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IMMUNOMODULATORY POTENTIAL OF PALLADIUM(II) COMPLEXES WITH (1E,6E)-1,7-BIS (3,4-DIMETHOXYPHENYL) HEPTA-1,6-DIENE-3,5-DIONE

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ABSTRACT. In the cancer chemotherapy the metal-based cytotoxic drugs are invariable components of therapeutic protocols, despite the biologic drugs expansion. A current tendency is to design metal-based drugs with highest efficacy and limited toxicity on normal human cells implicated in immune response. Therefore we synthesized and characterized two palladium(II) complexes with (1*E*,6*E*)-1,7-bis(3,4-dimethoxypenyl)hepta-1,6-diene-3,5-dione, a curcuminoid like ligand; their cytotoxicity towards tumor cells was tested, and their expected impact on T and B lymphocytes was measured *in vitro*. The lymphocytes treatment with the free ligand, and with the two complexes **1** and **2**, increased significantly the proportion of the T helper CD4 positive cell population, concomitant with the decrease of T effector CD8 positive cells. The B cells were not affected by **1**, but **2** has a minor inhibitory effect on CD19+ and CD45RA+ cells. The cells function was tested through the compounds modulator effect on membrane markers CD25 and GITR, both being slightly down regulated by **2**, compensating of CD4+ overexpression and CD8+ down regulation. Moreover, complex **1** displayed minimal interferences with the cellular antitumor immunity, acting as a selective inhibitor of cancer cells growth.

Keywords: *(1E,6E)-1,7-bis(3,4-dimethoxyphenyl)hepta-1,6-diene-3,5-dione, palladium complex, cancer therapy, immune response*

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INTRODUCTION

Several metal-based drugs were introduced in the clinical practice; among them the platinum complexes having cytotoxic properties are drugs of choice for systemic therapy of cancer [1]. When using cytotoxic drugs against different cancer localizations, besides the beneficial effect of tumor cell destruction, the normal cells are damaged as well, but more hazardous is the human immune systems damaging, because it cuts the human body self-defense mechanism.

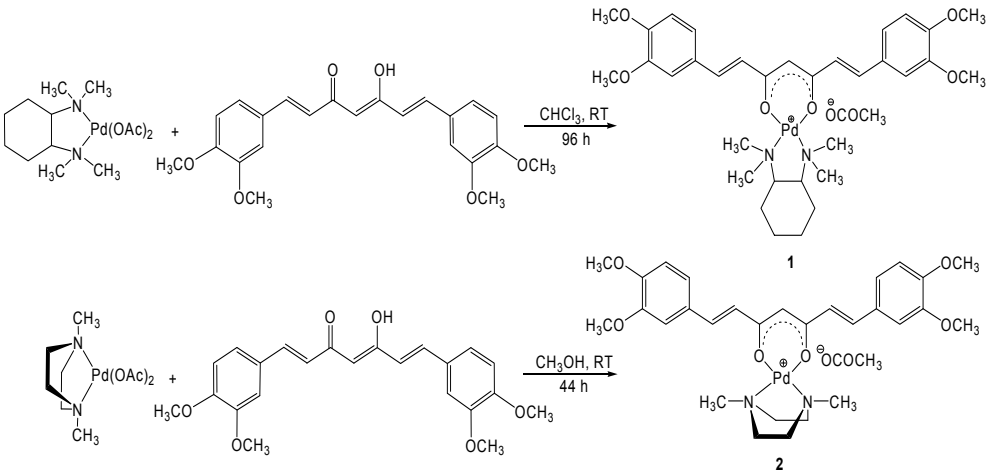
A new tendency in the drug discovery encourages the development of drugs which exhibit cytotoxicity and stimulate the antitumor immunity; in the case of metal compounds, a possible approach is the employment of appropriate ligands. The natural compound curcumin displayed anticancer properties [2] selectively against tumor cells [3] and it is immunostimulatory [4]. The metal complexes of curcumin exhibit as well antitumor properties [5, 6], palladium being one of the central metals which form biological active compounds with curcumin analogues [7,8].

In the present study the curcuminoid (1*E*,6*E*)-1,7-bis(3,4-dimethoxyphenyl)hepta-1,6-diene-3,5-dione, and its palladium(II) complexes were synthesized and characterized. T and B lymphocytes are implicated in the adaptive immunity, being the basis of cell-mediated antitumor immunity in humans [9, 10], responsible for the quality of the cellular immune response. We estimated *in vitro* the Pd(II) complexes capacity to influence the lymphocytes activation processes, in order to establish weather they will hinder the immune response of the host organism.

RESULTS AND DISCUSSION

The curcuminoid (1*E*,6*E*)-1,7-bis(3,4-dimethoxypenyl)hepta-1,6-diene-3,5-dione was synthesized following a former procedure [11]. Complexes **1** and **2** (Scheme 1) as well as the precursor palladium complexes were synthesized based on a procedure reported in our previous studies [8,9].

The curcuminoid and the complexes **1** and **2** were tested *in vitro* on two human tumor cell lines: A2780 ovary and the HT-29 colon carcinoma, and on a primary culture of normal lymphocytes. Cytotoxicity was expressed as the half inhibitory concentration (IC50), the concentration which reduce with 50% the amount of living cells. IC50 values were obtained using the sigmoidal dose-response relation, and they reflect the compounds cytotoxicity against tumor and normal cells (Table 1). The curcuminoid, **1** and **2** exhibit *in vitro* cytotoxicity against the tumor cells and they have a milder effect on normal human lymphocytes.



Scheme 1. Synthesis of palladium(II) complexes **1** and **2.**

Table 1. Half inhibitory concentrations IC50 of curcuminoid and palladium(II) complexes (SD standard deviations).

Cell type	A2780 ovary cancer cells		HT-29 colon cancer cells		Normal lymphocytes	
IC50 values (mM)	Median values	SD	Median values	SD	Median values	SD
Curcumin	2.082	0.240	4.325	0.248	6.375	0.419
Complex **1**	1.522	0.141	0.767	0.099	3.857	0.307
Complex **2**	0.944	0.091	0.577	0.027	5.512	0.071

The treatment with curcuminoid, **1** and **2** increases significantly the number of CD4 positive cells in the population (Figure 1), but the standard drug oxaliplatin raise even more the CD4+ cells percent. In CD8 positive population a significant decrease occurred in the cell number after the treatment with all compounds ($p < 0.01$), related to untreated cells. Complex **2** diminish at a lower extent the CD8+ population, and its effect is milder as of oxaliplatin.

No significant difference was observed in CD19 cells expression between the untreated cells and curcumin ($p > 0.5$) and **1**, and **2** cause a decrease with limited importance, as depicted in Figure 2 (not significant in 95% confidence interval). GITR-positive lymphocytes proportion decreases when treated with **2**, which has a weak effect (p 0.0498, significant decrease in 95% confidence interval), while curcumin (p 0.0844) and complex **1** (p 0.0823) show no significant effect on these population. The same tendency was observed in CD25 positive population.

CD45R transmembrane signaling molecule [13] is characteristic for mature B cells and also for some T cell populations. CD25 is a transmembrane protein present on activated T and B cells; CD4+CD25+ suppressor cells and CD8+CD25+ T regulatory cells are critical in clearance of tumor cells. GITR is the glucocorticoid-induced tumor necrosis factor related protein; its expression increase upon T-cell activation and is implicated in programmed cell death and stimulates the antitumoral responses [14].

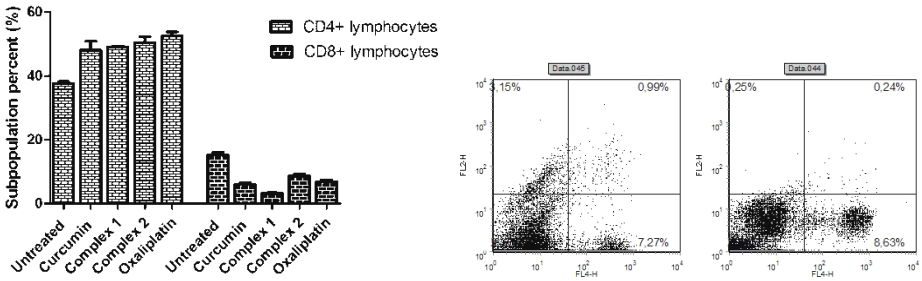


Figure 1. The effect of the complexes effect on CD4+ and CD8+ lymphocytes (left image) and histograms corresponding to flow cytometry measurements for complexes **1** (center) and **2** (right).

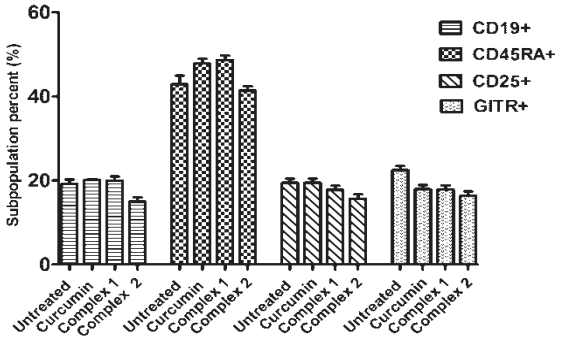


Figure 2. Modulation of CD19, CD45R, CD25 and GITR lymphocyte populations by the treatment with palladium (II) complexes.

The compounds stimulate the survival of the T helper CD4-positive cells in the whole population, and the 24-hours treatment of lymphocytes with the curcumin or his palladium complexes: **1** and **2** give rise to CD4+ enriched cell population.

Cytotoxic CD8-positive T cells are depleted as a consequence of the same treatment, but it is compensated by the enrichment in CD4+ (proportion of CD8 depletion and CD4 enrichment are interrelated). Therefore if non-self

cells destruction mechanism could be affected by novel compounds, the antibody recognition and antigen-presenting mechanisms will work better. The inhibitory effect against CD8+ lymphocyte have highest degree in complex **1** treatment, while the best proliferation in CD4+ lymphocytes is provided by **2**.

Only **2** decreases the survival of CD19 lymphocytes, curcumin and **1** does not interfere with B cells survival, therefore they will not influence the production of antibodies. **2** slightly inhibit B cells survival, but, the proportion of mature CD45R-expressing cells remains unaltered, therefore it couldn't significantly affect the immunoglobulin-secretor function of the B cells.

The activated lymphocytes proportion remains unchanged following the treatment with all compounds, CD25+ and GITR-expressing cells will be not affected, but **2** inhibit somewhat the GITR expression. The novel compounds will not influence lymphocyte activation, an important step in antitumor immune response of the host. Moreover, CD25+ proportion being unchanged and CD4+ increasing, it is obvious that among CD4 population the CD4+CD25+ suppressor cells involvement decreased which could enforce the antitumor response, and will lead to the amplification of effector function. Only in complex **2** effect it is observed a simultaneous increase in CD4+expression concomitant with CD25+ steadiness.

The patterns of CD19+ and CD45R+ cells modulation through curcumin compounds are similar; none of the compounds exert a significant effect on CD19, or CD45RA markers. CD8+CD45R+ cells are suppressor effectors cells [15]; it is very likely that their activity is amplified, because even if CD8+ population drop off, CD45RA+ cells are not affected, thus the proportion of CD8+CD45R+ suppressor effector cells ponder does not modify significantly, and despite the overall CD8 positive population depletion, the cytotoxic effect will be preserved a certain extent. GITR activate the CD4+ and CD8+ T cells [16], and induces tumor rejection [17]. Since curcumin and **1** does not affect GITR expression, and GITR+ cell survival exhibit a moderate decrease due to **2** action, the antitumor immune mechanisms involving CD4+ T cells and B cells will enhance, and the effect of CD8+ drop will be compensate once again by the reliability of GITR expression on the different cell populations, including CD8+ T-cells.

CONCLUSIONS

The curcuminoid and complexes **1** and **2** exhibit a very moderate toxicity against normal human lymphocytes; they do not inhibit the main cell subsets implicated in the signaling pathways of the antitumor response. The curcuminoid inhibits only the CD8+ cells, helps the CD4+ cells survival and it causes no impairment in the other epitopes expression in lymphocytes.

The immune response pathways are not affected by the treatment with the free ligand. Complex **1** action overall on the studied lymphocytes subsets indicates that he has many compensatory effect on the effector cells of the immune system, despite his inhibitory effect on CD8+ cluster, and we anticipate a positive modulation on immunity following *in vivo* administration. Complex **2** inhibitory effects on CD8+ cells and the slight decrease in CD19+ cell expression and GITR, can be a source of moderate immunodepression of the host organism *in vivo*. Overall, the curcuminoid and its palladium(II) complexes will be no source of severe side effects in a potential application as anticancer drugs.

EXPERIMENTAL SECTION

All chemicals for syntheses were of reagent grade and were used as they received; $Pd(OAc)_2$ was purchased from Sigma Aldrich.

All NMR spectra were measured on a Varian Gemini 2000 spectrometers at working frequencies 300 MHz (for ^{1}H-NMR) and 75 MHz (for ^{13}C-NMR). Spectra were measured in CD3OD, using as internal standard TMS. Infrared spectra were recorded on a FT-IR Impact 400 D spectrophotometer on KBr disks. Column chromatography was performed on silica gel (silica 0.035-0.070 mm 60 Å, Acros).

The measurements for biologic effect were made using FACSCalibur flowcytometer (Becton Dickinson, USA) and Synergy2 multiplate reader (BioTek, USA). A2780 and HT-29 tumor cell lines were from ECACC; lymphocytes were isolated on Histopaque separation media from the whole blood of a 42-years old female healthy donor following her informed written consent. We used RPMI-1604 and McCoy's5 cell culture media supplemented with 2mM glutamine, 10% fetal bovine serum, and penicillin-streptomycin solution for cell cultures, all media and supplements were provided by Sigma Aldrich Chemicals. Oxaliplatin was acquired from Actavis Pharma.

Syntheses: Complex **1**: 0.126 mmoles (0.05 g) of (1*E*,6*E*)-1,7-bis(3,4-dimethoxyphenyl)hepta-1,6-diene-3,5-dione were dissolved in 5 mL of dry chloroform. To this solution was added dropwise 0.126 mmoles (0.05 g) of Pd(II) complex containing (*R,R*)-*N,N,N',N'*-tetramethylcyclohexane-1,2-diamine dissolved in 5 mL of $CHCl_3$. Then, 30 µL of NaOH (1M) were added to the reaction mixture. After 96 hours of stirring at room temperature, the reaction mixture was checked by TLC and no unreacted starting curcuminoid was observed. Complex **1** was isolated as a yellow powder (0.06 g; 55%).

^{1}H-NMR (CD_3OD, 300 MHz) δ (ppm) 1.29-1.39 (m, 2H) 1.48-1.59 (m, 2H) 1.81 (m, 2H) 1.89 (s, 3H) 2.20 (m, 2H) 2.85 (s, 6H) 2.87 (s, 6H) 3.25 (m, 2H) 3.87 (s, 6H) 3.89 (s, 6H) 5.89 (s, 1H) 6.75(d, 2H) 6.98 (d, 2H) 7.21 (d, 2H) 7.25 (s, 2H) 7.47 (d, 2H). ^{13}C-NMR (CD_3OD, 75 MHz) δ (ppm) 25.89 (1C) 26.77 (2C) 27.36 (2C) 45.49 (4C) 58.25 (2C) 58.35 (2C) 70.90 (2C) 75.05 (2C) 113.28 (2C) 114.51 (2C) 125.83 (2C) 125.95 (2C) 131.49 (2C) 131.70 (2C) 143.49 (2 C) 152.67 (2C) 181.88 (2C). IR (KBr) ν_{max} (cm^{-1}) 3436, 2931, 2860, 1728, 1620, 1597, 1508, 1465, 1400, 1268, 1139, 1025, 997, 845, 697, 585.

Complex **2**: 0.20 mmoles (0.08 g) of (1*E*,6*E*)-1,7-bis(3,4-dimethoxyphenyl)hepta-1,6-diene-3,5-dione were dissolved in 10 mL of dry methanol. To this solution was added 0.20 mmoles of Pd(II) complex containing 1, 4-dimethylpiperazine dissolved in 5 mL of MeOH. The final product was isolated as a yellowish powder (0.05 g; 62.5%).

^{1}H-NMR (CD_3OD, 300 MHz) δ (ppm) 1.90 (s, 3H) 2.64 (s, 6H) 2.75 (d, 4H) 3.86 (s, 6H) 3.88 (s, 6H) 3.90 (d, 4H) 5.89 (s, 1H) 6.72 (d, 2H) 6.96 (d, 2H) 7.16 (dd, 2H) 7.21 (s, 2H) 7.49 (d, 2H). ^{13}C-NMR (CD_3OD, 75 MHz) δ (ppm) 24.98 (1C) 46.72 (2C) 56.45 (2C) 56.56 (2C) 59.62 (4C) 106.11 (1C) 111.40 (2C) 112.71 (2C) 123.92 (2C) 124.00 (2C) 129.77 (2C) 141.93 (2C) 150.87 (2C) 152.69 (2C) 179.94 (2C). IR (KBr) ν_{max} (cm^{-1}) 2929, 1618, 1597, 1580, 1503, 1451, 1393, 1257, 1136, 1021, 997, 996, 834, 797, 697.

Biologic activity: The cytotoxicity was assessed with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, from Sigma Aldrich) colorimetric method, as described earlier [18]. The treated lymphocyte subpopulations were analyzed using flow cytometry, before and after 24 hours incubation with 0.5 mM curcumin, **1** or **2**. We analyzed the proportion of different cell types among the whole lymphocyte population as described before [19] using antibodies conjugated with fluorochromes: CD4 FITC, CD8 APC, CD25 FITC, CD19PE, CD45R FITC and GITR FITC. The biostatistical analysis was performed with the Graph Pad Prism5 software (GraphPad, USA).

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