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Selective inhibition of the interaction of C1q with immunoglobulins and the classical pathway of complement activation by steroids and triterpenoids sulfates

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Abstract—Since undesirable activation of the complement system through the classical pathway is associated with tissue damage and other pathologic proinflammatory consequences at ischemia/reperfusion injury, autoimmune diseases, and rejection of allo- and xenografts, creation of selective inhibitors of the classical pathway leaving the alternative pathway intact is of great importance. Classical pathway is triggered by binding of its recognizing unit, protein C1q, to a number of targets like antibodies, pentraxins, apoptotic cells, and others. In order to obtain inhibitors blocking the first step of the classical cascade, synthesis of sulfates of steroids (Δ^5-3β-hydroxycholenic, Δ^5-3β-hydroxyetiocholenic, deoxycholic, and cholic acids) and triterpenoids (betulin, 20,29-dihydro-20,29-dichloromethylenbetulin, betulinic, ursolic, and oleanolic acids) has been performed. Testing of the compounds in classical pathway inhibition assay has displayed derivatives of triterpenoid betulin (betulin disulfate and betulinic acid sulfate) to be the most potent inhibitors. Further studies of the two compounds established that their activity to inhibit the classical pathway had been due to their capability to block the interaction of C1q with antibodies. Betulin disulfate and betulinic acid sulfate have shown weak inhibition of the alternative route of activation, what makes them promising inhibitors for the selective suppression of the classical complement pathway at the earliest possible level as well as perspective agents for blocking the interaction of C1q with its other targets.

1. Introduction

One of the most ancient parts of immunity is the complement system, which triggers a powerful, coordinated repertoire of antimicrobial reactions, including inflammation, opsonization, and direct cell lysis.[1–3] Thirty plasma and membrane components, factors, regulators, and receptors of the complement system are linked in biochemical cascades and can be activated via three pathways. The classical pathway of the complement is usually activated when the first complement component C1q binds to a complex of antigen and IgM or IgG antibody. Interaction of C1q with pentraxins, lipopolysaccharides, viral particles, apoptotic cells, and some other agents can also trigger the classical pathway. Activation of the lectin pathway is initiated by mannose-binding lectin (MBL) recognizing mannose on bacteria surface, by IgA or by structures exposed on damaged endothelium. The alternative pathway is activated by polysaccharides on microbial surfaces and complex polysaccharides, for example, yeast cell walls, endotoxins, and viral particles. Activation of the complement

Abbreviations: BSA, bovine serum albumin; *C*log*P*, hydrophobicity; EA, antibody-sensitized erythrocytes; EAC1q, antibody-sensitized erythrocytes with bound C1q; ED_{50}, concentration of a compound causing 50% hemolytic effect; Er, rabbit erythrocytes; IC_{50}, concentration of a compound causing 50% inhibition; K_i, inhibition constant; R1q, human serum depleted of C1q; TEBA, triethylbenzylammonium chloride.

Keywords: Complement system; Inhibitors; Betulin; Steroid; Triterpenoid; Sulfate.

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system via the alternative and the lectin pathways is a major mechanism in innate immunity and takes place upon direct contact with microorganisms.

The wide array of proinflammatory consequences of complement activation[4] makes the complement system an attractive target for therapeutic intervention and has led to the isolation, design, and synthesis of numerous complement inhibitors targeting in most cases all three complement pathways.[1,5–7] However, at many pathological conditions complement-mediated damage is initiated by only one complement route. Activation of the classical complement pathway is involved in tissue injury resulting from deposition of autoantibodies and immune complexes, which may occur in autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, myasthenia gravis, and hyperacute xenograft rejection.[8,9] Thus, the selective inhibition of the classical pathway would result in suppression of the injury, but not affect the alternative and lectin pathways, playing a key role in innate immunity against pathogens. That is why inhibitors of the first classical pathway component C1 (comprising C1q, C1r, and C1s) have particular perspectives for the therapeutic complement inhibition. Roos has described peptide 2J (CEGPFGPRHDLTFCW) selected from phage-displayed peptide library on the basis of binding to protein C1q.[8] It showed effective inhibition of the classical complement pathway (IC_{50} 2–6 μM) but no inhibition of the alternative complement pathway. A new series of potent and selective inhibitors of serine protease C1s described recently includes thiopheneamidine derivatives inhibiting the classical pathway of complement activation at IC_{50} 0.3 μM.[10]

This work focuses on studying of negatively charged low molecular weight inhibitors of protein C1q. The protein has six heterotrimeric globular domains (gC1q), which are responsible for binding of C1q to most of its targets: antibodies,[11] C-reactive protein,[12] β-amyloid peptide,[13] and other molecules. Recent studies dealing with mutant recombinant forms of C1q fragments and three-dimensional models of the C1q globular domain in complex with two of its physiological ligands, C-reactive protein and IgG, have revealed a central role for positively charged amino acid residues in the interaction of C1q with its targets.[11,14] It likely contributes to high effectiveness of negatively charged compounds in blocking the C1q functions.

Natural and synthetic polymers carrying sulfate groups (heparin, carrageenin, dextran sulfate, chondroitin sulfate, polyvinyl sulfate, and others) have been known to have strong complement-inhibiting properties for more than 65 years.[15,16] The reason for the fact that they are

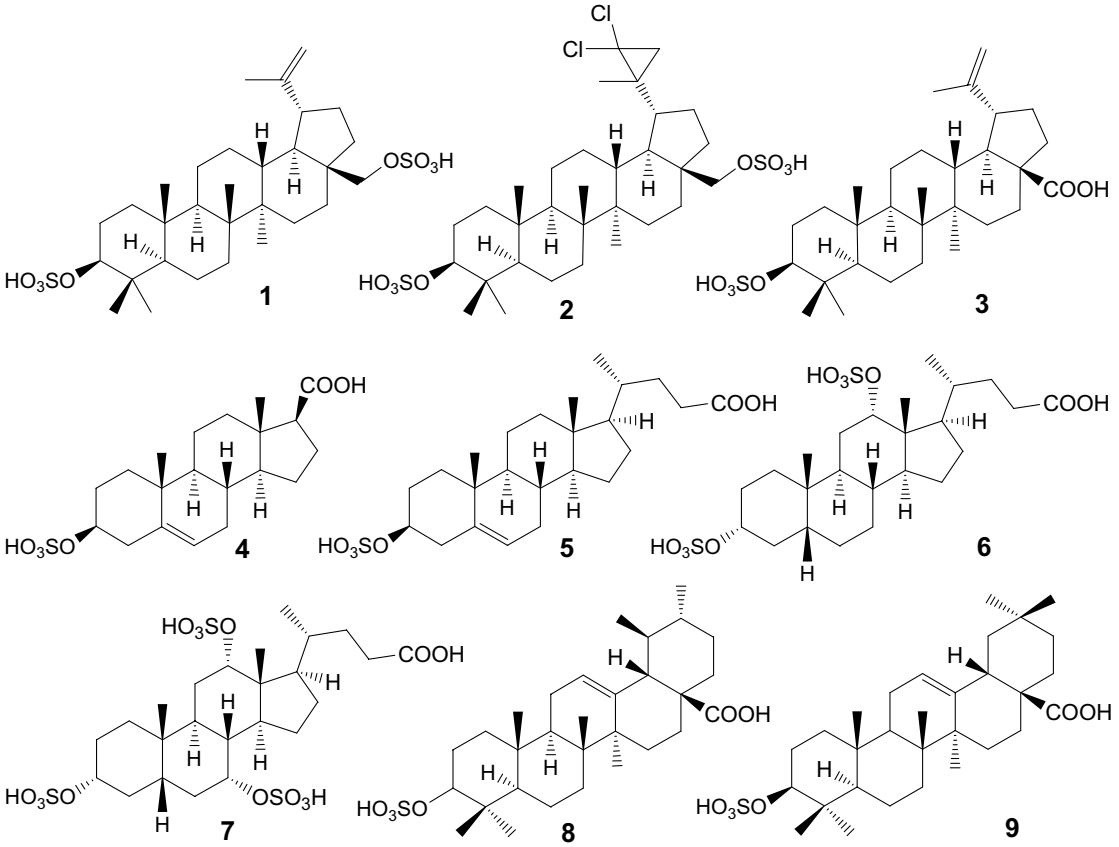


Figure 1. Studied compounds: betulin disulfate (**1**), 20,29-dihydro-20,29-dichloromethylenbetulin disulfate (**2**), betulinic acid sulfate (**3**), Δ^5-3β-hydroxyetiocholenic acid sulfate (**4**), Δ^5-3β-hydroxycholenic acid sulfate (**5**), deoxycholic acid disulfate (**6**), cholic acid trisulfate (**7**), ursolic acid sulfate (**8**), oleanolic acid sulfate (**9**).

still not used as therapeutic complement inhibitors is an anticoagulant effect of heparin-like compounds.[17] We proposed that low molecular weight compounds having at least two negatively charged groups would display comparable complement-inhibiting activity without mentioned side effects. A series of bisphenol disulfates (IC_{50} 40–250 μM)[18] was obtained on the basis of this hypothesis and here we describe a set of sulfated steroids and triterpenoids as effective complement inhibitors. As steroids and triterpenoids have been widely accepted to be low toxic compounds, their sulfo- and phosphoesters are supposed to have good therapeutic perspectives.

Some steroids and triterpenoids have already been studied for possible complement-inhibiting properties. Packard and Weiler have found a set of sulfates and phosphates of steroids inhibiting the alternative pathway of activation at high concentrations (0.6 mg/ml).[19] Assefa and collaborators have discovered the classical pathway-inhibiting properties of betulinic acid (IC_{50} 577 μM), oleanolic acid (IC_{50} 72.3 μM), and some negatively mono-charged derivatives (the most active compound had IC_{50} 31.8 μM)[20] and A/B-ring partial analogues (IC_{50} 488–633 μM) of oleanolic acid.[21] Ability of ursolic acid to inhibit the classical pathway (IC_{50} 54.7 μM) has been described by Master.[22] Potent inhibitor targeting the classical pathway, clionasterol (IC_{50} 4.1 μM), has been found as a result of study focusing on complement-inhibiting properties of a series of steroids.[23] This compound does not inhibit the alternative pathway at concentrations as high as 400 μM and has been shown to exert its effect interfering with complement component C1.

Here we describe synthesis and classical pathway-inhibiting properties of some steroids (Δ^5-3β-hydroxycholenic, Δ^5-3β-hydroxyetiocholenic, deoxycholic, cholic acids) and triterpenoids (betulin, 20,29-dihydro-20,29-dichloromethylenbetulin, betulinic, ursolic, and oleanolic acids) sulfates (Fig. 1). Activity of the most potent inhibitors, betulin disulfate and betulinic acid sulfate, was studied in more detail. They appeared to inhibit the first step of the classical pathway activation, the interaction of protein C1q with antibodies, what can be the basis of their classical pathway-inhibiting properties. The compounds showed no considerable activity with respect to the alternative pathway of complement activation.

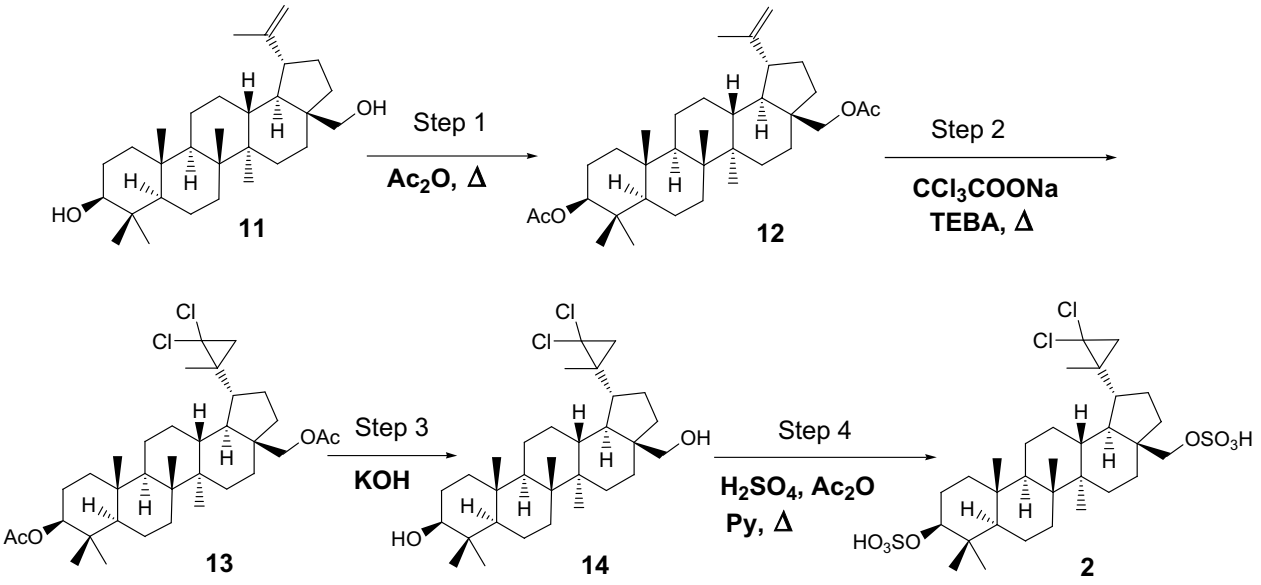
2. Results

2.1. Chemistry

20,29-Dihydro-20,29-dichloromethylenbetulin (**14**) was prepared by Makosza reaction[24] of dichlorocyclopropanation of olefins using sodium trichloroacetate as a carbon source and triethylbenzylammonium chloride (TEBA) as a phase-transfer catalyst (Scheme 1, Steps 1 and 2) followed by the deprotection of hydroxyl groups (Scheme 1, Step 3).[25]

For the obtaining of sulfates **1**–**9** from correspondent alcohols we developed method using sulfuric acid and acetic anhydride in pyridine as reagents (Scheme 1, Step 4).[18] This way is a good alternative for the 'classical' method of sulfation of steroids, when pyridine sulfur trioxide as a reagent is applied.[26] In that case, bad quality of pyridine sulfur trioxide often results in low conversion of starting alcohol, difficult purification of a product from polymeric byproducts, and the low yield of di-substituted derivative. The new method allows obtaining of sulfates of aliphatic and aromatic alcohols with high yield.

Betulin diphosphate was obtained using $POCl_3$ in acetone with following hydrolysis by water.



Scheme 1.

2.2. Hemolytic activity of steroids and triterpenoids sulfates depends on the morphology of supramolecular structures they form

Before the studying of complement-inhibiting properties of compounds **1**–**10** in complement-hemolytic system we determined their intrinsic hemolytic activity incubating various concentrations of them with sheep erythrocytes. Two of the compounds, 20,29-dihydro-20,29-dichloromethylenbetulin disulfate (**2**) and oleanolic acid sulfate (**9**), showed strong lytic properties (Table 1). High hemolytic activity of oleanolic acid sulfate was an unexpected result considering the fact that its close analogues, sulfates of betulinic (**3**) and ursolic acids (**8**), did not display lytic properties in diapason of concentrations below 900 μM. Working with compounds under research, we observed that in contrast to the rest of the inhibitors, sulfates **3**, **8**, **9** formed gels in veronal buffer. We determined the critical micelle concentration (CMC) in veronal buffer for these compounds (35, 276, 14 μM, correspondently) and hypothesized that their molecules could assemble into supramolecular structures of different morphology, which could explain their different hemolytic properties. In order to examine our hypothesis, we studied solutions of compounds **3**, **8**, and **9** by microphotography (Fig. 2). Interestingly, microphotos of structures derived by betulinic and ursolic acids sulfates (Fig. 2A and B) revealed the presence of thin long fibers of 4 and 10–40 nm thickness, correspondently. Solution of oleanolic acid sulfate turned out to be a thin suspension of nanocrystals (Fig. 2C). Apparently, hemolytic activity of compound **9** is connected with these nanocrystals, which might be able to damage the membranes of erythrocytes and cause thereby hemolysis.

2.3. Steroids and triterpenoids sulfates inhibit the classical pathway of complement activation

Ability of compounds **1**–**8**, **10** to inhibit the classical pathway of complement activation was assessed in hemolytic system using high-diluted guinea-pig serum as a complement source and antibody-sensitized sheep erythrocytes. It was established that all the compounds exert complement-inhibiting properties (Table 1) and the activity depends on the hydrophobicity of the inhibitors. Betulin disulfate (**1**) (Fig. 3), 20,29-dihydro-20,29-dichloromethylenbetulin disulfate (**2**), and betulinic acid sulfate (**3**), compounds having the highest hydrophobicity, displayed the best effect. More hydrophilic compounds, Δ^5-3β-hydroxyetiocholenic acid sulfate (**4**), deoxycholic acid disulfate (**6**), cholic acid trisulfate (**7**), were less active.

It was interesting to compare the complement-inhibiting activity of sulfates of Δ^5-3β-hydroxycholenic acid (**5**) (IC_{50} 23.7 μM), deoxycholic acid (**6**) (IC_{50} 185.6 μM), and cholic acid (**7**) (IC_{50} 100.8 μM) having the same structure of steroid skeleton but different quantity of charged groups. Surprisingly, the results of their testing

Table 1. Classical pathway-inhibiting and hemolytic properties of steroids and triterpenoids sulfates

No.	Compound	*C*log *P*	Classical pathway IC_{50}[a], μM	Hemolytic activity ED_{50}[b], μM
1	Betulin disulfate	9.55	6.9 ± 3.1	850
2	20,29-Dihydro-20,29-dichloromethylenbetulin disulfate	9.29	6.8 ± 0.2	85
3	Betulinic acid sulfate	9.21	9.9 ± 1.5	>900
4	Δ^5-3β-Hydroxyetiocholenic acid sulfate	5.03	409.5 ± 16.5	>1600
5	Δ^5-3β-Hydroxycholenic acid sulfate	6.63	23.7 ± 6.5	>400
6	Deoxycholic acid disulfate	5.20	185.6 ± 6.5	>1600
7	Cholic acid trisulfate	3.42	100.8 ± 12.9	>1400
8	Ursolic acid sulfate	9.28	25.2 ± 0.3	>900
9	Oleanolic acid sulfate	9.33	—	15
10	Betulin diphosphate	7.27	42.6 ± 2.9	>800

[a] To assess the capability of the compounds to inhibit the classical pathway, antibody-sensitized sheep erythrocytes were incubated with high-diluted guinea-pig serum (600-fold diluted) in presence of different concentrations of the compounds.
[b] To determine the hemolytic activity of the compounds, sheep erythrocytes were incubated with different concentrations of the compounds.

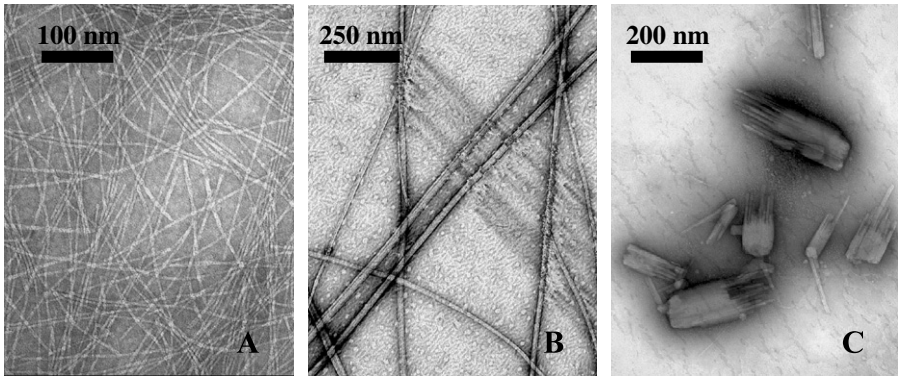


Figure 2. Negatively stained nanostructures in water: (A) betulinic acid sulfate (480 μM); (B) ursolic acid sulfate (360 μM); (C) oleanolic acid sulfate (360 μM).

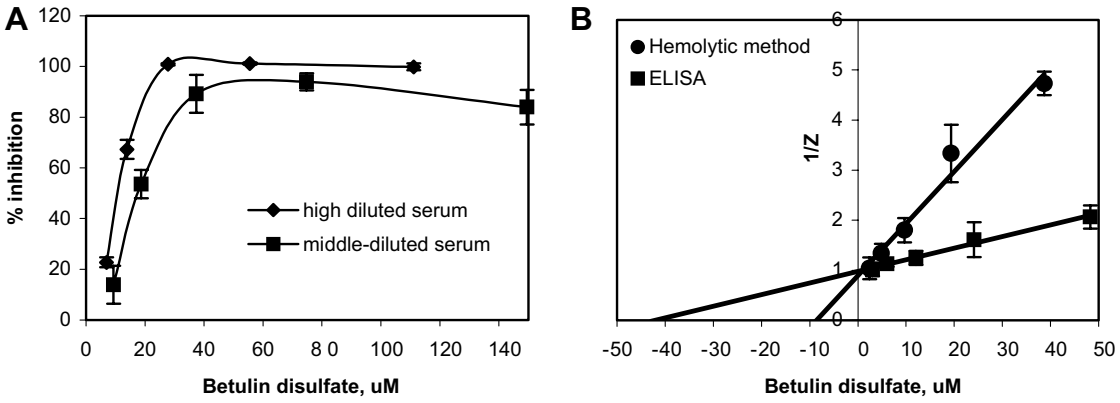


Figure 3. (A) Effect of betulin disulfate on activation of the classical pathway initiated by incubation of antibody-sensitized sheep erythrocytes with high-diluted serum (600-fold diluted guinea-pig serum) or middle-diluted serum (170-fold diluted human serum). (B) Effect of betulin disulfate on C1q enzyme activity assessed by ELISA and hemolytic method.

Table 2. Complement-inhibiting properties of betulin disulfate and betulinic acid sulfate

No	Compound	IC_{50}, μM			K_i, μM	
		Inhibition of the complement system activation			Inhibition of the C1q—immunoglobulin interaction	
		Classical pathway[a]		Alternative pathway[b]	ELISA[c]	Hemolytic method[d]
		High-diluted guinea-pig serum	Middle-diluted human serum			
1	Betulin disulfate	6.9 ± 3.1	14.6 ± 2.1	414.5 ± 13.5	42.2 ± 2.6	8.6 ± 1.8
3	Betulinic acid sulfate	9.9 ± 1.5	53.83 ± 5.43	640.0 ± 86.0	57.3 ± 12.0	14.6 ± 3.5

[a] Classical pathway—inhibiting properties were assessed by incubation of antibody-sensitized sheep erythrocytes with high-diluted guinea-pig serum (600-fold diluted) or middle-diluted human serum (170-fold diluted) in presence of the compounds.
[b] Activity to inhibit the alternative pathway was determined by incubation of rabbit erythrocytes with human serum in presence of the compounds.
[c] Microtiter wells were coated with IgG3 and incubated with C1q preincubated with the compounds.
[d] Cellular intermediates EAC1q were obtained by interaction of EA with C1q source in the presence of the inhibitors and incubated with serum devoid of C1q to develop complement-mediated lysis of cells.

show that the increase in quantity of charged groups does not provide more potent complement inhibition. Probably, the introduction of more than two charged groups leads to considerable decrease in hydrophobic portion of the inhibitors and as a consequence decreases the activity. Apparently, the reduction of the hydrophobicity is also the reason for the lower activity of betulin diphosphate (**10**) with respect to that of betulin disulfate (**1**).

Activity of the most promising inhibitors, betulin disulfate and betulinic acid sulfate, having the highest complement-inhibiting effect and low hemolytic properties was studied further in classical pathway-mediated hemolytic reaction using middle-diluted human serum (Table 2, Fig. 3). As expected, IC_{50} values were 2 and 5.5 times higher than those required in high-diluted serum assay for betulin disulfate and betulinic acid sulfate, correspondently.

2.4. Betulin disulfate and betulinic acid sulfate weakly inhibit the alternative pathway of complement activation

In order to estimate selectivity of the action of compounds **1** and **3** on the classical pathway, we determined their activity with respect to the alternative route of complement activation. Alternative pathway was induced by incubation of rabbit erythrocytes with human serum at the conditions excluding the classical pathway's activation. Sulfates of betulin and betulinic acid exerted weak inhibitory properties with respect to alternative pathway (Table 2) showing the possibility of their use for the selective inhibition of the classical complement pathway.

2.5. Betulin disulfate and betulinic acid sulfate inhibit the interaction of C1q with immunoglobulins

Activation of the classical pathway of complement system is triggered when recognizing protein C1q binds to antibodies of immune complexes. To study the ability of compounds **1** and **3** to block the interaction of C1q with immunoglobulins, we used two different methods: hemolytic method allowing to separate the first step of complement activation and to assess the inhibitors' activity with respect to C1q protein and enzyme-linked immunosorbent assay based on the interaction of immobilized IgG3 antibodies with C1q in the presence of the inhibitors.

According to the first method,[27] EA are incubated with an inhibitor and diluted human serum as a C1q source at the conditions (30 °C for 15 min) when complex C1

is dissociated on 95%[28] and C1q is capable to bind to immune complexes, but complement cascade does not develop further. The obtained EAC1q complexes are isolated by centrifugation, supernatant is removed, and cells are incubated with R1q serum (at 37 °C for 30 min). At these conditions the classical complement pathway develops further, membrane attack complex assembles on erythrocyte surface and causes lysis of the cells to the extent corresponding to the quantity of C1q bound to the EA in the presence of an inhibitor. Using this method we established that betulin disulfate and betulinic acid sulfate inhibit the C1q enzyme activity (K_i 8.6 and 14.6 μM, correspondently) and C1q-immunoglobulin interaction (Table 2, Fig. 3).

K_i values obtained for these compounds using ELISA (42.2 and 57.3 μM) (Table 2 and Fig. 3) are worse than those obtained by the first hemolytic method what can be caused by differences in methods and concentrations of the biological reagents used.

3. Discussion

Undesirable activation of the complement system through the classical pathway is associated with tissue damage and other proinflammatory consequences at ischemia/reperfusion injury, autoimmune diseases, and rejection of allo- and xenografts.[8,9] Recent data suggest that unwanted activation of the classical pathway may involve binding of C1q to in situ deposited self proteins, such as acute phase proteins of the pentraxin family, and β-amyloid fibrils in brain lesions in Alzheimer's disease.[29–32] That is why development of inhibitors capable to block C1q functions and its interaction with the targets without affecting the alternative and lectin pathways is of great importance. A number of specific molecules that can regulate the functional activity of C1q have been reviewed.[1,29] The natural C1q-binding proteins and their functional parts, monoclonal antibodies directed against C1q, several series of C1q-binding peptides, and competitive inhibitors derived from the sequence of C1q are among them. Authors of the reviews note that the main problems arising at the inhibition of the C1q functions by these molecules are their short half-life in circulation, possible immunogenic reactions, low effectiveness of such inhibitors, and risk of triggering C1 activation by the inhibitors in case they bind to the globular head of C1q (it especially concerns multimeric structures).

Effective and selective synthetic low molecular weight inhibitors of non-protein nature could be a good alternative for the inhibition of the C1q functions. It prompted us to conduct this study focusing on the synthesis and investigation of the complement-inhibiting properties of a set of steroids and triterpenoids sulfates (Fig. 1). Testing of compounds **1**–**8**, **10** in the classical pathway-induced assay showed that all the compounds inhibited the classical pathway and the most hydrophobic structures, derivatives of triterpenoid betulin **1**–**3**, exerted the most potent effect (Table 1). These observations are in line with our previous findings[18,33] and correspond to the results of some other researchers[5] pointing out the important role of the hydrophobicity for the complement-inhibiting activity.

Betulinic acid sulfate (**3**) and ursolic acid sulfate (**8**) in our assay showed more potent complement-inhibiting properties than betulinic and ursolic acids studied by other researchers (their activity is given in Introduction). As a rule, IC_{50} values highly depend on complement-inhibiting assay, source and concentration of serum used for the activity determination. Unfortunately, we did not find the possibility to assess the activity of betulinic and ursolic acids in our assay to make the results more comparable because of extremely poor solubility of the compounds even in DMSO-containing water.

Two compounds of the set, **2** and **9**, turned out to have unwanted strong hemolytic properties. Possible reason for the high lytic effect of oleanolic acid sulfate is formation of nanocrystals, which were displayed by microphotography (Fig. 2C). Low solubility and amphiphilic properties can explain the appearance of fibrillar supramolecular structures of sulfates of betulinic and ursolic acids (Fig. 2A and B), which most likely exist at acting concentrations. It is well known that polymers, including negatively charged polymers, have higher effectiveness at the inhibition of the protein C1q functions as compared with low molecular weight (or monomeric) compounds. It can be explained by the presence of six identical globular domains in C1q structure and the opportunity of polymers to interact with more than one globular head of this protein simultaneously. For example, conjugates of Trp-Tyr (peptide imitating Trp^{277}-Tyr^{278} residues of C_H2 domain of immunoglobulin, which were determined to be involved in C1q–IgG interaction[34]) coupled to BSA and dextrans of different sizes were obtained by Anderson showed more than 100-fold enhancement in activity as against monomeric Trp-Tyr peptide.[35] Taking the speculations into account it was logical to expect an increase of complement-inhibiting activity of compounds **3** and **8** with respect to other compounds' activity. However, we do not observe such regularity: the activity of the compounds is in the same diapason as other inhibitors have. Thinking about possible explanations for this, we can suggest that the fibrillar structures considered here are much less flexible than usual polymers or their charged groups are not exposed effectively enough for the interaction with the protein. It should be noticed that microphotographs of water solutions of betulin disulfate did not reveal the presence of any supramolecular structures at concentrations up to 3 mM.

After testing of the obtained compounds with high diluted guinea-pig serum and determination of their hemolytic properties, two most promising inhibitors, betulin disulfate and betulinic acid sulfate, were sorted out for the next experiments. They were tested in the classical pathway assay using middle-diluted human serum and showed expected less activity (Table 2, Fig. 3). Weak inhibition of the alternative pathway found for betulin disulfate and betulinic acid sulfate shows that compounds **1** and **3** do not inhibit considerably the last stages of complement cascade, common for the three

routes of complement activation, what makes possible the selective blocking of the classical pathway of activation by these compounds. Activity of some negatively charged compounds to inhibit more than one step of complement activation was described more than once. The bright example is suramin having six sulfo-groups, which blocks most steps of the complement cascade.[5]

Our further effort was put into determination of whether betulin disulfate and betulinic acid sulfate inhibit the interaction of C1q with antibodies preventing thereby the development of the classical complement pathway. We attempted to assess K_i for the inhibition of the C1q functions by two different methods and found that inhibition constant values obtained by the hemolytic method, supposing the formation of cellular intermediate EAC1q in the presence of an inhibitor, better correlate with IC_{50} values of classical pathway inhibition assay than those obtained by ELISA. However, the considerable inhibition of C1q functions found in these assays and weak activity of betulin disulfate and betulinic acid sulfate with respect to the alternative pathway suggest that the blocking of the interaction of C1q with immunoglobulins may be the main mechanism of action of the compounds.

Evaluating the possible therapeutic future of steroids and triterpenoids sulfates, it should be noticed that the initial compounds, betulin, betulinic, oleanolic, and ursulic acids, are low toxic compounds and were described to have hepatoprotective effect.[36] Their sulfation unlikely changes the toxic properties, as the conjugation with sulfate group is one of the physiologic processes of biotransformation. The bioavailability of betulin is 30–50% (our unpublished data), however, that of charged analogues might be lower. Formation of any supramolecular structures by the compounds can unpredictably change the bioavailability decreasing the probability of hydrolysis of sulfate groups in the acidic medium and making possible the absorption of the nanoparticles through M cells of Peyer's patch.[37]

In conclusion, in search of selective low molecular weight inhibitors of recognition stage of the classical pathway of the complement system activation a set of steroids and triterpenoids sulfates have been studied. Betulin disulfate and betulinic acid sulfate displayed the highest effect at the inhibition of the classical pathway activation and weak activity with respect to the alternative route. The compounds likely suppress the classical pathway of complement by blocking the first stage of its activation. The results make betulin disulfate and betulinic acid sulfate promising inhibitors for the selective blocking of the classical complement pathway at the earliest possible level as well as perspective agents for the inhibition of the interactions of C1q with its other targets.

4. Materials and methods

4.1. Reagents and materials

Betulin was isolated from the birch bark and betulinic acid was obtained by betulin oxidation in our laboratory as described previously.[38] Oleanolic, ursolic, and cholenic acids were obtained as a kind gift from Dr. E. Zvonkova (Russian Institute of Aromatic Herbs, Moscow). Deoxycholic and cholic acids are commercially available from Aldrich. Sheep erythrocytes and rabbit antibodies against them were obtained from 'Biolek' company (Kharkov, Ukraine). Rabbit erythrocytes, serums, reagent R1q, IgG3, C1q, anti-C1q antibodies were obtained from G.N.Gabrichevsky Research Institute of Epidemiology and Microbiology (Moscow, Russia).

The ^{1}H NMR spectra were recorded at 300 MHz on Bruker DPX-300 (Germany) instrument. Chemical shifts are reported as δ units (ppm), and signals are expressed as s (singlet), d (doublet), m (multiplet). The elemental analysis was performed by Thermo Finigan EA1112 CHNS analyzer (Italy). The Shimadzu (Japan) liquid chromatography single quadrupole mass-spectrometer (LCMS-2010A) with atmospheric pressure electrospray ionization (API-ESI) source was used for determination in a negative ionization mode. The operation conditions were: temperature of drying gas (250 °C), capillary voltage (4000 V), dry gas (nitrogen, 10 L/min), and nebulizing gas (nitrogen, 1.5 L/min), respectively. Quadrupole full scan analyses were conducted from *m*/*z* 200 to 1400. Mass-spectra of 10 ppm solutions of tested compounds were obtained by direct injection technique with methanol/water (4:1 w/w) as a solvent with flow rate 0.2 ml/min. A 20-μL sample volume was injected.

For electron microscopy the specimens were placed onto grids coated with freshly glow-discharged carbon-collodion film, negatively stained with 1% aqueous uranyl acetate for 1 min, and investigated using JEM-100CX electron microscope (JEOL) at 80 kV.

Buffers used were GVB, 5 mM sodium veronal, 145 mM NaCl containing 0.1% gelatin, 0.02% NaN_3, pH 7.4; GVB^{2+}, GVB containing 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$; GVBE, GVB containing 10 mM EDTA; PBS, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 145 mM NaCl, pH 7.4; PBST, PBS containing 0.05% Tween 20, pH 7.4; carbonate buffer, 15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6; citrate-phosphate buffer containing 0.006% H_2O_2, 38 mM citric acid, 67 mM $Na_2HPO_4 \cdot 12H_2O$, pH 5.0.

4.2. Chemistry

4.2.1. 20,29-Dihydro-20,29-dichloromethylenbetulin (14). Acetic anhydride (170 ml) was added to betulin (**11**) (20.0 g, 45.2 mmol) and heated to the complete dissolution. The reaction mixture was cooled, and the precipitated crystals were filtered, washed with water to pH 7, dried over P_2O_5, and recrystallized from isopropanol, to give betulin diacetate (**12**) as white acicular crystals; yield 15.3 g (64.3%); mp 223–224 °C. Sodium trichloroacetate (3.67 g, 19.8 mmol) and TEBA (38 mg, 0.2 mmol) were added to a solution of betulin diacetate (5.20 g, 9.90 mmol) in chloroform (10 ml) at room temperature. The reaction mixture was boiled and intensively stirred for 2 days, cooled to room temperature,

twice washed with water, and evaporated. The residue (5.00 g) was dissolved without further purification in a mixture of THF (50 ml) and methanol (13 ml) and after adding of 4 N NaOH (9 ml) was stirred at room temperature for 17 h. The reaction mixture was neutralized with 20% HCl to pH 7 and evaporated to dryness. The obtained crystals were dissolved in chloroform (100 ml), washed with water (3× 100 ml), dried with Na_2SO_4, and evaporated. The residue was recrystallized from a dichloromethane–isopropanol mixture to give betulin derivative (**14**) as white crystals; yield 3.50 g (67.3%); mp 201–202.5 °C; R_f 0.16 (chloroform); ^{1}H NMR ($CDCl_3$): 0.74, 0.81, 0.95, 0.98, 0.99, and 1.21 (all 3H, s, CH_3), 2.30 (1H, m, H19), 3.19 (1H, m, H3), 3.21 (1H, d, *J* 10.7 Hz, H28), 3.71 (1 H, d, *J* 10.7 Hz, H28).

4.2.2. Steroids and triterpenoids sulfates (1–9). Steroids and triterpenoids sulfates were obtained from correspondent alcohols. Standard procedure for starting compound carrying one hydroxyl group was followed. Sulfuric acid (0.16 ml, 3 mmol) and acetic anhydride (0.28 ml, 3 mmol) were mixed with dry pyridine (5 ml) and after 5 min of stirring at 50–55 °C a solution of alcohol (1 mmol) in 2 ml of pyridine was added. The mixture was stirred for 30 min at the same conditions, cooled to 0 °C, and 25% ammonia water (0.74 ml) was added. After 15 min of stirring, the precipitate was filtered and the filtrate was placed to a crystallizer for concentrating. After escaping of ammonium acetate, the mixture was chromatographed to yield ammonium salt of sulfate as a solid. The sodium salt was obtained by adding equivalent of sodium hydroxide in methanol and concentrating to dryness (yield 80–95%). Twofold and threefold quantity of sulfuric acid and acetic anhydride were used for obtaining di- and trisulfates.

The structures of the compounds obtained were confirmed by ^{1}H NMR- and mass-spectroscopy. ^{1}H NMR spectra in general corresponded to those for the starting compounds excepting the absence of signals of hydroxyl groups' protons and paramagnetic shifts of the protons' signals of groups adjacent to hydroxyl groups. ^{1}H NMR-spectra information (d_6-DMSO); number of compound: chemical shift (integral, multiplicity, constant of spin–spin interaction (if noted), number of carbon atom at standard numeration of steroid/triterpenoid skeleton): **1**: 3.75 (1H, d, *J* 10.0 Hz, H28), 3.86 (1H, m, H3), 4.39 (1H, d, *J* 10.0 Hz, H28); **2**: 3.28 (1H, d, *J* 10.0 Hz, H28), 3.61 (1H, m, H3), 3.83 (1H, d, *J* 10.0 Hz, H28); **3**: 3.59 (1H, m, H3); **4**: 3.83 (1H, m, H3); **5**: 3.82 (1H, m, H3); **6**: 3.93 (1H, m, H3); 4.34 (1H, m, H12); **7**: 3.78 (1H, m, H3); 4.12 (1H, m, H7), 4.37 (1H, m, H12), **8**: 3.64 (1H, m, H3), **9**: 3.61 (1H, m, H3). For recording mass-spectra negatively charged deprotonated molecular ions of obtained compounds were produced. Mass-spectra information; number of compound: product ions/negative charge—*m*/*z*: **1**: $(M-2H^+)/2-300.0$; **2**: $(M-2H^+)/2-342.0$; **3**: $(M-H^+)/1-535.4$; **4**: $(M-H^+)/1-397.3$; **5**: $(M-H^+)/1-453.3$; **6**: $(M-2H^+)/2-275.0$; **7**: $(M-2H^+)/2-323.0$, $(M-3H^+)/3-215.0$; **8**: $(M-H^+)/1-535.0$; **9**: $(M-H^+)/1-535.0$.

4.2.3. Betulin diphosphate (10). Solution of betulin (1 g, 2.26 mmol) in 6 ml of pyridine was dropped for 1 h to the mixture of $POCl_3$ (1.68 ml, 0.02 mol) and 8 ml of acetone at stirring and cooling to 0 °C. After evaporating, 30 ml of dioxane and 2 ml of water were added to the residue and the mixture was boiled for 1 h. The mixture was concentrated to dryness under reduced pressure, the residue was dissolved in 20 ml of mixture of chloroform–methanol, 3:1, and crystals containing desired product were precipitated by adding 15 ml of water. The precipitate was filtered, dried, and chromatographed in mixture of chloroform–methanol–pyridine–formic acid, 1:1:0.15:0.15, giving 1.43 g (69%) of solid; R_f 0.59 (methanol–pyridine–phosphoric acid, 2:0.5:0.04); ^{1}H NMR (CD_3OD): 0.87, 0.94, 1.04, 1.07, 1.13, and 1.74 (all 3H, s, CH_3), 2.48 (1H, m, H19), 3.77 (1H, d, *J*10.0 Hz, H28), 3.89 (1H, m, H3), 4.22 (1H, d, *J*10.0 Hz, H28).

Results of elemental analysis of all compounds were satisfactory.

4.3. Classical pathway hemolytic assay

Capability of the compounds **1**–**8**, **10** to inhibit the classical pathway-mediated hemolysis was measured using antibody-coated sheep erythrocytes (EA, 1.5×10^8/ml) and guinea-pig serum as a complement source at a dilution previously determined to lyse 70–80% of cells (resulting dilution ~1:600). Two hundred microliters of EA was mixed with 200 μL of guinea-pig serum and 600 μL of various concentrations of the inhibitors diluted in GVB^{2+}. To determine the spontaneous lysis of erythrocytes, 200 μL of EA was mixed with 800 μL of GVB^{2+}. Three uninhibited samples consisted of 200 μL of EA, 200 μL of guinea-pig serum, and 600 μL of GVB^{2+}. The reaction mixtures were incubated for 30 min at 37 °C and diluted with 3 ml of cold GVB^{2+} to stop the reaction. Cells were separated by centrifugation, and the absorbance at 414 nm of the supernatants was measured to quantify hemoglobin release. Values for the spontaneous lysis were substracted from sample values, and the fractional inhibition was determined relative to the uninhibited (no added compounds) sample.

To perform the classical complement inhibition assay with middle-diluted human serum, 10 μL of EA (1×10^9/ml) was mixed with 200 μL of various concentrations of the inhibitors diluted in GVB^{2+} and 290 μL of NHS diluted 1:100 in GVB^{2+} (resulting dilution ~1:170). After 30 min of incubation at 37 °C, the reaction was stopped by adding 500 μL of cold GVB^{2+}, and the results were determined as described above. Data are given as an average of three repetitions ± standard deviation.

4.4. Alternative pathway hemolytic assay

Inhibition of the alternative pathway-mediated hemolysis was determined by measuring of lysis of rabbit erythrocytes (Er) under the influence of human serum. Different concentrations of inhibitors diluted in 78 μL of GVB were mixed with 5 μL of 50 mM MgEGTA,

Table 3. Content of the tubes for hemolytic assay for the inhibition of the C1q-immunoglobulin interaction, μM

	Step 1				Step 2
	EA	GVB^{2+}	NHS	Inhibitor	
C_L	200	100			490 H_2O
C_R	200	100			490 R1q
C_S	200	90	10		490 R1q
Experimental tubes	200		10	90	490 R1q

7 μL of undiluted NHS, and 10 μL of Er (1×10^9/ml). The reaction mixtures were incubated at 37 °C for 20 min, and the reaction was stopped by adding 400 μL of cold GVBE. The tubes were centrifuged, and the percentage of hemolysis was determined as described above. Data are given as an average of three repetitions ± standard deviation.

4.5. Hemolytic assay for the inhibition of the C1q-immunoglobulin interaction

Antibody-sensitized sheep erythrocytes diluted in GVB^{2+} (1.5×10^8 cells/ml), NHS diluted 1:15 in GVB^{2+}, and various concentrations of the inhibitors were added to experimental tubes according to Table 3, column 'Step 1'. Tubes C_L and C_R are the controls of full, and R1q-reagent-mediated lysis of erythrocytes, correspondently. Tubes C_S represent uninhibited samples, which were run in triplicate. The reaction mixtures were incubated at 30 °C for 15 min and cells were separated by centrifugation. Supernatants were carefully removed and ingredients were added according to Table 3, column 'Step 2'. Dilution of reagent R1q was previously determined to lyse 70–80% of the erythrocytes. After 1 h of incubation at 37 °C, 3 ml of cold GVB^{2+} was added. To determine the extent of hemolysis, reaction mixtures were centrifuged and optical densities of supernatants were determined at 414 nm. Values z expressing the quantity of active C1q molecules were calculated from formula (I) taking into account the optical densities of supernatants of correspondent tubes. Inhibition constants were determined from Fig. 3B. Data are given as an average of three repetitions ± standard deviation.

$$z = \ln\left[\frac{C_L - C_R}{C_L - \mathrm{Exp}}\right] \qquad \text{(I)}$$

4.6. ELISA for the inhibition of the interaction of C1q with IgG3

Microtiter wells were coated with human IgG3 (0.5 μg/well) in carbonate buffer and any residual binding sites were blocked with 1% w/v BSA in PBS for 1 h at 37 °C. After washing, wells were incubated (1.5 h at 37 °C) with C1q, previously preincubated with betulin disulfate or betulinic acid sulfate in different concentrations diluted in PBST. After washing, wells were incubated with rabbit anti-C1q antibodies conjugated with horseradish peroxidase in suitable dilution. To assess the quantity of the bound antibodies, the color was developed using 3,3′,5,5′-tetramethylbenzidine in citrate–phosphate buffer containing 0.006% H_2O_2. Data are given as an average of three repetitions ± standard deviation.

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