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RESEARCH ARTICLE

Monosubstituted Coumarins Inhibit Epinephrine-induced Platelet Aggregation

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Abstract: ***Aim*:** The aim of this study was to evaluate the *in vitro* effect of coumarin and 15 monosubstituted derivatives on the inhibition of human platelet aggregation induced by various pro-aggregatory agonists, particularly by epinephrine.

***Background*:** The emergence of residual platelet reactivity during the use of conventional antiplatelet agents (acetylsalicylic acid and clopidogrel) is one of the main causes of double therapy´s therapeutic failure. Platelet adrenoceptors participate in residual platelet reactivity. Therefore, it is necessary to develop new antiplatelet agents that inhibit epinephrine-induced platelet aggregation as a new therapeutic strategy. Information on the antiplatelet activity of coumarins in inhibiting epinephrine-induced aggregation is limited.

***Objective*:** The objective of this study was to establish the structure-activity relationship (SAR) of coumarin derivatives with hydroxy, methoxy, and acetoxy groups in different positions of the coumarin nucleus to identify the most active molecules. Moreover, this study aimed to use *in silico* studies to suggest potential drug targets to which the molecules bind to produce antiplatelet effects.

***Methods*:** The platelet aggregation was performed using a Lumi-aggregometer; the inhibitory activity of 16 compounds were evaluated by inducing the aggregation of human platelets ($250 \times 10^3/\mu l$) with epinephrine (10 μM), collagen (2 μg/ml) or ADP (10 μM). The aggregation of control platelets was considered 100% of the response for each pro-aggregatory agonist.

***Results*:** Eleven molecules inhibited epinephrine-induced aggregation, with 3-acetoxycoumarin and 7-methoxycoumarin being the most active. Only coumarin inhibited collagen-induced platelet aggregation, but no molecule showed activity when using ADP as an inducer.

***Conclusions*:** *In silico* studies suggest that most active molecules might have antagonistic interactions in the α_2 and β_2 adrenoceptors. The antiplatelet actions of these coumarins have the potential to reduce residual platelet reactivity and thus contribute to the development of future treatments for patients who do not respond adequately to conventional agents.

Keywords: Antiplatelet agents, residual coumarin derivatives, epinephrine, molecular docking, platelet reactivity, SAR.

1. INTRODUCTION

The inhibition of platelet activity is an important therapeutic strategy for the prevention of arterial thrombosis, myocardial infarction, and strokes. The administration of acetylsalicylic acid combined with clopidogrel, also known as Dual Antiplatelet Therapy (DAT), reduces the incidence of cardiovascular events both in acute coronary syndrome and after percutaneous coronary intervention with coronary stent implantation [1]. However, during such treatment, a significant number of patients develop residual platelet reactivity, which is one of the main causes of therapeutic failure [2]. It has been suggested that α_2 adrenoceptors participate in residual platelet reactivity, and authors have proposed that their

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inhibition is an important pharmacological target that should be considered for the development of new antiplatelet agents [3, 4]. On the other hand, in recent clinical studies in patients with acute coronary syndrome who received DAT, non-selective β-antagonists reduced residual platelet reactivity compared to those who received selective β_1-antagonists [5, 6]. This evidence shows the importance of developing new antiplatelet agents that inhibit epinephrine-induced aggregation.

Coumarin is a molecule widely distributed in nature, which is consumed in the human diet and has been used in the perfume industry for decades ago [7]. In humans, the tolerable daily intake of coumarin is 0.1 mg/kg of body weight, but it has been seen that in winter, the German population exceeds this value significantly, without presenting evidence of toxicity [8]. However, there are susceptible subpopulations to coumarin that present hepatotoxicity, which is attributed to polymorphic differences of CYP2A6, which is the main enzyme that metabolizes coumarin to 7-hydroxycoumarin in humans [9]. It has been suggested that perhaps some of the biotransformation products of coumarin could be less toxic than coumarin itself or have different biological activities than the parent molecule [10].

Warfarin and dicumarol are the most common drug coumarin derivatives used as oral anticoagulants. These molecules require a hydroxyl group at position 4 and a nonpolar substituent at position 3 of the coumarin nucleus to inhibit vitamin K reductases, which is responsible for their anticoagulant actions [11]. However, the coumarin itself and some of its derivatives lack anticoagulant activity but have antiplatelet actions [12]. Previous studies have evaluated the antiplatelet effects of different coumarin derivatives on aggregation induced by arachidonic acid, collagen, ADP, thrombin, platelet-activating factor, thromboxane A_2 analog U-46619, and calcium ionophore A23187 [13-15]. Although moderate antiaggregant effects of coumarins have been observed in general, it has been reported that 5,7-dihydroxy-4-methylcoumarin inhibits the arachidonic acid pathway with a dual mechanism: 1) inhibiting cicloxigenase-1, and 2) antagonizing the receptor for thromboxane A_2, which could be particularly useful in therapeutics [16]. However, there is limited information on the inhibitory activity of simple coumarins in aggregation induced by other agonists, such as epinephrine.

It has been reported that coumarins can interact with carbonic anhydrase enzyme (CA) as suicide inhibitors [17]. Recently, it has been proposed that inhibition of CAII in platelets could be an additional therapeutic strategy that allows a synergistic effect of epinephrine, increasing the power of thrombin to induce platelet aggregation [18]. Since it is reported that epinephrine stimulates the activity of CAII [19, 20], it can be hypothesized that the direct inhibition of this enzyme by coumarins could perhaps cause inhibitory effects on platelet aggregation induced by epinephrine.

In silico methods are broadly used in drug discovery. Examples of applications are constructing three-dimensional models of molecular receptors (homology modeling) and predicting possible molecule-receptor interactions (molecular docking). One of the main goals of *in silico* approaches is to guide the discussion of experimental results and to generate novel hypotheses about the molecular mechanisms involved in physiological events or pharmacological effects [21].

The goal of the present work was to carry out a structure-activity relationship (SAR) study of coumarin and 15 monosubstituted derivatives with hydroxy, methoxy, and acetoxy groups in different positions of the coumarin scaffold. These molecules represent potential biotransformation products of coumarin. We evaluated the *in vitro* antiplatelet effect of the 16 compounds by inducing the aggregation of human platelets with epinephrine, collagen, and ADP. To assess the possible participation of CAII in the antiplatelet effect, a classic carbonic anhydrase inhibitor (acetazolamide) and a suicide inhibitor (*trans*-2-hydroxycinnamic acid, a product of coumarin hydrolysis) were also evaluated [17].

The tested molecules have greater antiplatelet activity in the epinephrine-induced aggregation. The docking studies have a good correlation with experimental results, showing several antagonistic interactions with the adrenoceptors α_2 (α_2-AR) and β_2- (β_2-AR). These actions could reduce residual platelet reactivity and contribute to the development of future treatments for patients who do not respond to conventional antiplatelet therapy.

2. MATERIALS AND METHODS

2.1. Reagents

Adenosine diphosphate (ADP), collagen, and epinephrine were obtained from Chrono-PAR Corporation (Havertown, PA, USA). Coumarin, 7-hydroxycoumarin, 4-hydroxycoumarin, acetazolamide, *trans*-2-hydroxycinnamic acid, and dimethylsulfoxide (DMSO) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). The other coumarin derivatives were synthesized in the Faculty of Chemistry of the National Autonomous University of Mexico (UNAM), as previously reported [10].

To perform the *in vitro* concentration-response curve of platelet aggregation, the tested compounds were dissolved in DMSO, and from each of these, dilutions were prepared in phosphate buffer (PBS) to reach final concentrations of 50, 100, 200, and 400 μM. The final concentration of DMSO was 0.4% v/v and did not modify the biological response compared to the control samples with PBS.

2.2. *In vitro* Platelet Aggregation

This study was approved by the Ethics Committee of Medicine School at UNAM (Protocol reference number 032-2019), and based on the directives of the Helsinki Declaration, all human volunteers provided informed consent. The blood samples were obtained from healthy normal donors in the blood bank at the National Institute of Cardiology "Ignacio Chávez". All donors had not ingested any alcohol for at least 24 hrs before the tests. Additionally, donors had not taken medication, especially anti-inflammatory analgesics, for at least 2 weeks before the tests.

For each assay, as previously described [22], blood was collected by venipuncture from four male subjects, aged 24-50 years, collected in plastic tubes containing an anticoagulant (0.109 M trisodium citrate). Platelet-rich plasma (PRP) was obtained as a supernatant by centrifugation from collected blood at 140 *g* for 5 min at room temperature (20-24°C). Platelet-poor plasma (PPP) was prepared by centrifugation of the remaining blood at 250 *g* for 20 min at room temperature (20-24°C). The platelet count was adjusted to 250×10^3/µl with platelet-poor plasma (PPP). The assays were carried out within 2 hrs after the blood had been drawn.

The platelet aggregation was performed using a Lumi-aggregometer (Model 560 CA and accompanying software Model 810 AGGRO/ LINK Chrono-log, Havertown, PA, USA). For each measurement, adjusted RPP (500 µl) was incubated for 5 min, at 37°C with 10 µl of PBS as control platelets, with 10 µl of DMSO 0.4% as control-solvent platelets or the corresponding concentration of each compound. Aggregation was induced by the addition of either ADP (final concentration 10 µM), epinephrine (10 µM), or collagen (2 µg/ml). Aggregation was measured as a percentage of light transmission relative to a PPP reference and was recorded for 6 min after the addition of the pro-aggregatory agonists. Data of control platelets were considered 100% of the response. We performed at least six independent experiments.

2.3. Statistical Analysis

All data were analyzed by unpaired one-way ANOVA with Dunnet-corrected post *hoc* analyses. Data are reported as the mean ± standard deviation (SD). Values of $P \leq 0.05$ were considered significant. Statistical calculations were performed using the GraphPad Prism software, version 5.0.

2.4. *In silico* Studies

Based on our results on the inhibitory effect of coumarins on epinephrine-induced platelet aggregation, homology modeling and molecular docking were carried out to explore putative interactions against α_2-AR and β_2-AR of the platelet. This was in order to guide the discussion of experimental results and generate new hypotheses for future works.

2.4.1. Homology Modeling of α_2- AR

Based on Marcinkowska *et al.* [4], the model of α_2-AR (UniProtKB/Swiss-Prot: P08913) was generated using YASARA software v.18.8.9 and as a principal template (reported in Protein Data Bank - PDB ID: 2RH1). Using the PSI-BLAST tool and the protein sequences with the highest reported homology were identified and aligned [23-25]. In a second step, the templates were classified according to the alignment score and structural quality according to the WHAT_CHECK algorithm. The missing loops were completed by *ab initio* calculations and subsequently optimized [26, 27]. The final models generated were qualified with PROCHECK based on the parameters: Root-mean-square deviation (RMSD), percentage of identity with the template, alignment coverage, and normality of the dihedral angles (Supplemental Fig. **S1**). For all tools, the default parameters were used. Finally, the best-qualified model was validated using the Ramachandran plot (Fig. **S1** on the supplementary material), which is the gold standard to validate a homology model since it allows to know exactly how many and which amino acids were not ideally modeled. The Ramachandran plot displays the main chain conformation angles of the protein (φ and Ψ). *i.e.*, is a representation of the tridimensional conformation of the model [28].

2.4.2. Protein and Ligand Preparation

The crystallographic structure of β_2-AR (PDB ID: 3SN6), GPVI (PDB ID: 2gi7), $P2Y_{12}$ (PDB ID: 4NTJ) were obtained from the Protein Data Bank (https://www.rcsb.org/) [29-31]. The hydrogen atoms were added, followed by a minimization step with the AMBER99 forcefield in Molecular Operating Environment (MOE) software (Chemical Computing Group, Montreal, QC, Canada) [32]. For the protein structure, structural waters and ligands were removed. The ligands were built and energy-minimized in MOE using the MMFF94x force field. The most stable protomers at physiological pH were identified [33].

2.4.3. Molecular Docking

Yasara software (v. 18.8.9) was used to add the solvent model and assign the Gasteiger atomic charges to proteins and ligands [34]. The grid was centered on the binding site of the proteins (15 $Å^3$). Using the scoring function of AutoDock Vina, the binding compounds were subjected to 25 search steps, and the default values were used for the other parameters. The clusters with an RMSD < 2 Å were visually explored. During the docking simulations, the receptors were considered rigid and the ligands flexible. The conformations with the lowest binding energy and that also replicated the previously reported key interactions were selected.

3. RESULTS

3.1. Aggregation Inhibition

In epinephrine-induced aggregation, the most active compounds were 3-acetoxycoumarin (IC_{50} = 131 ± 9.9 µM) and 7-methoxycoumarin (IC_{50} = 142 ± 7.9 µM). Only these compounds produced 90% of inhibitions concerning the control, even at the concentration of 200 µM (Fig. **1**). Coumarin, monohydroxycoumarins, 4-methoxycoumarin, 6-methoxycoumarin, and 8-methoxycoumarin had an intermediate inhibitory activity, with IC_{50} values between 222 and 243 µM (Table **1**). 3-Methoxycoumarin and the rest of the acetylated coumarins only produced inhibitions below 50%, therefore the corresponding IC_{50} could not be calculated. Acetazolamide and *trans*-2-hydroxycinnamic acid also showed low activity.

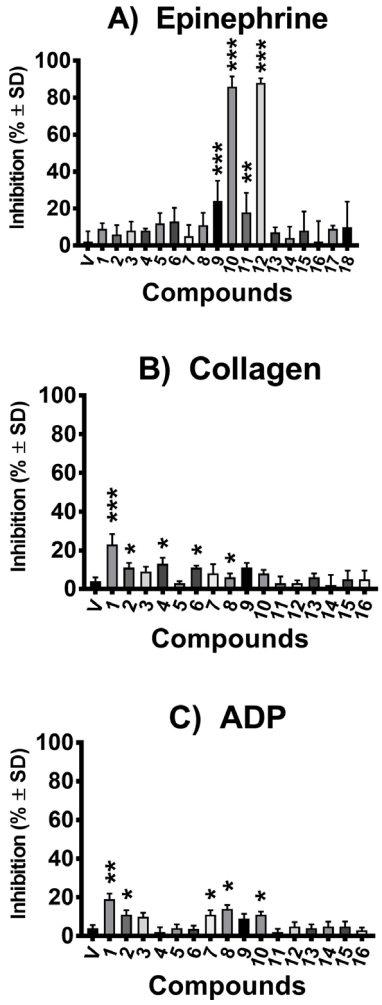

Fig. (1). Coumarin compounds inhibit agonists-induced platelet aggregation. Control platelets were incubated with PBS, solvent-control treated platelets (v = vehicle) were incubated with 0.4% DMSO, and treated platelets were incubated with the correspondent 200 µM of each compound listed in Table **1**. Aggregation was induced with the addition of epinephrine (10 µM), collagen (2 µg / ml), or ADP (10 µM), and the aggregation of controls was considered 100% of the response for each pro-aggregatory agonists. **A**) Epinephrine-induced aggregation inhibition: only 7-methoxycoumarin (10) and 3-acetoxycoumarin (12) produced inhibitions close to 90%. **B**) in collagen-induced aggregation inhibition: coumarin (1) produced a 23% inhibition with the highest significance compared to the vehicle. **C**) in ADP-induced aggregation inhibition: although some compounds produced statistically significant inhibitions compared to the vehicle, the magnitude of the responses was less than 20%. The inhibition percentages of the aggregation of each compound are expressed as the mean value ± SD; (n = 6). *: P <0.05, **: P <0.01, ***: P <0.001. *vs* DMSO - treated group.

In collagen-induced aggregation, coumarin was the only active molecule (IC_{50} = 211 ± 10 µM). At the concentration of 200 µM, coumarin produced a significant inhibition of 20% concerning the control, while all other molecules only produced inhibitions of smaller magnitude and significance.

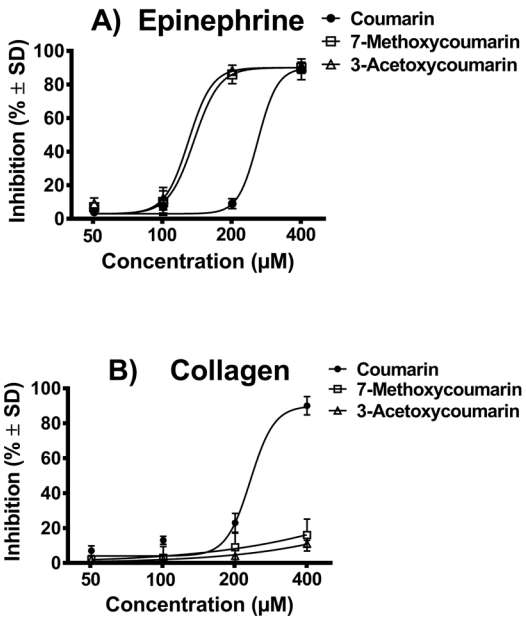

Fig. (2). Concentration-response curves of coumarin, 7-methoxycoumarin, and 3-acetoxycoumarin on the inhibition of agonists-induced platelet aggregation. **A**) Epinephrine-induced aggregation inhibition: 7-methoxycoumarin and 3-acetoxycoumarin were the most potent molecules, which showed an efficiency close to 90% from the concentration of 200 µM, while coumarin was only active at the concentration of 400 µM. **B**) Collagen-induced aggregation inhibition: only coumarin has an inhibitory activity at the highest concentration. The percentages of aggregation inhibition of each point represent the mean value ± SD; (n=6). Concentrations are represented on a logarithmic scale.

However, at the concentration of 400 µM (Fig. **2**), coumarin was the only molecule that reached 90% of efficacy.

Finally, in ADP-induced aggregation, no compound reached a 50% inhibition.

3.2. Molecular Docking

Based on the *in vitro* results, molecular docking was used to explore the binding potential of the study compounds with β_2-AR and α_2-AR. Two parameters were used to approximate the highest affinity: 1) binding score and 2) the number of binding modes by the molecular target.

Docking with β_2-AR showed that the most active compounds have contact with key amino acids VAL 114, PHE 193, and ASN 293 [35]. Docking with α_2-AR revealed that the most active compounds are distinguished by showing interactions with amino acids ASP 113, VAL 114, CYS 117, SER 204, and PHE 391 (Table **2**), although they also interact with other amino acids of the binding site, such as THR 118, SER 200, CYS 201, PHE 390, and TYR 394 (Fig. **3**).

GPVI docking results show that only coumarin has contacts with key amino acid ARG 46 [30], but there are no key interactions against $P2Y_{12}$ receptor [31, 36] with coumarin derivatives (see Supplementary Material).

Table 1. Inhibitory Concentration 50 (IC_{50}) of coumarin compounds on platelet aggregation induced by different agonists.

Inhibitory Concentration 50 (IC_{50} µM)				
Sr. No.	**Evaluated Compound**	**Epinephrine (10 µM)**	**Collagen (2 µg/mL)**	**ADP (10 µM)**
1	Coumarin	237 ± 1.9	211 ± 10	> 400
2	3-Hydroxycoumarin	224 ± 6.7	> 400	> 400
3	4-Hydroxycoumarin	230 ± 3.5	> 400	> 400
4	6-Hydroxycoumarin	236 ± 13	> 400	> 400
5	7-Hydroxycoumarin	243 ± 11	> 400	> 400
6	8-Hydroxycoumarin	222 ± 12	> 400	> 400
7	3-Methoxycoumarin	> 400	> 400	> 400
8	4-Methoxycoumarin	236 ± 4.2	> 400	> 400
9	6-Methoxycoumarin	229 ± 5.0	> 400	> 400
10	7-Methoxycoumarin	142 ± 7.9	> 400	> 400
11	8-Methoxycoumarin	233 ± 2.5	> 400	> 400
12	3-Acetoxycoumarin	131 ± 9.9	> 400	> 400
13	4-Acetoxycoumarin	> 400	> 400	> 400
14	6-Acetoxycoumarin	> 400	> 400	> 400
15	7-Acetoxycoumarin	> 400	> 400	> 400
16	8-Acetoxycoumarin	> 400	> 400	> 400
17	Acetazolamide	> 400	ND	ND
18	*Trans*-2-hydroxycinnamic acid	> 400	ND	ND

Note: The results are expressed as the mean value ± SD (n=6). IC50 was determined by GraphPad Prism software, version 5.0. ND = not determined.

Table 2. Overview of docking results against β_2 and α_2 adrenoreceptor.

Ligands	**β_2 (Å)**	***RMSD**	**Representative Interaction**	**α_2 (Å)**	***RMSD**	**Representative** Interactions**
Coumarin	-7.29	0.97	SER203	-6.85	1.21	------
3-Hydroxycoumarin	-7.83	1.89	VAL114	-6.76	1.72	VAL144/SER204
4-Hydroxycoumarin	-7.53	1.91	VAL114	-6.80	1.84	VAL144
6-Hydroxycoumarin	-7.54	1.69	SER207	-6.92	1.30	---
7-Hydroxycoumarin	-7.49	1.78	VAL114/ SER203	-7.07	1.95	---
8-Hydroxycoumarin	-7.54	1.92	VAL114	-7.29	1.73	VAL144
3-Methoxycoumarin	-7.66	1.55	VAL114	-6.83	1.23	VAL114
4-Methoxycoumarin	-7.45	1.47	---	-7.16	1.42	VAL114
6-Methoxycoumarin	-7.57	1.63	PHE193/SER203	-7.16	1.35	-----
7-Methoxycoumarin VAL114/CYS117	**-7.66**	**1.57**	**VAL114/PHE193/ASN293**	**-7.25**	**1.47**	**ASP113**
8-Methoxycoumarin	-7.02	1.66	---	-6.83	1.53	VAL114
3-Acetoxycoumarin VAL114/SER204	**-8.18**	1.49	**VAL114/PHE193/ASN293**	**-7.34**	**1.78**	-----
4-Acetoxycoumarin	-8.78	1.56	VAL114/PHE193/ASN293	-7.43	1.65	-----
6-Acetoxycoumarin	-8.08	1.64	PHE193/SER203	-7.68	1.62	------
7-Acetoxycoumarin	-7.87	1.57	PHE193	-7.46	1.57	VAL114
8-Acetoxycoumarin	-7.67	1.61	---	-6.88	1.43	VAL114
Trans-2-hydroxy	-7.93	3.41	VAL14/VAL117/SER203/SER207	-691	2.73	VAL114
cinnamic acid	----	----	PHE290/ASN293	----	----	----
***BI-167107	8.58	1.87	ASP113/SER203/SER207	----	----	----

Note: *Calculated values of the selected binding cluster; **Interactions computed with the PLIF algorithm (with MOE software); *** Ligands co-crystalized.

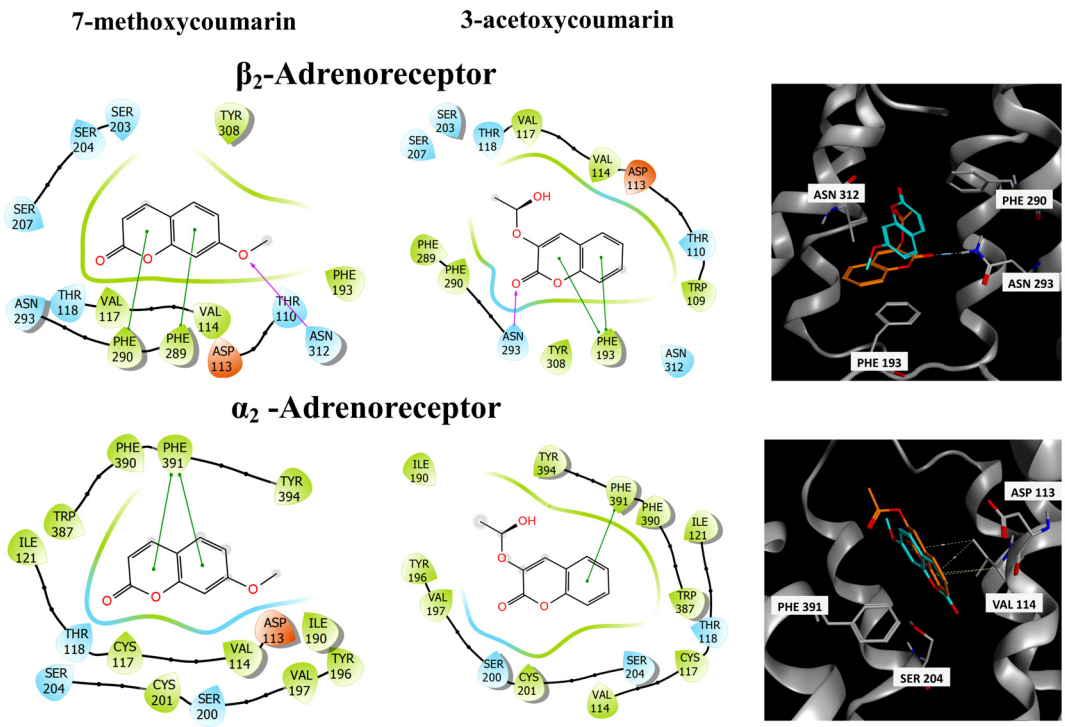

Fig. (3). Representative binding models of 7-methoxycoumarin and 3-acetoxycoumarin with the β_2 and α_2 adrenoreceptors, respectively. Binding score β_2: 7-methoxycoumarin (-7.66) and 3-acetoxycoumarin (-8.18). Binding score α_2: 7-methoxycoumarin (-7.25) and 3-acetoxycoumarin (-7.34). Representative poses were analyzed using Desmond software (Schrödinger) *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

4. DISCUSSION

Although the actions of catecholamines have been studied extensively by multiple research groups, the participation of different adrenoreceptors in the regulation of platelet aggregation remains somewhat unclear. In platelets, there is a higher proportion of α_{2A}-AR and α_{2B}-AR coupled to G_i proteins, but also a small amount of β_2-AR coupled to G_S [3, 37].

Different signaling pathways that increase intra-platelet calcium $[Ca^{2+}]_{ip}$ favor aggregation, while the cAMP and PKA pathways act as antiplatelet signals by decreasing levels of $[Ca^{2+}]_{ip}$ [38, 39]. Thereby, the activation of receptors coupled to G_i proteins contributes to decreasing cAMP signals and favors the elevation of $[Ca^{2+}]_{ip}$.

The aggregating effect of catecholamines is frequently explained through the activation of α_{2A}-AR and α_{2B}-AR. However, epinephrine has a greater affinity for β_2-AR, and the actions mediated by this receptor cannot be considered insignificant since, *ex vivo*, β-antagonists induce a slight but significant reduction in the intraplatelet cAMP content [38]. These data are consistent with recent clinical studies where it has been observed that non-selective β-antagonists, such as carvedilol, decrease residual peripheral reactivity in patients receiving double therapy [6].

Other groups have proposed antagonism of α_{2B}-AR as an alternative strategy for the development of new antiplatelet agents [3]. Recently, α_{2B}-adrenergic antagonists have been synthesized for antiplatelet purposes [4], confirming the importance of our study in identifying molecules that inhibit epinephrine-induced platelet aggregation. Our molecular docking results suggest that coumarin derivatives with higher activity might have antagonistic interactions with α_2-AR but might also have a greater affinity for β_2-AR.

It has been reported that coumarin, 7-methoxycoumarin, and *trans*-2-hydroxycinnamic acid are effective suicide inhibitors of CAII [17]. The activity of CAII is crucial for platelet aggregation since this enzyme catalyzes the reversible hydration of carbon dioxide to a bicarbonate anion and a proton, which regulates the exchange of ions across the membrane [18]. Under experimental conditions, activation of AR-α_2 with epinephrine has been observed to increase the entry of Cl^- to the platelet, while inhibition of CAII with acetazolamide and chlorthalidone reduces platelet aggregation and transport of Cl^- unmodified cAMP levels [19, 20]. However, in our aggregometry experiments, acetazolamide and trans-2-hydroxycinnamic acid only produced non-significant inhibitions by inducing epinephrine aggregation, indicating that the participation of CAII in the *in vitro* antiplatelet effect of coumarins is limited.

On the other hand, when the collagen activates its GPVI and GPIa/IIa receptors ($\alpha_2\beta_1$ integrin), $[Ca^{2+}]_{ip}$ is increased, which induces the release of intra-platelet granules and the formation of TXA_2, ultimately promoting the sustained acti-

vation of GPIIb/IIIa [40]. The activation of GPIIb/IIIa is a fundamental event that allows fibrinogen binding, which is essential for the formation of bridges between platelets and subsequent platelet aggregation. Zaragozá *et al.* [12] evaluated the antiaggregant activity of several flavonoids and coumarins in response to the induction of a calcium ionophore. This group reported that the magnitude of the response of coumarin and 6,7-dihydroxycoumarin (25.75 and 53.83% respectively) correlated directly with their ability to bind GPIb/IIIa.

Although our experimental results are not comparable with those of these authors, our data shows that coumarin does inhibit collagen-induced platelet aggregation, and perhaps the addition of two or more functional groups to the benzopyrone structure could increase the potency and efficacy of the molecule to inhibiting this pathway.

CONCLUSION

With our SAR study, we have concluded that a total of 11 coumarin derivatives inhibited epinephrine-induced aggregation. Docking simulations suggested that the most active molecules, 3-acetoxycoumarin and 7-methoxycoumarin, have antagonistic interactions with α_2-adrenoceptor (through ASP 113, VAL 114, CYS 117, and SER 204) and with β_2-adrenoceptor (through VAL 114, PHE 193, and ASN 293). Additionally, docking studies suggest a greater affinity of coumarins for β_2-AR than for α_2-AR. Only coumarin inhibited collagen-induced aggregation, which is consistent with a unique interaction against GPVI in ARG 46. The lack of activity of the coumarin derivatives in ADP-induced aggregation is probably due to the fact that there is no interaction between the tested molecules with the $P2Y_{12}$ receptor, as suggested by docking studies. CA inhibitors showed only low activity, indicating low participation of CAII in the *in vitro* antiaggregant effect of coumarin derivatives. The inhibition of the epinephrine-induced aggregation could reduce residual platelet reactivity and contribute to the treatment of patients who do not respond to conventional antiplatelet therapy.

However, it is necessary to evaluate other coumarins' activity with two or more substitutions to identify more potent molecules that inhibit the aggregation and establish a better SAR. In this sense, one of the perspectives of this work is to use the molecular docking predictions to generate massive virtual screens with the idea of identifying novel antiaggregant agents of the epinephrine pathway [41].

LIST OF ABBREVIATIONS

ADP	=	Adenosine-Diphosphate
CA	=	Carbonic Anhydrase
DAT	=	Dual Antiplatelet Therapy
PRP	=	Platelet Rich Plasma
SAR	=	Structure-Activity Relationship
α2-AR	=	α2-Adrenoceptor
β2-AR	=	β2-Adrenoceptor

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Ethics Committee of Medicine School of the National Autonomous University of Mexico, of México City (Protocol reference number 032-2019).

HUMAN AND ANIMAL RIGHTS

No Animals were used in this study. All the human procedures were is in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2013 (http://ethics.iit.edu/ecodes/node/3931).

CONSENT FOR PUBLICATION

All human volunteers provided informed consent. The blood samples were obtained from healthy normal donors in the blood bank of the Nacional Institute of Cardiology Ignacio Chávez.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analyzed during the current study are available from the corresponding author, Dr. Orozco, on reasonable request.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIALS

Supplementary material is available on the publisher's website along with the published article.

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