ZN(II)/AU(I) AND ZN(II)/AG(I) COMPLEXES WITH SALEN SCHIFF BASE EXPRESS PROMISING CYTOTOXIC ACTIVITY IN HUMAN CANCER CELLS

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ABSTRACT

Objective: The aim of our study was to evaluate the influence of two complexes of Zn(II)/Au(I) and Zn(II)/Ag(I) with Schiff base ligand (H_2Salen) obtained from the condensation reaction between salicylaldehyde and ethylenediamine (abbreviated ZnSalenAu, ZnSalenAg) on viability and proliferation of cultured human cancer cells.

Methods: The following cell lines were used as model systems: Human cervical carcinoma (cervical carcinoma), A549 (non-small cell lung cancer [NSCLC]), glioblastoma multiforme (8MGBA), and A431 (squamous cell carcinoma) and its multidrug-resistant (MDR) clones A431-MDR, A431-MRP, and A431-ABCG2 that express *mdr1*, *mrp1*, or *abcg2* gene, respectively. The investigations were performed by thiazolyl blue tetrazolium bromide test, neutral red uptake cytotoxicity assay, crystal violet staining, hematoxylin and eosin staining, double staining with acridine orange, and propidium iodide in short-term experiments (12–72 h, with monolayer cell cultures) as well as colony-forming method in long-term experiments (25 days, with three dimensional cancer cell colonies).

Results: The results obtained revealed that ZnSalenAu and ZnSalenAg decreased significantly viability and proliferation of the treated cells in a time- and concentration-dependent manner being more active as compared to the free ligand H_2Salen.

Conclusion: The present study demonstrates for the 1st time the ability of two heterometallic complexes ZnSalenAu and ZnSalenAg to decrease significantly viability and proliferation of cultured cell lines established from some of the most common and aggressive human cancers (NSCLC, carcinoma of uterine cancer, 8MGBA, and squamous cell carcinoma) as well as MDR cancer cells.

Keywords: Cytotoxic activity, Human cancer cell lines, H_2Salen, Schiff bases, Zn(II)/Au(I), Zn(II)/Ag(I) complexes.

INTRODUCTION

Despite undoubted success in the early diagnosis, prevention, and treatment of cancer (at least some kinds), neoplastic diseases continue to be among the leading causes of morbidity and mortality worldwide with approximately 14.1 million new cases and 8.2 death cases in 2012 [1,2]. Chemotherapy is an important therapeutic strategy for cancer treatment, but its success is limited due to various obstacles including tumor heterogeneity, intrinsic or acquired (multi) drug resistance (MDR) of cancer cells, and severe side/toxic reactions. Hence, there is a constant need to develop effective new antitumor agents [3,4].

The discovery of the antitumor properties of cisplatin, and later of carboplatin and oxaliplatin, encouraged efforts of scientists to search for other metals and metal compounds with pronounced antineoplastic activity and opened a new page in the development of medicinal chemistry and experimental oncopharmacology [5,6].

Gold and silver have been used for medicinal purposes for thousands of years. The antimicrobial activity of silver has been known since ancient times, but there are also data about the anticancer activity of silver compounds [7-11].

Even in the 19th century gold was still considered as "panacea." The modern age of gold in medicine began in the early 20th century when Robert Koch discovered the antituberculosis effect of K[Au(CN)$_2$] and

Jacques Forestier started to use thiolate gold salts for the treatment of rheumatoid arthritis [5,12]. Recent findings suggest that various Au(I) and Au(III) complexes can be potentially useful in cancer chemotherapy [13,14].

Zinc is an essential element required for proper activity of >300 enzymes, 3000 proteins, and over 1000 transcription factors. This metal plays an important role in key biological processes including DNA replication and transcription, gene expression and genome integrity, normal functioning of the immune, and central nervous system [15-18]. Zinc deficiency has been associated to various pathological conditions such as inflammation, infections, and cancer [17,19,20]. Many research groups demonstrate encouraging the antineoplastic potential of zinc compounds [21-23]. As an essential element, zinc is expected to be less toxic than platinum, for example, gold and silver compounds also show relatively low toxicity [24,25].

Schiff bases and their derivatives/metal complexes are also reported to express a wide range of biological activities, including antidiabetic and antitumor properties [26-30].

The aim of our study was to evaluate the influence of two heterometallic complexes ZnSalenAu, ZnSalenAg containing a Schiff base ligand (H_2Salen) obtained from the condensation reaction between salicylaldehyde and ethylenediamine, on viability and proliferation of

cultured human cancer cells. The X-ray structure, chemical and spectral characterization of the first complex ($_1^\infty$[{Zn$_3$(Salen)$_2$}{m-Au(CN)$_2$}$_2$] denoted ZnSalenAu) was reported in one of our previous paper [31]. For this reason, in this work, we report only the synthesis and physicochemical characterization of ZnSalenAg.

MATERIALS AND METHODS

Materials
Dulbecco's modified Eagle's medium (D-MEM) and fetal bovine serum (FBS) were purchased from Gibco-Invitrogen (UK). Dimethyl sulfoxide (DMSO), neutral red (NR), crystal violet (CV), and trypsin were obtained from AppliChem (Germany); purified agar (Difco) and thiazolyl blue tetrazolium bromide (MTT) were from Sigma-Aldrich Chemie GmbH (Germany). All sterile plasticware was from Orange Scientific (Belgium).

All starting materials for the synthesis of the complexes (salicylaldehyde, ethylenediamine, Zn(NO$_3$)$_2$•4H$_2$O, K[Au(CN)$_2$], K[Ag(CN)$_2$]) were reagent grade and used without purification. The trinuclear Zn(II) precursors were synthesized by reacting the Schiff base [H$_2$Salen - Scheme 1] obtained *in situ* by condensation of salicylaldehyde with ethylenediamine, with zinc(II) nitrate.
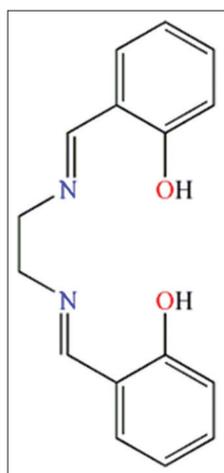
Synthesis
ZnSalenAg has been obtained following the same general synthetic procedure previously described for ($_1^\infty$[{Zn$_3$(Salen)$_2$} {m-Au(CN)$_2$}$_2$] (ZnSalenAu) [31], using K[Ag(CN)$_2$] instead of K[Au(CN)$_2$]. ZnSalenAg was obtained as a yellow precipitate (yield ca. 70%). Single crystals suitable for X-ray diffraction could not be obtained. Anal. Calcd for C$_{36}$H$_{28}$Ag$_2$N$_8$O$_4$Zn$_3$: 41.20% C, 2.67% H, 10.68% N. Found: 41.98% C, 3.20% H, 9.89% N. infrared (IR) bands (KBr, cm^{-1}): 3043 w, 3010 w, 2898w, 2185vs (vCN), 1641vs, 1599m, 1541s, 1474s, 1447s, 1392m, 1339s, 1303s, 1192w, 1152m, 1128s, 1064m, 1051w, 1033m, 997w, 946m, 903w, 852m, 751s, 738m, 647w.

Physical measurements
IR spectra were recorded as KBr pellets on a JASCO Fourier-transform IR 4100 spectrophotometer from 4000 to 400 cm^{-t}. Elemental analyzes of carbon, hydrogen, and nitrogen were carried out on a EuroEA elemental analyzer. Absorption spectra were recorded with a JASCO V-670 spectrophotometer. The photoluminescence measurements were carried out at room temperature using a JASCO FP 6500 spectrofluorometer.

Cell cultures and cultivation
The following human permanent cell lines were used as model systems in our study: Human cervical carcinoma (HeLa) (carcinoma of the uterine cervix), A549 (non-small cell lung cancer [NSCLC]), glioblastoma multiforme (8MGBA), and A431 (squamous cell carcinoma) and its MDR clones A431-MDR, A431-MRP, and A431-ABCG2 that express *mdr1*, *mrp1,* or *abcg2* gene, respectively. The freezing and thawing of the cells were performed according to the conventional methods [32,33]. The cells were grown as monolayer cultures in a D-MEM medium, supplemented with 5–10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. The cultures were maintained at 37°C in a humidified CO$_2$ incubator (Thermo Scientific, HEPA Class 100). For routine passages, the cells were detached using a mixture of 0.05% trypsin - 0.02% ethylenediaminetetraacetic acid. The experiments were performed during the exponential phase of cell growth.

Cytotoxicity assays
The cells were seeded in 96-well flat-bottomed microplates at a concentration of 1×104 cells/well. After the cells were grown for 24 h to a subconfluent state (~60–70%), the culture medium was removed and changed by media modified with different concentrations (0.1, 0.5, 1, 5, and 10 µg/ml) of the compounds tested. Each concentration was applied into 4–8 wells. Samples of cells grown in non-modified medium served as controls. After 12, 18, 24, 48, and 72 h of incubation, the effect of the compounds on cell viability and proliferation was examined by MTT test, NR uptake cytotoxicity assay, and CV staining.

The MTT colorimetric assay of cell survival was performed as described by Mosmann [34]. The method consisted of 3 h incubation with MTT solution (5 mg MTT in 10 mL D-MEM) at 37°C under 5% carbon dioxide and 95% air, followed by extraction with a mixture of absolute ethanol and DMSO (1:1, vol/vol) to dissolve the blue formazan.

The NR assay was based on the method of Borenfreund and Puerner [35]. To each well-containing corresponding cells a medium consisting of NR (50 µg/mL, 0.1 mL) was added. The plate was placed in the CO$_2$ incubator for 3 h for the uptake of vital dye. Thereafter, the medium with NR was removed, and the cells were washed with phosphate saline buffer (PBS, 0.2 mL/well), followed by the addition of 0.1 mL 1% acetic acid solution containing 50% ethanol to extract the dye from the cells.

The CV assay was based on the method of Saotome *et al.* [36]. After each well was washed with PBS, the cells were fixed and stained with 0.4% CV solution in methanol for 30 min. Optical density was measured at 540 nm using an automatic microplate reader (TECAN, Sunrise™, Austria). Relative cell viability, expressed as a percentage of the untreated control (100% viability), was calculated for each concentration. Concentration-response curves were prepared and the effective cytotoxic concentration (CC) of the compounds CC$_{50}$ (µM) and CC$_{90}$ (µM) causing, respectively, 50% and 90% reduction of cell viability was estimated from these curves. All data points represent an average of three independent assays.

Hematoxylin and eosin (HE) staining
The cells were grown on coverslips (3.0–3.5×10^5 cells/well) in 6-well plates in the presence of the compounds tested. Non-treated cells served as controls. After 24, 48, and 72 h of incubation, the coverslips were removed, washed with PBS, fixed in methanol (2×15 min) and stained with HE. Dehydration was performed in increasing concentrations of alcohols (70%, 80%, 96%, and 100% ethyl alcohol), followed by xylene treatment (2×15 min) and mounting in cedar oil. The microscopic preparations were examined under the light microscope (Leika DM 500B, Wetzlar, Germany).

Double staining with acridine orange (AO) and propidium iodide (PI)
The ability of compounds to induce cytopathological changes was assessed using double staining with AO and PI according to the standard procedures [37]. The cells were grown on coverslips and treated as it was described in the previous paragraph. After 24, 48, and 72 h of incubation, the coverslips were removed and washed with PBS for 2 min. Equal volumes of fluorescent dyes containing AO (10 µg/mL in PBS) and PI (10 µg/mL in bidistilled water) were added to the cells.

Scheme 1: H$_2$Salen structure

Fresh stained cells were placed on a glass slide and examined under a fluorescence microscope (Leika DM 500B, Wetzlar, Germany) within 30 min before the fluorescent color started to fade.

Colony forming method

Tumor cells (10^3 cells/well) suspended in 0.45% purified agar in D-MEM medium containing different concentrations of the compounds examined (ranging from 0.5 to 25 µg/mL) were layered in 24 well microplates. The presence/absence of colonies was registered using an inverted microscope (Carl Zeiss, Germany) during the period of 25 days. Colony inhibitory concentration (CIC, µM) at which the compounds tested inhibit completely the ability of tumor cells to grow in a semi-solid medium was determined.

Statistical analysis

The data are presented as mean±standard error of the mean. Statistical differences between control and treated groups were assessed using one-way analysis of variance followed by Dunnett *post hoc* test and Origin 6.1™.

RESULTS AND DISCUSSION

Physical and chemical properties

Due to the impossibility of obtaining monocrystals suitable for X-ray measurements, we tried to establish the molecular formula of ZnSalenAg by correlating the chemical analysis with spectral data.

The IR spectrum of ZnSalenAg (Fig. 1) contains some main bands which certify the formation of the complex: 3010 cm^{-h} (v_{asim}C-H), 2898 cm^{-H} (v_{sim}C-H), 2185 cm^{-H} (vCN bridge), 1641 cm^{-b} (vC=N azomethine), 1541, 1474, 1447 cm^{-c} (vC=C).

The other small bands are characteristic to the Schiff base ligand. Comparing the spectrum of ZnSalenAg with that of the free ligand, some slight displacements of the bands were observed, confirming its involvement in complexation. Another important information regarding the structure of the complex is given by the absence of the bands corresponding to nitrate anion that confirm its replacement by [Ag(CN)$_2$]$^-$ units.

The similarity between the data obtained for ZnSalenAg and those of ZnSalenAu [31] encouraged us to propose a similar molecular formula for ZnSalenAg: $_1^\infty$[{Zn$_3$(Salen)$_2$} {µ-Ag(CN)$_2$}$_2$].

The electronic spectrum of ZnSalenAg shows bands located in the 200–500 nm region (Fig. 2). These bands are must probably due to a superposition of π–π* transitions of the organic ligand that appears at higher energies and strong MLCT (d–π*) transitions characteristic for dicyanoargentate polymers.

It is known that silver(I) and gold(I) complexes have attracted attention in the field of the photophysics of metal complexes. The structures constructed through metallophilic interactions are known to be potentially luminescent materials [38]. For these reasons, the luminescent properties of Schiff-base ligand and ZnSalenAg (in solid state), at room temperature, have been investigated (Fig. 3). For H$_2$Salen a green emission was observed with the maximum at 504 nm by excitation with 370 nm.

The emission spectrum of ZnSalenAg presented in Fig. 3 exhibits luminescence with a peak maximum at λ_{em}=447 nm, slightly shifted compared with that observed for ZnSalenAu (464 nm) [31]. Compared with the free ligand, emission of ZnSalenAg is slightly shifted to lower wavenumber as a result of coordination of zinc(II) centers to the ligand, and the luminescence is most probably due to intraligand ^{1}(π*–π) fluorescence.

Cytotoxic activity

Short-term experiments

The investigations were performed in two steps: (1) The influence of the compounds investigated on viability and proliferation of cancer (HeLa, A549, 8MGBA, A431, and its clones) cells used as model systems in our study was evaluated initially by MTT test - the gold standard for cytotoxicity assays. The "concentration - response" curves were prepared (examples of such curves are presented in Figs. 4-7) and effective concentrations CC$_{50}$ and CC$_{90}$ that reduce cell viability by 50% and 90%, respectively, as compared to the control, were calculated from these curves where possible (Tables 1-3).

The results obtained reveal that ZnSalenAu and ZnSalenAg decrease significantly viability and/or proliferation of the treated cells in a time- and concentration-dependent manner being more active cytotoxic agents as compared to H$_2$Salen. For instance, CC$_{50}$ of ZnSalenAu and ZnSalenAg (determined by MTT, 72 h) were found to be <6.5 µM and <7.5 µM, respectively. The experimental design was extended using additional methods with different molecular/cellular targets and mechanisms of action * NR uptake cytotoxicity assay and CV staining
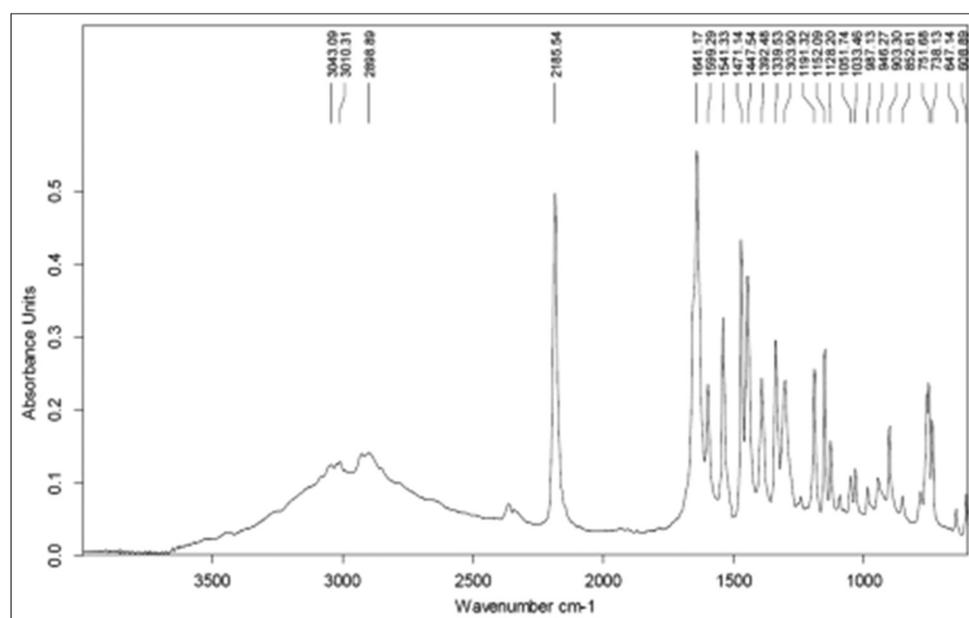

Fig. 1: Infrared spectrum of ZnSalenAg

Table 1: CC_{50} μM and CC_{90} μM of ZnSalenAu and ZnSalenAg for HeLa cell line

Treatment period (h)	Method	H2Salen	ZnSalenAu	ZnSalenAg
12	MTT	n.d.	0.52* (3.67)**	3.13 (4.70)
18		n.d.	0.46 (2.64)	2.99 (4.47)
24		n.d.	0.41 (n.d.)	2.89 (4.46)
48		n.d.	0.23 (0.41)	2.59 (4.33)
72	MTT	n.d.	0.28 (0.63)	1.76 (4.17)
	NR	n.d.	0.80 (3.40)	2.04 (4.18)
	CV	n.d.	2.94 (n.d.)	2.83 (n.d.)

*CC_{50} μM, **CC_{90} μM, MTT: Thiazolyl blue tetrazolium bromide test, NR: Neutral red uptake cytotoxicity assay, CV: Crystal violet staining, n.d. - CC_{50}: Respectively, CC_{90} was not determined because at all examined concentrations (0.1–10 μg/ml) the cell viability was >50%, respectively, >10%. CC: Cytotoxic concentration

Table 2: CC_{50} μM and CC_{90} μM of ZnSalenAu and ZnSalenAg for human A549 and 8MGBA cell lines

A 549 cell line				
Treatment period	Method	H2Salen	ZnSalenAu	ZnSalenAg
48	MTT	n.d.	2.50* (3.91)**	2.54 (7.60)
72		n.d.	6.46 (8.90)	7.32 (9.54)
8MGBA cell line				
24	MTT	n.d.	1.34 (8.15)	2.74 (4.49)
48		n.d.	0.45 (2.89)	2.75 (4.35)
72		n.d.	0.65 (3.34)	3.19 (4.48)

*CC_{50} μM, **CC_{90} μM, MTT: Thiazolyl blue tetrazolium bromide test, n.d. - CC_{50}, respectively, CC_{90}, was not determined because at all examined concentrations (0.1–10 μg/ml) the cell viability was >50%, respectively, >10%. CC: Cytotoxic concentration, 8MGBA: Glioblastoma multiforme

Table 3: CC_{50} μM and CC_{90} μM of ZnSalenAu and ZnSalenAg against human squamous cell carcinoma cell line A431 and its multidrug-resistant clones

Cell line	ZnSalenAu	ZnSalenAg
	MTT, 72 h	
A431	0.98* (3.42)**	3.10 (4.38)
A431-MDR1	2.46 (6.28)	2.06 (4.24)
A431-MRP1	2.67 (8.15)	2.28 (4.46)
A431-ABCG2	2.16 (3.60)	3.82 (4.58)

*CC_{50} μM; **CC_{90} μM, MTT: Thiazolyl blue tetrazolium bromide test, CC: Cytotoxic concentration, 8MGBA: Glioblastoma multiforme. CC: Cytotoxic concentration, MDR: Multidrug-resistant

Fig. 2: The UV–visible spectrum of ZnSalenAg

Fig. 3: Solid-state emission spectrum of ZnSalenAg at room temperature (λ_{exc}=370 nm) (inset - emission spectrum of H_2Salen)

on HeLa cervical carcinoma cell line, due to its high sensitivity to the cytotoxic activity of the examined compounds and wide application as a model system in laboratory practice.

The CC_{50} and CC_{90} values for HeLa cells measured by MTT, NR, and CV assays are presented in Table 1. The cytopathological changes (such as reduced cell number, vacuolization of cytoplasm, cell picnosis, and apoptotic bodies) in HeLa cells cultured in the presence of ZnSalenAu and ZnSalenAg are visualized by HE staining as well as double staining with AO and PI (Fig. 8).

Long-term experiments
The MTT, NR, CV, HE, and AO/PI assays demonstrate the "quick" (12–72 h) effect of the compounds tested on monolayer (two dimensional [2D]) cell cultures whereas the colony-forming method reveals their long-term ability to suppress the three dimensional (3D) growth of cancer cells. 3D cell cultures have been considered to be more physiologically relevant and predictive model systems than 2D cell cultures. Various differences between 2D and 3D experimental cell models have been reported including response to therapeutic agents, demonstrating the advantages of 3D cell culturing [39,40]. It has been found in our investigations that both metal complexes completely inhibit 3D growth of HeLa cells in a semi-solid medium administered
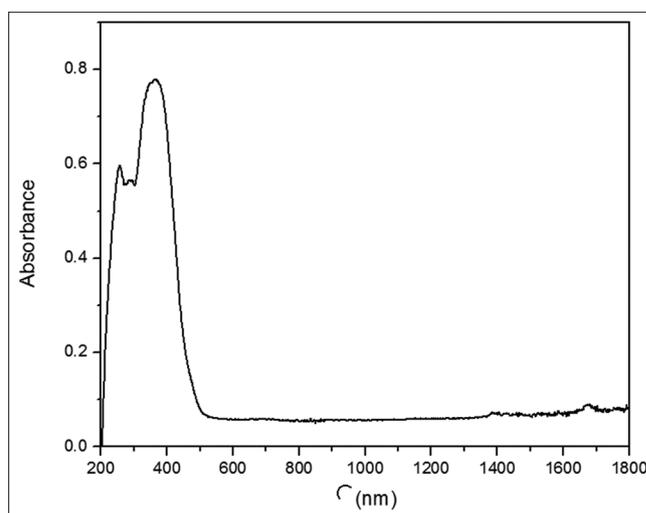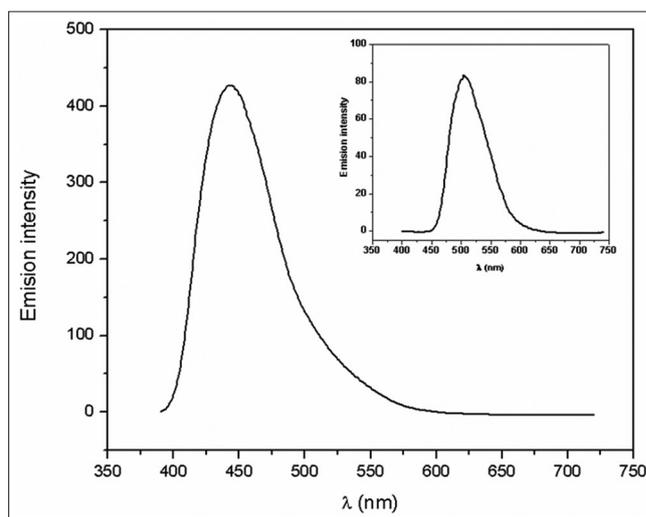
at concentrations ≥0.41 μM (0.5 μg/ml, ZnSalenAu) and ≥0.47 μM (0.5 μg/ml, ZnSalenAg). Applied independently, H_2Salen does not inhibit 3D colony-forming ability of HeLa cells.

In this study, we report for the 1st time the ability of ZnSalenAu and ZnSalenAg to inhibit the 2D- and 3D-growth of cultured human cancer cells. It's worth mentioning that our model systems used in our experiments provoke interest due to the following reasons: (i) Lung cancer is among the most frequent and deadly neoplastic diseases. NSCLC is the most common form of lung cancer representing about 85% of all cases. In 85–90% of the
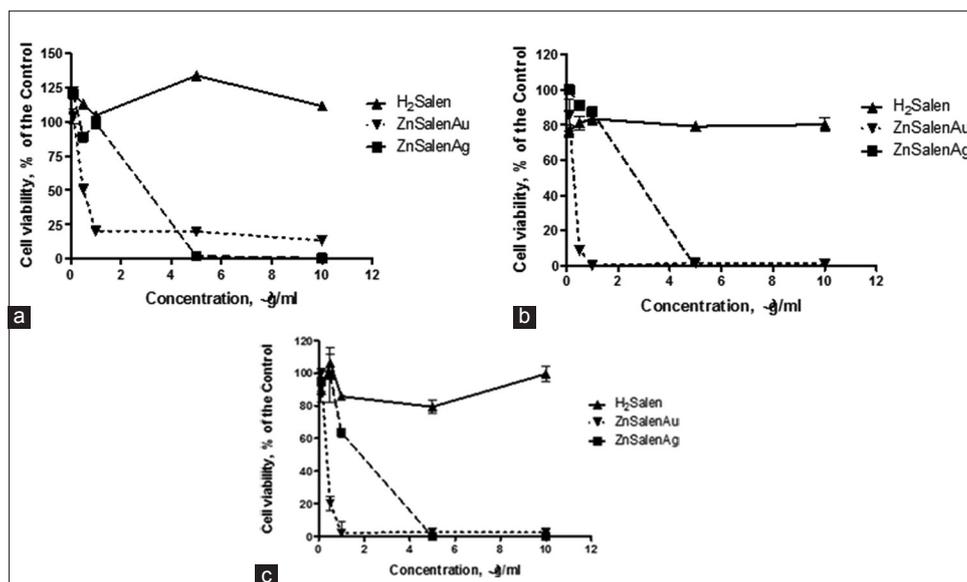
Fig. 4: Concentration-response curves of ZnSalenAu, ZnSalenAg and their ligand H$_2$Salen for human cervical carcinoma cells evaluated by thiazolyl blue tetrazolium bromide test after 24 h (a), 48 h (b), and 72 h (c) treatment period
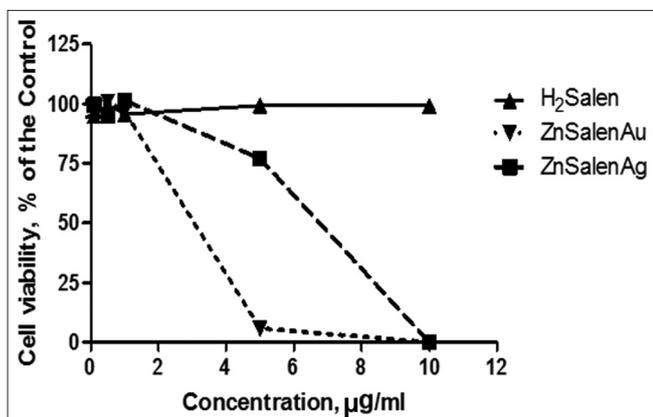

Fig. 5: Concentration-response curves of ZnSalenAu, ZnSalenAg and their ligand H$_2$Salen for human non-small cell lung cancer (A549) cells evaluated by thiazolyl blue tetrazolium bromide test after 48 h treatment period
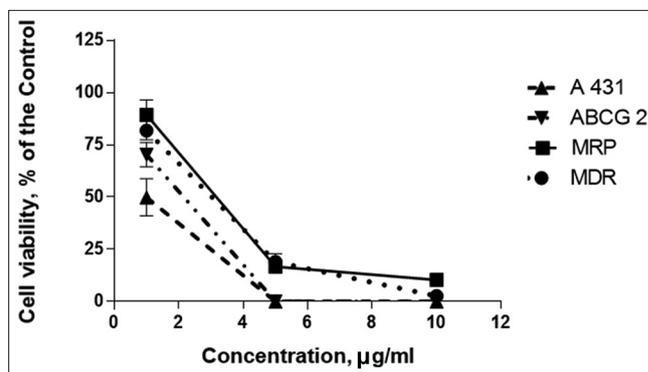

Fig. 7: Concentration-response curves of ZnSalenAu for human squamous cell carcinoma cells (A431 parental cell line and its drug-resistant clones) evaluated by thiazolyl blue tetrazolium bromide test after 72 h treatment period
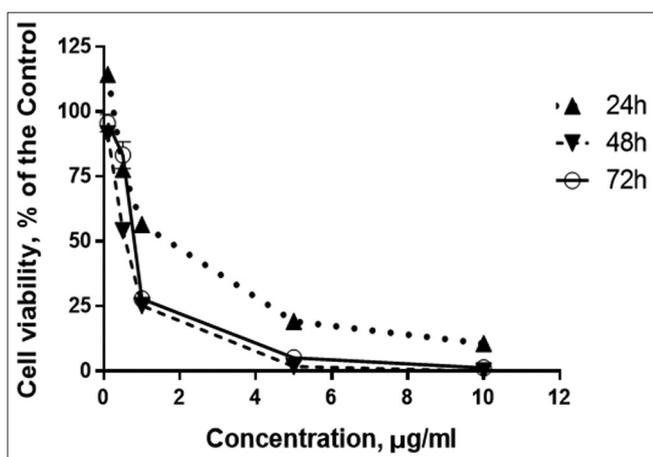

Fig. 6: Concentration-response curves of ZnSalenAu, for human glioblastoma multiforme cells evaluated by thiazolyl blue tetrazolium bromide test after 24 h, 48 h, and 72 h treatment period

cases, the disease is diagnosed later (IIIB and IV stages) when the patients are inoperable, and the main treatment is conservative - chemotherapy and/or radiotherapy [41]; (ii) 8MGBA is a highly aggressive cancer. The survival rate of people affected by this disease has remained virtually unchanged over the past few decades and is only 6-12-15 months after the diagnosis despite advances in surgical approaches, radiation, and chemotherapy, 5-year survival remains <3% [42,43]; (iii) drug resistance is the main obstacle preventing successful chemotherapy of cancer. The so-called ATP–binding cassette proteins transporters (such as the products of *mdr1*, *mrp1,* and *abcg2* genes) are key players in MDR phenomenon [44,45]; (iv) carcinoma of the uterine cervix is among the most frequent malignancies in women [46,47]. HeLa is the oldest and the most widely used cell culture model in biomedical research [48,49].

Although both metal complexes are active, the CC$_{50}$ and CC$_{90}$ of ZnSalenAu are lower than those of ZnSalenAg. Our preliminary investigations with Ru(III) complexes with H$_2$Salen reveal that they possess significantly lower cytotoxic activity for HeLa cells as compared to Zn(II)/Au(I) and Zn(II)/Ag(I) complexes with the same ligand (not published data).

CONCLUSION

In this study, we report for the 1st time the ability of two heterometallic complexes ZnSalenAu and ZnSalenAg containing a Schiff base
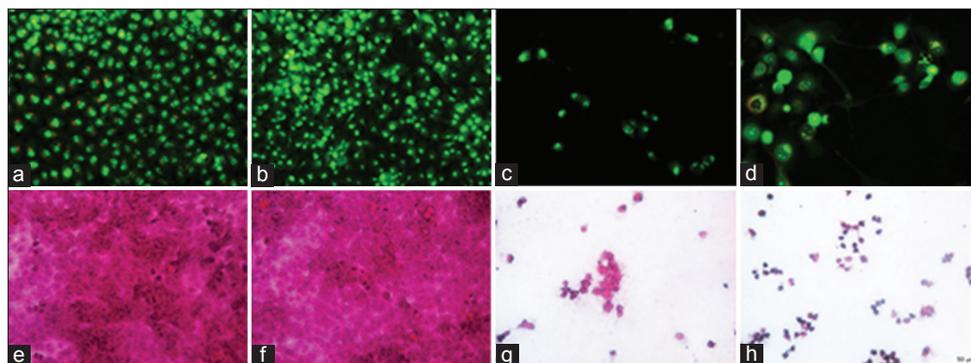
Fig. 8. (a) Non-treated HeLa cells - Control, (b-d) HeLa cells after 72 h of treatment with 10 µg/ml H₂Salen (b), 0.5 µg/ml ZnSalenAu (c), 5 µg/ml ZnSalenAg (d and e), untreated HeLa cells - Control, (f and g) HeLa cells after 72 h treatment with 10 µg/ml H₂Salen (f), 0.4 µg/ml ZnSalenAu (g), 2.5 µg/ml ZnSalenAg (h), double staining with propidium iodide and acridine orange (a-d), double staining with hematoxylin and eosin (e-h), Bar=50 µm

ligand (H₂Salen) obtained from the condensation reaction between salicylaldehyde and ethylenediamine, to decrease significantly viability and proliferation of cultured cell lines established from some of the most common and aggressive human cancers (NSCLC, carcinoma of the uterine cancer, 8MGBA, and squamous cell carcinoma) as well as MDR cancer cells. The cytotoxic activity of the compounds investigated was prove by in short-term and long-term experiments using 2D and 3D cell cultures and methods with different molecular/cellular targets and mechanisms of action. Additional investigations are underway to clarify better the antineoplastic potential of ZnSalenAu and ZnSalenAg.

AUTHORS' CONTRIBUTIONS

Gabriela Marinescu specifically contributed for the chemical part and Radostina Alexandrova for the biological part. All the authors contributed equally in publishing this manuscript.

CONFLICTS OF INTEREST

The author(s) declare(s) that there are no conflicts of interest regarding the publication of this article.

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