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Short communication

Cytotoxicity and effects of 1,10-phenanthroline and 5-amino-1,10-phenanthroline derivatives on some immunocompetent cells

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ABSTRACT

The biological activity of previously synthesized compounds $[(phen)_3(H^+)_2(NO_3^-)_2$ (**1**), Pd(5-NH_2-phen)$_2(NO_3)_2$ (**2**) and Pd(phen)$_2(NO_3)_2(H_2O)$ (**3**)] was investigated *in vivo*. The three compounds did not show any histological alterations in the observed lung, liver, spleen and lymph nodes of White Wistar rats. The propidium iodine staining did not discover any cytotoxic effect of the tested derivatives.

The tests for immunological response predominantly showed stimulation of the antibody-producing B-cells and lower or no stimulation of the T-cells. The LIF-test showed better stimulation of all lymphocytes with **1**, followed by **2** and **3**.

Substance **3** showed highest stimulating effect on B-cell blood lymphocytes in all doses (maximum in the lowest dose), whereas the impact of **2** is weaker and that of **1** is the weakest. The T-cell immune response after treatment with substance **1** is best influenced by dose of 1 mg in the spleen cell-fraction, followed by **3** (5 mg).

1. Introduction

The necessity to improve the structure of some compounds that are used as therapeutic drugs but show much or less side effects led many investigators to the task to compose some derivatives which escape this phenomenon. The platinum drugs represent a unique and important class of antitumour agents [1–3]. Palladium is quite similar to platinum in its chemical and physical properties. The cation Pd(II) is a structural analogue of Pt(II) – d^8-electron configuration. The well-known analogy between the chemical coordination of platinum(II) and palladium(II) compounds explained the interest of studying Pd(II) complexes as antitumour drugs [4,5].

The synthesis and anticancer activity of mixed-ligand complexes of palladium(II) with 1,10-phenanthroline or 5-nitro-1,10-phenanthroline was described in [5–11]. A series of mononuclear and dinuclear palladium derivatives of 1,10-phenanthroline were prepared and tested against L1210 and Bel7402 tumour cell lines and showed lower IC50 values than cisplatin [12]. Many types of chemotherapeutic agents showed to be effective against cancer and human cancer cell lines, but at the same time they are toxic and many of them also destroy the normal cells and possess immunotoxicity [9,13–15]. A series of derivatives of palladium(II) and 1,10-pnenathroline or 5-amino-1,10-phenantroline were prepared and a large diversity of effects such as stimulation of immunocompetent cells [6,8] or antivirus activity [16] were proved.

In previous papers we reported on the synthesis and biological activity of some novel 1,10-phenanthroline (phen) and 5-amino-1,10-phenanthroline (5-NH_2-phen) derivatives [17–19] with interesting biological activity. Substance Pd(5-NH_2-phen)$_2(NO_3)_2$ possesses a strong antitumour action against a cancerous (100% lethality) myeloid subcutaneous tumour (with Graffi-tumour origin) in hamsters [18]. The palladium complex Pd(phen)$_2$(H_2O)$(NO_3)_2$ shows high activity against *Escherichia coli* [19]. The bidentate group $Pd(phen)^{2+}$ is so stable toward dissociation that it can be considered as a new heterocyclic system [20].

The compound $(phen)_3(H^+)_2(NO_3^-)_2$ does not contain a metal ion, but nevertheless revealed an activity against both *E. coli* and *Pseudomonas aeruginosa* [19]. It is noteworthy that the described three substances do not show acute toxicity when administered intraperitoneally (*i.p.*) in rats at doses up to 10 mg [19].

Despite all impacts of the many substances used as chemotherapeutics, there are scarce data about the histological examinations when side effects are observed.

The aim of the present study is to investigate whether substances **1**–**3** damage the tissues of the experimental animals

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and whether they possess any activity on some parameters of the immunocompetent cells, as well as on their cytotoxicity.

2. Chemistry

The derivatives of 1,10-phenanthroline and 5-amino-1,10-phenanthroline, $(phen)_3(H^+)_2(NO_3^-)_2$ (**1**) [17], $Pd(5\text{-}NH_2\text{-}phen)_2(NO_3)_2$ (**2**) [18] and $Pd(phen)_2(NO_3)_2(H_2O)$ (**3**) [18], were synthesized as previously reported. Compound **1** was obtained by reaction of 1,10-phenanthroline·H_2O and $Be(NO_3)_2 \cdot 4H_2O$. It is a protonated form of the trimer of 1,10-phenanthroline compensated by NO_3^- and was obtained by a process of self-assembly. Palladium(II) complexes of (phen) and their derivative (5-NH_2-phen) with a composition of (**2**) and (**3**) were also synthesized. Compound **2** was obtained by reaction of 5-amino-1,10-phenanthroline·H_2O, $Ca(NO_3)_2 \cdot 4H_2O$ and $PdCl_2$; compound **3** – by reaction of 1,10-phenanthroline·H_2O, $Ca(NO_3)_2 \cdot 4H_2O$ and $PdCl_2$. It is known that palladium(II) cation (d^8-electron configuration) forms very strong covalent bonds with organic bidentate ligands. Its complexes have square-planar geometry.

3. Immunology

3.1. Experimental animals

The experiment was carried out on White Wistar rats with the aim to investigate above all whether the tested compounds possess any toxicity.

3.2. Animal treating

White Wistar rats were single treated *i.p.* with different concentrations of substances in phosphate buffered saline (PBS).

It was important to establish whether the studied compounds can cause T- and B-cells' response. The tests for that migration of lymphocytes, rosette forming and plaque forming tests, were accomplished on spleen cells, thymocytes and peripheral blood lymphocytes received from treated animals.

3.3. Migration, rosette forming and plaque forming tests

Migration, rosette forming and plaque forming tests were accomplished on spleen cells, thymocytes and peripheral blood lymphocytes using compounds **1**–**3** and the effect on T- and B-cells' response was estimated.

3.3.1. Rosette forming test

The rosette forming cells among the lymphocytes in the peripheral blood, spleen and thymus were counted following the procedure of Biozzi et al. [21].

3.3.2. Plaque forming test

Plaque cells (PFC-haemolysin producing B-cells) were detected by the method of Cunningham and Szenberg [22].

3.3.3. Leucocyte migration inhibition

Leucocyte migration inhibition was carried out by the procedure of Anders and Natvig [23], which allows the experimental leucocytes to follow the activation or inhibition of their migration.

3.3.4. Propidium iodine (PI) procedure

For PI-fluorescence of dead cells the method of PI-labelling of Inanami et al. [24] was used.

In all procedures the same cells from animals untreated with the substances were used as controls.

4. Histology

Histological observations were done on preparations from liver, lung, spleen and lymph nodes of treated with the substances animals and from the control ones.

5. Results and discussion

The propidium iodine labelling showed that none of the substances was toxic to the examined cells from peripheral blood, spleen or thymus.

Experiments with RFC- and PFC-tests showed that substances **1**–**3** gave predominantly well expressed stimulation of the antibody-producing B-cells (PFC-test) and lower or none stimulation of the T-cells (RFC-test) compared to the controls (Tables 1 and 2). Compound **1** induced higher T-spleen cell response in 1 mg concentration, while compound **3** induced higher T-cell response in 5 mg concentration for the spleen cells and thymocytes. This is understandable because T- and B-cells share the same region in the spleen.

All tested substances did not show any T-cell stimulation in the peripheral blood (Table 1).

In the lowest concentrations **3** gave the best B-response stimulation of all tested cells. The optimal stimulation concentrations (to B-cell response) for **2** and **1** are 5 and 10 mg respectively (Table 2).

LIF-test (Table 3) can reflect positive stimulation (intense migration of lymphocytes) when MI is >1. Well established positive migration was seen after using substance **1** in concentration 10 mg, followed by 0.1 mg. The other two substances seemed to have low influence on the migration ability of all lymphocytes, the optimal concentration of **2** was 0.5 mg and of **3**, 0.05 mg.

The above-mentioned facts indicate that the tested compounds have a general stimulating effect on the B-cells' response.

The propidium iodine staining did not discover any cytotoxic effect of the tested derivatives.

Histological observations (Figs. 1–4) showed total absence of pathological alterations in all examined organ tissues as a result of compound impact.

6. Conclusion

The synthesized derivatives of 1,10-phenanthroline and 5-amino-1,10-phenanthroline show preferably stimulation impact on the B-cell mediated immune response. It means that the antibody response could be great after application of the compounds to animals or humans. Substance **3**, which is a palladium(II) complex of 1,10-phenanthroline, gave the highest stimulating effect on B-blood lymphocytes in its lowest doses. The same impact on the B-immunocompetent cells is lower for **2** (at its highest dose) and lowest for **1** ($(phen)_3(H^+)_2(NO_3^-)_2$).

That fact provides evidence that in **2** the presence of an amino group, attached to the 1,10-phenanthroline aromatic system, is probably responsible for lower stimulation of the immunocompetent cells. The amino group is an electron donor substituent, thus increasing the electron density in the aromatic ring. The structure of **1** is quite different from that of **3** and **2**. It is a protonated trimer of 1,10-phenanthroline compensated by a NO_3^- anion.

As the lymphocyte migration is strongly stimulated by **1** (in its highest concentration), it is the most suitable cell immunostimulator among the three substances.

Our previous studies [17–19] demonstrated that these three compounds did not show acute toxicity and possess good antitumour and antibacterial activities. According to our present investigations these substances do not cause pathological alterations in all examined organ tissues of the tested animals, have no

Table 1
Effect of the compounds on the immunocompetent T-cells (rosette forming test) RFC/10^6 lymphocytes.

Cells	Substance/dose								
	1			**2**			**3**		
	10 mg	1 mg	0.1 mg	5 mg	0.5 mg	0.05 mg	5 mg	0.5 mg	0.05 mg
Blood									
Lymphocytes	0	0	0	0	1	0	0	1	1
Spleen cells	4	11	0	1	1	3	7	1	0
Thymocytes	1	1	0	1	0	1	4	0	0

cytotoxicity and in definite doses stimulate B- and T-immunocompetent cells.

7. Experimental protocols

7.1. Chemistry

7.1.1. Chemicals and apparatus

1,10-Phenanthroline·H_2O and $NaBF_4$ were obtained from Merck, Darmstadt. 5-Amino-1,10-phenanthroline·H_2O was prepared in our laboratory from 5-nitro-1,10-phenanthroline by reduction with hydrazine hydrate using Raney-nickel as a catalyst. All other chemicals used were of the highest available quality.

7.1.2. Synthesis of $(phen)_3(H^+)_2(NO_3^-)_2$ (**1**)

The compound was synthesized as described in [17].

7.1.3. Synthesis of palladium(II) complex of 5-amino-1,10-phenanthroline $Pd(5\text{-}NH_2\text{-}phen)_2(NO_3)_2$ (**2**)

The compound was synthesized as described in [18].

7.1.4. Synthesis of compound $Pd(phen)_2(NO_3)_2(H_2O)$ (**3**)

The compound was synthesized as described in [18].

7.2. Immunology

7.2.1. Experimental animals

White Wistar rats (200–250 g) were provided by the Breeding house of the Bulgarian Academy of Sciences (Sofia). Animals were given standard pellet diet and tabwater ad libitum and kept in rooms with controlled 12/12 h light and dark cycle and temperature (22 ± 2 °C), humidity 60 ± 10%. All experiments were carried out between 09.00 a.m./13.00 p.m. Only those with normal motor function were used.

7.2.2. Ethical aspects

The experiments followed a protocol approved by the Committee of Ethics for Animal Experiments at the Institute of Experimental Pathology and Parasitology (Bulgarian Academy of Sciences). All experiments comply with the Bulgarian National Veterinary Service (Reg. No. 11130007/19.01.2007–26.01.2012) according to the requirements of the National Institute of Health (NIH) Guide for the Care and Treatment of Animals (Bethesda, MD, USA). The experiments could not be done only in conditions *in vitro*.

Table 2
Effect of the compounds on the B-cells (plaque forming test) PFC/10^6 lymphocytes.

Cells	Substance/dose								
	1			**2**			**3**		
	10 mg	1 mg	0.1 mg	5 mg	0.5 mg	0.05 mg	5 mg	0.5 mg	0.05 mg
Blood									
Lymphocytes	21	0	0	30	3	12	21	63	154
Spleen cells	7.5	0	4.5	6	12	1.5	15	12	96
Thymocytes	1.5	0	1.5	33	6	1	0	60	36

Table 3
Effect of the compounds on the migration index (MI) of lymphocytes.

Cells	Substance/dose								
	1			**2**			**3**		
	10 mg	1 mg	0.1 mg	5 mg	0.5 mg	0.05 mg	5 mg	0.5 mg	0.05 mg
Blood									
Lymphocytes	20.46	0	7.56	0	1.25	0.48	0	0	0
Spleen cells	0.75	3.62	6.58	0	1.89	0	0	0	1.28
Thymocytes	13.62	0	4.97	0	0.61	0.61	0	0	0.607

7.2.3. Animal treating

The amount of the injected substance **1** was 10, 1 and 0.1 mg, substances **2** and **3** – 5, 0.5 and 0.05 mg. The animals were narcotized and sacrificed on the 10th day after treating.

7.2.4. Cell preparation

Cells from thymus and spleen were obtained by grinding the tissue in cold PBS, pH 7.2 in Potter homogenizer and filtering the cells through a 60 mesh. After 10 min centrifugation at 1200 rpm the thin white over layer was aspirated and used in the experiments. Peripheral blood lymphocytes were obtained from heparinized blood centrifuged at 1200 rpm for 15 min and the white ring was brought out after that, laid on LYMPHOPREP ("Nyccomed", Norway) and centrifuged for further 30 min at 20 °C and 2800 rpm. All lymphocytes (from spleen, thymus and peripheral blood) were finally washed twice in cold PBS. The cell-vitality was measured by 0.2% methylene blue. It was found to be 85–96%. The number of the immunocompetent cells (T-cells, i.e. rosette forming cells – RFCs and B-cells, i.e. plaque forming cells – PFCs) was calculated by 10^6 lymphocytes.

7.2.5. Rosette forming cells

Rosette forming cells among the lymphocytes was considered such lymphocyte surrounded by more than 4 sheep erythrocytes.

7.2.6. Plaque forming test

Plaque forming test was completed as follows: the experimental cells were mixed with complement and sheep red blood cells (SRBC) in definite concentrations and 50 μl – chambers were filled with them. After 24 h of storage at 37 °C and 5% CO_2 the PFCs were counted as haemolytic zone and compared to those of the healthy controls.

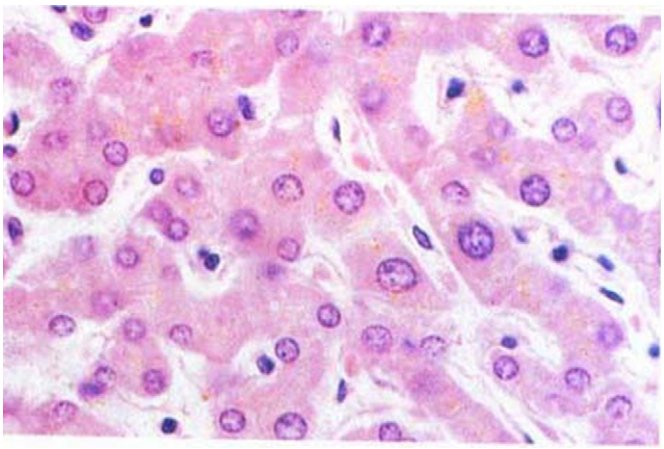

Fig. 1. Light microscopy of experimental liver; HE stain, magnification 450×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

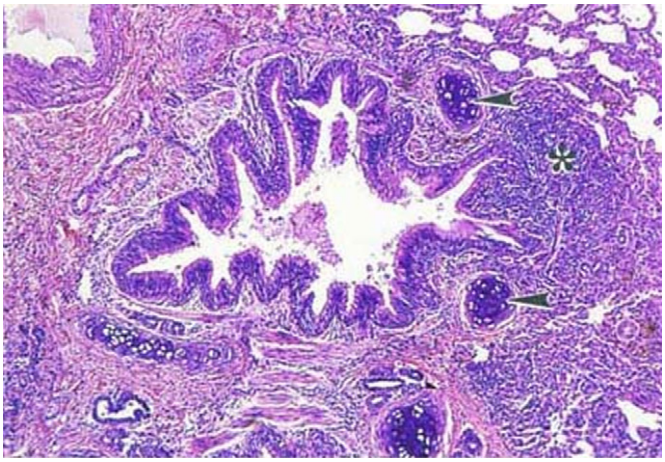

Fig. 2. Light microscopy of experimental lung; HE stain, magnification 200×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

7.2.7. Leucocyte migration inhibition

Leucocyte migration inhibition test was undertaken in 50 mm plastic Petri dishes with monolayer of agarose containing appropriate different components. 3-mm diameter holes in the agarose were filled with 10 μl of the examined cell suspension (10^6 cells/ml) and after 16 h of preservation at 37 °C and 5% CO_2 the migration area of the cells was calculated. The migration index (leucocyte migration factor – LIF) was the ratio between the area of migration of the experimental cells and the area of migration of the control ones.

7.2.8. Propidium iodine (PI) procedure

For *in vitro* estimation of cytotoxicity of the compounds on the examined cells, a part of the centrifuged at 1000 rpm/5′/4 °C cells (5×10^5) from each sample (blood, spleen and thymus) was washed in Ca^{2+}- and Mg^{2+}-free phosphate buffered PBS and fixed with 1% glutaraldehyde/PBS. For PI-fluorescence of dead cells the method of PI-labelling of Inanami et al. [24] was used and the microscopic observations were performed with "Carl Zeiss" (Germany) fluorescence microscope.

In all procedures the same cells from animals untreated with the substances were used as controls.

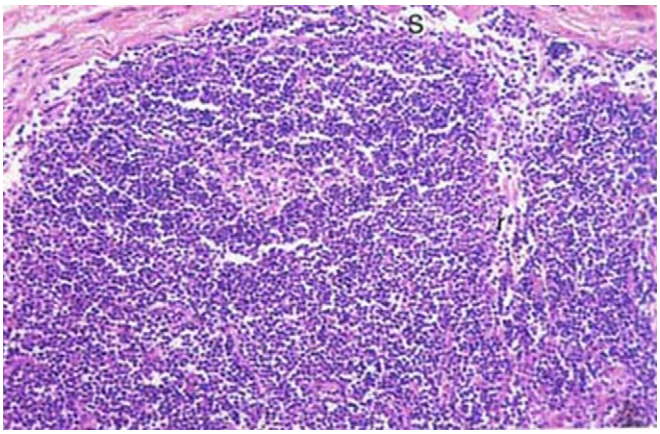

Fig. 3. Light microscopy of experimental lymph node; HE stain, magnification 100×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

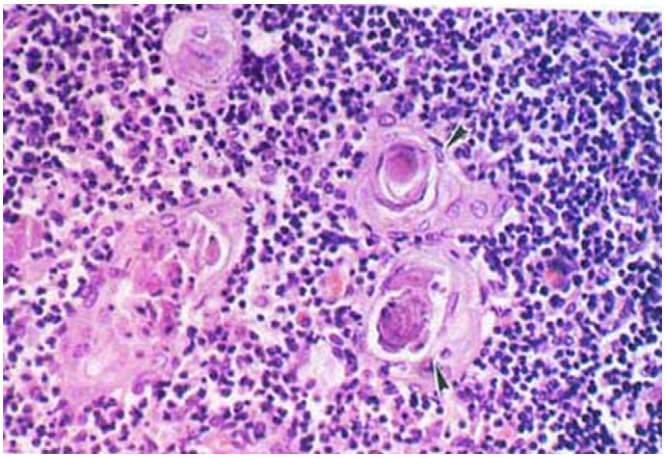

Fig. 4. Light microscopy of experimental thymus; HE stain, magnification 350×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

7.3. Histology

Histological observations were done on preparations from liver, lung, spleen and lymph nodes of treated with the substances animals and from the control ones. The tissue samples were fixed for 48 h in 4% buffered formaldehyde, embedded in paraffin and prepared in 5–8 μm sections stained with haematoxylin–eosin.

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