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# Thromboxane $A_2$ Receptor Antagonism by Flavonoids: Structure−Activity Relationships

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Thromboxane $A_2$ ($TxA_2$) is a strong platelet agonist involved in the pathogenesis of thrombotic diseases that elicits platelet aggregation and vasoconstriction through the activation of its specific membrane receptor (TP). Previous studies have demonstrated that certain flavonoids, naturally occurring phytochemicals, inhibit platelet function through several mechanisms, including antagonism of TP in these cells. However, the steric and inductive or mesomeric requirements underlying this effect are not fully understood. In this study, the ability of 20 naturally occurring flavonoids belonging to different structural subtypes to inhibit [$^3$H]-SQ29548 binding to platelet-rich plasma was compared to establish the structural basis explaining their TP antagonistic activity. The results show a key contribution of C7 and C8 carbons in the A ring, $\gamma$-pyrone structure conjugated with a double bond between C2 and C3 carbons in the C ring, and C2′, C3′, and C4′ carbons in the B ring as the structural determinants that create the active flavonoid skeleton in TP blockade. These data might help in the design of new TP antagonists with potential antithrombotic effects and provide additional evidence for the correlation between biological properties of flavonoids and their structure.

**KEYWORDS: Flavonoids; $TxA_2$ receptor; antagonism; platelets; SAR**

## INTRODUCTION

Thromboxane $A_2$ ($TxA_2$) is a labile prostanoid synthesized from arachidonic acid through the sequential actions of cyclooxygenase (COX) and $TxA_2$ synthase enzymes. This lipid mediator elicits a variety of important vascular responses such as platelet activation and vascular smooth muscle contraction (*1, 2*). $TxA_2$ has proved to be a key platelet agonist crucial in the amplification and maintenance of the platelet response and in the recruitment of new platelets to the growing thrombus (*3, 4*). High plasma levels of this metabolite have been implicated in the pathogenesis of thrombotic diseases, including myocardial infarction, unstable angina, pulmonary embolism, and atherosclerosis (*5*−*8*). Indeed, as established by current clinical guidelines, blockade of $TxA_2$ synthesis through COX inhibition by aspirin represents a valuable approach for managing high cardiovascular risk patients (*9*−*11*).

However, the use of aspirin is not exempt from problems. First, its continuous use may produce several undesirable side effects including an increasing risk of gastrointestinal bleeding and hemorrhagic stroke and inhibition of the synthesis of prostacyclin, a major product of COX-2-dependent metabolism of arachidonic acid in the endothelium that inhibits platelet function by increasing intracellular cAMP levels. The reduction in prostacyclin synthesis associated with the use of selective COX-2 inhibitors has been reported to increase the risk of myocardial infarction by 2-fold in patients at low risk for atherothrombosis (*5*). Second, a significant proportion of patients behave as aspirin nonresponders. This aspirin resistance, defined as the inability of aspirin to reduce platelet $TxA_2$ synthesis and to inhibit platelet activation, has been correlated with an increasing risk of recurrent cardiovascular events and represents an emerging clinical problem. Third, even in the absence of resistance, aspirin is unable to inhibit the formation of $F_2$-isoprostanes, which can partially activate $TxA_2$ signaling pathway by anchoring its specific membrane receptor, contributing to an increased endothelial and platelet activation and enhanced vasoconstriction (*5, 10, 12*). Given all of these facts, the development of new $TxA_2$ receptor antagonists capable of inhibiting the effects of both $TxA_2$ and isoprostanes would be of great clinical interest.

$TxA_2$ receptor (TP) is a member of the G-protein coupled receptors superfamily coupled to $G_q$ and $G_{12/13}$ protein activating pathways. Despite much effort, TP has not yet been crystallized, and the actual tertiary structure is still unavailable, making the development of antagonists an arduous task. Nevertheless,

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during the past few years several groups have examined the structure of TP extracellular domains in solution using NMR spectroscopy, site-specific antibodies, and mutational analysis, providing valuable data for the understanding of the ligand binding mechanisms. These studies have implicated TM3 and TM7 transmembrane domains as part of the ligand-binding pocket and EL2 and EL3 extracellular loops as potential regions for ligand coordination (*2, 13*−*15*).

A wide variety of plant-derived polyphenolic flavonoids are present in the human diet. Increasing attention has focused on these compounds due to their biological activities and low toxicity, which make them potential therapeutic agents of great interest (*16, 17*). In previous studies, we demonstrated that certain flavonoids behave as selective TP antagonists in the micromolar range in platelets and other cells (*18, 19*). We showed that, despite flavonoids being 30-fold less effective in competing for TP binding in the presence of plasma proteins compared with a washed platelet system, antagonism of TPs by flavonoids remains a feasible mechanism of platelet inhibition under physiological conditions (*18, 20*).

All flavonoid compounds contain a common benzo-$\gamma$-pyrone structure: two benzene rings (A and B) linked through a pyrone ring (C). They are divided into various subtypes, depending on their molecular substituents, as flavones, isoflavones, flavonols, flavanones, and flavan-3-ols, which can in turn also occur as glycoside forms (*21*).

Numerous studies have revealed the crucial role that the structure of flavonoids plays in their biological function. Thus, the number and position of substituents in the flavonoid basic structure extremely affects the antiproliferative, cytotoxic, antioxidant, and antienzymatic activities of such molecules (*22*−*27*).

We previously reported the presence of a double bond in C2═C3 and a keto group in C4 as important structural features for the antagonistic activity of flavonoids (*18*), but to date the relative importance of the different molecular substituents has not been assessed in depth. In this work we aimed to define the key flavonoid structural demands needed to exert such TP antagonism by measuring their ability to compete with the $TxA_2$ receptor antagonist, SQ29548, in platelet-rich plasma.

## MATERIALS AND METHODS

**Materials.** We selected a wide range of flavonoids to include representative compounds differing in presumed key chemical substituents of the molecule (**Figure 1**). All of the flavonoids used in this study were kindly provided by Nutrafur-Furfural Español S.A. (Murcia, Spain) except for genistein, which was from Sigma-Aldrich Química (Madrid, Spain). Flavonoids were solubilized in dimethyl sulfoxide (DMSO) and stored frozen until use. In all experiments, the final concentration of DMSO was <0.4%.

The TP agonist U46619 was from Calbiochem-Novabiochem AG (Lucerne, Switzerland). The TP agonist I-BOP and the stable synthetic TP antagonist SQ29548 were obtained from Cayman Chemical (Ann Arbor, MI). The tritium-labeled form [$^3$H]-SQ29548 (specific activity = 48.2 Ci/mmol) was purchased from Perkin-Elmer (Boston, MA).

**Platelet Preparation.** Human citrated blood samples were obtained by venous puncture from healthy volunteers who gave informed consent for this study, that was approved by the Ethical Committee. Platelet-rich plasma (PRP) was obtained by centrifugation at 150*g* for 12 min at room temperature and transferred into a separate tube. Platelet-poor plasma (PPP) was prepared by further centrifugation at 1000*g* for 10 min and used to adjust the PRP count.

**[$^3$H]-SQ29548 Binding Assays.** Binding experiments of [$^3$H]-SQ29548 to platelets were carried out essentially as described previously (*20*). Briefly, citrated platelet-rich plasma (PRP) adjusted to a final count of $150 \times 10^9$/L was incubated at room temperature with 5 nM [$^3$H]-SQ29548 in the absence or presence of a fixed concentration of flavonoid (250 $\mu$M) in a final volume of 250 $\mu$L of SQ buffer (10 mM Tris-HCl, 120 mM NaCl, 5 mM D-dextrose, 0.8 $\mu$M indomethacin, pH 7.4) for 30 min. Nonspecific binding was measured in the presence of 10 $\mu$M SQ29548. Platelet-bound ligand was separated by filtration through glass fiber filters (Millipore, Bedford, MA) under vacuum. Filters were subsequently rinsed with ice-cold SQ buffer and transferred to vials containing 5 mL of scintillation fluid. Radioactivity was measured in a liquid scintillation counter (Wallac, Turku, Finland) for 1 min. Results are reported as the percentage of [$^3$H]-SQ29548 specific binding inhibition, considering that 100% of inhibition was achieved in the presence of 10 $\mu$M unlabeled SQ29548. Results are expressed as mean ± SD from three experiments performed in different platelet samples.

## RESULTS AND DISCUSSION

We assessed the inhibitory effects of a panel of 20 flavonoids (250 $\mu$M) from different structural types on the specific binding of [$^3$H]-SQ29548 to human PRP. The structures of all the compounds studied are represented in **Figure 1**.

As shown in **Figure 2**, the order of the inhibitory potencies was apigenin = genistein > scutellarein > naringenin > chrysin > luteolin = catechin. The rest of the flavonoids exhibited minor inhibitory activities, except for the compounds trimethylflavone, neohesperidin dihydrochalcone (NHDC), and the phloroacetophenone (biosynthesis precursor), which did not show any inhibitory effect.

We confirmed that the TP agonists U46619 and I-BOP, but not the structurally related compound $PGE_1$, were able to displace [$^3$H]-SQ29548 binding almost completely at a concentration of 10 $\mu$M (data not shown).

The comparison among the abilities of the different flavonoids tested to compete with $TxA_2$ for binding to TP confirms that the most important structural features for their antagonistic activity, which all together would shape a global structure that fits the receptor, are (a) C7 and C8 carbons in the A ring; (b) $\gamma$-pyrone structure conjugated with a double bond between C2 and C3 carbons in the C ring; and (c) C2′, C3′, and C4′ carbons in the B ring. The affinity of the flavonoid skeleton for TP is also regulated by the presence of free hydroxyl groups in positions 7 and 4′ in the A and B rings, respectively.

The flavone apigenin and the isoflavone genistein, due to the presence of such a combination of structural features, are the flavonoids that show the highest TP antagonism among all of the compounds tested (**Figure 3A**). The comparison of certain elements of the $TxA_2$ molecule (i.e., the heterocyclic ring conjugated with a double bond and the adjacent hydroxyl group) and the $\gamma$-pyrone side of the A and C conjugated rings of apigenin or genistein reveals a close structural relationship, similar to an image and its specular reflection, that might justify the high TP antagonistic activity displayed by apigenin and genistein (**Figure 3B**).

As shown in **Figure 4**, the presence or absence of additional substituents in the flavonoid active core leads to three different types of structural consequences, which significantly affect the affinity for TP, as follows:

(1) Steric impediments to receptor binding may occur due to inclusion of new elements that increase the molecular volume. The addition in apigenin of a hydroxyl radical in position 3′ reduces its specific antagonistic activity by up to 52%, whereas the methylation of 7 and 4′ hydroxyl groups induces a 67% decrease. However, the most potent negative steric effect is glycosylation in position 7, as addition of a glucose−rhamnose dimer almost completely abrogates the antagonistic activity (92% reduction).

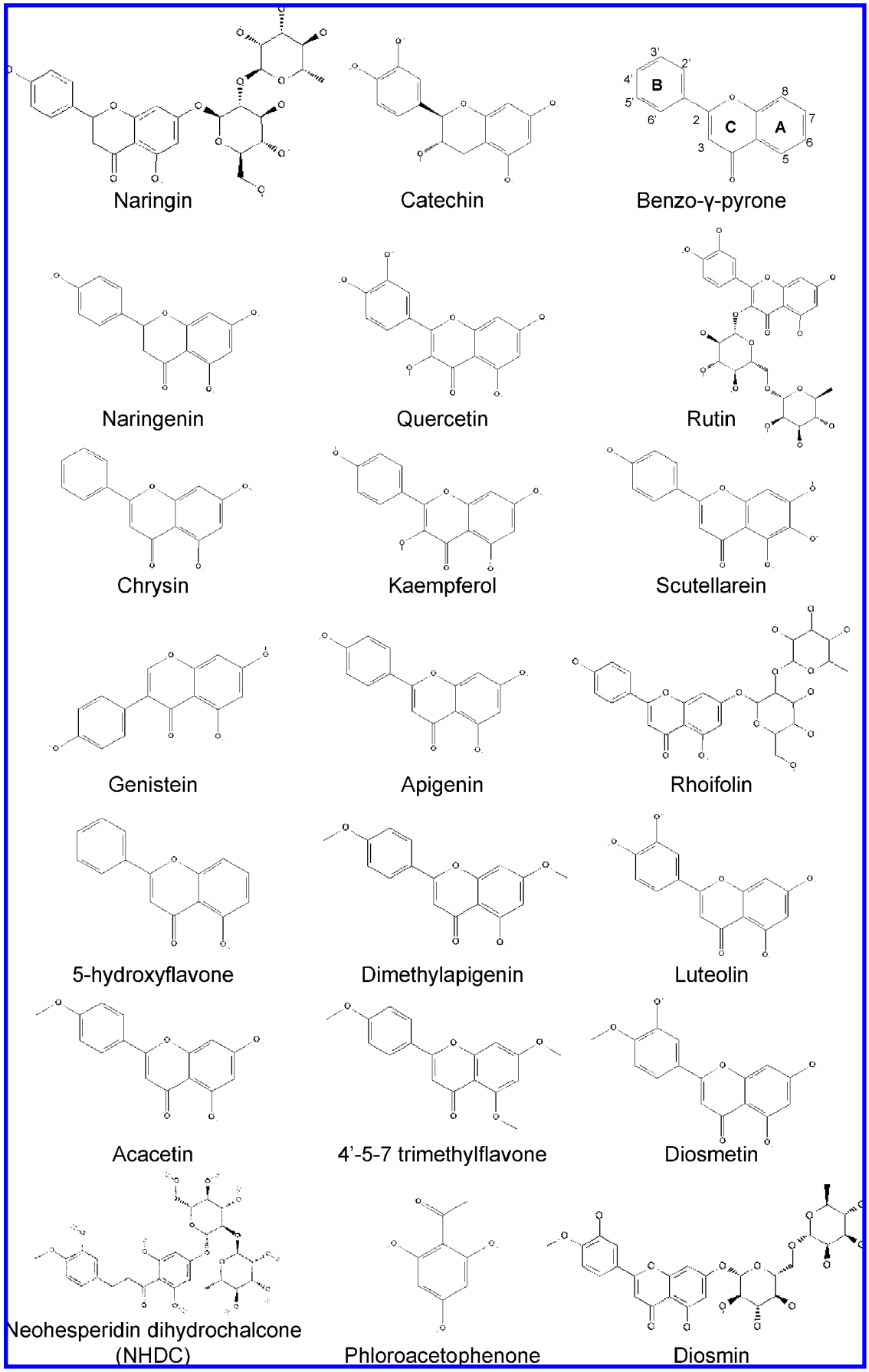


**Figure 1.** Structures of the different flavonoids used in this study.

(2) Disruption of the flavonoid anchor structure may occur due to removal of essential substituents. Indeed, the absence of the free hydroxyl group in the C4′ carbon (chrysin) diminishes the affinity for TP up to 40%. The antagonistic activity of

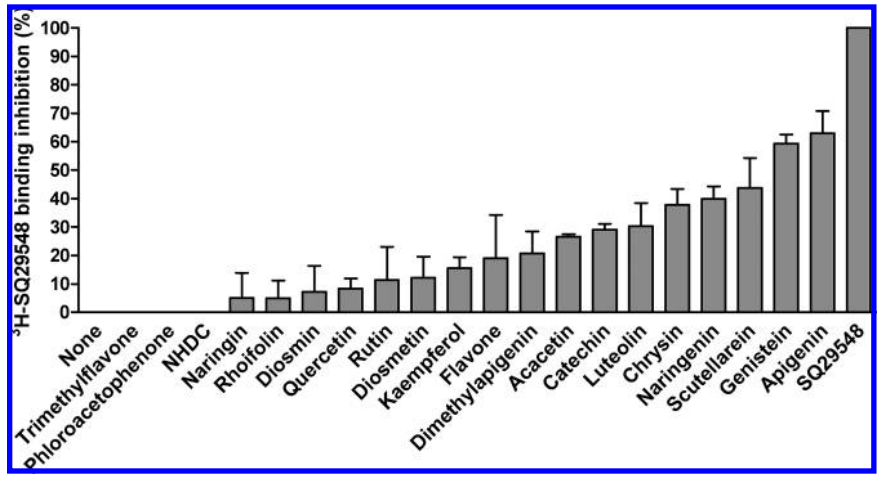


**Figure 2.** Effect of flavonoids on [$^3$H]-SQ29548 binding to platelet-rich plasma. Platelets ($150 \times 10^9$/L) were incubated with 5 nM [$^3$H]-SQ29548 in the absence or presence of different flavonoids (250 $\mu$M) or 0.4% DMSO as control for 30 min. Results are expressed as the percentage of [$^3$H]-SQ29548 specific inhibition, considering that 100% of inhibition was achieved in the presence of 10 $\mu$M unlabeled SQ29548. The plot represents mean $\pm$ SD from three experiments.

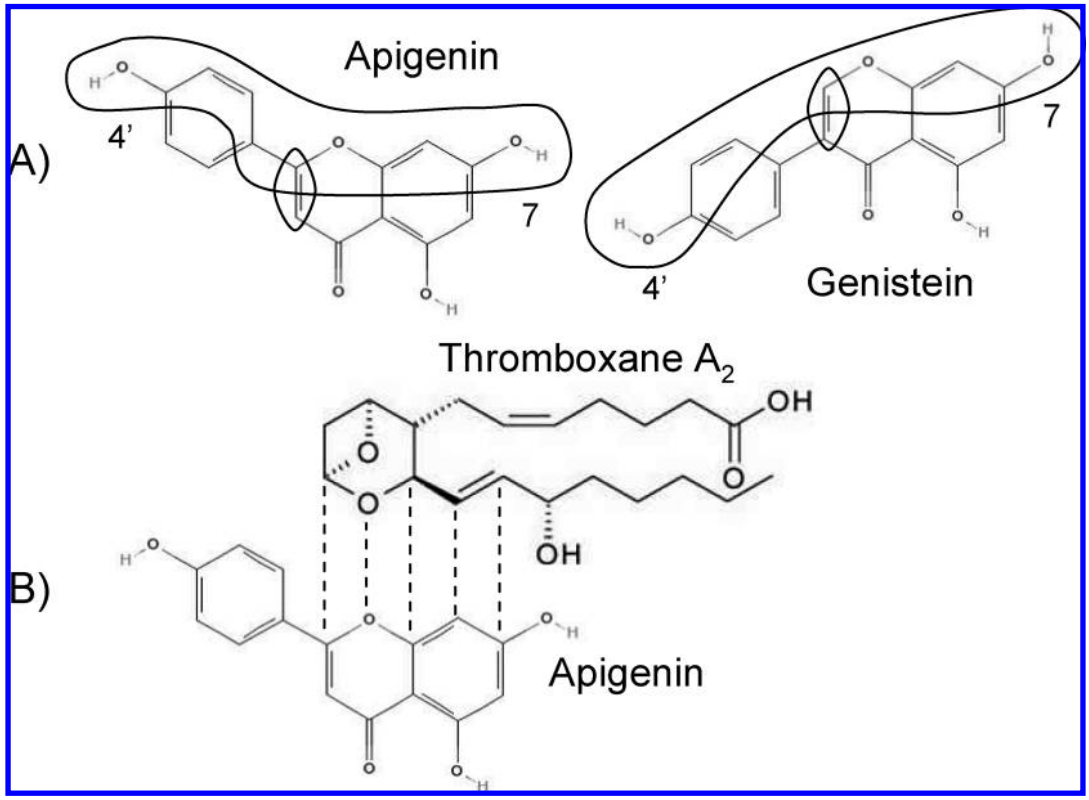


**Figure 3.** Structural features explaining the TP antagonistic activity of flavonoids: (**A**) flavonoid active core believed to interact with the thromboxane $A_2$ receptor; (**B**) specular relationship between certain elements within the structure of thromboxane $A_2$ (the heterocyclic ring conjugated with a double bond and the adjacent hydroxyl group) and apigenin ($\gamma$-pyrone side of the A and C conjugated rings).

5-hydroxyflavone, which lacks C4′ and C7 free hydroxyl groups, is reduced by 70% compared to apigenin.

(3) Inductive or mesomeric modifications may occur owing to the inclusion or absence of elements that alter the electronic disposition. Several structural substitutions alter the electronic distribution of the flavonoid skeleton, such as the transformation of flavone into flavonol by the addition of a hydroxyl group in position 3 of the C ring, which causes a 75% reduction in activity, or the inclusion of a hydroxyl radical in position 6, which produces a 30% decrease. The suppression of the C2═C3 double bond in the C ring, which modifies the electronic distribution and planarity of the flavone core, reduces its activity by up to 37%.

TP antagonism has proven to be a feasible strategy to treat pathological conditions such as asthma and allergic reactions. Indeed, seratrodast is a TP antagonist used for the treatment of asthma in Japan, whereas ramatroban, with antagonistic activities against TP and prostaglandin D2 receptors, is prescribed in the case of allergic conditions (*1*).

Additionally, TP antagonism is a promising new management strategy in antithrombotic therapy (*28*). Thus, picotamide, a TP antagonist, is an alternative to aspirin in secondary prophylaxis for patients suffering from peripheral arterial disease in the case of aspirin resistance or gastrointestinal intolerance (*29*). The potential value of TP antagonists as antithrombotic drugs has led to the development of new synthetic TP inhibitors such as

| Flavonoid | Systematic name | Reference | Group differing from apigenin structure | % Inhibition of [$^3$H]-SQ29548 binding at 250μM vs. 10μM SQ29548 (100%) | Reduction in activity vs. apigenin, % | Structural consequence |
|---|---|---|---|---|---|---|
| Apigenin | 4',5,7-trihydroxyflavone | | | 63% | 0% | |
| Scutellarein | 4',5,6,7-tetrahydroxyflavone | a | + 6-OH | 44% | - 30% | Inductive-mesomeric modification |
| Naringenin | 4',5,7-dihydroxyflavanone | b | - C2 = C3 | 40% | - 37% | Inductive-mesomeric modification |
| Chrysin | 5,7-dihydroxyflavone | c | - 4'-OH | 38% | - 40% | Disruption of the anchor structure |
| Luteolin | 3',4',5,7-tetrahydroxyflavone | d | + 3'-OH | 30% | - 52% | Steric impediment |
| Acacetin | 5,7-dihydroxy-4'-methoxyflavone | e | + 4'-$CH_3$ | 27% | - 58% | Steric impediment |
| Dimethyl apigenin | 5-hydroxy-4',7-dimethoxyflavone | e+f | + 4',7-$CH_3$ | 21% | - 67% | Steric impediment |
| Flavone | 5-hydroxyflavone | c+g | - 4',7-OH | 19% | - 70% | Disruption of the anchor structure |
| Kaempferol | 4',5,7-trihydroxyflavonol | h | + 3-OH | 15% | - 75% | Inductive-mesomeric modification |
| Rhoifolin | 4',5-dihydroxyflavone-7-rhamnoglycoside | i | + 7-glycoside | 5% | - 92% | Steric impediment |

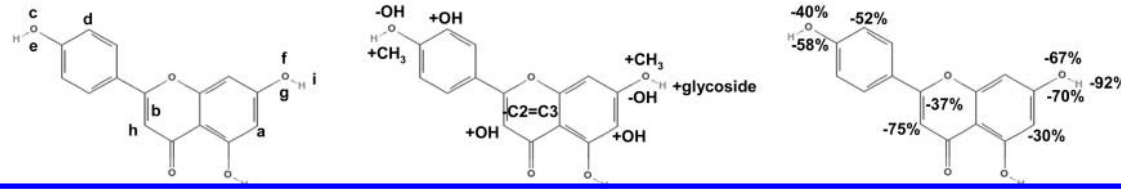


**Figure 4.** Structural characteristics and inhibitory potency of tested flavonoids with a significant TP antagonistic activity considering the effect of the addition or elimination of different structural elements from the flavonoid core on the TP antagonistic activity of apigenin.

S18886-terutroban or a series of *N*-alkyl-*N*′-[2-(aryloxy)-5-nitrobenzenesulfonyl]urea compounds (*7*, *8*). S18886 was able to improve endothelial function in patients with coronary artery disease (*30*), has been shown to be effective in animal models of thrombosis, atherosclerosis, and diabetic nephropathy (*31*, *32*), and is currently undergoing phase III development for the secondary prevention of acute thrombotic complications of atherosclerosis (*33*).

In platelets, certain flavonoids, especially apigenin and genistein, have been shown to abrogate a variety of responses dependent on $TxA_2$, mainly through the blockade of TP (*18*, *19*). Furthermore, our previous data demonstrated that platelets exposed in vivo to subinhibiting concentrations of aspirin displayed a fully abrogated $TxA_2$-dependent response when incubated with low apigenin concentrations (*20*). The increase in the ex vivo antiplatelet effect of aspirin in the presence of apigenin supports the therapeutic use of aspirin in combination with apigenin or other active flavonoid, which would prevent the activation of platelets due to $F_2$-isoprostanes or remaining $TxA_2$ molecules in the case of incomplete COX inhibition. Additionally, a combination of a TP antagonist such as apigenin with a COX-1 inhibitor may theoretically reach a cardioprotective level similar to that of aspirin while preserving prostacyclin formation.

Here, by showing that changes in the flavonoid active core affect its binding affinity to the G-protein coupled receptor TP, in a similar way to that described for adenosine receptors (*34*), we provide an additional example of flavonoid structure−activity relationships and establish the structural features needed for the TP inhibitory activity of flavonoids. Also, it is clear that only the ingestion of dietary flavonoids, such as apigenin, will be insufficient and, consequently, to obtain the necessary efficacy, we need to consider the use of pure flavonoids in pharmaceutical dosages.

Given that the exact tridimensional structure of the TP receptor is unknown, the analysis of TP receptor blockade by flavonoids can be a useful tool in the design of new and more selective synthetic antagonists, which might be used alone or in combination with low doses of aspirin or other COX inhibitors for the prevention of atherothrombotic episodes.

## ABBREVIATIONS USED

ADP, adenosine triphosphate; ApoE, apolipoprotein E; cAMP, cyclic adenosine monophosphate; COX-1, COX-2, cyclooxygenase 1/2; EL, extracellular loop; I-BOP, [1*S*-[1a,2a(*Z*),3b(1*E*,3*S**),4a]]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; LDL, low-density lipoprotein; NHDC, neohesperidin dihydrochalcone; NMR, nuclear magnetic resonance; $PGE_1$, prostaglandin $E_1$; SQ29548, ([1*S*-[1a,2a(*Z*),3a,4a]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid); TM, transmembrane domain; TP, $TxA_2$ receptor; $TxA_2$, thromboxane A2; U46619, 9,11-dideoxy-9a,11a-methanoepoxy-prosta-5*Z*,13*E*-dien-1-oic acid.

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