

Article

Investigating the Effect of Cyclodextrin Nanosponges and Cyclodextrin-Based Hydrophilic Polymers on the Chemical Pharmaceutical and Toxicological Profile of Al(III) and Ga(III) Complexes with 5-Hydroxyflavone

Claudiu Radu ¹, Andreea Alexandra Olteanu ^{2,*} , Corina Cristina Aramă ^{2,*} , Mirela Mihăilă ³ 
and Valentina Uivarosi ¹ 

¹ Department of General and Inorganic Chemistry, Faculty of Pharmacy, “Carol Davila” University of Medicine and Pharmacy, 6 Traian Vuia, 020956 Bucharest, Romania; claudiu.radu@drd.umfcd.ro (C.R.); valentina.uivarosi@umfcd.ro (V.U.)

² Department of Analytical Chemistry, Faculty of Pharmacy, “Carol Davila” University of Medicine and Pharmacy, 6 Traian Vuia, 020956 Bucharest, Romania

³ Stefan S. Nicolau Institute of Virology, 285 Mihai Bravu Street, 030304 Bucharest, Romania; mirela.mihaila@virology.ro

* Correspondence: andreea.olteanu@umfcd.ro (A.A.O.); corina.arama@umfcd.ro (C.C.A.)

Abstract: In the present study, the complexes of aluminum and gallium with 5-hydroxyflavone were evaluated for their interaction with cyclodextrin polymers, as well as for the pharmacological effect of their inclusion. The cyclodextrin polymers were synthesized using diphenylcarbonate as a crosslinking agent, resulting in a lipophilic nanosponge (DPCNS), and pyromellitic dianhydride, resulting in a hydrophilic polymer (PMDACD). The inclusion complexes were synthesized and characterized via IR spectrometry and thermal analysis. The effect on the solubility of the metal complexes was also studied, where the hydrophobic nanosponge did not lead to an increase in solubility, but on the contrary, in the case of Al, it decreased; meanwhile, in the case of the hydrophilic polymer, the solubility of the metal complexes increased with the amount of polymer added. The cytostatic effect of inclusion complexes was investigated on two cell lines with different localizations, human colon adenocarcinoma (LoVo) and human ovarian adenocarcinoma (SKOV-3). The cytostatic efficacy is increased compared to simple complexes with efficacy on LoVo cells. Compared between the two metals, gallium complexes proved to be more active, with the efficacy of gallium complexes with the PMDACD being approximately the same as that of cisplatin, an antitumor agent used in therapy.

Keywords: cyclodextrin; nanosponge; 5-hydroxyflavone; aluminum complex; gallium complex



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1. Introduction

Flavones are a class of naturally occurring compounds, resultants of plant metabolic processes [1,2], and found in a variety of plant products. Flavones fulfill different roles in protecting plants against various external factors such as microorganisms, herbivores, or UV radiation [3].

Flavones have sparked interest in broad medical research due to their antioxidant [4], antibacterial [5], antiviral [6], anti-inflammatory [7,8], anticancer [9,10], anti-ischemic [11], hypolipidemic [12], antimutagenic [13], and immune-stimulating properties [14].

From a chemical point of view, the basic structure is a benzopyran nucleus to which a benzene ring is grafted. The backbone structure is commonly described in the literature as C6-C3-C6 (Figure 1) [15–17].

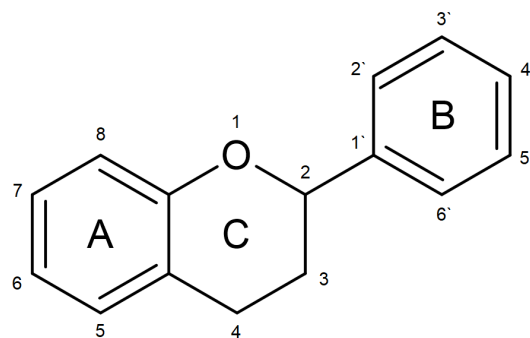


Figure 1. The general chemical structure of flavonoids.

5-Hydroxyflavone, or primuletin (5-hydroxy-2-phenyl-4*H*-1-benzopyran-4-one) (Figure 2), is a naturally occurring flavone identified in *Primula* [18] and *Dionysia* species [19] and has a single hydroxyl group at the 5 position on the flavone core. Thus, 5-hydroxyflavone has two adjacent centers that can donate electrons to chelate metal ions and, along with the planarity of the molecule, primuletin can act as a bidentate ligand in complexation reactions with metal ions [20,21].

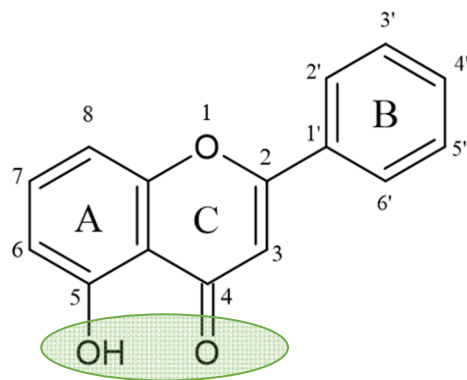


Figure 2. Structure of 5-hydroxyflavone.

Metal complexes of 5-hydroxyflavone with magnesium [22], zinc [22], copper [23], iron [23], chromium [24,25], samarium [21], europium [21], gadolinium [21], terbium [21], aluminum [26], gallium [26], and indium [26] have been described in the literature, revealing that through complexation processes, the pharmacological properties of ligands can be modified, with the effects of complexes being in most cases more pronounced than those of ligands alone [21,23,25,27–31].

As the limited solubility of complexes in aqueous media was considered a potential disadvantage [32,33], new techniques have been developed over time to overcome this impediment [34]. In particular, cyclodextrin-derived nanosponges as transport systems have been obtained through the polycondensation of cyclodextrin molecules with crosslinking agents. By joining cyclodextrin molecules, a three-dimensional network is formed. In the network cavities, substances are incorporated, thereby increasing the solubility [35–37].

For example, camptothecin is a substance with limited solubility in water and chemical instability due to the lactone ring [38]. To overcome these drawbacks, the active substance has been incorporated into nanosponges. The superior antitumor activity of the formed inclusion complex was demonstrated by in vivo and in vitro studies [39]. Cyclodextrin-derived nanosponges represent a biocompatible and nontoxic carrier agent solution for preclinical studies, improving the pharmacokinetic and pharmacodynamic profiles of the transported substances [40]. CRLX101 represents a novel nanosubstance synthesized using a novel method called “self-assembly”. Camptothecin is covalently linked to the cyclodextrin polymer using a crosslinker. This derivative polymer can additionally encapsulate

camptothecin molecules, and the potential of this nanomedicine has been studied in clinical trials on a diverse range of cancers such as lung, renal, and ovarian [41].

The purpose of the present study is to characterize the interactions between aluminum and gallium complexes with 5-hydroxyflavone and the three-dimensional structure of cyclodextrin nanosponges and evaluate the pharmacological properties of the complexes.

2. Materials and Methods

All reagents and solvents were of analytical reagent grade. 5-hydroxyflavone aluminum complex (Al-5HF) and 5-hydroxyflavone gallium complex (Ga-5HF) were prepared using 5-hydroxyflavone, $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$, and Ga_2O_3 purchased from Sigma Aldrich Chemical Co., Schnellendorf, Germany. Diphenyl carbonate (DPC) (Alfa Aesar GmbH & CO KG, Karlsruhe, Germany), pyromellitic dianhydride (PMDA), β -cyclodextrin (β CD, MW ~1135) (Sigma Aldrich, Darmstadt, Germany), dimethyl sulfoxide (DMSO), triethylamine (TEA), and acetone (Merk KGaA, Darmstadt, Germany) were used to obtain β -cyclodextrin polymer complexes.

Metal complexes Al-5HF and Ga-5HF were previously prepared and characterized by Munteanu et al. [26].

The pharmaceutical substances Cisplatin (CisPt), PBS/1 mM EDTA, L-Glutamine (Glu), Penicillin (100 units/mL), Streptomycin (100 $\mu\text{g}/\text{mL}$), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Propidium Iodide (PI), and RNase A were purchased from Sigma Aldrich (St. Louis, MO, USA). The Annexin V-FITC kit was purchased from Becton Dickinson Biosciences (San Jose, CA, USA).

The deionization of water was realized using a PureLab Chorus 1 Complete apparatus (Abu Dhabi, United Arab Emirates).

Al-5HF and Ga-5HF Assay

Al-5HF and Ga-5HF concentrations were determined using UV spectrometry at 302 nm, using an Able Jasco V-730 UV-Vis spectrophotometer (Budapest, Hungary). Measurements were performed in 1 cm quartz cells, against water, as the cyclodextrins and the cyclodextrin polymer do not absorb at the selected wavelength. The specific absorbance $A_{1\text{cm}}^{1\%}$ for Al-5HF was 488.34 and 414.17 for Ga-5FTH, respectively.

Cyclodextrin polymers preparation (PMDA-CD)

To prepare the β -cyclodextrin polymers, β -CD and PMDA were measured at a molar ratio of 1:4 and dissolved in 50 mL of DMSO at room temperature, using magnetic stirring. Subsequently, 2 mL of triethylamine was added, and stirring was maintained for 3 h. Precipitation of the polymer was started by adding acetone. Decantation (overnight) was used to remove acetone and the polymer was retrieved through vacuum filtration using a G4 filtration crucible and then dried in an oven at 30 °C. The method was described in a previously published protocol [42].

β -cyclodextrin nanosponges (DPCNS)

β -cyclodextrin and DPC were weighted at a molar ratio of 1:4 and dissolved in DMF. The solution was heated at 80 °C and stirred for 10 min. TEA solution was added, and the mixture was stirred for 3 h at 300 RPM. At the end of the reaction, gelation occurred. The reaction mass was cooled to room temperature and distilled water was added until the precipitation of the polymer. The polymer was recovered via vacuum filtration through a G4 filtration crucible, washed in ethanol to eliminate unreacted reagents, and then dried in the oven at 30 °C. The nanosponges were prepared by adapting a previously mentioned protocol, by Singh et al. [43].

Preparation of Al-5HF- and Ga-5HF-loaded nanosponges

The loaded nanosponges were prepared using two methods:

1. Al-5HF, Ga-5HF, and DPCNS (1:5) were added in a mortar and triturated using dichloromethane until solvent evaporation (further, on the following notations, Al-5HF-DPCNS1 and Ga-5HF-DPCNS1 will be used).

2. Al-5HF, Ga-5HF, and DPCNS (1:5) were suspended in 25 mL of water and the suspensions were stirred overnight. After decantation for 24 h, the supernatant was removed and the precipitates were dried in the oven for 24 h at 50 °C (further, on the following notations, Al-5HF-DPCNS2 and Ga-5HF-DPCNS2 will be used).

Preparation of Al-5HF- and Ga-5HF-loaded polymers

The preparation of PMDA CD polymers loaded with Al-5HF and Ga-5HF was performed via trituration and the procedure was similar to the one used for loading DPCNS nanosponges (from this point forward, the notations Al-5HF-PMDACD1 and Ga-5HF-PMDACD1 will be used).

Physicochemical characterization

Fourier-Transform Infrared Spectroscopy (FT-IR)

IR absorption spectra were recorded on a FT-IR Bruker Alpha spectrometer, equipped with ATR diamond crystal.

Thermal analysis

Thermal analyses, including differential scanning calorimetry (DSC), were conducted using equipment from Mettler Toledo, specifically the DSC3 module (Mettler Toledo International GmbH, Zürich, Switzerland). DSC measurements were performed under controlled atmospheric conditions (N₂ 20 mL/min) using 40 µL aluminum pans sealed with punched lids, within a temperature range of 25–500 °C and a temperature gradient of 10 °C. DSC samples were accurately weighed using an XSR105DU Mettler Toledo (Columbus, OH, USA) balance. Each sample was analyzed in triplicate.

Solubility studies

In total, 10 mg of Al-5HF and Ga-5HF were transferred individually into a 100 mL volumetric flask, and the volumetric flask was brought to volume with distilled water. The resulting suspensions were sonicated for 5 min and filtrated through a 0.45 µm Nylon filter membrane (Whatman® Puradisc™, Merk, Darmstadt, Germany). For each of the obtained solutions, the absorbance was measured at 302 nm and the water solubility of Al-5HF and Ga-5HF was calculated.

In a similar way, 4 mixtures have been prepared—10 mg of Al-5HF and 50 mg of PMDA (mixture 1); 10 mg of Al-5HF and 50 mg of DPCNS (mixture 2); 10 mg of Ga-5HF and 50 mg of PMDA (mixture 3); 10 mg of Ga-5HF and 50 mg of DPCNS (mixture 4) were transferred individually into a 100 mL volumetric flask and the volumetric flask was brought to volume with distilled water. The obtained suspensions were also sonicated for 5 min and filtrated through a 0.45 µm Nylon filter membrane.

Phase solubility diagrams

Phase solubility studies were conducted following the method outlined by Higuchi and Connors [44]. An excess amount of metal complex (approximately 10 mg) was added to increasing concentrations of cyclodextrin polymers and 2.5 mL of water, then shaken for 24 h at 25 ± 0.5 °C. The suspensions were allowed to equilibrate for 2 h. Afterward, samples were filtered through a 0.45 µm Nylon filter membrane (Whatman® Puradisc™, Merk, Darmstadt, Germany) and the absorbance was measured at 302 nm to calculate the concentration of Al-5HF and Ga-5HF.

Loading capacity and entrapment efficiency

The content of 5-hydroxyflavone (5-HF) in the β-cyclodextrin macromolecules was determined through a reverse-phase high-performance liquid chromatography (RP-HPLC) method. The loaded polymers and nanosponges (5 mg) were transferred into a 100 mL volumetric flask, dissolved in methanol, and brought to volume with the same solvent. The samples were sonicated for 5 min, then analyzed on Waters Alliance 2695 HPLC (Milford, MA, USA) with a PDA 996 detector using a C18 column (Inertsil ODS, GL Sciences, Tokyo, Japan, 150 mm × 4.6 mm × 5 µm).

The mobile phase was composed of water, acetonitrile (350:650, *v/v*), in a flow of 1 mL/min, and detection set at 270 nm.

The entrapment efficiency (EE%) and loading capacity (LC%) were calculated using Equations (1) and (2),

$$EE\% = \frac{W_{\text{recovered}}}{W_{\text{theoretical}}} \cdot 100 \quad (1)$$

$$LC\% = \frac{W_{\text{recovered}}}{W_{\text{loaded NS}}} \cdot 100 \quad (2)$$

where $w_{\text{recovered}}$ is the quantity determined using HPLC assay, $w_{\text{theoretical}}$ is the theoretical quantity of the metal complex found in the loaded nanosponges, and $w_{\text{loaded NS}}$ is the quantity of the loaded nanosponges.

Cell cytotoxicity

Cell culture and treatment

Human cancer cell lines of human colon adenocarcinoma (LoVo) and human ovarian adenocarcinoma (SKOV-3) were obtained from the American Type Culture Collection (ATCC). Human umbilical vein endothelial cells (HUVECs), a normal cell line, were used as a reference. Adherent cells were regularly maintained in DMEM: F12 medium, supplemented with 2 mM L Glutamine, 10% fetal bovine serum, 100 units/mL of penicillin, and 100 µg/mL of streptomycin. The cells were incubated at 37 °C in a humidified atmosphere with 5% CO₂. Once the cells reached approximately 60% confluence, they were treated with different concentrations of the compounds for various periods. After treatment, cells in the flasks were detached using a nonenzymatic PBS/1 mM EDTA solution, washed twice in PBS, and used for proliferation/cytotoxicity assays and apoptosis. Throughout the study, all untreated cells were designated as control cells.

Cytotoxicity assay

All experiments were carried out in triplicate using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). The reagent mixture used contained 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), inner salt, and phenazine ethosulfate (PES), which has high chemical stability and can be combined with MTS to form a stable solution. An MTS-based colorimetric assay was performed in 96-well microtiter plates with a flat bottom (Falcon). The assay was based on the ability of metabolically active cells to reduce MTS, a yellow tetrazolium salt, to the colored formazan that is soluble in the culture medium. Each well contained 1×10^4 cells in 100 µL of culture medium cultured for 24 h. After discarding the culture supernatants, normal and cancer cells were treated with increasing concentrations of compounds or oncolytic drugs for 24 and 48 h. After incubation, 20 µL reagents containing MTS and PES were added to each well. The plates were then incubated for 4 h at 37 °C with mild agitation every 15 min. The reduction in the tetrazolium compound to formazan was measured spectrophotometrically at $\lambda = 492$ nm using a Dynex plate reader (DYNEX Technologies MRS, Chantilly, VA, USA). The percentage of viability compared to untreated cells (considered 100% viable) was calculated using Equation (3).

$$\text{Cell viability (\%)} = (\text{absorbance of treated cells} - \text{absorbance of culture medium}) / (\text{absorbance of untreated cells} - \text{absorbance of culture medium}) \times 100 \quad (3)$$

The mean $\pm$ standard deviation (SD) of the experiments obtained in triplicate was used to calculate the percentage of viability compared to untreated cells [45,46].

Apoptosis analysis

The Annexin V-FITC kit from BD Biosciences performed the apoptosis assay, following the manufacturer's instructions. The cells were treated with an experimental compound. They were then resuspended in a cold binding buffer. To determine the percentage of apoptotic cells, each tube was completed with 5000 cells/sample and 400 µL of Annexin V

binding buffer for 15 min, in the dark, at room temperature. Finally, the cells were analyzed using a FACS CantoII flow-cytometer (Becton Dickinson—BD). Using DIVA 6.2 software, the analysis was performed in order to distinguish between viable cells (FITC-PI[−]), necrotic cells (FITC+PI⁺), early apoptosis (FITC+PI[−]), and late apoptosis [47,48].

3. Results and Discussion

Physicochemical Characterization

IR Spectra

In the FT-IR spectra (Figure 3a,b) of all loaded polymers (all IR spectra are presented in Supplementary Figures S1–S8), a broad band at 3400–3200 cm^{−1} characteristic of hydroxylic groups from cyclodextrin polymers was revealed. For DPCNS complexes, a band at approximately 1750 cm^{−1} was identified due to the stretching vibration of double bond C=O from the carbonate group of the crosslinker. For PMDACD complexes, a band at approximately 1720 cm^{−1} was observed, generated by the double bond C=O from the carboxylic free groups of the polymer. The band observed at 1650 cm^{−1} was determined by the keto group of the 5-hydroxyflavone ligand. The mentioned band suffered a displacement from 1638 cm^{−1} in the IR spectrum of metal complexes alone to 1650 cm^{−1} in the loaded polymers. The displacement was generated through the interaction of metal complexes with the macromolecular structure of β -cyclodextrin polymers.

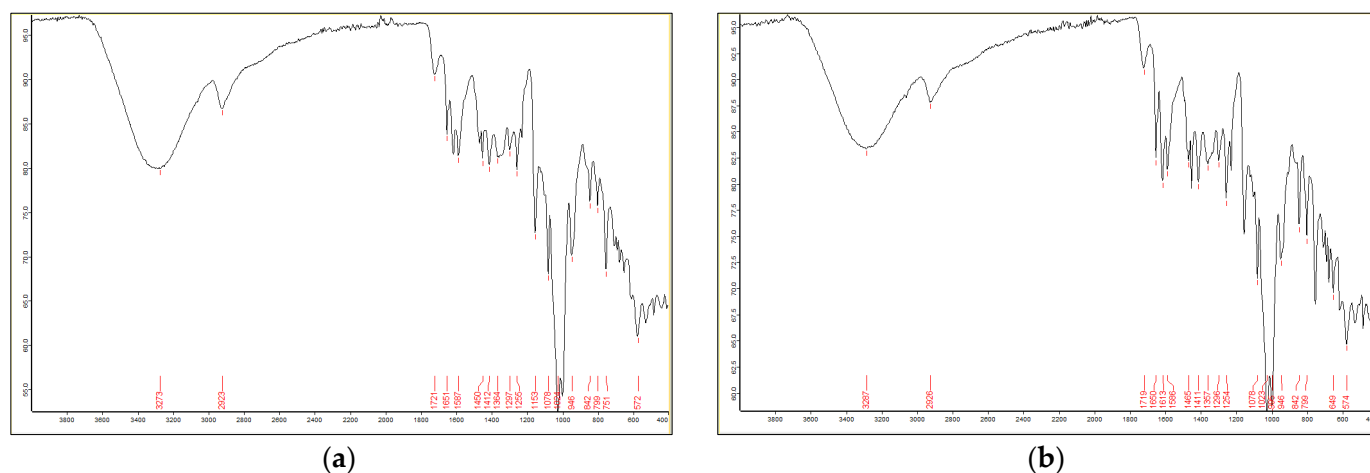


Figure 3. FT-IR spectra of (a) Al-5HF-PMDACD1 and (b) Ga-5HF-PMDACD1.

Thermal analysis

DSC analysis of Ga-5HF and Al-5HF revealed a flat stage between 25 and 300 °C and a sharp small endothermic peak visible at around 300 °C. When metal complexes are included in DPCNS and PMDACD, the DSC curves have a shape like the DSC curve of the polymer, revealing a wide endothermic region at 100–120 °C, corresponding to the loss of retained water. The small endothermic peak at 300 °C disappears, indicating the inclusion of Ga-5HF and Al-5HF in the cyclodextrin cavities and polymer pores (Figure 4). On the DSC curves of Ga-5HF-PMDACD1 and Al-5HF PMDACD1, a small endothermic peak appears at 150–200 °C, corresponding to the melting point of traces of 5-hydroxyflavone (probably formed during complex preparation).

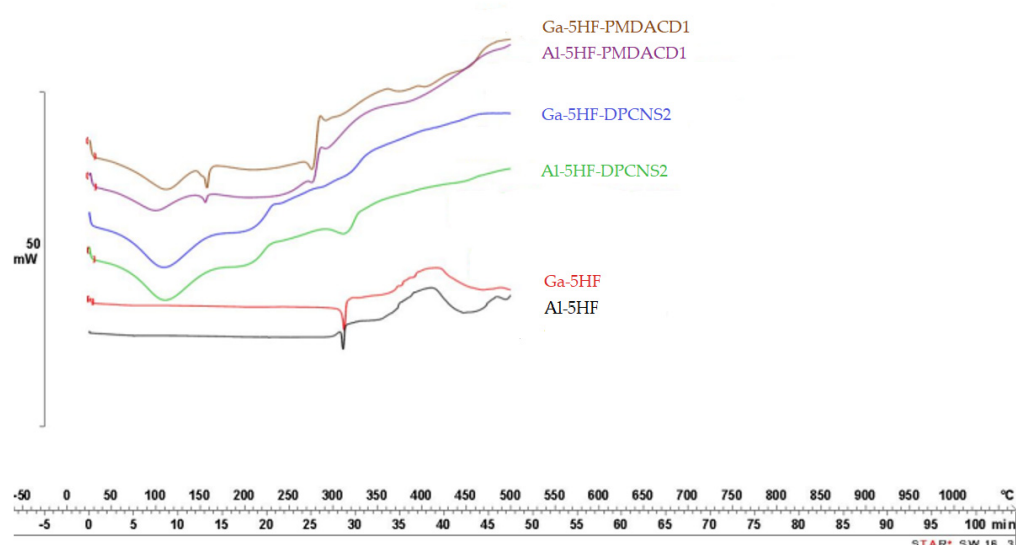


Figure 4. DSC curves of Ga-5HF (red), Al-5HF (black), Ga-5HF-DPCNS2 (blue), Al-5HF-DPCNS2 (green), Al-5HF-PMDACD1 (purple) and Ga-5HF-PMDACD1 (brown).

Solubility studies

Solubility data of the metal complexes in the presence of DPCNS and PMDACD are presented in Figure 5. An increase in solubility was observed for both metal complexes, as PMDACD is a hydrophilic polymer. The solubility of Al-5HF increased 9 times and Ga-5HF 16 times. In the case of DPCNS, a decrease in solubility can be observed due to the high degree of lipophilicity compared to the PMDACD polymer.

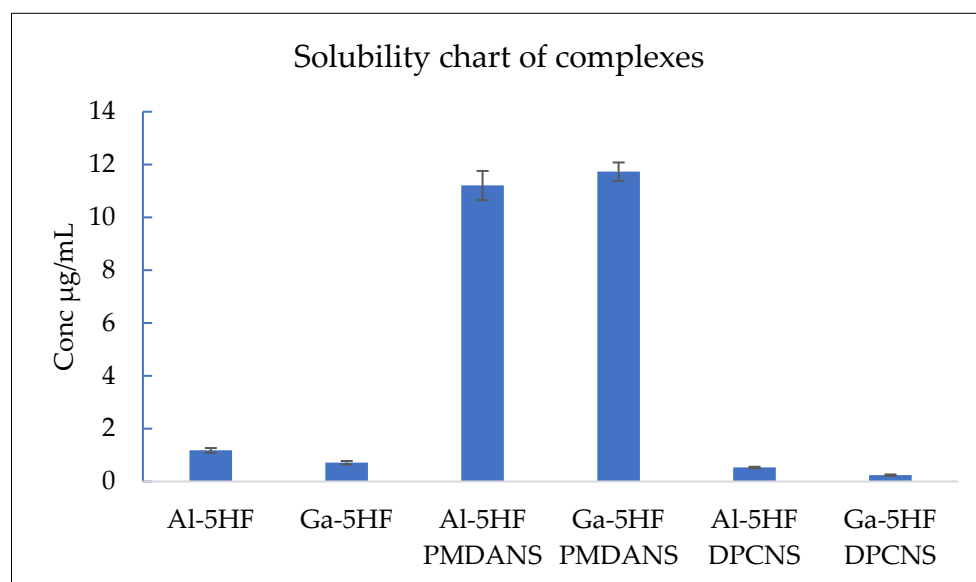


Figure 5. Concentration of the metal complexes in water, alone, and in the presence of cyclodextrin nanosponges.

Phase diagram studies

Solubility studies were performed according to the method reported by Higuchi and Connors [44]. The phase diagram study has been carried out to observe the evolution of the metal complexes in the presence of increasing amounts of cyclodextrin polymers.

Due to the hydrophobic character of the nanosponge, B-type phase solubility diagrams (Figure 6) were obtained for DPCNS loaded with Ga-5HF and Al-5HF, suggesting the

formation of limited solubility complexes. The solubility of the Al-5HF complex decreased with increasing amounts of NS added, while the solubility of the Ga-5HF complex increased with the increasing amounts of NS added up to a point where it reached a plateau [49].

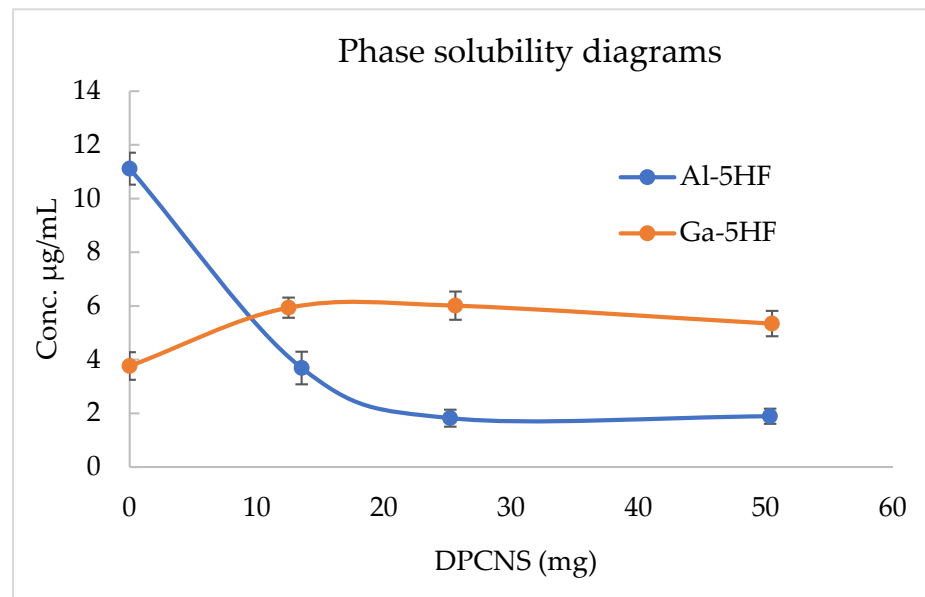


Figure 6. The variation of metal complexes concentration in the presence of increasing quantities of DPCNS.

The phase solubility diagrams for PMDADC loaded with Ga-5HF and Al-5HF are AL-type and indicate the formation of soluble complex. The solubility of both the Al-5HF and Ga-5HF complex increased with the increasing amount of PMDADC. The effect occurred due to the chemistry of the β -cyclodextrin polymer, which presents free carboxylic groups, thereby conferring a hydrophilic character. In the results presented in Figure 7, the dependence on the amount of polymer added is linear, with a correlation coefficient close to 0.99 for both metal complexes.

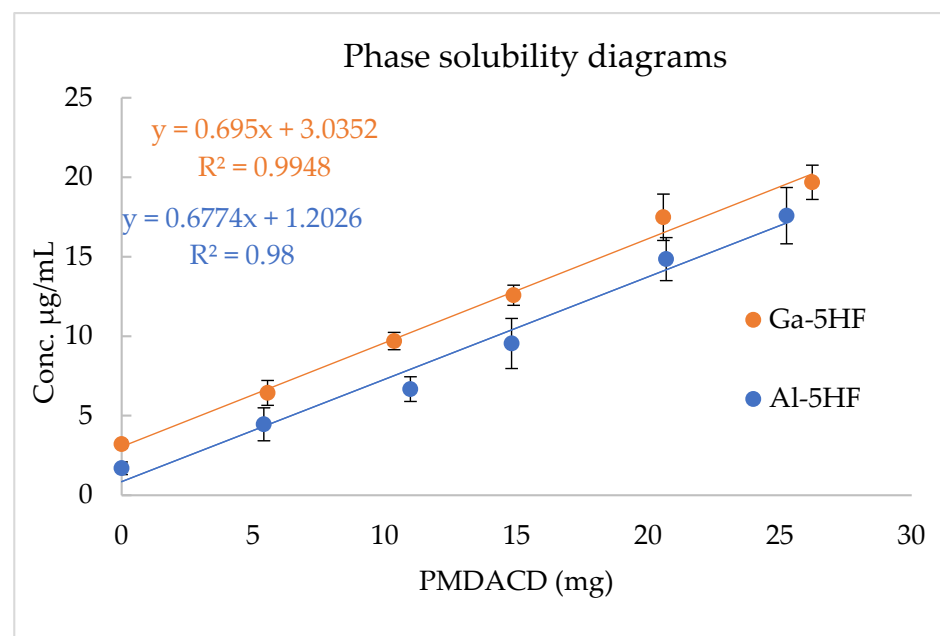


Figure 7. The variation in metal complex concentrations in the presence of increasing quantities of PMDANS.

The loading capacity and entrapment efficiency

The loading capacity and entrapment efficiency were determined based on Equations (1) and (2). The results presented in Table 1 show an entrapment efficiency between $64.96 \pm 1.92\%$ and $1.00 \pm 1.73\%$, with the highest value for Ga-5HF-DPCNS2. The loading capacity showed results between $10.43 \pm 0.31\%$ and $13.82 \pm 0.26\%$ with the highest value for the same compound. Moreover, it can be observed that in comparing the metal complexes, higher values were obtained for Ga compounds.

Table 1. Entrapment efficiency and loading capacity for loaded polymers.

Loaded Polymers	Entrapment Efficiency (%)	Loading Capacity (%)
Al-5HF-DPCNS1	69.63 ± 2.62	11.18 ± 0.42
Ga-5HF-DPCNS1	75.28 ± 0.78	11.43 ± 0.12
Al-5HF-DPCNS2	81.35 ± 1.11	13.07 ± 0.18
Ga-5HF-DPCNS2	91.00 ± 1.73	13.82 ± 0.26
Al-5HF-PMDACD1	64.96 ± 1.92	10.43 ± 0.31
Ga-5HF-PMDACD1	84.77 ± 3.04	12.87 ± 0.46

Cell cytotoxicity

The growth and spread of tumors are marked by an increase in cell proliferation and a decrease in the process of apoptosis. The signals that encourage cell proliferation result in uncontrolled growth, which is a significant characteristic of cancer. Apoptosis, or programmed cell death, on the other hand, is a process that typically disposes of damaged, unnecessary, or potentially harmful cells [50].

Specific mechanisms inside the cells can prevent cell apoptosis during tumor formation, which can lead to tumor cell proliferation. It is essential to develop chemical molecules that can trigger apoptotic processes in tumor cells, as it can help eliminate cancerous cells [51].

Our study aimed to investigate the effect of specific compounds on the LoVo and SKOV-3 human tumor cells compared to normal cell line HUVECs. We analyzed the impact of these compounds on cell proliferation and apoptosis. The study's positive control was the cytostatic CisPt, which is commonly used to treat colon and ovarian cancer. We exposed the cell lines to different concentrations of the compounds for 24 and 48 h. To determine the apoptotic process, we used double labeling with annexin V-FITC/PI and flow cytometry. This method helped us identify the population of cells in early or late apoptosis and those in necrosis. Furthermore, we compared the effects of the analyzed compounds on tumor cells to those on normal cells.

The obtained data revealed differences in the effectiveness and specificity between the compounds we studied compared and the clinically used cytostatic. In analyzing the impact of these compounds on the apoptotic processes and proliferation, we were able to gain a more comprehensive understanding of their mechanism of action.

The effect of the compounds on cell viability

According to viability tests, the studied compounds have either a toxic or antitumor effect on LoVo and SKOV-3 human tumor cells or HUVEC human normal cells. The percentages of cell viability were calculated for each compound and cell line, and the cytotoxic effects varied depending on dose, time, and cell type. We conducted dose–effect curves for each compound to determine the most effective concentration, testing concentrations ranging from 0.3125 to 50 $\mu\text{g/mL}$. The curves show how a compound's concentration affects cell viability.

The data indicate that tumor or normal cells' viability depends on the concentration. The cells were treated with the study compounds for either 24 h or 48 h at concentrations ranging from 0.3125 to 50 $\mu\text{g/mL}$. The findings can help select the optimal working dose and develop strategies to minimize the compound's side effects on normal cells.

The effects of the studied compounds were analyzed compared to Cisplatin, the classic drug with known antitumor effects [52,53]. HUVEC cells were treated with different

concentrations of the analyzed compounds for 24 h (Figure 8a) and 48 h (Figure 8b). The viability tests demonstrated that regardless of the structure, at 24 h, the analyzed compounds affected the viability of normal cells HUVEC only at concentrations equal to or higher than 12.5 $\mu\text{g/mL}$. In the case of the Ga complex and its derivatives, at 24 h, the viability of normal HUVEC cells is affected when cells are treated with concentrations higher than 6.25 $\mu\text{g/mL}$.

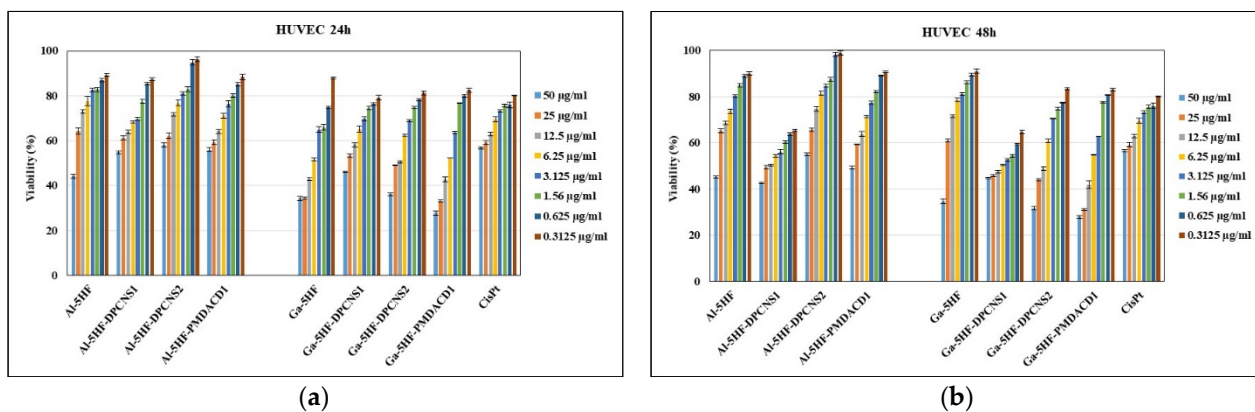


Figure 8. The impact of the tested compounds on the viability of normal HUVEC cells after (a) 24 h of treatment and after (b) 48 h of treatment.

The analysis of the obtained viability data following the treatment of the LoVo tumor line with the Ga complex shows that after 24 h of treatment, it acts like the reference cytostatic (CisPt), significantly inhibiting cell viability regardless of the concentration. The cells were treated with the study compounds for either 24 h (Figure 9a) or 48 h (Figure 9b), at concentrations ranging from 0.3125 to 50 $\mu\text{g/mL}$.

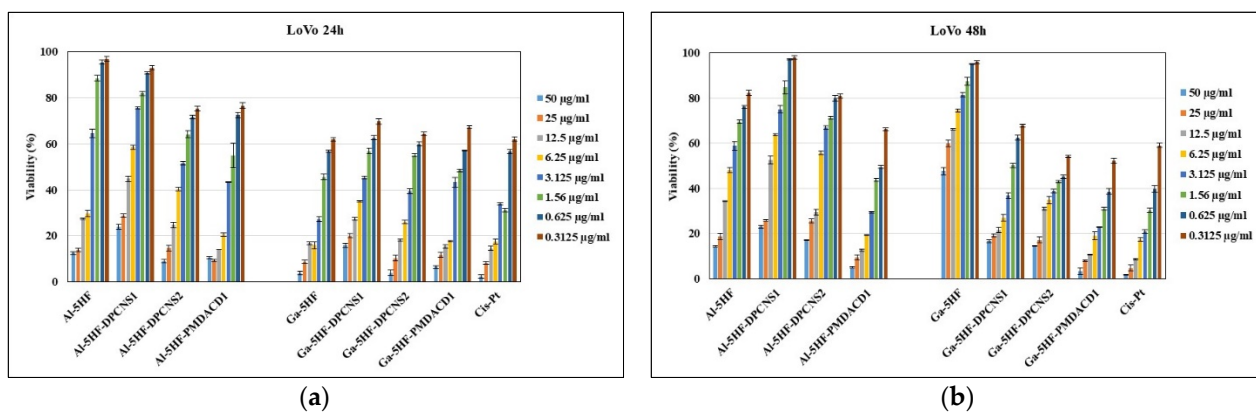


Figure 9. The impact of the tested compounds on the viability of LoVo colon cancer cells after (a) 24 h of treatment and (b) 48 h of treatment.

The treatment of LoVo tumor cells for 24 h with the analyzed compounds showed that the Al-5HF compound, at low concentrations (1.56 $\mu\text{g/mL}$), killed 11.2% of the tumor cells and the Al-5HF-DPCNS1 and Al-5HF-DPCNS2 complexes killed 18.1% and 35.9%, respectively (Figure 9a).

In the case of some compounds, such as Ga-5HF-DPCNS1, Ga-5HF-DPCNS2, and Ga-5HF-PMDACD1, their actions after 48 h treatment indicate a much stronger effect than that induced by Ga-5HF. The effect of the compound Ga-5HF-PMDACD1 is comparable to that of CisPt, inhibiting LoVo cell viability below 50%.

The SKOV-3 human ovary tumor cells were treated with analyzed compounds in different concentrations for 24 h (Figure 10a) or 48 h (Figure 10b).

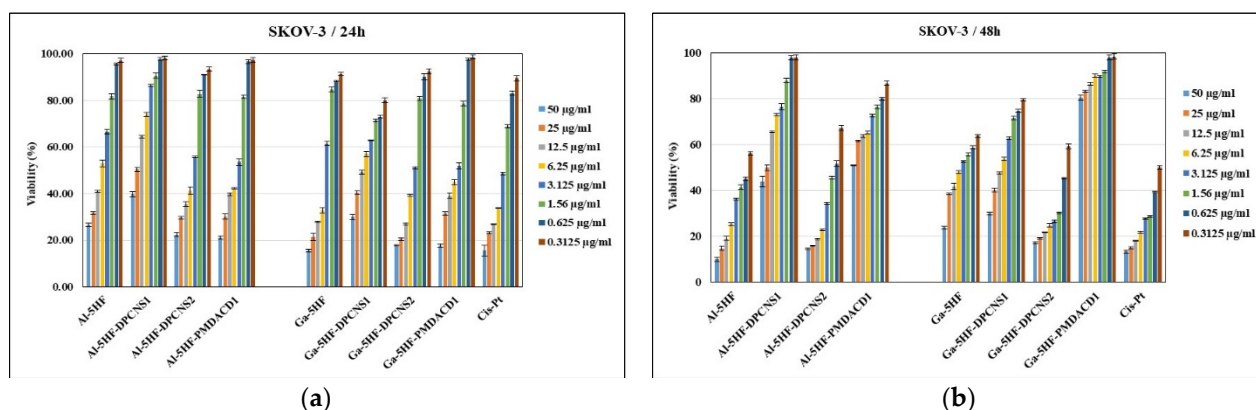


Figure 10. The impact of the tested compounds on the viability of SKOV-3 ovary cancer cells after (a) 24 h of treatment and (b) 48 h of treatment.

In the case of SKOV-3 ovarian tumor cells, the treatment for 24 h with the compounds Al-5HF-DPCNS1 and Al-5HF-DPCNS2 at a concentration of 5 µg/mL managed to kill about 40% and 50%, respectively, of the tumor cells. The compound Al-5HF-DPCNS2 at this concentration has a similar effect to Cisplatin. Treatment with the same compounds at a concentration of 0.5 µg/mL kills 5% and 10%, respectively, of the tumor cells.

Extending the treatment for 48 h with the Al-5HF-DPCNS2 and Ga-5HF-DPCNS2 compounds significantly reduces the viability of tumor cells depending on the concentration, similar to Cisplatin.

The inhibitory concentration values (IC) of the analyzed compounds or CisPt were calculated using the linear regression analysis from the cytotoxicity data of each compound and for different percentages of cell lysis corresponding to drug concentrations. Therefore, the corresponding drug concentrations required to induce the lysis of 50% (IC₅₀) of the cells were calculated when the cytotoxicity of compounds was assessed via MTS assay after 24 or 48 h of their incubation with HUVEC, LoVo, and SKOV-3 cells compared to cisplatin as positive control (Table 2).

Table 2. Values (µg/mL) of IC₅₀ for studied complexes and CisPt.

Compound	HUVEC		LoVo		SKOV-3	
	24 h	48 h	24 h	48 h	24 h	48 h
Al-5HF	9.05 ± 0.79	8.74 ± 0.77	4.75 ± 0.99	4.53 ± 0.80	5.53 ± 0.86	1.63 ± 1.14
Al-5HF-DPCNS1	9.03 ± 1.11	6.22 ± 0.67	5.61 ± 1.11	5.78 ± 1.04	7.38 ± 0.79	7.43 ± 0.85
Al-5HF-DPCNS2	9.52 ± 0.65	9.54 ± 1.17	3.92 ± 1.07	4.84 ± 1.09	5.07 ± 1.06	2.39 ± 0.99
Al-5HF-PMDACD1	9.13 ± 0.80	8.35 ± 1.05	3.38 ± 0.95	2.14 ± 0.94	5.16 ± 1.13	8.77 ± 1.12
Ga-5HF	5.41 ± 0.92	7.99 ± 0.84	2.21 ± 1.03	8.25 ± 0.83	4.73 ± 0.72	4.08 ± 0.82
Ga-5HF-DPCNS1	7.72 ± 0.58	5.40 ± 1.09	3.48 ± 1.01	2.96 ± 1.06	5.65 ± 0.90	5.55 ± 1.10
Ga-5HF-DPCNS2	6.47 ± 0.93	6.07 ± 1.02	2.88 ± 0.59	1.73 ± 1.16	4.69 ± 1.18	0.89 ± 0.87
Ga-5HF-PMDACD1	5.34 ± 1.02	4.27 ± 0.81	2.71 ± 0.70	0.49 ± 0.95	5.12 ± 1.05	20.03 ± 1.17
CisPt	10.07 ± 0.83	8.42 ± 0.79	2.00 ± 1.21	0.97 ± 0.92	4.39 ± 0.97	2.41 ± 1.20

The analyzed compounds were studied for their impact on the apoptotic process

The process of programmed cell death, known as apoptosis, is significantly obstructed in tumor cells. This ensures cell proliferation and tumor progression. To identify compounds with potential therapeutic effects, the study analyzes how certain substances affect the apoptotic process and selectively induce apoptosis in cancer cells (LoVo and SKOV-3) without harming normal cells. The effects of the studied compounds on the apoptotic process of both normal and tumor cells were analyzed using flow cytometry.

For this purpose, we analyzed the effect induced by Al and Ga complexes and their derivatives on the apoptotic process of LoVo and SKOV-3 tumor cells compared to normal

HUVEC cells. The analysis of the cell viability data led to the choice of two reference concentrations (0.5 and 5 $\mu\text{g/mL}$) for which the cells were treated for 24 h (Figure 11a) and 48 h (Figure 11b).

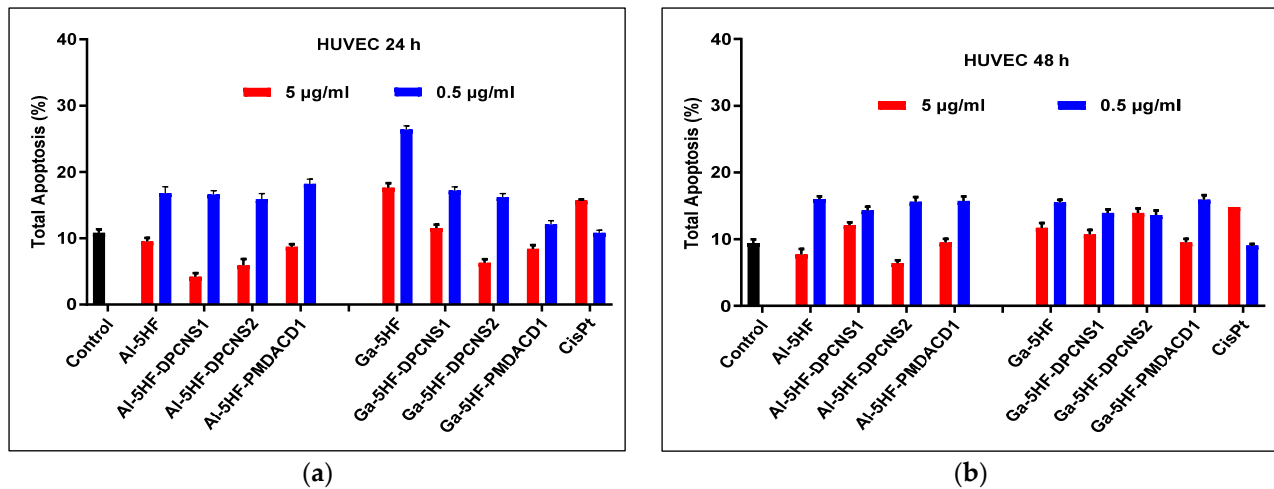


Figure 11. Effect of the analyzed compounds on the apoptotic process of the HUVEC normal cells after (a) 24 h of treatment and (b) 48 h of treatment.

Normal untreated HUVEC cells (control) showed a small percentage of apoptosis that was not influenced by the duration of treatment (24 h—10.8%; 48 h—9.4%).

Normal HUVEC cells were treated for 24 h with compounds Al-5HF, Al-5HF-DPCNS1, and Al-5HF-DPCNS2 at a concentration of 0.5 $\mu\text{g/mL}$, which induced an increase in apoptosis compared to untreated HUVEC cells (control).

The treatment of HUVEC cells with Ga-5HF-DPCNS2 for 48h did not influence apoptosis, regardless of the concentration used.

The 24 h treatment with Ga-5HF-DPCNS1 did not influence the apoptosis of LoVo cells, regardless of the concentration used (Figure 12a). Similar results were obtained when LoVo cells were treated for 48 h with Ga-5HF (Figure 12b).

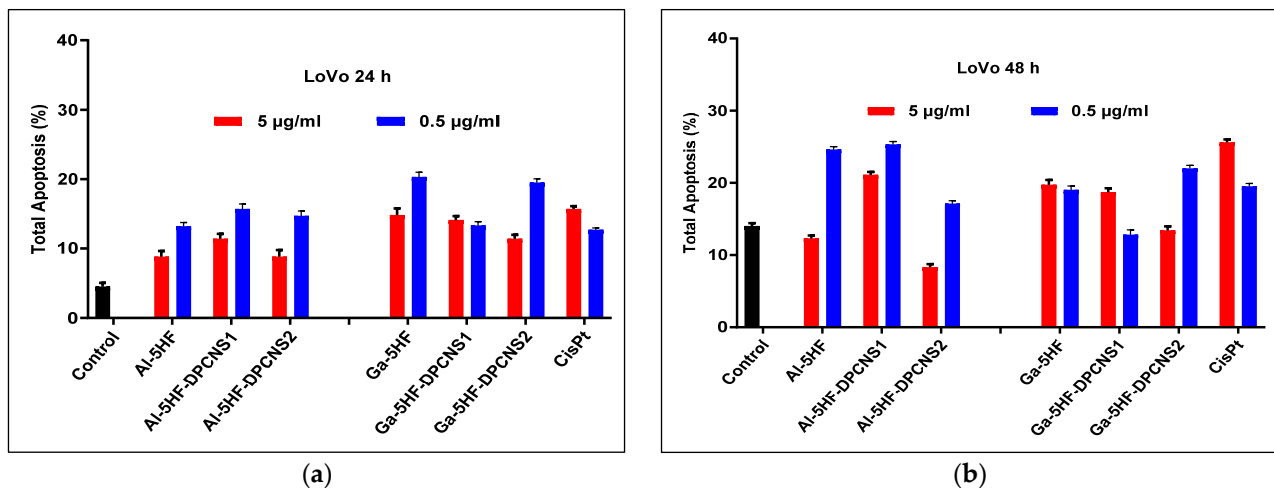


Figure 12. Effect of the analyzed compounds on the apoptotic process of the LoVo colon cancer cells after (a) 24 h of treatment and (b) 48 h of treatment.

The compounds Al-5HF and Al-5HF-DPCNS1, at 5 $\mu\text{g/mL}$ and 0.5 $\mu\text{g/mL}$, increased the percentage of apoptosis by two times and three times, when the LoVo tumor cells were treated for 24 h.

The results obtained after the treatment of LoVo tumor cells with the studied compounds, both at 24 and 48 h, are compared with those obtained in the HUVEC cell line. At 24 h, there are no significant differences in the apoptotic process compared to the normal cell line at the concentration of 0.5 $\mu\text{g/mL}$. Instead, the more extended treatment, up to 48 h, at the concentration of 0.5 $\mu\text{g/mL}$, affects the apoptotic process more significantly, inducing a 1.5-fold increase compared to 24 h.

The treatment of SKOV-3 tumor cells with the compound Al-5HF for 24 h did not affect the apoptotic process regardless of concentration.

Similar results were obtained when SKOV-3 cells were treated for 24 h with Al-5HF-DPCNS2 and Ga-5HF-DPCNS2 at a concentration of 5 $\mu\text{g/mL}$. The SKOV-3 tumor cells were treated with compounds Al-5HF-DPCNS2 and Ga-5HF-DPCNS2, at a concentration of 0.5 $\mu\text{g/mL}$, which induced a rise in apoptosis two times versus the control at 24 h treatment (Figure 13a).

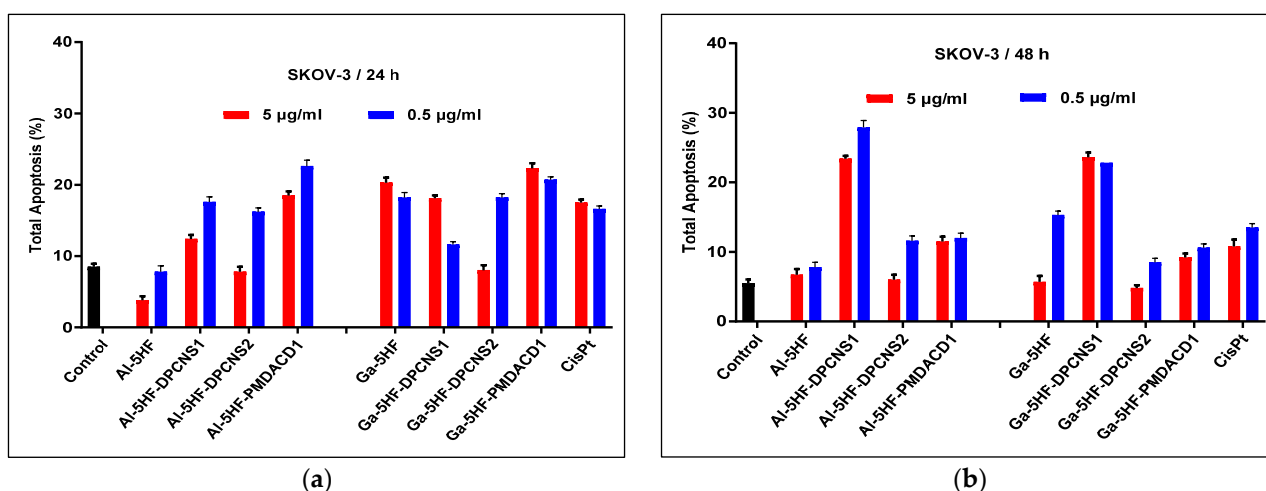


Figure 13. Effect of the analyzed compounds on the apoptotic process of the SKOV-3 ovarian cancer cells after (a) 24 h of treatment and (b) 48 h of treatment.

Treating SKOV-3 tumor cells with the compounds Al-5HF, Ga-5HF-DPCNS2, and Ga-5HF-PMDACD1 for 48h did not affect the apoptotic process regardless of concentration (Figure 13b).

The results obtained following the treatment of SKOV-3 tumor cells with the studied compounds at 24 and 48 h are compared with those obtained in the HUVEC cell line. At 24 h, significant differences in the apoptotic process are obtained compared to the normal cell line at the two concentrations used. A more extended treatment, up to 48 h, at both concentrations induces a decrease in the apoptotic percentage compared to HUVEC cells, except for the compounds Al-5HF-DPCNS1 and Ga-5HF-DPCNS1.

4. Conclusions

The data analysis obtained in the experiments performed on two tumor cell lines with different localizations showed that the complexes studied with Al and Ga acted differently on the tumor processes. The results emphasized that the complexes with Al and Ga can act as cytostatic agents depending on the concentration and time of treatment, as well as the type and origin of the tumor cells. In conclusion, the hydrophilic complexes Al-5HF-PMDACD1 and Ga-5HF-PMDACD1 significantly affect LoVo colon tumor cells. Simultaneously, the hydrophobic complexes Al-5HF-DPCNS2 and Ga-5HF-DPCNS2 were the most effective on SKOV-3 tumor cells, the results for both cell lines being comparable with CisPt.

Within the studies on target identification and structure optimization for increased cellular uptake, the possibility of entrapping the complexes in cyclodextrin-based nanosponges

or hydrophilic reticulate CD polymers is an actual and interesting approach. The inclusion complexes of Al-5-HF and Ga-5HF with DPCNS and polyPMDACD were characterized. When comparing the cytostatic activity of Ga-5HF and Al-5HF and their prepared supramolecular structures, the Ga complex and its derivatives proved to be much more effective than the Al complex.

The obtained data encourage the elaboration of further research directions of the study, implying the evaluation of cell cycle phases. The development of new molecular targets and the evaluation of their potential apoptotic effects through cellular and molecular mechanisms are considered future perspectives of the present research.

The studied compounds may open new pathways for cancer treatment leading to improved therapeutic response in ovarian or colorectal cancer.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/app14135441/s1>: Figures S1–S8: FT-IR spectra of Al-5HF, Ga-5HF, Al-5HF-PMDACD1, Ga-5HF-PMDACD1, Al-5HF-DPCNS1, Ga-5HF-DPCNS1, Al-5HF-DPCNS2, respectively, and Ga-5HF-DPCNS2.

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