



EXPERIMENTAL STRATEGY FOR IN VITRO EVALUATION OF CYTOTOXIC / ANTITUMOR PROPERTIES OF NEW METAL COMPLEXES WITH DIFFERENT LIGANDS

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ABSTRACT

PURPOSE. The aim of this study was to evaluate cytotoxic / antitumor properties of newly synthesized metal [Zn(II), Cu(II), Co(II), Ni(II), Zn(II)/Ag(I), Zn(II)/Au(I)] complexes with various ligands (Schiff bases, non-steroidal anti-inflammatory drugs, bile acids) and to introduce an optimized strategy for such investigations in our research activity.

MATERIALS AND METHODS. Human and animal tumor and non-tumor cells were used as model systems. Short-term (3 – 96h) and long-term (> 2 weeks) experiments were carried out using cytotoxicity assays, cytological / immunocytochemical, biochemical and molecular-biological methods to assess the influence of the compounds on cell viability and proliferation and their ability to induce apoptosis/necrosis and/or autophagy.

RESULTS. The examined metal complexes express cytotoxic activity that is time- and concentration-dependent and are more active than the corresponding ligands tested alone. Zn(II)/Au(I) and Zn(II)/Ag(I) complexes with Salen are found to be the most promising cytotoxic agents being effective also in multidrug resistant cancer cells. Their cytotoxic activity is higher than those of cisplatin, oxaliplatin and epirubicin.

CONCLUSIONS. A complex approach, based on a wide range of cell cultures and methods with different molecular / cellular targets and mechanisms of action was optimized and successfully applied for the assessment of cytotoxicity of new metal complexes.

Key words: Cell cultures, Metal compounds, Cancer, Cytotoxic / Anticancer activity, Antitumor agents, Apoptosis, Autophagy

INTRODUCTION

The need for effective new anticancer agents is one of the main challenges in modern biomedicine. The application of metal

compounds in cancer therapy started in the early 1960s with the discovery of antitumor properties of cisplatin. Today it is one of the most widely used antitumor drugs, being involved in the treatment of testicular, ovarian, cervical, bladder, head and neck cancers, melanoma and lymphomas (1). The successful story of cisplatin as well as of second (carboplatin) and third (oxaliplatin) generation of platinum preparations in clinical

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oncology has stimulated the search for other metal complexes with antitumor efficiency. Particularly attractive in this regard are the essential metals (such as zinc, copper, cobalt) that are involved in key physiological processes and are expected to be less toxic than platinum. On the other hand, many years of experience with chrysotherapy (treatment of rheumatoid arthritis with gold salt) have shown good biological tolerance for gold, and data on promising antitumor potential of gold compounds have been accumulated (2-4).

The aim of this study was to evaluate cytotoxic/antitumor properties of newly synthesized metal [Zn(II), Cu(II), Co(II), Ni(II), Zn(II)/Ag(I), Zn(II)/Au(I)] complexes with various ligands (Schiff bases, non-steroidal anti-inflammatory drugs, bile acids)

and to introduce an optimized strategy for such investigations in our routine research activity.

MATERIALS AND METHODS

Compounds

The cytotoxic / antitumor activity of newly synthesized metal [Zn(II), Cu(II), Co(II), Ni(II), Zn(II)/Ag(I), Zn(II)/Au(I)] complexes with various ligands (Schiff bases - Salen; non-steroidal anti-inflammatory drugs – Isoxicam, Meloxicam; bile acids - Ursodeoxycholic acid) was evaluated in our study (**Table 1**). The Schiff base Salen (H₂Salen) resulted from the condensation reaction between salicylaldehyde with ethylenediamine. Commercially available antitumor agents cisplatin, oxaliplatin and epirubicin were used as positive controls.

Table 1. Compounds investigated

Ligand	Metal	Structural formula	Abbrevia tion	Model system (Cell line)
Non-steroidal anti-inflammatory drugs				
Meloxicam	Cu(II)	[Cu(HMel) ₂ (H ₂ O) ₂]	Cu-Mel	HeLa, 8MGBA
	Zn(II)	[Zn(HMel) ₂ (H ₂ O) ₂]	Zn-Mel	
	Co(II)	[Co(HMel) ₂ (H ₂ O) ₂]	Co-Mel	
	Ni(II)	[Ni(HMel) ₂ (H ₂ O) ₂]·H ₂ O	Ni-Mel	
Isoxicam	Cu(II)	[Cu(HISOX) ₂ (H ₂ O) ₂]	Cu-Iso	LSCC-SF-Mc29- strain E7, LSR-SF-SR, MCF-7, HeLa, Lep-3
	Zn(II)	[Zn(HISOX) ₂ (H ₂ O) ₂]	Zn-Iso	
	Co(II)	[Co(HISOX) ₂ (H ₂ O) ₂]·3H ₂ O	Co-Iso	
	Ni(II)	[Ni(HISOX) ₂ (H ₂ O) ₂]	Ni-Iso	
Bile acids				
Ursodeoxycho- lic acid	Cu(II)	Cu(UDC) ₂ ·2H ₂ O	Cu-UDC	LSCC-SF-Mc29, LSR-SF-SR, MCF-7, HeLa, A549, HepG2, Lep3
	Zn(II)	Zn(UDC) ₂ ·3H ₂ O	Zn-UDC	
	Ni(II)	Ni(UDC) ₂ ·11H ₂ O	Ni-UDC	
Schiff bases				
Salen	Zn(II)/Au(I)	[Zn ₃ (Salen) ₂ {(μ-Au(CN) ₂) ₂ }]	ZnSalenAu	HeLa, A549, 8MGBA, A431, A431-MDR, A431-MRP, A431-ABCG2
	Zn(II)/Ag(I)	[Zn ₃ (Salen) ₂ {(μ-Ag(CN) ₂) ₂ }]	ZnSalenAg	

Model systems and culturing

The cell lines applied as model systems in our study are presented in **Table 2**. The cells were routinely grown as monolayer (2D) cultures in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 5-10% fetal bovine serum and antibiotics (100 U/ml penicillin and

100 µg/ml streptomycin). The cultures were maintained at 37°C in a humidified CO₂ incubator (Thermo Scientific, HEPA Class 100). For routine passages the cells were detached using a mixture of 0.05% trypsin – 0.02% ethylenediaminetetraacetic acid.

Table 2. Experimental cell model systems

Origin	Cell line	Established from
Human	HeLa	Carcinoma of the uterine cervix
	A549	Non-small cell lung cancer
	MCF-7	Luminal type A breast cancer
	MDA-MB-231	Triple negative breast cancer
	HepG2	Hepatocellular carcinoma
	8MGBA	Glioblastoma multiforme
	SAOS-2	Osteosarcoma
	A431	Squamous cell carcinoma
	A431-MDR	Multidrug resistant clone of A431, expressing <i>mdr1</i> gene
	A431-MRP	Multidrug resistant clone of A431, expressing <i>mrp1</i> gene
	A431-ABCG2	Multidrug resistant clone of A431, expressing <i>abcg2</i> gene
	Lep-3	Non-tumor lung fibroblastoid cells
Rat	LSR-SF-SR	Transplantable sarcoma induced by <i>Rous sarcoma virus</i> , strain <i>Schmidt-Ruppin</i> . The cells express <i>v-src</i> oncogene
Avian	LSCC-SF-Mc29	Transplantable chicken hepatoma, induced by the myelocytomatosis virus Mc29. The cells express <i>v-myc</i> oncogene

Cytotoxicity assays

Thiazolyl blue tetrazolium bromide (MTT) test (5), neutral red uptake cytotoxicity assay (NR assay) (6) and crystal violet staining (CV staining) (7) were performed as it was earlier described (8).

Identification of cytopathological changes and cell death

Haematoxylin and eosin staining (H&E) and / or double staining with acridine orange and propidium iodide (AO/PI) were carried out to visualize cytopathological changes and to identify the type of cell death (apoptosis / necrosis) (9). Annexin V/ FITC (ImmunoCRUZ Apoptosis/Necrosis detection kit, USA) or Annexin V/DAB (Rabbit polyclonal anti-Annexin V, abcam - ab140068; (Novolink TM Polymer Detection Systems, Catalog No. RE7200-CE, Leica Bio Systems, UK) were performed also to detect the presence and rate of early and late apoptosis and necrosis.

Expression of Ki67

The effect of the compounds on the expression of Ki67 marker for proliferative activity was immunocytochemically examined by administration of a monoclonal antibody (anti-human Ki-67, clone MM1; Catalog No. PA0118, Leica Bio Systems, UK). The

reaction was visualized according to the manufacturer's recommendations using a polymer-based system (Novolink TM Polymer Detection Systems, Catalog No. RE7200-CE, Leica Bio Systems, UK) with chromogen 3,3'-diaminobenzidine (DAB).

Single cell gel electrophoresis (Comet assay)

The ability of the compounds to induce single-stranded and/or double stranded DNA damages (genotoxic effect) was evaluated by alkaline variant and/or neutral variant of Comet assay, respectively (10, 11).

Detection of Autophagy

Autophagy was detected using confocal microscopy (Autophagy detection kit - Enzo Life Sciences, Inc., USA, confocal microscope Leica TCS SPE), electron microscopy (electron microscope JEM 1200 EX) and immunocytochemistry (Novolink TM Polymer Detection Systems, Catalog No. RE7200-CE, Leica Bio Systems, UK) and rabbit polyclonal antibody against LC3B protein (abcam 48394).

3D cell colony forming method

The influence of the compounds on the ability of cancer cells to grow as 3D colonies in semisolid medium was examined as it was earlier described (8, 9). Groups of at least 8-10 dividing cells were considered as colonies.

Statistical analysis

Significant differences between experimental groups and control groups were evaluated by ANOVA followed by Dunnett's post-hoc analysis. *GraphPad Prizm*, *GraphPad Software Inc.*, USA, 2000 and *Origin 6.1TM* programs were used.

RESULTS AND DISCUSSION

It has been suggested that a compound can be considered as toxic to cultured cells when one or more of the following conditions are met: prevents cell adhesion; causes morphological changes; inhibits proliferation; induces cell death (12).

Short-term experiments

Cytotoxicity assays

The assessment of cytotoxicity of the compounds examined started with MTT test - the gold standard for cytotoxicity assays. The first step was to fix the concentration range of the compounds starting with non-toxic concentrations (cell viability ≥ 80 -90% as compared to the control) and reaching concentrations where cell viability is ≤ 5 -10% as compared to the control.

MTT test, NR assay and CV staining possess different molecular/cellular targets – succinate dehydrogenase / mitochondria (MTT test), lysosomes (NR assay), proteins and DNA (CV staining), and mechanisms of action and provide complementary information on the cytotoxic activity of the compounds tested.

Experimental data obtained were used to prepare “concentration – response” curves for each compound (metal complex, ligand, anticancer agent) and cell line. An example of such a curve is presented in **Figure 1**. From these curves, concentrations were determined which decreased by 50% and by 90% the amount of surviving cells in the treated samples as compared to the control (where the cell viability is 100%) - the so-called cytotoxic concentration 50 (CC_{50}) and cytotoxic concentration 90 (CC_{90}), respectively. Performed at equal conditions cytotoxicity methods (MTT test, NR assay, CV staining) usually reveal similar cytotoxicity profile of the compound investigated. Differences / Fluctuations can be observed at concentrations / treatment intervals at which the toxic effect of the substance is less pronounced, in multinucleated cells (in this case CV staining may show increased cell viability) as well as due to technical problems (insufficient cell washing / dye removing), etc. Other possible source of errors is cell overgrowth - that is why cytotoxicity assays has to be started with monolayer density around 50-60%. The chemical / physicochemical properties of the compound tested must be also taken into account in order to avoid an incorrect measurement of extinction. Therefore, it is very important to choose the appropriate (“right”) method for each compound.

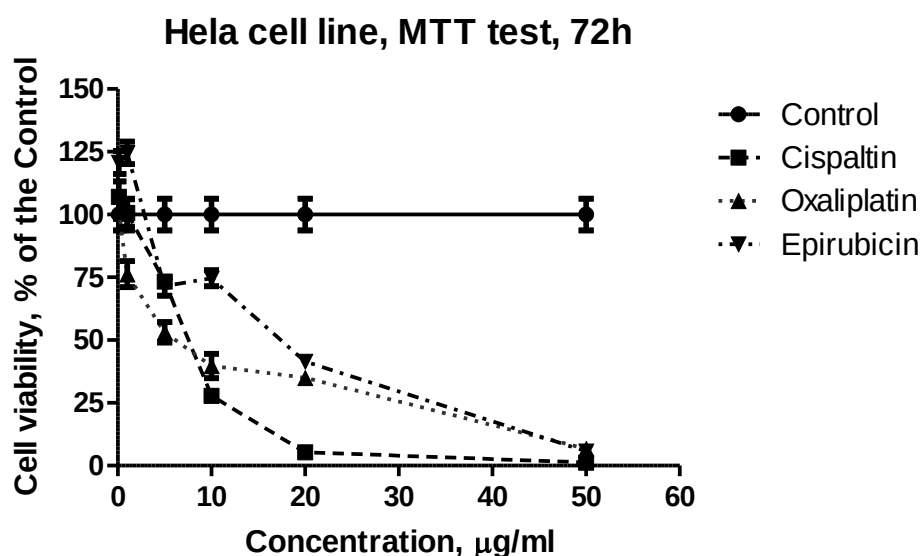


Figure 1. Effect of antitumor agents cisplatin, oxaliplatin and epirubicin on viability and proliferation of human HeLa cervical carcinoma cells. MTT test was performed after 72 h of treatment. CC_{50} were calculated by Origin 6.1TM - 8.46 µg/ml (28.20 µM) for cisplatin; 6.12 µg/ml (15.40 µM) for oxaliplatin and 17.38 µg/ml (31.98 µM) for epirubicin.

Regarding treatment periods, the cells were usually cultured for 24h, 48h and 72 h in the presence of the compounds tested. Shorter intervals (for example between 3 and 18 h) were also performed because of at least two purposes: i) to understand how long it takes for the cytotoxic effect to appear; ii) to compare the results obtained by different methods (for example MTT test and apoptosis/necrosis and/or autophagy detection). Something more, culturing cells in the presence of the compounds examined (for different periods of time) and then in non-modified culture medium (without compound) provide information on the possible reversibility of the cytotoxic effect. No such reversibility was observed in the studies we conducted.

The results obtained by us revealed that:

- The compounds investigated decrease viability and / or proliferation of the treated cells in a time- and a concentration-dependent manner. According to their cytotoxic effect (CC_{50} , μM) the compounds are divided into three groups: compounds with high, medium and low cytotoxicity. In **Table 3** are summarized data about cytotoxic activity of the examined compounds – newly synthesized metal complexes, ligands and antitumor agents, in HeLa cells – one of the most widely used model systems in experimental biomedical research.
- Cell-specific response was observed – cell lines exhibit different rate of sensitivity to the toxic effect of the compounds. This observation is not surprising and can be explained by at least two reasons: i) the cell lines were established from different histological types of cancer; ii) due to the tumor heterogeneity phenomenon each cell line is a unique biological system. According to their sensitivity to the

cytotoxic effect of the compounds tested, the cell lines were graded in hierarchical orders (Examples are presented in **Table 4**). LSCC-SF-Mc29 chicken hepatoma cells have been found to express high sensitivity to the cytotoxic activity of compounds with different chemical composition / structure and chemical / physicochemical properties. The increased sensitivity can be due to the expression of the viral *gag-myc* oncogene contained in these cells - it is known that the products of the *myc* genes are involved in a number of biological functions (often contradictory depending on the specific conditions) including increased sensitivity to apoptosis (13). It is worth noting that LSCC-SF-Mc29 are the only avian cells included in the experiments. In general, non-tumor human Lep-3 fibroblastoid cells also exhibit sensitivity to the cytotoxic effect of the compounds examined. Non-tumor cell lines (like Lep-3) usually are derived from embryonic tissues and organs. Embryonic cells are known to have some common characteristics with tumor cells, including high proliferative potential and expression of certain antigens. In addition, permanent cell lines have undergone a large number of passages in laboratory conditions, which result in various genetic / epigenetic and phenotypically expressed differences in comparison with the starting material from which they were obtained. This necessitates the development of more appropriate model systems for evaluating the cytotoxic effect of substances in healthy cells (14).

- Metal complex have been found to be more cytotoxic as compared to the corresponding ligands (Isoxicam, Meloxicam, Ursodeoxycholic acid, Salen) tested alone.

Table 3. Brief information about cytotoxic activity of the compounds investigated

Rate of cytotoxicity	Compounds	CC_{50} , μM	CIC, μM
High	ZnSalenAu, ZnSalenAg	0.1 – 5.0	≥ 0.5
Medium	ZnUDCA	100 – 200 (165.8)	-
Low	ZnMel, CuMel, CoMel ZnIso, CuIso, CoIso, Mel	300.0 – 650.0	Cu Mel ≥ 625 Mel ≥ 1424.5

CC_{50} (μM) were determined in HeLa human cervical carcinoma cells after 72 h of treatment by MTT test; CIC = Concentration (μM) that completely inhibited the formation of 3D cell colonies in semi-solid medium; In some cases CC_{50} (NiUDCA, CuUDCA, NiMel, NiIso, Iso, Salen, UDCA) was not calculated because at all concentrations examined cell viability was $> 50\%$ as compared to the control.

Table 4. Hierarchical orders of human cell lines, used as experimental models according their sensitivity to the cytotoxic effect of Zn(II)/Au(I) and Zn(II)/Ag(I)

Compound	Hierarchical order
ZnSalenAu	Lep-3 (0.08)* > HeLa (0.28) > LSCC-SF-Mc29 (0.34) > 8MGBA (0.65) > A431 (0.98) > A431-ABCG2 (2.16) > A431-MDR (2.46) > A549 (2.54) > A431-MRP (2.67)
ZnSalenAg	LSCC-SF-Mc29 (0.76) > HeLa (1.76) > A431-MDR (2.06) > A431-MRP (2.28) > A431 (3.10) > 8MGBA (3.19) > Lep3 (3.82) = A431-ABCG2 (3.82) > A549 (7.32)

The cell lines are graded based on CC₅₀ (μM) of metal compounds determined by MTT test after 72 h of treatment.

* CC₅₀ value is shown in brackets next to the name of cell line.

Expression of Ki67 marker of proliferation

The decreased amount of living cell estimated by MTT test or NR could be the result of cell death and/or suppressed cell proliferation. Distinguishing between these two processes when conducting such cytotoxicity methods is a difficult task. In this regard, immunocytochemical demonstration of Ki-67 expression provides valuable information on cell proliferative activity.

Cytopathological changes / Cell death and Genotoxicity

H&E staining and AO/PI staining visualize cytopathological changes (if any) induced by the compounds and their ability to stimulate apoptosis and/or necrosis. The concentration of the compound/ treatment period should be selected to ensure the presence of “enough” cells on the slide. It is a good idea to start this type of investigations with the well-known classical H&E staining technique because it is a basal method which provides information regarding morphology of cytoplasmic and nuclear organelles, relatively quick, low-cost, and not demanding any special equipment. The samples are long-lasting and can be evaluated many times microscopically. Methods based on the identification of Annexin V can not only distinguish between apoptosis and necrosis but also demonstrate early apoptosis. The best option here is a combination of flow cytometric analysis (allowing for quantification of cells in early and late apoptosis and necrosis) and immunocytochemistry (morphological changes can be observed). Genotoxicity is associated with a large number of commonly used anticancer agents in clinical oncology, including cisplatin, doxorubicin, cyclophosphamide and bleomycin. This fact is not surprising at all, having in mind their mechanism of action, but draws the attention of professionals to developing strategies for

their co-administration with antimutagenic factors (15).

Autophagy detection

The controversial role of autophagy in carcinogenesis is known – tumor preventing in the beginning and tumor-stimulating in the course of tumor progression. Autophagy is stimulated in the presence of stress factors and is a mechanism for cell survival. There are data indicating the connection between autophagy and apoptosis. Intensive research work is currently underway to develop cancer treatment strategies based on autophagy suppression. This provokes our interest in evaluating the ability of the compounds tested to induce autophagy. Although in the initial stages, the results obtained show some interesting details. Just one example - Co(II) and Ni(II) complexes of Meloxicam have been found to induce autophagy (when administered for 48 h at a concentration of 50 and 100 μg/ml) in MDA-MB-231 triple negative breast cancer cells. The viability of the treated MDA-MB-231 cells (examined by MTT test) remains 56% (CoMel) and 73% (NiMel) even when both compounds were applied at the highest concentration examined (500 μg/ml). At the same time ZnMel shows the highest cytotoxicity among the investigated complexes of Meloxicam, but does not induce autophagy at the conditions explored.

Long-term experiments

3D colony forming ability

The “rapid” cytotoxic effect of the compounds examined (bile acids, NSAIDs, their metal complexes, antitumor drugs) have been examined also in long-term experiments with 3D cancer cell colonies. Brief comparative information about short-term and long-term experiments performed by us is summarized in Table 5. The advantages and disadvantages of monolayer (2D) and 3D cell cultures as model systems in cancer research and experimental

oncopharmacology have been previously discussed (14).

Although in many cases decreased number of 3D cell colonies was observed, we looked for compounds and concentrations at which complete suppression of 3D cellular growth was observed (no dividing cells, not even groups of 2-3 cells). We decided to do it

because the presence of even single colonies of a small number of cells may be a result of the ability of individual cells to "escape" from the cytotoxic effect of the compounds tested. In clinical practice such persistent and / or dormant cancer cells are known to be responsible for relapses and metastases, as well as the subsequent death of patients.

Table 5. Brief description of short-term and long-term experiments

Parameters	Short-term experiments	Long-term experiments
Duration	3-96 h (usually 24-72 h)	> 2 weeks
Cell cultures	Monolayer, two-dimensional (2D)	Three dimensional (3D) cancer cell colonies
Methods and techniques	MTT test, NR assay, CV staining, H&E, AO/PI, AnnexinV/FITC, AnnexinV/DAB, Immunocytochemistry for Ki-67, Autophagy detection	3D colony forming method
Information provided	Quick cytotoxic effect of the compounds evaluated	Long-lasting cytotoxic effect
Advantage	Allows the application of a variety of methods and techniques, including the quantification of the number of cells of interest	Provide more "realistic" data about cytotoxic / antitumor activity of the compound
Challenges	The information about putative antitumor properties of the compounds investigated can be more "Optimistic"	Counting colonies is not easy and requires experience.

CONCLUSIONS

Zn(II)/Au(I) and Zn(II)/Ag(I) complexes with Salen have been found to be the most pronounced cytotoxic agents among the examined compounds. Applied at concentrations of 0.5 - 10 µg/ml (ZnSalenAu) and 2.5 - 10 µg/ml (ZnSalenAg), depending on the cell line, both compounds induce apoptosis and significantly decrease viability of the treated cells as well as completely inhibit their 3D growth. ZnSalenAg and ZnSalenAu are effective also in multidrug resistant human carcinoma cells. Their CC₅₀ are lower than those of the commercially available antitumor drugs cisplatin, oxaliplatin and epirubicin.

A complex approach for the assessment of metal complexes with various ligands based on methods with different molecular / cellular targets and mechanism(s) of action and a wide range of cell model systems have been optimized and successfully introduced in our routine research activity. This approach allows a more complete picture of the cytotoxic / antitumor properties of metal complexes and

other synthetic compounds and natural products and is useful in identifying novel agents with promising antitumor activity and biological safety.

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