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IN VITRO ANTICANCER ACTIVITY OF CHLORO(N-PHENYL-N'-[(PYRIDIN-2-YL)METHYLIDENE]CARBAMOHYDRAZONOTHIOATO) (4-AMINOBENZENE-1-SULFONAMIDE)COPPER

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This study was aimed to evaluate the antiproliferative activity of the mixed-ligand complex (chloro(N-phenyl-N'-[(pyridin-2-yl)methylidene]carbamohydrazonothioato)(4-aminobenzene-1-sulfonamide)copper) on several cancer cells of lines. It was established, that the copper(II) mixed-ligand complex exhibits the highest anticancer activity against MeW-164, HeLa, BxPC-3 and RD cells of lines with IC_{50} values of 1.0±0.2, 0.4±0.04, 1.7±0.2, 1.3±0.3 μM, respectively. A comparative study between the tested compound and DOXO in regard to cancer lines has established that the tested copper(II) mixed-ligand complex exhibits stronger inhibitory activity on cancer cells proliferation than doxorubicin and cisplatin.

Keywords: *mixed-ligand complex, doxorubicin, cisplatin, anticancer activity, cancer cells of line.*

***IN VITRO* ACTIVITATEA ANTICANCERIGENĂ A (CLORO(N-FENIL-N'-[(PIRIDIN-2-IL)METILIDEN] CARBAMOHIDRAZONTIOLATO) (4-AMINOBENZEN-1-SULFONAMID)-CUPRU)**

Acest studiu a avut ca scop studierea activității antiproliferative a complexului (cloro(N-fenil-N'-[(piridin-2-il)metiliden]carbamohidrazontiolato)(4-aminobenzen-1-sulfonamid)-cupru) pe linii de celule cancerigene. S-a stabilit că complexul investigat cu cupru (II) prezintă cea mai mare activitate anticancerigenă împotriva celulelor liniilor MeW-164, HeLa, BxPC-3 și RD cu valori IC_{50} de 1,0±0,2, 0,4±0,04, 1,7±0,2, 1,3±0,3, respectiv. Studiul comparativ al compusului testat cu DOXO în ceea ce privește liniile de celule cancerigene a arătat că complexul testat de cupru (II) prezintă o activitate inhibitoare mai puternică asupra proliferării celulelor cancerigene decât doxorubicina și cisplatina.

Cuvinte-cheie: *complex mixt-ligand, doxorubicină, cisplatină, activitate anticancerigenă, linii de celule cancerigene.*

Introduction

Cancer represents one of the most serious health problems and major causes of death around the world [1]. The principal need in the chemotherapy of cancer remains the discovery of new effective and safe agents, since the therapeutic applications of antiproliferative drugs are restricted due to their toxic potentials, resistance and genotoxicity [2]. Every cancer type requires a specific treatment regimen that encompasses one or more modalities such as surgery, radiotherapy, and chemotherapy.

Doxorubicin, a frontline drug being regarded as one of the most potent of the U.S. Food and Drug Administration approved chemotherapeutic drugs, has been used for treating cancer for over 30 years. While providing a cure in select cases, doxorubicin causes toxicity to most major organs, especially cardiotoxicity, which forces the treatment to become dose-limiting [3] It is known that DOXO cardiomyopathy carries a poor prognosis and is frequently fatal [4]. DOXO induces toxic damage to the mitochondria of cardiomyocytes contributing increased oxidative stress [5].

Platinum-based anticancer drugs play a leading role in the treatment of various malignant tumors, but severe side effects such as nephrotoxicity, neurotoxicity, and drug resistance have limited their wide range of clinical applications [6]. This has stimulated extensive research and has promoted chemists to establish alternative approaches on the basis of using endogenous metals to improve the pharmacological properties. Among the many bio-essential metals, copper complexes are regarded as promising alternatives to platinum complexes as anticancer drugs because copper is biocompatible and exhibits many significant roles in biological systems [7]. Also, copper shows the altered metabolism of cancer cells and differential response between normal and tumor cells [8]. It is proven that the concentration of copper in cancerous tissues exceeds that of normal tissue, and the sequestration of copper can prevent the establishment of new blood vessels [9]. Therefore, cancer cells may represent a suitable, selective target for copper-based agents [10]. In recent years, a large number of synthetic copper(II) complexes of thiosemicarbazones ligands have been

reported to act as pharmacological agents and as potential anticancer and cancer-inhibiting agents, and they have been found to be active both in vitro and in vivo [11]. Thiosemicarbazone is a class of organic compounds that possesses a wide spectrum of biological activities and medical properties. Thiosemicarbazones contain a wide range of donor atoms and, therefore, can form coordination compounds with transition metal ions [12,13].

In this study, we have compared the antiproliferative activity of the synthesized copper(II) mixed-ligand complex (chloro(N-phenyl-N'-[(pyridin-2-yl)methylidene]carbamohydrazonothioato)(4-aminobenzene-1 sulfonamide)copper) with that of doxorubicin and cisplatin in several human cancer cell lines: MeW-164 (human malignant melanoma), HeLa (human cervix adenocarcinoma), BxPc-3 (human epithelial pancreatic adenocarcinoma), RD (human muscle rhabdomyosarcoma).

Materials and methods

Characterization of the tested copper(II) mixed-ligand complex [Cu(Str)(L)Cl]

The copper(II) mixed-ligand complex chloro(N-phenyl-N'-[(pyridin-2-yl)methylidene]carbamohydrazonothioato)(4-aminobenzene-1-sulfonamide)copper [Cu(Str)(L)Cl] was synthesized in research Laboratory of Advanced Materials in Biopharmaceutics and Technics of the Moldova State University by acad. A. Gulea et al. The copper(II) mixed-ligand complex was synthesized by reaction between thiosemicarbazone ligand 2-formilpyridine N(4)-phenylthiosemicarbazone (HL) with $CuCl_2 \cdot 2H_2O$ and 4-aminobenzenesulfonamide (Str) [15,16]. The tested compound [Cu(Str)(L)Cl] and the positive controls doxorubicin (7S,9S)-7-[(2R,4S,5S, 6S)-4-amino-5-hydroxy-6-methyloxan-2-yl]oxy-6,9,11-trihydroxy-9-(2-hydroxyacetyl)-4 methoxy-8,10-dihydro- 7H-tetracene-5,12-dione and cisplatin (*cis*-diamminedichloridoplatinum(II)) were dissolved in dimethylsulfoxide DMSO to create 10 mM stock solutions that were stored at 7°C. The stock solutions were further diluted with cell culture medium to appropriate concentrations before use. The maximum final concentration of DMSO was <0.1% did not

In vitro antiproliferative activity

Cell Culture

Human melanoma cells of line MeW-164 was derived from cell line collection established from melanoma metastases, surgically removed from patients in the Warsaw Cancer Center. MeW-164 cells were routinely grown in suspension in 90% Minimum Essential Medium Eagle (Biomed-Lublin) containing L-glutamine and supplemented with 10% fetal bovine serum FBS (Invitrogen) and 1% penicillin/streptomycin in 75 cm^2 cell culture flasks.

Human epithelial pancreatic adenocarcinoma cells of line BxPC-3 (ATCC) was cultured as monolayer in Roswell Park Memorial Institute medium RPMI 1640 (ATCC), supplemented with FBS (ATCC) (10% v/v) and antibiotics penicillin-streptomycin (final concentration 100 U/ml penicillin and 100 µg/ml streptomycin sulfate) (Sigma).

Human cervical epithelial cells of line HeLa (ATCC), human muscle rhabdomyosarcoma spindle and large multinucleated cells of line RD (ATCC) were cultured in the Dulbecco's Modified Essential Medium (ATCC) with L-glutamine (4 mM), glucose (4.5 g/L), bovine albumin fraction (0,2% v/v), HEPES buffer (N-2 hydroxyethylpiperazine-N'-2-ethane sulfonic acid) (20 mM), antibiotics penicillin-streptomycin (final concentration 100 U/ml penicillin and 100 µg/ml streptomycin sulfate) and FBS (10% v/v). Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere in the incubator in 25-cm^2 culture dishes, and used for experiments between passage 7 and 9.

Cell proliferation MTT assay

The MTT method is a colorimetric assay for viable cell quantification, also referred to as mitochondrial reduction assay. It was used for determine the possible cytotoxic effect of tested compounds on cancer cell line MeW-164. The essence of this assay is based on the metabolic reduction of 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT), which is carried out by the enzyme mitochondrial succinate dehydrogenase from metabolically active mitochondria cells. Suspension cells were harvested, using centrifugation method. Adherent cells were released from their substrate by trypsinization. The dilutions of cells were prepared in culture medium RPMI 1640 at concentration $5x10^3$ cells/100 µl and plated out into 96 wells of a microtiter plate, in triplicate. After 2 h incubating cells, 10 µl of the tested compounds were added

for 24 h at 37^{0}C, 5% CO_2. Following each treatment, 10 µl of MTT reagent was added to each well and incubated in the dark for 4 h, at room temperature. The supernatant was discarded, and DMSO was added to dissolve the formazan crystals. The optical absorbance was measured at 540 nm on microplate reader. The percent of inhibition was calculated according to the following formula: I (%) = 100-[(Abs 540nm $_{sample}$ /Abs 540nm $_{control}$) ×100].

Cell proliferation Resazurin assay

Resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one) is a non-fluorescent indicator dye, is converted to highly red fluorescent resorufin via reduction reactions of metabolically active cells in the presence of NADPH dehydrogenase or NADH dehydrogenase. The amount of fluorescence produced is proportional to the number of living cells. Resazurin was dissolved in physiological buffer and added directly to the cells.

Cells of lines HeLa, BxPC-3, RD were trypsinized from subconfluent cultures by adding 3 ml of 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. The trypsin reaction was stopped by adding 10 ml of appropriate culture medium containing 10% FBS. The cell suspension was centrifuged at 750 rpm 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 triplicate wells of a 96-well microtiter plat (90 µl/well) and incubated at 37°C, 2% CO_2. After an initial 4 h period to allow cell attachment, 10 µl of the tested compounds and positive controls were directly added to the medium resulting. The plate was further incubated for 24 h at 37°C, 2% CO_2. Following each treatment, 20 µl resazurin was added to each well and incubated at 37°C, 2% CO_2 for 4 h. Subsequently, the absorbance was read by hybrid reader (Synergy H1, BioTek) with 570 nm and 600 nm filters. The percent of inhibition was calculated according to the formula: I (%) =100-[(Abs570nm$_{sample}$–Abs600nm$_{sample}$) / (Abs570nm$_{control}$– Abs600nm$_{control}$)×100].

Statistical analysis

The cell proliferation assay results were reported as the percent inhibition of the test and control substances. As an indicator of efficiency of the experimental compounds on proliferation of cell lines was used the half maximal inhibitory concentration (IC_{50}). All data are presented as means ± SD. The correlation coefficient (R^2) was obtained using a least means squared linear regression analysis. Probabilities $P < 0,05$ were considered significant. Statistical analysis was performed by using statistical software.

Results and discussion

This work represents a series of comparative antiproliferative studies on several cancer cells of the mixed-ligand complex chloro(N-phenyl-N'-[(pyridin-2-yl)methylidene]carbamohydrazonothioato)(4-aminobenzene-1-sulfonamide)copper [Cu(Str)(L)Cl] (Fig.1). The tested compound was synthesized by the methods described in the literature [15].

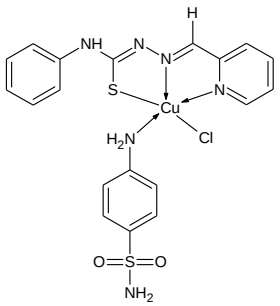

Fig.1. Structural formula of the mixed-ligand complex chloro(N-phenyl-N'-[(pyridin-2-yl)methylidene]carbamohydrazonothioato)(4-aminobenzene-1-sulfonamide)copper.

The morphological examinations of cancer cells were observed and photographed using the inverted microscope (LOMO). The morphological changes in MeW-164, HeLa, BxPC-3 and RD cells of lines after 24 h treatment with [Cu(Str)(L)Cl] at 10µM concentration are represented in Fig.2a-5a. The vacuolization of the cell membranes, small and round cells were observed. It is possible that, the tested compound significantly induced apoptosis in the cells.

Thus, the tested compound showed enhanced antiproliferative activity which is associated with increased induction of apoptosis, breaking structures of genomic DNA in the cell nucleus. Probably, the ability to induce high apoptotic effect of the mixed-ligand complex [Cu(Str)(L)Cl] at concentration 10 μM has resulted from its inner ligand environment properties, so the presence of an additional amino group 4-aminobenzene-sulfonamide in the internal sphere of the copper(II) mixed-ligand complex, characterized by the presence of a lone electron pair on the nitrogen atom, makes it easier to interact with the atoms of the grooves DNA molecules at the moment of replication or transcription.

The antiproliferative activity on MeW-164 cells of the mixed-ligand complex [Cu(Str)(L)Cl] was tested, using the MTT assay, which demonstrates mitochondrial activity of cells and is conventionally used as a measure of cell viability.

Evaluating antiproliferative effect on MeW-164 cells, increasing the mixed-ligand tested complex [Cu(Str)(L)Cl] and the positive control DOXO concentration to 10 μM resulted in a logarithmic increase in percentage inhibition with IC_{50} values of 1.0±0.2 μM and 1.0±0.1 μM, respectively. Curves were characterized by the correlation coefficients values (R^2) of 0.88, 0.99, for [Cu(Str)(L)Cl] and DOXO, respectively (Fig. 2b). Thus, both compounds showed high antiproliferative activity against line MeW-164.

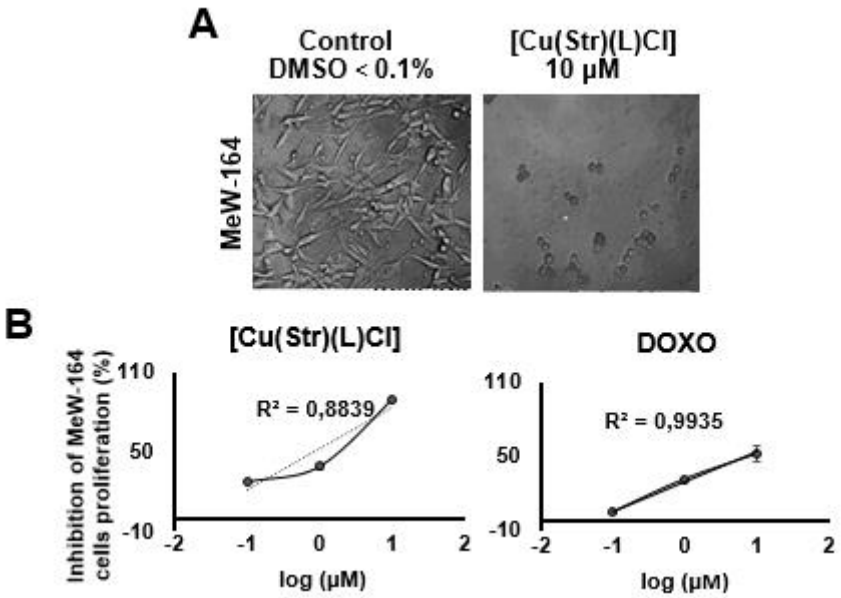

Fig. 2. Antiproliferative activity of the tested compound [Cu(Str)(L)Cl] and the positive control DOXO on human melanoma cells of line Mew-164. A: Phase-contrast images of MeW-164 cells after 24 h treatment with [Cu(Str)(L)Cl] at 10 μM. Untreated MeW-164 cells served as a control. B: Inhibitory effect of [Cu(Str)(L)Cl] and DOXO on the proliferation of line MeW-164. Mew-164 cells were treated with ([Cu(Str)(L)Cl] and DOXO at 0.1, 1 or 10 μM for 24h. Antiproliferative activity was tested by MTT-assay. All results are the means ± SD (n = 3).

The comparative study and concentration ranges identification of cytotoxic activity of the tested complex [Cu(Str)(L)Cl] and the positive controls DOXO, cisplatin in regard to human cancer cells of lines HeLa and BxPC-3 are shown in Fig.3b, 4b.

It was found, that [Cu(Str)(L)Cl] exhibited stronger inhibitory activity against HeLa cells, than DOXO and cisplatin, with IC_{50} values of 0.40±0.04μM for [Cu(Str)(L)Cl], 10.0±4.0 μM for DOXO and 4.0±0.3 μM for cisplatin, with the determination coefficients (R^2 = 0.9, 0.9 or 0.8), respectively.

The mixed-ligand complex [Cu(Str)(L)Cl] inhibits the formation and growth of pancreatic adenocarcinoma cells of line BxPC-3, that's demonstrate the capacity to inhibit the process of metastasis. It was founded, that the IC_{50} values of BxPC-3 cells are 1.7±0,2 μM for [Cu(Str)(L)Cl], 5,24±2.03 μM for DOXO and 11.2 ± 1.2 μM for cisplatin, with the high correlation coefficients (R^2 = 0.998, 0.99 or 0.78), respectively.

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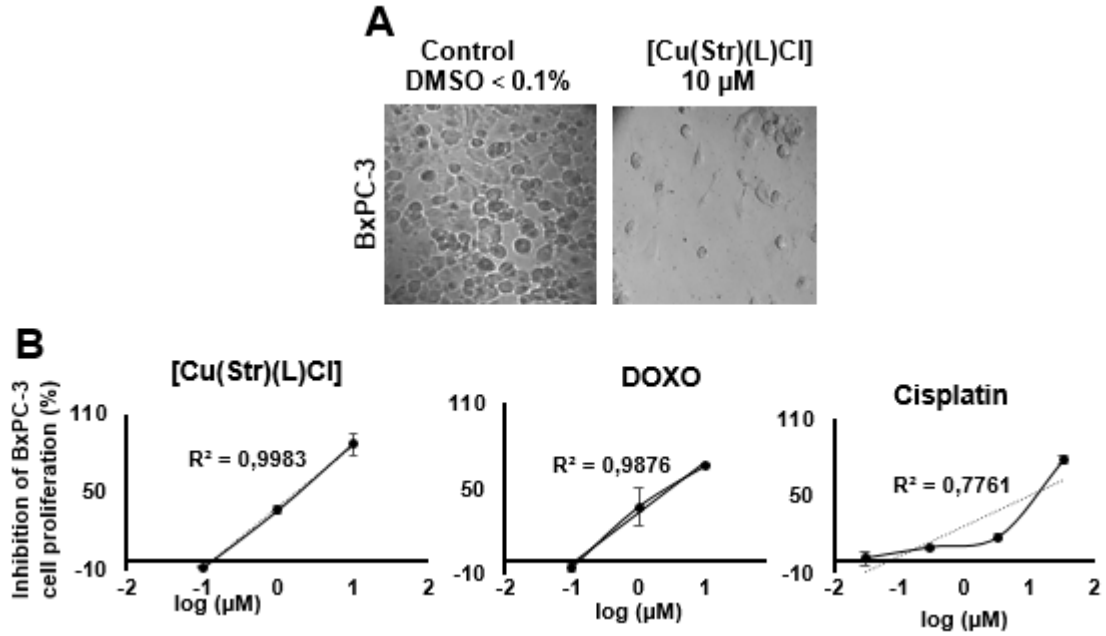

Fig.3. The antiproliferative activity of the tested compound [Cu(Str)(L)Cl] and the positive control DOXO on human epithelioid cervix carcinoma cells of line HeLa. A: Phase-contrast images of HeLa cells after 24 h treatment with [Cu(Str)(L)Cl] at 10 μM; Control - HeLa cells without treatment. B: Inhibitory effect of the tested compound [Cu(Str)(L)Cl] and the positive control DOXO, cisplatin on cells of line HeLa after 24 h exposure. Antiproliferative activity was estimated by resazurin method. All results are the means ± SD (n = 3).

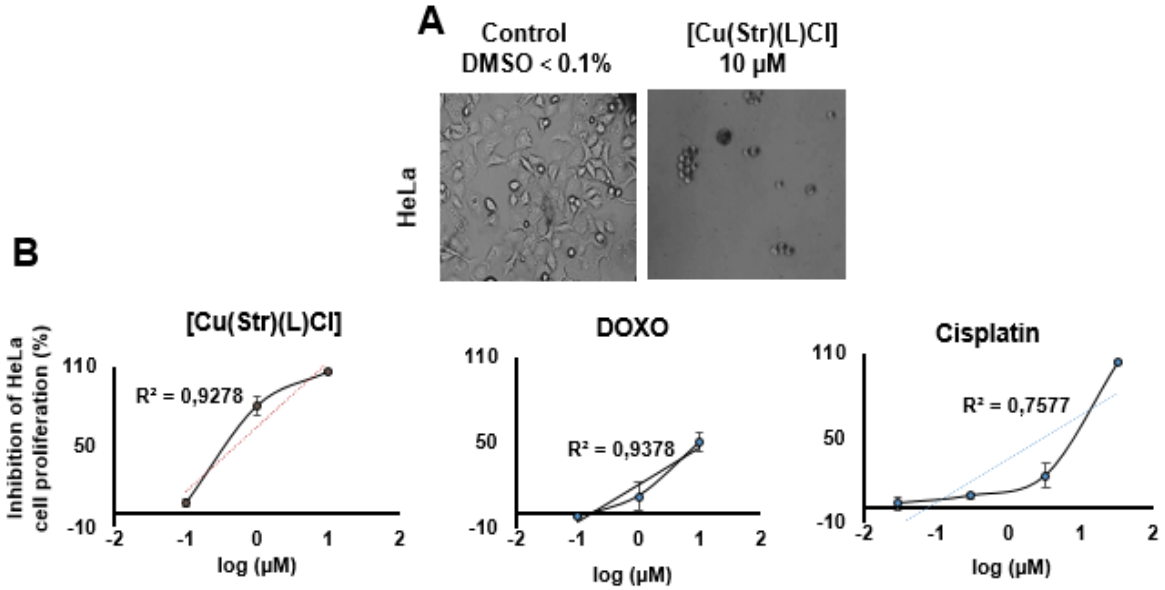

Fig.4. The antiproliferative activity of the tested compound [Cu(Str)(L)Cl] and the positive control DOXO on human epithelial pancreatic adenocarcinoma cells of line BxPC-3. A: Phase-contrast images of BxPC-3 cells after 24 h treatment with the tested compound [Cu(Str)(L)Cl] at 10 μM; Control - BxPC-3 cells without treatment. B: Inhibitory effect of the tested compound [Cu(Str)(L)Cl] and the positive controls DOXO, cicplatin on cells of line BxPC-3 after 24 h exposure by resazurin method. All results are the means ± SD (n = 3).

It was established that, the mixed-ligand complex [Cu(Str)(L)Cl] exhibits stronger inhibitory activity on human muscle rhabdomyosarcoma spindle and large multinucleated cells of line RD proliferation than DOXO. Thus, the IC_{50} values for RD are 1.3±0.3 μM for [Cu(Str)(L)Cl], and 2.3±0.9 μM for DOXO, with the high correlation coefficients (R^2 = 0.79 or 0.99), respectively (Fig.5b).

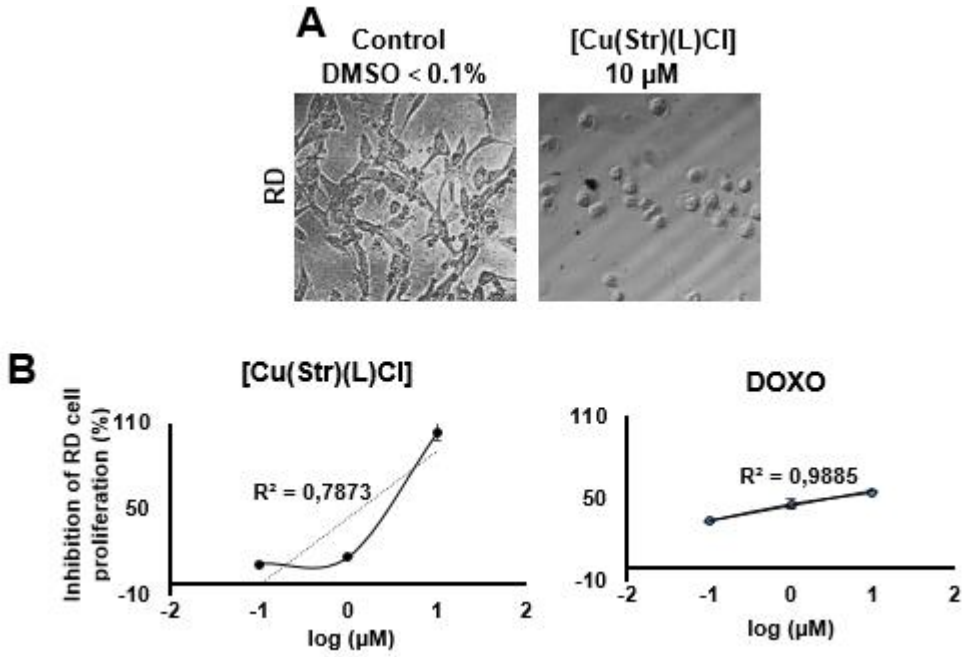

Fig.5. Antproliferative activity of the tested compound [Cu(Str)(L)Cl] and the positive control DOXO on human muscle rhabdomyosarcoma spindle and large multinucleated cells of line RD. A: The morphology of RD cells. Phase-contrast images of RD cells after 24 h treatment with [Cu(Str)(L)Cl] at 10 µM and control - RD cells without treatment. B: Inhibitory effect of the tested compound [Cu(Str)(L)Cl] and the positive control DOXO at 0.1, 1, 10 µM on RD cells after 24 h exposure by resazurin method. All results are the means ± SD (n = 3).

The antiproliferative activity experiments were displayed in a dose-dependent manner and showed concentration dependence between inhibitory effects of the mixed-ligand complex [Cu(Str)(L)Cl] at the micromolar range. The inhibitory rates of the mixed-ligand complex [Cu(Str)(L)Cl] on cancer cells were higher than cisplatin and DOXO as a positive control, which is used in the clinical management of a wide range of cancers. Table 1 describes statistically significant 95% confidence intervals (95% CI) of Log IC_{50} were obtained using unconditional logistic regression, which is an important aspect in determining therapeutic doses for preclinical and clinical studies.

Table 1

95% Confidence Intervals of LogIC_{50} of the mixed-ligand complex [Cu(Str)(L)Cl] for Mew164, HeLa, BxPC-3 and RD cells of lines

95% Confidence Intervals of LogIC_{50} (µM)	
Cells of line	[Cu(Str)(L)Cl]
Mew164	-6.403 to 6.448
HeLa	0.096 to 0.543
BxPC-3	-0.554 to 0.047
RD	-1.539 to 0.408

Conclusion

Analyzing the results of the antiproliferative activity it was observed that the the mixed-ligand complex (chloro(N-phenyl-N'-[(pyridin-2-yl)methylidene]carbamohydrazonothioato)(4-aminobenzene-1-sulfonamide) copper) exhibits stronger inhibitory activity on the tested cancer cells of line proliferation, than DOXO and cisplatin which are in the clinical management of a wide range of cancers. However, additional studies are needed to evaluate the mechanism of action of these compounds as well as the side effects in other bioassay systems and animal models.

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