Antiplatelet Effect and Selective Binding to Cyclooxygenase by Molecular Docking Analysis of 3-Alkylaminopropoxy-9,10-anthraquinone Derivatives

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In an effort to develop potent cytotoxic inhibitors of cyclooxygenase (COX), a series of cytotoxic 3-alkylaminopropoxy-9,10-anthraquinone derivatives was screened to evaluate their antiplatelet effect on washed rabbit platelets and human platelet-rich plasma (PRP). Thrombin, arachidonic acid (AA), collagen, and platelet-activating factor (PAF) induced platelet aggregations were potently inhibited by compounds 1, 2, and 3 (each at 300 μM). Of the compounds tested in human PRP, compounds 1, 8, and 10 showed significant inhibition of primary and secondary aggregation induced by epinephrine and had a weak inhibitory effect on cyclooxygenase-1 (COX-1). Molecular docking studies revealed that compounds, 1, 8, and 10 were bound in the active sites of COX-1. This indicated that the antiplatelet effect of these three compounds was partially mediated through the suppression of COX-1 activity and reduced thromboxane formation. It is concluded that the cytotoxic compounds 1, 8, and 10 may interfere the conversion of arachidonic acid to prostaglandin (PG)H_2 in the active site of COX-1.

Key words antiplatelet effect; cyclooxygenase; 3-alkylaminopropoxy-9,10-anthraquinone

Platelet aggregation plays an important role in both physiological haemostatic and pathological thrombotic processes. Platelets are activated by a number of physiological agonists such as collagen, arachidonic acid (AA), thrombin, and adenosine diphosphate (ADP), and undergo a complex cascade of events resulting in shape change, secretion of granules, thromboxane (TX) A_2 formation, and aggregation.[1,2] When exogenously applied to platelets, AA is metabolized by cyclooxygenase (COX) to a reactive intermediate, prostaglandin (PG)H_2, which is processed by a PG isomerase, yielding PGE_2, or by thromboxane synthase (TXS) to make TXA_2.[3] Therefore, TXA_2 is a metabolite of AA formed *via* the COX/TXS pathway. In platelets, TXA_2 binds to a G-protein-coupled receptor inducing phospholipase C activation, $[Ca^{2+}]_i$ increase, and aggregation. The abnormal activation of platelets will cause arterial thrombosis. Therefore, one rational approach in the development of antithrombotic drugs is to search for inhibitors of platelet aggregation. Antiplatelet compounds with an inhibitory effect on AA-induced platelet aggregation in washed rabbit platelets and secondary aggregation induced by epinephrine in human platelet-rich plasma (PRP) are mainly mediated through the suppression of COX activity and reduced thromboxane formation or owing to inhibition of thromboxane synthetase, leading to the reduction of thromboxane formation.[4—6]

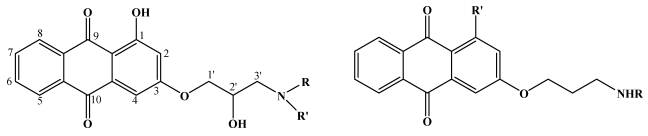
During the past few years, COX (COX-1 and COX-2) was discussed as new targets for several types of cancer, including breast cancer. COX-2 proteins increase new blood vessel formation and proliferation.[7] COX-1 selective inhibitors may also have therapeutic value, as it was shown that COX-1 is overexpressed in some ovarian cancer cells, where it stimulates antiogenesis.[8]

In an effort to develop potent cytotoxic inhibitors of COX, we studied the antiplatelet effects and the inhibitory effect on COX-1 activity of a series of cytotoxic 3-alkylpropoxy-9,10-anthraquinone derivatives and report these in the present paper. A more detailed understanding of COX isoform differences may aid in the design of more selective and potent inhibitors of both COX isoforms, thus, the molecular binding models of 3-alkylpropoxy-9,10-anthraquinone derivatives with COX-1 are also reported.

MATERIALS AND METHODS

Chemistry The syntheses of **1**—**10** have been reported previously.[9,10]

Platelet Aggregation Rabbit washed platelets were obtained from ethylenediaminetetraacetic acid (EDTA)-anticoagulated PRP according to the washing procedures described previously.[11] Platelet numbers were counted by Coulter Counter (Model ZM) and adjusted to 4×10^8 platelets/ml. The platelet pellets were suspended in Tyrode's solution con-



1 R = $CH(CH_3)_2$ R' = H
2 R = $(CH_2)_2CH_3$ R' = H
3 R = $CH(CH_3)CH_2CH_3$ R' = H
4 R = ⬡ R' = H
5 R = CH_2CH_3 R' = CH_2CH_3
6 R = $C(CH_3)_3$ R' = H

7 R = $(CH_2)_2CH_3$ R' = H
8 R = $CH(CH_3)_2$ R' = H
9 R = $(CH_2)_3CH_3$ R' = H
10 R = $CH(CH_3)_2$ R' = OH

Fig. 1. Structures of Anthraquinone Derivatives

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Table 1. Effect of Various 3-Alkylaminopropoxy-9,10-anthraquinone Derivatives on the Platelet Aggregation Induced by Thrombin, AA, Collagen, and PAF

Compound	(μM)	Platelet aggregation (%)			
		Thrombin (0.1 U/ml)	AA (100 μM)	Collagen (10 μg/ml)	(PAF 2 ng/ml)
Control		94.4±0.8	89.9±0.3	90.9±0.6	91.2±0.5
1	300	0.0±0.0***	3.2±1.7***	1.4±1.2***	0.0±0.0***
2	300	0.0±0.0***	4.6±4.0***	4.0±3.2***	0.0±0.0***
3	300	0.0±0.0***	19.6±7.3***	14.6±8.2***	0.0±0.0***
4	300	81.0±0.4***	80.5±1.0***	58.5±4.4***	75.6±3.0***
Aspirin	50	91.9±2.5	0.0±0.0***	85.4±3.9	90.5±1.2

Platelets were preincubated with compounds **1**, **2**, **3**, **4** (each at 300 μM) and aspirin (50 μM) or dimethylsulfoxide (0.5%, control) at 37 °C for 3 min, then inducer was added. Percentages of aggregation are presented as mean±S.E.M. (n=3—4). *** $p<0.001$: compared with the respective control value.

taining (mM) NaCl, 136.8, KCl, 2.8, $NaHCO_3$, 11.9, $MgCl_2$, 2.1, NaH_2PO_4, 0.33, $CaCl_2$, 1.0, and glucose, 11.2, with 0.35% bovine serum albumin. Human PRP was obtained from the supernatant after centrifugation of blood mix with 3.8% sodium citrate (1 : 9 to blood). All glassware was siliconized. Just 1 min before the addition of the aggregation inducer, PRP or the platelet suspension was stirred at 1200 rev/min. Aggregation was measured by turbidimetric method. The absorbance of PRP or the platelet suspension was taken as 0% aggregation and the absorbance of platelet-poor plasma or platelet-free Tyrode's solution as 100% aggregation. The aggregation was measured by a Lumi aggregometer (Chrono-Log Co., Havertown, PA, U.S.A.) connected to a dual channel's recorders and assay for aggregation of human PRP was performed by the method described previously.[4)]

COX-1 Activity The reaction mixture (0.1 M Tris–HCl, pH 8.0, 5 mM tryptophan, 8 mM hematin, test drugs and 10 μg/ml of ram seminal vesicles COX) was incubated for 3 min at 30 °C. The reaction was initiated by adding 100 μM AA. The velocity of oxygen consumption in the reaction mixture was monitored continuously with a Clark-type oxygen electrode using a YSI biological oxygen monitor (Model 5300).[12)]

Molecular Docking and Dynamic Study Docking experiments were performed using DS modeling 1.7 (Accelrys, San Diego, CA, U.S.A.). The coordinate for the X-ray crystal structure of the enzyme COX-1 was obtained from the Protein Data Bank (PDB ID: 1Q4G), crystal cell restriction was removed, and the hydrogens were added following typing of the whole structure in the CHARMm force field. The scaffolds of recruited compounds were constructed under ChemOffice 2006 software (Cambridge Scientific Computing, Cambridge, Massachusetts, U.S.A.) and each compound was minimized in the MM2 force field. After the protein was defined as a receptor and the binding site was defined depending on the position of the ligand, 2-(1,1′-biphenyl-4-yl)propanoic acid (BFL), in the crystal structure, the docking experiment on COX-1 was carried out. To use the LigandFit program the optimal binding orientation of each recruited ligand was obtained after being further minimized for 1000 iterations using the conjugate gradient method until the convergence of 0.001 kcal/mol-Å.[7)] The dock scores were determined and the remarkable compounds were chosen as candidates for the ligand-receptor complex dynamic cascade performance. Under the "Standard Dynamics Cascade" protocol, several consecutive simulations containing minimization, heating, equilibration, and production were executed for ligand-receptor complexes. Before this protocol running, the complex should be harmonically restrained. Two minimizations were carried out previously including "Steepest Descent" and "Adopted Basis NR" algorithms with the 0.1 and 0.0001 RMS gradients, respectively. Next, the whole system was heated to 310 K with 2000 steps based on 0.001 ps for each step. Equilibration was performed to stabilize the molecular system around the target temperature with 1000 steps for each 0.001 ps per step. Finally, the complex was produced based on the constant-temperature, constant-volume ensemble (NVT). Subsequently the interaction energies were calculated based on the Chemistry at Harvard Macromolecular mechanics (CHARMm) force field according to the following equation and the docking results were analyzed.[13)]

$$E_{\text{interaction}}=E_{\text{complex}}-(E_{\text{ligand}}+E_{\text{receptor}})$$

where E is energy and the complex contains ligand and receptor.

Table 2. IC_{50} Values of 3-Alkylaminopropoxy-9,10-anthraquinone Derivatives on the Platelet Aggregation Induced by Thrombin, AA, Collagen, and PAF

Compound	Platelet aggregation (%)			
	Thrombin (0.1 U/ml)	AA (100 μM)	Collagen (10 μg/ml)	PAF (2 ng/ml)
1	92.4	82.7	69.1	78.0
2	94.3	95.6	73.4	77.6
3	86.7	74.9	62.4	71.6

Data Analysis Data are presented as means±S.E.M. One-way analysis of variance was used for multiple comparison, and if there was significant variation between the treatment groups and the inhibitor-treated groups, they were then compared with the control by Student's t-test. Values of $p<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

The aggregation of washed rabbit platelets induced by thrombin, AA, collagen, and PAF was used to study the antiplatelet effects of compounds **1**—**4**. As shown in Table 1, compounds **1**—**3** at 300 μM had potent antiplatelet effects on all the four inducers used in Table 1. The inhibitory effects of **1**—**3** were concentration dependent (Table 2).

As shown in Tables 1 and 2, a branched alkyl group substi-

tuted at the side chain of N-atom, such as **3** revealed stronger antiplatelet effect than those of **2** and **4** on washed rabbit platelet aggregation induced by the four inducers used in Table 1.

A cyclic alkyl group substituted at the same side chain of N-atom did not enhance the antiplatelet effect on washed rabbit platelet aggregation induced by the same inducer. Compounds **1**, **2**, and **3** inhibited platelet aggregation induced by PAF, which did not cause thromboxane formation. This inhibition could be due to the calcium antagonizing effect or an inhibitor of intracellular calcium mobilization.[10] Further experiments are needed to elucidate the mechanism of action.

The antiplatelet effects of selective compounds **1**, **3**, and **5**—**10** on platelet aggregation induced by epinephrine (5 μM) in human PRP were also studied. As shown in Table 3 and Fig. 2, compounds **1**, **8**, and **10** had significant antiplatelet effects on epinephrine-induced platelet aggregation and the antiplatelet effects of **1**, **8**, and **10** appeared to be concentration-dependent while the other compounds did not reveal significant effect. It also indicated that compounds **8** and **10** showed stronger antiplatelet effect than that of **1** and an isopropyl group substituted at the side chain of N-atom of the 3-alkylpropoxy-9,10-anthraquinone derivatives showed stronger antiplatelet effect.

In epinephrine induced platelet aggregation in human PRP, compounds **1**, **3**, **8**, and **10** prevented secondary aggregations and suppressed the primary aggregation at high concentrations induced by epinephrine (Fig. 3). The above result suggested that the antiplatelet effects of **1**, **8**, and **10** were probably mediated through the suppression of COX activity and reduced thromboxane formation or due to the inhibition of thromboxane synthetase, leading to reduced thromboxane formation, and interference in the epinephrine-receptor interaction.[4,,6,14] For study of the mechanism of antiplatelet action of these compounds, the activity of fatty acid COX-1 from ram vesicular glands was measured in their presence. The result in Table 4 showed that compounds **1**, **8**, and **10** weakly inhibited the activity of COX-1; the above result clearly indicated that their antiplatelet action is partially mediated through the suppression of COX-1 activity and reduced thromboxane formation.

Further experiments are needed to elucidate the mechanism of action.

Before starting the docking study of **1**, **8**, and **10** with COX-1, the re-docking works showed the root mean square deviation (RMSD) of the indomethacin after docking compared to the initial one in 1PGF, 1PGG, and 2OYE, was 0.66 Å, 0.80 Å, 0.64 Å, respectively. Even the re-docking of alpha-methyl-4-biphenylacetic acid in 1Q4G of COX-1 crystal structure showed the RMSD of 0.49 Å. These RMSD values revealed reasonable results within 1.5 Å, so the docking method could be relied upon. In crystallization experiments, it was reported that all COX-1 inhibitors interacted with the putative catalytic amino acid residue Tyr 385 and formed hydrogen bonds with Arg 120 and Tyr 355.[15] When mapping compounds **8** and **10** into the COX-1 model (Figs. 4, 5), it could be observed that the N-atom, and the N-atom and OH-1 of **8** and **10,** respectively, accept a hydrogen bond from Arg 120 (2.00 Å), and two hydrogen bonds from Arg 120 (2.07 Å) and Ser 530 (2.12 Å), respectively. While mapping compound **1** into the COX-1 model (Fig. 6), it could be observed that the OH-1, OH-2′, and NH groups of **1** accept two hydrogen bonds from Ser 530 (2.06 Å, 2.17 Å), two hydrogen bonds from Arg 120 (2.02 Å, 2.42 Å), and two hydrogen bonds from Tyr 355 (2.17 Å) and Arg 120 (2.30 Å), respectively. As shown in Figs. 5 and 6, the OH-1 of **10** and **1** are in the vicinity of Tyr 385. This suggested that a compound with an OH-1 group oriented in the vicinity of Tyr 385, such as **1** and **10**, showed stronger inhibitory activity on COX-1 than a compound without an OH-1 group, such as **8**. The scoring values given by dock score and interaction energies in COX-1 are given in Table 5. The dock score could suggest that the inhibition of compounds **1**, **8**, and **10** relates this score, a higher dock score could allow more chances to bind to the target site. But for the interaction energy, they showed the re-

Table 3. Effect of Various 3-Alkylaminopropoxy-9,10-anthraquinone Derivatives and Aspirin on Epinephrine Induced Platelet Aggregation in Human PRP

Compound	(μM)	Aggregation (%)
Control		94.9±0.4
1	300	0.0±0.0***
	200	24.5±8.7**
	100	88.0±2.0
3	300	0.0±0.0***
	200	77.0±0.3**
5	300	100.0±0.0
6	300	100.0±0.0
7	300	35.1±6.2***
	200	100.0±0.0
8	300	0.0±0.0***
	200	4.4±2.5***
	100	88.4±1.9
9	300	100.0±0.0
10	300	0.0±0.0***
	200	0.0±0.0***
	100	76.5±7.0**
Aspirin	50	29.5±1.0**
	25	67.7±2.7***
	10	94.5±1.0

Platelets were preincubated with various concentrations of **1**, **3**, **5**—**10** and aspirin or dimethylsulfoxide (0.5%, control) at 37 °C for 3 min, then epinephrine was added, respectively. Percentages of aggregation are presented as mean±S.E.M. (n=3). ** p<0.01, *** p<0.001: compared with the respective control value.

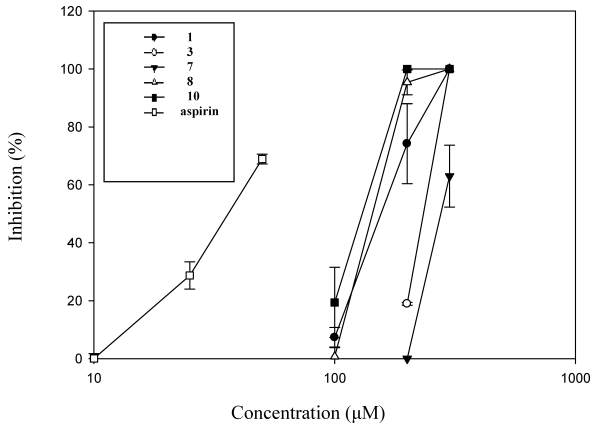

Fig. 2. Concentration-Dependent Inhibitory Effect of Compounds **1**, **3**, **7**, **8**, **10**, and Aspirin on Platelet Aggregation Induced by Adrenaline in Human PRP

Human PRP was incubated with various concentrations of compounds **1**, **3**, **7**, **8**, **10**, aspirin or dimethylsulfoxide (0.5%) at 37 °C for 3 min, and then adrenaline (5 μM) was added to trigger aggregation. Data are presented as means±S.E.M. (n=3).

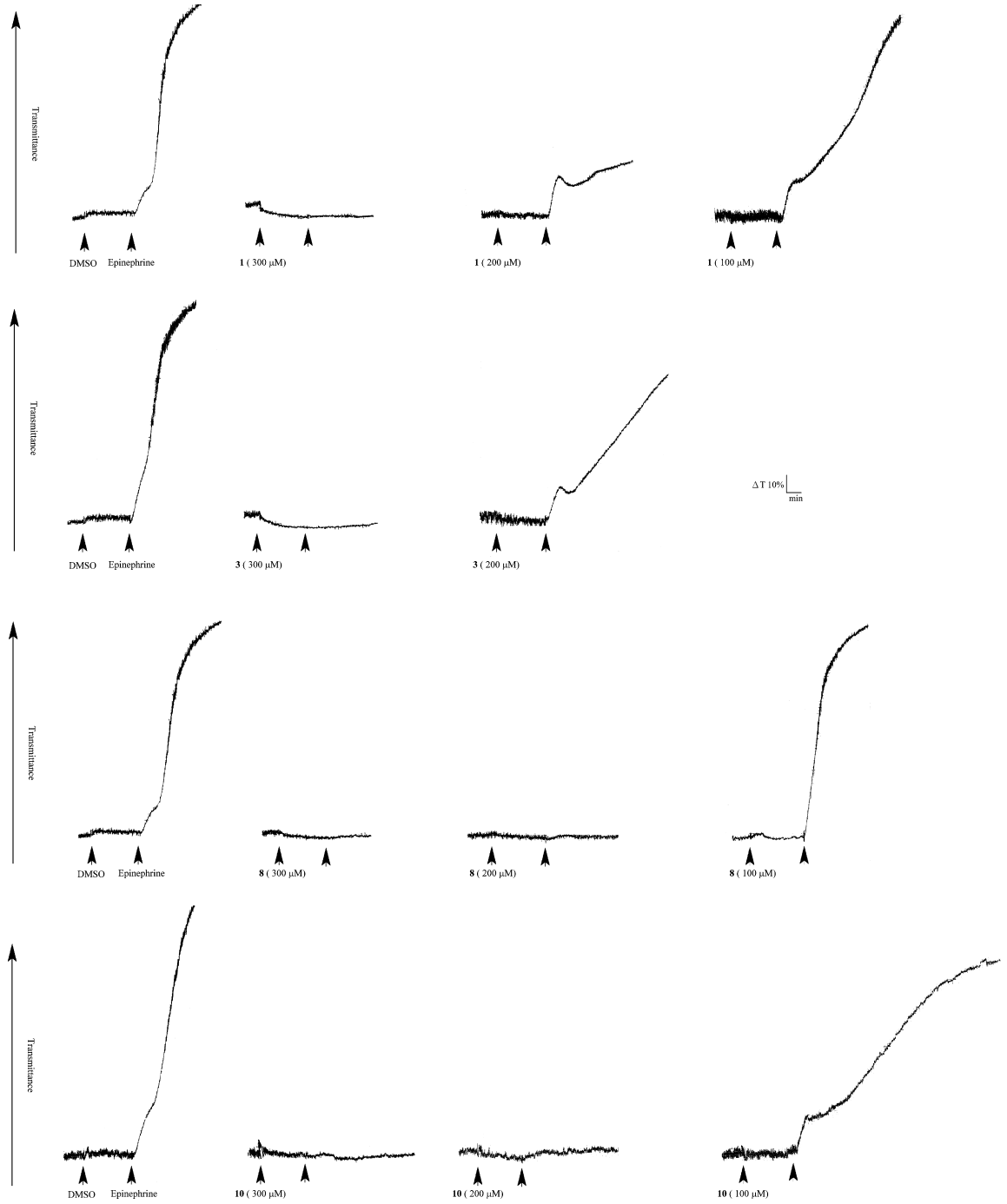

Fig. 3. Effect of **1**, **3**, **8**, and **10** on the Aggregation of Human PRP Induced by Epinephrine

Human PRP was preincubated with dimethylsulfoxide (0.5%) or various concentrations of **1**, **3**, **8**, and **10** at 37 °C for 3 min, and then epinephrine (5 μM) was added to trigger the aggregation.

verse, and the one with higher interaction energy would be less stable in the binding site.

Aspirin acetylates a serine hydroxyl group in the arachidonic acid binding channel (Ser 530 of COX-1) and blocks the approach of fatty acid substrate to the active side for its oxygenation,[15] while cytotoxic 3-alkylaminopropoxy-9,10-anthraquinones such as **1**, **8**, and **10** did not possess an acetyl moiety like that of aspirin for acetylating the serine hydroxyl group in the AA binding channel (Ser 530 of COX-1). Thus, the acetylation of OH-1 of **1** and **8** may enhance the inhibitory effect on COX-1 activity. Based on the above results, these compounds showed a partial mechanism of action similar to that of aspirin.

In summary, anthraquinones with an appropriate 3-alkylaminopropoxy side chain such as **1**, **2**, and **3** revealed a significant inhibitory effect on platelet aggregation of washed rabbit platelet induced by thrombin, AA, collagen, and PAF while anthraquinones with an isopropylaminopropoxy side chain such as **1**, **8**, and **10** showed an inhibitory effect on platelet aggregation of human PRP induced by epinephrine. Additionally, the inhibitory effect on COX-1 activity of **1**, **8**, and **10** indicated that a mode of action including interference

Table 4. Inhibitory Activity (O_2 Consumption) of Selective Compounds **1**, **8**, and **10** on COX-1

Compound	(μM)	COX-1 activity nmol/min (% inhibition)	IC_{50} (μM)
Control		10.9±1.1	
1	(10)	10.4±1.9 (8.4±7.3)	>100
	(100)	8.7±1.5 (23.1±5.1)	
8	(10)	10.4±1.0 (4.5±5.1)	>100
	(100)	9.0±1.3 (20.2±6.6)	
10	(10)	10.0±1.9 (8.4±7.3)	>100
	(100)	8.7±1.5 (23.1±5.1)	
Indomethacin	(0.3)	9.1±1.1 (20.6±6.2)	1.4±0.2
	(1)	5.6±1.0 (49.1±6.4)**	
	(3)	3.8±0.7 (61.9±5.2)**	

** $p<0.01$: compared with the respective control value.

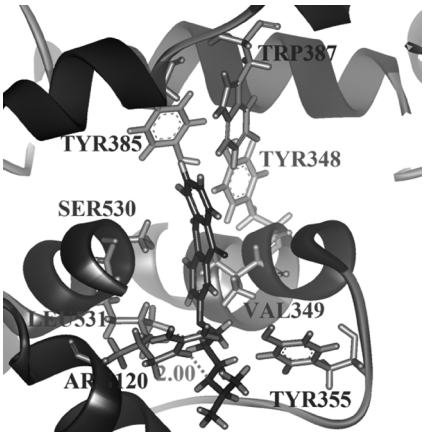

Fig. 4. Compound **8** Was Docked into the Active Site of COX-1 and Showed One Hydrogen Bond Presented as a Broken Line with ARG120 (2.00 Å)

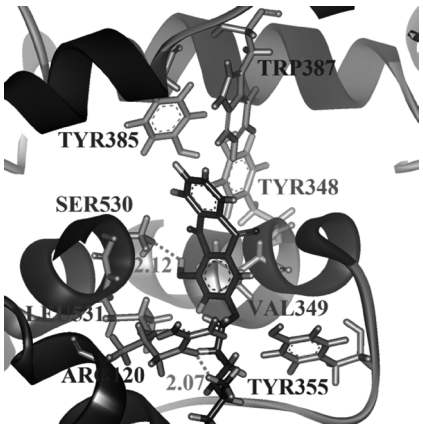

Fig. 5. Compound **10** Was Docked into the Active Site of COX-1 and Exhibited Two Hydrogen Bonds Presented as a Broken Line with ARG120 (2.07 Å) and SER530 (2.12 Å), Respectively

in the arachidonic acid binding channel might be possible. The docking results of **1**, **8**, and **10** can be used as a new pharmacophore for lead generation and optimization of novel antithrombotic and anti-inflammatory agents.

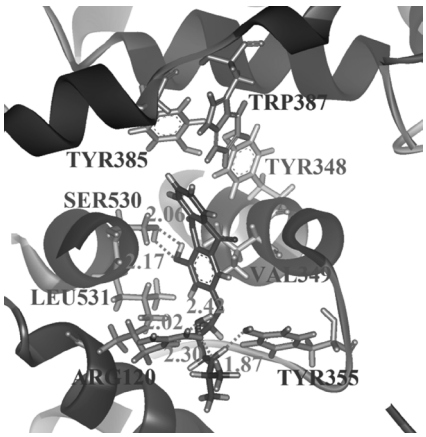

Fig. 6. Compound **1** Was Docked into the Active Site of COX-1 and Revealed Six Hydrogen Bonds Presented as a Broken Line with ARG120 (2.02 Å, 2.30 Å, 2.42 Å), with TYR355 (1.87 Å), and with SER530 (2.06 Å, 2.17 Å), Respectively

Table 5. The Molecular Simulation Results Showed the Dock Scores and Interaction Energies in COX-1

Compound	Dock score	E_b
1	10.95	−95.83
8	20.35	−83.65
10	29.63	−67.61

Dock score: −(ligand/receptor interaction energy+ligand internal energy). E_b: interaction energy, kcal/mol.

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