UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA

PhD THESIS

-SUMMARY-

DESIGN OF A NEW CLASS OF LIPOSOMES WITH POTENTIAL OSTEOTROPIC PROPERTIES

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SUMMARY

Recent progress in the field of nanotechnology made possible the creation of specific systems which allow the active principles to be delivered to their target, thereby enhancing their therapeutic efficacy and reducing their systemic toxicity. Among nanoparticles, our attention has been attracted by liposomes, nanostructured lipid vesicles that can be decorated with various functional groups in order to direct them to a certain biological site. This thesis has as goal the synthesis of two compounds that will be used for the preparation of liposomes that could target the bones and could therefore be employed in the treatment of bone-related diseases.

The thesis is structured in two parts:

1. **The first part** presents **the state-of-art in the approached research domain** and it contains two chapters:

Chapter I is entitled “Nannoparticles, liposomes, drug vectors”. In this chapter, general notions regarding the nanoparticles are exposed, with an emphasis on liposomes. A short

presentation of liposomes' applications, advantages and disadvantages of their use in the therapeutic domain has also been realised. Several aspects regarding the concept of drug vectorisation, active and passive targeting, liposomes' generations and their *in vivo* fate have also been presented.

Chapter II, entitled "The design of an original class of osteotropic systems", introduces the subject of the thesis: the preparation of two amphipathic compounds that will be used for the preparation of liposomes able to selectively recognise bone hydroxyapatite (HA).

After a short presentation of bone structure, the osteotropic functional groups reported by now are reviewed. Among them, our attention has been attracted by hydroxybisphosphonates. Three scientific papers have reported the preparation of liposomes decorated with these functional groups, having affinity for the anorganic part of bones.

The originality of this PhD thesis consists in the introduction of tartronic acid as osteotropic functional group for liposomes' functionalization. The choice of this group is motivated by the structural similarities between the hydroxybisphosphonic and hydroxybiscarboxylic acids, as well as by a series of scientific papers which report the fact that tartronic acid derivatives affect bone metabolism and have HA affinity.

This paper has as goal to obtain two amphipathic compounds that will be used for the preparation of liposomes able to recognize and selectively bind to hydroxyapatite:

a) **The new amphiphilic compound proposed by us: 1-hydroxy-(12-cholesteryloxy-4,7,10-trioxa)dodecan-1,1-biscarboxylic Acid**, that will be named, for an easier designation, „Tartronic **A**cid -**T**riethyleneglycol -**Cholest**erol" conjugate or AT-TEG-Cholest. It will be used in order to prepare liposomes for which the affinity for HA will be evaluated. TA-TEG-Cholest has an original structure (**Figure 1**-compound **15**).
b) **An amphiphilic compound that will serve as witness: 1-hydroxy-(12-cholesteryloxy-4,7,10-trioxa)dodecan-1,1-bisfosfonic Acid,** that will be named, for an easier designation, „**H**ydroxy**b**is**p**hosphonic **A**cid-**T**riethyleneglycol-**Cholest**erol" conjugate or HBPA-TEG-Cholest (**Figure 1**-compound **16**). Its structure is similar to the structure of other reported compounds, which have the capacity to bind liposomes to HA. Its role is to validate the affinity tests between liposomes and HA and to make a comparison between

the affinity of the new compound TA-TEG-Cholest and that of a compound with HBPA functional groups in the same experimental conditions.

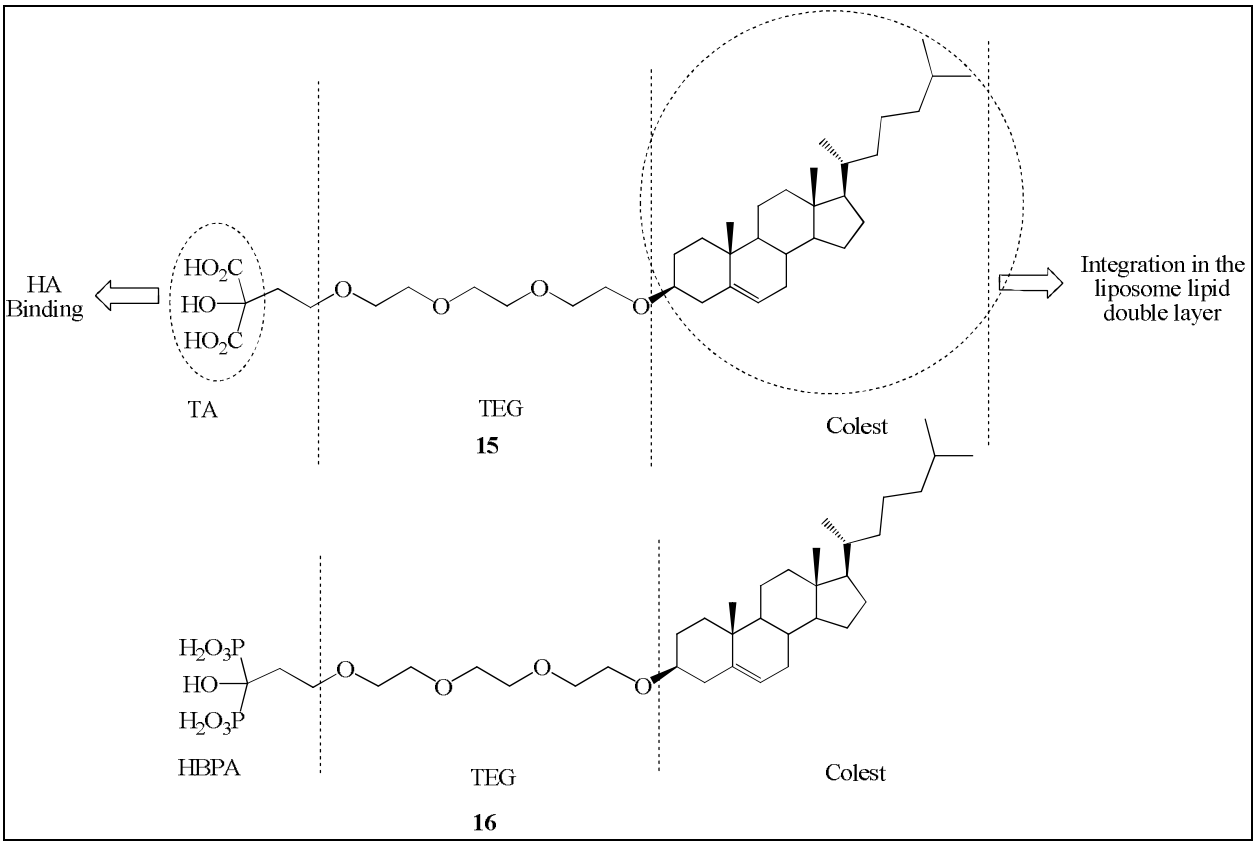

Figure 1: Chemical structure of the two amphiphilic compounds

The interaction between the liposomes decorated with osteotropic groups (in our case, HBPA, and, we hope, TA) with HA, and consequently with the bone, is schematically represented in a **Figure 2**. In order to increase the circulating time in blood, liposomes surface can be covered with polyethylene glycol (PEG), which is not represented in this figure for simplification.

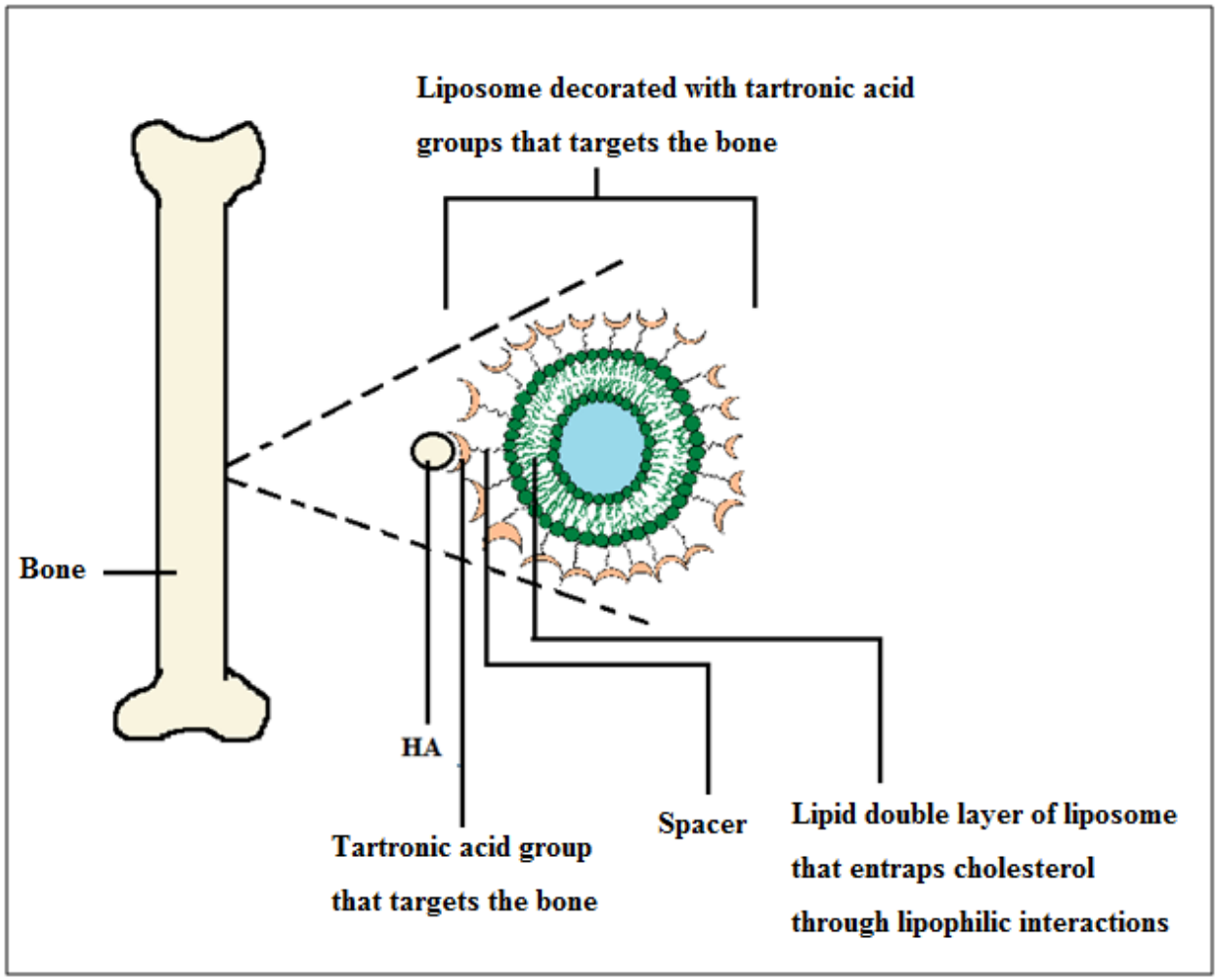

Figure 2: Schematic representation of the interaction between liposomes decorated with osteotropic groups with hydroxyapatite and bone

2. **The second part** is represented by **the personal contributions** and is structured in three chapters: the experimental part and two chapters containing the results and the discussions.

The experimental part represents the third chapter of the thesis.

The results and discussions part starts with the presentation of preliminary studies which consists in the comparative evaluation of the geometry, charge distribution and electrostatic potential generated by the two proposed compounds. These studies indicate that the two products have similar structure and physicochemical properties.

Chapter IV presents the preparation of HBPA-TEG-Cholest conjugate (**16**), through a 5 steps synthesis, realized using cholesterol as starting compound, with a global yield of 15%. All the intermediate compounds have been purified and analysed by MS, ^{1}H and ^{13}C NMR. Compound **16** has been analyzed by ^{1}H NMR, ^{31}P NMR and HPLC/MS, and these analyses have confirmed its structure.

The synthesized compound has a structure similar to that of other hydroxybisphosphonic acid derivatives reported in literature. The compound prepared during this PhD thesis is new (it contains a supplementary methylene group compared to a similar product reported by Hengst V. et al.), and its synthesis brings an element of originality when compared to the methods previously reported for the preparation of this compound, which corresponds to the 2 steps elongation of the alcoholic polyoxygenated linker with 3 carbon atoms and to the introduction of a carboxylic group. These two steps can be easily monitored by TLC, and they can be realized with basic laboratory equipment, because they do not involve any special experimental conditions.

Chapter V presents the preparation of TA-TEG-Cholest (**15**) through a 7 steps synthesis, starting from cholesterol. All the intermediate compounds have been isolated and characterized by mass spectrometry, ^{1}H and ^{13}C NMR. A series of problems appeared in the last step, of basic hydrolysis of the bromomalonate **23**, described in the thesis. Besides the desired conjugate **15**, this reaction leads to secondary products of decarboxylation and HBr elimination, which make difficult the purification of compound **15**. After many attempts to purify the compound by column chromatography, it has finally been purified by thin layer preparative chromatography. The obtained solid contains silica gel also, and the organic compound will be extracted in an appropriate solvent. The results obtained by analyzing the solid by LC/MS and its analysis by thin layer chromatography showed that it has been successfully isolated from the other organic compounds present in the mixture, this being actually the most difficult step of the purification.

Perspectives

After complete characterization of the two compounds **15** and **16**, *in vitro* tests of affinity between liposomes containing these ligands and HA can be realized. HA used in these tests can be a commercial powder or it can be obtained in laboratory by using different methods. In order

to determine the affinity between liposomes and HA, already reported methods can be employed, or new experimental procedures can be implemented. The tests will have as goal to quantify the interaction between:

- liposomes decorated with TA groups and hydroxyapatite;
- liposomes decorated with HBPA groups (witness liposomes) and hydroxyapatite;
- liposomes with cholesterol instead of ligand (control liposomes).

In order to study the interaction between functionalized liposomes and HA, the affinity tests can be realized by varying the percentages of incorporated ligands, of HA concentration, of liposomes.

The realization of affinity tests between liposomes functionalized with TA and hydroxyapatite is also important in order to respond to the question formulated by Hengst V., if the interaction of liposomes functionalized with HBPA and HA is caused simply by the negative charge found on liposomes' surface or by the HBPA group itself.

If the affinity tests prove to confirm the affinity of the new compound to HA, the research can afterwards advance to the consequent steps: (a) *in vivo* tests, in order to determine the percentage of liposomes that bind to bone; (b) the employment of tartronic acid in order to create other osteotropic systems. These can be obtained from the conjugation of TA with active principles, resulting products with bone targeting capacity, followed by the release of the active principle at the target by the activation of a group cleavable in certain conditions (for example, acidic conditions at tumor site). Other applications can consist in TA usage in order to direct other types of nanoparticles to bone: nanospheres, nanocapsules.

HBPA-TEG-Cholest **16** conjugate can be used in a series of individual tests. These tests can target: (a) the determination of quantitative interactions between liposomes functionalised with HBPA and HA by using a method that has not been used by now for this type of tests. In this purpose, the SPR ("Surface Plasmon Resonance") technology might be employed. The results could allow a comparison of the affinity obtained by using this new method with the affinities reported previously; (b) the study of quantitative interactions between liposomes decorated with HBPA and HA in presence and in absence of different PEG percentages that sterically stabilize liposomes' surface in order to prolong their half-life in blood.