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Interactions of pentacyclic triterpene acids with cardiolipins and related phosphatidylglycerols in model systems

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ABSTRACT

Pentacyclic triterpene acids (PTAs): betulinic (BAc), oleanolic (Ola) and ursolic (Urs) are potent pharmaceuticals applied in the therapy of cancer and bacterial infections. The mechanism of PTA action is multifactor, but the important step is their interaction with the lipids of mitochondrial and bacterial membranes. In our studies we applied the Langmuir monolayer technique to investigate the interactions between PTAs and cardiolipins (CLs) and phosphatidylglycerols (PGs). We applied two different mammalian mitochondrial CLs and one species extracted from the membrane of *Escherichia coli*. For comparison we performed the same experiments on the systems containing PTAs and 3 PGs strictly correlated structurally to the applied CLs. Our studies proved that PTAs can disturb the organization of CL-rich domains and affect the bacterial membrane fluidity by the interactions with phosphatidylglycerols, so anionic phospholipids are the targets of their membrane action. The thermodynamic interpretation of the results indicated that Urs has the highest membrane disorganizing potential among the 3 studied PTAs. The studies performed on model systems proved also that BAc can discriminate over structurally similar animal cardiolipin species, interacts specifically with BHCL — the main mammalian CL and can disturb its organization in the membrane. In contrast, Ola and Urs are much active as far as the interaction with bacterial CLs and PGs is concerned.

1. Introduction

Betulinic (BAc), oleanolic (Ola) and ursolic (Urs) acids are isomeric pentacyclic triterpene carboxylic acids (PTAs) of high pharmacological potential, which are isolated worldwide from different medicinal plants used in folk medicine [1–3]. The structures of these bioactive PTAs are summarized in Scheme 1.

As it is visible, PTAs are isomers which differ only in the structure of the last E ring. In BAc the ring E is five-membered and the carbon atoms C20, C29 and C30 are shifted to the isopropene substituent. In Ola and Urs ring E is six-membered cyclohexane. Ola and Urs differ in the location of the C30 carbon atom (CH_3 group). Additionally, in BAc all the rings are *trans*-fused leading to their coplanar location, whereas in Ola and Urs the junction between D and E rings is *cis* leading to the distortion of the E ring from the plane defined by A–D rings [4].

BAc, Ola and Urs are intensively investigated because of their multiple pharmaceutical activities combined with relatively low toxicity to normal eukaryotic cells. The most important here are the anticancer and chemopreventive properties [5–9]. Generally, PTAs are able to induce apoptosis of cancer cells on the mitochondrial pathway [10–13]. The mechanism of their action is not exactly elucidated but it is believed that their incorporation into mitochondria triggers the generation of reactive oxygen species (ROS) leading to the peroxidation of some mitochondrial membrane phospholipids and the following permeation [14]. The leak of mitochondrial complexes like cytochromes into the cytosol activates the caspase cascades leading finally to apoptosis. Taking into consideration the number of scientific papers, the next in the order of importance is the antimicrobial activity of PTAs [15,16]. Indeed, they proved to be bactericides both against Gram-positive and Gram-negative bacterial strains, and what should be here underlined, in some cases they were also active against bacterial strains exhibiting multidrug resistance [17,18]. PTAs were also promising antiviral substances, especially in HIV infections [19,20]. PTAs were also tested as drugs against diseases of different etiology from inflammation treatment [21], via obesity prevention [22] to Alzheimer disease therapy [23], to mention only some extremes of the research.

The described PTAs here differ significantly in their pharmaceutical potential, depending on the area of the research. In the field of anticancer therapy BAc turned out to be the most active and most versatile of them [8]. However, as far as the antibacterial activity is concerned, BAc is considered inactive in most of the performed studies in contrast

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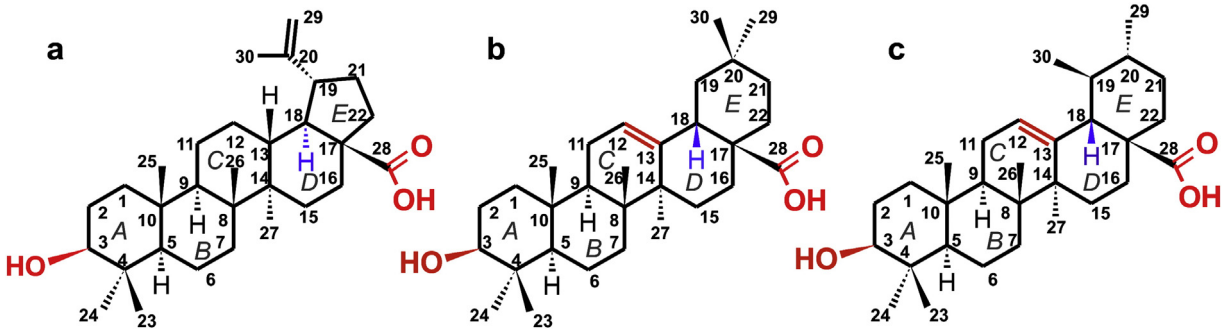

Scheme 1. Structural formulas of: a) BAc, b) Ola, and c) Urs. Black — skeletons of the ground triterpene alkanes: a) lupane, b) oleanane, and c) ursane. Red — polar groups and the additional double bond in ring C of Ola and Urs. Blue — the important chiral center on the ring D–ring E junction.

to isomers Ola and Urs [16]. Regarding Ola and Urs, they also differ mutually in their activity depending on the particular case. PTAs are surface active compounds which are structurally similar to steroids, originating also from squalene [24]. Therefore, their interactions with mitochondrial and bacterial membranes can be the crucial step in the mechanism of their action [25,26]. The hypothesis claiming that mitochondria evolved from bacteria is widely accepted, and one of the arguments of its supporters is the presence of unique phospholipids in the membranes of both mitochondria and bacteria [27]. These unique phospholipids which do not occur in normal cellular membranes are cardiolipins — dimeric phosphatidylglycerol molecules possessing 4 acyl chains bound to one head-group [28–30].

Mitochondrial and bacterial membranes are complicated multicomponent systems; therefore the reductive approach is here necessary and appropriate model environment should be applied. In our preliminary studies we do not intend to model the membranes but to investigate the interactions of the terpenes with cardiolipins and phosphatidylglycerols in simplified binary systems. To achieve this aim we applied Langmuir monolayers formed by these substances at the air/water interface as the versatile platforms enabling such investigation. Although Langmuir monolayers can be considered extremely artificial compared to real biomembranes, numerous studies regarding the interactions of various membrane active drugs with membrane phospholipids were performed with their application providing valuable results. The Langmuir monolayer technique is beneficial in some aspects compared with other membrane mimicking systems: the composition of the monolayer and the number of film forming molecules are strictly controlled, while by the film compression that required organization of the molecules can be achieved [31].

In our studies we applied three different cardiolipins: BHCL — tetra-linoleoyl CL, the cardiolipin species most populated in mammalian mitochondria, TOCL — tetraoleoyl CL, the cardiolipin dominating in human lymphoblasts and ECCL — bacterial CL extracted from *Escherichia coli*. Cardiolipins are not the only anionic phospholipids present in bacterial membranes. They are accompanied by phosphatidylglycerols (PGs) [32]. Therefore, in our studies we also investigated binary monolayers formed by the three PTAs and three different PGs. The PGs were selected in such a way that the investigated cardiolipins can be considered dimers of the particular PGs. Such an approach enabled us the comparison of the interactions of PTAs with CLs and PGs and the elucidation of the question which of the anionic phospholipids is targeted by the PTAs in bacterial membrane: CLs or PGs? In our studies we recorded surface pressure (π)–mean molecular area (A) isotherms for different compositions of the binary film. This technique was combined with the visualization of the investigated monolayers by Brewster angle microscopy (BAM). We also performed thermodynamic analysis of the registered data, calculating the excess functions of mixing. The combination of these methods enabled the thorough characterization of the interactions of anionic membrane phospholipids with the bioactive PTAs in the model environment.

2. Experimental

2.1. Materials

Betulinic acid (98%), oleanolic acid (99%) and ursolic acid (99%) were purchased from Sigma Aldrich. All the phospholipids were supplied by Avanti Polar Lipids. We bought 6 anionic lipids in the form of lyophilized powders of high (>99%) purity. There were: beef heart CL (BHCL, tetra-linoleoyl CL) extracted from the beef heart, tetraoleoyl CL (TOCL, synthetic sample), cardiolipin extracted from *E. coli* (ECCL), dilinoleoyl PG (DLPG, synthetic sample), dioleoyl PG (DOPG, synthetic sample) and the PG extracted from *E. coli* (ECPG). The exact names and structures of the investigated compounds as well as the information about the fatty acid distribution can be found in Supplementary materials and on the producer's website [33]. For the preparation of solutions we applied HPLC grade chloroform (99%) stabilized by ethanol and HPLC grade methanol (99.9%). As the subphase ultrapure water of the resistivity 18.2 $M\Omega \cdot cm^{-1}$ was applied, the ultrapure water was produced on site with the Millipore Synergy system.

2.2. Solutions

The investigated PTAs and anionic phospholipids were dissolved in chloroform/methanol 9/1 v/v mixture. The concentrations of the solutions oscillated between 0.2 and 0.3 mg/ml, which gives PTA molar concentrations from 4.4 to $6.6 \cdot 10^{-4}$ M, ca. 1.3 to $2.0 \cdot 10^{-4}$ M for CLs and from 2.7 to $4.0 \cdot 10^{-4}$ M for PGs. The binary mixtures were prepared from the stock solutions in darkened glass vials just before the given experiment. In this paper we present the data for 18 binary systems (6 phospholipids combined with 3 terpenes). The applied surfactants differ in the cross section, thus it was preferable to keep constant surface proportions of the molecules and not mole ratios. For each of the 18 binary systems we investigated 5 different surface proportions of the surfactants (terpene: phospholipid): 1:4, 1:2, 1:1, 2:1, and 4:1. In Supplementary materials the problem of the recalculation of the surface proportions into mole ratios is covered in details.

2.3. Langmuir technique

In our experiments we used a KSV (KSV, Helsinki, Finland) double-barrier Langmuir trough with nominal area of 273 cm^2. BAM experiments were performed on a larger instrument with an area of 840 cm^2 designated by KSV for microscopic experiments. Surface pressure was monitored with a Wilhelmy-type tensiometer with a filtration paper strap (Whatman, ashless) as the pressure sensor. Surface pressure was acquired with a 1 s time log and every π value is an average of 5 single measurements. The accuracy of the sensor was 0.1 mN/m.

Before an experiment the Langmuir trough was carefully cleaned, after which it was filled with ultrapure water. The appropriate volume of the chloroform/methanol solution of investigated surfactant(s) was

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