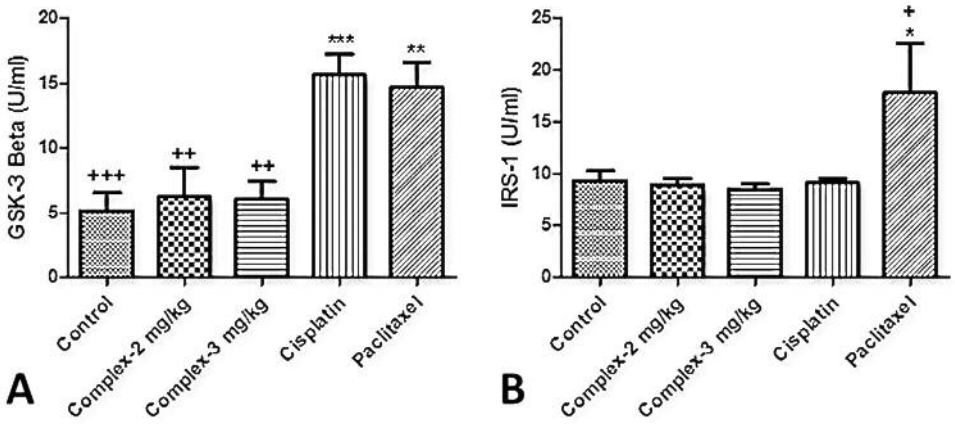

Figure 3. *Statistical comparison of GSK-3 Beta [pS9] (A) and IRS-1 [pS312] (B) according to luminex results. *p<0.05, **p<0.01, ***p<0.001, versus control group. +p<0.05, ++p<0.01, +++p<0.001, versus cisplatin group.*

with cytotoxic drugs (27, 28). Besides changes in expression of the protein were found to be associated especially with mammary, ovarian, testicle and prostate cancer which are related to the reproduction system (24). In this study similar to these findings, Bcl-2 expression decreased in all treatment groups. Bax expression increased in paclitaxel and complex-2 and -3 mg/kg groups. The increase of Bax expression in complex-2 mg/kg group that generally showed similar results with cisplatin group was striking. We presume that this difference resulted from administration dose of cisplatin and the chemical difference between cisplatin and complex.

P53 protein is a transcription factor that regulates the cell cycle. It has been shown that p53 protein takes part in many cell activities like control of cell cycle, regulation of gene expression, aging and programmed cell death (29). Under these qualifications p53 is an important protein in many organisms and has a role in suppression of cancer, protecting the genome (30, 31). When there is a damage in DNA, p53 stops the cell cycle at G_1 and G_2 control points, allowing the DNA repairing proteins to activate. If the damage is unrepairable then the Bax gene is activated, causing apoptosis (29, 32). Both Bcl-2 and Bax proteins are transcriptional targets for p53 tumor suppressor protein (29). In order for the cell to enter apoptosis the level of Bax must be higher than Bcl-2 (33-35). In other words, in the presence of p53 cells with low levels of Bcl-2 expression enter apoptosis (33, 36). At this point evaluation of p53 existence with Bcl-2 and Bax proteins is very important to investigate and to determine the pathway of apoptosis. Likewise to our previous study, we propose that decrease in p53 in treated groups results from perishing of the cells with mutant p53 during treatment (10). We can explain why mutant p53 cells decreased by the decrease of cytoplasmic staining in treated groups.

When compared to controls, p53 expression was respectively 1.40-fold lower in complex-2 mg/kg, 1.76-fold lower in complex-3 mg/kg, 1.28-fold in the cisplatin and 1.94-fold lower in paclitaxel group. Also complex-3 mg/kg was 1.37-fold lower than cisplatin, this shows that this dose is more effective then cisplatin group. Besides it was reported that another Pd(II) complex used by another research group was found to be cytotoxic for Bax and AC3 mutant cells (37). This result supports the antibody dependent results we obtained.

The caspase-3 method is a widely used method for determining apoptotic cells, depending on whether or not caspase-3 was responsible for fragmenting the DNA (38). The TUNEL method to determine the apoptotic cells is a technique that demonstrates DNA fragmentation in conventional tissue sections *in situ*, generally performed by using TdT enzyme and biotinylated dUTP. It is a widely used sensitive method that labels 180-200 bp and multiple DNA terminals resulting from apoptotic fragmentation, with deoxynucleotidyl transferase enzyme (TdT) (39). In AC3 and TUNEL techniques which demonstrate apoptotic cells in different phases close results were obtained especially in paclitaxel and complex-3 mg/kg groups. Also in the same complex dose the number of apoptotic cell labelled with AC3 staining was higher than cisplatin. Parallel to other finding complex-2 mg/kg which was administered as half dose of cisplatin resulted similar to cisplatin. We think that difference complex-3 mg/kg having highest results in AC3 staining and paclitaxel having highest results in TUNEL technique originated from each technique demonstrating different phases of apoptotic cells (40). Both doses of complex induced apoptosis in both methods.

Glycogen synthase kinase-3 β (GSK-3β) is a multi-functional serine/ threonine kinase. Its activity is negatively regulated by the phosphorylation of serine 9 (pGSK-3β-ser-9)

and positively by phosphorylation of tyrosine 216 (pGSK-3β-tyr-216). Cai *et al*. demonstrated that GSK-3β negatively regulated the expression of p53 (41). When the level of p53 is elevated it plays a role in directing the actions of GSK3β, and that GSK3β, in turn, contributes to an important facilitating role in promoting the actions of p53, suggesting that these two proteins co-operate as partners in controlling cellular responses to DNA damage (42). In this study, levels of pGSK-3β-ser-9 were found to be low in both complex doses compared to the cisplatin group (Figure 2). We think that this is because apoptosis was not induced through p53 and pGSK-3β-ser-9 co-operation, but through mitochondria with Bax and/or Bcl-2.

Insulin receptor substrate (IRS) proteins are docking proteins that couple growth factor receptors to various effector molecules (43). After activation with JNK, TNF-alpha and anisomysin, by linking IRS-1 it causes activation of Ser312 phosphorylation, results in inhibition of IRS-1 tyrosine phosphorylation (44-47). Overexpression of JNK significantly enhances Taxol-induced apoptosis and inhibits IGF-I survival effects. Furthermore, JNK attenuates anchorage-independent growth of MCF-7 cells. The inhibitory effect of JNK appears to be mediated by serine phosphorylation of IRS-1 (insulin receptor substrate) since both taxol and IGF-I treatment enhanced Ser312 IRS-1 phosphorylation, while LY294002 blocked IGF-I-mediated phosphorylation. Taken together, these data provide a mechanism whereby stress or growth factors activate JNK to reduce proliferation and/or survival in breast cancer cells (48). We think that taxol mediated an increased Ser312 IRS-1 phosphorylation-induced apoptosis and suppressed proliferation in this group. These effects were not observed in other groups, so we thought that it they were due to the property of used agents, doses of applied complexes and application interval. Also we thought that apoptotic effect of the complexes do not occur through IRS-1 Ser-312 phosphorylation.

After the application of the complex, p53, PCNA and Bcl-2 expression decreased, AC3, Bax expression and TUNEL data increased. These results are parallel with the observed decreased of EAC tumor sizes. With luminex that evaluate survival pathway no effect of complex's both doses were observed over Akt, GSK-3β, IGF-1R, IR, IRS-1, p70S6K, PRAS40 protein activity. In light of these findings we are able to say that complex-2 mg/kg has cisplatin-like, complex 3 mg/kg has paclitaxel-like effects on EAC tumors. Some Pd complexes were found to be more effective then cisplatin in MCF7, T-47D and MDA-MB-231 cell lines (49). This result was parallel to our findings for complex-3mg. Besides, this complex is very promising, the use of this complex in combination with anthelminthic drug Niclosamide (Nic) resulted in enhanced cytotoxic effect against breast cancer stem cells (50).

In conclusion, this palladium complex has a strong anticancer effect on EAC cells and by suppressing proliferation, inducing apoptosis through the intrinsic pathway displaying an effect like cisplatin and paclitaxel.

Conflicts of Interest

The Authors declare no conflict of interest.

Acknowledgements

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