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A novel antibody targeting the ligand binding domain of the thromboxane A_2 receptor exhibits antithrombotic properties *in vivo*

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ABSTRACT

In efforts to define new targets for antithrombotic purposes, there is interest in utilizing antibodies targeting ligand binding domains of platelet receptors. To this end, we have recently shown that an antibody (designated C-EL2Ab), which targets the C-terminus of the 2nd extracellular loop (C-EL2) of the thromboxane A_2 receptor (TPR), selectively blocks TPR-mediated platelet aggregation, under both *in vitro* and *ex vivo* experimental conditions. In the current studies we sought to determine whether C-EL2Ab exhibits *in vivo* antithrombotic activity, by employing a carotid artery injury thrombosis model. It was found that mice treated with C-EL2Ab, exhibited a significant increase in time for occlusion, when compared to controls such as normal rabbit IgG, or an antibody which targets a region separate from the ligand binding site (i.e., EL1). We next examined the effect of C-EL2Ab on hemostasis, and found no increase in tail bleeding times in C-EL2Ab treated mice, compared to the aforementioned controls. Collectively, these results clearly demonstrate that C-EL2Ab has anti-platelet/anti-thrombotic effects, and is devoid of increased bleeding risk. Moreover, the identification of a functionally active TPR sequence should significantly aid molecular modeling study predictions for organic derivatives which possess *in vivo* activity.

1. Introduction

Hemostasis is a physiological process that is orchestrated by the concerted response of platelets, the vessel wall and coagulation factors. The inappropriate activation of hemostatic mechanisms may lead to thrombosis, with potentially fatal consequences, such as stroke, and/or myocardial infarction (MI) [1,2]. Moreover, the development of thromboembolic events is closely related to unrestrained platelet activation and aggregation [3]. Consequently, better understanding of mechanisms of platelet activation, will not only define novel pharmacological approaches/agents, but may also reduce the adverse effects of current therapies.

Up regulation of signaling through platelet thromboxane A_2 receptor (TPR) has been implicated in the pathogenesis of multiple thrombosis-based diseases [4,5]. The role of TPRs in maintaining hemostasis is supported by the finding that a point mutation in human TPR results in bleeding disorders [6]. Thus, for the safe and effective targeting of this pathway, there must be a balance between platelet and systemic TPR antagonism (ideal thromboprotection). In this connection, clinical findings have shown a significant correlation between inhibition of platelet thromboxane A_2 (TXA_2) production (i.e., by aspirin therapy) [7,8], and the incidence of MI and stroke [8,9].

Despite the crucial role of TPR in platelet function, not a single TPR antagonist has been approved by the Food and Drug Administration (FDA), for clinical use. Regarding current antiplatelet agents, the thienopyridine class of $P2Y_{12}$ receptor antagonists (e.g., clopidogrel) makes up a large majority of therapies which target G-protein coupled receptors (GPCRs) [10].

Targeting with more efficiency, are the glycoprotein IIb/IIIa (GPIIb/IIIa) inhibitors like the monoclonal antibody abciximab. While these inhibitors eliminate physical platelet aggregation at fibrinogen binding sites [11], they result in significant increases in hemorrhagic events compared to traditional therapies [12,13].

Even the most classic of antiplatelet drugs, the COX-1 inhibitor aspirin, has adverse effects and complications with regards to GI bleeding, resistance issues, as well as a host of questions regarding efficacy in certain disease states [14,15].

Given the limitations of current therapies, there remains interest in defining new therapeutic approaches and/or targets for managing thromboembolic disorders. To this end, we have recently shown that an antibody (designated C-EL2Ab) which recognizes TPR's ligand binding domain (i.e., the C-terminus of the 2nd extracellular loop (C-EL2)), selectively blocked TPR-mediated (murine and human) platelet aggregation [16].

In the present manuscript, we characterized the *in vivo* pharmacological activity of C-EL2Ab by employing mouse models of hemostasis and thrombosis. Our results revealed that C-EL2Ab selectively blocked TPR-induced platelet aggregation (*ex vivo*),

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significantly prolonged the time for occlusion in a carotid artery injury thrombosis model, whereas (interestingly) it did not produce any detectable effects on the tail bleeding times. These findings make C-EL2Ab the first functional antibody with *in vivo* antithrombotic activity against platelet TPRs, and suggest that it may be the "ideal" antiplatelet agent. These findings underscore the notion that purposing C-EL2Ab as an antiplatelet agent has reasonable probability of clinical success; especially when compared to the GPIIb/IIIa antagonist abciximab.

2. Methods and materials

2.1. Reagents and materials

U46619, SQ29,548, and arachidonic acid were from Cayman Chemical (Ann Arbor, MI). ADP and other platelet disposables were from Chrono-Log (Havertown, PA). Thrombin receptor activating peptide 4 (AYPGKF-NH_2), isoflurane and indomethacin were from Sigma Aldrich (St. Louis, MO). Hanks phosphate buffered saline solution (HBSS), and sodium citrate (3.8% w/v), Whatman™ filter paper, ferric chloride [$FeCl_3$], Hepes/Tyrode's buffer: sodium chloride, potassium chloride, sodium dihydrogen phosphate, magnesium chloride, sodium bicarbonate, and D-dextrose were from Fisher Scientific (Hanover Park, IL). Bovine serum albumin (BSA) was from Equitech-Bio, Inc. (Kerrville, TX). The polyclonal antibodies C-EL2Ab and EL1Ab were a generous gift from Dr. Guy Le Breton, The University of Illinois at Chicago [17]. The C-EL2 peptide (CFLTLGAESGD) was custom made by United Biosystems Inc. (Cabin John, MD). Normal rabbit IgG was purchased from Cell Signal Technology (Danvers, MA).

2.2. Animals

C57BL/6 J mice were from Jackson Laboratories (Bar Harbor, ME). All mice used for experiments were 6–10 weeks of age. All experiments involving animals 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.

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

A group of six to eight mice (6–10 weeks old) received tail vein injections of C-EL2Ab (250–325 nM), EL1Ab (325 nM), IgG (325 nM), SQ29,548 (1–50 nM) and Hanks/HBSS vehicle (equal volume) once daily for 5 days, including the day of experiment/blood collection. Mice were anesthetized with 5% isoflurane and sacrificed using cervical decapitation, and blood was collected using 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 at 170 *g* for 10 min. Platelet counts were adjusted to 2–3 $\times$ 10^8/ml with Hepes/Tyrode's buffer. Note: we did not observe a difference in platelet count between C-EL2Ab and control treated animals. 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 (AA), 15 μM ADP, or 40 μM TRAP4.

2.4. Tail bleeding time assay

Mice 6–10 weeks old received tail vein injections of either C-EL2Ab (325 nM), EL1Ab (325 nM), IgG (325 nM), SQ29,548 (50 nM) or Hanks HBSS vehicle (equal volume) once daily for 5 days, including the day of experiment.

Hemostasis was measured using the tail transection technique [18]. Briefly, mice were anesthetized using isoflurane and then placed on a 37 °C heating blanket (Harvard Apparatus Limited, Edenbridge, KY, USA) before the tail was transected with a clean cut using a sterile scalpel at a distance of 5 mm from the tip. After transection, the tail was immediately immersed in warmed saline (37 °C, constant temperature). Bleeding was observed visually and recorded as the time from the tail transection to the moment the blood flow stopped and did not resume within 60 s from the initial cessation time. Normal bleeding times for murine specimens lasts between 1–3 min. However, when bleeding did not stop within 15 min, pressure was applied to the tail and styptic powder was used to help close the wound, thus avoiding excessive loss of blood. Bleeding times beyond 15 min were considered as the cut-off time for the purpose of statistical analysis.

2.5. Arterial thrombosis model protocol

These studies were performed as described previously [19]. Mice 6–10 weeks old received tail vein injections of either C-EL2Ab (325 nM), EL1Ab (325 nM), IgG (325 nM), C-EL2Ab preabsorbed with 100 μM of its cognate peptide, SQ29,548 (50 nM) or Hanks HBSS vehicle (equal volume), once daily for 5 days, including the day of experiment.

Operators were blinded to the drug or vehicle treatment while performing these studies. Animals were anesthetized with tribromoethanol (Avertin, 250 mg/kg, i.p. Sigma Aldrich. St. Louis, MO), and placed on a 37 °C heated surgical table under a stereo microscope (Leica Microsystems Ltd, CH-9435, Heerbrugg, Switzerland). A midline incision of the skin was made directly on top of the right common carotid artery region, and a segment of the left common carotid artery was exposed and cleaned. Baseline carotid blood flow was measured and recorded with a miniature Doppler flow probe (Model 0.5 VB, Transonic System, Ithaca, NY, US), interfaced with a flowmeter (model TS402, Transonic Systems). Thrombosis was induced by applying a saturated segment of filter paper (0.5 $\times$ 1 mm) in 7.5% ferric chloride, onto the carotid artery. After 3 min of exposure, the filter paper was removed. The carotid blood flow was continuously monitored for 15 min after ferric chloride application, and the data was registered by a computerized data acquisition program (LabChart®6, ADInstruments, Colorado Springs, CO, USA). Time to occlusion was calculated as the difference in time between the removal of the filter paper and stable occlusion, which was described as zero blood flow for 2 min. An occlusion time beyond 15 min was considered as the cut-off time for the purpose of statistical analysis.

2.6. Statistical analysis

All *ex vivo* experiments were performed at least three times, with blood pooled from at least six mice each time. Results displayed in the occlusion and bleeding time figures are measurements from individual mice; data from each group are expressed as the median value obtained. Values for time to occlusion, and tail bleeding time were compared by Mann Whitney non-parametric analysis using GraphPad PRISM statistical software (San Diego, CA), for the evaluation of differences in median. Significance was accepted at $P < 0.05$ (two-tailed P value), unless stated otherwise.

3. Results

3.1. C-EL2Ab selectively inhibits TPR-mediated platelet aggregation under ex vivo experimental settings

Our recently published results of *in vitro* and *ex vivo* aggregation data provided evidence that C-EL2Ab targets the C-EL2 domain of

TPR and has the capacity to selectively inhibit TPR-mediated platelet activation [16]. Therefore our initial goal was to repeat these *ex vivo* experiments, in order to properly select the dose of C-EL2Ab for the *in vivo* studies.

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 AA (Fig. 1A; AA data not shown), as was previously shown [16]. 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. 1B). Control experiments revealed that the classical TPR antagonist SQ29,548 produced dose-dependent inhibition of aggregation triggered by 1 μM U46619 (1–50 nM; data not shown). Further control experiments revealed that neither normal rabbit IgG, nor EL1Ab (325 nM) exerted any detectable effects on murine platelet aggregation stimulated by 1 μM U46619 or 0.5 mM AA (data not shown), as was previously reported [16].

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 aggregation in response to 15 μM ADP, or 40 μM TRAP4 (Fig. 1C; TRAP4 data not shown); as was previously shown [16].

These findings demonstrate that C-EL2Ab does indeed have the capacity to attenuate platelet aggregation in an animal model *ex vivo*, reinforces that the underlying mechanism involves TPR antagonism, and supports the use of 325 nM C-EL2Ab as a suitable dosage in the *in vivo* models of thrombosis and hemostasis.

3.2. C-EL2Ab prolongs the time for occlusion in a mouse carotid artery injury-induced thrombosis model

Our next goal was to investigate whether this antiplatelet activity of C-EL2A would translate into thromboprotective properties (*in vivo*). Therefore, a mouse carotid artery injury thrombosis model was utilized, and the time for occlusion was measured after injury was induced by $FeCl_3$. Our studies revealed that mice treated with 325 nM of C-EL2Ab exhibited a significant increase in the time for occlusion in comparison to the animals treated with the IgG control (900 s *versus* 188 s; median, $p < 0.001$; Fig. 2). To further establish the specificity of C-EL2Ab, cognate peptide preabsorption experiments were performed. It was found that preabsorbing C-EL2Ab with its cognate peptide reversed its capacity to prolong the time for occlusion (Fig. 2). Moreover, separate control experiments revealed that EL1Ab did not produce any detectable effects on the time for occlusion (Fig. 2), whereas 50 nM of the TPR antagonist SQ29,548 did prolong the time for occlusion ($p < 0.01$; Fig. 2). Taken together, these findings demonstrate that C-EL2Ab prolongs the time for occlusion, and hence provides thromboprotective effects. Furthermore, these data provide evidence that the C-EL2 domain of TPR plays an important role in the genesis of thrombosis, *in vivo*.

3.3. C-EL2Ab does not prolong tail bleeding time, in mice

Given the increased bleeding risk associated with antithrombotic therapies, and in order to define the clinical potential of C-EL2Ab, we next examined its effects with regards to hemostasis. To this end, tail bleeding time experiments were performed. Our studies revealed that, in comparison to the saline vehicle control (median bleeding time = 154 s; Fig. 3), mice treated with C-EL2Ab (325 nM) did not exhibit a statistically significant increase in tail bleeding time (median = 189 s; $p = 0.4307$; Fig. 3). Similar results were obtained with 325 nM EL1Ab (median bleeding time = 154 s; $p = 0.2421$; Fig. 3), and with animals treated with 325 nM IgG (median time = 190 s; $p = 0.4047$; Fig. 3). This finding indicates that inhibiting the C-EL2 region of platelet TPRs, does not appear to have any adverse effect on hemostasis. Separate control experiments revealed that SQ29,548 (50 nM) did prolong bleeding time ($p < 0.001$; Fig. 3).

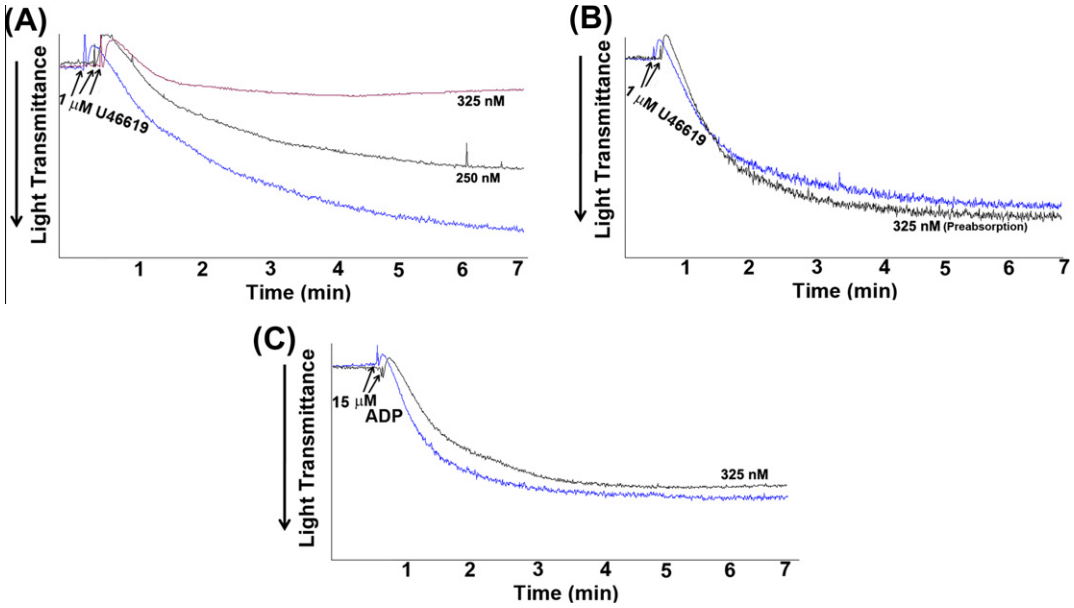

Fig. 1. C-EL2Ab selectively inhibits TPR-mediated mouse platelet aggregation *ex vivo*: (A) Indomethacin-treated mouse PRP prepared from animals injected with C-EL2Ab (250–325 nM; once daily for 5 days, including the day of experiment) was stimulated with 1 μM U46619 to generate a concentration-dependent inhibition curve. (B) Indomethacin-treated mouse PRP prepared from animals injected with C-EL2Ab (325 nM; once daily for 5 days, including the day of experiment) preabsorbed with 100 μM of its cognate peptide was stimulated with 1 μM U46619. (C) Indomethacin-treated mouse PRP prepared from animals injected with C-EL2Ab (325 nM; once daily for 5 days, including the day of experiment) was stimulated with 15 μM ADP. Each aggregation curve is representative of the traces obtained from three separate platelet preparations of 6–8 mice each.

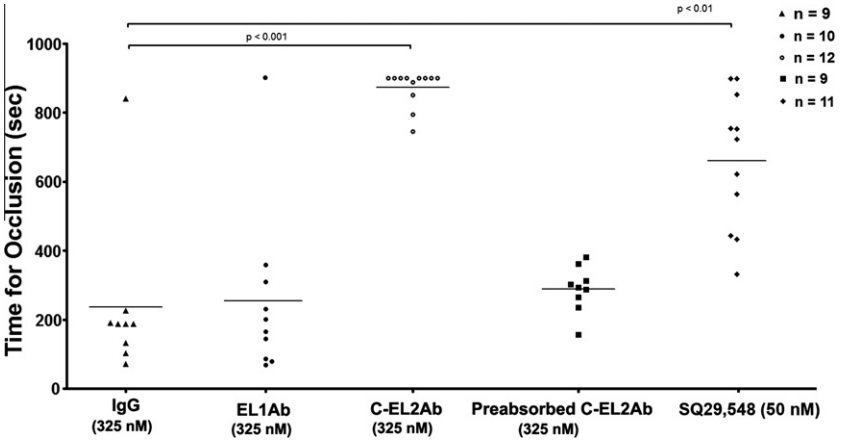

Fig. 2. C-EL2Ab delays formation of stable thrombi: Animals were divided into four groups, and injected (once daily for 5 days, including the day of experiment) with 325 nM IgG (▲), 325 nM EL1Ab (●), 325 nM C-EL2Ab (○), preabsorbed C-EL2Ab (■), and 50 nM SQ29,548 (♦).Each point represents a separate mouse.

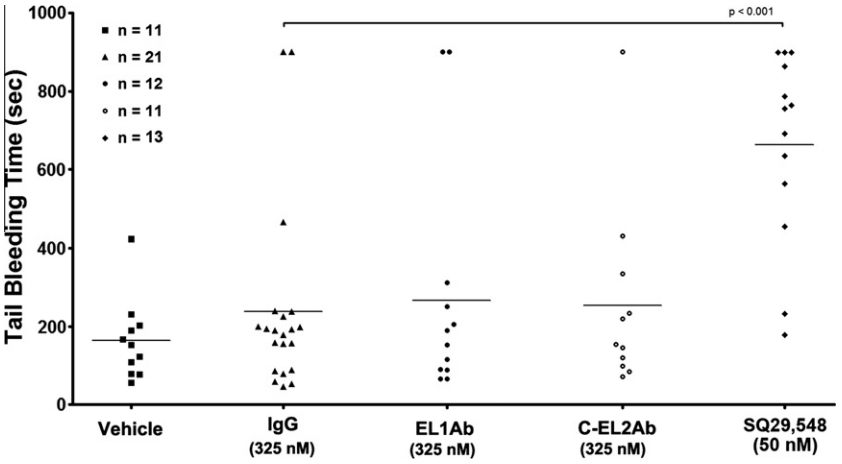

Fig. 3. C-EL2Ab does not prolong tail bleeding time: Animals were separated into four groups, and injected (once daily for 5 days, including the day of experiment) with saline (■),325 nM IgG treated group (▲), 325 nM EL1Ab treated group (●), 325 nM C-EL2Ab treated group (○), and 50 nM SQ29,548 (♦). Each point represents a separate mouse.

4. Discussion

Due to the limitations associated with aspirin use, there is renewed interest for defining additional TXA_2-associated drug targets. TPR antagonism would appear to be the most logical and attractive approach, from a basic pharmacology point of view. TPR antagonists developed as antiplatelet therapies and tested thus far have failed in various phases of drug development [3,20]. We hypothesized that the selective targeting of TPR's ligand-binding domain may produce antiplatelet effects. Indeed, we have recently shown that an antibody (C-EL2Ab) which targets TPR's ligand binding pocket, inhibited human and mouse platelet aggregation [16]. These findings laid the foundation for further *in vivo* analysis of the antithrombotic activity of C-EL2Ab. These studies are clearly warranted given that establishing the antiplatelet activity of drugs *in vitro*, does not necessarily guarantee *in vivo* effectiveness in managing thromboembolic disorders [21].

The present study initially established the *ex vivo* antiplatelet activity of C-EL2Ab, as it was found to selectively inhibit TPR-mediated platelet aggregation, in a dose-dependent fashion. These studies allowed a rational selection of the dose of C-EL2Ab, which was employed investigating the *in vivo* thromboprotective effects of this antibody, i.e., 325 nM was selected as it almost completely blocked aggregation. Thus, we next investigated the antithrombotic activity of C-EL2Ab, in order to address its clinical significance. By employing a carotid artery injury-induced thrombosis model, our results demonstrated that C-EL2Ab (and the classical TPR antagonist SQ29,548), prolonged the time for occlusion, relative to vehicle, indicating that it does indeed exhibit antithrombotic activity. Control studies confirmed the specificity of this inhibitory effect, as EL1Ab, normal rabbit IgG and preabsorbed C-EL2Ab were with no apparent activity. This result provides evidence that in mouse platelets, the C-EL2 of TPR plays an important role in thrombosis development *in vivo*.

As the main concern with all known antiplatelet treatments, we next examined the extent to which C-EL2Ab affects hemostasis, using the tail bleeding time experiments [18]. Our findings revealed no increase in bleeding time with C-EL2Ab, or either of the aforementioned controls, relative to the vehicle, whereas the TPR antagonist SQ29,548 did increase it. These finding indicates that while the C-EL2 region of TPR plays an important role in platelet function, targeting it with the C-EL2Ab does not appear to result in an adverse effect with regards to hemostasis.

The fact that there appears to be no enhanced bleeding risk with C-EL2Ab is rather interesting, given the presence of TPRs on vascular smooth muscle cells [22]. Nonetheless, our hypothesis as to why C-EL2Ab has no detrimental effects with regards to bleeding derives from the tight junctions, which act as protection and selective transport barrier from the lumen to the endothelium, having an estimated width of nearly 2 nm [23]. This is compared to the

estimated diameter of an IgG isotype from nearly 16 to 19 nm [24]. Thus, since C-EL2Ab is unlikely to reach the TPR sites on smooth muscle cells, it may not adversely affect vasoconstriction, and so will not allow for excessive bleeding.

In addition to the physical limitations of endothelium and C-EL2Ab interaction, genetic mutations to TPR has been identified to eliminate TPR coupling to Gq and reduce platelet functional responses but only have a mild bleeding phenotype [25]. Literature on the topic shows loss of TPR function with mild bleeding phenotype in an R60L mutation [26].

Taken together, our findings support a potential role for C-EL2Ab as a function-blocking reagent against platelet TPRs. Specifically, C-EL2Ab may provide an alternative to traditional TXA_2 suppression by aspirin, or serve as a substitute for the FDA-approved antiplatelet antibody abciximab [27].

To this end, and regarding antibody-based antiplatelet therapies, the direct GPIIb/IIIa inhibitor abciximab, which is a chimeric IgG [12], has been used for managing certain thromboembolic disorders. Abciximab allows for immediate treatment when emergency antithrombotic interventions are needed. However, one of the areas of concern with the use of abciximab, are the incidence of immune reactions, and thrombocytopenia [28], as well as deadly hemorrhagic events [29], and the limit to the IV route of administration [30]. While the route of administration limitation would also be shared by C-EL2Ab, there are potentially less bleeding events, as was observed with our tail bleeding time studies. This is especially true given that the C-EL2Ab antiplatelet activity is limited to TPRs, unlike the broad spectrum activity of abciximab. Regarding the concern for an immune response with C-EL2Ab, the use of single-chain variable-fragment (scFv) may be the avenue to address this issue, as it has already been investigated in the cancer therapeutics area [31]. Nonetheless, thrombocytopena does not appear to be an issue with C-EL2Ab considering that a bleeding phenotype was not observed, even after 5 days of its administration. Additionally, this notion is consistent with platelet counts prior to and after treatment with C-EL2Ab.

It's noteworthy that an antibody against the mouse protease activated receptor 3 (PAR3) have also been shown to exert antiplatelet effects, as it was found to inhibit platelet activation in response to thrombin, but not the TPR agonist U46619 [32]. Therefore, we believe that antibodies with function blocking activity (e.g., those which target a receptor's ligand binding domains), may define a new approach for targeting GPCRs, for managing multiple disease states.

In summary, the present manuscript provides evidence that: C-EL2Ab selectively inhibited TPR-mediated platelet activation; prolonged occlusion times (and thus provides antithrombotic effects); all without exerting detrimental effects on hemostasis. Our data also indicate that the C-EL2 region of TPRs play a vital role in platelet function, *in vivo*, and provide the basis for purposing a pharmacologically effective and potentially clinically relevant non-aspirin derivative, for targeting TPRs. Finally, the identification of a functionally active TPR sequence should significantly aid molecular modeling study predictions for organic derivatives which possess *in vivo* anti-TPR activity.

5. Disclosures

None.

Acknowledgments

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