https://doi.org/10.31925/farmacia.2021.3.7 **ORIGINAL ARTICLE**

SYNTHESIS, CHARACTERIZATION AND CYTOTOXICITY EVALUATION OF Ni(II), Cu(II) AND Zn(II) COMPLEXES WITH DEOXYCHOLATE LIGAND

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Manuscript received: June 2020

Abstract

Three new complexes of Ni(II), Cu(II) and Zn(II) with deoxycholate anion, of the type $M(DCA)_2 \cdot nH_2O$ (M = Cu(II) n = 4, M = Ni(II) n = 3 and M = Zn(II) n = 2) have been synthesized and characterized on the basis of elemental analysis, FTIR and UV-Vis spectroscopy, thermal analysis, molar conductivity and magnetic measurements. The cytotoxicity of the synthesized complexes was tested against HT29 human colorectal cancer cells. Applied at a concentration range of 10 - 200 µg/mL, sodium deoxycholate and its metal complexes have been found to decrease viability and growth of cultured HT29 cells in a time- and concentration-dependent manner in short-term and long-term experiments. The ability of the complexes to induce pathological changes, genotoxicity and apoptosis in the treated cells has also been proved. In addition, the complexes demonstrated a cytotoxic effect in HT29-OxPt (resistant to oxaliplatin) cells, allowing to conclude that they are more pronounced cytotoxic agents as compared to the sodium deoxycholate alone.

Rezumat

Trei noi complecși ai Ni(II), Cu(II) și Zn(II) cu anionul deoxicolat, de tipul $M(DCA)_2 \cdot nH_2O$ (M = Cu(II) n = 4, M = Ni(II) n = 3 și M = Zn(II) n = 2), au fost sintetizați și caracterizați pe baza analizei elementale, a spectroscopiei FTIR și UV-Vis, analizei termice, conductivității molare și a măsurătorilor magnetice. Citotoxicitatea complecșilor sintetizați a fost testată pe celule canceroase colorectale umane HT29. La concentrații de 10 - 200 µg/mL, s-a observat că deoxicolatul de sodiu și complecșii săi metalici scad viabilitatea și dezvoltarea celulelor colorectale umane HT29 cultivate, într-o manieră dependentă de timp și concentrație, în experimente pe termen scurt și pe termen lung. Capacitatea complecșilor de a induce modificări patologice, genotoxicitate și apoptoză în celulele tratate a fost de asemenea dovedită. În plus, complecșii au demonstrat un efect citotoxic asupra celulelor HT29-OxPt (rezistente la oxaliplatină), permițându-ne să concluzionăm că sunt agenți citotoxici mai puternici comparativ cu deoxicolatul de sodiu.

Keywords: deoxycholate complexes, transition metals, cytotoxicity, colorectal cancer cells

Introduction

It is well-known that metals accumulated in the liver cause serious problems. The use of chelating agents which facilitate urinary excretion may be a solution of these problems, but it has the disadvantage that kidneys damage could occur. An alternative way for excretion of such metals *via* bile and faeces could be the use of bile acids and its derivatives as chelating agents. Progress in understanding how bile salts interact with metal ions in solution depends on the knowledge of their coordination with metal ions [1-3].

Bile acids are steroid compounds usually containing one to three hydroxyl groups linked to a steroid nucleus and a short aliphatic side chain having a carboxyl group at the end [4]. Primary bile acids, including cholic and chenodeoxycholic acid are synthesized in the liver and constitute the main components of bile [5]. In the colon, intestinal microflora transforms primary bile acids into secondary bile acids, such deoxycholic, lithocholic and ursodeoxycholic acids [6]. The main roles of bile acids in the organism are solubilisation and adsorption of lipids, cholesterol and lipid-soluble

vitamins [6]. They also exhibit a broad spectrum of biological activities such as antimicrobial, anticancer, antiinflamatory, antifungal, etc. [4]. A significant number of published papers reports various chemical transformations of the bile acids including etherification, amidation [7, 8], esterification [9], replacement of hydroxyl with keto groups [10], etc. aiming to enhance their biological activities. In the last years, bile acids became important as drug vehicles for delivering platinum-related cytostatic drugs to the liver [11, 12] and also to modify the solubility of drugs [13]. Such kind of compounds containing transition metals (platinum, gold and palladium) and bile acids, designated as Bamet, were synthesized and characterized, and proved to have cytostatic and anti-tumour properties [14-16]. Bamet-UD2 is a cisplatin-ursodeoxycholate derivative with strong antitumour activity, marked hepatobiliary organotropism and reduced toxic side effects as compared to the parent drug cisplatin [17]. Bamet-R2, a complex of cisplatin with glycocholate, is a strong cytostatic and antitumour compound, probably due to its ability to modify DNA structure and to inhibit DNA synthesis [9].

The need to find a safe and highly selective cure for neoplastic diseases remains a major challenge for modern science. The discovery of the antitumour efficacy of cisplatin and some related platinum compounds (carboplatin, oxaliplatin) has stimulated the search for other metal complexes with antineoplastic properties. Various metal complexes, including zinc, copper and cobalt, are found to be promising substances for the design of new anticancer agents [18-21].

The goal of this research was to extend the range of coordination compounds of transition metals with bile acids and to evaluate their cytotoxic activity against HT29 human colorectal cells and HT29-OxPt (resistant to oxaliplatin) cells. Therefore, we decided to study the coordination compounds of Cu(II), Ni(II) and Zn(II) with the anion of deoxycholic acid, one of the most important secondary bile acids. It is well known that in the human body deoxycholic acid takes part in the emulsification of fats for the absorption in the intestine [22]. Outside the body it is used in experimental basis of cholagogues. Currently, it is part of predominantly gallstone reducing medicines (choleretics). Recently, deoxycholic acid has been approved as a food additive in some countries. Newer approaches are assumed that deoxycholic acid plays an important role in the immune system, possibly as an activator of macrophages in tumour tissue and in inflammation. Sodium deoxycholate (NaDCA), the sodium salt of deoxycholic acid, is frequently used in mesotherapy injections, mixed with phosphatidyl choline. Also, it is often used as a biological detergent to lyse cells and solubilize cellular and membrane components [23]. Some papers on the capacity of different bile acids to coordinate metal ions have also been published [3, 24-28].

Materials and Methods

Deoxycholic acid sodium salt (Calbiochem, Germany), nickel nitrate hexahydrate ($Ni(NO_3)_2 \cdot 6\ H_2O$), copper nitrate trihydrate ($Cu(NO_3)_2 \cdot 3\ H_2O$), zinc nitrate hexahydrate ($Zn(NO_3)_2 \cdot 6\ H_2O$) (Merck, Germany) were of analytical grade. The other materials Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum (FBS) were purchased from Gibco-Invitrogen (UK). Dimethyl sulfoxide (DMSO), neutral red, crystal violet and trypsin were obtained from AppliChem (Germany), thiazolyl blue tetrazolium bromide (MTT), purified agar and consumables for the Comet Assay were from Sigma Aldrich Chemie GmbH (Germany). All other chemicals of the highest purity commercially available were purchased from local agents and distributors. All sterile plastic and syringe filters were from Orange Scientific (Belgium). The fluorescent dye SYBR® Green I for fluorescent visualization of Comet Assay results was purchased from Sigma-Aldrich (S9430). Annexin V/FITC Apoptosis/Necrosis detection kit was purchased from ENZO (ALX-850-020-KI02).

Synthesis of the complexes

Stoichiometric amounts of metal salt and sodium deoxycholate were dissolved separately in water, at room temperature. The metal complexes were obtained as fine precipitates by adding sodium deoxycholate solution into the metal salt solution drop by drop, under stirring, at 50ºC. After 60 minutes, the precipitates were filtered, washed several times with distilled water and dried in desiccator over P_4O_{10}.

Methods

All solid complexes obtained were characterized by elemental chemical analysis, FTIR and UV-Vis spectroscopy, thermal analysis, magnetic and conductivity measurements. The results are presented in Table I.

The FTIR spectra were recorded on KBr pellets in the wavenumber region 4000 - 400 cm^{-1} using a Jasco FTIR-4700 spectrometer.

Table I

Elemental chemical analysis and physical properties of the compounds

Compound	Colour	Molecular weight	Yield %	Found/calcd. (%)		μ_{eff} (BM)	Molar conductivity (μS/cm)
				C	H		
CuDCA	Olive green	918.5	76	62.87/62.69	9.21/9.36	1.71	1.8
NiDCA	Light green	895.7	73	63.76/64.29	9.20/9.37	3.31	3.0
ZnDCA	White	884.4	79	64.06/65.11	9.23/9.26	Diamagnetic	1.8

CuDCA = $Cu(DCA)_2 \cdot 4\ H_2O$; NiDCA = $Ni(DCA)_2 \cdot 3\ H_2O$; ZnDCA = $Zn(DCA)_2 \cdot 2\ H_2O$

The electronic spectra were recorded on a Jasco V-750 spectrophotometer equipped with diffuse reflectance accessories. Magnetic measurements were performed by Faraday method with Mohr salt as a standard, at room temperature. Elemental analysis was performed on a Perkin Elmer CHNS/O Analyzer 2400 Series II. Thermal analysis (TG/DSC) of the compounds was performed with a Netzsch TG 449C STA Jupiter thermal analyser at a heating rate of 10°C/min, from room temperature to 900°C, in air. Molar conductivity of 10^{-3} M solutions in DMSO was measured at 17°C, on a Mettler Toledo SevenGo Duo SG23 conductivity meter.

Cell model systems and cultivation

The compounds were dissolved in dimethyl sulfoxide (the concentration of the compound in the stock solution was 1 mg/mL containing 2% DMSO) and then diluted in culture medium. The stability of the complexes dissolved in DMSO was checked by recording their UV-Vis spectra for a week, once every two days. No differences were observed between the spectra, which proved that the complexes in DMSO solution are stable over time.

Human permanent cell line HT29 established from a colorectal adenocarcinoma as well as its resistant to Oxaliplatin clone HT29-OxPt [29] were used as model systems in our study. The cells were grown in D-MEM medium supplemented with 5 - 10% foetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. The cell cultures were kept in a humidified incubator (Thermo Scientific, HEPA Class 100) at 37ºC under 5% CO_2 in air. For routine passages the cells were detached using a mixture of 0.05% trypsin and 0.02% EDTA. The cell lines were passaged 2 - 3 times per week (1:2 to 1:3 split). The lower sensitivity of HT29-OxPt cells to the cytotoxic effect of oxaliplatin was proved using MTT test – CC_{50} calculated after 72 h of treatment was found to be 60.5 µM for HT29 cell and 241.1 µM for HT29-OxPt cells. Prior to subsequent experiments, the HT29-OxPt cells were maintained in drug-free growth medium for at least one week [29].

Cytotoxicity assays

The cells were seeded in 96-well flat-bottomed micro-plates at a concentration of 1 x 10^4 cells/well. At the 24th hour, the culture medium was removed and changed with media containing different concentrations of the compounds tested (10, 50, 100 and 200 µg/mL). Each concentration was applied in 6 to 8 wells. Samples of cells grown in non-modified medium served as a control. The effect of the compounds on cell viability and proliferation was evaluated using thiazolyl blue tetrazolium bromide (MTT) test, neutral red uptake cytotoxicity assay (NR assay) and crystal violet staining (CV staining) after 24 - 72 h of incubation.

The MTT assay of cell viability was performed in accordance with the the Mossmann's procedure [30]. The cells were incubated for 3 h with MTT solution (5 mg MTT in 10 mL D-MEM) at 37ºC under 5% carbon dioxide in the air. The formed blue MTT formazan was extracted with a mixture of absolute ethanol and DMSO (1:1, vol/vol).

The neutral red (NR) cytotoxicity assay was based on the method of Borenfreund and Puerner [31]. A solution of NR (50 µg/mL, 0.1 mL) was added to each well containing cells. The plates were placed in CO_2 incubator for 3 h to ensure the uptake of vital dye. Next, the NR-containing medium was removed and the cells were washed with phosphate-buffered saline (PBS, pH 7.2; 0.2 mL/well). The addition of 0.1 mL 1% acetic acid solution containing 50% ethanol extracts the dye from the viable cells.

Crystal violet staining was performed as described by Saotome *et al*. [32]. The microplate with tested compounds was incubated for 72 h at 37ºC in 5% CO_2 atmosphere. After this period, the medium was removed and the cells were stained with 0.4% crystal violet solution in methanol for 30 min and carefully washed with distilled water.

The final step of cytotoxicity assays - quantitative analysis, was performed by absorbance measurements in an automatic microplate reader (TECAN, Sunrise^{TM}, Austria) at 540/620 nm (for MTT test and CV staining) and 540 nm (for NR assay). Relative cell viability, expressed as a percentage of the control, was calculated for each concentration of sodium deoxycholate and its metal complexes. Concentration–response curves were prepared and the effective concentrations of the compounds – CC_{50} (that decrease by 50% the amount of viable cells as compared to the control) and/or CC_{90} (causing a 90% reduction of cell viability as compared to the control) were estimated where possible. All data points represent an average of at least three independent assays.

Double staining with acridine orange and propidium iodide

The ability of compounds to induce cytopathological changes was evaluated using double staining with acridine orange (AO) and propidium iodide (PI) according to the standard procedures [33]. The cells were cultured on cover slips in 6-well plates in the presence of the compounds tested. Non-treated cells served as a control. After 48 h of incubation, the coverslips were removed and washed with phosphate buffered saline (PBS) for 2 min. A mixture, containing equal volumes (1:1) of fluorescent dyes AO (10 µg/mL in PBS) and PI (10 µg/mL in bi-distilled water) were added to the cells. Fresh stained cells were placed on a glass slide and examined under fluorescence microscope (Leika DM 500B, Wetzlar, Germany) within 30 min before the fluorescent colour started to fade.

Single cell gel electrophoresis (Comet assay)

The alkaline variant of the Comet assay, designed by Olive *et al*. [34], was used in the current research with minor modifications [35]. Gels were prepared by mixing the cells with 1.4% low-gelling agarose. The

cell-agarose suspension was spread onto microscopic slides followed by lysis of cells in a cold lysis solution (1 M NaCl, 50 mM EDTA pH 8, 30 mM NaOH, 0.1% N-lauroyl sarcozine; pH 10) for 1 hour at 10ºC. DNA was denatured by incubating the slides 3 x 20 min in a denaturing solution (30 mM NaOH, 10 mM EDTA; pH 12.6). Electrophoresis was conducted at 10°C for 20 minutes, at 0.45 V/cm in the same denaturing solution. The slides were washed with 0.5 M Tris-HCl, pH 7.5 to neutralize the alkali and were dehydrated by successive washes in 75% and 95% ethanol for 5 min each. Comets were observed under a Leitzepi-fluorescence microscope (Orthoplan, Vario Orthomat 2) using 450 - 490 nm band-pass filter following staining of DNA with SYBR green I. Pictures were taken with a digital photo camera, Olympus μ8, at a resolution of 3 Mpx. Quantification of results was conducted by calculating the percentage of comets in each sample.

Identification of cell death

Apoptosis detection was performed using Annexin V-FITC Certified Apoptosis/Necrosis detection kit as it was recommended by the manufacturer (ENZO, Romania). Briefly, cells were spun down at 400 g for 5 min at room temperature and were carefully resuspended in 1 mL cold 1 x PBS (2.68 mM KCl, 1.47 mM KH_2PO_4, 1.37 mM NaCl, 8 mM Na_2HPO_4), pH 7. Spinning down follows at the same conditions and the pellet was resuspended in 510 μL Dual Detection Reagent (500 μL 1 x binding buffer, 5 μL Apoptosis Detection reagent/Annexin V-Enzo Gold; 5 μL Necrosis Detection Reagent). Samples were incubated at room temperature for 10 min at dark and were analysed *via* cytometry using 488 nm laser at FL 2 and FL 3 channels for apoptosis and necrosis detection respectively. FACS analysis was performed on the model HT29 cells treated with the studied compounds at increasing concentrations. Cellular morphology was assessed and results are presented as four groups of cells with different cell size and granularity. Each value represents the percentage of cells with specific morphology as a percentage from the whole cell population. Results were quantified with FlowJo software. Population of live cells, and cells in early, late apoptosis and necrosis were calculated as a percentage of the whole cell population.

3D Colony forming method

Tumour cells (10^3 cells/well) suspended in 0.45% purified agar in D-MEM medium containing different concentrations of deoxycholic acid and its metal complexes (concentrations of 10 to 200 μg/mL) were layered in 24 well plates. Non-treated cells served as a control. The presence/absence of colonies and their number (counted in at least 4 - 5 different fields or each well) were registered using an inverted microscope (Carl Zeiss, Jena, Germany) during a period of 48 days (counted on 3 - 5-day intervals).

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 (ANOVA) followed by Dunnett post-hoc test. Effective concentrations of the compounds – CC_{50} and/or CC_{90} were estimated by Origin 6.1™. FACS data were quantified by FlowJo software. Comet Assay data were statistically evaluated using Comet-Score Software.

Results and Discussion

Physico-chemical characterization of the compounds

The complexes $M(DCA)_2 \cdot n\ H_2O$ (M = Cu(II) n = 4; M = Ni(II) n = 3 and M = Zn(II) n = 2) were prepared in high yields upon mixing aqueous solutions of metal salts and deoxycholic acid sodium salt, in a 1:2 molar ratio, at 50ºC. The compounds are soluble in warm DMSO and insoluble in water and most of the organic solvents. The molecular formulae of the complexes were established by correlating the elemental analysis results with thermal analysis, spectroscopic data, magnetic moments and conductivity values (Table I).

In order to obtain information about the coordination manner of the deoxycholate anion to the divalent transition metal ions, FTIR spectroscopic measurements were performed. The spectra of the complexes showed changes in the positions and intensities of some bands compared to those of the free ligand. The formation of the metal complexes was established by the presence of two strong bands in the following ranges: 1552 - 1598 cm^{-1} ($\nu_{asym(OCO)}$) and 1411 - 1417 cm^{-1} ($\nu_{sym(OCO)}$) which are assigned to the coordinated carboxylate group (Table II). The bands corresponding to the asymmetrical and symmetrical vibrations of the COO^- groups in complexes are slightly shifted as compared to sodium deoxycholate. The magnitude of separation ($\Delta\nu$) between frequencies of asymmetrical and symmetrical vibrations of the COO^- group is useful in defining the structure of complexes [36]. According to Shaheen [37], a $\Delta\nu$ value between 150 and 250 cm^{-1} suggests a bridging coordination, while $\Delta\nu$ below 150 cm^{-1} indicates a chelating mode of coordination. For the complexes of Ni(II) and Zn(II), $\Delta\nu$ is 142 and 141 cm^{-1} respectively, suggesting a bidentate chelate coordination mode. In the case of Cu(II) complex, two frequencies attributable to ν_{asCOO} were observed at 1598 and 1564 cm^{-1} indicating bidentate bridging and chelating coordination of the carboxylate groups to copper ion. The bands at ~ 2935 and 2864 cm^{-1} correspond to stretching vibrations of C-H bonds, while the broad and intense band between 3000 cm^{-1} and 3600 cm^{-1} range in all spectra corresponds to OH stretching vibrations, formation

of hydrogen bond network and also confirms the presence of water molecules in complexes.

Table II

The characteristic frequency data of FTIR spectra for coordination compounds and their assignments

NiDCA	CuDCA	ZnDCA	NaDCA	Assignments
3385	3401	3357	3406	νOH
2936	2936	2934	2935	νCH
2866	2865	2864	2864	νCH
1555	1598, 1564	1552	1563	$\nu_{as}COO^-$
1449	1449	1454	1449	νCH
1413	1417	1411	1407	ν_sCOO^-

The electronic absorption spectra are often very helpful in the evaluation of results furnished by other methods of structural investigation. The electronic spectral measurements were used to assign the stereo-chemistry of the metal ions in the complexes based on the positions and number of *d-d* transition peaks. The electronic absorption spectra of the ligand and its complexes were recorded in the 200 - 900 nm range, at room temperature.

The electronic spectrum of Ni(II) complex shows the characteristic features for (pseudo)octahedral Ni(II) complexes, with two bands at 555 nm and 1000 nm assigned to $^3A_{2g}$ (F) → $^3T_{1g}$ (F) (ν_2) and $^3A_{2g}$ (F) → $^3T_{2g}$ (F) (ν_3) transitions, respectively (Figure 1) [38, 39]. The shoulder at ~ 350 nm could be assigned to the $^3A_{2g}$ (F) → $^3T_{1g}$ (P) (ν_1) transition of Ni(II). The effective magnetic moment of 3.31 BM found for the Ni(II) complex suggests an octahedral arrangement around the metal ion [40].

The electronic spectrum of $Cu(DCA)_2 \cdot 4\,H_2O$ presents an asymmetric band (~ 687 nm) with a tail towards low energies, which is characteristic for square-pyramidal Cu(II) complexes (Figure 1) [38]. The Cu(II) complex shows a normal magnetic moment of 1.71 BM observed for a d^9 (S = ½) system with an unpaired electron. The band located in the region 200 - 400 nm in the absorption spectra of all complexes is most probably due to a π-π* transition of the organic ligand. The Zn(II) complex shows no d-d bands as is expected for a d^{10} system. This compound was found to be diamagnetic.

Conductivity measurements reveal that all metal complexes have low conductivity values characteristic for non-electrolytic compounds [41, 42].

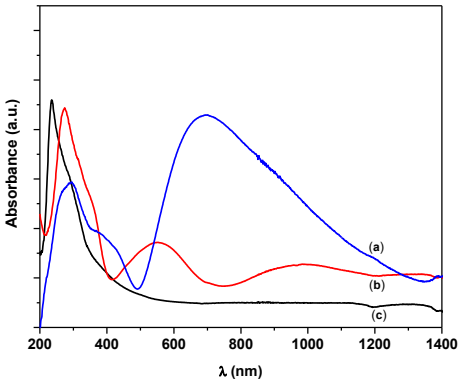


Figure 1.
UV-VIS spectra of (a) CuDCA, (b) NiDCA and (c) ZnDCA

Thermal analysis

In order to give more insight into the structure of the complexes, thermal analyses (TG/DSC) have been carried out from room temperature up to 900°C. The thermoanalytical curves are shown in Figure 2. The decomposition patterns are almost similar for all three complexes. From room temperature up to 170ºC the mass loss ranging from 3.46 to 5.48% corresponds to endothermic release of water molecules (both crystallization and coordinated water). The weight loss recorded in the second step, between 170ºC and 310ºC, could be most probably attributed to de-hydroxilation of the organic ligand. In the next step occurring between 310ºC and 450ºC for CuDCA, and the next two steps ranging from 310ºC and 495ºC for NiDCA and ZnDCA, all the three complexes undergo decomposition and oxidative degradation processes accompanied by overlapping exothermic effects. The fourth (for CuDCA) and the fifth (for NiDCA and ZnDCA) steps consist in complete pyrolysis of the remaining carbonaceous residue, the accompanying exothermic effects being very strong and broad. This step consists in transformation of the metallic intermediates into its stable oxides. The mass losses for each step are given in Table III.

Table III

Thermoanalytical data of the CuDCA, NiDCA and ZnDCA complexes

Complex	Temperature range (°C)	Thermal effect	Mass loss (Δm) (%)
CuDCA	25 - 170	Endothermic	3.46
	170 - 310	Endothermic	5.60
	310 - 450	Exothermic	46.48
	450 - 600	Exothermic	35.16
	Residue CuO		8.67
NiDCA	25 - 170	Endothermic	5.48
	170 - 310	Endothermic	1.56
	310 - 425	Exothermic	59.59
	425 - 495	Exothermic	8.26
	495 - 640	Exothermic	17.18
	Residue NiO		8.17

Complex	Temperature range (°C)	Thermal effect	Mass loss (Δm) (%)
ZnDCA	25 - 170	Endothermic	3.88
	170 - 310	Endothermic	1.75
	310 - 385	Exothermic	11.64
	385 - 495	Exothermic	59.20
	495 - 640	Exothermic	14.64
	Residue ZnO		8.53

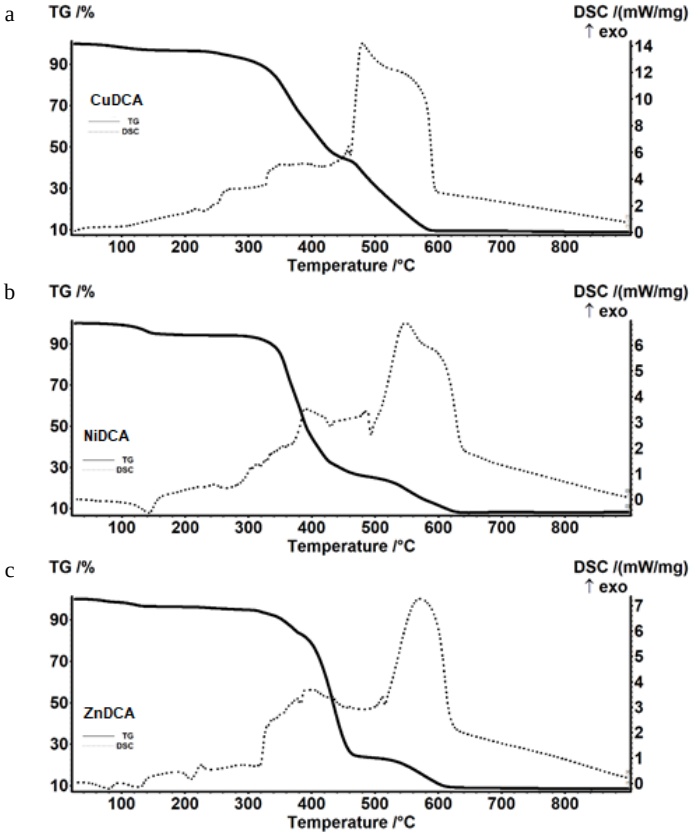
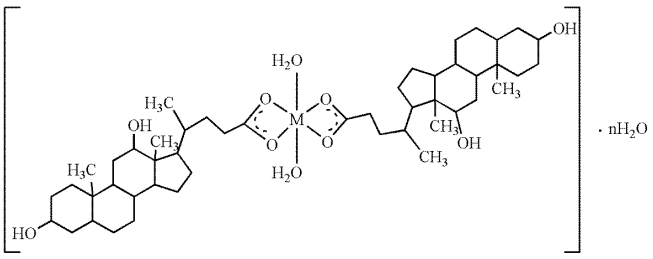
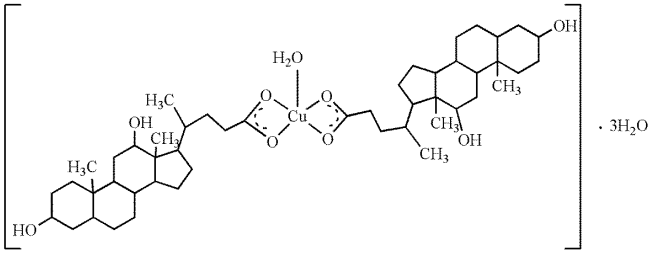


Figure 2.
TG/DSC curves for CuDCA (a), NiDCA (b) and ZnDCA (c)



(a)



(b)

Figure 3.
Proposed structures of the complexes: a) NiDCA and ZnDCA; b) CuDCA

Taking into account all the above data, the following structures of the complexes were proposed (Figure 3).

Biological evaluation

Studying the antitumour activity of a new compound requires conduction of various experiments at different levels: subcellular/molecular, cellular (cell cultures) and organismic (animal models). Demonstrating the ability of a test substance to exert a cytotoxic effect in cell cultures used as model systems is the first, but not the last step. For example, it is necessary to study the cytotoxic effect of the substance on a wide range of cell cultures: derived from different malignancies, from different subtypes of the same tumour, including cell lines of the same subtype (this is due to the heterogeneity of tumour cells, which turns each tumour/ tumour cell line into an unique system); to identify the type of cell death caused by them (apoptosis, necrosis) and their ability to induce autophagy; to determine their targets and the mechanism of their action. A comparative analysis with non-tumour cells is needed, as well as comparison with the action of antitumour drugs established in clinical practice. In this regard, the presented results are part of a comprehensive study on the cytotoxic and antitumour properties of sodium deoxycholate and its metal complexes, which includes a wide range of cell cultures (human and animal tumour and non-tumour cells). For comparative analysis, anti-tumour drugs widely used in clinical oncology, such as cisplatin, oxaliplatin, epirubicin and paclitaxel, were included.

The cytotoxic activity of deoxycholic acid sodium salt and its metal complexes was evaluated using the MTT test which measures the ability of the mitochondrial enzyme succinate dehydrogenase to convert MTT into formazan, NR assay which provides information about the incorporation of neutral red into the lysosomes of living cells, staining of DNA and nuclear proteins with crystal violet, Comet Assay for evaluation of genotoxicity and FACS analysis for quantitation of the type of induced cell death [43, 44].

Applied at a concentration range of 10 - 200 µg/mL for 24 - 72 h, the studied compounds decreased the viability and proliferation of the treated cells. Their cytotoxic activity was increased by increasing the concentrations and prolonging the treatment (Figures 4, 5 and 6). NaDCA and its metal complexes were found to decrease viability and growth of both HT29 (sensitive/ parental) and HT29-OxPt (resistant) human colorectal cancer cells (Figure 7).

The cytotoxic activity of DMSO alone has been studied in our previous investigations [45-47] in a wide variety of human and animal tumour and non-tumour cells, including HT29. It has been found that DMSO did not disturb significantly the viability and proliferation of the treated cells applied for 72 h at concentrations up to 0.8 - 1%. In this study, NaDCA and its metal complexes were applied in concentrations from 1 to 200 µg/mL, in which the content of DMSO was from 0.02 to 0.4%, respectively, and its cytotoxic effect is negligible.

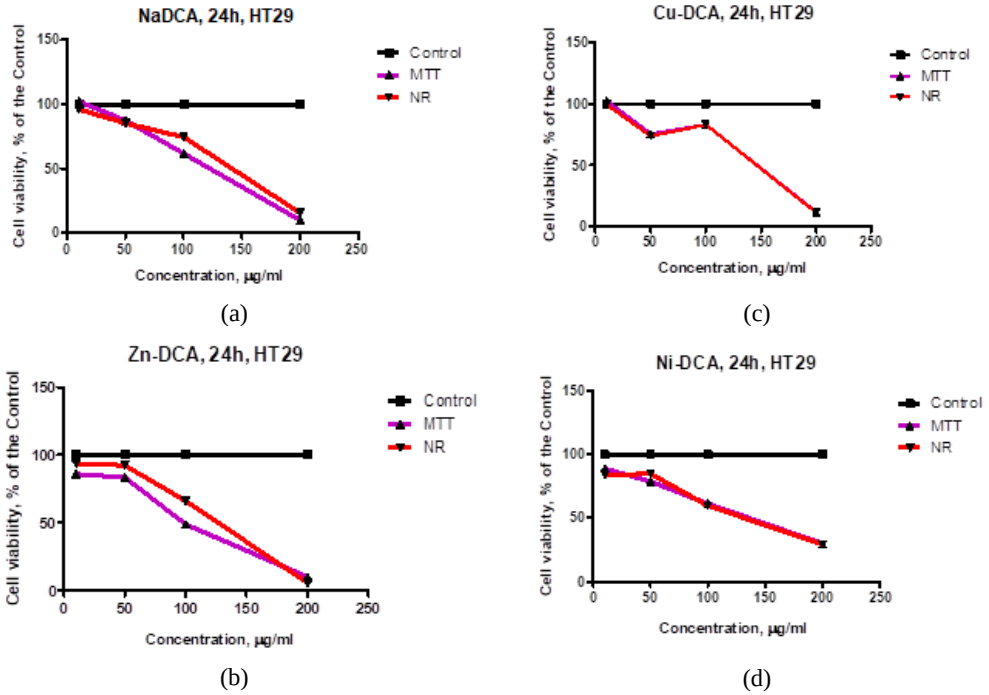


Figure 4.

Influence of NaDCA (a), ZnDCA (b), CuDCA (c) and NiDCA (d) complexes on viability and proliferation of cultured HT29 human colorectal cells

The compounds are applied at a concentration range of 10 - 200 µg/mL for 24 h. The investigation was performed by MTT test (MTT) and NR uptake cytotoxicity assay (NR).

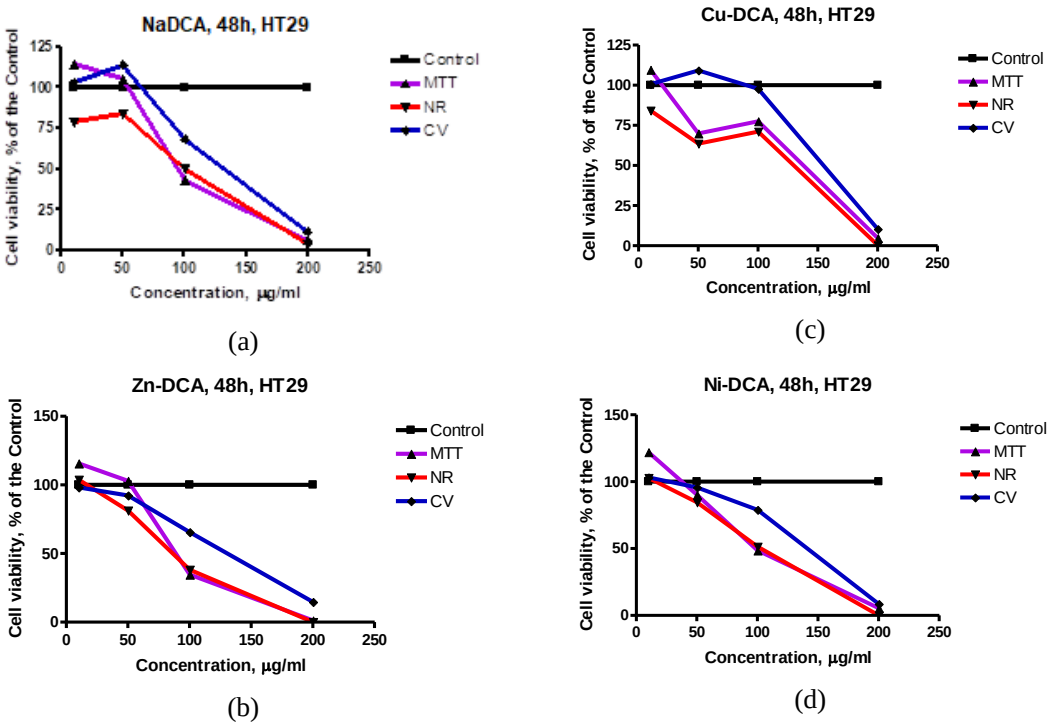


Figure 5.

Influence of NaDCA (a), ZnDCA (b), CuDCA (c) and NiDCA (d) on viability and proliferation of cultured HT29 human colorectal cells

The compounds are applied at a concentration range of 10 - 200 µg/mL for 48 h. The investigation was performed by MTT test, NR uptake cytotoxicity assay and crystal violet staining (CV).

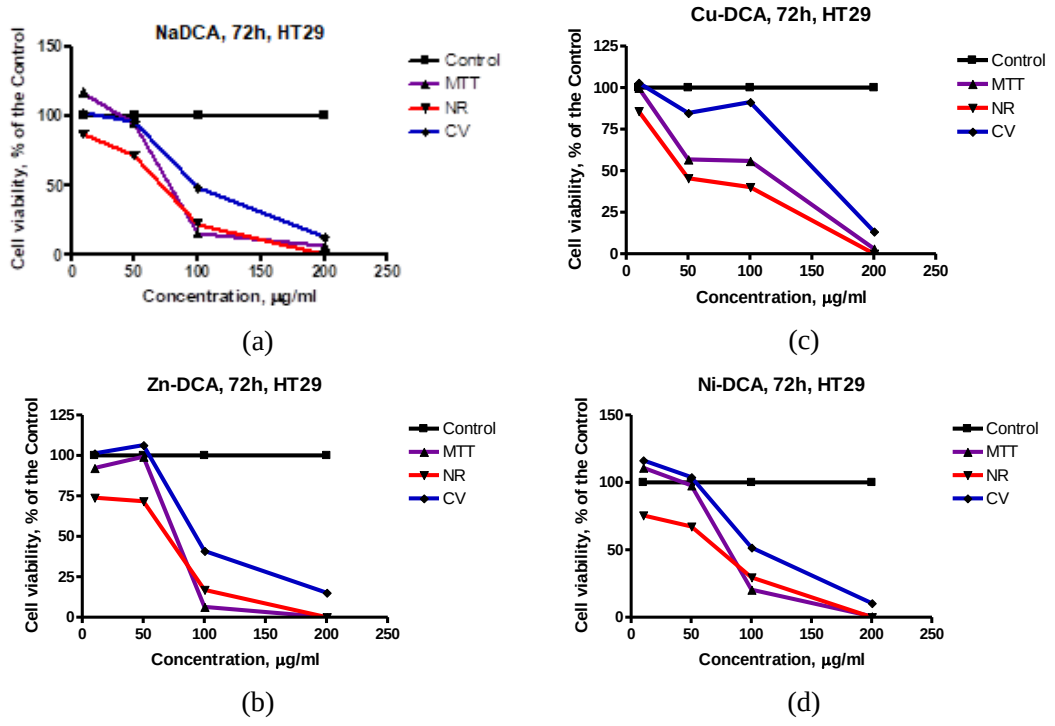


Figure 6.

Influence of NaDCA (a), ZnDCA (b), CuDCA (c) and NiDCA (d) on viability and proliferation of cultured HT29 human colorectal cells

The compounds are applied at a concentration range of 10 - 200 µg/mL for 72 h. The investigation was performed by MTT test, NR uptake cytotoxicity assay and CV staining.

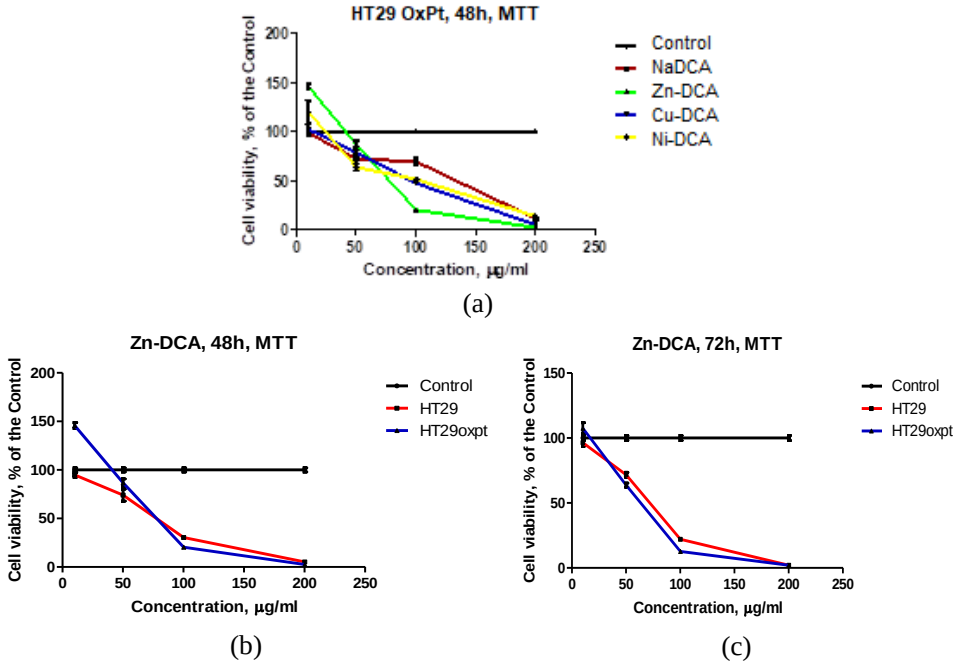


Figure 7.

Influence of NaDCA and its metal complexes on viability and proliferation of human colorectal carcinoma cells resistant to Oxaliplatin (the cell line HT29-OxPt) (a); Effect of ZnDCA on viability and proliferation of HT29 and HT29-OxPt human colorectal carcinoma cells

The compounds are applied at a concentration range of 10 - 200 µg/mL for after 48 h (a, b) and 72 h (c). The investigation was performed by MTT test.

CC_{50} (µM) and CC_{90} (µM) were calculated from "concentration-response" curves (Figures 4, 5, 6 and 7) and presented in Table IV. Based on their cytotoxic activity (CC_{50}, µM), the examined compounds are graded in hierarchical orders (Table V), showing that the metal complexes were pronounced cytotoxic agents as compared to sodium deoxycholate. The resistant HT29-OxPt cells demonstrated the highest sensitivity to the cytotoxic effect of ZnDCA.

The CC_{50} of oxaliplatin (65.4 µM) and epirubicin (45.4 µM) determined by the MTT test (after 72 hours of treatment) was found to be lower than the CC_{50} (determined at the same conditions) of NaDCA (321.4 µM) and its complexes with zinc (II) (138.2 µM), copper (II) (122.6 µM) and nickel (II) (125.2 µM).

Table IV

Cytotoxic activity (CC_{50}, µM and CC_{90}, µM) of NaDCA and its metal complexes on cultured human colorectal cancer cells

Cell line	Method	Treatment interval (hours)	CC_{50} (CC_{90})			
			Compound			
			NaDCA	Zn-DCA	Cu-DCA	Ni-DCA
HT 29	MTT	24	310.4 (506.3)	130.1 (221.7)	159.7 (-)	138.3 (-)
		48	340.0 (490.7)	142.4 (207.7)	150.1 (210.8)	122.0 (194.4)
		72	321.4 (490.7)	138.2 (204.2)	122.6 (203.1)	125.2 (187.5)
	NR	24	362.0 (-)	140.2 (213.5)	159.6 (-)	133.9 (-)
		48	289.4 (481.1)	118.1 (201.7)	140.6 (202.7)	104.3 (184.0)
		72	244.6 (457.1)	104.8 (199.3)	207.3 (192.7)	90.3 (181.7)
	CV	24	-	-	-	-
		48	336.8 (-)	144.5 (-)	169.0 (-)	143.9 (200.8)
		72	337.3 (-)	158.4 (-)	166.0 (-)	106.0 (-)

Cell line	Method	Treatment interval (hours)	CC_{50} (CC_{90}) Compound			
			NaDCA	Zn-DCA	Cu-DCA	Ni-DCA
HT29-OxPt	MTT	24	-	170.4 (-)	208.7 (-)	-
		48	341.8 (-)	86.5 (-)	119.4 (206.6)	105.0 (-)
		72	191.1 (-)	93.0 (200.1)	124.8 (212.2)	130.6 (-)

CC_{50} and CC_{90} (in brackets) concentrations (μM), in which the percentage of viable cells decrease by 50%, respectively 90%, compare to the control; symbol (-) refers to cases where cell survival remains > 50% or > 10%, respectively, at all tested concentrations (10 - 200 μg/mL)

Table V

Hierarchical orders of NaDCA and its metal complexes, reflecting the effect of the compounds on viability and proliferation of the treated cells

Cell line	Treatment interval (hours)	Hierarchical order
HT29	24	Zn-DCA > Ni-DCA > Cu-DCA > NaDCA
	48	Ni-DCA > Zn-DCA > Cu-DCA > NaDCA
	72	Cu-DCA > Ni-DCA > Zn-DCA > NaDCA
HT29-OxPt	24	Zn-DCA > Cu-DCA > (Ni-DCA; NaDCA)
	48	Zn-DCA > Ni-DCA > Cu-DCA > NaDCA
	72	Zn-DCA > Cu-DCA > Ni-DCA > NaDCA

The compounds investigated are ordered in descending order according to their ability to reduce the survival of the treated cells, based on CC_{50} (μM) determined by MTT test

Various cytopathological changes and apoptotic cell death were observed after double staining with acridine orange and propidium iodide in HT29 cells cultured in the presence of NaDCA and its metal complexes (Figure 8). Figure 8a (HT29 Control) reveals a monolayer of vital cells with similar morphology and presence of multiple mitoses. After treatment with 100 μg/mL NaDCA for 48 h (Figure 8b), a partially distorted monolayer of homogeneous in shape and size cells with a nuclear-distorted nuclear cytoplasmic index, pronounced nuclear polymorphism and fine grain chromatin, single cells in mitosis could be observed. Cells with impaired membrane integrity were predominant and were visibly permeable to small amounts of propidium iodide. By increasing the concentration of NaDCA to 200 μg/mL (Figure 8c), the HT29 cell monolayer was reduced to 80% relatively to the control and was represented by non-vital cells with late apoptosis signs: reduced size, scarce cytoplasm and heterogeneous pyknogenic chromatin nucleus, cell membrane with impaired integrity and presence on the cells surface of multiple apoptotic bodies. 100 μg/mL CuDCA treatment (Figure 8d) produced a disturbed cell monolayer, represented by rosette clustered enlarged, but vital cells with enlarged nucleus, large granulated chromatin and nucleolus. There were also small groups of diminished non-vital cells and apoptotic bodies on the surface of the cell membrane. When CuDCA was applied at a concentration of 200 μg/mL (Figure 8e), rosette clusters of cells with signs of late apoptosis can be observed. The cytoplasm was reduced, nucleus was with pyknotic chromatin, cell membranes were permeable to propidium iodide. When treatment was performed with 100 μg/mL NiDCA (Figure 8f), the HT29 cell monolayer was reduced by 50% in comparison to the control, and predominantly represented by vital cells with a core-shifted nucleotide-cytoplasmic index. A small number of cells have a permeability of the cell membrane, and disaggregated cell fragments were seen in the spaces between the preserved cell groups. By increasing the concentration of NiDCA to 100 μg/mL (Figure 8g), the cell monolayer was represented by small groups of non-viral cells with ballooned cytoplasm, deleted cytoplasmic and nuclear morphology. There were multiple extracellular fragments, the presence of which, along with the lack of signs of apoptosis, suggests necrotic cell death. After 100 μg/mL ZnDCA treatment (Figure 8h) groups of 15 - 20 non-vital cells can be observed, most of them with reduced size to untreated cells, with impaired membrane integrity and homogeneous cytoplasm without organelles. Intact nuclei in part of the cells with a coarse-grained chromatin and nucleolus appeared. When ZnDCA was applied at 200 μg/mL (Figure 8i), all HT29 cells became non-vital, with reduced size in comparison to the untreated cells, and completely deleted nuclear cytoplasmic morphology.

NaDCA and its metal complexes were proved by the method of Comet Assay to induce single- and double-stranded DNA breaks (Table VI). Comet assay data showed that with increasing concentrations of the tested compounds the percentage of comets, i.e. cells with damaged DNA increased. The most prominent was the genotoxicity of all compounds at 200 μg/mL, with ZnDCA and NiDCA (at 200 μg/mL) demonstrating the highest genotoxic activity on the cells.

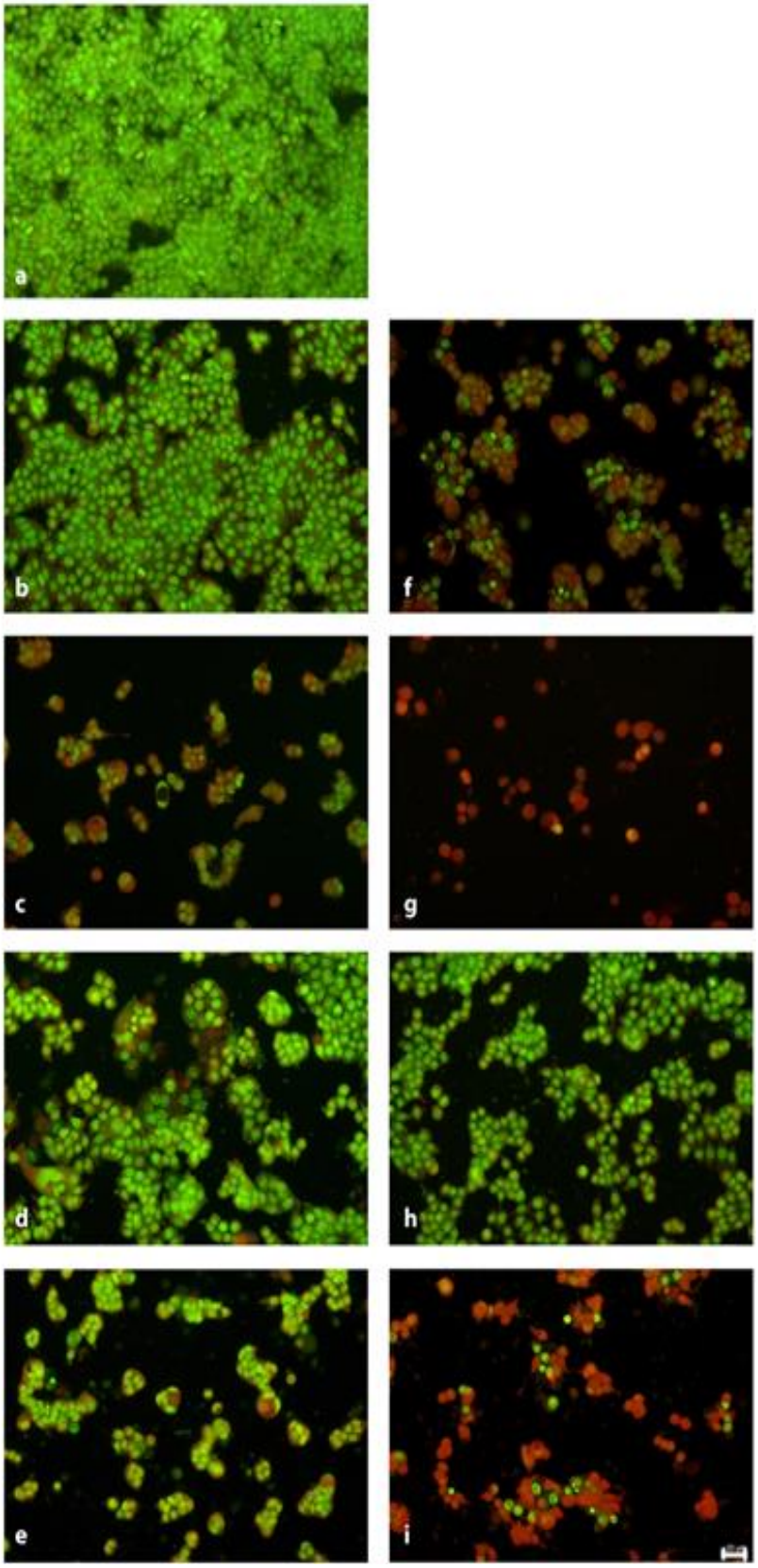


Figure 8.

Cytopathological changes in HT29 human colorectal cancer cells after treatment for 48 h with NaDCA and its metal complexes (scale bar = 20 μm): HT29 Control (a); 100 μg/mL NaDCA (b); 200 μg/mL NaDCA (c); 100 μg/mL CuDCA (d); 200 μg/mL CuDCA (e); 100 μg/mL NiDCA (f); 200 μg/mL NiDCA (g); 100 μg/mL ZnDCA (h); 200 μg/mL NiDCA (i)

Table VI

Genotoxicity of NaDCA and its metal complexes in HT29 human colorectal cancer cells

Compounds	Concentration (μg/mL)	Cell viability*, % of the Control	Comets**, % of the Control
NaDCA	100	15.3 ± 3.1	40.0*
	200	5.9 ± 1.3	66.7
ZnDCA	100	6.4 ± 0.5	47.0
	200	0.0 ± 0.0	80.0
CuDCA	100	55.8 ± 0.8	47.0
	200	3.1 ± 0.1	53.3
NiDCA	100	20.4 ± 3.8	60.0
	200	0.74 ± 0.02	73.3

*Cell viability was examined by MTT test, **Alkaline variant of Comet assay was performed to evaluate the genotoxic effect of the compounds. HT29 cells were cultured in the presence of NaDCA and its metal complexes for 72 hours. The results are expressed as mean ± standard error of the mean of four repetitions in each group. The differences of the individual groups relative to the control were determined by single-factor dispersion analysis (ANOVA), followed by the Dunnet test (*$P < 0.05$; **$P < 0.01$).

Cellular morphology was assessed by FACS analysis after staining of cells with propidium iodide (Table VII). FACS results showed that all tested compounds induced pathological changes in HT29 cellular morphology (compare to the % of cells with normal size and granularity for all probes). The portion of cells with normal morphology gradually decreased with the incubation of the cells with the compounds reciprocally to the applied concentrations. The most prominent was the effect of the compounds on the population of the cells detected with big size and high granularity. The percentage of cells with these pathological changes in the morphology was gradually increasing being the most pronounced for NaDCA, ZnDCA and CuDCA. FACS analysis was further used to study the type of cell damage. The cells were incubated with the tested substances and after that were submitted to FACS with Annexin V Apoptosis/Necrosis detection kit. Results are shown in Table VII. Cells were distributed in four groups depending on their FACS detected Annexin V characteristics: live cells, cells in early and late apoptosis and cells in necrosis. All tested compounds exerted cytotoxicity which was demonstrated in the decreasing number of live cells in all treated probes. FACS Annexin V data showed that all tested compounds induced late apoptosis and necrosis in the cells regardless concentration. This prompts apoptosis is one of the mechanisms of cell death induction in HT29 cells and requires further studies to elucidate this mechanism in details. The reduction in cellular viability was with 50% in comparison to the control cells.

Table VII

Ability of NaDCA and its metal complexes to induce changes in cellular morphology and apoptosis of HT29 human colorectal cancer cells

	Control	Na-DCA-100	Na-DCA-200	Zn-DCA-100	Zn-DCA-200	Cu-DCA-100	Cu-DCA-200	Ni-DCA-100	Ni-DCA-200
Normal size/normal granularity	53.20	45.6	32.00	39.10	32.40	52.00	38.70	48.60	41.30
Normal size/high granularity	1.29	2.88	2.17	2.17	2.99	1.65	1.71	1.17	0.87
Big size/high granularity	35.20	44.00	62.00	53.10	63.00	32.10	53.50	38.20	49.70
Big size/normal granularity	10.40	7.48	3.81	5.65	1.62	14.20	6.16	12.00	8.06
Live cells	70.00	34.40	22.00	25.60	13.80	39.30	26.40	34.20	28.70
Early apoptosis	11.90	1.47	0.81	0.92	0.14	1.00	1.67	0.97	1.04
Late apoptosis	13.20	37.10	33.42	41.20	25.30	30.10	40.10	32.90	35.10
Necrosis	5.99	27.00	43.70	32.30	60.80	29.60	31.80	32.00	35.20

The long-term cytotoxic effect of the compounds tested was studied by 3D colony-forming method. The obtained experimental data (Table VIII) indicate that applied for 14 days and 48 days at a concentration of 200 μg/mL, NaDCA, ZnDCA and CuDCA completely inhibited 3D growth of HT29 cells in a semisolid medium. Undoubtedly, these data are of interest, as they suggest that the cytotoxic/cytostatic effect of the compounds persists over time. In addition, three-dimensional cell colonies hold number of advantages over traditional monolayer (2D) cell cultures, as they more adequately represent biology and behaviour of tumour cells.

Table VIII

Influence of NaDCA and its metal complexes on 3D colony-forming ability of HT29 human colorectal cancer cells

Compounds	Concentration, µg/mL	HT29		
		Colonies, % of the Control		
		6 day	14 day	48 day
Control		100 ± 0.66	100 ± 1.87	100 ± 1.66
NaDCA	10	100 ± 0.29	96.11 ± 0.00	83.49 ± 1.38***
	50	28.57 ± 0.58***	75.68 ± 2.84***	18.90 ± 0.58***
	100	42.86 ± 0.64***	37.24 ± 0.85***	1.57 ± 0.25***
	200	28.57 ± 0.58***	0.00 ± 0.00***	0.00 ± 0.00***
CuDCA	10	42.86 ± 0.96***	90.10 ± 1.25***	42.53 ± 0.48***
	50	71.43 ± 0.64***	40.84 ± 1.26***	39.38 ± 0.48***
	100	50.00 ± 0.25***	19.22 ± 0.82***	6.30 ±0.71***
	200	14.28 ± 0.29***	0.00 ± 0.00***	0.00 ± 0. 00***
ZnDCA	10	57.14 ± 0.71***	96.11 ± 0.00	45.68 ± 1.03***
	50	50.00 ± 0.48***	90.10 ± 1.25**	33.08 ± 0.75***
	100	21.43 ± 0.48***	33.64 ± 1.78***	14.18 ± 0.95***
	200	28.58 ± 0.41***	0.00 ± 0.00***	0.00 ± 0.00***
NiDCA	10	85.71 ± 0.41***	102.11 ± 1.25	51.98 ± 1.49***
	50	57.14 ± 0.71***	60.07 ± 1.89***	40.96 ± 1.19***
	100	50.00 ± 0.63***	60.07 ± 1.44***	36.23 ± 0.48***
	200	71.43 ± 0.5***	81.69 ± 0.71***	26.78 ± 0.75***

3D cell colonies were observed by an inverted light microscope (Carl Zeiss, Jena, Germany) and counted in 4 - 5 independent visual fields at 6^{th}, 14^{th} and 48^{th} day. The experimental data are presented as mean ± standard error of the mean of four repetitions in each group. The differences of the individual groups relative to the control were determined by single-factor dispersion analysis (ANOVA), followed by the Dunnet post hoq test (***$P < 0.01$).

In the recent years, potential anticancer properties of NaDCA have gained increasing attention because of at least two reasons: (i) NaDCA has been reported to inhibit proliferation and to induce apoptosis in cells obtained from human colon cancer [48, 49] and gastric carcinoma [50]; (ii) coupling with bile acid (such as deoxycholic acid) moieties has been suggested to be a promising strategy for liver-targeted drug delivery due to the presence of bile acid receptors on hepatocytes [51]. Deoxycholic acid modified conjugates have been developed and proposed as suitable novel carriers for anticancer drugs including epirubicin and paclitaxel [52, 53]. Transport and apoptosis induction was reported in hepatocellular carcinoma cells as a result of treatment with camptothecin-deoxycholic acid derivate [54].

According to the literature available, in this study we report for the first time the ability of deoxycholic acid sodium salt and its metal Zn(II), Cu(II) and Ni(II) complexes to decrease viability and 2D/3D growth of human HT29 and HT29-OxPt colorectal cells.

Conclusions

In conclusion, three new complexes of Cu(II), Ni(II) and Zn(II) with deoxycholate ligand were synthesized and characterized on the basis of elemental analysis, FTIR, UV-Vis, thermal analysis, conductivity and magnetic measurements. Biological investigations have demonstrated that these complexes manifest more pronounced cytotoxic activity both in human colorectal cancer cells and in resistant to oxaliplatin malignant cells as compared to the free ligand. Our findings represent a goal for a promising experimental treatment of colorectal cancer with metal complexes with deoxycholate as ligand and deserve further investigations.

Acknowledgement

This study was supported by (Project DFNP-17-89 by the Bulgarian Ministry of Education and Science under the National Research Programme "Young scientists and postdoctoral students" approved by DCM # 577 / 17.08.2018; National Science Fund, Bulgarian Ministry of Education and Science (Grant №ДКОСТ 01-10 from 22.10.2018); COST Action CA16119 "CellFit" and a bilateral project between Bulgarian Academy of Sciences and Romanian Academy.

Conflict of interest

The authors declare no conflict of interest.

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