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Structure, stability, and antiplatelet activity of O-acyl derivatives of salicylic acid and lipophilic esters of acetylsalicylate

Ilya B. Zavodnik[1,3], Elena Lapshina[1], Elena Sudnikovich[1], Magdalena Boncler[2], Bogusława Luzak[2], Marcin Różalski[2], Magdalena Helińska[2], Cezary Watała[2]

[1]Department of Bioregulators, Institute of Pharmacology and Biochemistry, National Academy of Sciences, Grodno, Belarus

[2]Department of Haemostasis and Haemostatic Disorders, Medical University of Łódź, Łódź, Poland

[3]Department of Biochemistry, Yanka Kupala State University, Grodno, Belarus

Correspondence: Cezary Watała, e-mail: cwatala@csk.umed.lodz.pl

Abstract:
The anti-thrombotic activity of acetylsalicylic acid (ASA) has been shown to be due to specific irreversible acetylation of blood platelet cyclooxygenase. The aim of our study was to investigate the associations between the antiplatelet activities of derivatives of both ASA and salicylic acid (SA), as well as the structure, stability, and molecular properties of these compounds. Homologous series of O-acyl derivatives of salicylic acid (propionyl-, butyrylsalicylic acids, PSA, BSA) and lipophilic dodecyl (C12)-, hexadecyl (C16)-, and cholesteryl acetylsalicylates were synthesized and tested for structure-activity relationships. The molecular properties (heat of formation, molecular surface area, dipole moment) of ASA and SA derivatives obtained by theoretical calculations changed with the increasing length of the acyl or alkyl residue. The inhibition of whole blood platelet aggregation and the reduction in thromboxane (TX) generation by O-acyl derivatives were concentration-dependent and decreased along with increasing the length of acyl chain. These effects correlated with the extent of platelet reactivity and P-selectin expression inhibition in collagen-activated platelets. In contrast to ASA and O-acyl derivatives of SA, none of the lipophilic ASA derivatives had a significant inhibitory effect on platelet aggregation.
In conclusion, all SA and ASA derivatives studied under *in vitro* conditions showed much lower antiplatelet activities than ASA itself, despite their higher affinity to plasma proteins or membrane components and their equivalent ability to acetylate protein free amino groups. We suggest the significance of the carboxylic group, dipole moment, geometry, and size of these pharmaceuticals in their ability to bind to the active site of cyclooxygenase and their antiplatelet efficacy.

Key words:
acetylsalicylic acid, O-acyl derivatives of salicylic acid, lipophilic esters of acetylsalicylate, platelet aggregation, platelet protein acylation, thromboxane generation

Abbreviations: AM1 – Austin Model 1, ASA (C2SA) – acetylsalicylic acid, aspirin, BSA (C4SA) – butyrylsalicylic acid, C12 – dodecyl ester of acetylsalicylic acid, C16 – hexadecyl ester of acetylsalicylic acid, Chol-ASA – cholesteryl ester of acetylsalicylic acid, COX – cyclooxygenase, PSA (C3SA) – propionylsalicylic acid, QSAR – Quantitative Structure-Activity Relationship, SA – salicylic acid, TX – thromboxane, TXA_2 – thromboxane A_2, TXB_2 – thromboxane B_2

Introduction

Acetylsalicylic acid (ASA, aspirin) inhibits the synthesis of thromboxane A_2 in blood platelets *via* the irreversible acetylation of the serine-530 residue close to the active site of cyclooxygenase (COX) [16]. This serine-530 residue is thought to constitute the molecular mechanism underlying the non-enzymatic inhibition of the cyclization of arachidonic acid [25, 27]. Such an acetylation may be mediated by various acetyl donors, including ASA; it is not confined to cellular cyclooxygenase but may also target other free amino (and hydroxyl) groups of a variety of proteins in blood cells and plasma [28].

The chemical structure of various salicylic acid (SA) derivatives that target COX plays an essential role in their stereochemistry of binding to the active center of COX [12, 26]. Further, the biochemical pharmacological activities of these SA derivatives may offer promising alternatives to overcome the so-called "aspirin-resistance", which is encountered to a major extent in special groups of patients at risk for cerebro- and cardiovascular complications [18].

Different lines of aspirin derivatives have been synthesized and the mechanism of their action evaluated. One study by Casadebaig et al. demonstrated that different 3- or 5-substituted salicylate derivatives blocked the inhibitory effect of ASA on platelet aggregation and, at the same concentrations, induced an inhibition of platelet aggregation. Based on these data, the mechanism involving two non-independent sites (the enzymatic site and the binding site) on COX catalytic activity was proposed [3]. Given that the selectivity of ASA as an anti-thrombotic agent has been postulated to be due to selective antiplatelet effects in the portal circulation, it has been suggested that more lipophilic, membrane-permeable, and highly extractable analogs of ASA may be potentially more selective antithrombotic agents than ASA itself [12].

The aim of the present study was to evaluate the structure-activity associations for some salicylate derivatives. For this purpose, the homologs of more lipophilic O-acyl derivatives of SA (propionyl- and butyrylsalicylic acids) and dodecyl- (C12-ASA), cetyl- (C16-ASA), and cholesteryl-acetylsalicylates (chol-ASA) were synthesized and compared to the standard reference compound ASA, with respect to the capabilities considered to be the most crucial underlying refractoriness to 'aspirin therapy' observed in clinical practice. These capabilities include antiplatelet activity, stability and vulnerability to hydrolysis in blood plasma, as well as interactions with proteins (human serum albumin) and membranes (human red blood cell membranes). Using quantum-mechanical methods, the molecular geometries of ASA and SA derivatives were optimized and their charge distributions evaluated, in order to explain the observed experimental characteristics of the pharmaceuticals studied.

Materials and Methods

Reagents

SA (2-hydroxybenzoic acid), ASA, human serum albumin, 1-anilinonaphthalene-8-sulfonate (ANS), trichloroacetic acid (TCA), cholesterol, hexadecyl (C16, cetyl) and dodecyl (C12, lauryl) alcohols, thionyl chloride, pyridine, and benzene were obtained from Sigma-Aldrich (St. Louis, MO, USA or Steinheim, Germany). Pyrene, propionyl chloride, and butyryl chloride were obtained from Fluka (Buchs, Switzerland). Collagen fibrils (type I) from equine tendons, adenosine-5-diphosphate, and arachidonate for whole blood platelet aggregometry were obtained from Chrono-Log Corp. (Havertown, PA, USA). Mouse monoclonal antibodies *anti*-human platelet GPIIIa (*anti*-CD61, fluorolabeled with fluorescein isothiocyanate, CD61/FITC), mouse monoclonal antibodies *anti*-human platelet P-selectin (*anti*-CD62P, fluorolabeled with phycoerythrin, CD62P/PE), and CellFix (phosphate buffered fixative containing 10% v/v formaldehyde and 1% w/v sodium azide) were from BD Biosciences (San Jose, CA, USA). Tubes for blood collection containing 0.105 M sodium citrate (*Vacutainer tubes)* were from BD Diagnostics (Plymouth, UK). All other chemicals, unless otherwise stated, were from POCh (Gliwice, Poland). Water used for solution preparation and glassware washing was passed through an Easy Pure UF water purification unit (Thermolyne Barnstead, IA, USA).

Synthesis of SA and ASA derivatives

The short chain O-acyl derivatives of SA (propionyl- and butyrylsalicylic acids; PSA and BSA, respectively) were prepared from the corresponding acyl chlorides in the presence of pyridine, according to Hung et al. [14]. Dodecyl (C12), hexadecyl (C16),

and cholesteryl (chol) esters of acetylsalicylates were synthesized from the chlorine anhydride of ASA and corresponding alcohols, according to a common procedure [1]. Chlorine anhydride of ASA was synthesized from ASA and thionyl chloride ($SOCl_2$), following the general procedure [11].

The synthesized derivatives were identified and characterized by the methods of infrared (IR)- and ultraviolet (UV)-spectroscopy, thin-layer chromatography, and melting point range determination. IR-spectra were recorded in KBr discs on a Specord M80 IR-spectrophotometer (Carl Zeiss, Jena, Germany), UV-spectra were recorded in phosphate buffer, pH 7.4, on a Cary 100 UV-VIS spectrophotometer (Varian, Australia), and uncorrected melting point ranges were determined in capillary tubes with an Electrothermal apparatus (VEB Analytik, Dresden, Germany). The IR-spectra of the short chain O-acyl derivatives of SA showed the presence of C–H, ester C=O, and acid C=O moieties; those of the lipophilic esters of ASA showed the presence of C–H and ester C=O moieties. The UV-spectra of ASA and SA esters were similar to those of ASA and did not show contamination by SA.

Table 1 presents the melting points of the synthesized ASA and SA derivatives. These melting point values of O-acyl derivatives of SA were very close to those reported earlier by Hung et al. [14].

Theoretical calculations

The molecules of ASA and SA derivatives were considered theoretically by performing both semi-empirical molecular orbital theory calculations and *ab initio* calculations. Preoptimization was carried out by the molecular mechanics method. The Austin Model 1 (AM1) semi-empirical method within the restricted Hartree-Fock (RHF) formalism was considered to optimize fully the geometry of the systems involved [9]. The electronic structure of the studied molecules was estimated according to the *ab initio* RHF scheme [9]. Geometry optimization with AM1 or *ab initio* methods generally yields geometries corresponding to energy minima. Since minimization calculations cannot cross or penetrate potential energy barriers, the molecular structure found during an optimization may be a local and not a global minimum. We performed five calculations for every derivative and took the conformation with minimal energy. All the calculations were performed by the use of the HyperChem 6.0 software.

Tab. 1. The melting points of ASA derivatives

ASA derivative	Melting point, C
ASA (C2SA)	135
PSA (C3SA)	97
BSA (C4SA)	82
C12ASA	54–55
C16ASA	67–68
CholASA	76–77

Data are averaged values of four to six measurements

Blood collection and preparation of platelets

We used blood from healthy young volunteers to test the *in vitro* effects of ASA and SA derivatives on the blood platelets' ability to generate thromboxane, to aggregate and release α granule contents. This group consisted of 35 healthy individuals (16 men and 19 women, mean age of 28.2 ± 11.6 years).

Blood was collected from a peripheral vein of each subject into a tube containing 0.105 M buffered sodium citrate (the final citrate: blood ratio was 1:8 v/v for platelet isolation or 1:9 for aggregometry and flow cytometry studies). Care was taken to avoid undesirable artifactual platelet activation: donors rested for 20–30 min prior to the blood collection to minimize mental stress, and the first 0.5 ml was discarded. All platelet reactivity measurements were performed within two hours after blood withdrawal.

Platelet aggregation assays

The ability of the platelets to aggregate was monitored in whole blood with the use of a Multiplate Platelet Function Analyzer (Dynabyte, Munich Germany). Whole blood was diluted 1:1 with 0.85 % saline, incubated for 10 min at 37°C with esters, and then supplemented with collagen (1 μg/ml) or arachidonic acid (0.5 mM) to stimulate platelet aggregation. The impedance, reflecting the extent of platelet aggregation, was monitored for 15 min.

Due to the differences in solubilities of the compounds, working solutions of SA and ASA derivatives were freshly prepared in either absolute ethanol (lipophilic ASA derivatives) or 30% ethanol (O-acyl derivatives of SA). The aliquots of whole blood were supplemented with working solutions of the esters to give the

relevant concentration maintained, within the range of 50–500 μM. Concentration of ethanol in the examined samples did not exceed 1.25% for the lipophilic ASA derivatives and 0.3% for the remaining agents (0.8% if tested at the concentration of 500 μM). In each case, we simultaneously monitored platelet function in control samples containing the same concentrations of ethanol.

Flow cytometry measurements

All flow cytometry experiments were carried out on whole blood platelets, which reduced the risk of undesirable, artifactual platelet activation. Immediately after venipuncture, 10 μl aliquots of whole blood were added to a 10-fold volume of CellFix, mixed and left at room temperature (RT) for 2 h or at 4°C overnight. These samples were used to evaluate the extent of platelet activation in the circulating blood.

Aliquots of fresh whole blood were supplemented with ASA and SA derivatives at final concentrations of either 20 μM for C12-, C16-, chol-ASA, and ASA (as a reference) or 100 and 200 μM for PSA, BSA, and ASA (as a reference). The samples were incubated at 37°C for either 10 or 60 minutes. The concentration of ethanol in these samples did not exceed 0.2%. After incubation, all samples were stimulated with collagen (20 μg/ml, 5 min), then both resting and activated platelets were diluted 10-fold with 1% CellFix. Cells were fixed for 2 h at RT or at 4°C overnight, and stained with *anti*-CD61 and *anti*-CD62P/PE antibodies. Fluorescence of 3,000 platelets was measured with a LSR II Flow Cytometer (Becton Dickinson). The fractions of the specific fluorescence-positive platelets were obtained after subtraction of non-specific fluorescence in the control samples (labeled with *anti*-CD61/FITC alone). All flow cytometry measurements were fluorescence-compensated on a daily basis for each set of measured samples using calibration beads (Becton Dickinson), to ensure that there was no considerable green, orange, and red fluorescence overlap.

Thromboxane generation assay

To monitor the ability of SA or ASA derivatives to hamper the generation of platelet TX in whole blood, we adopted the experimental model described earlier by Catella et al. [4], with our own novel modifications. Briefly, an aliquot of whole blood (anticoagulated with buffered 0.105 M sodium citrate, 9:1 v/v) was supplemented with 500 μM ASA immediately upon withdrawal (10 min). The blood was then centrifuged (3,000 × g, 10 min, 4°C), separated, and platelet-poor plasma was frozen (–70°C) for use in further analyses (these samples were designated the baseline TXB_2 concentrations). Another blood sample was supplemented with 50, 100, or 200 μM of a given SA or ASA derivative, and either incubated at RT for one hour (the static model of thromboxane (TX) generation protocol) or agitated at RT for one hour on a rotary mixer (the dynamic model of TX generation protocol). Samples were then treated with 500 μM ASA (to inhibit COX) and processed for further analyses as described above. Furthermore, based on our preliminary data, we analyzed the time-dependent inhibitory effects of PSA and ASA (at the concentration of 50 μM) on TX generation in whole blood. In this case, baseline TX was measured in either the static model (following 1 or 6 h incubation at RT), the dynamic model (samples agitated on a rotary mixer, 60 min, RT), or the mixed static-dynamic model (1 h agitation preceded by 5 h incubation without agitation, RT) [2, 4, 21].

The enzymatic measurements of TXB_2 concentration were performed using a previously validated enzyme linked immunoassay technique (sensitivity 0.004 ng/ml, cross-reactivity with other prostanoids < 0.25%) [17, 20].

Enzymatic and spontaneous hydrolysis of salicylic acid and acetylsalicylate ester derivatives

We monitored the extent of spontaneous and enzymatic hydrolysis (the activities of ester-hydrolyzing enzymes in blood plasma) of esters by the intrinsic fluorescence of the hydrolysis product, SA, using a Perkin Elmer LS50-B instrument (Perkin Elmer, Wellesley, MA, USA; excitation wavelength 305 nm, emission wavelength 405 nm). The amounts of hydrolyzed ASA were equimolar to the amount of liberated SA.

To distinguish between esterase I (aspirin esterase I, AE I, albumin fraction) and II (aspirin esterase II, AE II or butyrylcholine esterase, BchE), two different buffers were used: citrate buffer (200 mM; pH 5,5) for AE I and Tris/HCl buffer (200 mM; pH 7,4) for AE II. Stock solutions of ASA or SA derivatives (500 mM) were prepared in absolute ethanol. Working solutions (6.25 mM) were prepared in either Tris/HCl (pH 7.4) or citrate buffer (pH 5.5). Due to the very poor solubility of ASA esters (cholesteryl, hexadecyl [C16, cetyl] and dodecyl [C12, lauryl]), we were not able to monitor their hydrolysis in these experiments. In all

variants of the experiments on ester hydrolysis in blood plasma, the following procedure was employed. A 40 µl aliquot of platelet-poor plasma (PPP) was diluted with 160 µl of the working solution of the SA ester (a final substrate concentration 5 mM). Samples were incubated at 37°C for 2 h. To stop the reaction, the samples were supplemented with 100 µl of 10% (w/v) ice-cold TCA followed by centrifugation at 2,500 × g, 0°C for 10 min. Then, 50 µl aliquots of the supernatants were mixed with 950 µl of either Tris/HCl or citrate buffer before the SA concentration was measured spectrometrically. Blanks for monitoring spontaneous ester hydrolysis were prepared in either Tris/HCl or citrate buffer as described above, but PPP and TCA were added only after the incubation. The amount of SA formed enzymatically in plasma was calculated by subtracting the fluorescence of the blank (spontaneous hydrolysis) from the total fluorescence (enzymatic plus spontaneous hydrolysis) after a 2 h incubation. SA solutions for the standard curve were prepared in 200 mM Tris/HCl, pH 7.4 or 200 mM citrate buffer, pH 5.5. A 40 µl aliquot of PPP was diluted with 160 µl of SA solution (at a final concentration of 0.1, 0.2, 0.4, 0.8, 1.6, 2.4, 3.2, or 4.5 mM) and supplemented with 100 µl of 10% (w/v) ice-cold TCA. This was followed by centrifugation at 2500 × g, 0°C for 10 min. Finally, 50 µl portions of the supernatants were mixed with 950 µl of either Tris/HCl or citrate buffer to determine SA concentrations.

Binding of ASA and SA derivatives to albumin, polylysine, and erythrocyte membrane component

Human erythrocytes were isolated from fresh blood by centrifugation at 4°C, 2,000 × g and purified by washing with phosphate-buffered saline (PBS, 150 mM NaCl, 10 mM phosphate buffer, pH 7.4). Erythrocyte ghosts were prepared from washed cells according to the method of Dodge et al. [6], using 0.5 mM phenylmethylsulfonyl fluoride (PMSF) as proteolytic inhibitor. The fluidity of the erythrocyte membrane lipid bilayer was assessed with the use of the fluorescent dye pyrene incorporated into the membrane [5] by measuring the ratio of the fluorescence intensities of the pyrene excimer and monomer, emitting at 465 nm and 392 nm, respectively, when excited at λ_{ex} = 313 nm. Pyrene was added as a concentrated ethanolic solution (1 µl/ml) to the membrane suspension (in PBS pH 7.4, 22°C, protein concentration 0.1 mg/ml) to give a final dye concentration of 5 µM. The fluorescence spectra of ANS (6 µM, λ_{ex} = 360 nm, λ_{em} = 465 nm) bound to human serum albumin (10 µM in PBS, pH 7.4, 22°C) were monitored to study the interactions of ASA and SA derivatives with the protein.

The irreversible acetylation of the poly-L-lysine (poly-L-Lys, MW > 70 kDa) by lipid derivatives of ASA or its acylation by O-acyl SA derivatives was examined following the incubation (poly-L-lysine, 20 µg/ml) with 100 µM (final concentration) of SA and ASA derivatives (PBS, pH 7.4, 1 h at 37°C). The content of free amino groups in poly-L-Lys was determined according to the method in Sashidhar et al. using L-lysine and L-glutamic acid as standards [24]. The reduction of free amino acid groups after the incubation was considered to result from irreversible acetylation/acylation. Results are expressed as µmol free amino groups per mg of poly-L-Lys.

Statistical analysis

The results are expressed as the arithmetic mean ± standard deviation/standard error of mean (SD/SE) or median (*Me*) and interquartile range (IQR: from lower quartile, Q1 to upper quartile, Q3). The data used for analysis are replicates of two to four single determinations of each studied sample. The differences were determined with either the Student's *t*-test or various models of one-way and two-way ANOVAs or the Mann-Whitney *U* test for nonparametric data. Pearson's linear correlations were used to assess simple associations. Multiple regression was used for *post-hoc* data standardization (for the presence of confounding variables) and to reason on partial effects (controlling for other accompanying variables), variable contribution (R^2_{corr}), and the collinearity of variables. Spearman's (R_S), Kendall's, or gamma rank correlations were used to assess the associations between variables departing from the normal distribution.

Results

Theoretical consideration of ASA and SA derivatives

In order to understand better the antiplatelet drug structure-activity relationships for the selection and

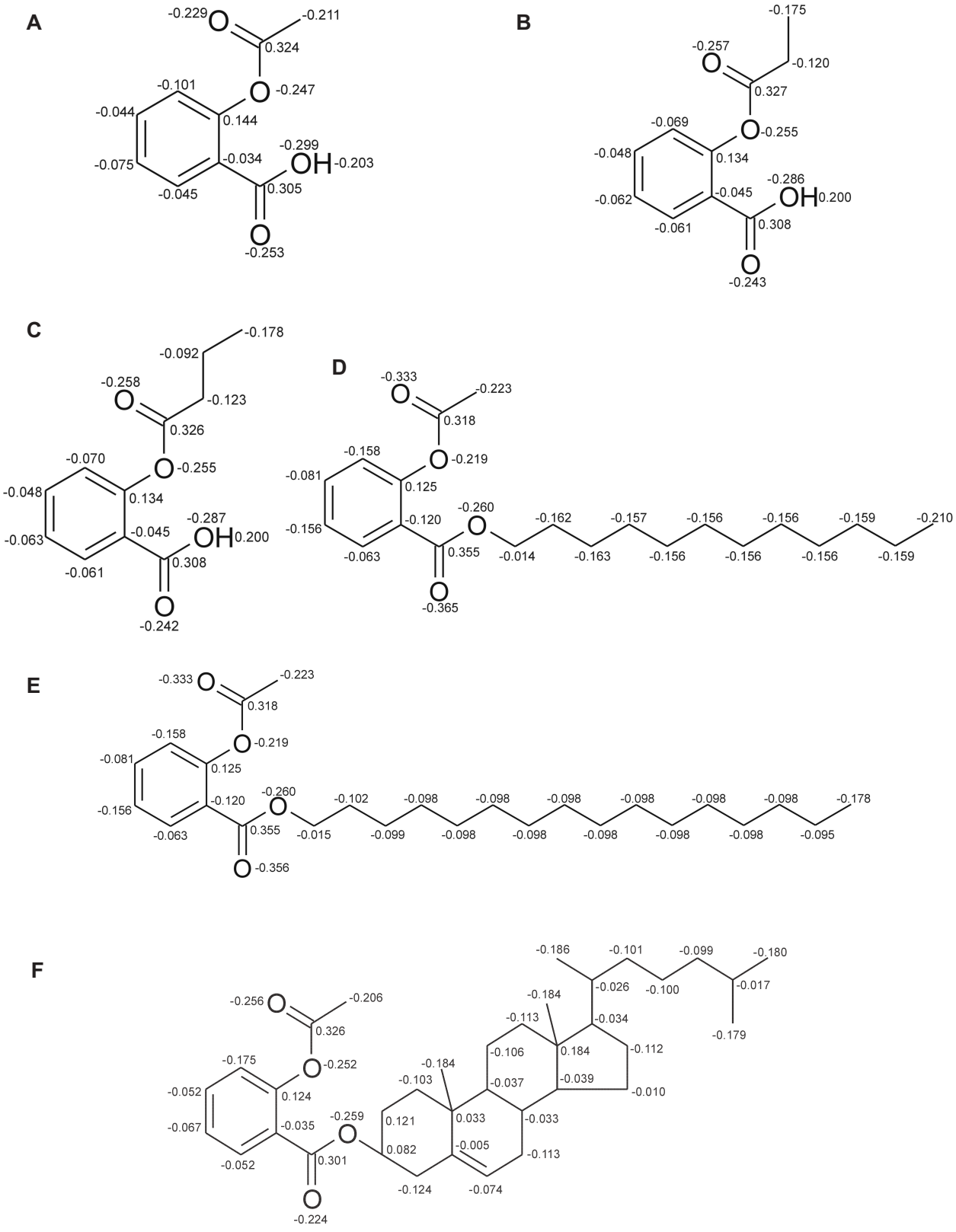

Fig. 1. The planar chemical formulae of the molecules of O-acyl derivatives of SA and lipophilic ASA esters. (**A**) ASA, (**B**) PSA, (**C**) BSA, (**D**) dodecyl-ASA ester, (**E**) hexadecyl-ASA ester, (**F**) cholesteryl-ASA ester. The excess charges evaluated on the atoms of C and O (omitted on H atoms) have been calculated for optimized structures by the ab initio method. Optimization was performed by the AM1 method using Hyperchem 6.0 software.

Tab. 2. Molecular properties, energy values, and dipole moments of ASA and SA derivatives

	ASA	PSA	BSA	C12ASA	C16ASA	cholASA
QSAR properties						
Surface area (Grid) [Å^2]	348.8	385.9	414	651.9	822.5	840.6
Volume [Å^3]	543.1	592.5	646.9	1114.8	1395.0	1571.6
Hydration energy [kcal/mol]	–8.32	–8.86	–8.44	1.46	3.73	0.52
AM1						
Total energy [kcal/mol]	–58 671	–62 260	–65 853	–101 773	–116 156	–152 421
Binding energy [kcal/mol]	–2335	–2612	–2894	–5696	–6832	–9296
Electronic energy [kcal/mol]	–288345	–319515	–353763	–780447	882326	–1646107
Nuclear energy, [kcal/mol]	229674	257255	287910	678674	766170	1493686
Heat of formation [kcal/mol]	–142.3	–143.8	–150.7	–202.2	–237.3	–196.2
Ab initio						
Total energy [kcal/mol]	–399508	–423711	–447921	–690010	–786680	–1049473
MP2 correlation contribution [kcal/mol]	–480.5	–478.1	–510.6	–836.9	–964.8	–1336.8
Electronic energy [kcal/mol]	–890870	–971231	–1053838	–2036150	–2295931	–4025806
Nuclear energy, [kcal/mol]	491362	547520	605917	1346140	1509071	2976334
Highest occupied molecular orbital energy (HOMO) [eV]	–7.62	–7.77	–7.72	–7.59	–7.47	–7.48
Lowest unoccupied molecular orbital energy (LUMO) [eV]	5.63	5.83	5.84	5.78	5.88	6.38
E (HOMO–LUMO) [eV]	13.25	13.59	13.57	13.37	13.35	13.96
Dipole moment [Debye]	1.01	3.17	3.14	1.53	1.30	2.64

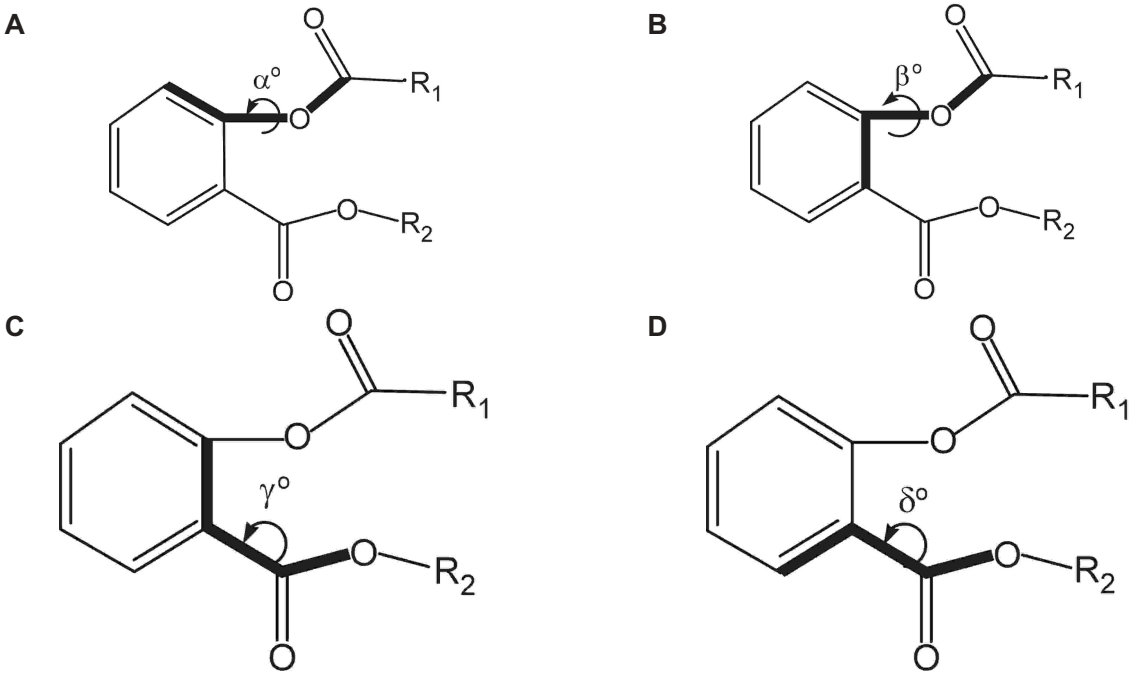

Fig. 2. Torsion angles α (**A**), β (**B**), γ (**C**), and δ (**D**) of the optimized structures of SA and ASA derivatives. R_1 = CH_3, CH_2CH_3, $CH_2CH_2CH_3$; R_2 = $(CH_2)_{11}CH_3$ (dodecyl), $(CH_2)_{15}CH_3$ (hexadecyl), $C_{27}H_{45}$ (cholesteryl)

Tab. 3. Torsion angles in molecules of ASA and SA derivatives

	α	β	γ	δ
ASA (C1SA)	108.0	–76.8	–174.1	5.9
PSA (C3SA)	73.9	–111.0	–143.6	38.6
BSA (C4SA)	70.2	–115.0	–143.6	38.9
C12ASA	51.0	–134.4	10.0	–170.4
C16ASA	–63.5	122.0	–21.8	158.5
cholASA	–46.1	138.4	–122.6	62.6

The definition of torsion angles α, β, γ, and δ in molecules of ASA and SA derivatives is shown in Fig. 2

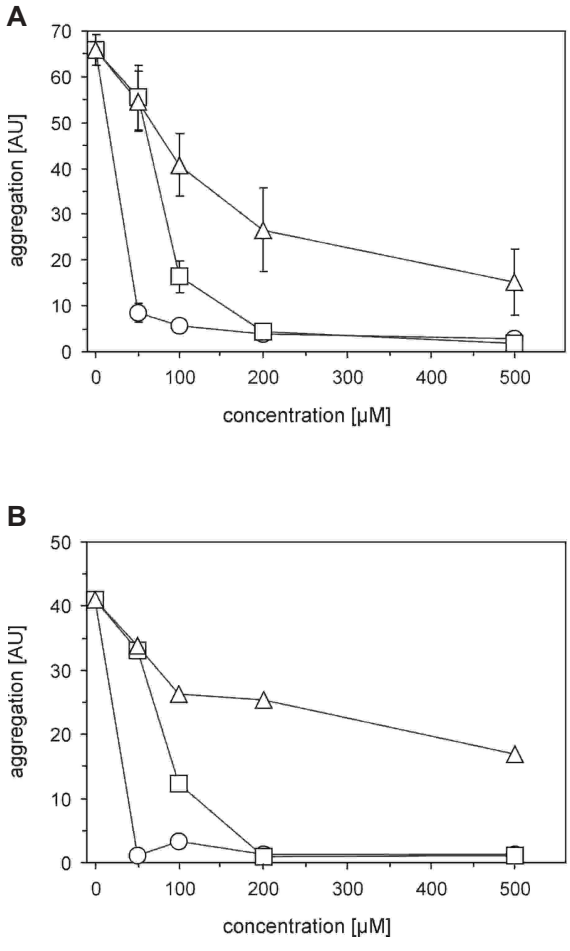

Fig. 3. The inhibition of the collagen (**A**)- or arachidonic acid (**B**)-induced platelet aggregation in human whole blood by O-acyl derivatives of salicylic acid. Data represent the mean ± SE, n = 7 for ASA (circles), PSA (squares), and BSA (triangles). Statistical significance, estimated by means of a *post-hoc* Tukey's test for multiple comparisons, was: (**A**) $\mu_{control} \neq \mu_{ASA\ 50,\ 100,\ 200,\ 500\ \mu M}$ $p < 0.0002$; $\mu_{control} \neq \mu_{PSA\ 100,\ 200,\ 500\ \mu M}$ $p < 0.0002$; $\mu_{control} \neq \mu_{BSA\ 500\ \mu M}$ $p < 0.002$, and (**B**) $\mu_{control} \neq \mu_{ASA\ 50,\ 100,\ 200,\ 500\ \mu M}$ $p < 0.0002$; $\mu_{control} \neq \mu_{PSA\ 100,\ 200,\ 500\ \mu M}$ $p < 0.0002$; $\mu_{control} \neq \mu_{BSA\ 500\ \mu M}$ $p < 0.025$

optimization of drug candidates, we studied the molecular structure and stoichiometric properties of ASA and its synthesized derivatives. Figure 1 presents the optimized structure (by the AM1 method) and the calculated excess charge on the atoms (by the *ab initio* method) of O-acyl SA derivatives and C12-ASA. Some of the molecular properties, energy values, and dipole moment values of the systems (O-acyl SA derivatives and lipophilic ASA derivatives) are listed in Table 2. According to the estimates of these calculations, ASA exhibits the lowest heat of formation (–142.3 kcal/mol) and the lowest dipole moment (1.0 D) among all of the derivatives considered (Tab. 2). According to the *ab initio* calculations, the gap of the highest occupied molecular orbital to the lowest unoccupied molecular orbital (HOMO-LUMO) is lowest for the ASA molecule (13.3 eV) and is elevated for all other derivatives.

Torsion angles (Fig. 2) determined from AM1-optimized geometries of ASA and SA derivatives are given in Table 3. Different substitutions in the drug molecule cause different relative orientations of acyl side chain or alcoholic moieties and the aromatic ring (Fig. 1), changing the torsion angles in comparison with the ASA molecule (Tab. 3). The torsion angles determined for PSA and BSA are close to each other, and different from those determined for ASA.

The effects of ASA and SA derivatives on platelet aggregation

ASA and O-acyl esters of SA exhibited similar concentration-dependent inhibitory effects on platelet aggregation induced by collagen or AA. Of all the tested agents, ASA remained the most potent inhibitor of platelet aggregation: it blocked platelet aggregation almost completely at a concentration of 50 μM (inhibition of 87% and 97%, respectively, in collagen-and AA-induced platelet aggregation, $p < 0.0002$). O-acyl derivatives of SA were effective in the inhibition of platelet aggregation at higher concentrations. PSA significantly affected platelet aggregation at 100 μM (for both agonists inhibition approx. 70%, $p < 0.0002$), whereas BSA was a highly effective inhibitor only at the highest tested concentration of 500 μM (79%, $p < 0.002$ and 49%, $p < 0.025$ for aggregation stimulated by collagen and AA, respectively) (Fig. 3). In contrast to ASA and O-acyl derivatives of SA, none of the lipophilic ASA derivatives had a significant in-

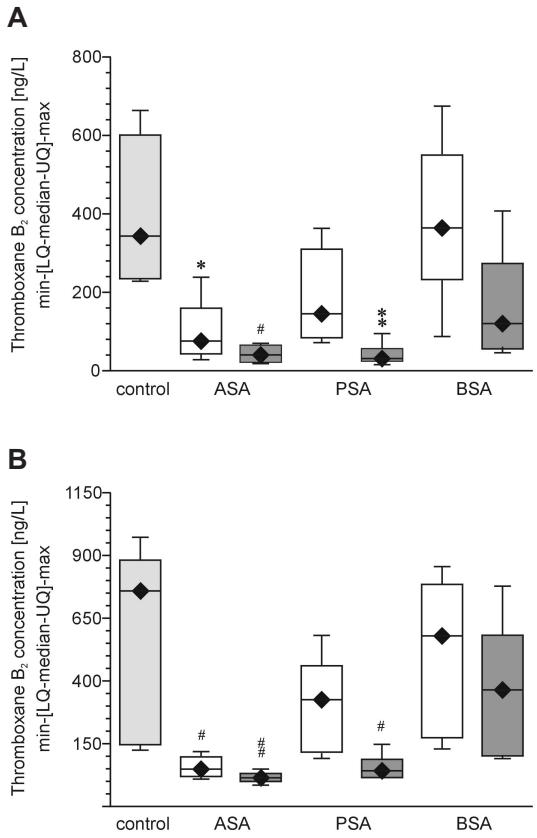

Fig. 4. The inhibition of thromboxane synthesis in human whole blood by O-acyl derivatives of salicylic acid. Thromboxane generation was monitored as the blood plasma concentration of TXB_2 after 1 h (RT) incubation (**A** – static model) or agitation (**B** – dynamic model) of the whole blood sample with ASA, PSA, or BSA at a concentration of 50 μM (white bars) or 200 μM (dark grey bars) (for details, see Materials and Methods). Data are presented as median, quartiles, and the range given by minimum/maximum values, n = 6. Statistically significant differences between ASA, PSA, BSA, and control (ANOVA) were: * $p < 0.04$, ** $p \leq 0.02$, # $p < 0.015$, ## $p < 0.004$

Tab. 4. The Stern-Volmer constants K_{sv} for the fluorescence quenching of ANS bound to albumin by SA and ASA derivatives

ASA derivative	K_{SV}, 10^{-4}, M^{-1}
ASA (C2SA)	0.057 ± 0.003
PSA (C3SA)	0.068 ± 0.004
BSA (C4SA)	0.083 ± 0.005
C12ASA	1.46 ± 0.07
C16ASA	0.36 ± 0.03
cholASA	1.64 ± 0.07

Data presented are the means ± SE, n = 4–6

hibitory effect on platelet aggregation (data not shown).

The inhibition of TXB_2 synthesis by ASA derivatives

The mechanism of platelet function inhibition by ASA depended on the inhibition of platelet TX synthesis. We compared the effect of ASA and its derivatives on the generation of TXA_2 in whole blood by measuring the concentration of its metabolite, TXB_2.

A one hour incubation of whole blood led to significantly increased thromboxane generation (for the static and dynamic model, respectively, TX generation increased by 7-fold, $p < 0.012$ and 10-fold, $p = 0.01$ *vs*. baseline values). The extent of the reduction in TXB_2 concentrations by O-acyl derivatives was concentration-dependent and decreased along with the increasing length of the acyl chain of the compounds. The significant inhibitory effect was seen only for ASA and PSA. ASA at a concentration of 50 μM effectively influenced TXB_2 synthesis (by 62%, $p < 0.04$ and 82%, $p < 0.015$ in the static and dynamic model of Tx generation, respectively), whereas PSA equivalently inhibited thromboxane generation only at 200 μM (Fig. 4). Furthermore, it was demonstrated that the inhibition of thromboxane generation by 50 μM ASA or PSA did not depend on the model of thromboxane generation (MANOVA $R_{Rao} = 1.84$, $p = 0.113$). The lipophilic ASA derivatives used at the concentrations of up to 100 μM failed to prevent thromboxane generation.

Enzymatic and spontaneous hydrolysis of ASA and SA derivatives

We studied the susceptibility of SA O-acyl derivatives and ASA lipophilic derivatives to hydrolysis by monitoring the fluorescence of the released product of hydrolysis, SA, in the course of enzymatic and spontaneous hydrolysis in the blood plasma. As shown in Figure 5, the rates of enzymatic hydrolysis of acyl derivatives of SA were higher at pH 7.4 than pH 5.5. The rate of enzymatic hydrolysis of these compounds at both pH values increased with the increasing length of the acyl chain in the following order: ASA < PSA < BSA. The rate of spontaneous hydrolysis was not affected significantly by pH in the range of 7.4–5.5 and increased in the following order: BSA < ASA < PSA (Fig. 5).

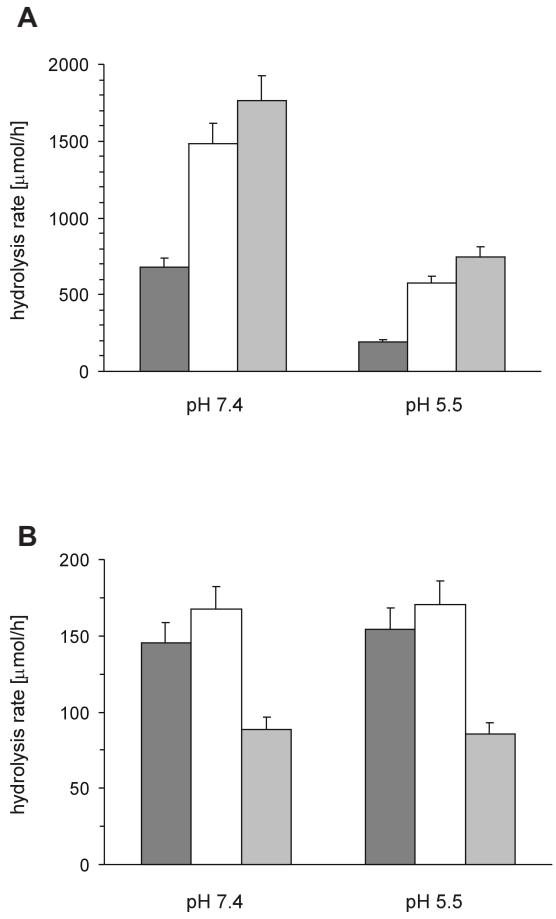

Fig. 5. The apparent rates of the enzymatic (**A**) and spontaneous (**B**) hydrolysis of O-acyl derivatives of salicylic acid. Data are presented as the mean ± SE, n = 11. Hydrolysis rates of ASA (dark grey), PSA (white), and BSA (light grey) were monitored at pH 5.5 (200 mM citrate buffer) or 7.4 (200 mM Tris-HCl). The ester was dissolved in platelet-poor plasma (enzymatic hydrolysis, **A**) or buffer (spontaneous hydrolysis, **B**) at a final concentration of 5 mM and incubated at 37°C for 2 h. Statistically significant differences between ASA, PSA, and BSA, estimated by means of a one-way ANOVA and *post-hoc* Tukey HSD test for multiple comparisons, were: (**A**) enzymatic: pH 7.4, ASA < PSA = BSA, $p < 0.0001$; pH 5.5, ASA < PSA = BSA, $p < 0.0001$; (**B**) spontaneous: pH 7.4, BSA < ASA = PSA, $p < 0.0001$; pH 5.5, BSA < ASA = PSA, $p < 0.003$

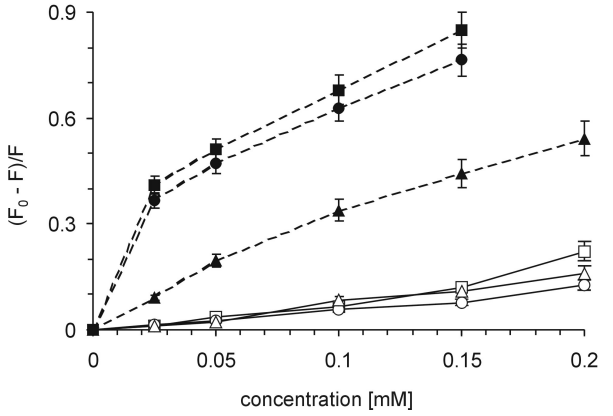

Fig. 6. Stern-Volmer plots of the fluorescence quenching of ANS bound to human serum albumin by O-acyl salicylic acids (solid line) and acetylsalicylate derivatives (dashed line). Data are presented as the mean ± SE, n = 4–5. F_0, ANS fluorescence in the absence of a quencher, F, ANS fluorescence at increasing concentrations of ASA (○), PSA (□), BSA (△), dodecyl-ASA ester (●), hexadecyl-ASA ester (▲), or cholesteryl-ASA ester (■); ANS 6 µM, albumin 10 µM in PBS, pH 7.4, 22°C; λ_{ex} = 360 nm, λ_{em} = 465 nm, slits 5 nm. Statistically significant differences between ASA and SA derivatives, estimated for the examined concentration ranges with MANOVA for planned comparisons with Bonferroni correction, were: ASA = PSA = BSA < * hexadecyl-ASA ester < ** dodecyl-ASA ester = cholesteryl-ASA ester, * $p < 0.04$, ** $p < 0.01$

The expression of the platelet surface membrane activation marker – P selectin (CD62)

The expression of CD62 (P selectin), a surface membrane marker of platelet granule secretion, was monitored in collagen-induced platelets. In all samples, ASA significantly inhibited the collagen-induced surface membrane expression of CD62; this effect was not significantly dependent on the ASA concentration (71.7 ± 18.6% *vs*. 27.4 ± 14.7%, 24.0 ± 8.5%, 24.5 ± 13.8% for control *vs*. 20, 100 or 200 µM ASA, respectively, after 10 min of incubation). The inhibition of the platelet activation was also observed in PSA-treated samples (38.1 ± 18.0% and 30.8 ± 12.0% for 100 and 200 µM PSA, respectively, after 10 min of incubation). For longer incubations (60 min.), 200 µM PSA remained as potent as ASA (57.3 ± 12.2% for control *vs*. 19.2 ± 6.5% and 16.5 ± 5.8%, respectively, for 200 µM ASA and 200 µM PSA). BSA only slightly inhibited platelet reactivity (60.0 ± 14.7 and 69.8 ± 10.9 for 100 and 200 µM BSA, respectively, *vs*. 71.7 ± 18.6% for control); this effect was significantly lower than the ASA inhibitory action ($p << 0.0001$), independently of ASA or BSA concentrations and the incubation time. ASA derivatives, including C12-, C16-, and cholASA, did not influence P-selectin expression in collagen-activated platelets.

Membrane and protein binding of SA and ASA derivatives

To evaluate the affinities of proteins and membrane components for various SA and ASA derivatives differing in lipophilicity, we measured their binding to human serum albumin and human erythrocyte membranes using spectrofluorescence methods. Figure 6

represents the averaged Stern-Volmer plots of the fluorescence quenching of ANS bound to albumin. Whereas the lipophilic esters of ASA efficiently quenched ANS fluorescence, ASA itself and other O-acyl derivatives of SA interacted with albumin with lower efficacies (Fig. 6).

Alterations in erythrocyte membrane lipid microviscosity were estimated based on the changes in the ratio of the intensities of fluorescence emitted by the monomeric and excimeric forms of pyrene incorporated into the lipid bilayer. When incorporated into the membrane lipid bilayer, both types of SA derivatives studied changed the dynamic properties of red cell membranes. Lipophilic ASA esters effectively increased the fluidity of the erythrocyte membrane lipid bilayer, and this effect increased with the increased hydrophobicity of the studied ASA esters. In contrast, the short chain acyl salicylates slightly decreased membrane lipid bilayer fluidity (Fig. 7).

The level of acetylation/acylation of poly-L-lysine by the studied SA and ASA derivatives was estimated by determination of residual free ε-amino groups. It was found that all of the studied derivatives at a concentration of 100 μM (1 h incubation at 37°C) significantly reduced the content of free amino groups (the content of free amino groups in control: 5.76 ± 0.87 μmol/mg of poly-L-Lys; reduced by 16.6 ± 2.7% for ASA, 18.0 ± 3.1% for BSA, 16.6 ± 3.5% for PSA, 13.0 ± 5.5% for C12, 10.0 ± 1.0% for C16; $p < 0.05$ by ANOVA and Tukey HSD test). The effect of irreversible acetylation/acylation by ASA and SA derivatives was not significantly different from that of ASA; similarly, there were no significant differences found between various derivatives.

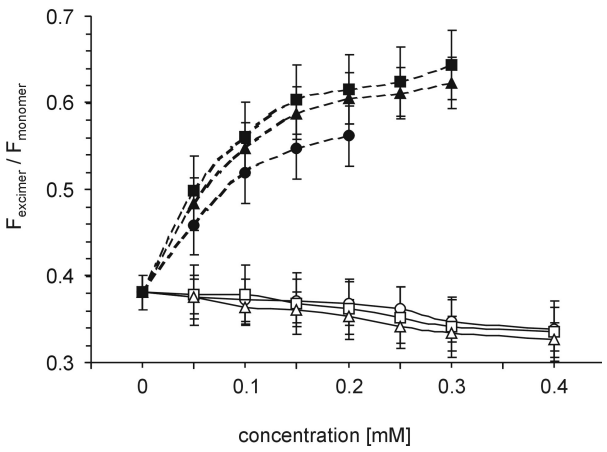

Fig. 7. Lateral mobility of pyrene in the lipid bilayer of erythrocyte membranes in the presence of O-acyl salicylic acids (solid line) and acetylsalicylate derivatives (dashed line). Data are presented as the mean SE, n = 4–5. $F_{excimer}$ and $F_{monomer}$, fluorescence intensities for pyrene excimers and monomers at increasing concentrations of ASA (o), PSA (□), BSA (△), dodecyl-ASA ester (●), hexadecyl-ASA ester (▲), or cholesteryl-ASA ester (■); membrane protein and pyrene concentrations were 0.1 mg/ml and 5 μM, PBS, pH 7.4, 22°C; respectively; λ_{exc} = 313 nm, $\lambda_{exc\ monomer}$ = 392 nm, $\lambda_{exc\ excimer}$ = 465 nm, slits 5 nm. Statistically significant differences between ASA and SA derivatives, estimated for the examined concentration ranges with MANOVA for planned comparisons with Bonferroni correction, were: ASA = PSA = BSA < * dodecyl-ASA ester < ** hexadecyl-ASA ester = cholesteryl-ASA ester, * $p < 0.0001$, ** $p < 0.04$

Discussion

The molecular mechanisms of ASAs pharmacological effects are still not completely understood. The analysis of the structure-activity relationship is the basis of the search for novel drugs possessing high pharmacological activity and lacking side effects. Importantly, the stereochemistry of ASA passage across biological membranes and its targeting intraplatelet COX-1 also remain elusive. Though there is a plethora of experimental evidence confirming both the biological and clinical effects of ASA, we are still far from understanding the molecular basis of how the acetyl group of ASA is transported into the cell interior to target COX and other intracellular proteins. Additionally, synthesized novel ASA derivatives may be potentially used as a tool for studying the topography of the active center of COX. In the present work, we synthesized and compared the stability and structure-activity relationships of O-acyl derivatives of SA and lipophilic ASA derivatives with a carboxylic group esterified by dodecyl alcohol, hexadecyl alcohol, or cholesterol.

Our present study shows that ASA remains the most potent inhibitor of platelet aggregation, whereas other O-acyl derivatives of SA were effective in the inhibition of whole blood platelet aggregation only at much higher concentrations. On the other hand, in contrast to ASA and O-acyl derivatives of SA, none of the lipophilic ASA derivatives had a significant inhibitory effect on platelet aggregation. In parallel to the outcomes of suppressed platelet aggregation, the reduction in TXA_2 generation by O-acyl derivatives was also concentration-dependent and decreased with the increasing length of the acyl chain.

Previously, Hung and co-workers have demonstrated that lipophilic O-acyl derivatives of SA inhibited human platelet aggregation and thrombin-stimulated rat serum TXA_2 production by platelets, both *in vitro* and *in vivo*, almost as effectively as ASA. Accordingly, the authors have suggested that the irreversible acylation of the platelet COX by SA derivatives is independent of the carbon chain length in the O-acyl moiety, and that the replacement of the O-acetyl group by a longer chain O-acyl moiety does not change the activity of the derivatives as inhibitors of platelet aggregation [13]. At the same time, the authors have revealed a linear relationship between the number of carbon atoms and the preincubation time needed for the inhibition of platelet aggregation. This observation has supported the conclusion that these non-acetyl salicylic acids might therefore represent a novel class of antiplatelet drugs that are able to bind to, or combine with, the same site on the platelet COX as ASA itself [13]. These findings are consistent with the earlier study by Mehler and Gerhards, who described correlations between anti-inflammatory activities and the energy and charge density distributions of a series of salicylic acids. These authors subsequently suggested that the binding of the drug to its receptor involves a charge transfer between the receptor and the aromatic group of the drug [19]. On one hand, we have learned from the models of the receptor for anti-inflammatory drugs built on the basis of structure-activity relationships, that the aromatic and carboxylic groups of the drugs and their relative orientations are likely to play important roles in the binding of the drug to COX [10, 22]. On the other hand, however, Santana et al. opposed the above reasoning by showing that neither the optimized molecular geometries nor the frontier orbital charge distributions of arylalkanoic acids (e.g., indomethacin, naproxen, or ibuprofen) were useful molecular parameters for the prediction of activity as inhibitors of prostaglandin biosynthesis [23]. This apparent controversy has led us back to early works by Dupin et al., who showed that none of the functional groups used to replace the original acetyl group is able to retain the original anti-aggregating activity of ASA. The antiaggregating activity is considerably enhanced when the carboxylic group is replaced with a hydroxyl or acetylated hydroxyl group. This is probably why diacetylated benzene derivatives appear to be more potent antiaggregating agents than ASA itself [7, 8].

The extent of *in vitro* platelet reactivity inhibition by O-acyl derivatives decreased in the present study with increasing O-acyl moiety length, as measured by the impedance method in whole blood samples. Likewise, the inhibitory effect of O-acyl salicylic acids toward the *in vitro* formation of TXB_2 decreased with increasing O-acyl chain length, as did the inhibition of platelet reactivity and degranulation (P-selectin expression) in collagen-activated platelets. Only PSA at higher concentrations (200 μM) was as potent as ASA in the reduction of TX generation and inhibition of platelet degranulation. The lipophilic C12-, C16-, and cholesteryl esters of SA showed low or no antiplatelet activity. These findings, consistent in supporting the reduced efficacy of ASA and SA derivatives, clearly suggest that ASA with its "small" acetyl group shows a much higher stochastic chance of blocking the COX active site than other acyl derivatives of SA with longer chain acyl groups. Hence, we are encouraged to suggest a role of steric hindrance for the lipophilic groups in these SA derivatives in accessing the active site in platelet membrane COX. On the other hand, all of the studied ASA and SA derivatives irreversibly acylated free amino groups of poly-L-Lys with an efficacy close to that of ASA. This finding remains consistent with the observations that the exposure of proteins to ASA results in an irreversible protein acetylation, preferably *via* the modification of essential lysine residues [15, 28].

At the same time, more hydrophobic derivatives of ASA have a much higher affinity for cell membrane proteins and lipids compared to ASA itself. As cyclooxygenase is a membrane-bound enzyme, we characterized the interactions of the studied esters with cell membrane proteins as well as a model protein, human serum albumin. The lipophilic esters of ASA bound more effectively to human serum albumin than did the short-chain acyl derivatives of SA. By analyzing the quenching of the albumin-bound ANS fluorescence by ASA derivatives, we revealed markedly increased values of the apparent Stern-Volmer constants, K_{SV}, along with the increased lipophilicity of the derivatives. Of the agents studied, cholASA had the highest affinity for the protein; ASA, PSA, and BSA showed similar but much lower affinities. With regard to the earlier report by Hung et al. [14], we evidence herein that the strength of protein binding to organic acids increases in proportion to the lipophilicity of the O-acyl derivatives of SA. Similarly, the interactions of C12-, C16-, and cholASA esters with cell membranes

and their fluidizing effects on membrane lipid bilayer effectively increased with the higher lipophilicity of the derivatives. In contrast, the short chain O-acyl salicylic acids slightly decreased membrane lipid bilayer fluidity; this finding remains consistent with our earlier observations of fluidized erythrocyte membranes and lipid bilayer disturbances as a result of exposure to free long chain fatty acids [29]. One may argue that the anchoring of fatty acid or cholesterol side moieties of ASA esters in the lipid bilayer should decrease the accessibility of these derivatives to the active site of COX. The very significant interaction of lipophilic ASA esters with the membrane lipid bilayer and the accompanying very weak inhibition of COX observed here might be interpreted as an indicator of a distal location of the COX active site, relative to the core of membrane lipid bilayer. Taking into account the observation of the decreased mobility of membrane bilayer lipids upon interaction with the short chain SA derivatives, one may speculate on the possible significance of lipid bilayer physico-chemical characteristics in effective ASA or acetyl penetration of the cellular membrane, when targeting intracellular COX.

It has been reported that the *in vitro* non-enzymatic hydrolysis rate constants generally decreased and the strength of the binding of ASA analogues to BSA increased with the elongation of carbon chain length of SA derivatives. These findings lead to the conclusion that the hydrolysis rate may be retarded by the presence of albumin [14]. In our present work, the spontaneous hydrolysis rate of O-acyl derivatives of SA was not affected by pH and was highest for PSA (C3SA) and lowest for BSA (C4SA). Hung et al. have suggested previously that the reduced electron density at the ester carbonyl group increases the susceptibility of acyl salicylates to hydrolysis [14]. On the contrary, the rate of enzymatic hydrolysis of O-acyl derivatives of SA, probably dependent on the activity of specific blood plasma butyrylcholine esterase, is much more efficient at higher pH values.

Overall, in our experiments, the modification of the ASA molecule resulted in a marked (in the case of the elongation of the O-acyl chain length in O-acyl derivatives of SA) or complete (for the esterification of the carboxylic group by lipophilic moieties) abolition of antiplatelet activity. Such modifications caused changes in the orientations of acyl side chain or alcoholic moieties and the aromatic ring of the ASA derivatives as well as modifications of their molecular properties (e.g., dipole moment, molecular volume, surface area, and heat of formation). Carboxylic group esterification and increased lipophilicity reduced the melting temperature. Importantly, the antiplatelet activity decreased as the steric bulkiness of the O-acyl or alcoholic substitutions increased, which might point to a crucial role of the carboxylic group and the bulkiness of substitution (and hence also the bulkiness of the drug molecule) in binding to the active site of COX and anti-COX activity [14].

In conclusion, it appears that the structure of ASA is optimal for binding to the active site of COX and efficient enzyme inhibition. Molecule geometry and size likely play the most important roles amongst all the possible contributors to ASA effectiveness in biological systems. Other factors relating to ASA penetration of cells (e.g., interactions with cellular membrane proteins and lipids) or bioavailability (e.g., susceptibility to degradation) appear to be much less important.

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