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SYNTHESIS AND ANTIAGGREGANT AND ANTICOAGULANT ACTIVITY OF AMINO-ACID SALTS AND COMPUTER SIMULATION OF THE INTERACTION OF THEIR STRUCTURES WITH THE SURFACE OF CYCLOOXYGENASE

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Effective drugs with antiaggregant and anticoagulant activity are known to possess side effects. It was hypothesized that compounds synthesized from natural amino acids would possess these pharmacological activities without many of the side effects. In this regard, a number of amino-acid salts of alkali and alkaline-earth metals were synthesized and studied. The newly synthesized compounds were identified using PMR spectra, IR spectroscopy, and elemental analysis. Laboratory studies carried out with isolated blood of healthy volunteers identified seven compounds exhibiting antiaggregant properties. The antiaggregant effects of these compounds on the maximum amplitude of aggregation was comparable to that of acetylsalicylic acid and exceeded it in terms of inhibition of the platelet release reaction. Computer simulation of the interaction of the most active amino-acid salts with the surface of cyclooxygenase confirmed their possible participation in the inhibition of this enzyme.

Keywords: amino acids, salts, synthesis, antiaggregant activity, anticoagulant activity, cyclooxygenase, computer simulation.

Salicylic acid and its derivatives are used medically as efficacious nonsteroidal anti-inflammatory drugs (NSAIDs) [1, 2]. The most well-known salicylate, acetylsalicylic acid (ASA), exhibits anti-inflammatory, antioxidant, antipyretic, and analgesic activity and is used to prevent and treat cardiovascular diseases [3, 4] because of its excellent capability to suppress platelet aggregation. The antithrombic effect of ASA is explained by its acetyl group, which irreversibly inhibits cyclooxygenase 1 (COX-1) via acetylation and, as a result, suppression of thromboxane A2 formation [5]. Although ASA possesses a broad spectrum of pharmacological activity, adverse side effects limit its use. Like other NSAIDs, ASA can cause both local (irritation of mucous membranes) and systemic damage (reduced prostaglandin synthesis) to gastrointestinal tract mucous membranes after peroral administration and can also disrupt liver and kidney functioning [6]. These side effects make it unfeasible to modify salicylates and retain their main therapeutic effects. The preparation of various salicylic acid derivatives is one research strategy for increasing the efficacy and decreasing the adverse effects [7]. In this respect, amino-acid derivatives

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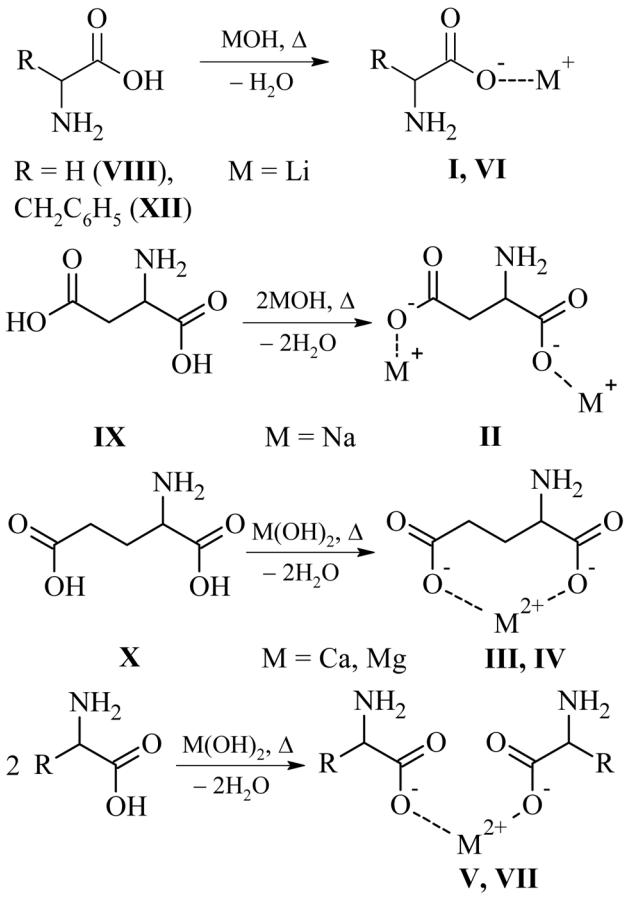
Scheme 1



and salts are enormously interesting because of their high reactivity and wide use in medical practice [8]. Several amino acids act as intermediates in the transmission of nerve impulses [9]. Currently, the synthesis, structure, and biological properties of various derivatives of amino acids and aromatic hydroxyacids are rather well studied. However, it is noteworthy that various amino-acid salts and salicylic acid ethers and esters are poorly studied with respect to antiaggregant and anticoagulant activity.

Therefore, the aim of the present research was to synthesize and study the antiaggregant and anticoagulant activity of a series of amino-acid salts containing alkali and alkaline-earth metal ions, i.e., lithium glycinate (**I**), sodium aspartate (**II**), magnesium glutamate (**III**), calcium glutamate (**IV**), calcium methioninate (**V**), lithium phenylalaninate (**VI**), and magnesium phenylalaninate (**VII**), and to perform computer simulation of the interactions with the surface of the COX enzyme of the structures of the most active amino-acid salts for which the PASS online program predicted the corresponding types of biological activity.

Compounds **I-VII** were synthesized in one step via reactions of the amino acids [glycine (**VIII**), *L*-aspartic acid (**IX**), *L*-glutamic acid (**X**), *L*-methionine (**XI**), *L*-phenylalanine (**XII**)] with alkali and alkaline-earth metal hydroxides (Scheme 1) [10].

IR spectra of **I-VII** showed characteristic absorption bands for carboxylate COO stretching vibrations (1552 – 1677 cm^{-1}), methylene CH_2 bending vibrations (1402 – 1423), and coordinated water (3200 – 3400), which were absent in spectra of starting amino acids **VIII-XII**.

PMR spectra of **I-VII** contained resonances for protons of the corresponding structural fragments of the amino-acid anions, which were close to the chemical shifts of proton resonances of the starting amino acids but smaller because of the deprotonation of the α-amine, and singlets for protons of coordinated water molecules at 4.65 ppm.

EXPERIMENTAL CHEMICAL PART

PMR spectra were recorded on a Bruker AM 400 instrument at operating frequency 400 MHz (TMS internal standard). IR spectra were taken on a FT-801 FT-IR spectrometer with an ATR accessory. Elemental analyses were performed on a system for express gravimetric determination of the elements [11]. Elemental analyses agreed with those calculated. Melting points of crystalline compounds in sealed capillaries were measured on a PTOP-2 apparatus (Russia).

Compounds **I-VII** were synthesized by the published methods [10].

Lithium glycinate (I). A solution of glycine (5.00 g, 66 mmol) in H_2O (20 mL) was treated in portions with $LiOH·H_2O$ (2.77 g, 66 mmol). The reaction mixture warmed to 30°C. The homogeneous solution was heated to 40°C for 20 min, held at room temperature for 1 h, and evaporated. The viscous mass was cooled, rinsed with EtOH, crystallized from H_2O, and dried at room temperature. Yield 6.50 g (83%). mp 198°C (H_2O, dec). IR spectrum (ν, cm^{-1}): 1417 δ(CH_2), 1584 (COO^-), 3353 (H_2O, NH_2). PMR spectrum (DMSO-d_6, δ, ppm): 3.44 (s, 2H, α-CH_2), 4.65 (s, 4H, $2H_2O$). Found, %: C 20.41; H 7.02; N 12.06. $C_2H_8LiNO_4$ (dihydrate). Calc., %: C 20.53; H 6.89; N 11.97.

Sodium aspartate (II). A suspension of *L*-aspartic acid (4.50 g, 34 mmol) in H_2O (25 mL) at 80°C was treated with NaOH (2.70 g, 68 mmol) and stirred for 10 min until the reagents dissolved completely. The homogeneous solution was heated at 40°C for 25 min, left at room temperature for 1 d, and evaporated. The solid was rinsed with EtOH and dried. Yield 6.12 g (85%). IR spectrum (ν, cm^{-1}): 1416 δ(CH_2), 1569 – 1584 (COO^-), 3367 (H_2O, NH_2). PMR spectrum (DMSO-d_6, δ, ppm): 2.59 (dd, 2H, β-CH_2), 4.00 (dd, 1H, α-CH), 4.66 (s, 4H, $2H_2O$). Found, %: C 22.49; H 4.39; N 6.47. $C_4H_9NNa_2O_6$ (dihydrate). Calc., %: C 22.55; H 4.26; N 6.57.

Magnesium glutamate (III). A suspension of *L*-glutamic acid (3.00 g, 20 mmol) in H_2O (20 mL) was treated with $Mg(OH)_2$ (1.13 g, 20 mmol). The reaction mixture was held at 35°C for 40 min. The homogeneous solution was evaporated. Yield 3.70 g (90%). IR spectrum (ν, cm^{-1}): 1410 δ(CH_2), 1632, 1682 (COO^-), 3406 (H_2O), 3211 (NH_2). PMR spectrum (DMSO-d_6, δ, ppm): 2.07 – 2.13 (m, 2H, β-CH_2), 2.34 – 2.36 (m, 2H, α-CH_2), 3.77 (q, 1H, γ-CH), 4.61 (s, 12H, 6H_2O). Found, %: C 21.57; H 7.02; N 5.13. $C_5H_{19}MgNO_{10}$ (hexahydrate). Calc., %: C 21.64; H 6.90; N 5.05.

Calcium glutamate (IV). A suspension of *L*-glutamic acid (5.00 g, 34 mmol) in H_2O (25 mL) at 80°C was treated with CaO (1.84 g, 34 mmol). The reaction mixture was held at 35°C for 40 min. The hot solution was filtered. The filtrate was evaporated. Yield 5,10 g (81%). mp 165 – 170°C (H_2O, dec). IR spectrum (ν, cm^{-1}): 1407 δ(CH_2), 1628, 1677 (COO^-), 3400 (H_2O), 3204 (NH_2). PMR spectrum (DMSO-d_6, δ, ppm): 2,14 (m, 2H, β-CH_2), 2,32 (m, 2H, γ-CH_2), 3,79 (q, 1H, α-CH_2), 4,59 (s, 4H, 2H_2O). Found, %: C 20,57; H 6,82; N 5,13. $C_5H_{19}CaNO_{10}$ (hexahydrate). Calc., %: C 20,48; H 6,53; N 4,78.

Calcium methioninate (V). A suspension of *L*-methionine (5.00 g, 34 mmol) in H_2O (25 mL) at 65°C was treated with $Ca(OH)_2$ (1.30 g, 17 mmol). The reaction mixture was held at 95°C for 40 min. The precipitate of unreacted calcium hydroxide was filtered hot. The homogeneous filtrate was evaporated. Yield 3.95 g (70%). IR spectrum, ν, cm^{-1}: 1423 δ(CH_2), 1571, 1658 (COO^-), 2915 (CH_3), 3051 (H_2O, NH_2). PMR spectrum (DMSO-d_6), ppm: 1.76 – 1.88 (m, 2H, β-CH_2), 2.07 (s, 2H, CH_3), 2.45 (m, 2H, γ-CH_2), 3.36 (t, 1H, α-CH), 4.65 (s, 4H, 2H_2O). Found, %: C 26.84; H 7.43; N 6.21. $C_{10}H_{32}CaN_2O_{10}S_2$ (hexahydrate). Calc., %: C 27.02; H 7.26; N 6.30.

Lithium phenylalaninate (VI). A solution of *L*-phenylalanine (1.65 g, 10 mmol) in H_2O (15 mL) was treated with LiOH (0.479 g, 20 mmol). The reaction mixture warmed to 30°C. The homogeneous solution was heated to 40°C for 20 min, held at room temperature for 1 h, and evaporated. The viscous mass was cooled, rinsed with EtOH, crystallized from H_2O, and dried at room temperature. Yield 2.12 g (85%). IR spectrum, (ν, cm^{-1}): 1408 δ(CH_2), 1497 (C-C, Ar), 1557 (COO^-), 3200 – 3400 (H_2O, NH_2). PMR spectrum (DMSO-d_6, δ, ppm): 2.96 (dd, 1H, β-CH_2), 3.13 (dd, 1H, β-CH_2), 3.83 (dd, 1H, α-CH), 4.65 (s, 4H, 2H_2O), 7.17 (d, 2H, H2, H2′, Ar), 7.24 (dd, 1H, H_4, Ar), 7.37 (s, 2H, H3, H3′, Ar). Found, %: C 52.01; H 6.98; N 6.87. $C_9H_{14}LiNO_4$ (dihydrate). Calc., %: C 52.18; H 6.81; N 6.76.

Magnesium phenylalaninate (VII). A solution of *L*-phenylalanine (1.65 g, 10 mmol) in H_2O (15 mL) at 80°C was treated with $Mg(OH)_2$ (0.58 g, 10 mmol). The reaction mixture was held at 90 – 95°C for 30 – 35 min. The $Mg(OH)_2$ dissolved. The homogeneous solution was evaporated. Yield 2.20 g (70%). mp 180 °C (H_2O, dec). IR spectrum (ν, cm^{-1}): 1402 δ(CH_2), 1552 (COO^-), 3200 – 3400 (H_2O, NH_2). PMR spectrum (DMSO-d_6, δ, ppm): 2.91 (dd, 1H, β-CH_2), 3.08 (dd, 1H, β-CH_2), 3.72 (dd, 1H, α-CH), 4.65 (s, 4H, 2H_2O), 7.19, 7.27 (m, 5H, Ar). Found, %: C 55.81; H 6.37; N 7.28. $C_{18}H_{24}MgN_2O_6$ (dihydrate). Calc., %: C 55.62; H 6.22; N 7.21.

Both experimental methods *in vitro* with peripheral blood cells as a model and molecular modeling *in silico* were used to evaluate the antiaggregant activity of the amino-acid salts.

EXPERIMENTAL BIOLOGICAL PART

The experiments were conducted in compliance with Good Laboratory Practice rules of the Eurasian Economic Union for circulation of drugs.

Antiaggregant and anticoagulant activities were evaluated under *in vitro* conditions using isolated blood samples from 35 healthy male volunteers 18 – 24 years old. The study was approved by the Ethics Committee of Bashkir State Medical University, Ministry of Health of Russia (No. 1 of Feb. 20, 2019). Informed consent was obtained from all study participants before collecting blood from them.

The effects of the compounds on platelet aggregation were studied by the Born method [12] on an AT-02 aggregometer (NPF Medtech, Russia). Antiaggregant activity of the studied compounds and reference drugs was evaluated at a final concentration of 1×10^{-3} M with incubation for 5 min. Adenosine diphosphate (ADP) at a concentration of 20 μg/mL and collagen at a concentration of 5 mg/mL (Tekhnologiya-Standard, Russia) were used as aggregation inductors. The effects of the compounds on the maximum amplitude of aggregation (MA), aggregation rate, and time to reach MA for platelet aggregation induced by ADP were studied. The latent period of aggregation was estimated in the collagen-induced aggregation test and corresponded to the release of platelets. The reference drug was ASA (substance powder; Shandong Xinhua Pharmaceutical Co., Ltd., China) [13].

Anticoagulant activity was determined from clotting tests [14] on a Solar CGL 2110 turbidometric hemocoagulometer (CSC Solar, Belarus). The final concentration of the studied compounds and the reference drug was $5 \cdot 10^{-4}$ g/mL. The parameters of activated partial thromboplastin time (APTT), prothrombin time (PT), and fibrinogen concentration were studied according to Clauss. Heparin sodium (heparin sodium, 5000 IU/mL, solution for injection, 1-mL ampuls; JSC Sintez, Russia) was used as the reference drug.

Statistical analysis used the Statistica 10.0 program suite (StatSoft Inc., USA). The normalcy of distributions was checked using the Shapiro–Wilk criterion. The median, 25, and 75 percentiles and minimum and maximum values were calculated to describe the variational series. One-factor dispersion analysis (if the dataset obeyed normal distribution laws and dispersions of all sets were equal; F-criterion) or

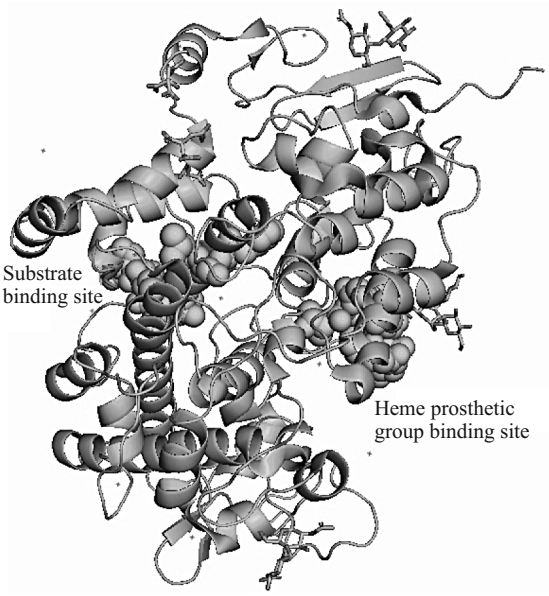


Fig. 1. Crystal structure of COX-1 (human) (PDB code 6Y3C). Asymmetric unit of *h*COX-1: sites of glycosylation of asparagine residues are shown. Spheres show surface excluded by solvent (determined for a solvent radius of 1.4 Å) of heme and substrate binding (arachidonic acid). Data are taken from the literature [18].

the Kruskal–Wallis test (if datasets did not obey normal distribution laws; A-criterion) was used. The critical significance level p for statistical criteria was taken as 0.05.

Computational part (in silico)

Molecular modeling. The PatchDock v.1.3. Molecular Docking Algorithm (Bioinfo$_3$D) was used to simulate interaction of the ligand with the target protein COX-1. It allowed simulation of the interaction of the molecules to be performed *in silico* considering the geometric patches of the surface (concave, convex, flat portions) [15]. The molecular modeling resulted in the overlaying of these patches on each other using algorithms for comparing shapes. The COX-1 enzyme model was obtained from an open database (Protein Data Bank; structure code 6Y3C).

RESULTS AND DISCUSSION

The research results established that several compounds exhibited antiaggregant activity at the level of ASA according to the maximum amplitude parameter (Table 1). However, compounds **I**, **III**, **VI**, and **VII**, in contrast to ASA, statistically significantly increased the lag time corresponding to release of platelets. The platelet aggregation rate decreased for all new derivatives, like the effect of ASA. This parameter was lowered most effectively through the action of **III**, **IV**, **V**, and **VI**.

It is noteworthy that all compounds caused hypocoagulation, increasing the APTT by 4.8 – 9.4% as compared to the control, and did not affect the fibrinogen concentration and prothrombin time. The studied compounds were much less potent than heparin, which increased the APTT by 20.3%.

Thus, the maximum antiaggregant activity in combination with the maximum prolongation of the latent time was recorded for lithium glycinate (**I**); the maximum antiaggregant activity in combination with the strongest decrease of platelet aggregation rate, for calcium glutamate (**IV**). Therefore, computer simulation of the interaction of them with the COX enzyme surface was performed to evaluate a possible mechanism of action of the antiaggregant activity.

Molecular modeling of the interaction of the studied compounds with COX-1

TABLE 1. Effect of Synthesized Compounds and Reference Drug on Platelet Aggregation and Hemostasis Coagulation Stage, Me (0.25 – 0.75)

No.	Compound	Latent period, % of control	Maximum amplitude, % of control	Aggregation rate, % of control	Time to reach MA, % of control	APTT, % of control
I	Lithium glycinate	+13.1 (12.5 – 15.7)*,#	– 12.3 (9.6 – 13.9)*	– 5.4 (4.3 – 7.4)*	+10.3 (8.9 – 13.1)*	+9.4 (8.2 – 11.5)*,†
II	Sodium aspartate	+3.1 (2.3 – 4.5)#	– 7.5 (5.8 – 7.6)*	– 12.3 (11.6 – 14.7)*	+10.7 (7.8 – 4.5)*	+7.5 (6.3 – 8.7)*,†
III	Magnesium glutamate	+8.3 (7.6 – 11.7)*,#	– 9.4 (7.8 – 11.4)**	– 14.3 (13.5 – 16.4)**,#	+10.7 (7.6 – 12.5)*	+5.9 (3.8 – 7.8)*,†
IV	Calcium glutamate	– 4.5 (3.6 – 6.1)#	– 11.5 (8.9 – 12.7)*	– 15.7 (13.2 – 18.9)*,#	+10.2 (8.1 – 12.6)*	+4.8 (3.2 – 6.5)*,†
V	Calcium methioninate	– 5.7 (4.3 – 6.2)#	– 8.3 (5.8 – 10.4)*	– 13.6 (11.4 – 16.7)*,#	+10.2 (7.9 – 12.3)*	– 5.1 (4.7 – 7.6)*,†
VI	Lithium phenylalaninate	+9.2 (7.4 – 12.7)*,#	– 10.3 (8.2 – 12.4)**	– 14.6 (12.5 – 17.7**,#	+9.1 (6.8 – 12.3)*	+5.7 (4.1 – 7.4)*,†
VII	Magnesium phenylalaninate	+10.0 (9.5 – 13.4)*,#	– 11.6 (8.3 – 14.2)*	– 10.6 (6.4 – 12.8)*	+10.2 (8.1 – 13.5)*	+5.4 (4.3 – 7.8)*,†
	Aspirin	– 2.1 (1.1 – 2.6)	– 13.7 (10.8 – 16.4)*	– 10.5 (7.6 – 12.3)*	+10.5 (8.7 – 13.4)*	+1.1 (0.5 – 1.9)†
	Heparin sodium	–	-	–	-	+20.3 (19.7 – 21.4)**

Note: Latent period given for platelet aggregation induced by collagen; other parameters, for ADP-induced platelet aggregation. $^{*}p \le 0.05$, $^{**}p \le 0.001$ vs. the control; $^{\dagger}p \le 0.05$ vs. heparin sodium; $^{\#}p \le 0.05$, $^{\#\#}p \le 0.001$ vs acetylsalicylic acid. "–", no data, $n = 6$.

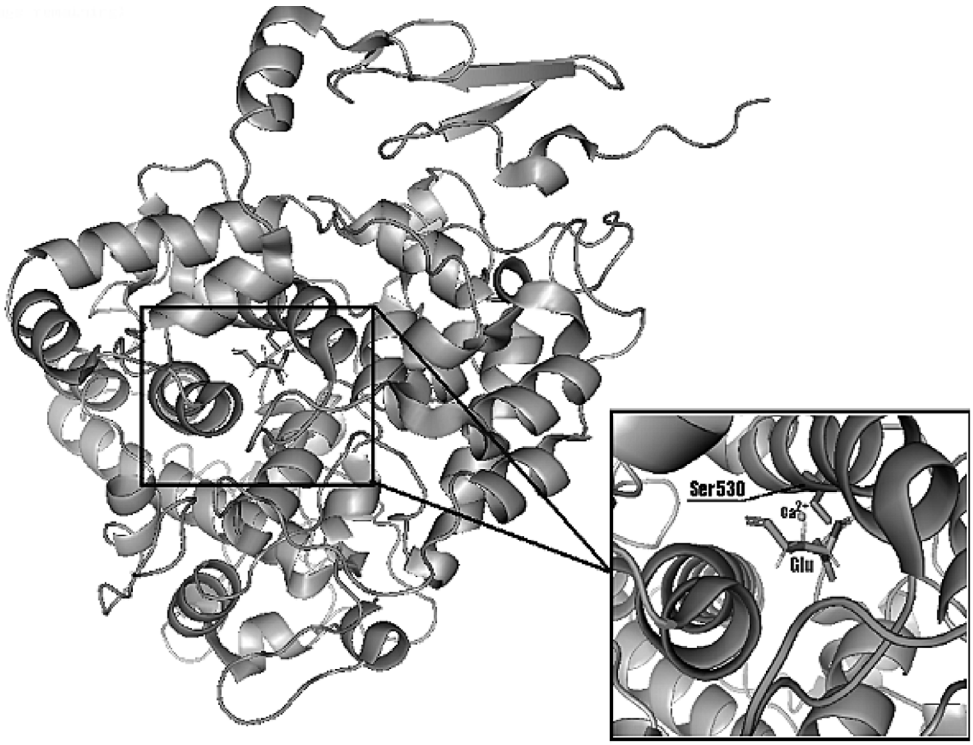


Fig. 2. Complex of **IV** (in center) with COX-1 enzyme. The Ser530 residue is shown.

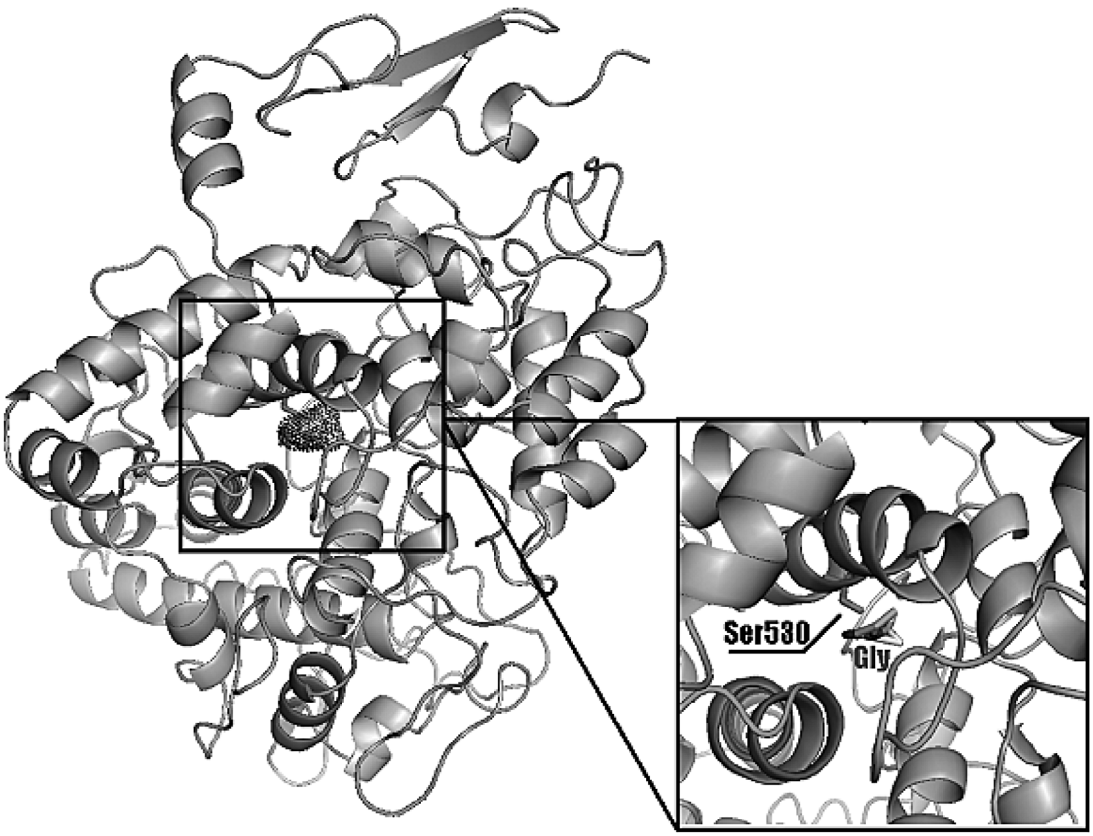


Fig. 3. Complex of **I** (in center) with COX-1 enzyme. The Ser530 residue is shown.

The active site of COX is a long hydrophobic channel formed by α-helices (Fig. 1). Evidence supporting several aspirin-like drugs such as flurbiprofen inhibiting COX-1, preventing arachidonic acid from filling this channel, was reported before [16]. The Tyr385 and Ser530 residues are located at the entrance to the long channel of the enzyme active site. Aspirin irreversibly inhibits COX-1 via acetylation of the Ser530 hydroxyl group, thereby preventing arachidonic acid from binding [17]. The carboxyl group of the *S*-(–)-stereoisomer of flurbiprofen interacts with Arg120. The inhibitor is bound in the channel pore through van-der-Waals forces of the second phenyl ring with Tyr385. It needs to be understood if several other drug-binding subsites could exist in this narrow channel.

Thus, the molecular modeling problem consisted of establishing patterns of the amino-acid salts containing patches of the COX-1 active site channel, i.e., a substrate site. Interactions of the active compounds (Table 1) with the COX-1 enzyme were simulated *in silico*. Figure 2 shows the sterically possible favorable positioning of ligand **IV** in the COX-1 active site channel pore.

Figure 2 shows that calcium glutamate is situated very close of the aspirin-binding domain. The ligand is presumably held in the active-site channel by H-bonds between the glutamic-acid amine and the Ser530 hydroxyl group.

Analogous binding could be observed for ligand **I** (Fig. 3).

Thus, amino-acid salts were prepared. They were evaluated biologically as compared to ASA. The analysis showed that several amino-acid salts lengthened the latent period in a collagen-induced platelet aggregation model (thrombocyte release response) and reduced the platelet aggregation rate. Molecular modeling of the interaction with the COX-1 surface for calcium glutamate and lithium glycinate agreed with data from an experiment on the *in vitro* platelet aggregation parameters. The results emphasized the importance of further studies of the mechanisms of action of amino-acid salts, their possible anti-inflammatory properties, and the design of antiplatelet drugs based on them.

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