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# Phospholipidic Colchicinoids as Promising Prodrugs Incorporated into Enzyme-Responsive Liposomes: Chemical, Biophysical, and Enzymological Aspects

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**Abstract:** Enzyme-responsive liposomes release their cargo in response to pathologically increased levels of enzymes at the target site. We report herein an assembly of phospholipase A2-responsive liposomes based on colchicinoid lipid prodrugs incorporated into lipid bilayer of the nano-sized vesicles. The liposomes were constructed to addresses two important issues: (i) the lipid prodrugs were designed to fit the structure of the enzyme binding site; and (ii) the concept of lateral pressure profile was used to design lipid prodrugs that introduce almost no distortions into the lipid bilayer packing, thus ensuring that corresponding liposomes are stable. The colchicinoid agents exhibit antiproliferative activity in sub-nanomolar range of concentrations.

**Keywords:** colchicinoids • lipid prodrugs • stimuli-responsive liposomes • phospholipase A2 • anticancer therapy

# Introduction

Colchicine (1), an alkaloid isolated from *Colchicum autumnale*, was the first discovered tubulin destabilizing agent. At the molecular level, it prevents self-assembly of tubulin by distorting the interface between  $\alpha/\beta$ -tubulin heterodimers<sup>1–4</sup>. Interaction between colchicine and tubulin results in both antimitotic activity and suppression of cell motility<sup>5</sup>. In clinical practice, colchicine has been approved for treatment of gout, familial Mediterranean fever<sup>6,7</sup>, and, recently, for several other diseases, such as amyloidosis, progressive systemic scleroderma, cirrhosis, and Behcet's disease<sup>8–12</sup>. The ability of colchicine to accumulate within the immune system cells, leading to the suppression of inflammatory reactions, opened the possibility of its application for the treatment of cardiovascular disease, such as acute pericarditis and atrial fibrillation caused by inflammation<sup>13–16</sup>. Usage of colchicine in treatment of malignant tumors is prevented by rather high general toxicity resulting in renal, hepatic, circulatory, and central nervous system injury (see for example<sup>17–19</sup>).

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#### **Bioconjugate Chemistry**

One way to reduce general toxicity of the drug and improve its biodistribution upon systemic administration is to design a carrier-linked drug delivery system<sup>20–25</sup>. Liposomes have been the first nano-sized drug delivery system to be successfully translated into clinical practice<sup>26</sup>. To enhance the ability of a liposomal nanocarrier to target specific tissues, ligand-targeted and stimuli-responsive liposomes have been proposed<sup>27</sup>. Ligands attached on surface of liposomes guide them to corresponding receptor on target tissue thus enhancing liposome uptake by the tissue, while stimuli-responsive liposomes do not release their cargo unless they are exposed to an endogenous or exogenous trigger at the target site. Particularly, enzyme-responsive liposomes are sensitive to pathologically increased levels of enzymes at the target site<sup>28</sup>.

Secreted phospholipase A2 (sPLA2) level is elevated in various inflammatory diseases, atherosclerosis, and cancers (for example, prostate, breast, and pancreatic cancers)<sup>29</sup>. This enzyme hydrolyzes lipids specifically at the *sn*-2 position, which can lead to either direct release of the active drug or degradation of liposome resulting in its unloading. Therefore, sPLA2-responsive liposomes can be considered as promising carriers for targeted delivery of therapeutic agents to pathological tissues (see reviews on the topic<sup>30,31</sup>). In the case of colchicinoid-type agents, we expect that such carries should manifest anti-inflammatory, cardioprotective, and anticancer properties at the site of inflammation or in tumor tissues.

This manuscript addresses the design and synthesis of colchicine-based therapeutic agents and corresponding phospholipid prodrugs, construction of enzyme-responsive therapeutic liposomes for delivery of the colchicinoids, investigation of the drug–lipid interactions inside the liposomal carrier, liposomes' stability in human serum, and drug release under the effect of PLA2.

## **Results and Discussion**

### **Design of Colchicinoid-Derived Phospholipid Prodrugs**



Figure 1. Structural design of a colchicinoid-containing phospholipid.

Based on the evidence of enhanced PLA2 levels at sites of inflammation and in tumors, we present a concept of PLA2-degradable prodrug where the active compound is bound to distal end of the fatty acid chain at *sn*-2 position of a phospholipid. Such a prodrug releases the active

agent upon two cleavage events: (1) PLA2 cleaves the drug-conjugated fatty acid chain from the phospholipid and (2) the drug is cleaved from the fatty acid chain (Figure 1).

For a drug derivative to be cleavable by PLA2, it has to fit the enzyme binding site, while leaving the drug moiety, especially as bulky as the colchicinoid moiety, outside. Earlier the ability of PLA2 to hydrolyze various substrates was studied<sup>32,33</sup>. In assays of hydrolysis of phospholipids naturally occurring in humans, secretory PLA2 did not show any specificity for individual substrate in the *sn*-2 position<sup>34</sup>. However, this is not true for fluorescently labeled or other modified acyl chains<sup>35</sup>. Particularly, the active site of sPLA2 IIA has intricate shape; therefore, a substrate should be flexible enough to adopt appropriate conformation. To reach the same binding efficiency as natural phospholipids, a modified phospholipid should accommodate the bulky moiety farther than 10 carbon atoms away from the beginning of the *sn*-2 chain. This is supported by the data on cobra venom PLA2, which accommodates first 10 or so carbons of the *sn*-2 acyl chain of phospholipids in its catalytic site, where they are exposed to a very hydrophobic environment<sup>34</sup>. Locating of a bulky group more than 10 carbon atoms away from the beginning of the *sn*-2 chain has been successfully used to improve sensitivity of PLA2 specific fluorescent probes<sup>36</sup>.

The linker between the drug moiety and the fatty chain was chosen to promote release of the active substance *in vitro* and *in vivo*. Particularly, ester bond is easily hydrolyzed by low-specificity esterases found in abundance in most tissues<sup>37</sup>.

### **Colchicinoid Design and Synthesis of Lipid Prodrugs**

The presence of an easily oxidizable 7-membered *C*-cycle and sensitive tropolone ether moiety in the colchicine **1** skeleton limits the possibilities of its direct chemical modification (Figure 2). Colchifoline **2** (Figure 2) is one of colchicine metabolites known for its strong binding to tubulin and higher antitumor activity than that of colchicine; it possesses a hydroxymethyl moiety in an amide side-chain suitable for easy functionalization<sup>38–40</sup>. However, it reveals the same level of general toxicity as the parent colchicine molecule<sup>41–43</sup>. Allocolchicine **3**<sup>44</sup>, as well as indole- and furane-derived allocolchicine analogs<sup>45–50</sup>, have demonstrated higher activity as tubulin polymerization and cell proliferation inhibitors compared to colchicine, while their general cytotoxicity was relatively low. Therefore, two colchicinoids were chosen for conjugation with a phospholipid: allocolchicine derivative bearing a hydroxymethyl group in the amide fragment (**4**)<sup>40</sup> and corresponding dihydrofurane congener of allocolchicine (**5**) (Figure 2).



Figure 2. Colchicine and related compounds.

Synthesis of functionalized allocolchicinoid 7 started from the conversion of commercial colchicine 1 into deacetylallocolchicine 6 by a three-step cleavage of the acetamide group performed in 56% yield according to a known protocol<sup>51</sup> (Scheme 1). At the next step, the amino

group in 6 was acylated with glycolic acid under Steglich conditions to give allocolchifoline 4 in good yield.

Intermediate 10 necessary for the synthesis of hydrofurano-allocolchicinoid 5 is available from colchicine 1 exploiting electrocyclic rearrangement/oxidation of colchicine into *N*-acetyliodocolchinol  $8^{52}$ . The phenolic hydroxyl underwent allylation to afford colchicinoid 9 in 63% yield. It was converted then into corresponding amine 10 (50% overall yield) *via* three-step deacetylation procedure. The latter was subjected to Steglich acylation with glycolic acid followed by catalytic intermolecular Heck reaction to afford desired hydrofurano-allocolchicinoid 5 with *exo*-double bond in *D*-cycle in 69% yield over two steps.

One should note (see section "Cytostatic Properties") that colchicinoid 4 shows slightly lower level of cytotoxic activity in comparison with colchifoline 2, while colchicinoid 5 demonstrates the same range of cytotoxicity as compound 2. Both allocolchicinoids 4 and 5 inhibit proliferation of pancreatic cells at low nanomolar or even at subnanomolar concentrations. High cytotoxic activity of mentioned colchicinoids and increased level of sPLA2 in pancreatic tumors<sup>34,53–57</sup> are important features for creating sPLA2-responsive liposomes as a promising tool for pancreatic cancer treatment.



Scheme 1. Synthesis of colchicinoids 7 and 11. *Reaction conditions: a)*  $Boc_2O$ , 4-DMAP,  $Et_3N$ , MeCN, 3h, 100 °C, 78%; *b)* MeONa, MeOH, 40 min, 40 °C, 99%; *c)* TFA, DCM, 1,5 h, 25 °C, 95%; *d)* glycolic acid, DIC, NHS,  $Et_3N$ , DCM, 20 h, 25 °C, 64%; *e)* 4-pentynoic acid, 2,4,6-trichlorobenzoyl chloride (TCBC), 4-DMAP,  $Et_3N$ , DCM, 24 h,  $0 \rightarrow rt$ ; 51% and 49% for compounds 7 and 11 respectively; *f)* 0,1 M HCl, AcOH, 100 °C, 3h, 98%; *g)* I<sub>2</sub>, KI, NaOH, H<sub>2</sub>O, 0–5 °C, 1h, 95%; *h)* allyl bromide, Na<sub>2</sub>CO<sub>3</sub>, DMF, 60 °C, 24 h, 63%; *i)* Boc<sub>2</sub>O, 4-DMAP,  $Et_3N$ , MeCN, 3h, 80 °C, than cooling to rt, 20 h, 86%; *j)* MeONa, MeOH, 40 °C, 1 h, 94%; *k)* TFA, DCM, 30 °C, 24 h, 62%; *l)* glycolic acid, DIC, NHS,  $Et_3N$ , DCM, 20 h, 25 °C, 79%; *m)* Pd(dppf)Cl<sub>2</sub>, AcOK, DMSO, 75 °C, 20 h, 87%.

All attempts to connect colchicinoids **4** and **5** directly with *lyso*-PC **12** using the linkers on the basis of dicarboxylic acids (>C12) failed. Particularly, different ways of acylchloride formation, as well as the Steglich acylation, Yamaguchi, Mitsunobu esterification, and mixed anhydrides, were used. It can be explained by strong coordination of carboxylic group and its further deactivation. Therefore, allocolchicinoids **4** and **5** were functionalized by 4-pentynoic acid applying Yamaguchi esterification giving alkyne building blocks **7** and **11** in 51 and 49% yields, respectively (Scheme 1).

Modified phosphatidylcholine 14 was prepared by Steglich acylation of lysophosphatidylcholine (lyso-PC) 12 with 11-azidoundecanoic acid 13<sup>58</sup> (Scheme 2) in 78% yield. Alkynes 7 and 11 were used in the copper-mediated 1,3-dipolar cycloaddition<sup>59</sup> with azidocontaining phosphatidylcholine building block 14 leading to target colchicinoid-containing conjugates 15 and 16 in 56 and 62% yields, respectively. The first example of exploiting the powerful click-conjugation tool in colchicine functionalization was performed by N. Nicolaus<sup>59</sup>.



Scheme 2. Synthesis of colchicinoid phospholipid conjugates.

# **Incorporation of Colchicinoid Prodrugs into Lipid Bilayer**

Inclusion of alien conjugates in the lipid bilayer may induce a disturbance of its ordered structure, which can affect both the loading capacity of liposomal formulations and their stability in the biological milieu. This fact, for example, has been established for taxol (another tubulinbinding agent)<sup>60</sup>. When content of the drug in the bilayer exceeds 2.8 mol. %, taxol forms aggregates, which results in destabilization of the lipid bilayer and precipitation of the drug <sup>60</sup>. Indeed, pharmacokinetics of the formulation in rats is consistent with losses of taxol associates from liposomes in the circulation<sup>61</sup>.

To investigate the behavior of colchicinoid conjugates in the lipid bilayer, we focused on the spatial and orientational distribution of colchicinoids in the lipid layer, lipid packing differences, and prodrug-loaded liposome stability in serum.

We describe the drug moiety as an interbilayer inclusion that has shape and dimensions. In our case it is attached to lipid host molecules and incorporated into the bilayer (Table 1). The geometrical parameters of the interbilayer inclusions were obtained by quantum chemistry optimization (see SI section "Quantum Chemistry"). It turned out that inclusions of both compounds 15 and 16 had very close molecular volumes and projection areas (Table 1).

	15	16
Minimal projection area, Å <sup>2</sup>	52.68	57.88
Maximal projection area, Å <sup>2</sup>	98.01	95.63

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#### **Bioconjugate Chemistry**

Important characteristics of a conjugate inclusion are its spatial and orientational distributions in the membrane leaflet (lipid monolayer). The distributions depend on the molecular structures of the inclusion, as well as membrane properties. The lipid tail region in monolayer is stressed, which is usually described by a so-called lateral pressure profile, i.e. the dependence of the 2D pressure on the distance from the head-group region. The lateral pressure inside the bilayer tends to exclude the molecule from the tightly packed areas. At the same time, the inclusion tends to hide its hydrophobic surface from water into the hydrophobic region. The equilibrium position is the result of the effect of these two forces.

Lateral pressure profile can be obtained from molecular dynamics simulation (MD)<sup>62</sup>, application of lipid probes (pyrene)<sup>63</sup>, or analytically<sup>64,65</sup>. Here, for the quantitative estimation, lateral pressure profile produced in MD simulations<sup>66</sup> was used. Molecular geometries obtained by quantum chemistry optimization were used for quantitative description of the molecule shape. The optimized molecular geometries and coordinate system location are shown in Figure 3. The coordinate system is oriented with XY plane being parallel to the membrane plane.

Colchicinoids are connected with the lipid core through a polar linker (structures **15** and **16** on Scheme 2; Figures 1 and 3). Nitrogen and oxygen atoms of the linker are involved in hydrogen bond formation and polar interactions with surrounding lipid head groups. Through these interactions, the linker binds with the polar head region. From this we deduce that the colchicinoid fragment should be integrated in the polar head region and the linker can be fixed in the XY plain inside the polar head region. The rest of the molecule is non-polar—it should be located inside the hydrophobic area of the membrane.

The energy of intermonolayer inclusion can be calculated as a work for expansion of the area of lipid layer against the pressure profile

$$W = \int_{\text{Vmol}} P dV = \int_{z1}^{z2} P(z) dz, \qquad (1)$$

where z is the coordinate along the axes parallel to average lipid tail direction, z = 0 corresponds to the lipid tail-head group connection region; P(z) is the lateral pressure profile; integration is performed over the region occupied by the molecule in the membrane. (This approach was recently successfully applied to the design of fluorescent lipid probes non-disturbing lipid bilayer<sup>67</sup>.) The inclusion should rearrange into optimal layout to minimize its energy, W. Since the area occurs in the integral as a linear term (equation 1) and the inclusion volume is constant, the optimum position for the inclusion is to expose its largest projection area to the minimum pressure.

Taking that colchicinoid group is bound to the polar region, energy minimization could be achieved through rotation of the inclusion around the z-axis (angle  $\phi$ ) and XY-plane (angle  $\Theta$ ). Due to lateral homogeneity of the monolayer, rotation around the z-axis does not vary the system's state and energy. Therefore, we can exclude rotation around the z-axis from the analysis and focus on the possible distributions of the colchicinoid moiety over angle  $\Theta$  (Figure 4A). The probability distributions were calculated under the assumption that the latter is proportional to Boltzmann factor

$$e^{\frac{-W}{k_BT}}$$
.

Two most preferred orientations were around  $\Theta = 90^{\circ}$  and  $\Theta = 0^{\circ}$  (or 180°) for both conjugates **15** and **16**. Note that the probability allocations are not totally symmetrical (probabilities at  $\Theta = 0^{\circ}$  and 180° are not equal). The latter is explained by low symmetry of molecular structures of the inclusions. Polar groups of both conjugates (**15** and **16**) are arranged

either parallel to the bilayer surface or perpendicular to it (see Figure 3B for schematic representation). We suggest that these orientations should be in a dynamic equilibrium.



Figure 3. A. Optimized geometry of the colchicine moiety in conjugates 15 and 16. B. Most probable orientations of colchicine moiety in compound 16 inside the bilayer. Hydrocarbon chain structure is not shown for clarity.



Figure 4. A. The probability to find colchicinoid moiety aligned along the tilt angle  $\Theta$ . B. Surface pressure diagrams.

The colchicinoid moiety localizing near the lipid head groups seems unexpectable due to presumably tight packing in that region. However, it has been shown that the bilayer surface is not homogeneous even in single-component membranes. There are hydrophobic, hydrophilic, and neutral areas at the bilayer surface. Hydrophobic regions are created by acyl chains of lipids "snorkeling" near the membrane–water interface. These hydrophobic "spots" correspond to regions between the lipid heads<sup>68</sup> covering up to 20% of the bilayer surface according to molecular dynamic simulations<sup>68</sup>. Thus, colchicine moieties in conjugates **15** and **16** can be forced out towards head group region and populate these hydrophobic surface spots, which are otherwise inhabited by hydrophobic chains.

To study the effect of conjugates **15** and **16** on lipid packing, we recorded surface pressure isotherms for pure prodrugs, as well as mixed 1-palmitoyl-2-oleoylphosphatidylcholine (POPC)–conjugate **15** and POPC–conjugate **16** monolayers (Figure 4). Both conjugates have increased area per lipid comparing to POPC, while area per lipid of conjugate **16** is lower than

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that of conjugate **15**. The higher is the content of the conjugates, the greater is the difference between the two. However, mixtures of 10% mol. of compound **15** or **16** with POPC demonstrate almost the same surface pressure isotherm profiles as pure POPC, which evidences that at 10 mol. % the conjugates practically do not disturb the lipid bilayer packing.

# **Calcein Release Assay**

Colchicine moieties lying on the surface of the bilayer should alter the surface properties. Thus, lipid bilayer of liposomes can suffer from lipid–protein interactions upon contact with human serum and protein corona formation. This in turn can limit application of liposomes as a delivery system for drug targeting. We examined stability of liposomes with colchicine derivatives in PBS and in human serum using calcein release assay. In the absence of any colchicinoid derivatives, ePC liposomes demonstrated very slow release of calcein (the degree of calcein release (CR) was approximately 3% after 24 h of incubation) (Figure 5A), which can be explained by simple diffusion of calcein through the fluid lipid bilayer<sup>69</sup>. The single-component liposomes incubated in serum demonstrated more intense, but still gradual calcein release, with CR reaching 10% by the end of the incubation (Figure 5A). The result may be a consequence of progressive degradation of the fluid lipid bilayer under the action of enzymes in serum or even albumin adsorption on liposomes and increase of permeability of the bilayer<sup>70</sup>.



Figure 5. Leakage of calcein from liposomal formulations in 80% human serum (solid lines) or PBS (dashed lines) at 37 °C. Plots were obtained for the following formulations: **A**, ePC; **B**, ePC–conjugate 15; **C**, ePC–conjugate 16. Plotted are mean  $\pm$  SD values for three repetitive measurements.

Liposomes with 5% of conjugate **16** demonstrated similar CR kinetics in both PBS and serum (Figure 5C), releasing slightly more calcein than the ePC liposomes by the end of incubation (24 h). Addition of 5% of conjugate 15 did not change the liposome stability in PBS (compared to ePC liposomes), but led to a dramatic change in liposome stability in serum, with 27% CR. The difference could not be explained by electrostatic forces, as  $\zeta$ -potential for the ePC–conjugate **15** and ePC–conjugate **16** formulations was almost the same (Table 2).

In order for calcein to be released from a liposome, it should pass three stages: bind the internal bilayer surface; get across the bilayer; and desorb from the external bilayer surface. Allocolchicinoid moieties floating on the bilayer interface change the pattern of hydrophobic/hydrophilic area distribution on surface of liposomes. Therefore, (1) they alter the sorption/desorption constants of calcein on the bilayer surface. It appears that conjugate 16 promotes calcein binding to the bilayer surface more readily than conjugate 15 (compare CR plots produced in PBS; Figures 5B and 5C). (2) Allocolchicinoid moieties hinder access of serum proteins, such as albumin, to oxygen atoms of phosphate and carbonyl groups, which are important for initial binding of albumin to the bilayer. However, in case of the conjugate 15, carboxymethyl group may create new possibilities for interaction thus promoting membrane destabilization upon contact with proteins, while conjugate 16 provides no such possibility.

Table 2. Liposonie proper	ues	
Liposomes	Size, nm <sup>a</sup>	ζ-potential, mV
5% conj. <b>15</b> –95% ePC	$111.0 \pm 0.3$	-5.4
5% conj. <b>16–</b> 95% ePC	111.1 ± 0.3	-3.5

# Table 2. Liposome properties

[a] See SI section on liposomes for DLS instrument reports and TEM images.

#### **PLA2 Responsivity**

To check whether the prodrugs in liposomes are susceptible to hydrolysis with PLA2, we subjected them to the effect of two secreted PLA2: porcine pancreas PLA2 (ppPLA2) and PLA2 from *Vipera ursinii* venom (vuPLA2). The former is homologous to human pancreatic PLA2 and the latter to non-pancreatic enzyme<sup>71</sup>. As a group I PLA2, ppPLA2 has the so-called surface loop, i.e. a helix region, which is a part of interfacial binding surface (IBS) of the enzyme (see Figure 6). Deletion of the surface loop enhances the enzyme activity<sup>72</sup>. vuPLA2 belongs to group II PLA2 and has no surface loop: shortened by 10 amino acids, the region has a random structure, yet two of its amino acids are still involved in interaction with the bilayer surface. They are Lys60 (corresponds to Tyr69 in ppPLA2) and Thr61 (Thr70).



**Figure 6.** Visualization of PLA2 structures. **A**, ppPLA2 (PDB ID 3O4M); cyan helix with indigo selection indicate amino acid residues of interfacial binding surface and light blue selection, the active site (His48, Tyr52, Tyr73, and Asp99<sup>73</sup>). **B**, vuPLA2 homolog (ammodytoxin A from *Vipera ammodytes ammodytes* [https://www.uniprot.org/uniprot/P00626]; >90% homology; PDB ID 3G8G); lilac helix with dark purple selection represents amino acid residues of IBS and magenta selection (His48, Tyr52, Tyr73, Asp99<sup>71</sup>), the active site.



**Figure 7.** Densitometry of TLC plates upon development with phosphate group reagent; areas under the curves are proportional to relative content of POPC and the prodrugs (peak on the left) and lyso-PC (peak on the right) upon treatment of the ePC–conjugate **15** (solid line) or ePC–conjugate **16** (dashed line) liposomes with vuPLA2 (A, B) and ppPLA2 (C, D) for 1 (A, C) or 24 h (B, D). Figures represent relative areas of the peaks (in %). For more information, see Supporting Information.

During the first hour of incubation of vuPLA2 with the liposomes, hydrolysis of both ePC-conjugate 15 and ePC-conjugate 16 liposomes was well pronounced (Figure 7A). After 24 h, lyso-forms of lipids became dominating (Figure 7B, see also SI). Notably, hydrolysis of the ePC-conjugate 16 liposomes proceeded faster than that of the ePC-conjugate 15 liposomes. PLA2 is an interfacial enzyme that acts at the lipid-water interface. Initially PLA2 interacts with the membrane by its interfacial binding surface (IBS) and subsequently the lipid is hydrolyzed in the active site of the enzyme<sup>74</sup>. Thus, difference in the rate of hydrolysis between two conjugates might reflect either differences in surface structure of the liposomes, particularly, distribution of hydrophobicity over the surface, or hydrolysis rate of the conjugates in the active sites of the enzymes. In other words, either the structure of conjugate 16 favors hydrolysis by PLA2 or, when inserted in the ePC bilayer, creates an interface beneficial for PLA2 binding (compared to conjugate 15). We assume that the two structures should be indistinguishable for PLA2, since the colchicinoid moieties are far enough from the active site of the enzyme. This implies that vuPLA2 binds surface of the ePC-conjugate 16 liposomes more efficiently than that of liposomes with conjugate 15. The fact that the ePC-conjugate 15 liposomes were less stable in serum (Figure 5) suggests that interaction with other serum proteins destabilizes liposome membranes more actively than PLA2.

Hydrolysis of liposomes with ppPLA2 is almost negligible during the first hour (Figure 7C). Small amount of hydrolyzed lipids can be detected for the ePC–conjugate **16** sample. The difference in hydrolysis between two samples increases after 24 h (Figure 7D). The slow rate of hydrolysis of the prodrugs by ppPLA2, which is most likely due to the presence of the surface

loop in the enzyme structure, suggests that the prodrug formulations will be more efficient in treatment of conditions characterized by elevated group II and not group I (pancreatic) PLA2.

#### Cytotoxic Properties of the Colchicinoids and Colchicinoid-Containing Liposomes

According to our concept, the liposomal forms of phospholipids 15 and 16 are to be hydrolyzed selectively at the *sn*-2 position by PLA2. The enzyme acts at the lipid–water interface on the surface of liposomes resulting in release of colchicine-derived fatty acids 17 and 18, respectively (see Figure 8). These compounds were synthesized for a comparative biological assay (see details in SI). In turn, acids 17 and 18 should undergo further hydrolysis of biodegradable ester bonds with non-specific esterases releasing intact therapeutic colchicinoids 4 and 5.



Figure 8. Stepwise hydrolysis of conjugates 15 and 16 by PLA2 and non-specific esterases.

Cytotoxic properties of compounds **4**, **5**, **15–18**, and therapeutic liposomes loaded with conjugates **15** and **16** were tested against pancreatic cell lines PANC-1, BxPC-3, Colo-357, and HaCaT (non-transformed immortalized keratinocytes) using colchifoline **2** as a reference (see Table 3). The choice of cell lines was due to the fact that in 90% of human pancreatic cancers elevated PLA2 GIIA levels were detected in serum; also, 65 and 84% tissues are PLA2 GIIA positive according to immunohistochemistry and RNA blotting of tissue extracts, respectively<sup>75</sup>.

Intact colchicinoids 4 and 5, as well as reference compound 2, demonstrated high cytotoxic activity in nanomolar concentration range against all tested cell lines. Colchicinoid 5 appears to be the most active compound. Notably, this trend persisted for its derivatives in the form of fatty acid 18 and the ePC–conjugate 16 liposomes, especially in the case of the PANC-1 cells (compare corresponding  $IC_{50}$  values with acid 17 and the ePC–conjugate 15 liposomes in Table 3).

Lipid-conjugate colchicinoid prodrugs **15** and **16** lost 1.5–2 orders of magnitude of activity compared to intact molecules **4** and **5**, which is due to delay needed for the enzymatic release of active agents. Fatty acids **17** and **18** exhibited intermediate or borderline cytotoxic activity between intact therapeutic agents **4** and **5** and their lipidic conjugates **15** and **16** (Table 3). The ePC–conjugate **15** and ePC–conjugate **16** liposomes showed higher cytotoxicity in comparison with conjugates **15** and **16** as such and even a bit higher values of activity than acids **17** and **18** after incubation with cells during 72 h (except for the BxPC-3 cells).

Such an intricate pattern of cytotoxic activity produced by various allocolchicinoid-based preparations against different cell lines should be the result of an interplay between several factors. (1) Interaction of the preparations with cell surface and further internalization depend strongly on specific features of cell surface of a given cell line. (2) Also, morphologies produced

by the allocolchicinoids (liposomes for conjugates **15** and **16** vs micelles or some other kind of aggregates for compounds **15–18** in aqueous phase) may promote or hinder the process of internalization. (3) Drug release inside cell is enzyme-dependent. As for PLA2, it is a surface-dependent enzyme and liposomes present the most convenient substrate therefor, which may ascribe for higher activity of prodrug-loaded liposomes compared to the prodrugs themselves. (4) Decreasing the incubation time from 72 to 48 h could have limited the possibility of liposomes to unload and decay with subsequent partial cleavage of the ester bonds in lipid conjugates **15** and **16** to release active compounds **4**, **5**, **17**, and **18**, resulting in the significant decrease in the cytotoxicity of liposomes ePC–conjugate **15** and ePC–conjugate **16** (Table 3, results marked with the "b" index).

Rather high cytotoxicity of conjugates **15** and **16** in liposomal form indirectly implies that liposomes are well internalized and processed by cells with the release of the parent drugs **4** and **5**, respectively<sup>76</sup>. On the other hand, considering future experiments *in vivo*, it is required to augment the formulations' performance with some stabilizing components, such as phosphatidylinositol<sup>77,78</sup>, cholesterol<sup>79–81</sup>, PEG-derived molecules<sup>82</sup>.

**Table 3.** Cytotoxicity of colchifoline 2, colchicinoids 4 and 5, conjugates 15 and 16, corresponding fatty acids 17 and 18, and therapeutic liposomes containing phospholipidic conjugates 15 and 16 ( $IC_{50}^{a}$ , nM)

Compound	PANC-1	Colo-357	BxPC-3	НаСаТ
2	6	10	-	3
4	80	16 (16) <sup>b</sup>	16 (16) <sup>b</sup>	16 (16) <sup>b</sup>
5	16	3 (3) <sup>b</sup>	3 (3) <sup>b</sup>	3 (3) <sup>b</sup>
15	400	400 (400) <sup>b</sup>	400 (400) <sup>b</sup>	400 (400) <sup>b</sup>
16	400	160 (400) <sup>b</sup>	80 (80) <sup>b</sup>	400 (400) <sup>b</sup>
17	240	80	16	80
18	30	16	16	16
ePC–conj. <b>15</b> , 95 : 5 liposomes	119	38 (200) <sup>b</sup>	38 (200) <sup>b</sup>	38 (40) <sup>b</sup>
ePC–conj. 16, 95 : 5 liposomes	38	38 (50) <sup>b</sup>	15 (40) <sup>b</sup>	8 (40) <sup>b</sup>
ePC liposomes	Non toxic	Non toxic	Non toxic	Non toxic

[a]  $IC_{50}$  concentration is concentration inducing 50% inhibition of cell growth after incubation during 72 h. [b] Values in brackets are given for cell incubation during 48 h.

# Conclusions

We present two novel colchicinoid-containing phospholipid prodrugs encapsulated into phosphatidylcholine-based enzyme-responsive liposomes. The prodrugs induce minimal distortions into lipid packing and corresponding liposomes appear to be stable in human serum. At the same time, being exposed to elevated levels of phospholipases A2, especially in the case of sPLA2 analog, the liposomes release colchicinoid-containing fatty acids. The latter undergo further hydrolysis by non-specific esterases with release of the active colchicinoid species. Our data on *in vitro* cytotoxicity of liposomes loaded with the new phospholipid conjugates evidence feasibility of such a scenario. For further *in vivo* studies the composition of therapeutic liposomes should be changed to improve their stability towards opsonisation and to prevent their premature decay in biological media. *In vivo* investigations are to be carried out in the nearest future.

# Experimental Section Synthesis

# General information

<sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were recorded on an *Agilent DDR2 400* spectrometer at 25°C. Chemical shifts ( $\delta$ ) are reported in ppm for the solution of compound in MeOD, CDCl<sub>3</sub>, and DMSO-*d*<sub>6</sub> with internal reference TMS and *J* values in Hz. MALDI spectra were recorded on *Bruker Microflex LT* spectrometer. Elemental analysis was performed using an *Elementar* (*Vario Micro Cube*) apparatus; final compounds have a purity of >95%. Separation by column chromatography was performed using *Merck Kieselgel 60 (70–230 mesh*). All reactions were performed with commercially available reagents (Aldrich, Alfa Aesar, Acros, Serva). Solvents were purified according to standard procedures. The petroleum spirit refers to the fraction with distillation range 40–70 °C.

# Synthesis of intermediate compounds

(Atomic numeration is given only for NMR assignment, for details see supplementary information.)

# Synthesis of N-deacetylallocolchicine 6

Synthetic procedure was carried out according to literature protocol<sup>39</sup>.

Colchicine 1 (3.000 g, 1 eq., 7.5 mmol) was treated with 4-DMAP (1.830 g, 2 eq., 15 mmol), triethylamine (0,756 g, 1 eq., 7.48 mmol, 1.043 ml), and Boc<sub>2</sub>O (5.700 g, 3.46 eq., 26 mmol) in MeCN at 100 °C for 2.5 h; after that another portion of Boc<sub>2</sub>O (3.200 g, 1.96 eq., 14.67 mmol) was added and the solution was heated at 100°C for 1 h. When the reaction was complete, volatiles were evaporated and the product was isolated by column chromatography with AcOEt–acetone (4 : 1) as red-brownish foam with78% yield (2.925 g).

<sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  7.27 (s, 1H, 8-H), 7.11 (d, J = 10.7 Hz, 1H, 11-H), 7.02 (d, J = 10.9 Hz, 1H, 12-H), 6.77 (s, 1H, 4-H), 4.90 (dd, J = 12.4, 6.0 Hz, 1H, 7-H), 3.87 (s, 3H, OMe), 3.83 (s, 3H, OMe), 3.77 (s, 3H, OMe), 3.54 (s, 3H, OMe), 2.69 (m, 1H, 6-H), 2.34–2.25 (m, 1H, 5-H), 2.22 (s, 3H, NAc), 1.96–1.85 (m, 2H, 5-H, 6-H), 1.49 (s, 9H, NBoc).

<sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>) δ 178.31, 170.79, 163.95, 153.55, 153.43, 150.91, 148.69, 141.26, 135.20, 134.96, 134.28, 132.16, 126.03, 112.59, 108.15, 84.80, 61.27, 61.09, 60.20, 56.48, 56.27, 32.24, 29.68, 27.72.

MALDI (neg. mode): 494.4 (100%), 484.3 (52%), 474.6 (20%), 497.4 (19%). Elemental analysis: for  $C_{27}H_{33}NO_8$  calcd.: C, 64.92; H, 6.66; found: C, 64.71; H, 6.82.

mp 105 °C

*N*-Boc-colchicine (2.000 g, 1 eq., 4 mmol) was dissolved in dry methanol, sodium methoxide (896 mg, 4 eq., 16 mmol) was added into the solution and the mixture was stirred at 40 °C for 40 min–1 h. When the reaction was complete, saturated solution of NH<sub>4</sub>Cl (26 mL) was added and methanol was removed under reduced pressure. The aqueous solution was extracted with AcOEt (3 × 50 mL); combined organic layer was washed with brine and dried over Na<sub>2</sub>SO<sub>4</sub>. Solvent was evaporated under reduced pressure and *N*-Boc-deacetylallocolchicine was obtained as beige solid with 99% yield (1.813 g) and used without further purification. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  7.69 (d, *J* = 7.8 Hz, 1H, N<u>H</u>Boc), 7.21 (s, 1H, 8-H), 7.10 (d, *J* = 10.6 Hz, 1H, 10-H), 7.02 (d, *J* = 10.9 Hz, 1H, 11-H), 6.76 (s, 1H, 4-H), 4.08 (dt, *J* = 13.7, 7.0 Hz, 1H, 7-H), 3.87 (s, 3H, OMe), 3.83 (s, 3H, OMe), 3.79 (s, 3H, OMe), 3.54 (s, 3H, OMe), 2.55 (dd, *J* = 13.3, 6.0 Hz, 1H, 6-H), 2.17 (td, *J* = 13.1, 7.0 Hz, 1H, 5-H), 2.06 – 1.93 (m, 1H, 5-H),

1.87 – 1.77 (m, 1H, 6-H), 1.32 (s, 9H, NHBoc).

 13C NMR (101 MHz, DMSO-*d*<sub>6</sub>) δ 178.00, 163.50, 154.50, 152.94, 151.03, 150.36, 140.69, 135.10, 134.39, 134.26, 130.51, 125.31, 112.12, 107.65, 60.83, 60.64, 56.01, 55.84, 54.90, 52.87, 40.78, 35.80, 28.14. MALDI (pos. mode): 458.2 (M+H<sup>+</sup>, 72%), 401.2 (39%), 356.1 (11%).

Elemental analysis: for  $C_{25}H_{31}NO_7$  calcd.: C, 65.63; H, 6.83; found: C, 65.82; H, 6.69. mp 151 °C.

*N*-Boc-deacetylallocolchicine (1.800 g, 1 eq., 3.93 mmol) was placed into round-bottom flask and dissolved in 30 ml DCM; 9 mL TFA were added subsequently and the solution was stirred for 1.5 h at room temperature. The acid was neutralized by addition of NaHCO<sub>3</sub> saturated solution until pH became 8. The aqueous layer was extracted with DCM ( $3 \times 70$  mL), the combined organic layer was washed with brine and dried over Na<sub>2</sub>SO<sub>4</sub>. Solvent was evaporated under reduced pressure and *N*-deacetylallocolchicine **5** was obtained as pale-beige solid with 95% yield (1.407 g) and used without further purification.

<sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  7.64 (s, 1H, 8-H), 7.05 (d, J = 10.6 Hz, 1H, 10-H), 6.99 (d, J = 10.7 Hz, 1H, 11-H), 6.74 (s, 1H, 4-H), 3.86 (s, 3H, OMe), 3.83 (s, 3H, OMe), 3.76 (s, 3H, OMe), 3.55 (s, 3H, OMe), 3.46 (dd, J = 10.5, 5.8 Hz, 1H, 7-H), 2.37–1.91 (m, 4H, 6-H, 5-H).

<sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>) δ 178.20, 163.26, 153.82, 152.73, 150.15, 140.47, 135.68, 134.88, 133.76, 131.83, 125.40, 111.84, 107.34, 60.66, 60.53, 55.90, 55.84, 53.11, 39.64, 29.87. MALDI (pos. mode): 357.2 (100%), 341.2 (49%), 326.1 (18%).

Elemental analysis: for C<sub>20</sub>H<sub>23</sub>NO<sub>5</sub> calcd.: C, 67.21; H, 6.49; found: C, 67.40 2; H, 6.21. mp 133 °C.

# Synthesis of allocolchifoline 4

*N*-deacetylallocolchicine **6** (1.400 g, 1 eq., 3.91 mmol) was mixed with glycolic acid (297 mg, 1 eq., 3.91 mmol) and *N*-hydroxysuccinimide (341 mg, 0.76 eq., 2.97 mmol). The mixture was dissolved in dry DCM with further addition of triethylamine (1.184 g, 3 eq., 11.73 mmol, 1.634 mL) and *di*-isopropylcarbodiimide (DIC) (680 mg, 1.5 eq., 5.86 mmol, 835  $\mu$ L). The mixture was stirred at room temperature for 20 h. The solvent was removed under reduced pressure and the product was isolated using column chromatography with the mixture of DCM–methanol (18 : 1) as eluent. Allocolchifoline **4** was obtained as pale-yellow solid with 64% yield (1.038 g).

<sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  8.47 (d, J = 7.7 Hz, 1H, NH), 7.17 (s, 1H, 8-H), 7.11 (d, J = 10.6 Hz, 1H, 10-H), 7.02 (d, J = 10.9 Hz, 1H, 11-H), 6.77 (s, 1H, 4-H), 5.51 (t, J = 5.8 Hz, 1H, OH), 4.41 (dt, J = 11.6, 7.2 Hz, 1H, 7-H), 3.87 (s, 3H, OMe), 3.83 (s, 3H, OMe), 3.83–3.80 (m, 2H, 2'-H), 3.79 (s, 3H, OMe), 3.52 (s, 3H, OMe), 2.59 (dd, J = 13.2, 6.0 Hz, 1H, 6-H), 2.25–2.16 (m, 1H, 5-H), 2.14–2.03 (m, 1H, 5-H), 1.97 (dd, J = 12.7, 6.3 Hz, 1H, 6-H).

<sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>) δ 177.99, 172.78, 171.39, 163.52, 152.93, 150.50, 150.45, 140.71, 135.18, 134.28, 130.76, 125.48, 112.09, 107.72, 61.39, 60.79, 60.70, 56.03, 55.86, 50.85, 29.27, 25.23.

MALDI (neg. mode): 400.2 (100%), 415.2 (88%).

Elemental analysis: for C<sub>22</sub>H<sub>25</sub>NO<sub>7</sub> calcd.: C, 63.61; H, 6.07; found: C, 63.45 2; H, 6.24. mp 123 °C.

# Synthesis of (a*R*,5*S*)-*N*-(3-carboxymethyl-9,10,11-trimethoxy-6,7-dihydro-5*H*-dibenzo[a,c]-cycloheptene-5-yl)(pent-4-yn-1-oyl)acetamide 7

4-Pentynoic acid (25 mg, 1.5 eq., 0.252 mmol) was treated with Yamaguchi reagent TCBC (82 mg, 2 eq., 0.336 mmol, 53  $\mu$ L) and triethylamine (85 mg, 5 eq., 0.84 mmol, 117  $\mu$ L) in dry DCM for 5 h at 0–5 °C. Than the mixture of allocolchifoline 4 (70 mg, 1 eq., 0.168 mmol) and 4-DMAP (61 mg, 3 eq., 0.504 mmol) in DCM was added dropwise and the mixture was stirred for 20 h at room temperature. When the reaction was complete, solvent was removed under reduced pressure and the product was isolated using column chromatography with the mixture of petroleum–ethyl acetate–ethanol (5 : 1 : 1) as eluent. Compound 7 was isolated as pale-beige solid with 51% yield (42 mg).

<sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  8.74 (d, J = 7.4 Hz, 1H, NH), 7.11 (d, J = 11.3 Hz, 1H, 10-H), 7.10 (s, 1H, 8-H), 7.03 (d, J = 11.1 Hz, 1H, 11-H), 6.77 (s, 1H, 4-H), 4.53 (d, J = 1.5 Hz, 2H, 2'-H), 4.39–4.31 (m, 1H, 7-H), 3.87 (s, 3H, OMe), 3.83 (s, 3H, OMe), 3.78 (s, 3H, OMe), 3.52 (s, 3H, OMe), 2.80 (t, J = 2.6 Hz, 1H, 7'-H), 2.58 (t, J = 7.2 Hz, 3H, 4'-H, 5'-H), 2.42–2.39 (m, 1H, 5'-H), 2.27–2.17 (m, 2H, 6-H, 5-H), 2.07–1.96 (m, 1H, 5-H), 1.95–1.85 (m, 1H, 6-H).

<sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>) δ 177.94, 170.87, 166.04, 163.53, 152.97, 150.42, 150.11, 140.74, 134.97, 134.45, 134.12, 130.45, 130.04, 125.32, 112.10, 82.98, 71.67, 62.79, 62.25, 60.82, 60.69, 56.06, 55.85, 51.13, 35.67, 32.41, 29.15.

MALDI (DCTB, pos. mode): 518.2 (M+Na<sup>+</sup>, 49%), 495.4 (98%), 332.2 (100%). Elemental analysis: for  $C_{27}H_{29}NO_8$  calcd.: C, 65.44; H, 5.90; found: C, 65.77; H, 6.02. mp 53 °C.

#### Synthesis of iodo-colchinol 8

Synthesis of iodo-colchinol 8 was carried out according to literature protocol<sup>51</sup>.

Colchicine 1 (1200 mg, 3 mmol) was dissolved in 11.5 ml of glacial acetic acid, 70 mL 0.1 *N* hydrochloric acid was added, and the mixture was stirred for 3 h at 100 °C. When the reaction was over, the mixture was cooled to room temperature, neutralized with NaHCO<sub>3</sub> to pH 6, and extracted with CHCl<sub>3</sub> (60 mL  $\times$  3); combined organic layer was dried over Na<sub>2</sub>SO<sub>4</sub>; the solvent was removed under reduced pressure. Colchiceine was obtained as yellowish-green foam with 98% yield (1132 mg) and used at the next step without further purification.

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  7.49 (d, J = 7.9 Hz, 1H, NHAc), 6.87 (s, 1H, 8-H), 6.54 (s, 1H, 4-H), 5.91 (d, J = 8.1 Hz, 1H, 11-H), 5.75 (d, J = 8.1 Hz, 1H, 12-H), 4.81–4.72 (m, 1H, 7-H), 3.92 (s, 3H, OMe), 3.88 (s, 3H, OMe), 3.53 (s, 3H, OMe), 2.54–2.26 (m, 4H, 5-H, 6-H), 2.04 (s, 3H, NHAc).

<sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>): δ 175.59, 170.36, 153.47, 151.57, 150.69, 141.32, 136.37, 134.31, 125.71, 122.53, 119.95, 107.22, 61.27, 61.22, 55.94, 52.61, 37.26, 29.64. mp 151 °C (lit. 150 °C).

Colchiceine (1068 mg, 2.7 mmol, 1 eq.), obtained on the previous step, was dissolved in 26 ml H<sub>2</sub>O, NaOH (1106 mg, 27 mmol, 10 eq.) was added and the mixture was cooled to 0 °C. NaI (9.3 g, 62.1 mmol, 23 eq.) and I<sub>2</sub> (2.057 g, 8.1 mmol, 3 eq.) were dissolved in 116 mL H<sub>2</sub>O. This mixture was added dropwise to solution of colchiceine during 1 h with temperature control (<5 °C). After complete addition, the mixture was stirred for 1 h at 0 °C. When the reaction was over (TLC control), the mixture was allowed to warm to ambient temperature. After removing the excess of iodine by addition of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, the mixture was acidified with HCl (conc.) to pH 2 and extracted with AcOEt (50 mL × 3); organic layers were collected and dried over Na<sub>2</sub>SO<sub>4</sub>. The solvent was removed under reduced pressure and iodo-colchinol **8** was obtained as yellow powder with 95% yield (1.244 g). The product was used without further purification.

 <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  10.28 (s, 1H, OH), 8.38 (d, J = 8.0 Hz, 1H, N<u>H</u>Ac), 7.56 (s, 1H, 8-H), 6.86 (s, 1H, 11-H), 6.76 (s, 1H, 4-H), 4.40–4.33 (m, 1H, 7-H), 3.82 (s, 3H, OMe), 3.77 (s, 3H, OMe), 3.48 (s, 3H, OMe), 2.50–2.44 (m, 1H, 6-H), 2.16–2.07 (m, 2H, 5-H), 1.93–1.88 (m, 1H, 6-H), 1.87 (s, 3H, NHAc).

<sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>) δ 168.31, 155.63, 152.22, 150.17, 142.41, 140.51, 139.18, 134.77, 126.60, 123.24, 110.05, 108.07, 81.42, 60.55, 60.53, 55.82, 48.23, 37.91, 29.98, 22.62. mp 238 °C (lit. 238 °C)

# Synthesis of (*aR*,5*S*)-*N*-(3-allyloxy-4-iodo-9,10,11-trimethoxy-6,7-dihydro-5*H*-dibenzo-[*a*,*c*]cycloheptene-5-yl)acetamide 9

Iodo-colchinol **8** (750 mg, 1 eq., 1.546 mmol) was treated with allyl bromide (374 mg, 2 eq., 3.09 mmol, 267  $\mu$ L) and potassium carbonate (640 mg, 3 eq., 4.64 mmol) in DMF under inert atmosphere at 60 °C. The reaction was carried out for 20 h, the solvent was removed under reduced pressure. To the residue, 50 mL of distilled water was added and the mixture was extracted with AcOEt (2 × 70 mL); combined organic layer was washed with brine and dried over Na<sub>2</sub>SO<sub>4</sub>. Solvent was removed under reduced pressure and the residue was purified using column chromatography with the mixture of petroleum–ethyl acetate–ethanol (7 : 1 : 1) as eluent. Compound **9** was isolated as pale-beige solid with 63% yield (511 mg).

<sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  8.40 (d, J = 8.5 Hz, 1H, NHAc), 7.68 (s, 1H, 8-H), 6.98 (s, 1H, 11-H), 6.78 (s, 1H, 4-H), 6.08 (ddd, J = 17.5, 10.5, 5.1 Hz, 1H, 2'-H), 5.56 (d, J = 17.2 Hz, 1H, 3'-H), 5.33 (d, J = 10.5 Hz, 1H, 3'-H), 4.66 (br.s., 2H, 1'-H), 4.49 (dt, J = 12.0, 7.9 Hz, 1H, 7-H), 3.83 (s, 3H, OMe), 3.78 (s, 3H, OMe), 3.50 (s, 3H, OMe), 2.55–2.50 (m, 1H, 6-H), 2.23–2.12 (m, 1H, 5-H), 2.12–2.00 (m, 1H, 5-H), 1.89 (s, 3H, NHAc), 1.87–1.76 (m, 1H, 6-H).

<sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>) δ 168.48, 155.69, 152.46, 150.15, 142.51, 140.51, 139.43, 134.82, 133.20, 128.32, 122.85, 117.55, 108.20, 107.79, 83.24, 69.13, 60.60, 60.55, 55.82, 48.16, 38.39, 30.00, 22.64.

MALDI (DCTB, pos. mode): 523.1 (100%), 332.2 (29%), 242.3 (23%). Elemental analysis: for C<sub>23</sub>H<sub>26</sub>INO<sub>5</sub> calcd.: C, 52.78; H, 5.01; found: C, 52.55; H, 5.14. mp 82 °C.

# Synthesis of (a*R*,5*S*)-*N*-(3-Allyloxy-4-iodo-9,10,11-trimethoxy-6,7-dihydro-5*H*-dibenzo-[*a*,*c*]cycloheptene-5-yl)amine 10

Into Schlenk flask #1 compound **9** (496 mg, 1 eq., 0.95 mmol) and 4-DMAP (115 mg, 1 eq., 0.95 mmol) were put, the flask was filled with argon and acetonitrile was added under inert atmosphere. Into Schlenk flask #2 Boc<sub>2</sub>O (826 mg, 4.6 eq., 3.79 mmol) was placed and acetonitrile was added under inert atmosphere. A half of Schlenk #2 contents was added into the first mixture followed by triethylamine (191 mg, 2 eq., 1.89 mmol, 264  $\mu$ L) pouring. The mixture was stirred for 3 h at 75 °C, then the second part of Schlenk #2 contents was added, the solution was cooled to ambient temperature and stirred overnight. When the reaction was complete, solvent was evaporated and the residue was purified using column chromatography with the mixture of petroleum–ethyl acetate–ethanol (12 : 1 : 1) as eluent. (a*R*,5*S*)-*N*-(3-Allyloxy-4-iodo-9,10,11-trimethoxy-6,7-dihydro-5*H*-dibenzo[*a*,*c*]cycloheptene-5-yl)(*tert*-butoxycarbamoyl)acetamide was obtained as reddish foam with 86% yield (509 mg).

<sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  7.71 (s, 1H, 8-H), 6.93 (s, 1H, 11-H), 6.79 (s, 1H, 4-H), 6.10– 6.02 (m, 1H, 2'-H), 5.49 (d, J = 17.2 Hz, 1H, 3'-H), 5.31 (d, J = 10.6 Hz, 1H, 3'-H), 5.13 (dd, J = 9.1, 2.5 Hz, 1H, 7-H), 4.62 (dd, J = 34.5, 10.7 Hz, 2H, 1'-H), 3.83 (s, 3H, OMe), 3.78 (s, 3H, OMe), 3.44 (s, 3H, OMe), 2.68–2.57 (m, 2H, 6-H, 5-H), 2.28 (s, 3H, NAc), 2.18–2.04 (m, 2H, 5-H, 6-H), 1.50 (s, 9H, NBoc).

<sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>) δ 155.54, 153.30, 152.58, 150.36, 140.66, 140.19, 139.47, 134.67, 133.22, 128.54, 122.84, 117.37, 109.17, 108.18, 83.87, 83.73, 69.14, 69.11, 63.36, 60.61, 60.60, 55.80, 36.22, 34.64, 30.10, 27.46.

MALDI (DCTB, pos. mode): 623.2 (100%), 332.3 (27%), 486.6 (19%).

Elemental analysis: for  $C_{25}H_{34}INO_7$  calcd.: C, 53.94; H, 5.50; found: C, 53.81; H, 5.62. mp 69 °C.

(a*R*,5*S*)-N-(3-Allyloxy-4-iodo-9,10,11-trimethoxy-6,7-dihydro-5*H*-dibenzo[*a*,*c*]-

cycloheptene-5-yl)(*tert*-butoxycarbamoyl)acetamide (485 mg, 1 eq., 0.78 mmol) was treated with MeONa (13 mg, 0.3 eq., 0.23 mmol) in methanol at 40 °C for 1 h. When the reaction was complete, the solvent was evaporated and the product was isolated using short-column silica-gel chromatography (petroleum–ethyl acetate–ethanol, 15 : 1 : 1 as eluent) with 94% yield (425 mg) as a beige oil.

<sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  7.66 (s, 1H, 8-H), 7.52 (d, J = 8.5 Hz, 1H, N<u>H</u>Boc), 6.99 (s, 1H, 11-H), 6.77 (s, 1H, 4-H), 6.10 (ddd, J = 15.5, 10.2, 4.9 Hz, 1H, 2'-H), 5.55 (d, J = 15.1 Hz, 1H, 3'-H), 5.32 (d, J = 10.5 Hz, 1H, 3'-H), 4.64 (tt, J = 13.3, 6.5 Hz, 2H, 1'-H), 4.17 (dt, J = 15.8, 8.0 Hz, 1H, 7-H), 3.83 (s, 3H, OMe), 3.77 (s, 3H, OMe), 3.54 (s, 3H, OMe), 2.46 (d, J = 5.7 Hz, 1H, 6-H), 2.16 (dd, J = 12.3, 6.3 Hz, 1H, 5-H), 2.00 (dt, J = 19.8, 9.8 Hz, 1H, 5-H), 1.92–1.82 (m, 1H, 6-H), 1.35 (s, 9H, NH<u>Boc</u>).

<sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>) δ 155.66, 154.74, 152.49, 150.08, 142.85, 140.49, 139.44, 134.84, 133.05, 128.25, 122.75, 117.60, 108.12, 107.77, 83.21, 77.91, 69.11, 60.66, 60.50, 55.80, 50.13, 38.31, 29.99, 28.19.

Elemental analysis: for C<sub>26</sub>H<sub>32</sub>INO<sub>6</sub> calcd.: C, 53.71; H, 5.55; found: C, 53.66; H, 5.70. MALDI (DCTB, pos. mode): 581.4 (100%), 242.3 (58%), 332.2 (29%), 396.2 (17%).

(aR,5S)-N-(3-Allyloxy-4-iodo-9,10,11-trimethoxy-6,7-dihydro-5H-dibenzo[a,c]-

cycloheptene-5-yl)*tert*-butoxycarbamate (425 mg, 1 eq., 0.73 mmol), obtained at the previous step, was dissolved in a mixture of DCM (30 mL) and concentrated hydrochloric acid (8 mL) was added. The solution was stirred overnight at 30 °C. When the reaction was complete, the mixture was neutralized with NaHCO<sub>3</sub> sat. solution and extracted with DCM ( $3 \times 50$  mL), combined organic layer was washed with brine and dried over Na<sub>2</sub>SO<sub>4</sub>. Solvent was removed under reduced pressure and the residue was purified using column chromatography with the mixture of petroleum–ethyl acetate–ethanol (6 : 1 : 1) as eluent. Compound **10** was isolated as beige oil with 62% yield (218 mg).

<sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  7.70 (s, 1H, 8-H), 7.32 (s, 1H, 11-H), 6.80 (s, 1H, 4-H), 6.13 (ddd, J = 15.5, 10.0, 4.7 Hz, 1H, 2'-H), 5.58 (d, J = 15.1 Hz, 1H, 3'-H), 5.32 (d, J = 10.6 Hz, 1H, 3'-H), 4.70 (s, 2H, 1'-H), 3.83 (s, 3H, OMe), 3.76 (s, 3H, OMe), 3.69 (dd, J = 11.2, 6.7 Hz, 1H, 7-H), 3.54 (s, 3H, OMe), 2.56–2.50 (m, 1H, 6-H), 2.45–2.33 (m, 1H, 5-H), 2.10–1.99 (m, Hz, 1H, 5-H), 1.90–1.82 (m, 1H, 6-H).

<sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>) δ 155.66, 152.57, 150.11, 140.85, 140.52, 139.58, 134.73, 133.10, 128.24, 122.48, 117.52, 108.41, 108.17, 83.75, 69.31, 60.66, 60.51, 55.86, 50.47, 29.84, 28.21.

Elemental analysis: for C<sub>21</sub>H<sub>24</sub>INO<sub>4</sub> calcd.: C, 52.40; H, 5.03; found: C, 52.23; H, 5.17. MALDI (DCTB, pos. mode): 481.0 (100%), 332.2 (45%), 465.0 (36%).

# Synthesis of (a*R*,5*S*)-*N*-(9,10,11-trimethoxy-6,7-dihydro-5*H*-dibenzo-[a,c]cycloheptene-5-yl-[2,3-*f*]-3'-methyleno-2'-hydrofuranyl)2-hydroxyacetamide 5

Substrate **10** (200 mg, 1 eq., 0,41 mmol), glycolic acid (32 mg, 1 eq., 0,41 mmol), and *N*-hydroxysuccinimide (36 mg, 0.76 eq., 0.31 mmol) were dissolved in dry DCM under inert atmosphere. Et<sub>3</sub>N (124 mg, 3 eq., 1.23 mmol, 171  $\mu$ L) and DIC (58 mg, 1.5 eq., 0.62 mmol, 99  $\mu$ L) were subsequently added and the mixture was stirred at room temperature for 20 h. After the completion of the reaction, the solvent was removed under reduced pressure and the residue was purified using column chromatography with the mixture of petroleum–ethyl acetate–ethanol (8 : 1 : 1) as eluent. Acylated product was isolated as beige oil with 79% yield (175 mg).

<sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  8.34 (d, J = 8.8 Hz, 1H, NH), 7.67 (s, 1H, 8-H), 6.98 (s, 1H, 11-H), 6.79 (s, 1H, 4-H), 6.08 (ddd, J = 15.6, 10.4, 5.1 Hz, 1H, 2'-H), 5.56 (dd, J = 17.3, 1.4 Hz, 1H, 3'-H), 5.52 (s, 1H, OH), 5.32 (d, J = 10.6 Hz, 1H, 3'-H), 4.67 (d, J = 11.7 Hz, 2H, 13-H), 4.60–4.53 (m, 1H, 7-H), 3.83 (s, 3H, OMe), 3.78 (s, 3H, OMe), 3.50 (s, 3H, OMe), 2.55–2.51 (m, 1H, 6-H), 2.14–2.02 (m, 3H, 6-H, 5-H).

<sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>) δ 171.32, 155.61, 152.48, 150.18, 142.28, 140.52, 139.32, 134.89, 133.17, 128.31, 122.86, 117.67, 108.23, 108.16, 83.28, 69.12, 61.56, 60.60, 60.58, 55.83, 47.77, 37.97, 30.03.

MALDI (DCTB, pos. mode): 412.1 (100%), 562.0 (M+Na<sup>+</sup>, 77%), 539.0 (69%), 465.0 (38%). Elemental analysis: for  $C_{23}H_{26}INO_6$  calcd.: C, 51.22; H, 4.86; found: C, 51.33; H, 4.99. mp 124 °C

Substrate, obtained at the previous step (170 mg, 1 eq., 0.32 mmol) was treated with Pd(dppf)Cl<sub>2</sub> (11 mg, 0.05 eq., 0.016 mmol) and AcOK (93 mg, 3 eq., 0.945 mmol) in DMSO. The mixture was stirred for 20 h at 75 °C. When the reaction was over, distilled water (50 mL) was added to the mixture and the resulting solution was extracted with AcOEt ( $3 \times 70$  mL). Combined organic layer was washed with brine and dried over Na<sub>2</sub>SO<sub>4</sub>. Solvent was removed under reduced pressure and the residue was purified using column chromatography with the mixture of petroleum–ethyl acetate–ethanol (9 : 1 : 1) as eluent. Compound **5** was isolated as beige oil with 87% yield (114 mg).

<sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  8.26 (d, J = 8.8 Hz, 1H, NH), 7.41 (s, 1H, 8-H), 6.88 (s, 1H, 11-H), 6.78 (s, 1H, 4-H), 5.49 (d, J = 6.0 Hz, 1H, 10-H), 5.46 (t, J = 3.1 Hz, 1H, OH), 5.14 (d, J = 2.7 Hz, 2H, 9-H), 5.02 (d, J = 2.5 Hz, 1H, 10-H), 4.61–4.52 (m, 1H, 7-H), 3.84 (d, J = 1.8 Hz, 2H, 13-H), 3.83 (s, 3H, OMe), 3.79 (s, 3H, OMe), 3.48 (s, 3H, OMe), 2.19–1.99 (m, 4H, 5-H, 6-H).

<sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>) δ 171.23, 162.48, 152.21, 150.35, 143.65, 143.27, 140.57, 134.81, 126.56, 124.31, 123.62, 122.00, 108.05, 105.61, 99.64, 74.92, 61.52, 60.59, 60.56, 55.83, 48.12, 37.75, 29.98.

MALDI (pos. mode): 411.0 (100%), 379.0 (11%), 353.0 (7%).

Elemental analysis: for C<sub>23</sub>H<sub>25</sub>NO<sub>6</sub> calcd.: C, 67.14; H, 6.12; found: C, 67.27; H, 6.02. mp 157 °C.

# Synthesis of (a*R*,5*S*)-N-(9,10,11-trimethoxy-6,7-dihydro-5*H*-dibenzo-[a,c]cycloheptene-5-yl-[2,3-*f*]-3'-methyleno-2'-hydrofuranyl)(2-pent-4-oyl)acetamide 11

4-Pentynoic acid (36 mg, 1.5 eq., 0.364 mmol) was treated with Yamaguchi reagent TCBC (118 mg, 2 eq., 0.486 mmol, 76  $\mu$ L) and triethylamine (123 mg, 5 eq., 1.215 mmol, 169

 $\mu$ L) in dry DCM for 5 h at 0–5 °C. Then the mixture of compound 5 (100 mg, 1 eq., 0.243 mmol) and 4-DMAP (89 mg, 3 eq., 0.729 mmol) in DCM was added dropwise and the mixture was stirred for 20 h at room temperature. After completion of the reaction, solvent was removed under reduced pressure and the product was isolated using column chromatography with the mixture of petroleum–ethyl acetate–ethanol (10 : 1 : 1) as eluent. Compound **11** was isolated as pale-beige solid with 49% yield (58 mg).

<sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  8.52 (d, J = 8.2 Hz, 1H, NH), 7.42 (s, 1H, 8-H), 6.85 (s, 1H, 11-H), 6.78 (s, 1H, 4-H), 5.47 (br.s, 1H, 10-H), 5.15 (s, 2H, 9-H), 5.03 (br.s, 1H, 10-H), 4.56 (d, J = 3.2 Hz, 2H, 2'-H), 4.52–4.46 (m, 1H, 7-H), 3.83 (s, 3H, OMe), 3.79 (s, 3H, OMe), 3.48 (s, 3H, OMe), 2.81 (s, 1H, 7'-H), 2.60 (t, J = 7.2 Hz, 2H, 4'-H), 2.42–2.39 (m, 2H, 5'-H), 2.22–1.97 (m, 3H, 6-H, 5-H), 1.97–1.88 (m, 1H, 6-H).

<sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>) δ 172.88, 170.96, 165.93, 162.53, 152.24, 150.34, 143.24, 143.22, 140.60, 134.65, 126.51, 124.21, 123.75, 122.12, 108.06, 105.36, 99.74, 83.53, 82.99, 74.92, 71.67, 71.31, 60.58, 55.82, 48.58, 37.92, 33.03, 32.47.

MALDI (DCTB, pos. mode): 491.1 (100%), 394.2 (37%), 354.2 (26%) Elemental analysis: for C<sub>28</sub>H<sub>29</sub>NO<sub>7</sub> calcd.: C, 68.42; H, 5.95; found: C, 68.23; H, 6.07. mp 67 °C.

# 1.3 Synthesis of target conjugates 15 and 16

# Synthesis of 1-palmitoyl-2-(11-azidoundecanoyl)-sn-glycero-3-phosphocholine 14

11-Azidoundecanoic acid (125 mg, 6 eq., 0.546 mmol), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids, Inc.) (45 mg, 1 eq., 0.091 mmol), and 4-DMAP (67 mg, 6 eq., 0.546 mmol) were dissolved in dry CHCl<sub>3</sub>. DIC (92 mg, 8 eq., 0.728 mmol, 113  $\mu$ L) was added subsequently and the mixture was stirred for 36 h at room temperature. Then solvent was removed and the product was isolated using column chromatography with the mixture of CHCl<sub>3</sub>–EtOH (6 : 4)  $\rightarrow$  CHCl<sub>3</sub>–EtOH–H<sub>2</sub>O (10 : 10 : 1) as a colorless oil with 78% yield (50 mg).

<sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD)  $\delta$  5.20 (dd, J = 6.2, 2.9 Hz, 1H, 2-H), 4.39 (dd, J = 12.0, 3.1 Hz, 1H, 4-H), 4.23 (br.s, 2H, 5-H), 4.13 (dd, J = 12.0, 6.9 Hz, 1H, 4-H), 3.98 (t, J = 6.2 Hz, 2H, 1-H), 3.61 – 3.56 (m, 2H, 3-H), 3.24 (t, J = 6.9 Hz, 2H, 2'-H), 3.19 (s, 9H, NMe<sub>3</sub>), 2.29 (q, J = 7.7 Hz, 4H, 2''-H, 11''-H), 1.61 – 1.53 (m, 6H, 3'-H, 3''-H, 10''-H), 1.32 – 1.20 (m, 36H, alkyl chains), 0.85 (t, J = 6.7 Hz, 3H, 16'-H).

<sup>13</sup>C NMR (101 MHz, CD<sub>3</sub>OD) δ 174.46, 174.05, 70.89, 70.81, 66.88, 66.82, 64.23, 64.18, 63.08, 59.65, 59.60, 54.47, 51.90, 34.64, 34.52, 32.37, 30.13, 30.11, 30.09, 29.95, 29.90, 29.82, 29.80, 29.75, 29.72, 29.58, 29.52, 29.27, 27.16, 25.34, 25.33, 23.10, 14.29.
<sup>31</sup>P NMR (162 MHz, CD<sub>3</sub>OD) δ –0.80.

# General procedure for conjugation

Alkyne-source (1.2 eq.) and azido-containing phosphatydylcholine **14** (1 eq.) were dissolved in DMF under inert atmosphere. To this mixture, solution of  $CuSO_4 \cdot 5H_2O$  (0.2 eq.), sodium ascorbate (0.2 eq.), and tris(benzyltriazolylmethyl)amine (TBTA) (0.4 eq.) in water was added dropwise. The resulting solution was stirred for 2 h at 55 °C. After completion of the reaction, solvents were removed under reduced pressure and the target conjugate was isolated using column chromatography with the mixture of CHCl<sub>3</sub>–EtOH (6 : 4)  $\rightarrow$  CHCl<sub>3</sub>–EtOH–H<sub>2</sub>O (10 : 10 : 1) as eluent.

Compound 15

 Isolated as white solid with 56% yield.

<sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD)  $\delta$  7.58 (s, 1H, 18-H), 7.54 (d, J = 1.4 Hz, 1H, NH), 7.37 (d, J = 10.8 Hz, 1H, 10-H), 7.32 (s, 1H, 8-H), 7.01 (d, J = 10.9 Hz, 1H, 11-H), 6.61 (s, 1H, 4-H), 5.20 (dd, J = 6.5, 2.9 Hz, 1H, 2'-H), 4.63 (d, J = 6.6 Hz, 1H, 4'-H), 4.41 – 4.37 (m, 1H, 4'-H), 4.28 (d, J = 7.1 Hz, 2H, 5'-H), 4.23 (s, 2H, 13-H), 4.13 (dd, J = 12.0, 6.8 Hz, 1H, 7-H), 3.98 (s, 3H, OMe), 3.95 (d, J = 9.4 Hz, 2H, 1'-H), 3.90 (s, 3H, OMe), 3.89 (s, 3H, OMe), 3.59 (s, 3H, OMe), 3.58 (d, J = 3.2 Hz, 2H, 3'-H), 3.20 (s, 9H, NMe<sub>3</sub>), 3.04 (d, J = 4.6 Hz, 2H, 15-H), 2.81 (t, J = 6.9 Hz, 2H, 16-H), 2.59 (dd, J = 13.7, 6.3 Hz, 1H, 11b-H), 2.39 (dd, J = 13.2, 6.5 Hz, 1H, 11b-H), 2.29 (dt, J = 13.8, 7.3 Hz, 6H, 3a-H, 3b-H, 10b-H), 2.21 (dd, J = 12.5, 6.3 Hz, 1H, 2a-H), 2.13–2.06 (m, 1H, 2a-H), 1.87–1.82 (m, 2H, 2b-H), 1.59–1.55 (m, 4H, 5-H, 6-H), 1.27–1.23 (m, 36H, alkyl chains), 0.85 (br.s, 3H, 16a-H).

<sup>13</sup>C NMR (101 MHz, CD<sub>3</sub>OD) δ 174.51, 174.07, 172.65, 168.48, 165.67, 154.28, 153.07, 151.53, 141.95, 137.86, 136.87, 135.16, 133.12, 126.01, 117.97, 117.92, 114.28, 108.12, 78.32, 72.39, 70.88, 70.79, 66.88, 66.78, 64.55, 63.12, 63.03, 61.75, 61.68, 59.97, 59.90, 56.77, 56.44, 54.48, 52.86, 51.07, 49.50, 49.29, 49.07, 36.52, 34.66, 34.55, 33.64, 32.45, 30.65, 30.31, 30.20, 30.16, 30.01, 29.87, 29.82, 29.75, 29.64, 29.56, 29.46, 26.92, 25.39, 23.17, 20.93, 14.31.

- <sup>31</sup>P NMR (162 MHz, CD<sub>3</sub>OD) δ –0.64.
- HRMS: found: 1200.6859

mp 47 °C.

# Compound 16

Isolated as white solid with 62% yield.

<sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD)  $\delta$  7.57 (d, J = 5.5 Hz, 1H, NH), 7.55 (s, 1H, 18-H), 7.46 (s, 1H, 8-H), 6.73 (s, 1H, 11-H), 6.62 (s, 1H, 4-H), 5.37 (br.s, 1H, 10-H), 5.22–5.19 (m, 1H, 2'-H), 5.09 (s, 2H, 9-H), 4.97 (s, 1H, 10-H), 4.76–4.71 (m, 1H, 4'-H), 4.55 (dd, J = 4.5, 1.4 Hz, 1H, 4'-H), 4.39 (t, J = 9.1 Hz, 1H, 7-H), 4.23 (t, J = 6.9 Hz, 4H, 15-H, 16-H), 4.14 (dd, J = 11.9, 6.8 Hz, 2H, 1'-H), 3.97 (s, 2H, 13-H), 3.90 (s, 3H, OMe), 3.88 (s, 3H, OMe), 3.58 (br.s, 2H, 3'-H), 3.52 (s, 3H, OMe), 3.19 (s, 9H, NMe<sub>3</sub>), 3.09–3.05 (m, 2H, 5'-H), 2.85–2.81 (m, 2H, 11b-H), 2.54–2.42 (m, 2H, 2a-H), 2.31–2.27 (m, 6H, 2b-H, 3b-H, 10b-H), 1.84–1.79 (m, 2H, 3a-H), 1.59–1.55 (m, 4H, 5-H, 6-H), 1.32–1.16 (m, 36H, alkyl chains), 0.85 (t, J = 4.6 Hz, 3H, 16a-H).

<sup>13</sup>C NMR (101 MHz, CD<sub>3</sub>OD) δ 174.46, 174.00, 172.77, 168.31, 155.43, 153.06, 141.97, 141.57, 136.90, 135.85, 129.18, 121.39, 115.92, 108.28, 106.30, 94.51, 84.37, 78.27, 70.70, 70.63, 66.69, 64.81, 63.36, 62.85, 61.62, 61.32, 60.20, 56.40, 56.25, 54.44, 51.16, 50.21, 50.16, 49.71, 49.50, 49.29, 49.07, 48.86, 38.78, 34.62, 34.52, 33.50, 32.42, 31.08, 30.42, 30.17, 30.13, 29.98, 29.84, 29.79, 29.68, 29.61, 29.50, 29.34, 26.79, 25.36, 25.32, 25.01, 23.14, 20.85, 14.30.

- $^{31}$ P NMR (162 MHz, CD<sub>3</sub>OD)  $\delta$  –4.57.
- HRMS: found: 1196.6882

mp 41 °C.

Synthesis and characterization of carboxyl-containing compounds for comparative analysis are given in supplementary information

# **Quantum Chemistry**

Geometries of colchicinoids were optimized by the Hartree–Fock method with the def2-SVP basis set. Calculations were carried out using the ORCA software<sup>83</sup>. Calculations were performed for compounds 4 and 5 on the assumption that the resulting colchicinoid structures will be equivalent to those in 15 and 16.

Van der Waals distances listed in<sup>84</sup> were used to calculate the volumes, dimensions, and projections for the rigid part of the colchicinoid molecule (without the HO–CH<sub>2</sub>–CO– fragment).

#### **Monolayer Surface Area Experiments**

In the monolayer experiments, we used 0.5 mg/mL POPC solution in chloroformmethanol (5 : 1, v/v) (Merck KGaA and Macron Fine Chemicals, correspondingly) or the POPC solution mixed with 0.3 mg/mL colchicinoid solution in chloroform. To create lipid film, we used a Hamilton syringe to place the lipid solution on the surface of 10 mM KCl (Sigma-Aldrich) prepared in triple distilled water. To let the solvent evaporate, the measurements were performed in about 20 min (room temperature of about 20 °C) after applying the lipid film. Experimental pressure–area diagrams were obtained simultaneously with the Volta potential measurements. The experiments were performed using a Microtrough XS equipment operating under FilmWareX-4.0 software (both Kibron Inc. Finalnd) with a PTFE trough and two POM barriers. Before the measurements, a cleaning procedure was performed, and the system was tested for the stability of pressure (within 0.1 mN/m) and potential (within 10 mV) over the entire area of the trough. We used the compression speed of 10 mm<sup>2</sup>/min.

# Liposome Preparation and Characteristics Liposome Preparation

Liposomes (large unilamellar vesicles) were prepared as described earlier<sup>85</sup>. Briefly, lipid films were obtained by co-evaporation of aliquots of stock solutions in chloroform–methanol (2 : 1) in round-bottom flask on a rotary evaporator, with subsequent drying for 45 min at 5 Pa. The resulting compositions were ePC–conjugate **15**, 95 : 5; ePC–conjugate **16**, 95 : 5; ePC, 100 % (by mol). Then lipid films were hydrated in phosphate buffered saline (PBS, pH 7.4) for MTT experiments or TrisHCl/NaCl buffer for TLC (pH = 8.2, with addition 5 mM Ca<sup>2+</sup>), subjected to seven cycles of freezing/thawing (liquid nitrogen/+40 °C), and extruded 20 times through polycarbonate membrane filters (Nucleopore, USA) with a pore size of 100 nm on a Mini-extruder by Avanti. Concentrations of **15** and **16** were controlled by UV spectrophotometry after liposome disruption with ethanol ( $\lambda_{15}$  max = 350 nm,  $\varepsilon \sim 10200$  M<sup>-1</sup> cm<sup>-1</sup>,  $\lambda_{16}$  max = 250 nm,  $\varepsilon \sim 16500$  M<sup>-1</sup> cm<sup>-1</sup>).

To prepare liposomes with self-quenched calcein, lipid films were hydrated in PBS with 80 mM calcein and processed as described above. After extrusion, non-encapsulated calcein was removed by size exclusion chromatography on a Sephadex G-50 column (1.3 × 18 cm) equilibrated in PBS: 200- $\mu$ L aliquots of calcein-containing liposome dispersions were applied onto the column, the void volume (4.5 mL) was discarded, and then 150–200  $\mu$ L fractions were collected. Three or four peak liposomal fractions were combined. **15**, **16**, and calcein concentrations were evaluated by measuring UV spectra of decomposed liposomal dispersions in ethanol ( $\lambda$  max calcein 504 nm,  $\epsilon \sim$ 74,000 M <sup>-1</sup> cm <sup>-1</sup>). Liposome dispersions were stored at +4°C and used for experiments within 5 days.

# **Zeta Potential**

For reliable measurements, liposome samples were prepared as described earlier<sup>85</sup>. Briefly, lipid films were hydrated in 10 mM KCl, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 1mM K<sub>2</sub>HPO<sub>4</sub> solution (pH 7.35), and after freezing–thawing procedure they were extruded 20 times through 200 nm

polycarbonate membrane filters to gain liposome diameter of 200 nm. Zeta potential values were obtained using ZetaPALS analyzer (Brookhaven Instruments Corp., Holtsville, NY; provided by the CoreFacility of the Institute of Gene Biology, Russian Academy of Sciences). Samples of liposomes (1.5 mL, 1 mg/mL total lipids) were equilibrated for 1 min in cuvettes before 10 runs of 25 cycles per sample were performed at 25 °C. Zeta potential values were calculated using Smoluchowski approximation.

# **Dynamic Light Scattering and TEM**

Liposome size upon preparation was controlled in diluted suspensions (1 mg total lipids/mL PBS) by dynamic light scattering using a ZetaPALS analyzer (Brookhaven Instruments Corp., Holtsville, NY; provided by the CoreFacility of the Institute of Gene Biology, Russian Academy of Sciences) equipment in at least ten runs per sample.

The liposome suspensions were studied under a Jeol JEM-100-CX-II transmission electron microscope. To prepare a sample, a droplet of dispersion was placed on a copper carbon-coated grid and left for 2 min. The excess of liquid was then carefully removed with an edge of a filter paper and the grid was stained with a droplet of 2% uranyl acetate (aqueous solution) for 2 min. After the removal of the stain droplet, the grid was air-dried. Transmission electron microscopy was performed using an acceleration voltage of 80 kV.

# PLA2 Hydrolysis

# **General Information**

Liposomes (ePC; ePC–conjugate **15**; ePC–conjugate **16**) with total lipid concentration 5 mM were treated with PLA2s from *Viperae ursinii* and porcine pancreas. Enzyme concentration in every sample was 1  $\mu$ M. Liposome suspension was incubated with PLA<sub>2</sub> for 15, 30, 60 min, or 24 h. Then PLA<sub>2</sub> activity was inhibited by 8-fold excess of EDTA, compared to Ca<sup>2+</sup> concentration. The samples were diluted with ethanol, evaporated on a rotary evaporator, and vacuum dried for 4 h at 3 Pa. Lipid components were extracted with the mixture of CHCl<sub>3</sub> and CH<sub>3</sub>OH, 1 : 1, using ultrasound treatment.

Insoluble material was pelleted and discarded (800 g, 10 min). Supernatant was analyzed by TLC on aluminium sheets precoated with silica gel (Kieselgel 60, Merck, Darmstadt, Germany). Eluent: CHCl<sub>3</sub>–CH<sub>3</sub>OH–NH<sub>4</sub>OH 28%, 65 : 25 : 4. Detection: molybdenum blue reagent by Vaskovsky<sup>86</sup>.

# Cytotoxicity Determination Cell Cultures

Pancreatic cell lines BxPC-3, PANC-1, COLO-357, and HaCaT were grown in DMEM medium supplemented with 10% fetal calf serum (FCS), pen-strep-glut (all from PanEco, Moscow, Russian Federation). All cell lines used were routinely tested for mycoplasma. Adherent cells were detached using 0.05% trypsin–EDTA (PanEco, Moscow), counted, and sub-cultured. Twenty four hours before assays, cells were seeded in appropriate plates (96- or 24-well plates), adjusted to  $3 \times 10^5$  cells/mL, and incubated overnight to achieve standardized growth conditions.

# **MTT Assay**

Cytotoxic effect of the allocolchicinoids was estimated by a standard 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT, Sigma) test as was described earlier<sup>87</sup>. The compounds were dissolved in dimethylsulfoxide to 20 mM concentration and stored at -20 °C until the assay. Different dilutions of the compounds in the DMEM medium from 20  $\mu$ M to 0.1 nM were prepared separately and transferred in 100  $\mu$ L to the plates with the cells. Liposome dispersions containing equivalent amounts of conjugates **15** and **16** were prepared similarly. Non-treated cells served as controls. Plates were incubated for 72 h. For the last 6 h, 5 mg/ml of MTT was added in the amount of 10  $\mu$ L to each well. After the incubation, culture medium was removed and 100  $\mu$ L dimethylsulfoxide was added to each well. Plates were incubated at shaking for 15 min to dissolve the formed formazan product. Optical density was read on spectrophotometer Titertek (UK) at 540 nm. Results were analyzed in Excel package (Microsoft). Cytotoxic concentration giving 50% of the maximal toxic effect (IC<sub>50</sub>) was calculated from the titration curves.

# Acknowledgements

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# **Author Contributions**

I.B. designed the study. I.B., Ek.S., A.F., and E.V. designed conjugates **15**and **16**. Ek.S. synthesized both **15** and **16** molecules. Ek.S., D.T., and A.A. performed PLA2 responsivity studies. Y.U. isolated enzymes. I.B. performed quantum chemistry calculations T.G. calculated the lateral stress profile. Y.E. performed monolayer experiments. N.K., El.S. performed MTT tests. A.A. and V.C. performed TEM analysis. All authors contributed to data analysis and manuscript writing.

# **Supporting Information**

Complete characteristics of synthesized compounds; parameters of quantum chemistry calculations; liposome characterization; PLA2 hydrolysis raw data. The Supporting Information is available free of charge on the ACS Publications website at DOI: xxx.

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