IN VITRO ANTIPROLIFERATIVE ACTIVITY AND ANTIOXIDANT CAPACITY OF NEW ORGANOMETALLIC COORDINATION COMPOUNDS, RESULTS CORRELATION ANALYSIS

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Rezumat

A fost examinată și testată capacitatea inhibitorilor organometalici activi CMJ-33, CMSA-4, de a induce apoptoza în celulele epiteliale de carcinom de col uterin linia HeLa. Viabilitatea celulelor canceroase a fost determinată prin testul Alamar Blue fiind folosit un indicator redox – resazurin. S-a depistat activitate antitumorală mai mare a substanțelor testate decât la cea luată în calitate de etalon: - doxorubicina (DOXO). În scopul de a detecta eventuala prezenţă a efectelor adverse concomitente legate cu proprietățile pro-oxidante ale compușilor coordinativi, a fost folosită metoda cantitativă ORAC (Oxygen Radical Absorption Capacity). Rezultatele au demonstrat că substanțele testate CMT-67 şi CMSA-4 posedă activitate antioxidantă mai mare decât Trolox și DOXO. În această lucrare, noi constatăm prezența unei corelații pozitive semnificative (coeficient de corelație - 0,98) între efectele anti-proliferative și anti-oxidante pentru compuşii CMT-67, CMSA-4 și DOXO.

Cuvinte cheie: ORAC; activitate antioxidantă; resazurina; activitate antiproliferativă; activitate antitumorală.

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Introduction

Cervical cancer is the second most common cause of female-specific cancer after breast cancer accounting for around 8% of both total cancer cases and total cancer deaths in women. Currently, surgery, radiotherapy, and doxorubicin - based chemotherapy are the primary methods for treating cervical cancer. Actually, neoadjuvant chemotherapy reduces the gross tumor volume, extends the 5-yr survival rate, decreases the recurrence rate, and has thus attracted extensive attention for various studies [1–3].

The antiproliferative effects of compounds were determined using epithelioid cervix carcinoma cells of line HeLa, due to the fact that HeLa cells are incredibly malignant, compared to other cancer cells [4]. We used Resazurin assay which works as a cells viability and proliferation indicator, through the conversion of resazurin to resorufin.

Generally, prooxidants produce adverse modifications to cell components, such as lipids, proteins, and DNA, resulting in cell damage [5]. They induce oxidative stress, which contributes to many pathological conditions, including cancer, neurological disorders [6], atherosclerosis, hypertension, ischemia/perfusion [7], diabetes, acute respiratory distress syndrome, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease [8], and asthma [9]. Therefore, the anticancer substances shouldn't be prooxidants that causes additional dangerous side effects [10].

The antioxidant capacity of experimental complexes was measured by ORAC assay and their potency was compared with that of the positive control, Trolox, a water-soluble vitamin E analogue. The ORAC assay is a sensitive method based on the detection of chemical damage to fluorescein through the decrease in its fluorescence emission by peroxyl radicals that are generated in situ by the thermal decomposition of the free radical initiator AAPH [11].

Thus, we evaluated the antioxidant potential and proliferative activity of some new organometallic coordination compounds CMT-67 and CMSA-4 of various concentrations in experiments in vitro.

Materials and methods

Cell Culture conditions

Epithelioid cervix carcinoma cells of line HeLa, passage 7 was used (Figure 1). The cells were cultured as monolayer in Dulbecco's Modified Eagle Medium (DMEM) high glucose (Invitrogen) containing L-glutamine, bovine albumin fraction (V7.5%) 0,2%v/v (Invitrogen), HEPES buffer (N-2 hydroxyethylpiperazine-N'-2-ethane sulfonic acid) 20mM (Invitrogen), antibiotics penicillin-streptomycin (final concentration 100 U/ml penicillin and 100 µg/ml streptomycin sulfate) (Invitrogen) and supplemented with fetal bovine serum (FBS-irradiated) 10% v/v (Cambrex) in 75 cm2 falcon culture flasks (Cellstar) and incubated in 2% CO_2, 78% air in humidified atmosphere at 37^0C with medium renewal every 2–3 days.

Cell proliferation Resazurin assay. Resazurin is a non-fluorescent indicator dye, is converted to highly red fluorescent resorufin via reduction reactions of metabolically active cells. The amount of fluorescence produced is proportional to the number of living cells. Resazurin was dissolved in physiological buffers (resulting in a deep blue colored solution) and added directly to cells in culture in a homogeneous format. Usually, in the presence of NADPH dehydrogenase or NADH dehydrogenase as the enzyme, NADPH or NADH is the reductant that converts resazurin to resorufin (Figure 2) [12].

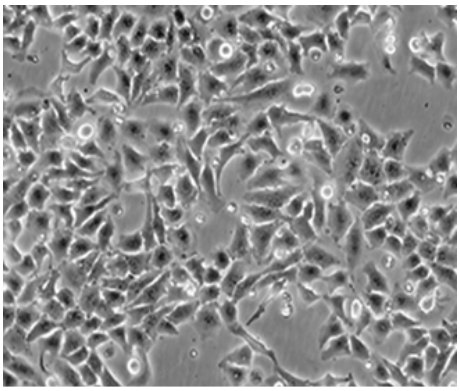

Figure 1. HeLa cell line in culture

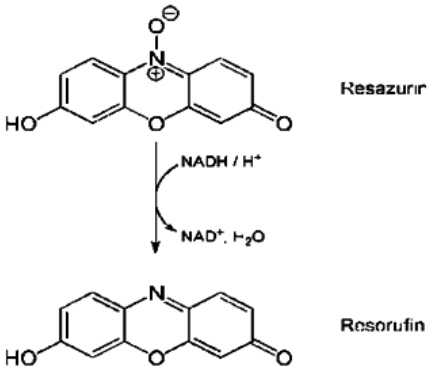

Figure 2. Reduction of resazurin to resorufin in living cells with NADH.

Cells were trypsinized from subconfluent cultures by adding 3 ml of trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) 0.05% (Invitrogen) to 50 ml falcon flasks with confluent cells followed by 5 min incubation at 37°C with regular gentle shaking and counted under an inverted microscope (OLYMPUS). The trypsin reaction

was stopped by adding 10 ml of appropriate culture medium containing 10% FBS. The cell suspension was centrifuged at 750rpm for 10 min at 25°C. The cell pellet was suspended in 2 ml of medium with 10% FBS and thoroughly mixed. Cells were counted and brought to a concentration of 1×10^5 cells/ml. The resulting cell suspension was seeded into duplicate wells of a 96-well microtiter plat (Becton Dickinson and Company, Lincoln Park, NJ, USA) (90µl/well) and incubated at 37°C, 2% CO_2. After an initial 4 h period to allow cell attachment, 10 µl compounds CMJ-33and CMSA-4 were directly added to the medium resulting. The plate was further incubated for 24 h at 37°C, 2% CO_2. Coordinative compounds and Doxorubicin hydrochlorid (SC Balkan Pharmaceuticals SRL) was dissolved in dimethyl sulfoxide (DMSO) to prepare the stock solution of 10 mM/L, which were used as reference at final concentrations ranging from 10, 1, 0,1 µM/L. These compounds and doxorubicin was incubated for 24 hours. Following each treatment, 20 µl resazurin indicator solution was added to each well and incubated at 37°C, 2% CO_2 for 4 hours. Subsequently, the absorbance was read with 570 nm and 600 nm filters. The measurement was made by imaging hybrid reader (Synergy H1, BioTek).

The inhibition (%) was calculated according to the formula:

$$100-((Abs\ 570nm_{sample}–Abs\ 600nm_{sample}) / (Abs\ 570nm_{control}– Abs\ 600nm_{control})\times100)$$

As an indicator of efficiency of the experimental compounds on proliferation of cancer cells was used the half maximal inhibitory concentration (IC_{50}), which is a quantitative indicator of the activity of antagonists test reactions in vitro in the pharmacological studies. The IC_{50} values were calculated according to the Hill equation [13], using the software.

Oxygen Radical Absorbance Capacity (ORAC-Fluorescein) assay

The ORAC assay is unique in that its reactive oxygen species (ROS) generator, AAPH produces a peroxyl free radical upon thermal decomposition that is commonly found in the body, making the reaction biological relevant. Furthermore, since AAPH is reactive with both water and lipid soluble substances it can be used to measure the total antioxidant potential. The radical can oxidize fluorescein to generate a product without fluorescence. Antioxidants suppress this reaction, by a hydrogen atom transfer mechanism, inhibiting the oxidative degradation of the fluorescein signal [14].

Reagents. Chemicals and reagents used were of analytical grade and obtained from Sigma: (3',6'-dihydroxy-spiro[isobenzofuran-1[3H], 9'[9H]-xanthen]-3-one) (FL) Fluorescein disodium; 2,2'-Azobis (2-methylpropionamidine) dihydrochloride (AAPH), and 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox). Doxorubicin hydrochlorid ($C_{27}H_{29}NO_{11}HCl$) (Naprod India). DMSO (dimethylsulfoxide), methanol and (PBS) phosphate-buffered saline (pH7.4) were purchased from local suppliers. Deionized water was obtained, Adrona Water treatment system Crystal E HPLC.

ORAC-method. The method was adapted to hybrid reader (Synergy H1, BioTek). The reaction was carried out in 10 mM/L PBS (pH 7.4) and the final reaction mixture was 200µl. Were prepared dilutions of 2 experimental substances CMT-67 and CMSA-4 in DMSO (solutions stock 10 mM/L) and next dilutions were prepared in 10 mM/L PBS (pH 7.4). After that, 25 µl of each experimental complexes dilution were transferred in

a 96 wells black microtitre plate (Nunc™ black microwell, Denmark) and 150 µl of working solution of fluorescein (15 nM/L final concentration) were dispensed with dispense module of hybrid reader (Synergy H1, BioTek). The mixture was preincubated for 30 min at 37°C prior with shaking at 700 rpm (Shaker-thermostat, Sky Line, Elmi ST-3L). After that, 25 µl AAPH solution (240 mM/L final concentration) was dispensed with dispense module of hybrid reader (Synergy H1, BioTek). The plate was immediately placed in the hybrid reader (Synergy H1, BioTek) and the fluorescence recorded every minute for 100 min. The plate was automatically agitated prior each reading. Fluorescence measures were carried out at 37°C. Excitation and emission filters were 485-P and 528-P, respectively. A blank was used phosphate buffer instead of the antioxidant solution. Trolox and DOXO were used for standards. Trolox (2 mM/L methanolic stock solution) was used as reference at dilution concentrations ranging from 3.125 to 100 µM/L in PBS (pH 7,4). DOXO (10mM/L) prepared in DMSO for use as a stock standard was used as reference at concentrations ranging from 6,25 to 50 µM/L in PBS (pH 7,4).

The antioxidant capacity, expressed as the area under curve (AUC), was calculated by a statistical program following formula:

$$AUC=1+(RFU_1/RFU_0)+(RFU_2/RFU_0)+(RFU_3/RFU_0)+\ldots+(RFU_n/RFU_0)$$

where RFU_0 = relative fluorescence units at time point zero and RFUn = relative fluorescence units at time points. The Net AUC was calculated by subtracting the blank AUC from the AUC of each sample, the standards, and the positive control: Net AUC = AUCsample – AUCblank.

The correlation coefficient (R^2) was obtained using a least means squared linear regression analysis. The slope was calculated as follows: Slope (m) = dY / dX. To determine Trolox equivalents (TE) of each sample range the ratio of the slope (m) of the linear regression analysis of the compound to the slope of the linear regression of Trolox was used:

$$TE = m_{compound} / m_{Trolox}$$

Results and discussion

Comparative study and concentration ranges identification of cytotoxic activity of organometallic complexes CMT-67, CMSA-4 in vitro in regard to cervical cancer cell line HeLa cells are shown in Figure 3.

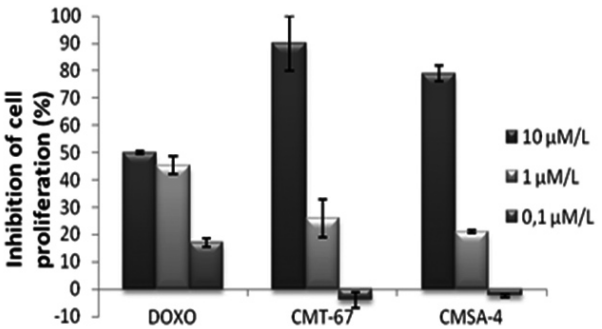

Figure 3. Inhibitory effect of CMT-67, CMSA-4 and DOXO on the proliferation of cervical cancer cells line HeLa.

Antiproliferative activity experiments showed that there is the concentration dependence between inhibitory effects of the complexes in the micromolar range.

Complexes CMT-67 and CMSA-4 inhibit the formation and growth of HeLa cell line, that's demonstrate the capacity of experimental substances to inhibit the process of metastasis. Was founded, that the IC_{50} values are 2.1 ± 5 μM/L for CMT-67; 3 ± 0,15 μM/L for CMSA-4 and 6 ± 0,1 μM/L for DOXO. Thus was established that, coordination compounds CMT-67 and CMSA-4 exhibit stronger inhibitory activity on HeLa cells proliferation than DOXO, a commonly used chemotherapeutic agent (Figure 4).

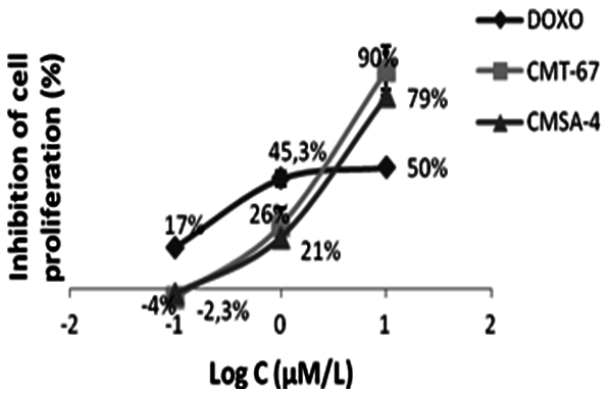

Figure 4. Dose-response curves of the cytotoxicity of complexes CMT-67, CMSA-4 and DOXO on line HeLa cervical cancer cells at three concentrations. Each value represents the mean ± SED.

The antioxidant capacity of a substance CMT-67, CMSA-4 and DOXO was estimated by comparison to the standard curve of Trolox. Figure 5 depicts the kinetic curves of Trolox demonstrate concentration dependent protection of fluorescein against oxidative degradation by AAPH. The progress of each reaction was followed in real-time using the current state option.

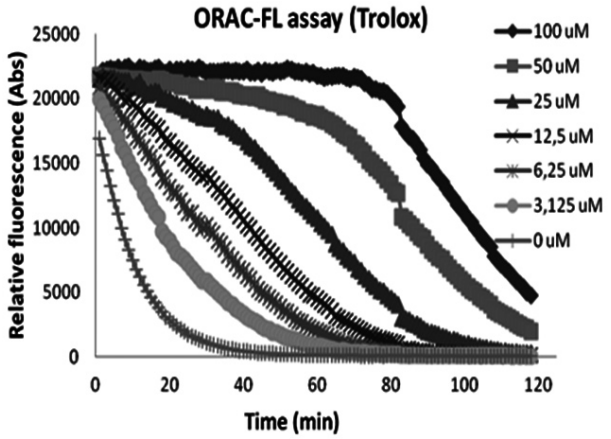

Figure 5. Plots of Trolox kinetic curves. Representative curves from ORAC assay of varying concentrations of Trolox antioxidant standards ranging from 0 to100 μM/L.

The Net AUC calculated by equation is graphically indicated in Figure 6 (A. Trolox; B. DOXO; C. CMT-67; D. CMSA-4). The correlation coefficients are all fairly high (R^2 - 0.99) for trolox; 0.98 for DOXO; 0.88 for CMT-67; 0.95 for CMSA-4.

Antioxidant properties for experimental substances, Trolox and DOX were determined by the ratio of the slope of the linear regression curve. Slope (m) values are 3,5 for CMT-67; 2,18 for CMSA-4; 0,5 for DOX and 1,2 for Trolox. The calculated Trolox equivalents (TE) were used for comparative analysis of the antioxidant capacity of complexes CMT-67, CMSA-4 and DOX.

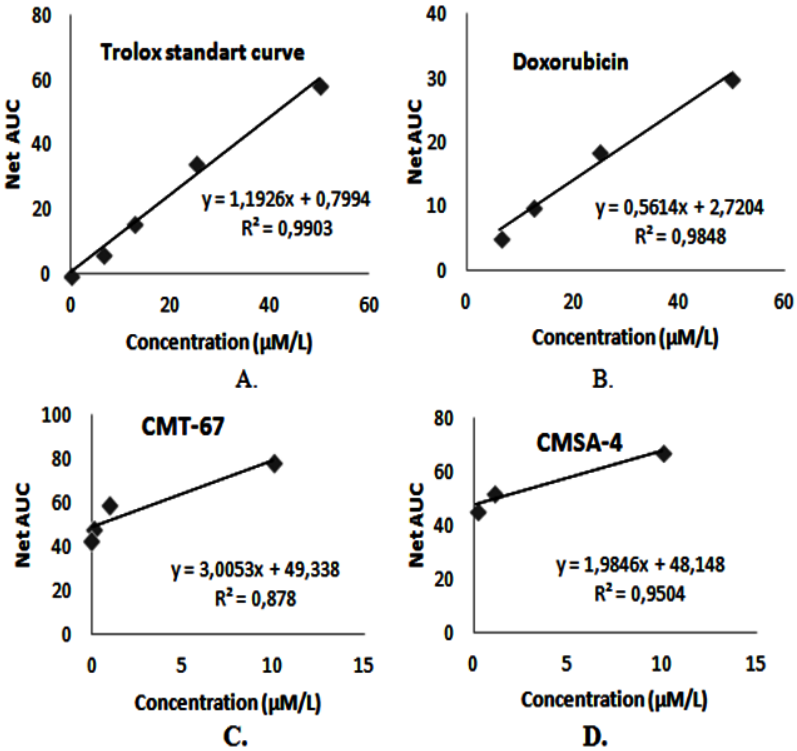

Figure 6. The Net AUC of varying concentrations of: A. Trolox antioxidant standards; B. DOXO; C. CMT-67 and D. CMSA-4.

The highest antioxidant activity is shown the complex CMT-67, where slope (m) = 3,5 (Table 1).

Table 1. Antioxidant Capacity

Name	m	R^2	TE
CMT-67	3,5	0,88	2.92
CMSA-4	2,18	0,95	1.82
DOXO	0,5	0,99	0.42
Trolox	1,2	0,99	

Analyzing the ORAC results, we observe that compounds CMT-67, CMSA-4 showed the best antioxidant activity compared with Trolox and DOX.

In order to establish the potential connection of the antioxidant and antiproliferative activities of experimental compounds was calculated correlation coefficient. Positive correlation between the antiproliferative activity on HeLa cell line and total antioxidant capacity of substances CMT-67, CMSA-4 and DOX was 0.98, which is statistically significant evidence of strong positive correlation between the proliferative and antioxidant activities (Figure 7).

Since the experimental substances CMT-67, CMSA-4 possess as pronounced antiproliferative activity, and so antioxidant capacity, the chemical complexes are of vital interest for further study for subsequent use their as antitumor agents.

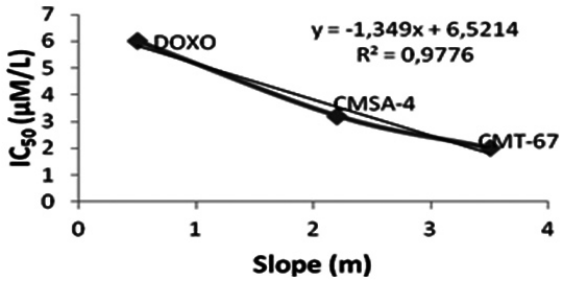

Figure 7. Correlation coefficient between the antiproliferative and antioxidant activity of substances CMT-67, CMSA-4 and DOXO.

Conclusions

- A comparative study of compounds CMT-67, CMSA-4 on the induction of apoptosis revealed that the 24-hour exposure to substances on the HeLa cell line can significantly inhibit proliferation at concentrations that are significantly less than doxorubicin, so the half maximal inhibitory concentration for CMT-67 is 3 times less and CMSA-4 is 2 times less than DOXO.

- Is very important that the anticancer substances due to their antioxidant potential may exert effects by interfering with oxygen free radicals, which act as second messengers in the critical signaling pathways for cancer cells proliferation.

- In this paper, we present the positive strong correlation (correlation coefficient - 0.98) which was obtained between antiproliferative and antioxidant effects of the substances CMT-67, CMSA-4 and DOXO

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