Biochemical Pharmacology 83 (2012) 88–96

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

The C-terminal segment of the second extracellular loop of the thromboxane A_2 receptor plays an important role in platelet aggregation

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ARTICLE INFO

Article history:
Received 23 August 2011
Accepted 3 October 2011
Available online 8 October 2011

Keywords:
Thromboxane A_2 receptor
Platelet
Function-blocking antibody
Antagonist
Ligand binding domain

ABSTRACT

There is considerable interest in discovering novel antiplatelet approaches with an enhanced safety profile. To this end, in our efforts to define new targets for antithrombotic activity, we investigated the utility of antibodies which recognize the ligand binding domains of the platelet thromboxane A_2 receptor (TPR). We hypothesized that an antibody (abbreviated as C-EL2Ab), which interacts with the C-terminus of the second extracellular loop (C-EL2; i.e., ligand binding domain) of TPR exhibits antagonistic activity. Our findings demonstrate that C-EL2Ab did indeed inhibit TPR-mediated platelet aggregation. However, it was devoid of any apparent effects on aggregation triggered by ADP or the thrombin receptor activating peptides 1 or 4. Furthermore, results from radiolabeled ligand binding studies indicate that C-EL2Ab competitively displaced the classical TPR antagonist [^{3}H]SQ29,548 from its binding sites. On the other hand, control experiments indicated that normal rabbit IgG and an antibody which targets a TPR domain separate from those involved in ligand recognition, failed to inhibit aggregation in response to TPR activation. Collectively, these findings demonstrate that C-EL2 of TPR plays a critical role in platelet activation, and establish C-EL2Ab as a function blocking antibody. Furthermore, our data suggest a potential for the therapeutic application of C-EL2Ab, which may serve either as an alternative to, or a complement for current treatments. Finally, the identification of a functionally active TPR sequence should aid molecular modeling study predictions for organic derivatives which possess *in vivo* activity.

1. Introduction

TPRs' clear role in normal hemostasis is supported by the finding that patients have a bleeding disorder as a result of a point mutation in the receptor protein [1]. On the other hand, upregulated signaling through TPR has been implicated in the pathogenesis of multiple cardiovascular and thrombosis-based diseases [2–6]. Consistent with this concept are clinical findings indicating that inhibition of platelet TXA_2 production provides therapy for thromboembolic diseases [4,7–13]. Indeed, the importance of TXA_2 in the thrombotic process serves as the rationale for the use of aspirin in myocardial infarction and in stroke [13,14].

Consequently, the TXA_2 pathway has been targeted for pharmacological intervention either to inhibit its formation or to modulate binding to its receptor. In light of this fact, several possibilities have emerged to achieve this goal with COX inhibitors and thromboxane synthase (TS) inhibitors being the initial lead candidates. However, even though the COX inhibitor aspirin is in clinical use, these compounds are associated with inherent limitations. Thus, aspirin was found to lack selectivity, cause gastric ulcers, redirect arachidonic acid metabolism to isoprostanes, and is associated with sensitivity and an increasing rate of resistance worldwide [15]. The TS inhibitors, on the other hand, exhibited minimal activity [16] because the immediate precursor of TXA_2, i.e., PGH_2 binds to the same receptor [17–21] and can therefore induce platelet aggregation [22]. On this basis, it became clear that more selective means of blocking TXA_2-mediated platelet aggregation needed to be developed, and receptor blockade seemed to be the most logical and promising approach. Of note, evidence obtained using mouse models suggests that, in certain disease states, therapeutic intervention using a TPR antagonist, with or without aspirin, is superior to aspirin therapy alone [23].

To this end, a number of TPR antagonists were designed throughout the years and tested for biological activity [17,24–31]. While *in vitro* results were encouraging, the *in vivo* effectiveness of these molecules was limited by short biological half-life, toxicity or limited tissue distribution. One apparent reason for this failure is because these agents were empirically designed based on the

Abbreviations: TXA_2, thromboxane A_2; TPR, thromboxane A_2 receptor; TS, thromboxane synthase; TRAP1, thrombin receptor-activating peptide 1; TRAP4, thrombin receptor-activating peptide 4; PAR, protease activated receptor; C-EL2, C-terminus of the second extracellular loop; C-EL2Ab, antibody targeting the C-terminus of the second extracellular loop; PRP, platelet rich plasma; PPP, platelet poor plasma.

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doi:10.1016/j.bcp.2011.10.002

complex structures of PGH_2 and/or TXA_2, with little information concerning the actual TPR binding domains. In this connection, research efforts [32,33] by us and others to map the TPR ligand binding domain revealed the following: (1) the ligand binding domain resides in the C-terminus of the second extracellular loop (C-EL2; C^{183}–D^{193}) of the receptor protein; and (2) this extracellular segment contains amino acid coordination sites (F^{184}, T^{186}, S^{191}, D^{193}, and S^{201}), for agonists and antagonists. Additionally, solution structure and molecular modeling of TPR and its ligand binding domains demonstrated an interaction between EL2 and a TPR ligand, which resulted in a conformational change in EL2 [34]. Separate work utilizing "NMR experiment guided mutagenesis" to define TPR's ligand binding pocket showed that the EL2 does in fact play a very important role in ligand binding, receptor activation and subsequent signal transduction [35,36].

Driven by an interest in identifying alternative targets (e.g., TPR), or discovering new agents for inhibiting platelet aggregation, we believe there is potential in using antibodies designed to recognize receptor ligand-binding sites as a direct approach to effective inhibition of signaling. Based on these considerations, we hypothesize that C-EL2 plays an important role in TPR-mediated platelet activation. We further hypothesize that an antibody targeting this specific domain (referred to as C-EL2Ab) can be employed as a function blocking reagent for platelet activation by TPRs. Our characterization of C-EL2Ab revealed that this biological reagent: (1) selectively inhibits human (*in vitro*) and murine (*in vitro* and *ex vivo*) platelet aggregation stimulated by TPR, but not by ADP or the thrombin receptor activating peptide 4 (TRAP4); and (2) displaces binding of the radiolabeled TPR antagonist [^{3}H]SQ29,548. On the other hand, a control antibody which targets a region separate from the ligand binding pocket (i.e., the first extracellular loop; referred to as EL1Ab), and normal rabbit IgG were found to be devoid of any detectable antiplatelet activity in response to TPR activation. Furthermore, the C-EL2Ab was found to lose its biological activity when it was preabsorbed with its cognate peptide. These findings make C-EL2Ab the first functional antibody against platelet TPRs, and highlight the value of its purposing as an antiplatelet, with a reasonable likelihood of clinical success, similar to the GPIIb/IIIa antagonist abciximab.

2. Materials and methods

2.1. Reagents

U46619 (15(S)-hydroxy-11,9-epoxymethanoprosta-5Z,13E-dienoic acid) and arachidonic acid were from Cayman Chemical (Ann Arbor, MI). ADP, stir bars and other disposables were from Chrono-Log Corporation (Havertown, PA). The C-EL2Ab and EL1Ab, previously characterized using ELISA and flow cytometry, were a generous gift from Dr. Guy Le Breton, The University of Illinois at Chicago [37,38]. The C-EL2 peptide was custom made (Research Resource Center, University of Illinois at Chicago). Normal rabbit IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Thrombin receptor activating peptides 1 and 4 (SFLLR-NH2 and AYPGKF-NH_2, respectively), isoflurane and indomethacin were from Sigma Aldrich (St. Louis, MO). The radiolabeled SQ29,548 ([^{3}H]SQ29,548) was purchased from PerkinElmer (Waltham, MA). Hepes/Tyrode's buffer: sodium chloride, potassium chloride, sodium dihydrogen phosphate, magnesium chloride, sodium bicarbonate, and D-dextrose was obtained from Fisher Scientific (Hanover Park, IL) and bovine serum albumin (BSA) was obtained from Equitech-Bio, Inc. (Kerrville, TX). Hanks phosphate buffered saline solution (HBSS), sodium citrate (3.8%, w/v) solution, 60 ml syringes, 50 ml conical tubes were obtained from Fisher Scientific (Hanover Park, IL). The C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME). *Note*: The chemical name for SQ29,548 is [1S-[1a,2b(5Z),3b,4a]]-7-[3-[[2-[(phenyl-amino) carbonyl]hydrazino]-methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid. Human platelets were from healthy volunteers who denied taking any medication at least 10 days prior to collection, and who gave their informed consent before they were allowed to donate blood.

2.2. In vitro analysis of antibody effects on human platelet aggregation

Blood was withdrawn by venipuncture of the cephalic vein and collected into 60 ml syringes (Kendall) from human volunteers. Coagulation was inhibited by addition of 3.8% (w/v) sodium citrate solution in a ratio of 9 parts blood to 1 part citrate. The collected blood was spun at $170 \times g$ for 10 min in a 50 ml centrifuge tube, and the supernatant/platelet rich plasma (PRP) was collected. Platelet poor plasma (PPP) was prepared by spinning blood at $2000 \times g$ for 15 min, and used to dilute PRP to adjust platelet count to $2–3 \times 10^8$ platelets/ml. To ensure thromboxane A_2 was prevented from being generated, all experiments were performed after incubation with 10 μM indomethacin for 2 min except when arachidonic acid was used as the aggregating agonist. Baseline light transmission was established for roughly 1 min before control traces were obtained by the addition of vehicle, U46619, arachidonic acid, ADP, TRAP1 or TRAP4 to PRP. For platelet inhibition functional studies, PRP was incubated with 100–250 nM C-EL2Ab, 250 nM EL1Ab, 250 nM IgG, or vehicle (PBS) for 5 min prior to stimulation with 1 μM U46619, 0.5 mM arachidonic acid, 15 μM ADP, 60 μM TRAP1, or 40 μM TRAP4. For the preabsorption experiment, C-EL2Ab was incubated with 100 μM of its cognate peptide prior to addition to PRP. The effect of these agents was measured using the turbidimetric method with a model 700 whole blood lumi-aggregometer and the aggregation traces were captured using the Aggrolink8 software (Chrono-Log Corporation; Havertown, PA).

2.3. In vitro analysis of antibody effects on murine platelet aggregation

Six to eight mice (6–8 weeks old) were anesthetized with 5% isoflurane and sacrificed using cervical decapitation. Blood was collected into 50 ml conical tubes containing 3.8% sodium citrate as an anticoagulant (9 parts blood to 1 part citrate) before being pooled. Platelet rich plasma (PRP) was then isolated by differential centrifugation of pooled blood at $170 \times g$ for 10 min. Platelet counts were adjusted to $2–3 \times 10^8$/ml with Hepes/Tyrode's buffer. For examining antibody inhibition of platelet aggregation, PRP was incubated with 100–250 nM of C-EL2Ab, EL1Ab, IgG or PBS vehicle for 5 min. These experiments were performed in the presence of 10 μM indomethacin (incubated for 2 min) to prevent TXA_2 generation (except when arachidonic acid was used as the aggregating agonist). After establishing baseline light transmission for 1 min, platelets were stimulated with 1 μM U46619, 0.5 mM arachidonic acid, 15 μM ADP, or 40 μM TRAP4. For the preabsorption experiment, C-EL2Ab was incubated with 100 μM of its cognate peptide prior to incubation with PRP. All experiments were performed in compliance with the relevant laws and institutional guidelines, and were approved by the Western University of Health Sciences Institutional Animal Care and Use Committee.

2.4. Radiolabeled displacement of SQ29,548 in human platelets by C-EL2Ab

Resuspended platelets were prepared as previously described from human volunteers [39]. The platelet suspension (1×10^9 platelets/ml) was incubated with the radiolabeled [^{3}H]SQ29,548

(1 nM) at RT for 10 min, and then increasing concentrations of the displacing antibody C-EL2Ab (0.1–1000 nM) were added for an additional 45 min. The [^{3}H]SQ29,548 bound platelets were captured by running through 0.45 μm Millipore filter over a vacuum suction unit. For the preabsorption experiment, C-EL2Ab was incubated with 100 μM of its cognate peptide. The filters were then washed once and counted for radioactivity in a Beckman LS 6000 liquid scintillation counter. To calculate the non-specific binding, the same concentration of radioligand was competed against 1000-fold excess of unlabeled SQ29,548.

2.5. Ex vivo analysis of antibody effects on murine platelet aggregation

Four groups of six to eight mice (6–8 weeks old) were utilized to perform *ex vivo* experiments to analyze effects of C-EL2Ab, EL1Ab, or IgG versus Hanks/HBSS vehicle on platelet aggregation. Mice received tail vein injections of C-EL2Ab (250–325 nM), EL1Ab (325 nM), and IgG (325 nM) once daily for 5 days, including the day of experiment/blood collection. Mice were anesthetized with 5% isoflurane and sacrificed using cervical decapitation. Blood was collected into separate 50 ml conical tubes containing 3.8% sodium citrate as an anticoagulant (9 parts blood to 1 part citrate) before being pooled. Platelet rich plasma (PRP) was then isolated by differential centrifugation of pooled blood at 170 × *g* for 10 min. Platelet counts were adjusted to 2–3 × 10^8/ml with Hepes/Tyrode's buffer. Aggregation experiments were performed in the presence of 10 μM indomethacin (incubated for 2 min) to prevent TXA_2 generation (except when arachidonic acid was used as the aggregating agonist). After establishing baseline light transmission for 1 min, platelets were stimulated with 1 μM U46619, 0.5 mM arachidonic acid, 15 μM ADP, or 40 μM TRAP4. For the preabsorption experiments, C-EL2Ab was incubated with 100 μM of its cognate peptide prior to injection into mice. All experiments were performed in compliance with the relevant laws and institutional guidelines, and were approved by the Western University of Health Sciences Institutional Animal Care and Use Committee.

2.6. Analysis of data

All experiments were performed at least three times, with blood obtained from at least three different donors, or with blood pooled from at least 6 mice each time. Data were analyzed using GraphPad PRISM statistical software (San Diego, CA). Results were compared using unpaired 2-tailed Student's *t* test, with $p < 0.05$ considered to be statistically significant. The displacement binding curve was generated using non-linear regression analysis.

3. Results

3.1. C-EL2Ab inhibits TPR-mediated aggregation of human and murine platelets in vitro

We hypothesized that C-EL2 plays an important role in TPR-mediated platelet activation, and that an antibody (referred to as C-EL2Ab) targeting this specific domain can be employed as a function blocking reagent. Our initial studies demonstrated that addition of 1 μM U46619 (to PRP) produced a typical aggregation response (Fig. 1A), and that 100 nM C-EL2Ab produced a significant inhibition of U46619-induced platelet aggregation (Fig. 1A). Furthermore, it was found that this inhibitory effect was dose dependent (100–250 nM; Fig. 1A). The specificity of this effect was demonstrated by loss of C-EL2Ab ability to inhibit aggregation after preabsorption with 100 μM of its cognate peptide (Fig. 1B). These findings demonstrate that C-EL2Ab does indeed have the capacity to "specifically" attenuate platelet aggregation. To examine if the underlying mechanism for this inhibitory effect involves TPR antagonism, we investigated the effects of C-EL2Ab on aggregation induced by the TXA_2 precursor arachidonic acid. It was found

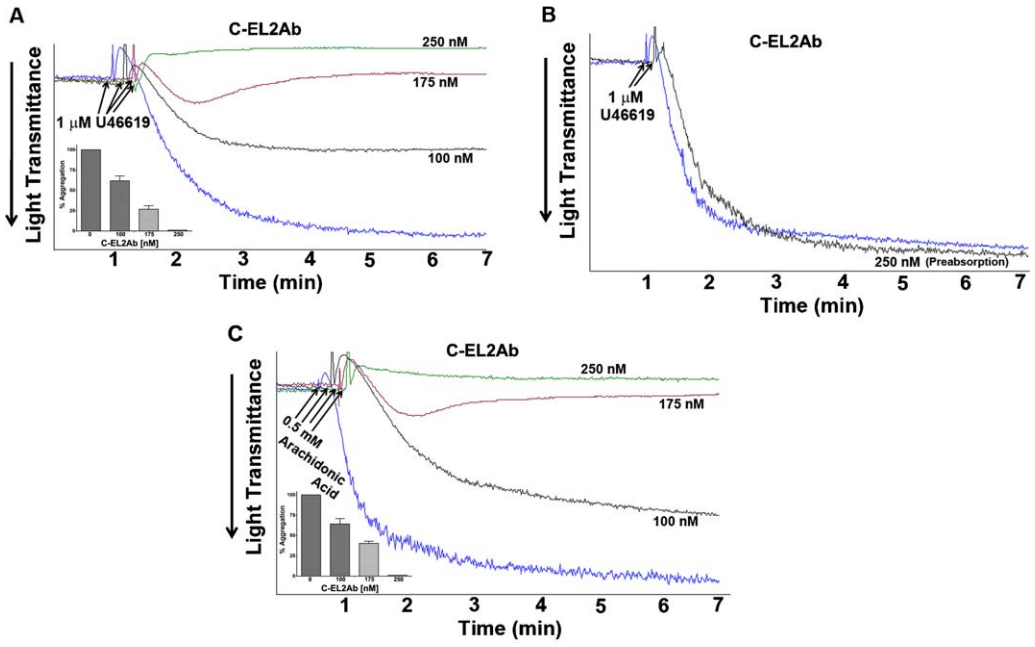

Fig. 1. Inhibition of TPR-mediated human platelet aggregation by C-EL2Ab *in vitro*. (A) Indomethacin-treated human PRP was stimulated with 1 μM U46619 in the absence or presence of increasing concentrations of C-EL2Ab (100–250 nM) to generate a concentration-dependent inhibition curve (inset shows quantification of the C-EL2Ab concentration-dependent inhibition curve of the U46619-induced aggregation response). (B) Indomethacin-treated human PRP was stimulated with 1 μM U46619 in the absence or presence of 250 nM C-EL2Ab that was preabsorbed with 100 μM of its cognate peptide. (C) Human PRP was stimulated with 0.5 mM arachidonic acid in the absence or presence of increasing concentrations of C-EL2Ab (100–250 nM) to generate a concentration-dependent inhibition curve (inset shows quantification of the C-EL2Ab concentration-dependent inhibition curve of the arachidonic acid-induced aggregation response). Each aggregation trace is representative of multiple traces obtained from three separate platelet preparations from three different human blood donors.

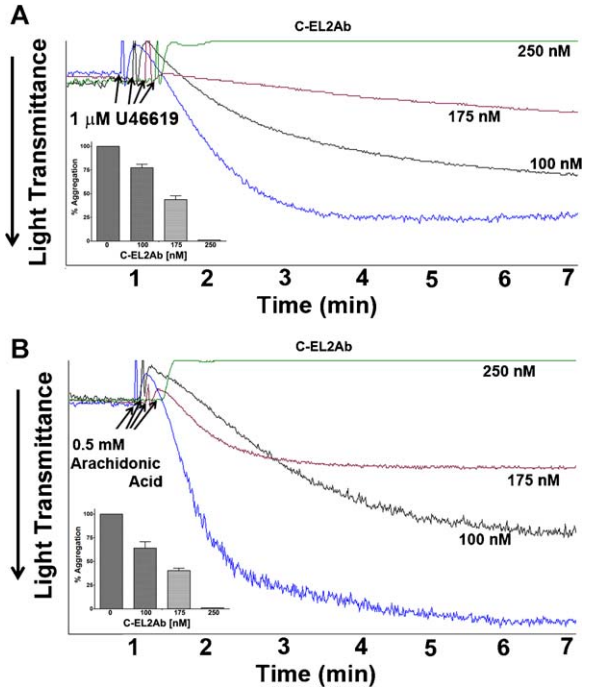

Fig. 2. Inhibition of TPR-mediated mouse platelet aggregation by C-EL2Ab *in vitro*. (A) Indomethacin-treated mouse PRP was stimulated with 1 μM U46619 in the absence or presence of increasing concentrations of C-EL2Ab (100–250 nM) to generate a concentration-dependent inhibition curve (inset shows quantification of the C-EL2Ab concentration-dependent inhibition curve of the U46619-induced aggregation response). (B) Mouse PRP was stimulated with 0.5 mM arachidonic acid in the absence or presence of increasing concentrations of C-EL2Ab (100–250 nM) to generate a concentration-dependent inhibition curve (inset shows quantification of the C-EL2Ab concentration-dependent inhibition curve of the arachidonic acid-induced aggregation response). Each aggregation trace is representative of multiple traces obtained from three separate platelet preparations from three groups of (6–8) mice.

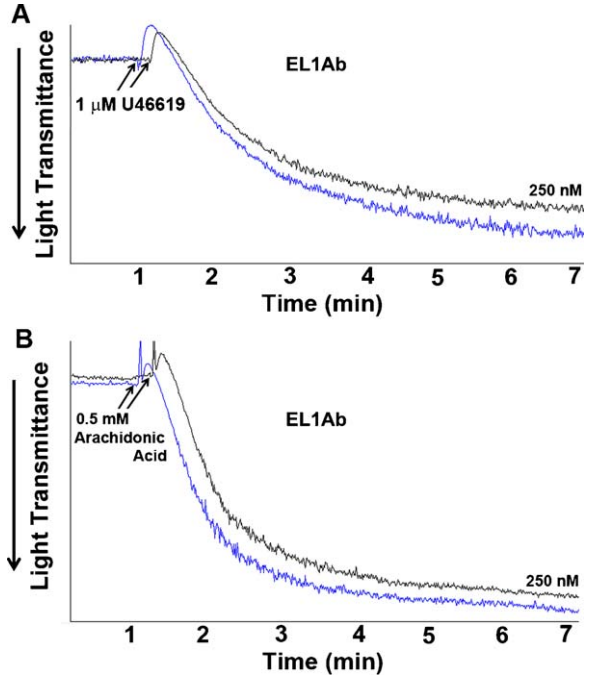

Fig. 3. Effect of EL1Ab on TPR-mediated platelet aggregation. (A) Indomethacin-treated human PRP was stimulated with 1 μM U46619 in the absence or presence of EL1Ab (250 nM). (B) Human PRP was stimulated with 0.5 mM arachidonic acid in the absence or presence of EL1Ab (250 nM). Each aggregation curve is representative of multiple traces obtained from three separate platelet preparations, from three different blood donors.

that C-EL2Ab also inhibited aggregation triggered by 0.5 mM arachidonic acid, dose-dependently (100–250 nM; Fig. 1C). Similar results were observed using murine platelets (Fig. 2). Specifically, C-EL2Ab inhibited aggregation triggered by U46619 and arachidonic acid, in a dose-dependent fashion (Fig. 2A and B, respectively); such an effect was not present when C-EL2Ab was preabsorbed with its cognate peptide (data not shown).

In order to verify the selectivity of inhibiting the C-EL2 region of TPR, the biological activity of normal rabbit IgG and an antibody targeting a region that does not participate in ligand binding (i.e., the first intracellular loop (EL1Ab)) was also analyzed. Our data demonstrated that neither EL1Ab (250 nM), nor normal rabbit IgG (250 nM) exert any detectable effects on aggregation stimulated by 1 μM U46619 or 0.5 mM arachidonic acid in human or murine platelets (Fig. 3A and B respectively; IgG and murine data not shown). This finding supports the notion that, unlike the EL1 domain, the C-EL2 site plays a key role in TPR-dependent platelet function, and harbors an important ligand binding site. We next tested the selectivity of C-EL2Ab inhibition of aggregation for the TPR pathway.

3.2. C-EL2Ab does not inhibit ADP induced aggregation of human and murine platelets in vitro

To confirm that C-EL2Ab does not exert inhibitory effects on aggregation mediated by stimulatory pathways independent of TPR, we analyzed its effects on ADP induced aggregation. It was found that 100 nM C-EL2Ab did not produce any detectable inhibition on platelet aggregation induced by 15 μM ADP (data not shown). In addition, even when used at a concentration as high as 250 nM, which completely blocked U46619- and arachidonic acid-induced aggregation, C-EL2Ab lacked any inhibitory effect on human platelet aggregation mediated by ADP (Fig. 4A). Similar results were obtained using murine platelets (Fig. 4B). These findings suggest that the mechanism by which C-EL2Ab inhibits

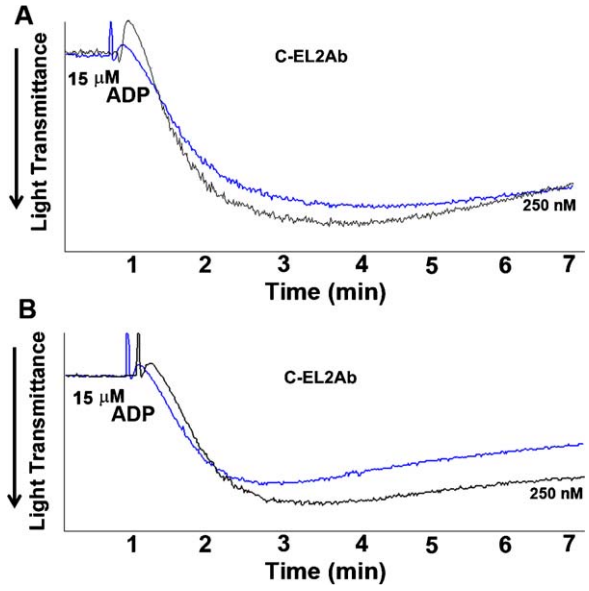

Fig. 4. Effect of C-EL2Ab on ADP-induced human and mouse platelet aggregation. (A) Indomethacin-treated human PRP was stimulated with 15 μM ADP in the absence or presence of C-EL2Ab (250 nM). (B) Indomethacin-treated mouse PRP was stimulated with 15 μM ADP in the absence or presence of C-EL2Ab (250 nM). Each aggregation curve is representative of multiple traces obtained from three separate platelet preparations, from three different blood donors or from three groups of (6–8) mice.

platelet aggregation is independent of ADP receptors (i.e., $P2Y_1$ and $P2Y_{12}$).

Furthermore, control antibodies EL1Ab (250 nM) and normal rabbit IgG (250 nM) did not produce any significant inhibition of aggregation induced by 15 μM ADP (data not shown). Similar findings were also observed with murine platelets (data not shown).

3.3. C-EL2Ab does not inhibit TRAP1- (human) and TRAP4- (human and murine) induced aggregation of platelets in vitro

We next assessed the effect of C-EL2Ab and control antibodies on the protease-activated receptor platelet activation pathway. It was found that C-EL2Ab, even when used at 250 nM, did not exert any inhibition on human platelet aggregation induced by 60 μM TRAP1 (Fig. 5A). Separate analysis was performed using 40 μM TRAP4 to target the PAR4 pathway in human and murine platelets, and C-EL2Ab was found to be devoid of any inhibitory effects under these conditions as well (Fig. 5B; human data not shown).

Comparable with the control untreated PRP, EL1Ab at 250 nM and IgG at 250 nM produced no detectable effect on the aggregation response to TRAP1 and TRAP4 (data not shown). These findings support our hypothesis that the effects of C-EL2Ab on platelet function are rather TPR specific. We next sought to investigate the underlying mechanism for the inhibitory effects C-EL2Ab exerts on platelet activation by TPRs. Thus, to confirm C-EL2Ab interaction with TPR's C-EL2, radiolabeled binding studies were performed to establish its ability to displace the classic TPR antagonist [^{3}H]SQ29,548.

3.4. C-EL2Ab competitively displaces radiolabeled [^{3}H]SQ29,548 from TPR ligand binding sites

In order to define the molecular mechanism by which C-EL2Ab selectively blocks TPR-mediated platelet aggregation, a radiolabeled ligand binding assay was performed. This is based on the notion that since this antibody was raised against a region (C-EL2) found to be part of the ligand binding pocket, it would be expected to effectively compete with other ligands for receptor binding. Thus, platelets were incubated with the radiolabeled TPR antagonist [^{3}H]SQ29,548, and increasing concentrations of C-EL2Ab were added (0.1–1000 nM). We indeed observed a dose dependent displacement of [^{3}H]SQ29,548 from its ligand binding site on TPR, by C-EL2Ab (Fig. 6A) in human platelets, which was not observed when C-EL2Ab was preabsorbed with its cognate peptide (Fig. 6B). Similar results were observed in mouse platelets (Fig. 6C and data not shown). On the other hand, neither EL1Ab nor IgG (data not shown) was with any apparent effect on [^{3}H]SQ29,548 binding.

We next examined whether the observed TPR-specific *in vitro* antiplatelet activity of C-ELAb would still hold in an animal model.

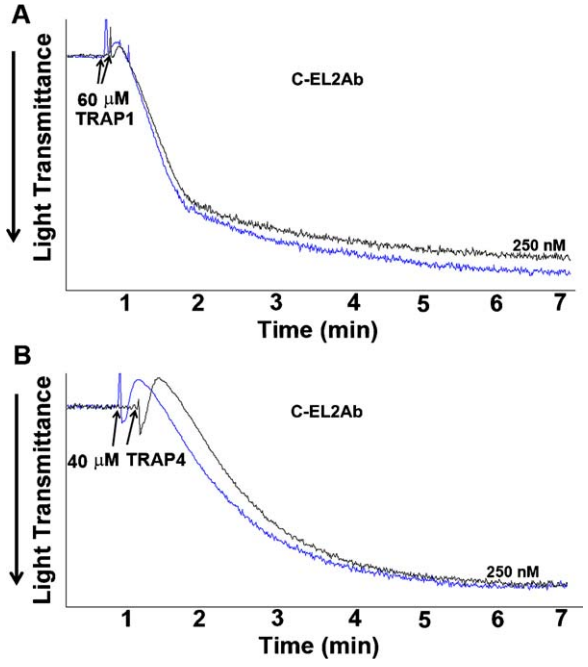

Fig. 5. Effect of C-EL2Ab on TRAP1- (human) and TRAP4- (mouse) induced platelet aggregation. (A) Indomethacin-treated human PRP was stimulated with 60 μM TRAP1 in the absence or presence of C-EL2Ab (250 nM). (B) Indomethacin-treated mouse PRP was stimulated with 40 μM TRAP4 in the absence or presence of C-EL2Ab (250 nM). Each aggregation curve is representative of multiple traces obtained from three separate platelet preparations, from three different blood donors or from three groups of (6–8) mice.

3.5. C-EL2Ab inhibits TPR-mediated aggregation of murine platelets ex vivo

Thus far, our experiments clearly show that C-EL2Ab has the capacity to inhibit platelet aggregation induced by TPR agonists under *in vitro* experimental conditions. Our next goal was to test for a potential pharmacologic/antithrombotic activity for C-EL2Ab in an animal model (*ex vivo*). Our studies demonstrated that C-EL2Ab injections into live animals resulted in a dose-dependent (250–325 nM) inhibition of platelet aggregation stimulated by the TPR agonist U46619 (1 μM), or 0.5 mM of the TXA_2 precursor arachidonic acid (Fig. 7A and B, respectively). The specificity of this effect was demonstrated by loss of C-EL2Ab ability to inhibit TPR-mediated aggregation after preabsorption with 100 μM of its cognate peptide (Fig. 7C and D). Further control experiments revealed that neither EL1Ab nor normal rabbit IgG (325 nM) exerted any detectable effects on murine platelet aggregation stimulated by 1 μM U46619 or 0.5 mM arachidonic acid (Fig. 8; IgG and arachidonic acid data not shown).

These findings demonstrate that C-EL2Ab does indeed have the capacity to attenuate platelet aggregation in an animal model *ex vivo*, and reinforces the notion that the underlying mechanism involves TPR antagonism.

3.6. C-EL2Ab does not inhibit ADP or TRAP4-induced aggregation of murine platelets ex vivo

To further confirm the selectivity of C-EL2Ab injections in our animal model studies, we analyzed its effects on ADP- or TRAP4-induced aggregation. It was found that administration of C-EL2Ab even at a concentration as high as 325 nM did not attenuate platelet aggregation in response to 15 μM ADP or 40 μM TRAP4 (Fig. 9A and B, respectively). These data support the notion that the effects of C-EL2Ab on platelet function are TPR specific, not only *in vitro*, but also *ex vivo*.

In summary, the present studies demonstrate that C-EL2Ab has the capacity to exert TPR-specific inhibitory effects on platelet function, as demonstrated by: (1) selective blockade of U46619- and arachidonic acid-induced aggregation; (2) displacement of binding of the classical TPR antagonist [^{3}H]SQ29,548; and (3) lack of effect on ADP or TRAP1/TRAP4 stimulated platelet aggregation. On the other hand, EL1Ab and normal rabbit IgG were unable to produce these aforementioned inhibitory effects.

Collectively, these findings provide evidence that the C-EL2 domain of TPR plays a critical role in platelet activation/aggregation, and that it may serve as a target for therapeutic interventions. Furthermore, C-EL2Ab itself may define a new therapeutic approach for managing thrombosis-based disorders.

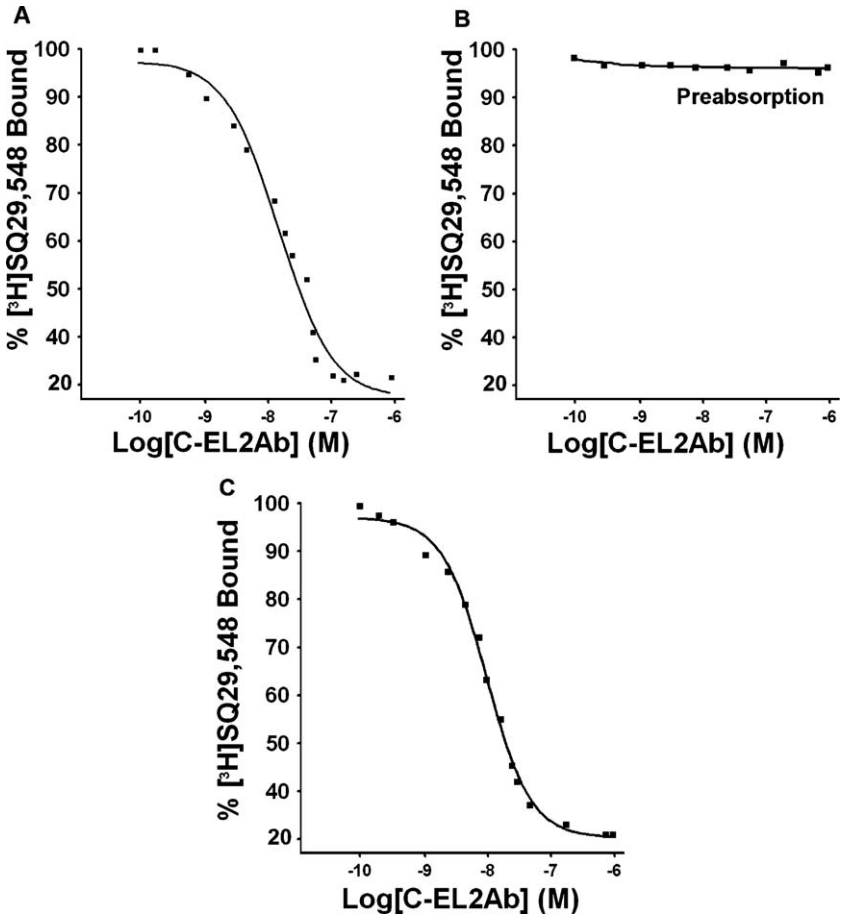

Fig. 6. Displacement binding of [^{3}H]SQ29,548 by C-EL2Ab, in intact human and mouse platelets. (A) Binding displacement of 1 nM [^{3}H]SQ29,548 with increasing concentrations of C-EL2Ab (0.1–1000 nM), in human platelets. (B) Binding displacement of 1 nM [^{3}H]SQ29,548 with increasing concentrations of C-EL2Ab (0.1–1000 nM) which was preabsorbed with 100 μM of its cognate peptide, in human platelets. (C) Binding displacement of 1 nM [^{3}H]SQ29,548 with increasing concentrations of C-EL2Ab (0.1–1000 nM), in mouse platelets. Results are the average of at least three different experiments, from blood obtained from three separate donors, or from three groups of (6–8) mice.

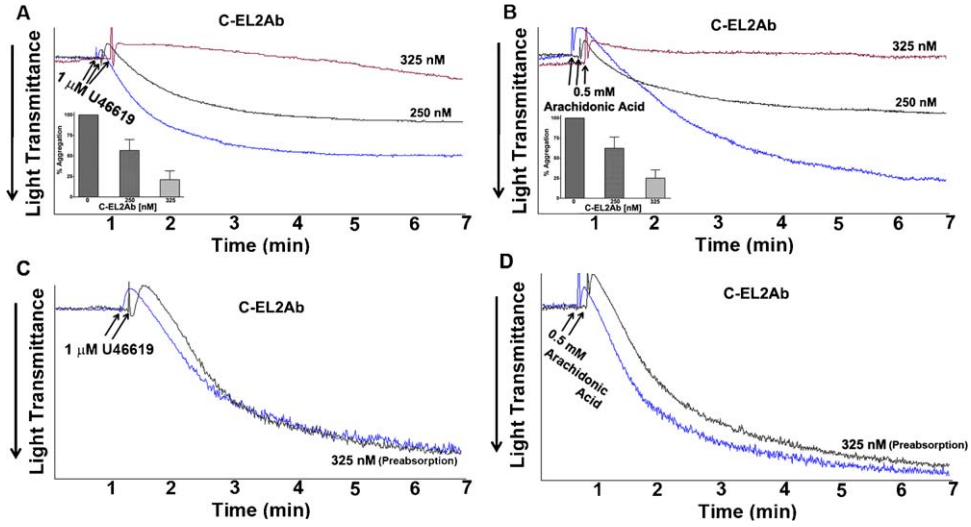

Fig. 7. Inhibition of TPR-mediated mouse platelet aggregation by C-EL2Ab, *ex vivo*. (A) Indomethacin-treated mouse PRP prepared from animals injected with C-EL2Ab (250–325 nM) was stimulated with 1 μM U46619 to generate a concentration-dependent inhibition curve (inset shows quantification of the C-EL2Ab concentration-dependent inhibition curve of the U46619-induced aggregation response). (B) Mouse PRP prepared from animals injected with C-EL2Ab (250–325 nM) was stimulated with 0.5 mM arachidonic acid to generate a concentration-dependent inhibition curve (inset shows quantification of the C-EL2Ab concentration-dependent inhibition curve of the arachidonic acid-induced aggregation response). (C) Indomethacin-treated mouse PRP prepared from animals injected with C-EL2Ab (325 nM) preabsorbed with 100 μM of its cognate peptide was stimulated with 1 μM U46619. (D) Mouse PRP prepared from animals injected with C-EL2Ab (325 nM) preabsorbed with 100 μM of its cognate peptide was stimulated with 0.5 mM arachidonic acid. Each aggregation curve is representative of multiple traces obtained from three separate platelet preparations from three groups of (6–8) mice.

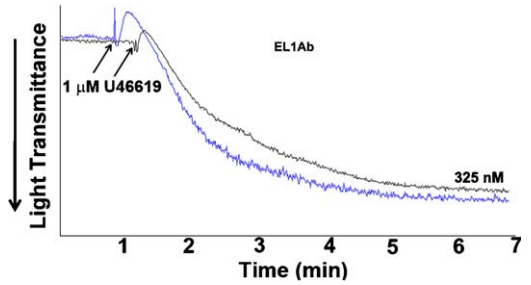

Fig. 8. Effect of EL1Ab on TPR-mediated platelet aggregation *ex vivo*. Indomethacin-treated mouse PRP prepared from animals injected with EL1Ab (325 nM) was stimulated with 1 μM U46619. Each aggregation curve is representative of multiple traces obtained from three separate platelet preparations from three groups of (6–8) mice.

4. Discussion

Current antiplatelet agents function by targeting certain GPCRs (e.g., $P2Y_{12}$ receptor) [40] and integrins, which reside on the surface of platelets, or by inhibiting endogenous biological processes such as TXA_2 production (via COX-1 inhibition) [13]. Nonetheless, to define alternatives to current antiplatelet therapies, the various activation pathways of platelets must be thoroughly examined. Currently, there is potential for targeting several signaling pathways on platelets, such as the ADP $P2Y_1$ receptor, thrombin receptors, Von Willebrand and collagen receptors [41]. Equally important is the TPR pathway, which is activated by the lipid TXA_2, currently targeted by aspirin therapy. To this end, given the limitations of aspirin (e.g., ulcers, GI bleeding, and resistance), there is considerable interest in means for the selective targeting of this receptor (e.g., antagonist), which would be expected to reduce adverse effects. Thus far, the discovery of antiplatelet drugs which directly inhibit TPR is yet to find FDA clearance and clinical success. On this basis, we explored the use of function blocking antibodies as an avenue for defining a new and a novel approach to target TPRs. In this connection, work using antibodies against antagonists for TPR has been documented, as far back as the early 1990s. Thus, monoclonal anti-idiotypic antibodies to the antagonist SQ29,548 were developed and tested for activity. Indeed, some of these antibodies displayed minor attenuation of aggregation in response to TPR specific agonists [25], thereby providing further incentive to study the TPR ligand binding domains, and pursue antibodies for therapeutic purposes.

Given that C-EL2 of TPR was previously shown to contain an important ligand binding site, as well as amino acids which participate in ligand recognition [32,38], we sought to investigate whether an antibody targeting this region (referred to as C-EL2Ab) would exhibit pharmacological activity, much like a classical antagonist would. This approach would provide a benefit of enhanced ability to target a particular receptor, and minimize adverse interactions with side targets. The use of antibodies (e.g., C-EL2Ab) to decrease platelet aggregation and treat thrombotic disorders would represent a specifically tailored treatment that would ideally reduce the risk of bleeding, that other antiplatelet therapies possess [42], particularly because of their limited tissue distribution.

We characterized the antiplatelet activity of C-EL2Ab under *in vitro* (human and mouse) and *ex vivo* (mouse) experimental conditions. Our results indicate that C-EL2Ab dose-dependently blocked aggregation mediated by TPR activation. The specificity of targeting C-EL2 was underscored by the findings that C-EL2Ab "lost" its ability to inhibit aggregation after preabsorption with its cognate, and that EL1Ab (and normal rabbit IgG) was devoid of any inhibitory effects on TPR-mediated platelet aggregation. Taken together, these findings indicate that, unlike EL1, C-EL2 plays an important role in TPR-dependent platelet activation, which is not surprising given that C-EL2Ab targets a domain that is critical for ligand binding [32,38].

The potential for homology to exist between receptors is not uncommon, and the use of homology of structures is utilized to help identify new therapeutic targets [43,44]. This being the case, coupled with existence of multiple platelet feedback activation pathways, there is a chance that attenuation of aggregation in response to TPR agonists may be due to recognition of separate receptors involved in platelet aggregation. Such receptors include the purinergic family $P2Y_1$ and $P2Y_{12}$ receptors which bind to ADP, and the protease activated receptors (PARs) which bind to thrombin [45]. Further analysis revealed that C-EL2Ab did not inhibit platelet aggregation stimulated by either ADP or TRAP4 (and TRAP1 in human platelets). Furthermore, as expected, separate control studies showed that EL1Ab and normal rabbit IgG did not inhibit aggregation under conditions of stimulation with ADP and TRAP4. These data indicate that C-EL2Ab has the capacity to inhibit TPR-dependent platelet function. These data further suggest that the underlying mechanism for the observed inhibitory effect is antibody interaction with a TPR ligand binding domain, i.e., the C-EL2 region.

To investigate the mechanism of action of C-EL2Ab, radioligand displacement binding studies were performed using a selective TPR antagonist, SQ29,548. Our results revealed that C-EL2Ab competitively displaced [^{3}H]SQ29,548 from its platelet TPR binding sites. This finding does indeed confirm that C-EL2Ab exerts its inhibitory effects by directly binding to TPR's ligand binding pocket, thereby preventing TXA_2 from binding to and activating the receptor protein.

Currently marketed antibody therapies include the monoclonal antibody abciximab, which was derived from a murine antibody, and developed as a chimeric IgG [46]. It acts by binding to the platelet GPIIb/IIIa integrin sites, thereby inhibiting the binding of fibrinogen, an essential factor for platelet adhesion. This "new"

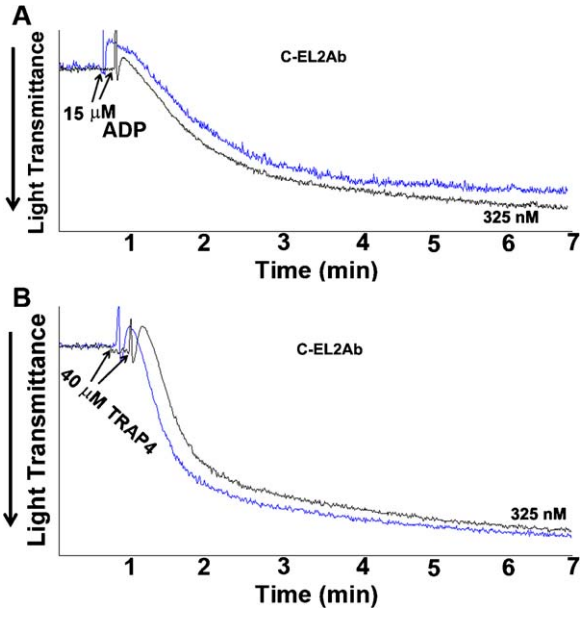

Fig. 9. Effect of C-EL2Ab on ADP- and TRAP4-induced mouse platelet aggregation, *ex vivo*. (A) Indomethacin-treated mouse PRP was stimulated with 15 μM ADP in the absence or presence of C-EL2Ab (325 nM). Each aggregation curve is representative of multiple traces obtained from three separate platelet preparations from three groups of (6–8) mice. (B) Indomethacin-treated mouse PRP was stimulated with 40 μM TRAP4 in the absence or presence of C-EL2Ab (325 nM). Each aggregation curve is representative of multiple traces obtained from three separate platelet preparations from three groups of (6–8) mice.

class of antiplatelet drugs allows for immediate antiplatelet treatment in cardiovascular intervention surgeries, however, like in all antiplatelet therapies, possible adverse effects must be noted. One of the areas of concern surrounding the administration of abciximab, is the incidence of thrombocytopenia [47], which has been associated with deadly bleeding events [48].

Additionally, the use of abciximab has been limited due to its intravenous route of administration. While the intravenous route of administration may also limit the use of C-EL2Ab, one may predict that bleeding and thrombocytopenia, which are associated with abciximab, are not going to be an issue. Thus, C-EL2Ab may offer a ''safer'' alternative when immediate antiplatelet activity is warranted.

These factors alone should be sufficient enough to keep searching for perhaps a better target for inhibition of platelet aggregation, such as TPRs. To this end, since an Ab-based biological has been approved by the FDA as an antiplatelet therapeutic, it is possible that C-EL2Ab would also have clinical applications, e.g., in acute conditions. This proposed therapeutic approach may be superior to a classical antagonist because its effects would, most likely, be predominantly limited to platelet TPRs. This is because the distribution of antibodies to compartments other than the vascular would be, in general, restricted due to poor penetration of the endothelial cell layer. Thus, this approach may allow us to address bleeding time and thrombosis under conditions of selective modulation of the platelet TPRs, but not those of the smooth muscle (which are known to affect bleeding time).

The basis for therapeutic treatment using antibodies is not completely novel. In total, nearly 300 monoclonal antibodies are in clinical development for a variety of targets including 10 which are designated for specific cardiovascular treatments. The focus of most research in this area has involved using monoclonal antibodies which are aimed to target various cancers. In fact, nearly 15 monoclonal antibodies have been approved by regulatory agencies around the world specifically for cancer treatments [49]. In addition to cancer therapy, there has been success in research combating antibody-mediated autoimmune diseases with the use of experimental drug rituximab [50]. Additionally, improving technology has produced interest in creating dual specificity antibodies [51]. The intrigue of antibody treatment is in its selectivity and specificity which can differentiate it from traditional small molecule drugs. With emerging bioengineering techniques, current progress in antibody research has the potential to produce many novel antibody molecules with therapeutic applications [49]. Based on these considerations, this methodology can easily be translated to new antiplatelet drugs which can help reduce the issue of acquired platelet dysfunction, attributed to overmedication for cardiovascular and thrombotic disorders with drugs such as aspirin, clopidogrel, as well as the broad range GPIIb/IIIa antagonists abciximab, eptifibatide, and tirofiban [52].

Other antibodies characterized as potential antiplatelet therapy include an antibody to PAR3 on mouse platelets. It was found that pre-treating mouse platelets with this antibody, inhibited activation of platelets in response to thrombin, but not U46619 [53]. Thus, antibodies with function blocking activity (which target receptor ligand binding domains) may define a new paradigm for targeting other GPCRs, and for managing multiple disease states.

We are currently in the process of characterizing the antiplatelet activity of C-EL2Ab under *in vivo* experimental settings (e.g., a carotid artery injury thrombosis models). These studies will determine whether the observed *in vitro*/*ex vivo* effects of C-EL2Ab do translate into thromboprotective properties. If C-EL2Ab produces significant effects on platelet function *in vivo*, efforts will be made to generate monoclonal antibodies against this ligand recognition sequence.

Our present studies provide evidence that the C-EL2 region of TPRs plays a vital role in platelet function, and that an antibody targeting this critical domain (C-EL2Ab) has the capacity to selectively inhibit TPR-mediated platelet activation, and displace antagonist binding. Further studies are warranted to investigate the clinical implications of our results. Finally, the identification of a functionally active TPR sequence will aid molecular modeling study predictions for organic derivatives which possess *in vivo* activity. This is an important consideration because in spite of the clear involvement of TPR signaling in occlusive vascular disease, aspirin is still the only clinically effective drug for the prevention of TPR-mediated platelet activation. Thus, the availability of a pharmacologically effective non-aspirin derivative with anti-TPR activity could have widespread therapeutic applications, given the limitations of aspirin therapy.

Acknowledgements

This research was supported by funds provided by the Regents of the University of California, Tobacco-Related Disease Research Program, Grant Number 19KT-0030 (to F.T.K.). The opinions, findings and conclusions herein are those of the author and not necessarily represent those of the Regents of the University of California, or any of its programs. This work was also supported by Intramural Funding from the College of Pharmacy at Western University of Health Sciences (to F.T.K.).

This work has been approved by Institutional Animal Care and Use Committee at Western University of Health Sciences.

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