UNIVERSITY OF MEDICINE AND PHARMACY

„CAROL DAVILA”

BUCHAREST

DOCTORAL SCHOOL

PHARMACY

Study of supramolecular inclusion systems based on metal complexes with biological action

PHD THESIS ABSTRACT

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1. The fundamental problem

One of the leading causes of death is cancer. Globally, about one in six people fall victim to the disease. By 2022, nearly 20 million new cancer cases and 9.7 million deaths have been reported. Estimates suggest that about one in five men or women develop cancer in their lifetime, while about one in nine men and one in 12 women die from malignant neoplasms [1,2].

2. Hypothesis

The antitumor activity of flavones is well known and well documented in the literature. These simple molecules exhibit a multitude of mechanisms by which they exert their anticancer properties, namely, modulate the activities of reactive oxygen species (ROS) scavenging enzymes, induce apoptosis, participate in cell cycle arrest and autophagy, suppress cancer cell proliferation and invasiveness [3,4].

By complexation of flavones with metal ions, pharmacokinetic and pharmacodynamic profiles may be altered, with increased potency of pharmacological effects [5–7]. The disadvantage of flavone metal complexes is low solubility in water. Therefore, carrier systems can be used to counteract this disadvantage, which may improve the pharmacokinetic properties of poorly soluble substances.

The research hypothesis was based on the attempt to maximize the antitumor properties of flavones by improving their pharmacokinetic and pharmacodynamic properties by incorporating metal complexes into carrier systems.

3. Objectives

In the global context in which the discovery of new anticancer treatments is needed, the research conducted in this paper aligns with modern trends in the development of new strategies in antitumor therapy. Starting from the promising antitumor effect of 5-hydroxyflavone compared to other derivatives of the same class [8], as well as its complexes with Al^{3+} and Ga^{3+}, characterized by low water solubility, during this work we evaluated the possibilities of improving the pharmacokinetic and pharmacodynamic profile of the mentioned complex combinations by incorporating these compounds in two transport systems, obtaining supramolecular inclusion systems.

4. Research methodology

The research methodology aimed to synthesize complexes of 5-hydroxyflavone with aluminium and gallium and to characterize these complexes. These two complexes were

subsequently incorporated into two types of carrier systems, namely cyclodextrin based nanosponges and liposomal systems, forming inclusion complexes. These were characterized and subjected to a cytotoxicity and apoptosis study on two standardized human cell lines derived from ovarian malignant cells (SKOV-3) and colorectal tumors (LoVo).

5. Metal complexes of aluminium and gallium with 5-hydroxyflavone

Synthesis of complex combinations of 5-hydroxyflavone with Al^{3+} and Ga^{3+} metal ions was carried out using a general procedure consisting of mixing solutions of metal salt and 5-hydroxyflavone in ethanol in a molar ratio of 1:3, adjusting the pH of the final solution to 6 to favor ligand deprotonation, followed by heating the mixture of reactants under reflux for 3 hours.

To characterize the metal complexes, infrared (FT-IR), ultraviolet-visible (UV-VIS) spectra, and thermal analysis: differential scanning calorimetry (DSC) and thermogravimetry (TGA) were evaluated. These techniques were used to determine the interactions that occur to form the metal complexes. *SwissADME* web service was used for *in silico* evaluation of the metal complexes, thus estimating some properties influencing the pharmacokinetic profile of the molecules. The metal complexes violate two Lipinski rules, exhibiting a logP greater than five and a molecular weight greater than 500 Da, but according to Veber's computational model, the metal complexes comply with the rules for oral bioavailability assessment.

6. Cyclodextrin based nanosponges as drug carrier systems for complexes of aluminium and gallium with 5-hydroxyflavone (inclusion complexes)

The synthesis of nanosponges is based on the condensation reaction of cyclodextrins with a crosslinking agent. The cyclodextrin used was β-cyclodextrin, and the crosslinking agents were: diphenylcarbonate (DPC) resulting in a predominantly hydrophobic nanosponge (DPCNS), and pyromellitic dianhydride (PMDA), resulting in a more hydrophilic polymer (PMDACD). Due to these macromolecules with different degrees of lipophilicity, we wished to observe the influence of this variable on the solubility and pharmacodynamic effects of the analyzed complexes.

The effect of cyclodextrin based nanosponges on the solubility of metal complexes was determined. The water solubility of the complexes is low (1.18 μg/mL for Al-5HF and 0.71 μg/mL for Ga-5HF). In the presence of cyclodextrin nanosponge DPCNS, the solubility is even lower, due to the hydrophobic character of the macromolecule. Regarding the pyromellitic

dianhydride polymer, the condensation of the cyclodextrin molecules with the cross-linking agent leaves free carboxyl groups, which increase the hydrophilicity of the macromolecule. Therefore, by the interaction of the metal complexes with the PMDACD polymer, the water solubility of the aluminium complex increased by about 9.5 times, and in the case of gallium by about 16.5 times.

Solubility phase diagrams were also determined according to Higuchi and Connors model [9]. The quantitative determination of the analytes was performed spectrophotometrically. In the case of Al-5HF complex in the presence of DPCNS a decreasing trend was observed, the solubility of the formed inclusion complex is very low, thus in the presence of nanosponge the metal complex starts to precipitate immediately, reaching a plateau at about 2 µg/mL. In the case of gallium complex a B_s-type, phase-solubility diagram was observed, where the nanosponge initially increases the solubility of the complex, reaching a plateau, after which a decreasing trend follows.

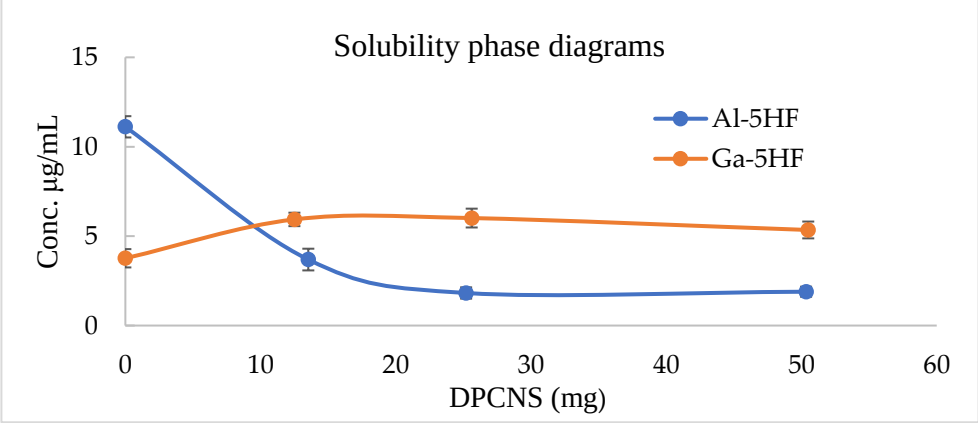

Figure 1. Solubility phase diagrams in the presence of DPCNS

In the presence of PMDACD polymer, the solubility of Al-5HF and Ga-5HF metal complexes increases with the amount of PMDACD added. Thus, the phase-solubility diagrams are A_L type, where the dependence is linear with a correlation coefficient R^2 of 0.9805 for aluminium and 0.9948 for gallium.

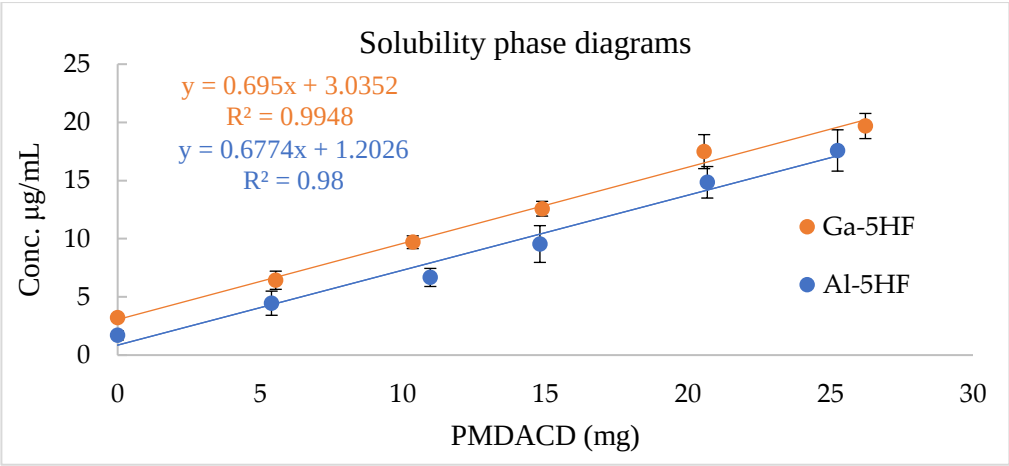

Figure 2. Solubility phase diagrams in the presence of PMDACD

Inclusion complexes were prepared by including aluminium and gallium metal complexes into the cross-linked three-dimensional network of the nanosponge. This inclusion was performed in aqueous suspension, followed by drying, resulting in Al-5HF-DPCNS2 and Ga-5HF-DPCNS2, and mechanochemically by grinding, resulting in Al-5HF-DPCNS1 and Ga-5HF-DPCNS1, respectively Al-5HF-PMDACD1 and Ga-5HF-PMDACD1. The inclusion complexes were characterized by FT-IR spectrometry, UV-VIS spectrometry and thermal DSC and TGA analysis. By corroborating these techniques, interactions occurring between the metal complexes and nanosponge macromolecules were demonstrated.

In order to evaluate the inclusion process, the loading capacity and the encapsulation efficiency of the nanosponges were determined by a reversed-phase high-performance chromatographic technique (RP-HPLC). The encapsulation efficiency showed values between 64.96 ± 1.92 for the Al-5HF-PMDACD1 inclusion complex and 91.00 ± 1.73 for Ga-5HF-DPCNS2. In close correlation are also the results from the loading capacity, which ranged between 10.43 ± 0.31 for Al-5HF-PMDACD1 and 13.82 ± 0.26 for Ga-5HF-DPCNS2. Moreover, higher values can be observed for gallium complexes compared to aluminium complexes.

7. Liposomes as drug carriers for complexes of aluminium and gallium with 5-hydroxyflavone

The liposome preparation method was based on the lipid layer hydration technique, and was based on the solubilization of lipophilic substances in organic solvents, followed by their evaporation to obtain a lipid film. The resulting film was subsequently rehydrated with an

appropriate solution to form multilamellar liposomal vesicles (MLVs), which were repeatedly extruded through polycarbonate membranes to obtain unilamellar liposomal vesicles (SUVs).

Two liposomal formulations were prepared for each complex. The first formulation contains the studied complex entrapped in a matrix of Phospholipon 90G and Tween 80 as surfactant. The denominations of these liposomal formulations in the present study were: Al-5HF-lip1 (for aluminium metal complex) and Ga-5HF-lip1 (for gallium metal complex). The second formulation additionally contains cholesterol. The denominations of these liposomal formulations in the present study were: Al-5HF-lip2 (for aluminium metal complex) and Ga-5HF-lip2 (for gallium metal complex). The cholesterol added in the Al-5HF-lip2 and Ga-5HF-lip2 formulations is intended to confer additional structural stability to the liposomes. Cholesterol intercalates into the phospholipid bilayer structure, reducing membrane fluidity and increasing the mechanical strength of the liposomes.

Physicochemical characterization of the obtained liposomal formulations was carried out by evaluating the particle sizes of the resulting systems and the polydispersity index (PDI) using the DLS (dynamic light scattering) technique, as well as by evaluating their electrokinetic potential.

Liposomes of average sizes between 134 nm and 230 nm were obtained, with the Ga-5HF-lip2 formulations exhibiting the smallest sizes (133.98±8.34 nm). The PDI values obtained ranged between 0.2161±0.011 and 0.3220±0.027, indicating a uniform size distribution, with the most homogeneous formulation being Ga-5HF-lip2. This result suggests that this formulation has similar particle sizes, which is crucial for the stability and reproducibility of liposomes in clinical applications. The electrokinetic potential of the formulations ranged from -6.42±0.79 mV to -8.56±0.21 mV, indicating adequate electrostatic stability. Ga-5HF-lip2 exhibited the most negative electrokinetic potential (-8.56±0.21 mV), suggesting a higher colloidal stability, essential for preventing aggregation and maintaining a stable suspension.

8. Evaluation of in vitro cytotoxicity and apoptotic process of the studied complexes on human tumor cell lines and normal human cells

Two standardized human cell lines derived from ovarian malignant cells (SKOV-3) and colorectal tumors (LoVo) were used for cytotoxicity determination. The study aimed to investigate the effect of the studied compounds on LoVo and SKOV-3 tumor cells compared to the normal HUVEC cell line. We analyzed the impact of these compounds on cell proliferation

and apoptosis. The positive control of the study was the cytostatic cisplatin (CisPt), which is commonly used to treat colon and ovarian cancer. We exposed cell lines to different concentrations of the compounds for 24 and 48 hours, at concentrations ranging from 0.3125 to 50 µg/mL.

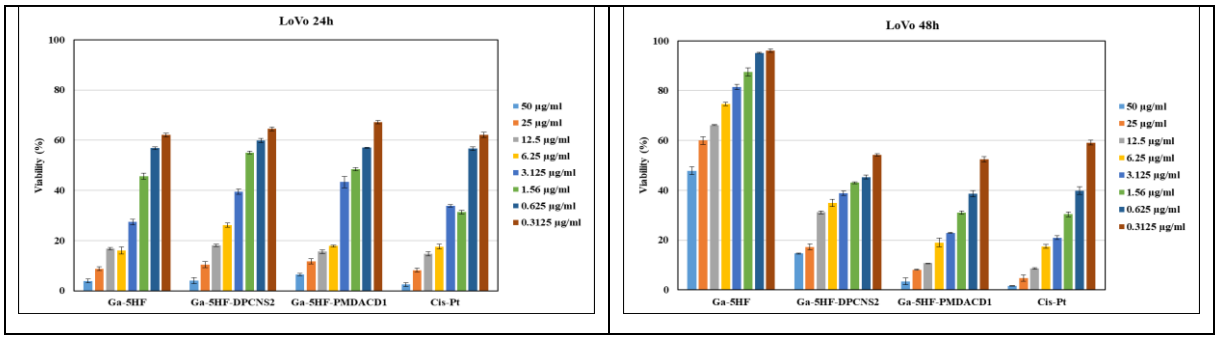

Figure 3. Impact of gallium compounds on the viability of LoVo colon tumor cells.

Analysis of the viability data obtained after the treatment of LoVo tumor line with the Ga-5HF-PMDACD1 complex shows that after 24 hours of treatment, it acts as the reference cytostatic (CisPt), significantly inhibiting cell viability at any concentration (Fig. 3).

A much more potent effect than that induced by Ga-5HF was observed after 48 hours of treatment with nanosponge complexes of gallium. The effect of the Ga-5HF-PMDACD1 compound is comparable to that of cisplatin, inhibiting the viability of LoVo cells below 50%.

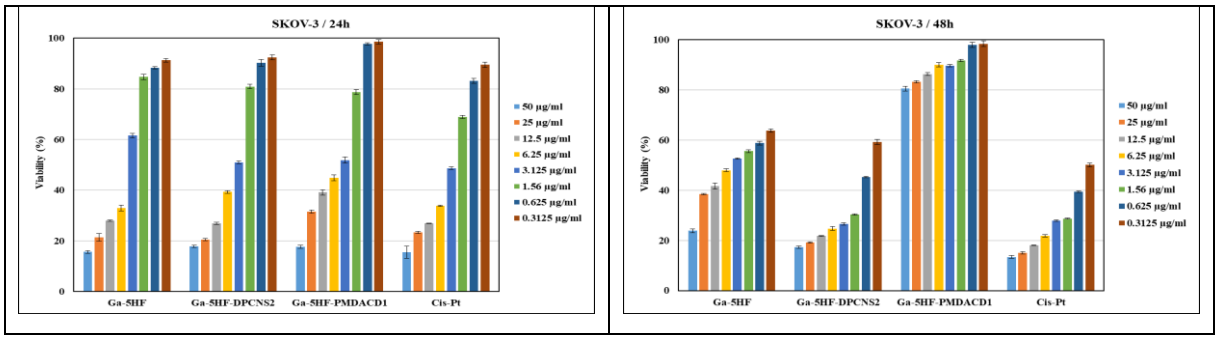

Figure 4**.** Impact of gallium compounds on SKOV-3 tumor cell viability.

In the case of SKOV-3 tumor cells, a large difference can be observed between the two types of nanosponges. The complexes of the hydrophilic polymer being much weaker in potency compared to those of the lipophilic DPCNS nanosponge, regardless of the metal ion. Comparing in terms of metal ions, a more pronounced activity is observed for the gallium complexes. Moreover, the Ga-5HF-DPCNS2 complex exhibits antitumor effects on this cell line similar to those of the anticancer agent used in therapy.

Regarding the apoptotic process, the most effective complexes were the liposomal complexes of gallium. Following treatment of SKOV-3 ovarian tumor cells with Ga-5HF-lip1 and Ga-5HF-lip2 compounds for 48 hours at the two concentrations, an increase in apoptotic percentages, almost twice as high as the cytostatic, is recorded (Fig. 5).

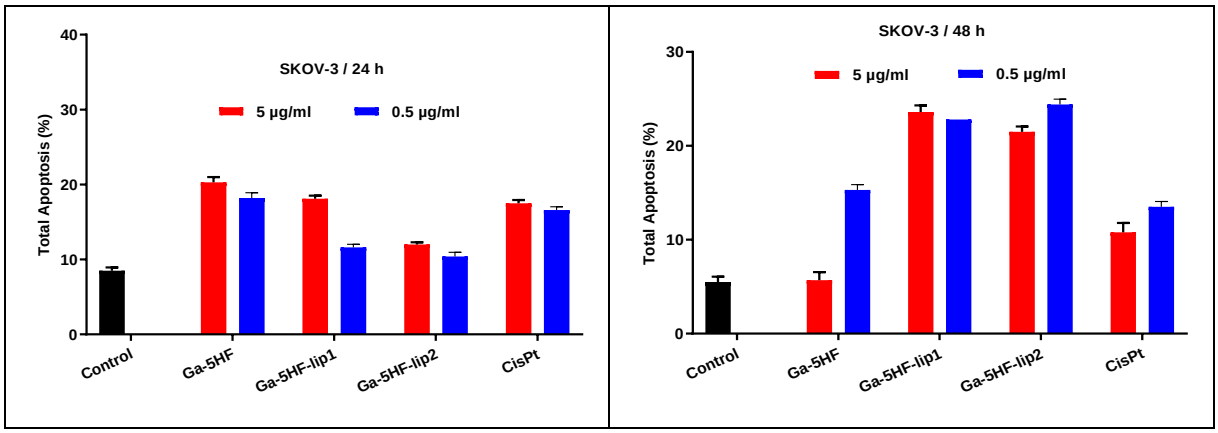

Figure 5. Effect of liposomal gallium complexes on the apoptotic process of SKOV-3 tumor cells

9. Conclusions and personal contributions

9.1. Conclusions

In the present research work we aimed to improve the pharmacokinetic and pharmacodynamic profiles of complexes of the natural substance 5-hydroxyflavone with the metal ions Al^{3+} and Ga^{3+}.

The studied metal complexes are described in the literature showing promising antitumor effects. In the present work, we synthesized the compounds based on literature methods and verified their identity and purity by FT-IR, UV-VIS and thermal analysis: TGA and DSC methods. Using *in silico* methods, in the present work we performed a predictive analysis of some pharmacokinetic properties of the studied metal complexes.

In order to maximize the pharmacodynamic effects and pharmacokinetic profiles of these compounds, we synthesized two types of carrier systems: cyclodextrin based nanosponges and liposomes.

We prepared two types of nanosponges, one hydrophilic and one hydrophobic using pyromellitic dianhydride and diphenylcarbonate as cross-linking agents, respectively. The confirmation of the formation of inclusion complexes was demonstrated by two different techniques, namely FT-IR and thermal TGA and DSC analysis.

In the solubility studies that were carried out, we demonstrated that inclusion in PMDACD hydrophilic nanosponge leads to a significant increase in the solubility of the metal complexes. A linear dependence of the complex concentration in the presence of increasing amounts of PMDACD nanosponge was observed through phase-solubility diagrams.

The second type of drug carriers was liposomal systems. We prepared two types of liposomes, in which the metal complexes of aluminium and gallium complexes with 5-hydroxyflavone were embedded.

Both types of supramolecular systems were studied for cytotoxicity and apoptotic process on LoVo and SKOV-3 human tumor cells compared to the normal HUVEC cell line. The positive control of the study was the cytostatic cisplatin, which is commonly used to treat colon and ovarian cancer.

PMDACD hydrophilic nanosponge inclusion complexes significantly affect LoVo colon tumor cells with a cisplatin-like effect, in the case of the gallium complex even superior to the antitumor on *in vitro* studies performed.

The hydrophobic nanosponge inclusion complexes Al-5HF-DPCNS2 and Ga-5HF-DPCNS2 were the most effective on SKOV-3 ovarian tumor cells, with cytostatic effects comparable to cisplatin.

In both transport systems studied, gallium complexes were found to have a more pronounced effect than aluminium complexes.

By comparing the two delivery systems, on both cell types, cyclodextrin-derived nanosponges were found to have superior cytotoxic action to liposomal systems.

Concerning the study of the apoptotic process, on LoVo tumor cells, all prepared supramolecular systems induced a cisplatin-like increase in apoptosis. SKOV-3 tumor cells underwent a different increase in apoptotic process: in the case of aluminium and gallium complexes with PMDACD hydrophilic nanosponge, the increase is similar to that induced by cisplatin, and in the case of liposomal complexes it is even higher than that induced by the antitumoral agent.

9.2. Personal contributions

The research work presents personal contributions to the development of novel carriers of molecules with antitumor effects, by this method improving certain properties that influence the pharmacokinetic and pharmacodynamic profile.

The originality is constituted by the realization of inclusion complexes formed between the metal complexes and the two studied transport systems, this association not being cited in the literature.

The original contributions of the PhD thesis include the completion of the physicochemical characterization of the studied metal complexes *(chapter 5),* the synthesis of nanosponges and nanosponge-inclusion complexes and their characterization *(chapter 6)*, the preparation and characterization of liposomal complexes *(chapter 7),* and the *in vitro* evaluation of the pharmacological effect of the formulations obtained in the PhD thesis *(chapter 8)*. The HPLC method for the quantitative determination of the ligand in *chapter 6* is also original.

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List with published articles

Papers published in ISI indexed journals (Clarivate) with impact factor

1. **Radu C**, Olteanu AA, Aramă C, Mihăilă M, Uivaroși V. 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. *Appl. Sci.*, 14, 5441, 2024. https://doi.org/10.3390/app14135441

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Factorul de impact (IF)/2023: 2,5 (Q1 - JCR Category Engineering, Multidisciplinary)

Article from the chapters 6 and 8 (pages 57-94 and 108-135).

2. **Radu C**, Olteanu AA, Arama CC, Uivarosi V. Hydroxiflavone Complexes with Biogenic and Abiogenic Metals. *Farmacia*, 72, 751–64, 2024. https://doi.org/10.31925/farmacia.2024.4.3

Indexing: Science Citation Index Expanded, SciVerse Scopus, Chemical Abstracts Service, EMBASE, SCImago Journal&Country Rank, IPA, British Library, SSCI, A&HCI, CPCI-S, CPCI-SSH, BKCI-S, BKCI-SSH, ESCI, CCR-EXPANDED, IC.

Impact factor (IF)/2023: 1,6.

Article from chapter 1 (pages 13-39).

Papers published as posters

1. **Radu C**, Olteanu AA, Uivarosi V, Aramă C. 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. European Cyclodextrin Conference, edition 7, 5-8 September, 2023, Bugapest, Hungary.
2. **Radu C**, Anuța V, Dinu-Pirvu CE, Mihăilă M, Uivarosi V. Investigating the effect of liposomal inclusion on the in vitro anticancer activity of new Al (III) and Ga (III) complexes with 5-hydroxyflavone. National Pharmacy Congress, edition XIX, 27-29 September, Cluj-Napoca, Romania.