This article has not been copyedited and formatted. The final version may differ from this version.
JPET Fast Forward. Published on September 16, 2013 as DOI: 10.1124/jpet.113.205591

JPET #205591

Title Page

The effects of an inhibitor of diglyceride lipase on collagen-induced platelet activation

Elke C.G. Jackson, Giorgio Ortar and Archie McNicol[†]

Department of Oral Biology (E.C.G.J., A.McN.) and Pharmacology & Therapeutics (A.McN.), University of Manitoba, Winnipeg, Manitoba, Canada; Dipartimento di Chimica e Tecnologie del Farmaco Università di Roma 'Sapienza', Roma, Italy (G.O.)

Running Title Page

Running Title: Effects of diglyceride lipase inhibition on platelet activation

Address for correspondence:

Dr. A. McNicol, Department of Oral Biology, 780 Bannatyne Avenue,

Winnipeg, Manitoba, Canada, R3E 0W2

Tel: (204) 789-3527; FAX: (204) 789-3913; E-mail: mcnicol@ms.umanitoba.ca

Number of text pages: 11 (Abstract, introduction, materials & methods, results and discussion)

Number of tables: 0

Number of figures: 6

Number of references: 42

Number of words in the Abstract: 231

Number of words in the Introduction: 725

Number of words in the Discussion: 1268

Non-standard Abbreviations: $AACOCF_3$, arachidonyl trifluoromethyl ketone; COX, cyclooxygenase; $cPLA_2$, cytosolic phospholipase A_2; DG, 1,2 diacylglycerol; DGL; diglyceride lipase; FcRγ, EIA, Enzyme Immunoassay; ELISA, Enzyme-Linked Immunosorbent Assay; ERK, p42/p44 isoform of mitogen-activated protein kinase; Fc-receptor γ-chain; GP, glycoprotein; IgG-HRP, Horseradish peroxidase-conjugated anti-immunglobulin G-conjugated antibody; inositol (1,4,5) trisphosphate; ITAM, immunoreceptor tyrosine-based activation motif; OMDM-188, *N*-formyl-L-isoleucine-(1*S*)-1-[[(2*S*,3*S*)-3-hexyl-4-oxo-2-oxetanyl]methyl]dodecyl ester; PLC, phospholipase C; PLD, phospholipase D; Tx, thromboxane; PLC; $p38^{MAPK}$, p38 isoform of mitogen-activated protein kinase.

Recommended section: Cellular and molecular

Abstract

Human platelet activation by collagen occurs in a dose-dependent manner. High concentrations of collagen bind to a pair of receptors, the α2ß1 integrin and GPVI/FcRγ, which stimulate a cascade of events including Syk, LAT, Btk , Gads and phospholipase Cγ2, leading to calcium release and protein kinase C (PKC) activation. Calcium and PKC are responsible for a range of platelet responses including exocytosis and aggregation, as well as the cytosolic phospholipase A_2 ($cPLA_2$)-mediated release of arachidonic acid which is converted to thromboxane (Tx) A_2. In contrast low concentrations of collagen are acutely aspirin sensitive, and calcium release and aggregation are TxA_2–dependent. Under these conditions $cPLA_2$ is not involved and it has been suggested that phospholipase C generates diglyceride (DG) from which arachidonic acid is liberated by diacylglycerol lipase (DGL). Here a novel DGL blocker (OMDM-188), inhibited collagen-, but not arachidonic acid-, induced aggregation and TxA_2 synthesis. Furthermore OMDM-188 inhibited collagen-induced arachidonic acid release. Finally OMDM-188 inhibited collagen-induced $p38^{MAPK}$, but not ERK, phosphorylation, with no effect on the phosphorylation of either enzyme in response to arachidonic acid. Taken together these data suggest a role for a pathway involving PLC liberating DG from membrane phospholipids in response to minimally-activate concentrations of collagen. The DG serves as a substrate for DGL, potentially under the regulations of $p38^{MAPK}$, to release arachidonic acid which is subsequently converted to TxA_2 which mediates the final platelet response.

JPET Fast Forward. Published on September 16, 2013 as DOI: 10.1124/jpet.113.205591
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Collagen is the most thrombogenic component of the subendothelial matrix. Endothelial damage results in the exposure of the collagen to circulating blood, in particular to platelets, resulting in haemostatic or atherothrombotic consequences. Multiple collagen receptors have been identified on the platelet surface including glycoprotein (GP)IV the α2ß1 integrin, immunoglobulin superfamily member GPVI, which is non-covalently coupled to the Fc-receptor γ-chain (FcRγ), the receptor-like protein tyrosine phosphatase CD148 and C-Type Lectin-Like Receptor 2 (CLEC-2) (Tandon et al., 1989; Nieswandt and Watson, 2003; Suzuki-Inoue et al., 2006; Farndale et al., 2007; Surin et al., 2008; Ellison et al., 2010). These individual receptors likely play specific roles to mediate collagen-induced platelet adhesion, activation and consolidation (Li et al., 2010; Kauskot and Hoylaerts, 2012).

Post-receptor signaling associated with collagen-induced platelet activation is particularly complex, partially concentration-dependent and influenced by transcellular secondary mediators thromboxane (Tx) A_2 and ADP (Nieswandt and Watson, 2003). The current evidence suggests that primary signaling occurs following the engagement of GPVI (Stalker et al., 2012). The interaction of collagen with GPVI leads to the phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) on FcRγ by the src family kinases Fyn and Lyn (Ezumi et al., 1998). The phosphorylated ITAM binds to and activates the tyrosine kinase Syk which in turn phosphorylates multiple downstream regulators, including LAT, Btk , Gads and phospholipase C (PLC)γ2 (Daniel et al., 1994; Gross et al., 1999; Atkinson et al., 2003; Hughes et al., 2008). The phosphorylation, and accompanying activation, of PLCγ2 leads to the formation of the inositol (1,4,5) trisphosphate (IP_3) and 1,2 diacylglycerol (DG) which, via the liberation of intracellular Ca^{2+} and activation of protein kinase C (PKC), mediate the platelet response, including the exocytosis of dense (δ) and alpha (α) granules, the expression of a pro-coaggulant surface, the synthesis and release of thromboxane (Tx) A_2 and the expression of adhesive receptors, all culminating in platelet aggregation (Stegner and Nieswandt, 2011).

However many of these observations have been made at high (≥20μg/ml) concentrations of collagen. At lower concentrations (1-2μg/ml), collagen-induced PLC activity, liberation of intracellular Ca^{2+} and aggregation are all sensitive to cyclo-oxygenase inhibition and are thus TxA_2-mediated (Narita et al., 1985; Kito et al., 1986; Pollock et al., 1986; Elvers et al., 2012),

JPET Fast Forward. Published on September 16, 2013 as DOI: 10.1124/jpet.113.205591
This article has not been copyedited and formatted. The final version may differ from this version.

reflecting the highly aspirin sensitive nature of platelet activation *in vivo* (Seymour et al., 1984; Gerrard et al., 1989). Agonist-induced synthesis of TxA_2 involves the release of arachidonic acid from membrane phospholipids followed by the sequential actions of cyclooxygenase (COX) and Tx-synthetase. Börsch-Haubold and colleagues demonstrated that cytosolic phospholipase A_2 ($cPLA_2$) was responsible for arachidonic acid release in response to high doses of collagen and this was modulated by pathways involving several kinases including the p38 isoform of mitogen-activated protein kinase ($p38^{MAPK}$) (Borsch-Haubold et al., 1995; Borsch-Haubold et al., 1997). In contrast, although inhibition of $cPLA_2$ by a pharmacological inhibitor, $AACOCF_3$, attenuated TxA_2 synthesis in response to low collagen concentrations (McNicol et al., 1998), there was no accompanying effect on arachidonic acid release (Lockhart et al., 2001), consistent with an action on COX rather than on $cPLA_2$ (McNicol and Nickolaychuk, 1995; Leis and Windischhofer, 2008). Similarly inhibition of $p38^{MAPK}$ had no effect on TxA_2 synthesis or arachidonic acid release in response to low collagen concentrations (Saklatvala et al., 1996; McNicol et al., 1998). Taken together it is therefore unlikely that a Ca^{2+} /$p38^{MAPK}$/ $cPLA_2$ pathway accounts for the release of arachidonic acid in response to low collagen concentrations.

Arachidonic acid can also be liberated from platelet membranes by the action of a second enzyme, diglyceride lipase (DGL), on DG (Bell et al., 1979; Rittenhouse-Simmons, 1980). Two potential DG-generating pathways are present in platelets; firstly the PLC pathway and secondly the consecutive actions of phospholipase D (PLD) and phosphatidic acid phosphohydrolase. Although both pathways have been reported to be engaged in platelets activated by low concentrations of collagen (Chiang, 1994; Lockhart et al., 2001), the significance of DGL in collagen-induced platelet arachidonic acid release and subsequent aggregation remains unclear. In part this is due to a paucity of pharmacological tools, notably the non-selective nature of DGL inhibitors (Bross et al., 1983; Oglesby and Gorman, 1984). However, a novel tetrahydrolipstatin analogue OMDM-188 has recently been synthesized (Ortar et al., 2008) and shown to potently inhibit DGL (Min et al., 2010). Therefore in the current study we have evaluated the effects of OMDM-188 on low dose collagen-induced platelet activation.

JPET Fast Forward. Published on September 16, 2013 as DOI: 10.1124/jpet.113.205591
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and methods

Materials.

OMDM-188 (*N*-formyl-L-isoleucine-(1*S*)-1-[[(2*S*,3*S*)-3-hexyl-4-oxo-2-oxetanyl]methyl]dodecyl ester) was synthesized as previously reported (Ortar et al., 2008). Arachidonic acid ELISA and TxB_2 EIA kits were purchased from Caymen Chemicals (Ann Arbour, MI), collagen and arachidonic acid were purchased from Helena Laboratories (Beaument, TX), and thrombin and BSA were purchased from Sigma-Aldrich Canada (Oakville, Ont.). Antibodies to $p38^{MAPK}$ and phospho-$p38^{MAPK}$ were purchased from New England Biolabs (Beverly, MA), and antibodies to ERK and phospho-ERK, as well as anti-mouse IgG-HRP and anti-goat IgG-HRP, were all obtained from Santa Cruz Biotechnology (Santa Cruz, CA). ECL reagents and hyperfilm were obtained from Amersham Pharmacia Biotechnology (Baie D'Urfé, Que.), and all elecrophoresis and immunoblotting supplies were from Bio-Rad (Mississauga, Ont.). All other chemicals and materials were of the highest grade available.

Blood collection.

The study was approved by the Research Ethics Board of the University of Manitoba and informed consent was obtained from all volunteers. Blood was collected by venipuncture of human volunteers who had denied taking medication known to interfere with platelet function within the previous two weeks, into acid/citrate/dextrose anti-coagulant (ACD; 3.8 mM citric acid, 7.5 mM trisodium citrate, 125 mM dextrose; 1.8 mL anti-coagulant/8.2 mL whole blood). Plasma-free platelet suspensions were obtained, in appropriate buffers, as previously reported (McNicol et al., 1998; McNicol and Jackson, 2003; Jackson and McNicol, 2010).

Platelet aggregation

Platelet aggregation in HEPES-buffered Tyrodes solution (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 1 mM HEPES, 5 mM dextrose; pH 7.4) was measured as increased light transmission in a Payton dual channel aggregometer, as previously described (McNicol et al., 1998; McNicol and Jackson, 2003; Jackson and McNicol, 2010) and analysed by OriginPro 8 software (Northampton, MA, U.S.A.).

Arachidonic acid release

Aggregation was terminated by the addition of an equal volume of 2mM EDTA, 100 µM indomethacin and the platelets removed by centrifugation (McNicol et al., 1998). The

JPET Fast Forward. Published on September 16, 2013 as DOI: 10.1124/jpet.113.205591
This article has not been copyedited and formatted. The final version may differ from this version.

supernatants were analysed for arachidonic acid, by ELISA following the manufacturer's instructions.

Thromboxane synthesis

Aggregation was terminated by the addition of an equal volume of ACD and the platelets removed by centrifugation (McNicol et al., 1998). The supernatants were analysed for TxB_2, the stable metabolite of TxA_2, by EIA following the manufacturer's instructions.

Immunoblotting

Aggregation was terminated by the addition of an equal volume of reducing buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% (w/v) bromophenol blue) and the samples denatured by boiling for 10 minutes. Proteins were separated on a 10% polyacrylamide/SDS vertical slab gel and transferred to nitrocellulose. Levels of MAP kinase phosphorylation were determined as previously described (McNicol and Jackson, 2003; Jackson and McNicol, 2010). Following blocking in a 5% non-fat powdered Carnation milk solution in TBS (20 mM Tris base, 130 mM NaCl, pH 7.4) containing 0.1% Tween-20 (TBS-T), the blots were incubated with antibodies to either phospho-$p38^{MAPK}$ or phospho-ERK then to the corresponding HRP-conjugated secondary antibody and the proteins visualized using the ECL Western Blotting Detection system. The immunoblot was subsequently stripped and re-probed with an antibody to the corresponding non-phosphorylated protein (ERK or $p38^{MAPK}$) to ensure equal sample loading, as previously reported (McNicol and Jackson, 2003; Jackson and McNicol, 2010).

Statistical analysis

Inter-experiment comparisons in platelet aggregation and detection of soluble factors for the different conditions were analysed by means of the paired t- test or Tukey`s multiple comparison test. All values are reported as means ± SEM and a P value <0.05 was considered to be significant.

JPET Fast Forward. Published on September 16, 2013 as DOI: 10.1124/jpet.113.205591
This article has not been copyedited and formatted. The final version may differ from this version.

Results

The effects of OMDM-188 on agonist-induced platelet aggregation.

The addition of sub-maximal agonist concentrations of 1 μg/ml collagen and 60 μM arachidonic acid caused platelet aggregation (Figure 1), as previously reported (McNicol and Jackson, 2003). Previous studies have shown that 2 μM OMDM-188 has a selective inhibitory action on DGL activity (Min et al., 2010). Therefore the effects of similar concentrations of OMDM-188 on agonist-induced platelet aggregation were examined.

Preincubation (2 minutes) with 3 μM and 10 μM OMDM-188 inhibited collagen-induced aggregation was by 12±4% (n=5; $p<0.05$) and 87±10% (n=5; $p<0.01$) respectively. In contrast 3 μM OMDM-188 had no effect on arachidonic-acid-induced aggregation, whereas 10 μM OMDM-188 inhibited arachidonic-acid-induced aggregation by 50±16% (n=5; $p<0.05$) (Figure 1).

The effects of OMDM-188 on agonist-induced arachidonic acid release

Collagen (1μg/ml) stimulated the release of a significant amount of arachidonic acid when compared to the saline control (1.05±0.02 ng/ml vs 1.43±0.14 ng/ml; $p<0.05$, n=3), which was equivalent to the release elicited by 0.1U/ml thrombin (1.37±0.08 ng/ml). The arachidonic acid release in response to collagen was significantly inhibited by pretreatment for 2 minutes with 10 μM OMDM-188 (1.12±0.05 ng/ml; $p<0.05$) but not with either 1μM OMDM-188 (1.39±0.14 ng/ml) or with 3μM OMDM-188 (1.23±0.15 ng/ml) (Figure 2).

The effects of OMDM-188 on agonist-induced thromboxane release

There were negligible levels of TxB_2, the stable metabolite of TxA_2, in the releasates of platelets stirred with saline alone. Collagen (1μg/ml) stimulated the release of TxB_2 which was significantly inhibited by pretreatment for 2 minutes with 5 μM OMDM-188 but not with 1 μM OMDM-188. The addition of arachidonic acid (60μM) to platelets also led to the formation of significant levels of TxB_2 however in this case the TxB_2 levels were unaffected by pretreatment of up to 100 μM OMDM-188, consistent with OMDM-188 having no effect on cyclo-oxygenase/thromboxane synthetase (Figure 3). There was an apparent increase in TxB_2, but not of arachidonic (Figure 2), release in the presence of the low (1 μM) concentration of OMDM-188. The reason for this apparent paradox is unclear and requires further examination.

The effects of OMDM-188 on agonist-induced protein phosphorylation

JPET Fast Forward. Published on September 16, 2013 as DOI: 10.1124/jpet.113.205591
This article has not been copyedited and formatted. The final version may differ from this version.

Collagen (1µg/ml) caused the phosphorylation of $p38^{MAPK}$ (Figure 4), as previously reported (McNicol and Jackson, 2003; Jackson and McNicol, 2010). This phosphorylation was unaffected by pretreatment for 2 minutes with 1 µM OMDM-188 but significantly reduced by pretreatment with 10 µM OMDM-188. In contrast $p38^{MAPK}$ phosphorylation by arachidonic acid was unaffected by pretreatment with OMDM-188 at concentrations of up to 10 µM (Figure 4).

Collagen (1µg/ml) and arachidonic acid (60µM) each caused the phosphorylation of ERK which was unaffected by pretreatment (2 minutes) with OMDM-188 at concentrations of up to 1 mM (Figure 5).

Discussion

Collagen is accepted to be the most thrombogenic component of the subendothelial matrix. Engagement of collagen receptors trigger intracellular signaling pathways leading to the final platelet response. The current evidence suggests that the primary signaling occurs via GPVI. Activation of GPVI leads to the phosphorylation of FcRγ which sequentially leads to the activation of Syk and PLCγ2 (Stalker et al., 2012). The effects of collagen are promoted by the release of arachidonic acid by $cPLA_2$ and its subsequent conversion to TxA_2 by COX/TxA_2 synthetase (Stalker et al., 2012). The upstream mediators of $cPLA_2$ are likely to include intracellular calcium and $p38^{MAPK}$ (Borsch-Haubold et al., 1995; Borsch-Haubold et al., 1997; Stalker et al., 2012) (Figure 6)

However studies both *in vivo* and *in vitro* have shown that platelet responses to low doses of collagen are highly aspirin-sensitive, and therefore TxA_2-mediated (Seymour et al., 1984; Narita et al., 1985; Kito et al., 1986; Pollock et al., 1986; Gerrard et al., 1989). The signaling pathways associated with platelets stimulated by low, sub-maximal doses of collagen are not well elucidated. Both PLC activity and the elevation of intracellular Ca^{2+} are inhibited by aspirin and therefore do not precede, but rather are a consequence of, TxA_2 synthesis (Narita et al., 1985; Kito et al., 1986; Pollock et al., 1986). Similarly pharmacological inhibition of neither $p38^{MAPK}$ nor $cPLA_2$ had any effect on low dose collagen-induced arachidonic acid release (McNicol et al., 1998; Lockhart et al., 2001), thereby dissociating a $p38^{MAPK}$/$cPLA_2$ pathway from low dose collagen-induced arachidonic acid release.

A second potential arachidonic acid liberating process is by the action of DGL on DG. Moriyama and colleagues have purified and characterized DGL in platelets (Moriyama et al., 1999). Of particular note DGL is active at basal Ca^{2+} (Moriyama et al., 1999) and therefore would not require a PLC-mediated generation of IP_3 and subsequent elevation of intracellular Ca^{2+} for activity. In the same study RHC-80267 was shown to inhibit DGL (Moriyama et al., 1999), however other studies have shown that, due to its relative non-selective activity, RHC-80267 cannot be used to determine the role of DGL in intact platelets (Bross et al., 1983; Oglesby and Gorman, 1984).

More recently novel tetrahydrolipstatin analogues with inhibitory effects on DGL have been synthesized (Ortar et al., 2008; Min et al., 2010). In the current study one of these analogues, OMDM-188, inhibited low dose collagen-induced aggregation of human platelets and this was

JPET Fast Forward. Published on September 16, 2013 as DOI: 10.1124/jpet.113.205591
This article has not been copyedited and formatted. The final version may differ from this version.

associated with an inhibition of both arachidonic acid release and TxB_2 formation. At similar concentrations OMDM-188 had significantly less of an inhibitory effect on arachidonic acid-induced aggregation and had no effect on arachidonic acid-induced TxB_2 formation. Taken together these data demonstrate that OMDM-188 has an inhibitory action on collagen-induced platelet activation prior to the conversion of arachidonic acid to TxA_2 by COX, likely by an action on DGL. This would suggest that DGL is a key enzyme in the pathway underlying platelet activation in response to low doses of collagen.

This supports the study of Moriyama and colleagues (Moriyama et al., 1994) who postulated that the action of DGL distal to PLC, at resting intracellular Ca^{2+}, to release arachidonic acid was a critical early step in collagen-induced platelet activation. Similarly, a role for a PLC, but not for $cPLA_2$, in collagen-induced platelet arachidonic acid release, via DGL, was postulated in a study utilizing a battery of pharmacological inhibitors (Lockhart et al., 2001) (Figure 6).

A second potential DGL-mediated pathway of liberating arachidonic acid in response to low-doses of collagen is by the sequential actions of PLD and DGL, as suggested by Chiang (Chiang, 1994). There are two isoforms of PLD in platelets, PLD1 and PLD2, both of which hydrolyse phosphatidylcholine to generate phosphatidic acid which, by the action of phosphatidic acid phosphohydrolase, synthesises DG (Vorland and Holmsen, 2008; Vorland et al., 2008). As reviewed by Vorland and colleagues, these two PLD isoforms are stimulated by collagen and inhibited by protein kinase A, suggesting a role in the signaling pathway, are found in different locations in the platelet and likely play differing roles during activation (Vorland et al., 2008). These functions may include lysosomal secretion and actin polymerization (Vorland et al., 2008); the DG generated may provide a source of archidonic acid via the action of DGL is possible but has not been addressed (Figure 6). Interestingly however a recent study using a novel PLD inhibitor, FIPI, as well as *Pld*$^{-/-}$ murine platelets has suggested that PLD plays a negative role in platelets signaling in response to a variety of agonists, including low-dose collagen (Elvers et al., 2012). Therefore it appears that a PLC/DLG, rather than a PLD/phosphatidic acid phosphohydrolase/DLG is the more likely pathway engaged (Figure 6).

The identity of the collagen receptor involved in PLC activation is open to speculation and further investigation. Clearly a role for GPVI/ FcRγ, as occurs at higher collagen concentrations (Li et al., 2010), is a possibility. An intriguing potential alternative is CD148, the only receptor-like protein tyrosine phosphatase on the platelet surface (Senis et al., 2009). Engagement of

JPET Fast Forward. Published on September 16, 2013 as DOI: 10.1124/jpet.113.205591
This article has not been copyedited and formatted. The final version may differ from this version.

CD148 may remove the restraints on the phosphorylated FcRγ ITAM leading to the activation of Syk and subsequent downstream regulators including PLCγ2. In both cases however the levels of DG formed would have to provide sufficient substrate for DGL to initiate the arachidonic acid/TxA_2 cascade without liberating enough IP_3 to trigger the Ca^{2+}-mediated $cPLA_2$ pathway. The α2ß1 integrin is also critical to the interaction of platelets with collagen, however it is likely that it plays a role as an adhesive receptor which requires an initial wave of internal signaling (Stalker et al., 2012).

The effects of OMDM-188 on MAP kinase activity in response to collagen were assessed to address any relationship between these enzymes and DGL function. Previous studies have shown that inhibition of the MEK/ERK pathway had no effect on platelet aggregation in response to a variety of agonists, including low doses of collagen (McNicol and Jackson, 2003). Therefore it is unlikely that this pathway is engaged in response to collagen, leading to DGL activity, arachidonic acid release and aggregation. Indeed the observed inhibition of aggregation by OMDM-188 combined with the lack of a corresponding effect on ERK phosphorylation supports the dissociation between these two events.

In the current study OMDM-188, at concentrations which blocked aggregation, inhibited collagen-induced $p38^{MAPK}$ phosphorylation. Studies by Saklavala and colleagues demonstrated that the effects of collagen at concentrations that were minimally required to induce aggregation of platelets were sensitive to inhibition of $p38^{MAPK}$ (Saklatvala et al., 1996), suggesting that $p38^{MAPK}$ precedes arachidonic acid release under these conditions. Taken together these observations potentially implicate $p38^{MAPK}$ in the DGL activating pathway, however this proposition requires to be addressed further (Figure 6).

Therapeutically inhibition of DGL is an attractive anti-thrombotic target as it would affect the release of arachidonic acid/TxA_2 without a corresponding action on endothelial COX-2-mediated arachidonic acid/prostacyclin release. However the concentrations of, OMDM-188 is the most potent and selective DGL inhibitor reported (Ortar et al., 2008) to date, required are high for this to be a viable agent. In addition tetrahydrolipstatin has a low permeability through gastrointestinal tract, which limits its oral bioavailability and it is probable that this will also affect OMDM118 (and other tetrahydrolipstatin analogues). Hence, it is necessary for DGL inhibitors of other chemical classes to be developed.

JPET Fast Forward. Published on September 16, 2013 as DOI: 10.1124/jpet.113.205591
This article has not been copyedited and formatted. The final version may differ from this version.

In conclusion the current study is consistent with a role for a pathway involving the liberation of DG from membrane phospholipids in response to minimally-activate concentrations of collagen. The DG serves as a substrate for DGL, potentially under the regulations of $p38^{MAPK}$, to release arachidonic acid which is subsequently converted to TxA_2 which mediates the final platelet response.

Acknowledgements

This study was funded by a grant from the Heart and Stroke Foundation of Canada.

Author Contributions

E.C.G.J. carried out all experiments, G.O. provided materials and A.McN. conceptualized the study, designed all experiments, carried out all statistical analysis and wrote the manuscript.

References

Atkinson BT, Ellmeier W and Watson SP (2003) Tec regulates platelet activation by GPVI in the absence of Btk. *Blood* **102**:3592-3599.

Bell RL, Kennerly DA, Stanford N and Majerus PW (1979) Diglyceride lipase: a pathway for arachidonate release from human platelets. *Proc Natl Acad Sci U S A* **76**:3238-3241.

Borsch-Haubold AG, Kramer RM and Watson SP (1995) Cytosolic phospholipase A_2 is phosphorylated in collagen- and thrombin-stimulated human platelets independent of protein kinase C and mitogen-activated protein kinase. *J Biol Chem* **270**:25885-25892.

Borsch-Haubold AG, Kramer RM and Watson SP (1997) Phosphorylation and activation of cytosolic phospholipase A_2 by 38-kDa mitogen-activated protein kinase in collagen-stimulated human platelets. *Eur J Biochem* **245**:751-759.

Bross TE, Prescott SM and Majerus PW (1983) RHC 80267 does not inhibit the diglyceride lipase pathway in intact platelets. *Biochem Biophys Res Commun* **116**:68-74.

Chiang TM (1994) Activation of phospholipase D in human platelets by collagen and thrombin and its relationship to platelet aggregation. *Biochim Biophys Acta* **1224**:147-155.

Daniel JL, Dangelmaier C and Smith JB (1994) Evidence for a role for tyrosine phosphorylation of phospholipase Cγ2 in collagen-induced platelet cytosolic calcium mobilization. *Biochem J* **302 (Pt 2)**:617-622.

Ellison S, Mori J, Barr AJ and Senis YA (2010) CD148 enhances platelet responsiveness to collagen by maintaining a pool of active Src family kinases. *J Thromb Haemost* **8**:1575-1583.

Elvers M, Grenegard M, Khoshjabinzadeh H, Munzer P, Borst O, Tian H, Di Paolo G, Lang F, Gawaz M, Lindahl TL and Falker K (2012) A novel role for phospholipase D as an endogenous negative regulator of platelet sensitivity. *Cell Signal* **24**:1743-1752.

Ezumi Y, Shindoh K, Tsuji M and Takayama H (1998) Physical and functional association of the Src family kinases Fyn and Lyn with the collagen receptor glycoprotein VI-Fc receptor gamma chain complex on human platelets. *J Exp Med* **188**:267-276.

Farndale RW, Slatter DA, Siljander PR and Jarvis GE (2007) Platelet receptor recognition and cross-talk in collagen-induced activation of platelets. *J Thromb Haemost* **5 Suppl 1**:220-229.

Gerrard JM, Docherty JC, Israels SJ, Cheang MS, Bishop AJ, Kobrinsky NL, Schroeder ML and Israels ED (1989) A reassessment of the bleeding time: association of age, hematocrit, platelet function, von Willebrand factor, and bleeding time thromboxane B_2 with the length of the bleeding time. *Clin Invest Med* **12**:165-171.

Gross BS, Melford SK and Watson SP (1999) Evidence that phospholipase C-γ2 interacts with SLP-76, Syk, Lyn, LAT and the Fc receptor gamma-chain after stimulation of the collagen receptor glycoprotein VI in human platelets. *Eur J Biochem* **263**:612-623.

Hughes CE, Auger JM, McGlade J, Eble JA, Pearce AC and Watson SP (2008) Differential roles for the adapters Gads and LAT in platelet activation by GPVI and CLEC-2. *J Thromb Haemost* **6**:2152-2159.

Jackson EC and McNicol A (2010) Cyclic nucleotides inhibit MAP kinase activity in low-dose collagen-stimulated platelets. *Thromb Res* **125**:147-151.

Kauskot A and Hoylaerts MF (2012) Platelet receptors. *Handb Exp Pharmacol*:23-57.

Kito M, Narita H, Takamura H, Park HJ, Matsuura T and Tanaka K (1986) Dissociation of Ca2+ mobilization from breakdown of phosphatidylinositol 4,5-bisphosphate in activated human platelets. *J Biochem* **99**:1277-1280.

Leis HJ and Windischhofer W (2008) Inhibition of cyclooxygenases 1 and 2 by the phospholipase-blocker, arachidonyl trifluoromethyl ketone. *Br J Pharmacol* **155**:731-737.

Li Z, Delaney MK, O'Brien KA and Du X (2010) Signaling during platelet adhesion and activation. *Arterioscler Thromb Vasc Biol* **30**:2341-2349.

Lockhart LK, Pampolina C, Nickolaychuk BR and McNicol A (2001) Evidence for a role for phospholipase C, but not phospholipase A_2, in platelet activation in response to low concentrations of collagen. *Thromb Haemost* **85**:882-889.

McNicol A and Jackson EC (2003) Inhibition of the MEK/ERK pathway has no effect on agonist-induced aggregation of human platelets. *Biochem Pharmacol* **65**:1243-1250.

McNicol A and Nickolaychuk BR (1995) Inhibition of collagen-induced platelet activation by arachidonyl trifluoromethyl ketone. *Biochem Pharmacol* **50**:1795-1802.

McNicol A, Philpott CL, Shibou TS and Israels SJ (1998) Effects of the mitogen-activated protein (MAP) kinase kinase inhibitor 2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one (PD98059) on human platelet activation. *Biochem Pharmacol* **55**:1759-1767.

Min R, Testa-Silva G, Heistek TS, Canto CB, Lodder JC, Bisogno T, Di Marzo V, Brussaard AB, Burnashev N and Mansvelder HD (2010) Diacylglycerol lipase is not involved in depolarization-induced suppression of inhibition at unitary inhibitory connections in mouse hippocampus. *J Neurosci* **30**:2710-2715.

Moriyama T, Urade R and Kito M (1999) Purification and characterization of diacylglycerol lipase from human platelets. *J Biochem* **125**:1077-1085.

Moriyama T, Wada K, Oki M, Matsuura T and Kito M (1994) The mechanism of arachidonic acid release in collagen-activated human platelets. *Biosci Biotechnol Biochem* **58**:93-98.

Narita H, Park HJ, Tanaka K, Matsuura T and Kito M (1985) Insufficient mobilization of calcium by early breakdown of phosphatidylinositol 4,5-bisphosphate for aggregation of human platelets by collagen. *J Biochem* **98**:1063-1068.

Nieswandt B and Watson SP (2003) Platelet-collagen interaction: is GPVI the central receptor? *Blood* **102**:449-461.

Oglesby TD and Gorman RR (1984) The inhibition of arachidonic acid metabolism in human platelets by RHC 80267, a diacylglycerol lipase inhibitor. *Biochim Biophys Acta* **793**:269-277.

Ortar G, Bisogno T, Ligresti A, Morera E, Nalli M and Di Marzo V (2008) Tetrahydrolipstatin analogues as modulators of endocannabinoid 2-arachidonoylglycerol metabolism. *J Med Chem* **51**:6970-6979.

Pollock WK, Rink TJ and Irvine RF (1986) Liberation of [^{3}H]arachidonic acid and changes in cytosolic free calcium in fura-2-loaded human platelets stimulated by ionomycin and collagen. *Biochem J* **235**:869-877.

Rittenhouse-Simmons S (1980) Indomethacin-induced accumulation of diglyceride in activated human platelets. The role of diglyceride lipase. *J Biol Chem* **255**:2259-2262.

Saklatvala J, Rawlinson L, Waller RJ, Sarsfield S, Lee JC, Morton LF, Barnes MJ and Farndale RW (1996) Role for p38 mitogen-activated protein kinase in platelet aggregation caused by collagen or a thromboxane analogue. *J Biol Chem* **271**:6586-6589.

Senis YA, Tomlinson MG, Ellison S, Mazharian A, Lim J, Zhao Y, Kornerup KN, Auger JM, Thomas SG, Dhanjal T, Kalia N, Zhu JW, Weiss A and Watson SP (2009) The tyrosine phosphatase CD148 is an essential positive regulator of platelet activation and thrombosis. *Blood* **113**:4942-4954.

Seymour RA, Williams FM, Oxley A, Ward A, Fearns M, Brighan K, Rawlins MD and Jones PM (1984) A comparative study of the effects of aspirin and paracetamol (acetaminophen) on platelet aggregation and bleeding time. *Eur J Clin Pharmacol* **26**:567-571.

Stalker TJ, Newman DK, Ma P, Wannemacher KM and Brass LF (2012) Platelet signaling. *Handb Exp Pharmacol*:59-85.

Stegner D and Nieswandt B (2011) Platelet receptor signaling in thrombus formation. *J Mol Med (Berl)* **89**:109-121.

Surin WR, Barthwal MK and Dikshit M (2008) Platelet collagen receptors, signaling and antagonism: emerging approaches for the prevention of intravascular thrombosis. *Thromb Res* **122**:786-803.

Suzuki-Inoue K, Fuller GL, Garcia A, Eble JA, Pohlmann S, Inoue O, Gartner TK, Hughan SC, Pearce AC, Laing GD, Theakston RD, Schweighoffer E, Zitzmann N, Morita T, Tybulewicz VL, Ozaki Y and Watson SP (2006) A novel Syk-dependent mechanism of platelet activation by the C-type lectin receptor CLEC-2. *Blood* **107**:542-549.

Tandon NN, Kralisz U and Jamieson GA (1989) Identification of glycoprotein IV (CD36) as a primary receptor for platelet-collagen adhesion. *J Biol Chem* **264**:7576-7583.

Vorland M and Holmsen H (2008) Phospholipase D in human platelets: presence of isoenzymes and participation of autocrine stimulation during thrombin activation. *Platelets* **19**:211-224.

Vorland M, Thorsen VA and Holmsen H (2008) Phospholipase D in platelets and other cells. *Platelets* **19**:582-594.

JPET Fast Forward. Published on September 16, 2013 as DOI: 10.1124/jpet.113.205591
This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends

Figure 1: Effects of OMDM-188 on collagen- and arachidonic acid-induced aggregation.

Washed human platelets were incubated for 2 minutes with OMDM-188 at the concentrations indicated, or saline control, prior to the addition of 1 μg/ml collagen (n=5) or 60 μM arachidonic acid (n=5) . Aggregation was monitored continuously as an increase in light transmission for 3 minutes. The extent of aggregation was quantified using OriginPro 8 software and data expressed as a percentage of the saline control (n=5; * $p<0.05$; **$p<0.01$). Insert is a representative example of the effects of OMDM-188 on collagen-induced aggregation.

Figure 2: Effects of OMDM-188 on collagen-induced arachidonic acid release.

Washed human platelets were incubated for 2 minutes with OMDM-188 at the concentrations indicated, or saline control, prior to the addition of 1 μg/ml collagen. Aggregation was monitored continuously as an increase in light transmission for 3 minutes as outlined in figure legend 1. Release was terminated and arachidonic acid determined by ELISA. Data expressed as mean±SEM and analysed by Tukey`s multiple comparison test (*$p<0.05$ with respect to saline control; # $p<0.05$ with respect to saline + collagen; n=3).

Figure 3: Effects of OMDM-188 on collagen- and arachidonic acid-induced TxB_2 release.

Washed human platelets were incubated for 2 minutes with OMDM-188 at the concentrations indicated, or saline control, prior to the addition of 1 μg/ml collagen or 60 μM arachidonic acid. Aggregation was monitored continuously as an increase in light transmission for 3 minutes as outlined in figure legend 1. Release was terminated and TxB_2 determined by EIA. Data expressed as mean±SEM and analysed by Tukey`s multiple comparison test (*$p<0.05$ with respect to saline control; # $p<0.05$ with respect to saline + agonist; n=3).

Figure 4: Effects of OMDM-188 on collagen- and arachidonic acid-induced $p38^{MAPK}$ phosphorylation.

Washed human platelets were incubated for 2 minutes with OMDM-188 at the concentrations indicated, or saline control, prior to the addition of 1 μg/ml collagen or 60 μM arachidonic acid. Aggregation was monitored continuously as an increase in light transmission for 3 minutes as outlined in figure legend 1. Proteins were extracted, transferred to nitrocellulose and

JPET Fast Forward. Published on September 16, 2013 as DOI: 10.1124/jpet.113.205591
This article has not been copyedited and formatted. The final version may differ from this version.

immunoblotted using anti-phospho-specific $p38^{MAPK}$. The blots were stripped then reprobed with anti-$p38^{MAPK}$ to confirm equal loading. The blots were quantified by densitometry. Data expressed as mean±SEM and analysed by Tukey`s multiple comparison test (* $p<0.05$ w.r.t. saline control; $p<0.05$ w.r.t agonist alone; n=4).

Figure 5: Effects of OMDM-188 on collagen- and arachidonic acid-induced ERK phosphorylation.

Washed human platelets were incubated for 2 minutes with OMDM-188 at the concentrations indicated, or saline control, prior to the addition of 1 μg/ml collagen or 60 μM arachidonic acid. Aggregation was monitored continuously as an increase in light transmission for 3 minutes as outlined in figure legend 1. Proteins were extracted, transferred to nitrocellulose and immunoblotted using anti-phospho-specific ERK. The blots were stripped then reprobed with anti-ERK to confirm equal loading. The blots were quantified by densitometry. Data expressed as mean±SEM and analysed by Tukey`s multiple comparison test (* $p<0.05$ w.r.t. saline control; $p<0.05$ w.r.t agonist alone; n=4).

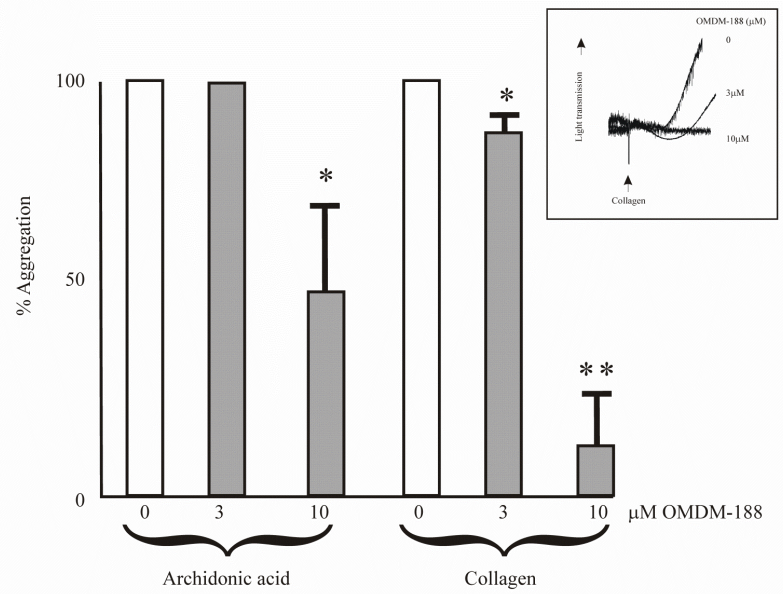
Figure 6: Diagrammatic representation of potential pathways of TxA_2 generation in response to high and low concentrations of collagen.

High concentrations of collagen bind to GPVI which forms a complex with FcγR and initiates a signaling pathway leading to the activation of PLCγ2 with the resultant generation of DG and IP_3. These in turn activate PKC and elevate Ca^{2+}. Ca^{2+} -induced $cPLA_2$ activation is modulated by both PKC and $p38^{MAPK}$. The released arachidonic acid is converted to TxA_2 which further stimulates platelets.

The receptor bound by low concentrations of collagen is unclear. A role for GPVI cannot be ruled out, although CD148 provides an interesting alternative. This in turn may lead to the release of DG either by the action of PLC or PLD. DGL, under the regulation of $p38^{MAPK}$, liberates arachidonic acid which is converted to TxA_2.

See text for further abbreviations and further details.

Figure 1



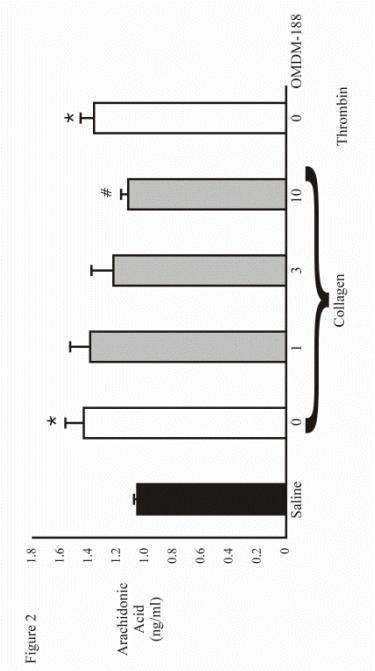


Figure 3

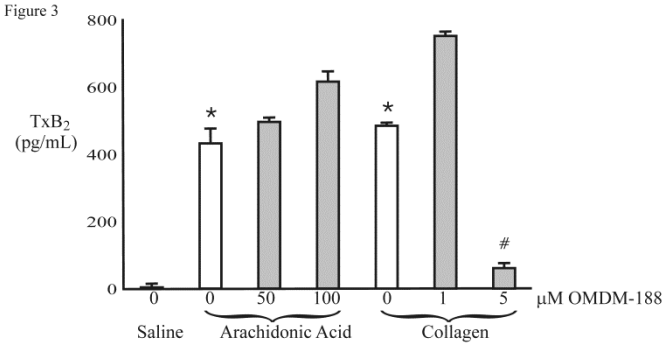


Figure 4

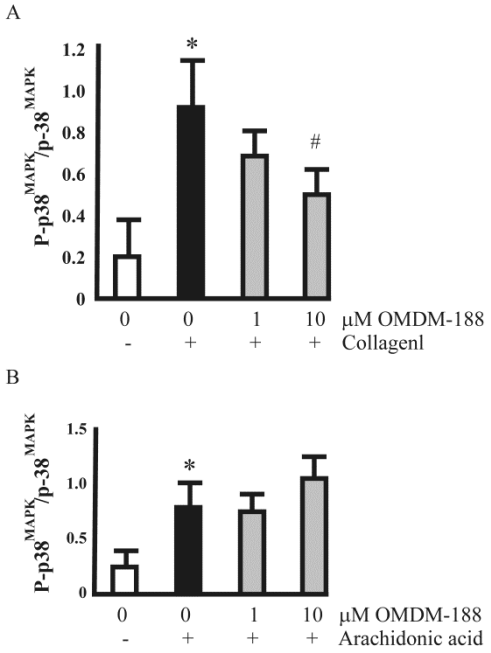


Figure 5

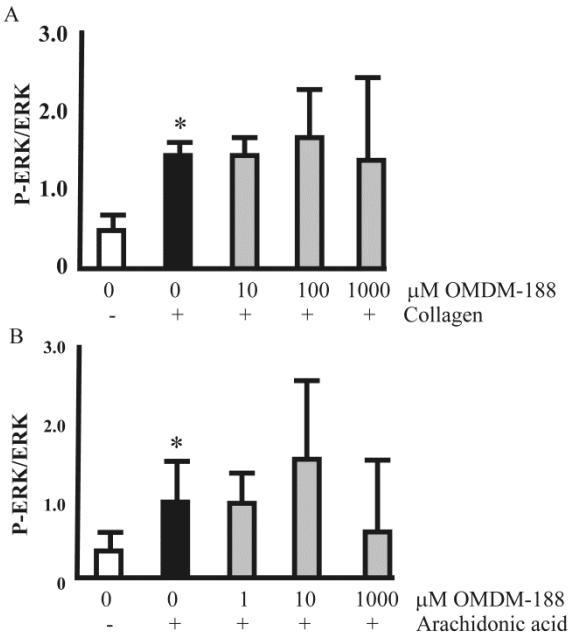


Figure 6