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Evaluation of Cytotoxic Effects of a DinuclearPalladacycle Derivative, Biphosphinic Complex, on Cisplatin-Resistant HT-29 Cells

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Previous studies showed that palladacycle complexes with three phenyl phosphine ligands and piperidine or biphosphinic based palladacycle complexes were cytotoxic on K562, HT29, and Hela cell lines. In the present study, in order to evaluate the efficacy of the compound on cisplatin resistant cells, first we made HT-29 cells resistant to cisplatin, and then evaluated the potential cytotoxicity of the mentioned plladacylce complex on them. In addition, it was evaluated whether cytotoxic effect of the compound is mediated via apoptosis or necrosis death mechanisms. In this regard, Annexin V/PI by staining followed by flow cytometric analysis was performed. Results showed that palladacycle complexes were 45 times more cytotoxic than cisplatin ($P<0.05$) on the resistant HT-29 cells. Flow cytometry results also revealed that apoptosis induction was the major cell death mechanism of these compounds. Therefore, it could be concluded that these compounds might be effectively cytotoxic for cisplatin resistant cells. However, further *in vitro* and *in vivo* preclinical studies on evaluation of specific and non-specific cytotoxic characteristics of these complexes are necessary.

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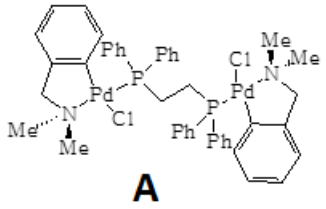
Introduction

Cancer is an uncontrolled growth of undifferentiated cells, which is almost always invasive and can spread into all parts of body with severe major health consequences such as nephrotoxicity, neurotoxicity and ototoxicity [1]. Actually, cancer is thought to be the major public health problem in many parts of the world and is expected to overcome the position of heart diseases as the leading cause of death in the next few years [1-4].Although the cancer incidence is accelerated through these years, but cancer death rates have been continuously declining in the past two decades (decreased by 22% between 1991 and 2011), [5-7].

Some traditional methods for cancer treatment, including surgery, chemotherapy, and radiation therapy are recognized as efficient methods. In addition, in the past decades, some novel arrays were used as diagnostic, preventive or treatment modalities for cancer. These include targeted therapy, immunotherapy, hyperthermia, stem cell transplantation, photodynamic therapy, and eventually blood product donation [8]. However, beside the new strategies applied for effective application of various types of cancer, many efforts have been made to improve the effectiveness of traditional approaches of cancer treatment. Based on their chemical structures, traditional chemotherapeutic agents can be classified into several groups including alkylating agents, antimetabolites, anti-tumor antibiotics, mitotic inhibitors, and topoisomerase inhibitors [9-10].

Alkylating agents gently damage DNA in all phases of cell cycle to prevent cell division. In this regard, the anticancer platinum-based complexes have been under intense investigations as novel alkylating agents. In fact, in the history of inventing successful anticancer agents, traditional platinum-based complexes (such as cisplatin, carboplatin and oxaliplatin) are the neutral platinum (II) complexes with two amine ligands and two additional ligands which are able to bind DNA covalently. The main mechanism of action of the platinum based complexes is their addition to DNA via nucleotide excision repair, followed by consequence interference of cellular processes, and induction of apoptosis. Since 1970 which platinum based compounds were clinically approved, some tumors became naturally resistant to these agents. It seems that altered expression of key proteins involved in cell control or in signal transduction and decreased drug uptake, increased efflux or inactivation via sulfhydryl molecules have a rule in the platinum based complex resistance. Therefore, production of new non-platinum drugs as tumor inhibiting metal complexes are necessary for overcoming drug resistant ceases of cancers. Gallium, gold, ruthenium and titanium-based complexes have exhibited efficacy in clinical trials [11,12]. Since palladium-based complexes (**A**) are structurally and thermodynamically similar to the platinum based complexes, they would be promising alternatives for platinum based complexes [12].



A

Scheme 1. Palladium-based complexe.

It seems that palladium-based complexes are able to generate more active species than platinum based complexes, hence, these compounds are considered more cytotoxic than their platinum congeners. We previously showed cytotoxicity of palladacycle complexes of three phenyl phosphine ligand and piperidine or biphosphinic complexes against K562, HT29, and Hela cell line [13]. However, resistance as a natural cellular self-defense mechanism will be a major obstacle for platin and its derivatives clinical effectiveness. This phenomenon occurred in multiple mechanisms including reduced accumulation, increased level of glutathione, enhanced DNA repair etc. by activation of many different pathways. Therefore compounds with platin or palladium moieties will be valuable in clinic where they affect against resistant cells. In this regard and to evaluate the effectiveness of synthesized compounds against resistant cells, in the present study, we first prepared cisplatin resistant HT-29

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cells and then evaluate the cytotoxicity of palladacycle complex on these cells. In addition, it was verified if the mechanism of induced cell death is via apoptosis induction or necrosis.

Materials and Methods

Cell lines and culture conditions

The human colorectal cancer cell lines, HT-29 were purchased from National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). The cells were cultured and maintained in high glucose DMEM supplemented with 10% heat-inactivated bovine serum and 1% penicillin/streptomycin. All cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and sub-cultured when the cells reached more than 85% confluency.

Preparation of cisplatin resistant cells

Cisplatin resistant (HT-29R) subline cells were derived from original HT-29 cell line by continuous exposure to increasing concentrations of cisplatin (0.5 µM -12 µM) over 72 h, using modified already published methods [14,15]. Selected doses were under the CC_{50} of cisplatin for HT29 cells and also chosen according to the dose range of cisplatin clinical applications.

Cytotoxic effect of palladacycle complex against HT-29

MTT colorimetric assay was performed to evaluate cytotoxic effect of the complex. HT-29 cells were seeded in wells of a 96-well culture plate (10000 cells/well) and allowed to attach for 24 hours before treatment. Subsequently, cells were treated with different concentrations of the synthetic dinuclearpalladacycle derivative for 48 hours. In the following, to evaluate cell survival, 20 µL of MTT solution (5mg/ml) was added to each well and incubated for 3h. Then, the media were carefully discarded and 150 µL DMSO was added to each well to dissolve insoluble formazan crystals. The formazan absorption was then measured at 570 nm using a microplate reader (Statfix 2000, Awareness, USA). All experiments were performed in triplicate. The percentage of cell survival was calculated comparing to untreated cells (negative control) that was assumed as 100% viability, using the following equation:

$$Percent\ of\ cell\ survival = \frac{Mean\ absorbance\ of\ treated\ groups - Mean\ absorbance\ of\ blank}{Mean\ absorbance\ of\ negative\ control - Mean\ absorbance\ of\ blank} \times 100 \quad (1)$$

Cytotoxicity evaluation of cisplatin and palladium complex on HT-29R cells

Briefly, 180 µl of HT-29R cells (1×10^5 cells/ml) were seeded into 96-well plate and incubated for 24h (37°C, 5% CO_2 air humidified). Then, 20µl of different concentrations of cisplatin (10, 25, 35, 40, 45 µM) and palladium complex (0.125, 0.25, 0.5, 1, 2, 3 µM) were added to specified wells and incubated for another 48h. The cell survival percentage was calculated for each compound as mentioned above.

Apoptosis assay

In order to evaluate cell death mechanism induced by palladium complex, flow cytometry analysis was performed using Annexin V/PI staining kit (Roche®, Germany). In this regard, HT-29 cells were treated for 22 hours with three different concentrations of palladium complex (0.8, 1 and 1.2 µM), and cisplatin (43 µM) as positive control. Then, the cells were washed in PBS by gentle shaking or pipetting up and down and resuspended in 200 µl of binding buffer. According to kit instruction, 5 µl of Annexin V solution was added to 195 µl of the HT-29 cell suspension (5×10^5 cell/ml), and incubated for 10 minute at room temperature. Then, the cells were washed, and resuspended in 190 µl of binding buffer. Finally, 10 µl of Propidium Iodide (20 µg/ml) was added to the cells and subjected to

flow cytometry assay using BD FACS caliber Partec™ instrument (USA).

Result

Verification of resistant HT-29 cells

To show the resistance of prepared HT-29R cells to cisplatin, the cytotoxic effect of different concentrations of cisplatin were evaluated against sensitive (HT-29S) and resistant (HT-29R) cells for 48 h and cell viability was measured by MTT assay. As shown in **Error! Reference source not found.**, the viability of sensitive HT-29 cells was less than 50% at concentrations ≥40.5μM, while for HT-29R cells at this concentration viability was more than 65%.

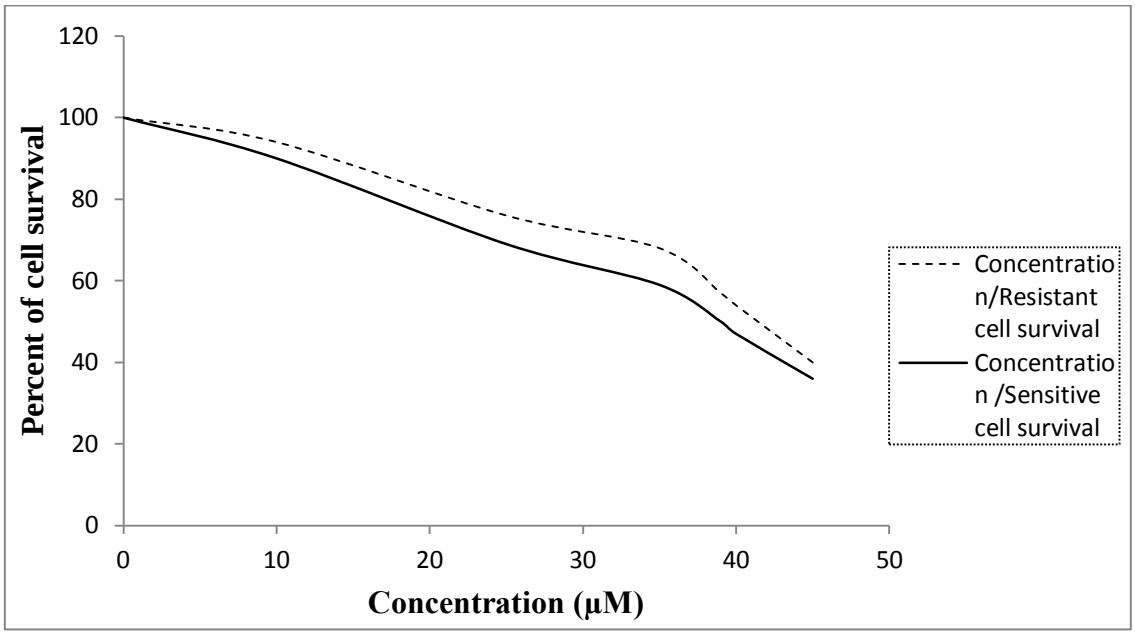

Fig. 1. The effect of cisplatin against HT-29R and HT-29S cells. HT-29 cells were exposed to increasing concentration of cisplatin (0.5 to 12 μM) during a period of 6 months. Both sensitive and resistant HT-29 cells (5×10^5 cell/ml) were exposed to different concentrations of cisplatin for 48 h and viability was evaluated using MTT assay. Data were represented as mean ± SD (p<0.05, n=3).

Cytotoxicity evaluation of cisplatin and palladium complex on HT-29R

The cytotoxic activity of cisplatin was evaluated against HT-29R cells. CC_{50} of cisplatin showed to be 43 μM (Fig. 2A) and 39 μM (Fig.1) against HT-29R and HT-29S cells, respectively. As depicted in **Fig.1**, the palladium complex revealed a $CC_{50}$of 0.94 μM on the resistant cells, showing noticeable cytotoxic effects against resistant HT-29 cells. According to these results the palladium complex was approximately 45 times more potent than cisplatin against the established HT-29R cells.

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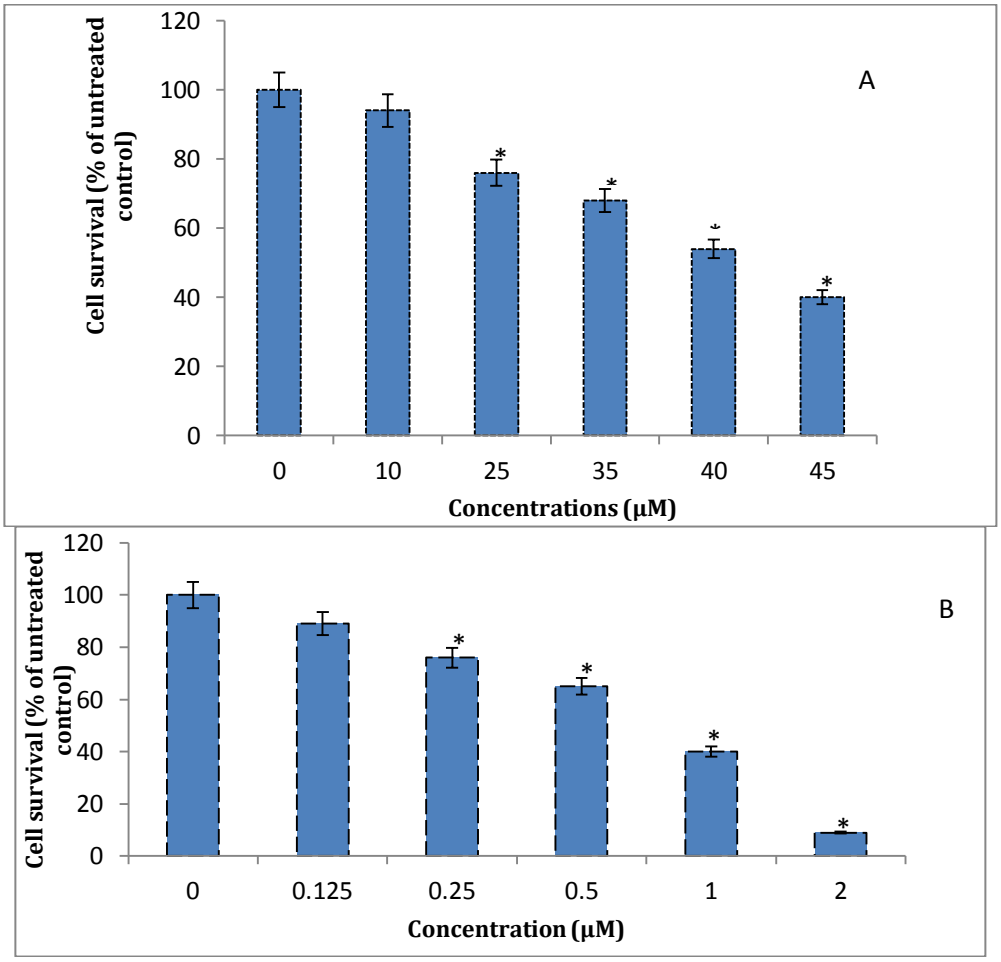

Fig.1. Cytotoxic effect of cisplatin (A) and palladium complex (B) on HT-29R cells. Resistant HT-29 cells (5×105 cell/ml) were exposed to different concentrations of the compounds for 48h and cells viability were evaluated using MTT assay. Data were presented as mean ± SD (*p <0.05, n=3).

Evaluation of cell death mechanism induced by the palladium complex

Identification of the mechanism of death induced by cisplatin or the palladium complex against HT-29 cells was performed by Annexin V/PI staining and subsequent flow cytometry as illustrated in figure 3. Similar to the pro-apoptotic effect induced by cisplatin, it was also showed that the palladium complex mediated its cytotoxic effects via induction of apoptosis, as compared to non-treated control cells.

Flow cytometry results (**Fig.2**) showed 91% viability and almost 4% of apoptotic cells in negative control. However, for the palladium complex the percent of apoptotic cells increased to about 46% at 1.2 µM concentrations, which confirmed apoptosis as the induced cell death mechanism of palladium complex.

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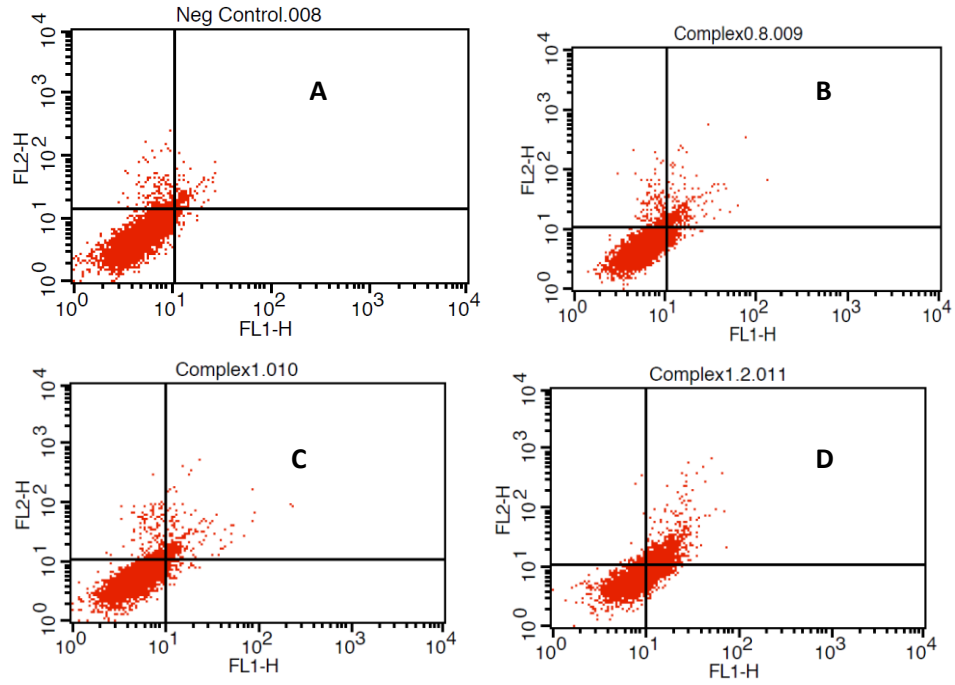

Fig.2.Apoptosis/Necrosis analysis in HT-29 cells by flow cytometry. Negative control cells (A) and cells treated with 0.8 (B), 1 (C) and 1.2 (D) μM of the palladium complex for 12 h, followed by staining with Annexin V and PI.

Discussion

Anti-cancer chemotherapeutic agents such as cisplatin were widely used to treat different types of cancers [16]. In recent years, resistance to anticancer drugs, such as cisplatin, has been investigated as a major problem in cancer treatment [17]. Mechanisms including less susceptibility to natural killer cells in cisplatin resistant cells, decreasing cisplatin accumulation, increasing drug inactivation and deficiency in the apoptotic pathway have been studied for creation of drug resistance in cancer cells [18,19]. It was demonstrated that susceptibility to lysis by natural killer cells was reduced through some elements in multi-drug resistance [20]. Because of the importance of multi drug resistance to better known the anticancer drugs, researchers investigated new compounds with more toxicity [5]. In recent years, several studies demonstrated that palladium complexes had remarkable cytotoxic effects, and therefore could be used for the treatment of many human cancers (e.g., leukemia), efficiently [21,22].

In consistent with these findings, Marques demonstrated that palladium complex [Pd2 (C, N-N, N-dimethylaminebenzylamine) 2 (μ-1,2(diphenylphosphinoe)ethane)(Cl)2] had a significant cytotoxic activity toward the HT-29 cell line that was more potent than cisplatin (12). Considering the previously obtained results, in the present study, HT-29 cells were made resistant to cisplatin by their cultivation in the presence of increasing doses of cisplatin to maximum concentration of 12 μM. Matsunaga*et al* also developed cisplatin resistant HT-29 cells by this method, however, it must be noted that the final resistant concentration they obtained was 10 μM [15]. Data obtained from our MTT assay showed that cell survival percent after 48h exposure to cisplatin in case of resistant or sensitive HT-29 cells at concentration of 10 μM was almost 80% and 64%, respectively. According to this data, cell survival percent in resistant cells was 1.3 fold more than sensitive cells. However, both sublines

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responded to increasing doses of cisplatin in a dose dependent manner. In addition, the CC_{50} of cisplatin against HT-29R and HT-29S was measured to be 43 and 39 μM, respectively. This increased cell survival indicated that HT-29 cells became resistant to cisplatin. In the present studies palladium complexes were chosen because of their structural similarity to Pt (II) complexes. In consistent with our previous results [13], it was shown that palladium complexes in comparison to cisplatin were more potent against cancer cells, and as it was shown by Rodrigues *et al.* may be less toxic toward normal cells [23]. Data obtained from MTT analysis in the present study showed cytotoxic effect of palladium at much more lower concentration against resistant cells comparing to the effective concentration of cisplatin. In fact, CC_{50} of the palladium complex and cisplatin against HT-29R cells were 0.94 μM and 43 μM, respectively, that mean the palladium complex was 45 times more potent than cisplatin. In this regard, other researchers indicated that some synthesized palladium complexes showed more cytotoxic effects than cisplatin. This higher toxicity was related to the nature as well as the geometry of the alkyl groups on the coordinated amine moieties [24-26].

Many anticancer agents including cisplatin induce apoptosis in cancer cell lines [22-27]. Several methods exist to assess the mechanism of cell death which the Annexin V/PI staining method is a commonly used assay for discriminating apoptotic and necrotic cell death [28].

In the present study, we evaluated cell death mechanism induced by palladium complex, comparing to cisplatin as positive control against the HT-29R subline. HT-29R cells were treated with concentration of 0.8, 1, and 1.2 μM of palladium complex, and 43 μM of cisplatin. In the negative control (without any treatment), the percent of viable cells was almost 91%. As shown in Fig. 3, treatment with 1.2 μM of the palladium complex showed 46% apoptotic cells. Therefore, the cytotoxic activity of the palladium complex was performed via induction of apoptosis, and this effect was observed at a concentration 36 times lower than cisplatin.

Conclusion

In conclusion, it could be said that newly synthesized palladium complex were cytotoxic against regular and cisplatin resistant HT-29 cell line mostly via apoptosis induction at a concentration which is remarkably less than cisplatin. The significant cytotoxic effect of the palladium complex on sensitive and the developed resistant HT-29 sublines make it a good candidate for replacing cisplatin. However, further *in vitro* and preclinical evaluations regarding safety and efficacy of the palladium complex are necessary.

Acknowledgement

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Conflict of interest

Authors certify that there is no actual or potential conflict of interest in relation to this article.

References

[1] Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin.2011;61:69-90.

[2] Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA Cancer J Clin. 2013;63:11-30.

[3] Li Z, Yu T, Zhao P, Ma J. Immunotoxins and cancer therapy. Cell Mol Immunol. 2005;2:106-12.

[4] Previati M, Lanzoni I, Corbacella E, Magosso S, Guaran V, Martini A, Capitani S. Cisplatin-induced apoptosis in human promyelocytic leukemia cells. Int J Mol Med. 2006;18:511-6.

[5] Lee Y, Kim YJ, Choi YJ, Lee JW, Lee S, Chung HW. Enhancement of cisplatin cytotoxicity by benzyl isothiocyanate in HL-60 cells.Food Chem Toxicol. 2012;50:2397-406.

[6] Traverso N, Ricciarelli R, Nitti M, Marengo B, Furfaro AL, Pronzato MA, Marinari UM, Domenicotti C. Role of glutathione in cancer progression and chemoresistance. Oxid Med Cell Longev. 2013;2013:972913.

[7] Giaccone G. Clinical perspectives on platinum resistance. Drugs. 2000;59:9-17.

[8] ArrueboM, VilaboaN, Sáez-GutierrezB , LambeaJ, TresA, ValladaresM , FernándezÁG. Assessment of the evolution of cancer treatment therapies. Cancers (Basel). 2011;3:3279–3330.

[9] Abu-Surrah AS, Kettunen M. Platinum group antitumor chemistry: design and development of new anticancer drugs complementary to cisplatin. Curr Med Chem. 2006;13:1337-1357.

[10] Casas JS, Castellano EE, Ellena J, García-Tasende MS, Pérez-Parallé ML, Sánchez A, Sanchez-Gonalza A, Sordo J, Touceda A. New Pd (II) and Pt (II) complexes with N, S-chelated pyrazolonate ligands :molecular and supramolecular structure and preliminary study of their in vitro antitumoral activity. J InorgBiochem. 2008;102:33-45.

[11] Caires AC. Recent advances involving palladium (II) complexes for the cancer therapy. Anticancer Agents Med Chem. 2007;7:484-91.

[12] Marques MPM. Platinum and palladium polyamine complexes as anticancer agents: the structural factor. ISRN Spectroscopy. 2013;1-29.

[13] Karami k,Hosseinikharat M, Sadeghi-Aliabadi H, Lipkowski J, Mirian M. Dinuclear bridged biphosphinic and mononuclear cyclopalladated complexes of benzylamines: Synthesis, structural characterization and antitumor activity. Polyhedron. 2012;50:187-92.

[14] Barr MP, Gray SG, Hoffmann AC, Hilger RA, Thomale J, O'Flaherty JD, Fennell DA, Richard D, O'Leary JJ, O'Byrne KJ. Generation and characterization of cisplatin-resistant non-small cell lung cancer cell lines displaying a stem-like signature. PLoS One. 2013;8:e54193.

[15] Matsunaga T, Hojo A, Yamane Y, Endo S, El-Kabbani O, Hara A.Pathophysiologicalroles of aldo-ketoreductases (AKR1C1 and AKR1C3) in development of cisplatin resistance in human colon cancers.ChemBiol Interact. 2013;202:234-242.

[16] Florea A-M, Büsselberg D. Cisplatin as an anti-tumor drug: cellular mechanisms of activity, drug resistance and induced side effects. Cancers (Basel). 2011;3:1351-1371.

[17] Chang XB. Molecular mechanism of ATP-dependent solute transport by multidrug resistance-associated protein 1. Methods Mol Biol.2010;596:223-249.

[18] Dedoussis GVZ, Menounos P, Papadopoulos N, Karameris A. Cisplatin (CDDP)-resistant K562 cell lines became less susceptible by NK lysis. Anticancer Res. 1998;18:1081-1085**.**

[19] Basu A, Krishnamurthy S. Cellular responses to cisplatin-induced DNAdamage. J Nucleic Acids. 2010;2010:201367.

[20] Yanovich S, Hall RE, Weinert C. Resistance to natural killer cell-mediated cytolysis by a pleiotropic drug-resistant human erythroleukemia (K562-R) cell line. Cancer Res. 1986; 46(9): 4511-4515.

[21] Moro AC, Mauro AE, Netto AV, Ananias SR, Quilles MB, Carlos IZ, Pavan FR, Leite CQ, Hörner M. Antitumor and antimycobacterial activities of cyclopalladated complexes: X-ray structure of [Pd (C 2, N-dmba)(Br)(tu)](dmba= N, N-dimethylbenzylamine, tu= thiourea). Eur J Med Chem. 2009;44:4611-4615.

[22] Divsalar A, Saboury A, Yousefi R, Moosavi-Movahedi A, Mansoori-Torshizi H. Spectroscopic and cytotoxic studies of the novel designed palladium (II) complexes: β-Lactoglobulin and K562 as the targets. Int J Biol Macromol. 2007;40:381-386.

[23] Rodrigues EG, Silva LS, Fausto DM, Hayashi MS, Dreher S, Santos EL, Pesquero JB, Travassos LR, Caires AC. Cyclopalladated compounds as chemotherapeutic agents: antitumor activity against a murine melanoma cell line. Int J Cancer. 2003; 107:498-504.

[24] Zhao G, Sun H, Lin H, Zhu S, Su X, Chen Y. Palladium (II) complexes with N,N'-dialkyl-1,10-phenanthroline-2,9-dimathanamine: synthesis, characterization and cytotoxic activity. J InorgBiochem.1998;72:173-177.

[25] Trávnícek Z, Szucová L, Popa I. Synthesis, characterization and assessment of the cytotoxic properties of cis and trans-[Pd(L)(2)Cl(2)] complexes involving 6-benzylamino-9-isopropylpurine derivatives. J InorgBiochem. 2007; 101:477-492.

[26] Huang CR, Jin ZX, Dong L, Tong XP, Yue S, Kawanami T, Sawaki T, Miki M, Iwao H, Nakajima A, Masaki Y, Fukushima Y, Tanaka M, Fujita Y, Nakajima H, Okazaki T, Umehara H. Cisplatin augments FAS-mediated apoptosis through lipid rafts. Anticancer Res. 2010;30:2065-2071.

[27] 27. Suzuki M, Endo M, Shinohara F, Echigo S, Rikiishi H. Enhancement of cisplatin cytotoxicity by SAHA involves endoplasmic reticulum stress-mediated apoptosis in oral squamous cell carcinoma cells. Cancer ChemotherPharmacol. 2009;64:1115-1122.

[28] Van Engeland M, Nieland LJ, Ramaekers FC, Schutte B, Reutelingsperger CP. Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry. 1998;31:1-9.

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