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New insights into flavonoid binding to the thromboxane A_2 receptor

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Key Points

- Genistein and tectorigenin inhibit TP receptor via extracellular loop H-bonding; mutation of key residues abolishes inhibition, preserving TxA_2 activation.
- Genistein synergizes with aspirin to inhibit platelet aggregation and thrombus formation, suggesting benefit in aspirin resistance.

Aspirin treatment irreversibly inhibits cyclooxygenase activity, blocking thromboxane A_2 (TxA_2) synthesis and reducing thromboxane-prostanoid (TP) receptor–induced platelet activation. Aspirin resistance refers to the failure of regular aspirin doses to produce the expected antiplatelet effect. Aspirin-induced bleeding complications are frequently observed in patients receiving excessive doses or combined antiplatelet and anticoagulant therapy. Although selective TP receptor antagonists have been tested clinically, they are ineffective due to persistently high prostaglandin levels, causing unexpected signaling effects in platelets and other cell types. Hydroxyflavonoids are bioactive phytonutrients with antithrombotic effects, inhibiting TxA_2-mediated platelet signaling and preventing abnormal platelet activation in cardiovascular diseases without major side effects. Using a recently published TP receptor crystal structure and docking studies with different flavonoid classes, including genistein and tectorigenin, we found that flavonoids occupy similar ligand-binding surface of TxA_2, thereby preventing TxA_2 binding. To evaluate our bioinformatics data, a mutant TP receptor variant was overexpressed in HEK293 cells, deleting interacting amino acids with hydroxyflavonoids, and stimulated with the TxA_2 analog U46619. Intracellular Ca^{2+} responses were measured in Fura-2–labeled cells. Amino acid substitutions in the extracellular domain, which did not influence U46619 binding, abolished hydroxyflavonoid-mediated inhibition of the TP receptor. These results suggest that hydroxyflavonoids directly bind to and inhibit the TP receptor. Using aggregometry and flow chamber assays, we confirmed that genistein and tectorigenin synergize with aspirin to inhibit platelet aggregation and thrombus growth in human and mouse platelets. Whether combined genistein-aspirin therapy improves antiplatelet responses in aspirin-resistant patients requires further investigation.

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Introduction

Thromboxane A_2 (TxA_2) is a potent platelet agonist that regulates hemostasis and thrombosis through its reversible interaction with the thromboxane-prostanoid (TP) receptor. TxA_2 is produced by platelet cyclooxygenase (COX) and thromboxane synthase and released into the circulation, triggering autocrine and paracrine signaling through the TP receptor, which leads to platelet aggregation and induces diverse biological effects in blood and endothelial cells.[1] Studies targeting the first extracellular loop (ECL1) of the TP receptor with antibodies showed unaltered TxA_2 binding and platelet aggregation. However, amino acid substitutions of the ECL2 or the use of blocking antibodies showed moderate or strong inhibitory effects on TxA_2 binding and consequent inhibition of TP receptor–mediated platelet aggregation.[2-5]

Several studies proposed that TxA_2 and TP receptor inhibitors could share and occupy the same ligand-binding pocket located between the ECL1 and ECL2 regions (Figure 1A,B). This binding pocket is stabilized by disulfide bridges located between cysteine 11 (N terminus)/102 (ECL1) and cysteine 105 (ECL1)/183 (ECL2). Mutation of these cysteine residues or modification of the disulfide bridges alters the size and shape of the ligand-binding pocket, thereby strongly inhibiting TP receptor function.[6,7] Therefore, oxidative/reductive processes triggered by reactive oxygen species and enzymatic reactions can dynamically change the disulfide bonds and consequently the shape of the ligand-binding pocket.[8] In addition, a binding site for TxA_2 was identified in a

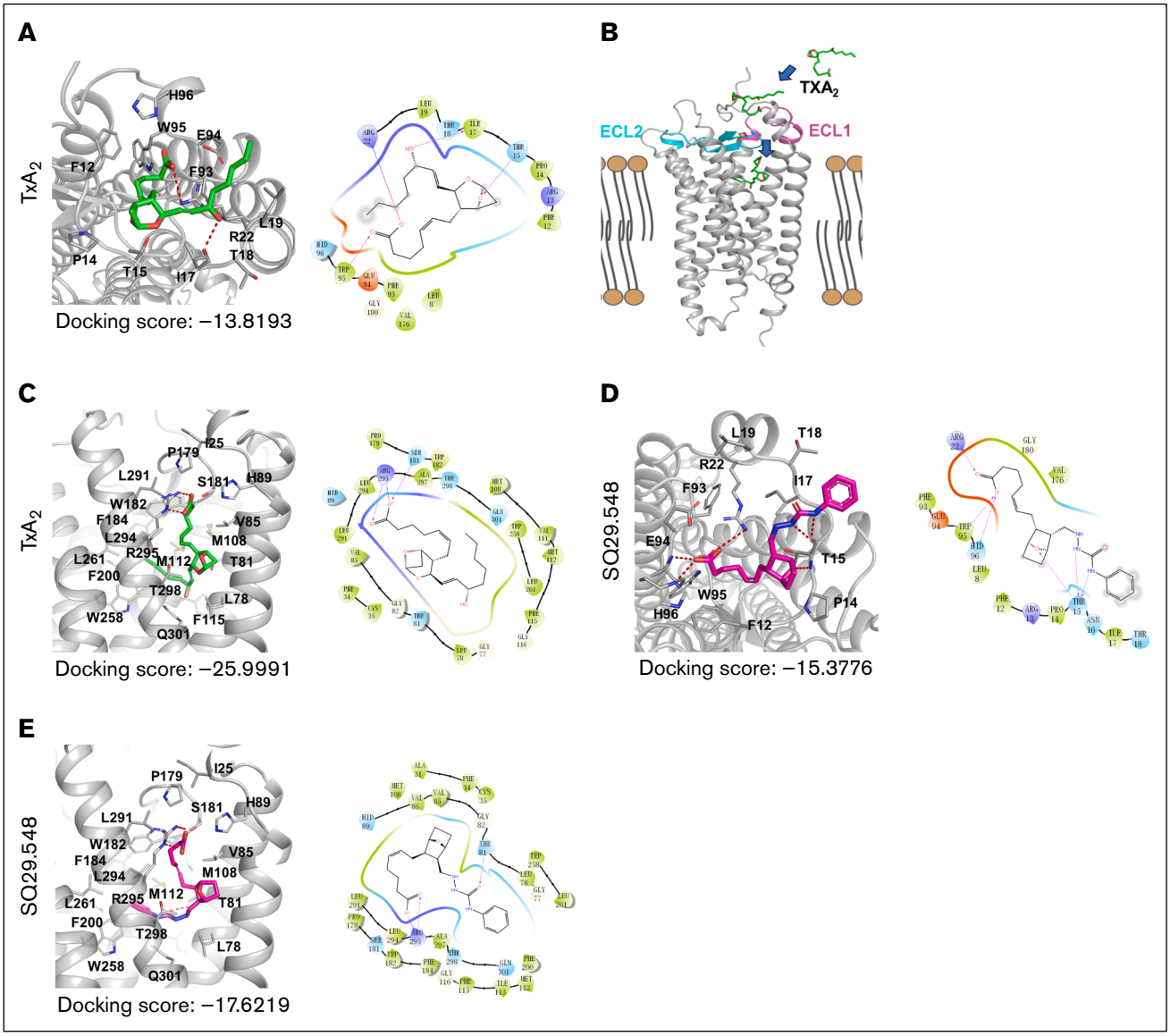


Figure 1. Comprehensive analysis of TxA_2 and SQ29.548 binding in the human TP receptor α. Putative binding sites of TxA_2 and SQ29.548 are visualized in the ECL (A,D) and TM region (C,E). Hydrogen bonds are indicated by arrows. (B) Schematic picture visualizes 2 TxA_2-binding pockets located in the ECL and TM regions. TxA_2 binds the ECL domain first, and moves down to the TM region. Ligand recognition and movement of TxA_2 into ligand-binding and TM-binding pockets are indicated by blue arrows.

lipophilic environment. The TxA_2 binding domain was mapped in the transmembrane domain 7 (TM7),[1] which interacts with the carboxyl group of TxA_2 (Figure 1B,C).

To identify the interacting surface of TxA_2, several amino acid substitutions were created in the TM7 region between L291 and W299, resulting in a complete loss of binding activity.[9] Based on structure-activity relationship results, it has been proposed that the initial binding of TxA_2 to the ligand-binding pocket located between ECL1 and ECL2 triggers a conformational change, leading to the movement of TxA_2 into the TM region, thereby activating downstream signaling pathways of G protein–coupled receptors.[7,8] Over the past decades, several TP receptor antagonists have been developed. However, due to issues with efficacy and cytotoxicity, a few have been used in clinical trials.[10] Ramatroban, initially developed against thrombosis and coronary artery disease, only blocks TxA_2 and prostaglandin D2–induced bronchoconstriction against allergic rhinitis, and is not effective in pathological context of cardiovascular diseases.[11] Another compound, daltroban, was also discontinued in clinical trials (phase 3; BM 13.505, SKF 96148). Interestingly, prostanoid-like ligands (SQ-29.548, U46619), as well as ramatroban and daltroban, bind to the TP receptor using a similar binding pocket. The interacting amino acids (T81, H89, S181, R295, and Q301) have been recently identified.[7] Although many sulfonamide derivatives have selective inhibitory effects on the TP receptor, drug development was terminated due to a lack of effectiveness compared with aspirin.[12] Therefore, another therapeutic strategy needs to be developed for the treatment of aspirin resistance.

Flavonoids are a group of bioactive phytonutrients with antithrombotic effects, isolated from many different fruits, vegetables, and herbs.[13] Flavonoids are well-known inhibitors of TxA_2-mediated signaling in platelets.[14] Although some flavonoids can directly bind and inhibit TP receptor function, others can inhibit COX activity and consequent TxA_2 production,[15] or directly bind TxA_2, enlarging the size of this second wave mediator.[16] Additionally, flavonoids with antioxidant properties can scavenge reactive oxygen species and reduce oxidative stress–mediated signaling.[17] Flavonoids also increase the production of nitric oxide in the vasculature, thereby inhibiting platelet aggregation and thrombus growth.[17] Flavonoids also inhibit different groups of kinases and thiol isomerases.[18] All these results suggest that flavonoids have multiple antithrombotic effects not only in platelets but also in immune and endothelial cells to prevent thromboinflammation and cardiovascular diseases.

Flavonoids have a 15-carbon structure with 2 phenyl rings (A and B) linked by a heterocyclic pyrone ring (C). Several hydroxyl and methoxy groups are linked to the rings at different positions, which can modify their chemical properties and biological activities.[19] Based on structural differences, they are classified as flavones, flavonols, flavanones, flavan-3-ols, and anthocyanins. Synthetic flavonoids have been developed and tested in platelets to enhance their inhibitory effects.[20] Structural and physiological comparisons between different classes of flavonoids showed that hydroxyflavonoids exhibit more potent antithrombotic effects than methoxyflavonoids. Indeed, changing 2 hydroxyl groups at R7 and R4′ to methoxy groups in apigenin strongly reduced the inhibitory potential on arachidonic acid (AA)–induced platelet activation.[21] The change of hydroxyl group of luteolin at R4′ to a methoxy group also inhibited the biological activity. These results indicate that R7 and R4′ hydroxyl groups are involved in the regulation of TxA_2-mediated signaling, probably through inhibition of the TP receptor. In line with this, hydroxylated flavones and isoflavones could strongly inhibit TP receptor function in a reversible and competitive manner.[22,23] Therefore, it was concluded that hydroxyl groups of flavonoids, as hydrogen bond donors, are essential for ligand recognition and inhibitory potential. Furthermore, it was proposed that the double bond in C2-C3 and the keto group in the C4 position on the C ring are important for TP receptor binding.[24] Without this structure, many flavonoids have less or no inhibitory potential on the TP receptor. Interestingly, glycosylated flavonoids with a larger size cannot properly bind and inhibit TP receptor function, due to the limited size of the ligand-binding pocket.[16]

Although several studies have described the structure of the TP receptor ligand-binding pocket, the direct interacting amino acids with different hydroxy and keto groups of flavonoids have not been evaluated. Using the human TP receptor crystal structure,[7] we performed docking studies with different flavonoid groups and identified key interacting residues for genistein and tectorigenin in the N terminus and ECL domain. Overexpression studies with a mutant form of TP receptor in HEK293 cells, or using aggregometry and flow chamber assays, we identified that genistein and tectorigenin directly bind the TP receptor and synergize with aspirin to inhibit platelet aggregation and thrombus growth. These findings propose genistein as a potential candidate to combine with aspirin for targeting TP receptor signaling in patients with aspirin resistance.

Materials and methods

Computational method: structure derivation, preparation, and docking

Ligand structures were prepared using the LigPrep module implemented in the Schrödinger Suite (release 2020-1). Protonation states and ionization forms were generated at a physiological pH 7.0 ± 2.0 using Epik, and low-energy 3-dimensional conformations were obtained. All other parameters were kept at their default settings. The crystal structure of the TP receptor was retrieved from the Protein Data Bank (identity: 6IIU). The protein structure was prepared using the Protein Preparation Wizard, including the assignment of bond orders, addition of hydrogen atoms, optimization of hydrogen-bonding networks, and restrained energy minimization. Missing side chains and loops, if present, were modeled using Prime. To assess cross-species conservation of the ligand-binding site, the predicted structure of the mouse TP receptor (obtained from the AlphaFold Protein Structure Database) was structurally aligned with the human TP receptor (Protein Data Bank identity: 6IIU) using standard protein alignment tools implemented in Schrödinger. Structural alignment analysis was performed to compare the overall fold and binding pocket architecture. Conserved residues within the flavonoid-binding pocket were identified. The potential impact of these substitutions on ligand binding was further evaluated through docking analyses. A receptor grid was generated by centering on the cocrystallized ligand-binding site. The grid box dimensions were set to 30 Å × 30 Å × 30 Å to ensure full coverage of the binding pocket. Default parameters were used for grid generation unless otherwise specified. Molecular docking was performed using Glide (version

8.7) in "standard precision" mode. Ligand sampling was conducted flexibly, and the top-ranked binding poses were retained for further analysis based on GlideScore.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (version 8.0; GraphPad Software, La Jolla, CA). Statistical differences between groups were analyzed using a 1-way analysis of variance followed by the Tukey post hoc test. Data are presented as mean ± standard deviation. P values $< .05$ were considered to be significant (*$P < .05$; **$P < .01$; ***$P < .001$; and ****$P < .0001$).

Results

Structural analysis of flavonoid-binding pocket in the extracellular domain of the TP receptor

A synthetic small molecule SQ29.548 is regularly used in experimental studies to investigate the role of TP receptor–induced signaling in platelets. Hence, it directly blocks the TP receptor, preventing TxA_2 binding in platelets without altering COX activity and TxA_2 production. Using our docking studies, we confirmed the binding sites of both TxA_2 and SQ29.548 within the ECL and TM7 domains of human TP receptor α (Figure 1). As expected, we identified 2 distinct binding pockets in the ECL region and TM7 domains of TP receptor (Figure 1B). In agreement with a previous study, our structural analysis showed that both TxA_2 (Figure 1A,C) and SQ29.548 (Figure 1D-E) bind well-defined ECL and TM7 binding pockets. Interestingly, different classes of flavonoids occupy similar ligand-binding pockets, thereby preventing TxA_2 binding to the TP receptor (Figure 2). Using hydroxyl or keto groups, many flavonoids interact with the N terminus and ECL1 region of TP receptor, creating several hydrogen bonds with common pharmacophores (F12, R22, E94, and W95) critical for TP receptor engagement (Figure 2). The lack of the keto group in flavan-3-ol and anthocyanins (Figure 2E-F) probably decreases the binding affinity to TP receptor. Furthermore, we found that flavonoids lack covalent binding with the ECL2 region; only hydrophobic interactions were observed with amino acids of V^{176}-GYP-G^{180} (Figure 2). Changing the conformation of the ECL1 region with several hydrogen bonds is a unique feature of flavonoids, whereas other ligands (TxA_2, SQ29.548) have been shown to bind to the ECL2 region between V^{176}/L^{187}.[25] Interestingly, we found additional interactions of TxA_2 (T15, I17, R22, and W95; Figure 1A) and SQ29.548 (T15, R22, W95, and H96; Figure 1D) within the N terminus and ECL1 region of TP receptors as well, closely related to the pharmacophores of flavonoids.

Structural analysis of flavonoid-binding pocket in the TM domain of TP receptor

Early studies showed that several TP receptor variants with point mutations at W299R, W299L, R295Q, or L291F, located at the TM7 domain, lost binding affinity to SQ29.548, suggesting the involvement of TM7 in forming a ligand-binding pocket with TxA_2.[9] We observed that both TxA_2 and SQ29.548 forms hydrogen bonds with R295 (Figure 1C,E), validating our docking model and confirming previous results.[26] However, we found additional interacting amino acids at position T81 and S181 for SQ29.548 and TxA_2, respectively. Nuclear magnetic resonance studies previously supported 4 residues (V176, L185, T186, and L187) within the ECL2 region, which are important residues for the ligand recognition of TP receptor.[25] In addition, our results suggest that S181 is also located in this ligand-binding pocket, forming hydrogen bonds and stabilizing TxA_2 binding. Although T81 residue has not been studied in TP receptor, in rat prostaglandin F2α receptor (sharing 30% homology with TP receptor), H81 was identified in the TM2 domain, located in close proximity to R291 (TM7), suggesting the involvement of TM2 in the ligand-binding pocket.[27] On the basis of structural similarity, we predict that T81-mediated hydrogen bonding with SQ29.548 is probably involved in the ligand-binding pocket. Next, we performed docking studies to identify flavonoid-binding sites in the TM7 domain (Figure 3). Interestingly, many flavonoids interact with hydrogen bonds at position T81 and S181, and also with π-cation at position R295. Hydrophobic interactions were also observed with the amino acid residues located in the L291-W299 region. These results suggest that flavonoids bind a similar binding pocket to TxA_2 at the TM7 domain of TP receptor. This conclusion is further supported by studies with ramatroban, building hydrogen bonds with similar amino acid residues (T81, H89, S181, R295, and Q301) in the binding pocket of TP receptor.[28]

Structural analysis of genistein- and tectorigenin-binding pockets in the TP receptor

To experimentally confirm the antiplatelet potential of flavonoids, mouse and human platelets were stimulated with a high dose of TxA_2 analog U46619 (1.5μM) in the presence or absence of tectorigenin and genistein. We preincubated mouse and human platelets with tectorigenin or genistein for only 1 minute to avoid intracellular action of the compounds. As expected, both flavonoids could inhibit the aggregation response in a dose-dependent manner in mouse (Figure 4A-D) and human platelets (Figure 4E-H). These results support that both flavonoids have antiplatelet effects, blocking TP receptor function on the platelet surface. Of note, we found that tectorigenin strongly inhibits U46619-induced human platelet aggregation (50μM; Figure 4G-H), whereas genistein has similar effect at higher concentration (100μM; Figure 4E-F).

Next, we performed a docking study, mapping the binding pocket of 2 hydroxyflavonoids, genistein and tectorigenin, in the ECL and TM7 regions using the crystal structure of human TP receptor α (Figure 5). In the N terminus and ECL1 domain, both flavonoids bind similar amino acid residues with hydrogen bonds (R13, H96, and E94) but with different π-cation interaction (tectorigenin: F12 and H96 vs genistein: H96; Figure 5A-B). This minor difference may modify the binding affinity of tectorigenin to the TP receptor, even in the presence of the methoxy group, located on the A-ring at position 6. In the TM7 domain, we could not observe major differences; both flavonoids form hydrogen bonds using similar amino acid residues (R295, T298) and π-cation interactions (W182; Figure 5C-D). These results suggest that both flavonoids bind a similar binding pocket in the TP receptor, which partially overlaps with TxA_2-binding sites.

Using different bioinformatics and docking studies, the predicted protein structure of the mouse TP receptor was aligned with the human TP receptor, confirming that the flavonoid-binding pocket is conserved between species (supplemental Figure 1A; human, white; mouse, gold). Several residues are conserved in the genistein- and tectorigenin-binding pocket, including F12 and R13

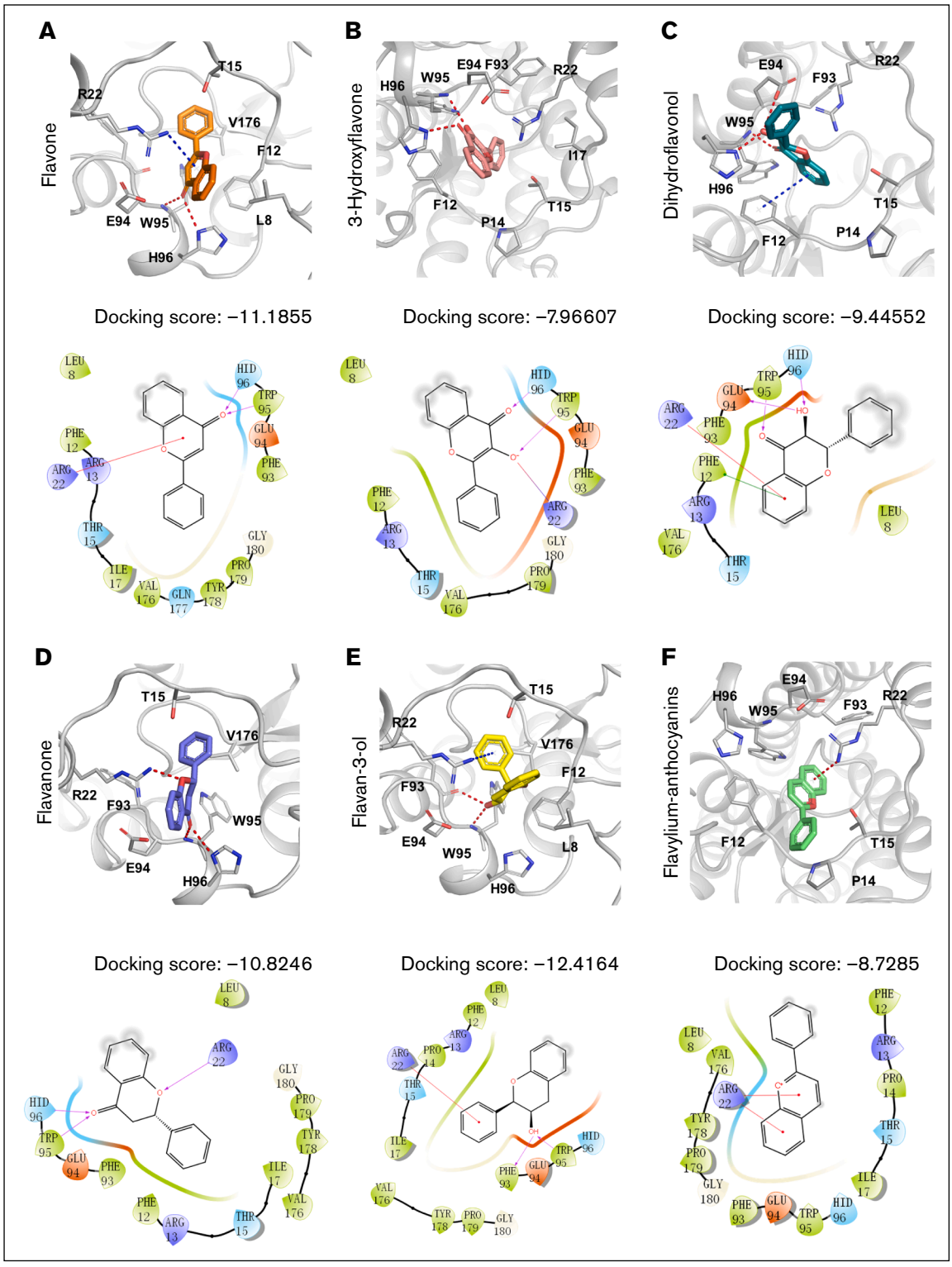


Figure 2. Comprehensive analysis of flavonoid binding to the extracellular domain of the human TP receptor α. (A-F) Interactions of different groups of flavonoids with indicated amino acids located in the ECL domain of the TP receptor. Hydrogen bonds are indicated by arrows.

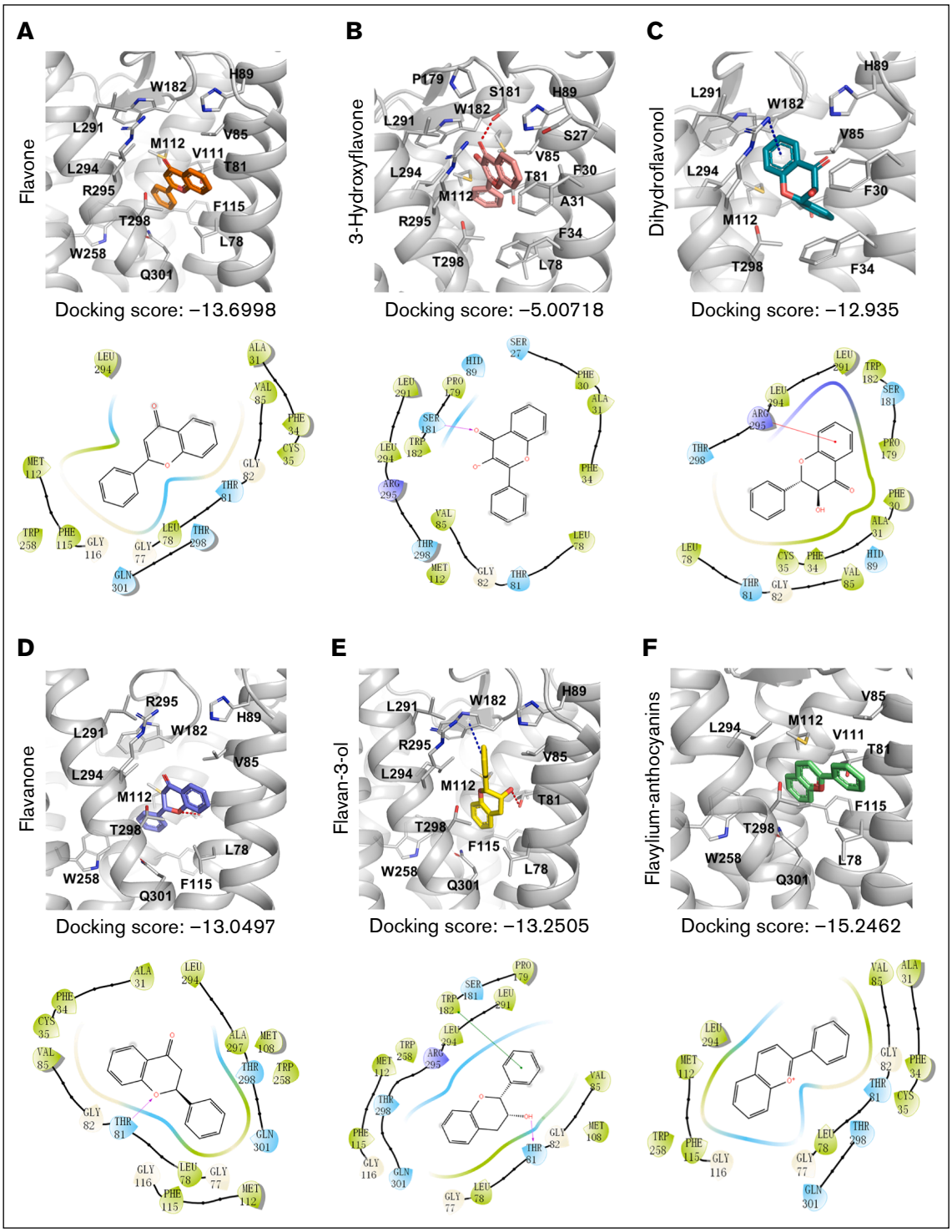


Figure 3. Comprehensive analysis of flavonoid binding to the TM domain of the human TP receptor α. (A-F) Interactions of different groups of flavonoids with indicated amino acids located in the TM7 domain of the TP receptor. Hydrogen bonds are indicated by arrows.

(supplemental Figure 1B-C). We observed that E94 and H96 amino acids exhibit species-specific substitutions, changed to D93 and R95 in the mouse and rat orthologs, respectively (supplemental Figure 2A). However, both amino acids interact with hydroxyl groups of genistein and tectorigenin in mouse TP receptor, thereby stabilizing the complex (supplemental Figure 1B-C). The observed structure similarity and preserved inhibitory effects observed in mouse and human platelet aggregation assays suggest that the overall binding mode and functional outcome remain largely conserved between species.

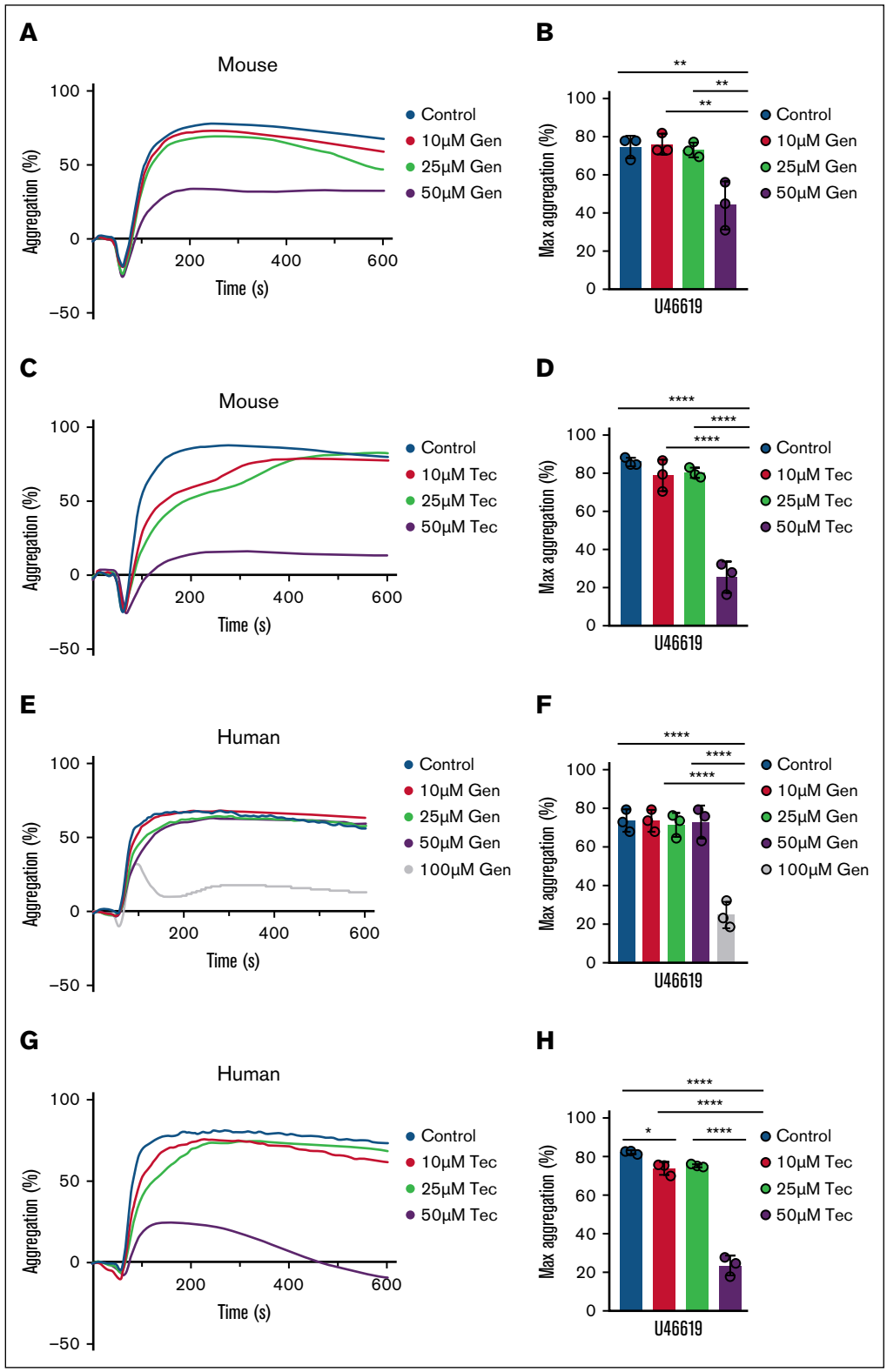


Figure 4. Comprehensive analysis of Gen- and Tec-mediated antiplatelet effects in mouse and human platelets. (A-D) Mouse platelets were stimulated with a high dose of TxA_2 analog U46619 (1.5μM) in the presence or absence of Gen (A-B) or Tec (C-D). Preincubation time was 1 minute before adding U46619. Concentrations of Gen or Tec are indicated. Aggregation responses were measured for 10 minutes. Maximal aggregations were statistically analyzed using 1-way analysis of variance (ANOVA) and Tukey post hoc test (*$P < .05$; **$P < .01$; ****$P < .0001$) and visualized in bar graphs. (E-H) Similar experiments with Gen and Tec were performed with human platelets using platelet-rich plasma. Higher concentration of Gen (100μM) was used to observe similar inhibitory effect to mouse platelets. Gen, genistein; Tec, tectorigenin.

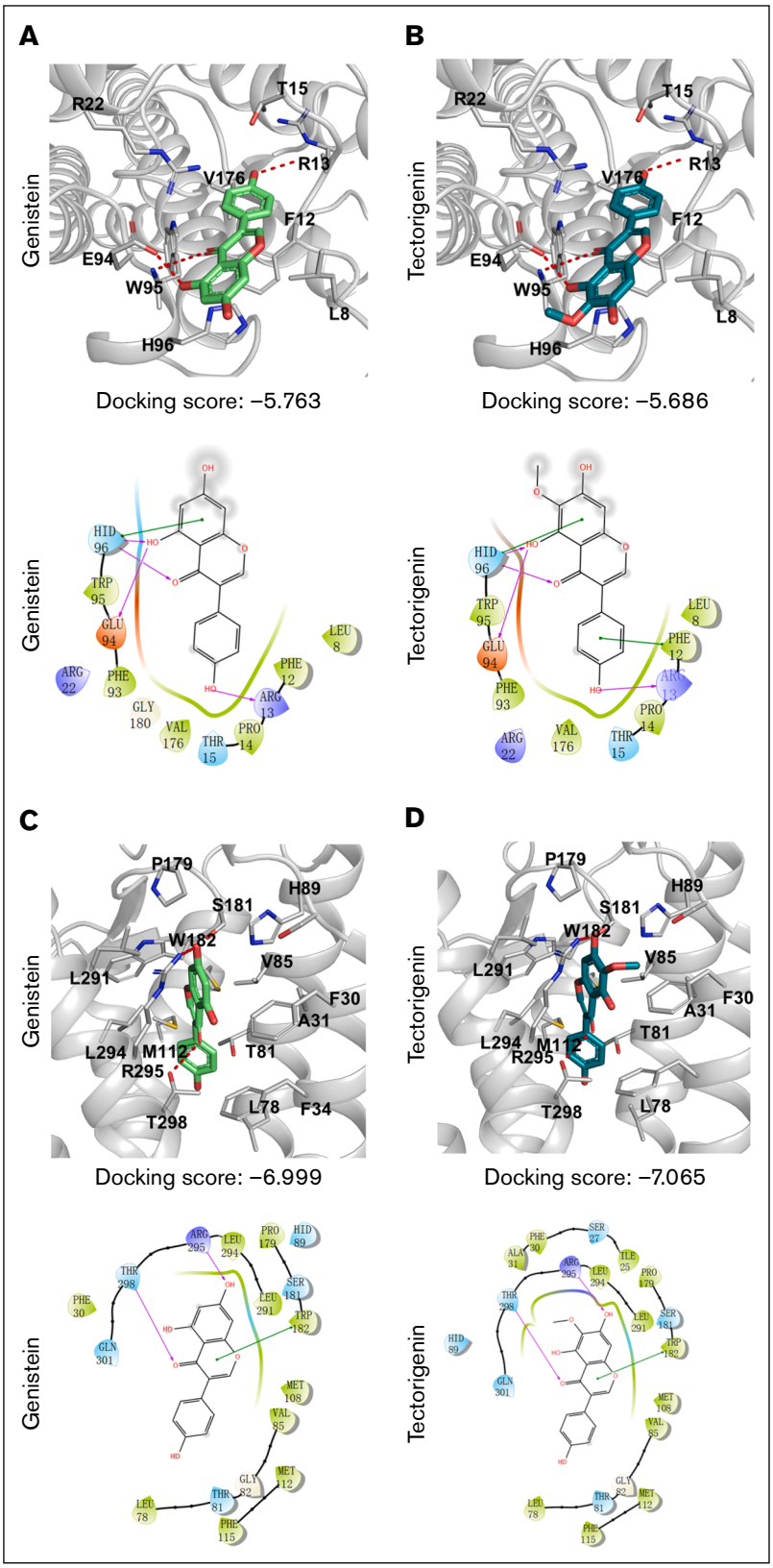


Figure 5. Comprehensive analysis of genistein and tectorigenin binding to the TP receptor α. Schematic representation showing the interacting amino acids of genistein and tectorigenin in the ECL domain (A-B) and TM domain (C-D) of TP receptor α. Hydrogen bonds are indicated by arrows.

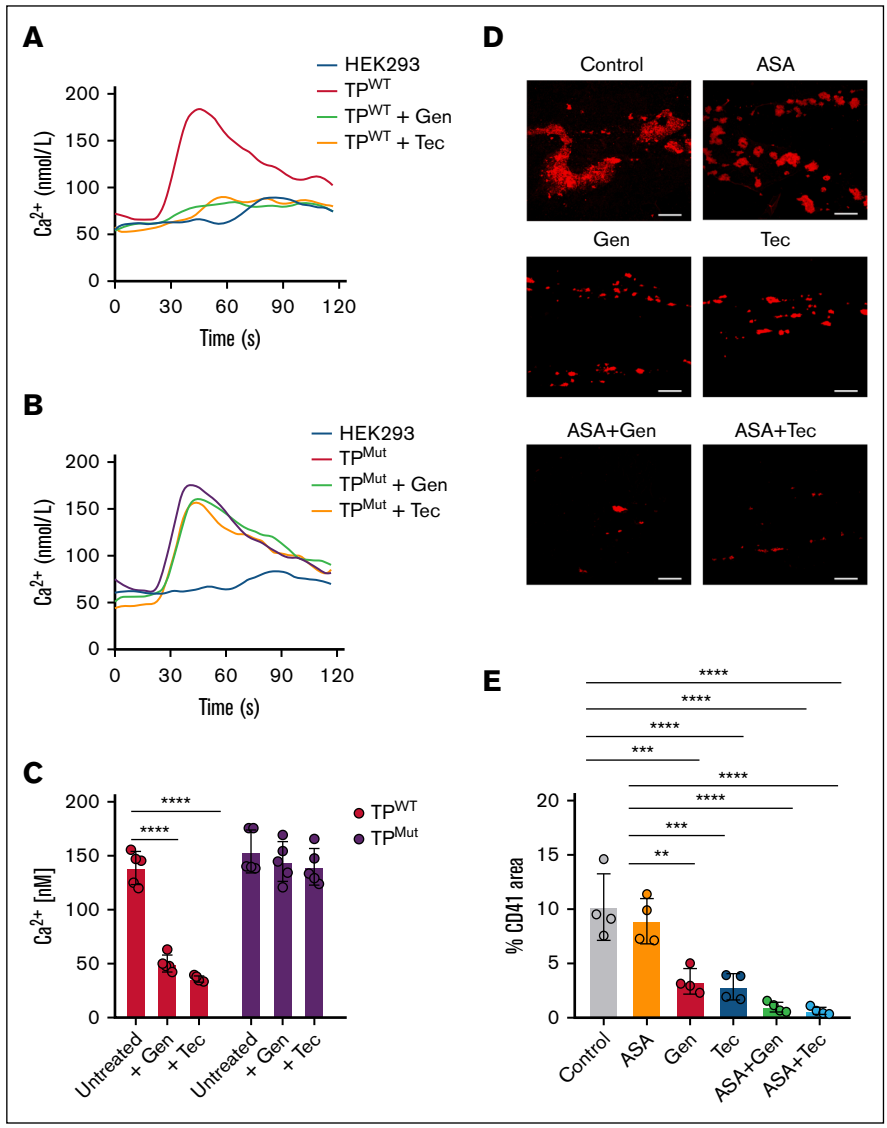


Figure 6. U46619-induced intracellular Ca^{2+} mobilization in HEK293 cells overexpressing human TP receptor α. (A) Fura-2–loaded parental HEK293 cells (blue line) show no response after U46619 stimulation. Human TP receptor–transfected HEK293 cells (TP^{WT}) showed elevated cytoplasmic Ca^{2+} concentrations (nanomolar). Gen or Tec pretreatment (100μM, 1 minute) abolishes TP receptor–mediated Ca^{2+} increase. (B) Mutant form of TP receptor (TP^{Mut}) shows transient cytoplasmic Ca^{2+} elevation in the presence or absence of hydroxyflavonoids. Experiments were performed in the absence of extracellular Ca^{2+}. Therefore, the detected cytoplasmic Ca^{2+} increase is caused by Ca^{2+} store depletion, stimulated by inositol trisphosphate receptor through activation of TP receptor/G protein–coupled receptors/phospholipase C-β signaling route. (C) The red bar graphs indicate responses of HEK293 cells expressing the human TP^{WT}, and the purple bar graphs indicate responses of HEK293 cells expressing the TP^{Mut} variant. Maximum changes in Ca^{2+} levels are quantified in nanomolar (nM). Data are presented as mean ± standard deviation (SD); 1-way ANOVA test and Tukey post hoc test were used for statistical analysis. ****$P < .0001$. (D-E) Hydroxyflavonoid-mediated antithrombotic effects in the presence of ASA under flow. (D) Representative fluorescence images show platelet adhesion and aggregate formation under flow conditions (1000 s^{-1}) in the presence of ASA (25μM), Gen (25μM), Tec (25μM), or their combinations (ASA + Gen; ASA + Tec). Staining: anti-human CD41 using Cy-3-conjugated anti-rabbit IgG. Scale bars, 50 μm. (E) Quantification of platelet aggregates is presented as mean platelet surface coverage (% CD41 area). Low-dose ASA treatment alone does not influence aggregate formation, mimicking aspirin resistance. Combined treatment results in a significant reduction. Data are presented as mean ± SD; 1-way ANOVA test and Tukey post hoc test were used for statistical analysis. **$P < .01$; ***$P < .001$; ****$P < .0001$. ASA, aspirin; Gen, genistein; Mut, mutant; Tec, tectorigenin; WT, wild-type.

Experimental validation of the genistein- and tectorigenin-binding pocket in the extracellular domain of TP receptor

We predicted several hydrogen bonds form between flavonoids and the TP receptor, which may modify the structure of the ECL-binding pocket, thereby inhibiting TxA_2 movement toward the TM7 region and subsequently preventing signaling mediated by Gq protein–coupled receptors at the cytoplasmic tail of the TP receptor. We identified key amino acids in human TP receptor including F12, R13, E94, and H96 that form hydrogen bonds or π-cation interactions with genistein and tectorigenin but that are not directly involved in TxA_2 binding. To destabilize flavonoid binding to the ECL1 and ECL2 domains of the human TP receptor, we mutated F12, R13, E94, H96, and V176 to alanine, followed by transfection into HEK293 cells. Positive clones were selected with puromycin. Using western blotting with a TP receptor–specific antibody, we confirmed stable protein expression (supplemental Figure 2B). We used these cell lines expressing comparable amounts of wild-type and mutant form of the TP receptor. After TP receptor stimulation with U46619 (20μM), we monitored intracellular Ca^{2+} mobilization in Fura-2-loaded control and TP receptor–transfected HEK293 cells (Figure 6A-C). Notably, parental HEK293 cells do not express the TP receptor (supplemental Figure 2B) and do not respond to U46619 (Figure 6A-B) whereas cells transfected with the wild-type TP receptor exhibited Ca^{2+} transients (Figure 6A). The mutant variant of the TP receptor was not affected by genistein or tectorigenin (Figure 6B), whereas the U46619-stimulated wild-type receptor was inhibited by these flavonoids (Figure 6A), suggesting an active role of the ECL domain in flavonoid binding. We conclude that the indicated amino acid substitutions selectively abolished the inhibitory effects of genistein and tectorigenin without affecting TxA_2 binding (Figure 6C).

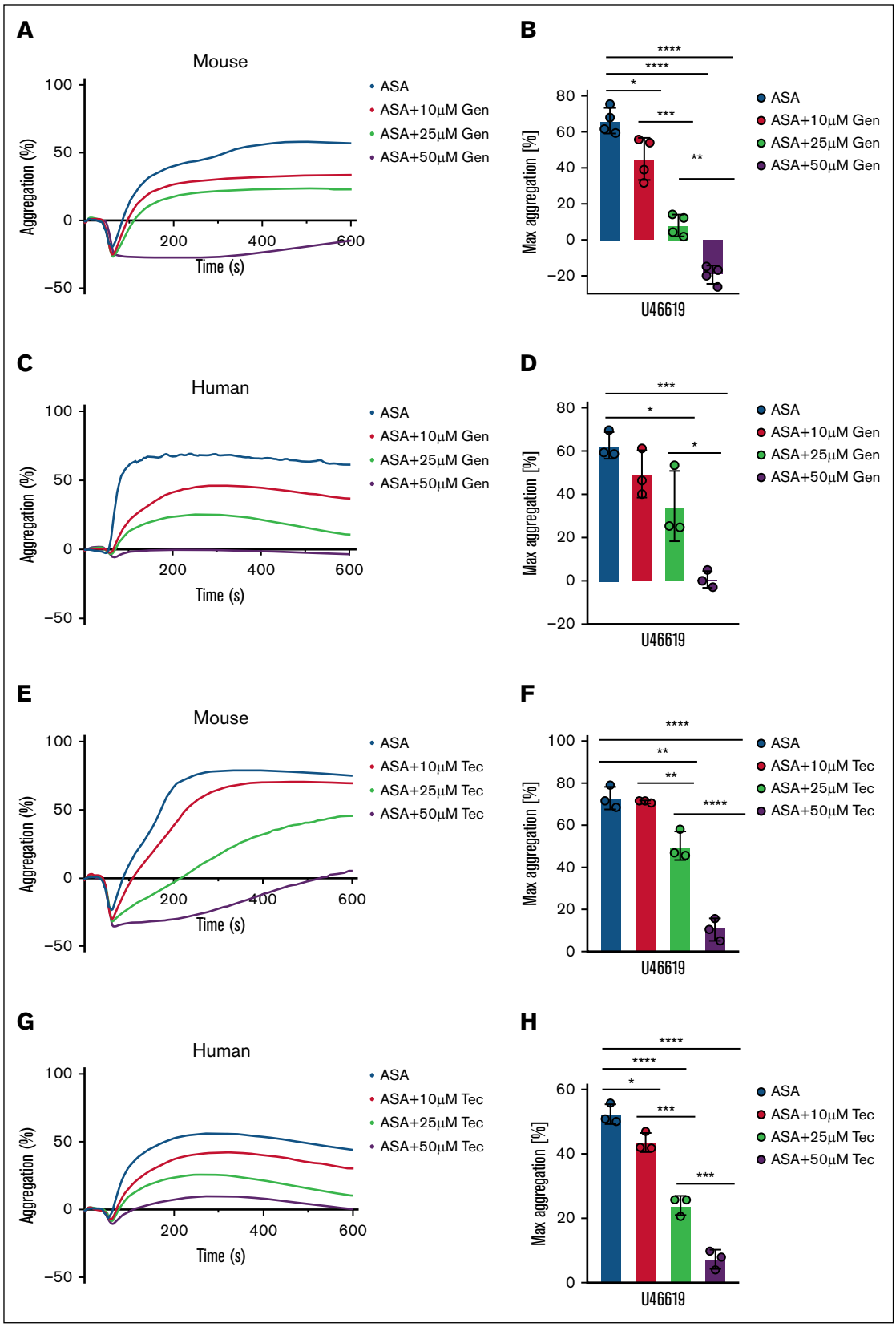


Figure 7. Synergistic effect of genistein and tectorigenin in aspirin-treated mouse and human platelets. (A-B,E-F) Mouse and (C-D,G-H) human platelets were pretreated with ASA (mouse, 4mM); human, 8mM) for 10 minutes, and stimulated with a high dose of U46619 (1.5μM). (A-B) Pretreatment of mouse and (C-D) human platelets

Synergistic effects of genistein and tectorigenin with aspirin in platelet aggregation and thrombus formation under blood flow

Aspirin reduces platelet aggregation in a dose-dependent manner at static and shear flow conditions.[29] To evaluate the combined antiplatelet effects of flavonoids with aspirin, first, platelets were preincubated with increasing concentrations of aspirin (0mM, 2mM, 4mM, and 8mM) for 10 minutes, and stimulated with a high dose of U46619 (1.5μM). We found that both mouse and human platelet aggregations were only partially inhibited by aspirin at 4mM and 8mM, respectively (supplemental Figure 3). Therefore, we used these concentrations of aspirin to mimic aspirin resistance in vitro. Next, different doses of genistein or tectorigenin (0μM, 10μM, 25μM, and 50μM) were applied in combination with aspirin in mouse and human platelets (Figure 7). At submaximal concentrations of aspirin, coapplication of genistein or tectorigenin markedly suppressed platelet aggregation (Figure 7), indicating synergistic effects on TxA_2-mediated pathways. To measure the direct inhibitory effects, TP receptor–mediated aggregation responses were followed after adding flavonoids at 120 seconds. Rapid disaggregation was observed during the observation time periods in both mouse and human platelets (supplemental Figure 4). The inhibitory effect was similar in both human and mouse platelets, suggesting a conserved mechanism across species.

Next, we tested the antithrombotic effects of genistein and tectorigenin in human whole blood under shear flow conditions. Using collagen-coated surface under shear rate of 1000 s^{-1}, first, we pretreated human whole blood with a low dose of aspirin (25μM) for 5 minutes to obtain no inhibitory effect in thrombus formation, thereby mimicking partial inhibition of COX activity, as observed in patients with aspirin resistance. The combination of aspirin with genistein (25μM) or tectorigenin (25μM) significantly reduced thrombus formation compared with individual treatments (Figure 6D-E). These findings demonstrate that genistein and tectorigenin enhance the antithrombotic efficacy of aspirin by targeting complementary pathways of TxA_2 signaling. The observed synergy suggests that combined treatment may represent a promising strategy to improve platelet inhibition in patients with aspirin resistance.

Discussion

In clinical settings, different degrees of aspirin resistance have been detected in 20% to 30% of patients with cardiovascular disease or diabetic and metabolic syndromes.[30,31] These patients do not respond to standard doses of aspirin, and long-term use of high-dose aspirin may lead to complications such as gastrointestinal bleeding. Although aspirin inhibits COX activity and TxA_2 production and subsequent TP receptor activation, it does not inhibit synthesis of isoprostanes, which are alternative TP receptor agonists.[32] Isoprostanes are generated via oxidative stress–dependent AA peroxidation, which could effectively activate TP receptor.[33] Increased levels of isoprostanes frequently observed in oxidative stress–related diseases, probably contributing to aspirin resistance.[34] In the cardiovascular system, 8-isoprostaglandin F2α is particularly active form of isoprostanes, interacting with the TP receptor and leading to platelet aggregation that is not inhibited by aspirin.[35] Despite clinical testing of several TP receptor antagonists (eg, ramatroban), their efficacy remains limited. Therefore, understanding the molecular interaction between the TP receptor and alternative TP receptor antagonists is essential for the development of effective antiplatelet therapies. The results presented in this article provide novel molecular insights into flavonoid binding to the TP receptor, which may be valuable information for rational drug design and new therapeutic approach. Our functional data showed that genistein and tectorigenin acted synergistically with aspirin to inhibit TP receptor–mediated aggregation in both human and mouse platelets. In addition, flow chamber assays demonstrated that cotreatment of genistein or tectorigenin with aspirin reduced thrombus formation under blood flow compared with individual treatments, supporting the translational relevance of this combined antiplatelet strategy. Of note, tectorigenin treatment may have therapeutic limitations in vivo, due to poor water solubility, limited absorption in the gut, and renal cytotoxicity.[36] The methoxy group of tectorigenin changes the lipophilicity and consequent membrane interaction whereas the hydroxyl group of genistein increases transient hydrogen bonds with membrane phospholipids, supporting membrane binding and diffusion.[36,37]

Using our docking studies, we confirmed many of the previously identified interacting amino acid residues in the ECL2 and TM7 domains. However, we also observed the interacting residue of S181, which is probably involved in the TxA_2-binding pocket. This is strongly supported by the finding that ramatroban also binds this amino acid,[7] and competing with TxA_2 binding. Although the antiplatelet effects of flavonoids on TxA_2 signaling were identified several years ago, the lack of a TP receptor crystal structure has limited our understanding of the inhibitory mode of flavonoids on this receptor. Here, we demonstrate that each class of flavonoids displays a common pharmacophore around the N terminus, ECL1, and TM7 regions, which are critical for TP receptor engagement. Although flavonoids mimic overlapping contact points with TxA_2, they also exhibit key differences, thereby inhibiting TP receptor function. Our findings indicate that hydroxyflavonoids such as genistein and tectorigenin have high binding affinity for both human and mouse TP receptors by forming hydrogen bonds and π-cation interactions with amino acids located in the N terminus and ECL1 domain, contributing to both polar and hydrophobic stabilization. In contrast to flavonoids, ramatroban forms strong salt bridges and hydrogen bonds with R295 and H296, while also occupying a different binding interface involving residues in the ECL2 domain (I193, L123, Y115, and W199). This extensive binding footprint explains its potent and competitive inhibitory properties against TxA_2 binding to the TP receptor and this inhibitory action differs from flavonoids.[7]

Based on our comparative analysis, we speculate that the hydrogen bonds formed by flavonoids may alter the shape of the ligand-binding pocket and modify the spatial relationship between

Figure 7 (continued) with Gen (10μM, 25μM, and 50μM) or (E-H) Tec (10μM, 25μM, and 50μM) was performed 1 minute before U46619 stimulation. (B,F,D,H) Maximal aggregation responses are indicated in mouse (B,F) and human (D,H) platelets in bar graphs. Data are presented as mean ± SD; 1-way ANOVA test and Tukey post hoc test were used for statistical analysis. *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$. ASA, aspirin; Gen, genistein; Tec, tectorigenin.

the N terminus and ECL regions, thereby interfering with TxA_2 binding. The binding pocket of TxA_2 is stabilized by 2 pairs of disulfide bridge located between cysteine 11 and 102, and between cysteine 105 and cysteine 183, in the ECL region. Mutation of these cysteine residues or modification of the disulfide bridges alters the size and shape of the ligand-binding pocket, thereby strongly inhibiting TP receptor function. Interestingly, we found that genistein and tectorigenin interact with R13, forming a hydrogen bond. We speculate that this hydrogen bond does not modify disulfide bonds directly but may modulate the stability or geometry of the disulfide bridge, influence redox potential, or assist in proper disulfide formation by guiding cysteine positioning. In addition, both tectorigenin and genistein form π-π stacking with F12, close to cysteine 11, which may indirectly affect the relative position or orientation of this cysteine residue or influence local electron transfer processes, indirectly affecting the oxidation or reduction of the disulfide bridge. We mutated both F12 and R13 amino acid residues, thereby disrupting these interactions and destabilizing flavonoid binding to the TP receptor. We could not predict interacting residues near the second disulfide bridge (C105 and C183), suggesting that this disulfide bridge may remain intact. Depending on the oxidative/reductive environment of the ligand-binding pocket, genistein and tectorigenin may also indirectly affect disulfide bridge stability, potentially binding to protein disulfide isomerase (PDI) and inhibiting its enzymatic activity in platelets, thereby interfering with thiol-disulfide exchange at cysteine 11 in the TP receptor. This idea is supported by the finding that genistein binds to the substrate-binding site of PDI and inhibits its chaperone function, affecting the protein folding process.[38] Nonetheless, further investigation is necessary to determine whether genistein or tectorigenin influences PDI activity and the subsequent folding of the disulfide bridge in platelets.

During the last decade, several studies have highlighted the therapeutic potential of genistein in thrombotic and cardiovascular diseases. In addition to its inhibitory function on the TP receptor, genistein also inhibits several tyrosine kinases, and serotonin secretion in collagen- and U46619-stimulated platelets, whereas thrombin-induced platelet activation remains largely intact.[39,40] Furthermore, our flow chamber results also support previous observation that genistein significantly reduces thrombus formation in heparinized blood samples under low and high shear conditions on collagen-coated surfaces ex vivo.[41] Although genistein is a well-established protein tyrosine kinase inhibitor, our platelet studies demonstrated that genistein inhibits U46619-induced platelet activation and rapidly disaggregates platelets, and due to the short incubation time, this effect attributed primarily to TP receptor antagonism in our experimental settings, rather than tyrosine kinase inhibition. Although our docking and functional experiments strongly support a direct TP receptor–dependent mechanism, off-target effects in vivo cannot be excluded. Under long-term treatment, genistein may modulate additional signaling pathways, including tyrosine kinase activity, oxidative stress responses, and inflammatory signaling cascades in whole blood and the vasculature. In the context of thromboinflammatory diseases, these polypharmacological properties of genistein may be therapeutically advantageous rather than detrimental. Thus, genistein may simultaneously target TP receptor signaling and complementary pathways involved in thrombus formation and vascular inflammation. To support this concept, our docking study shows that genistein has probably an overlapping binding pocket with 8-isoprostaglandin F2α, thereby inhibiting this alternative activator mode of TP receptor (supplemental Figure 5). Further studies are necessary to show whether genistein treatment may suppress isoprostane production and related TP receptor signaling in oxidative stress–related diseases.

Earlier, it has been shown that genistein has strong inhibitory effects on AA- and TxA_2 analog U46619-induced platelet aggregation, suggesting a potential role in TP receptor signaling.[16,39] Interestingly, tectorigenin, which only differs from genistein by a methoxy group at the R6 position, also strongly inhibits AA-induced platelet aggregation with better antiplatelet potential than aspirin.[21] However, tectorigenin has weak inhibitory action on COX-1 and exhibits poor bioavailability and dose-dependent cytotoxicity, and no effect on thromboxane A_2 synthase, suggesting a limited therapeutic potential.[21,36] Moreover, tectorigenin cannot rapidly enter into the platelet cytoplasm, suggesting a direct blockade of TP receptor function on the platelet surface. In our study, both genistein and tectorigenin potentiated the inhibitory effect of aspirin on U46619-induced platelet aggregation, indicating that TP receptor blockade by hydroxyflavonoids may help compensate when aspirin does not fully suppress thromboxane-dependent signaling.

Interestingly, the genistein isomer apigenin, or low-dose aspirin alone, did not inhibit AA-induced platelet aggregation, whereas their combined use strongly reduced aggregation responses.[42] Therefore, we speculate that genistein may correct the incomplete effects of aspirin by blocking the TP receptor and other signaling events in patients with aspirin resistance. Our aggregometry and flow chamber data support this concept, as submaximal concentrations of aspirin combined with genistein or tectorigenin produced stronger inhibition of platelet aggregation than either treatment alone in both human and mouse samples. Interestingly, isoprostane (8-isoprostaglandin F2α) levels are elevated in patients with aspirin resistance under different pathological conditions through oxidative stress pathways.[43] Therefore, antioxidants such as hydroxyflavonoids may represent a promising strategy to reduce the levels of isoprostanes, thereby enhancing the efficacy of aspirin. Of note, synthetic genistein has been tested, supplementation is well tolerated without any significant toxicity in healthy volunteers.[44-46] These results suggest that synthetic genistein could be safely used in combination with aspirin to enhance antiplatelet therapy, particularly in patients with aspirin resistance with elevated levels of isoprostanes.

Several patients with TP receptor (TBXA2R) mutations show mild to moderate bleeding. W29C and N42S impair receptor dimerization, causing platelet dysfunction and postsurgical bleeding, although lifelong mucocutaneous bleeding is absent. Heterozygous relatives show no symptoms.[47-49] R60L mutation, located in the first intracellular loop, causes dominant bleeding disorder by inhibiting wild-type receptor function, impairing Gq coupling and Ca^{2+} signaling despite normal ligand binding. These patients report lifelong mucosal bleeding and easy bruising without major bleeding episodes.[50,51] D304N mutation reduces TxA_2 binding, leading to mild childhood bleeding. These results suggest that mutations reducing ligand binding or dimerization do not cause severe bleeding.[52] So far, no patients have been identified carrying mutations in the ECL1- or ECL2-binding pocket of the TP receptor, reducing TxA_2 or other ligand binding. Altogether, these results suggest that reduced

ligand binding or dimerization of the TP receptor does not cause severe bleeding complications in humans.

Altogether, we propose to further evaluate genistein in combination with low-dose aspirin in different in vivo mouse models of oxidative stress–related diseases. Using blood samples from a cohort of patients with aspirin resistance with high levels of isoprostanes, the synergistic antiplatelet effects of genistein on TP receptor should be further analyzed. This evaluation represents an important step toward developing safe and effective combined therapy for patients with aspirin resistance.

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Authorship

Contribution: C.H. and P.R. performed computational modeling, cloning, and overexpression of thromboxane-prostanoid receptor in HEK293 cells and analyzed data; C.H., X.C., P.L., and B.X. performed western blotting, Ca^{2+} measurement, platelet aggregometry, and flow chamber assays; A. Bassot, E.C., and T.G. analyzed data and commented on the manuscript; and E.M.-B. and A. Braun designed and conceived the study, analyzed data, and wrote the manuscript.

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