Non-genomic effects of nuclear receptors: insights from the anucleate platelet

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Abstract

Nuclear receptors (NRs) have the ability to elicit two different kinds of responses, genomic and non-genomic. Although genomic responses control gene expression by influencing the rate of transcription, non-genomic effects occur rapidly and independently of transcriptional regulation. Due to their anucleate nature and mechanistically well-characterized and rapid responses, platelets provide a model system for the study of any non-genomic effects of the NRs. Several NRs have been found to be present in human platelets, and multiple NR agonists have been shown to elicit anti-platelet effects by a variety of mechanisms. The non-genomic functions of NRs vary, including the regulation of kinase and phosphatase activity, ion channel function, intracellular calcium levels, and production of second messengers. Recently, the characterization of mechanisms and identification of novel binding partners of NRs have further strengthened the prospects of developing their ligands into potential therapeutics that offer cardio-protective properties in addition to their other defined genomic effects.

Keywords

Nuclear receptors • Non-genomic • Platelets • Thrombosis

1. Introduction

Nuclear receptors (NRs) represent the family of mammalian proteins associated with the transcriptional regulation in human tissues and include the androgen receptor (AR), oestrogen receptor (ER), glucocorticoid receptor (GR), farnesoid X receptor (FXR), liver X receptor (LXR), peroxisome proliferator-activated receptors (PPARs), retinoic acid receptor (RAR), retinoid X receptor (RXR), and the vitamin D receptor (VDR). Upon activation by their lipophilic ligands, NRs regulate several fundamental biological processes, such as cell proliferation, differentiation, metabolism, and homeostasis (Table 1). Any deviation from their normal function can lead to the pathological manifestations, such as cancer, diabetes, arthritis, and obesity.

NRs have the ability to function in both genomic and non-genomic ways. Whilst historically associated with the regulation of transcription and control of gene expression (genomic), more recently non-genomic roles for the NRs have been identified that occur independently of transcriptional regulation. Unlike the genomic functions, which can occur over minutes or hours, these events occur in the time frame of seconds to a few minutes, which is considered too rapid to be attributed to the biosynthesis of mRNA or protein, and is often unaffected by the inhibitors of transcription or translation. The non-genomic functions of NRs vary and whilst it is thought these functions are initiated by physical interactions of NRs with cofactors and binding partners that initiate rapid signalling events, the exact mechanisms are not well understood. One possible explanation is that the cellular localization of NRs influences the availability of cofactors and substrates, which leads to varying combinations of binding partner interactions. For instance, localization of NRs to the cytosol, plasma membrane, or other intracellular organelles such as mitochondria increases the likelihood of initiation of non-genomic effects, whilst genomic functions may be restricted to when NRs are localized in the nucleus. The formation of different multi-protein signalling complexes with different localization and distribution patterns across different cell types could offer a high degree of cell and tissue selective action for the NRs but as of yet these are poorly defined.

Given the underlying differences between the genomic and non-genomic activities, non-genomic effects are more easily observed in cell types that lack a functional nucleus such as erythrocytes and platelets.

Emerging evidence indicates that platelets are also involved in roles beyond those described in haemostasis and thrombosis. For instance, granule secretion following platelet activation results in the release of an array of chemokines, cytokines, growth factors, anti-inflammatory factors, and several other biologically active molecules into the vicinity of injured tissues that contribute towards the progression of numerous diseases including inflammatory conditions (e.g. atherosclerosis and rheumatoid arthritis), type 2 diabetes, and cancer cell metastasis.
Thus, platelets are highly active cells with diverse functions despite lacking genomic DNA. Although devoid of a nucleus, platelets still contain different forms of RNA (mRNA, rRNA, tRNA, and miRNA) and components of the transcription and translation machinery that are derived from megakaryocytes during thrombopoiesis. There is a growing consensus that these RNAs are not subjected to a random transfer by megakaryocytes but are specifically sorted and are competent for translation within platelets. Moreover, there is evidence to suggest that platelet-derived microparticles may deliver platelet mRNAs into other nucleated cells, such as monocytes and endothelial cells, where they then undergo translation. Components of this transcription machinery found to be present inside platelets include the intracellular NRs. Due to their anucleate nature and mechanistically well characterized and rapid responses, such as aggregation and adhesion, platelets provide an excellent model system to study the acute non-genomic effects of the NRs.

### 2. NRs are acute regulators of platelet function

On the basis of the mechanisms of action in the nucleated cells, NRs are classified into two classes: type I or the steroid hormone receptors and type II or the non-steroid receptors. Platelets are known to express both the classes of these receptors. This includes the AR, ER, GR, FXR, LXR, PPARs, RAR, RXR, and VDR. Both natural and synthetic ligands for these NRs have been shown to alter platelet function through a variety of mechanisms as described below and summarized in Table 2.

### 3. Type I NRs

Mechanisms by which type I NRs (GR, ER, and AR) regulate platelet functions are poorly understood. This might be attributed to the variations in the plasma levels of the steroid hormones targeting these NRs (especially in females and under certain pathological conditions). This might lead to an inaccurate assessment of the role type I NRs play in modulating platelet functions in acute vs. chronic studies and might account for the existing contradictory published data. The effects of ligands of type 1 NRs on platelet function currently published are described below.

#### 3.1 Glucocorticoid receptor

GRs are activated by glucocorticoid and anti-inflammatory hormones that regulate inflammation and glucose homeostasis. Prednisolone, a synthetic glucocorticoid derived from cortisol, has been shown to

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**Table 1 NRs and their biological functions**

| Nuclear receptor | Ligands | Biological function |
|------------------|---------|---------------------|
| GR               | Natural: glucocorticoid | Lipolysis |
|                  | Synthetic: RU38486, A348441 | Glucose metabolism |
| ER               | Natural: oestrogen, including oestrone (E1), oestradiol (E2) and oestriol (E3) | Development of the female reproductive system and secondary sexual characteristics |
| AR               | Natural: dihydrotestosterone, testosterone | Development of the male reproductive system and secondary sexual characteristics |
|                  | Synthetic: mibolerone | |
| LXR              | Natural: oxysterols | Lipid and carbohydrate metabolism |
|                  | Synthetic: T0901317, GW3965 | |
| FXR              | Natural: bile acids | Bile acid homeostasis |
|                  | Synthetic: GW4064, farnesol, CDCA | |
| PPAR\xa | Natural: polyunsaturated fatty acids | Fatty acid oxidation and lipid metabolism |
|                  | Synthetic: fibrates (gemfibrozil, fenofibrate, clofibrate) | |
| PPAR\xb | Natural: unsaturated/saturated fatty acids, eicosanoids, prostacyclin | Cholesterol metabolism |
|                  | Synthetic: GW501516 | |
| PPAR\xc | Natural: 15-deoxy-12, 14 prostaglandin J2 (15d-PGJ2) | Lipid and glucose metabolism |
|                  | Synthetic: thiazolidinedione (ciglitazone, pioglitazone, rosiglitazone) | |
| RAR              | Natural: atRA | Cell growth, differentiation and organogenesis |
| RXR              | Natural: 9-cis-retinoic acid, docosahexaenoic acid | Cellular proliferation and differentiation, glucose, fatty acid and cholesterol metabolism |
|                  | Synthetic: methoprene acid, rexinoids (LG100268) | |
| VDR              | Natural: calcitriol | Calcium homeostasis, cell proliferation and differentiation |
|                  | Synthetic: maxacalcitol, calcipotriol | |
Table 2 A summary of NRs identified in platelets and their modes of action

| Nuclear receptor | Ligands | Effect on platelet function | Mechanisms of action |
|------------------|---------|-----------------------------|----------------------|
| GR27,28          | Prednisolone | Negative regulation of platelet secondary mediator regulated effects (ADP and TXA2) | Mechanism is unknown |
|                  |         | In vitro                    |                      |
|                  |         | Human platelets             |                      |
| ER25,26          | Oestrogen—oestrone (E1), oestradiol (E2) and oestril (E3) | Reduction in platelet responsiveness however, conflicting results exist | Mechanism is unknown |
|                  |         | In vitro, ex vivo, in vivo  |                      |
|                  |         | Human, mouse platelets      |                      |
| AR46–48          | Testosterone, Dihydrotestosterone | Potentiation of platelet aggregation | Mechanism is unknown |
|                  |         | In vitro                    |                      |
|                  |         | Human and rat platelets     |                      |
| LXR31            | GW3965, T0901317, 24(S)-OH-cholesterol, 27-OH-cholesterol | Inhibition of platelet function and thrombosis | Reduced phosphorylation of early GPVI signalling components—Syk, LAT and PLC/2 |
|                  |         | In vitro                    | Increase LXR-Syk and LXR-PLC/2 interaction |
|                  |         | Human and mouse platelets   | Formation of coated platelets, including PS exposure, mitochondrial membrane depolarization (see Figure 1) |
|                  |         | Conversion of platelets to the procoagulant state |                      |
|                  |         | In vitro                    |                      |
|                  |         | Human platelets             |                      |
| FXR29            | GW4064, Chenodeoxycholic acid, 6α-ethyl-chenodeoxycholic acid | Inhibition of platelet function, thrombosis and haemostasis | Cyclophillin D-dependent formation of coated platelets and closure of surface integrins |
|                  |         | In vitro and in vivo        | Associated with PS exposure and mitochondrial membrane depolarization |
|                  |         | Human, mouse platelets      | Augmented cGMP levels which promote PKG activity and phosphorylation of VASP S239 (see Figure 1) |
|                  |         | Conversion of platelets to the procoagulant state |                      |
|                  |         | In vitro                    |                      |
|                  |         | Human platelets             |                      |
| PPARα33          | Fenofibrate, Statins | Inhibition of platelet function | Increase in cAMP levels |
|                  |         | In vitro                    | PPARα–PKCα interaction and attenuation of PKCα (see Figure 2) |
|                  |         | Human, mouse platelets      |                      |
| PPARβ/δ35        | GW0742, L-165041 | Inhibition of platelet function | Increase in cAMP levels |
|                  |         | In vitro                    | PPARα–PKCα interaction and attenuation of PKCα (see Figure 2) |
|                  |         | Human, mouse platelets      |                      |
| PPARγ38,39       | 15d-PGJ2, Thiazolidinediones (rosiglitazone, ciglitazone, pioglitazone) | Inhibition of platelet function, thrombosis and haemostasis | Inhibition in phosphorylation of Syk and LAT to reduce GPVI signalling |
|                  |         | In vitro and in vivo        | Reduced PPARγ–Syk and PPARγ–LAT interaction upon PPARγ ligand treatment |
|                  |         | Human, mouse platelets      | Negative regulation of integrin αIIbβ3 outside-in via up-regulation of PKA activity and inhibition β3 phosphorylation (see Figure 2) |
| RAR41            | atRA    | Inhibition of cytoskeletal rearrangements and platelet spreading | Disruption of RARα–Arp2/3 interactions. (see Figure 3) |
|                  |         | In vitro                    |                      |
|                  |         | Human platelets             |                      |
Table 2

| Nuclear receptor | Ligands | Effect on platelet function | Mechanisms of action |
|------------------|---------|----------------------------|----------------------|
| RXR<sup>42,43</sup> | • 9-cis-retinoic acid • Methoprene acid • Docosahexaenoic acid | • Inhibition of platelet function, thrombosis and haemostasis • *In vitro* and *in vivo* • Human, mouse platelets | • RXR–Gq interaction and negative regulation of Rac activation to inhibit GPCR-mediated platelet activation • Up-regulation of PKA activity and phosphorylation of VASP S157 in cAMP- and NFκB-dependent manner (see Figure 3) Mechanism is unknown |
| VDR<sup>45</sup> | • Vitamin D and its metabolites | • Low vitamin D plasma levels cause high mean platelet volume, a marker of platelet hyperactivity • *In vivo* • Human platelets | |

attenuate platelet function. Prednisolone-treated platelets displayed reduced aggregation and thromboxane B<sub>2</sub> (TxB<sub>2</sub>) release in response to stimulation by either ADP or the TXA<sub>2</sub> mimetic U46619, which was reversed following treatment with a GR antagonist mifepristone. This inhibition was not found to be associated with up-regulation of cyclic nucleotides—cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP), key inhibitory mediators of platelet activity. Platelet adhesion and thrombus formation under flow on collagen in vitro were also found to be diminished following prednisolone treatment which is likely an outcome of reduced platelet responses to ADP and TXA<sub>2</sub>, secondary mediators of platelet activation that support platelet adhesion and thrombus growth. Prednisolone has also shown to modulate platelet–monocyte interactions following stimulation by ADP, which is attributed to an attenuation of platelet activity and not to inhibition of monocytes. It should be noted, however, that alternative GR ligands—dexamethasone, fludrocortisone, and triamcinolone have not been shown to elicit anti-platelet effects under the experimental conditions used in these studies. This difference in activation is thought to be due to the formation of a heterodimeric complex between GR and the mineralocorticoid receptor that is susceptible to the differential activation by specific receptor ligands. The mechanism underlying the negative regulation of secondary mediator signalling by GR and its ligand prednisolone is yet to be fully explored although evidence suggests that this might be mediated through the regulation of signalling events downstream of the P2Y<sub>12</sub> receptor.

### 3.2 Oestrogen receptor

Oestradiol-17β (E2) and ERs are not only well known for their role in reproductive and sexual development but also known to directly influence cardiovascular health. Human platelets have been shown to express ERβ but not ERα. Studies investigating the effects of several forms of oestrogen, including oestrone (E1), oestradiol (E2), and oestriol (E3) on platelet function have yielded conflicting results. In one study, acute treatment of platelets ex vivo with either E1 or E3 was found to increase aggregation to adrenaline or ADP. In contrast, chronic treatment with oestrogen, in an alternative study, investigating oestrogen replacement therapy (3 months), found a significant decrease in adrenaline-induced platelet aggregation and ATP release in patients receiving the therapy compared to the control groups. In further support of this, chronic treatment with high levels of oestradiol in mice was found to cause a marked decrease in platelet responsiveness both ex vivo and in vivo, with both an increase in bleeding time and resistance to thromboembolism being observed. However, it is important to note these effects on platelet reactivity are due to modulation of expression of platelet proteins (such as β1 tubulin) during haematoipoiesis that then alter platelet production and activation, rather than a direct non-genomic effect on platelet function.

### 3.3 Androgen receptor

The AR, activated by either testosterone or dihydrotestosterone, has been identified in platelets, but little is known regarding its potential role in the regulation of platelet function. Some studies have specified that the aggregation response of platelets isolated from male rats was stronger in comparison to female rats owing to higher levels of androgenic steroids as platelet aggregation was found to be reduced following castration in male rats and the reversal of these effects following treatment with testosterone. Pilo et al. also reported that acute treatment of rat or human PRP with testosterone potentiates platelet aggregation induced by ADP, adrenaline, collagen, arachidonic acid, and calcium ionophore indicating its rapid non-genomic responses. Two independent studies also confirmed that testosterone causes a significant increase in TXA<sub>2</sub> receptor density on the platelet surface, thereby, indirectly increasing platelet responsiveness. However, inhibition of platelet aggregation has also been observed following treatment with testosterone, although this was found to be attributed to endothelial NO synthesis and therefore not necessarily a direct effect of testosterone on the platelet AR and platelet activity.

### 4. Type II NRs

#### 4.1 Liver X receptor

LXR receptors are implicated in the regulation of fatty acid, cholesterol, and glucose homeostasis. Endogenous ligands for the LXR receptors include oysterosters such as 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, 27-hydroxysterol, and several synthetic ligands including GW3965 and T0901317 have also been developed. Like some of the other NRs, ligands for LXR have been described to have anti-inflammatory and atheroprotective properties, and LXRβ has been shown to be expressed in platelets. Treatment of platelets with the
synthetic agonist GW3965 results in inhibition of platelet activation, with attenuation of aggregation, calcium mobilization, secretion and integrin activation observed following stimulation by collagen, collagen-related peptide (CRP-XL; a GPVI collagen receptor-specific agonist), and thrombin. In analysis of thrombosis, GW3965-treated mice were also found to form smaller, less stable thrombi following laser injury of the cremaster arterioles. LXR has also been shown to interact with several components of the GPVI signalling pathway following treatment with GW3965, including Syk and PLCγ2, and treatment with LXR ligands is associated with decreased phosphorylation and signalling. In support of this, another study reported the ability of endogenous LXR ligand 22(R)-OH-cholesterol [but not its stereoisomer 22(S)-OH-cholesterol] to inhibit collagen-induced platelet aggregation and shape change.

During thrombus formation, two distinct populations of platelets appear, coaggregated platelets, which support thrombus growth, and loosely attached procoagulant platelets that expose phosphatidylserine and support coagulation. Conversion to the procoagulant state is also thought to be associated with platelet hyper-reactivity, a trait often observed in patients with an increased risk of thrombosis including those with pathological conditions, such as hyperlipidaemia, obesity, and high plasma cholesterol levels. Treatment of platelets with LXR ligands, GW3965 and T0901317, and natural ligands, 27-OH-cholesterol and 24-(S)-hydroxyl-cholesterol, has also been shown to cause platelet inhibition to several agonists through the conversion of platelets to procoagulant coated platelets. LXR ligand-stimulated coated platelets not only expose phosphatidylserine at the membrane surface but also retain high levels of fibrinogen (which is converted to fibrin) and other alpha granule components at the platelet membrane (Figure 1). Conversion to the coated platelet state is thought to support coagulation but renders the platelet, through closure of integrin αIIbβ3, unresponsive to platelet agonists, which was also observed in platelets following treatment with LXR agonists. The mechanism by which this occurs in LXR agonist (GW3965)-

Figure 1 LXR and FXR ligands negatively regulate platelet function through inhibition of platelet signalling and formation of procoagulant coated platelets. Treatment of platelets with LXR ligands results in reduced tyrosine phosphorylation of key GPVI signalling molecules Syk, LAT and PLCγ2. An increase in the level of LXR–Syk and LXR–PLCγ2 interaction is also observed. Exposure to LXR ligands also renders platelets into a procoagulant state characterized by the exposure of phosphatidylserine and α-granule contents on the platelet surface, which is coupled with depolarization of the mitochondrial membrane potential, reduced calcium mobilization and down-regulation of the affinity of integrin αIIbβ3, ultimately resulting in the inhibition of platelet aggregation. Similarly, incubation of platelets with FXR ligands can lead to platelet swelling and conversion to procoagulant coated platelets which is dependent on cyclophilin D activity. Additionally, FXR ligands are able to increase cGMP levels that promotes the activity of PKG and phosphorylation of VASP S239 and thereby suppresses platelet activation.
treated platelets appears to be via deregulation of intracellular calcium signalling, depolarization of the mitochondrial membrane potential independently of cyclophilin D, and generation of reactive oxygen species (ROS). It is therefore possible that the platelet dysfunction observed in patients with high cholesterol, hyperlipidaemia, metabolic syndrome, and obesity could be attributed to altered LXR signalling in platelets.

4.2 Farnesoid X receptor
The bile acid receptor, FXR, which is recognized to regulate bile acid and cholesterol homeostasis has been identified in both human and mouse platelets. Treatment of platelets with synthetic FXR ligand GW4064 was found to cause a decrease in sample turbidity, which was later confirmed to be due to platelet swelling and conversion of platelets to a procoagulant state, forming coated-platelets. Synthetic and natural FXR ligand-dependent formation of coated platelets, prior to platelet agonist stimulation, results in phosphatidylserine exposure, retention of fibrinogen, fibrin and alpha granule proteins at the platelet surface, cyclophilin D-dependent depolarization of the mitochondrial membrane, sustained calcium signalling, generation of reactive oxygen species, and closure of integrins at the platelet surface. This closure of platelet integrins is believed to underlie the observed reduction in platelet aggregation to platelet agonists. Although the initial kinetics of thrombus formation was increased in mouse in vivo models of thrombosis, consistent with a procoagulant state, thrombus stability was significantly decreased following treatment with the FXR ligand GW4064, in agreement with reduced integrin function, which is essential for stable thrombus formation. Treatment with FXR ligands was also found to be associated with an increase in intracellular levels of cGMP in platelets, indicative of deregulation of intracellular signalling (Figure 1). Platelets from FXR-deficient mice were found to be unresponsive to the actions of FXR agonists, confirming the selective non-genomic actions of these ligands to the FXR.

4.3 Peroxisome proliferator-activated receptors
PPARs represent three NR isoforms, PPARα, PPARβ, and PPARγ, which are involved in cell development, differentiation, cholesterol and fatty acid metabolism, and glucose homeostasis. All three isoforms of PPARs, upon binding to their ligands, are capable of heterodimerizing with the RXR and all have been identified to have acute, non-genomic, negative-regulatory effects in human platelets.

4.3.1 PPARα
The treatment of platelets with ligands of PPARα such as fenofibrate or statins (simvastatin) has been shown to inhibit ADP-stimulated platelet activation by increasing intracellular levels of cAMP via a PPARα-dependent mechanism. In support of this, the observed inhibition can be reversed following treatment with PPARα antagonist GW6471. This dependence on PPARα is further reinforced by experiments that show fenofibrate-induced inhibition of platelet activation and increased bleeding time in mice does not occur in mice deficient in PPARα. Fenofibrate-induced inhibition of platelet activity was found to be mediated through up-regulation of cAMP levels via inhibition of PKCα, a key mediator of platelet signalling, through interaction between PPARα and PKCα. This interaction is believed to sequester PKCα away from its substrates and thereby attenuates platelet functions (Figure 2). These findings identify PPARα as a key mediator of statin and fenofibrate-mediated anti-platelet activity.

![Figure 2](image-url) Non-genomic regulation of platelets by PPAR ligands. PPARα ligands, fenofibrate or simvastatin and PPARβ/δ ligands, GW0742 and L-165041 cause a reduction in intracellular calcium mobilization and platelet activation. This inhibition was found to be mediated by augmented levels of cAMP and attenuation of PKCα through its interaction with PPARα or PPARβ/δ, which limits its availability to facilitate signalling downstream of PKCα. Treatment with PPARγ ligands inhibits platelet activation to collagen through an inhibition in phosphorylation of Syk and LAT that mediate signalling initiated by the collagen receptor GPVI. Negative regulation of integrin αIIbβ3 outside-in signalling was observed as an outcome of up-regulation of PKA activity and inhibition in phosphorylation of β3 and subsequent downstream signalling molecules—Syk, PLCγ2, PKC substrates, FAK and PI3K substrates.
4.3.2 PPARβ/δ

Studies using synthetic ligands for PPARβ/δ, GW0742, and L-165041 have identified negative-regulation of platelet activity arbitrated through PPARβ/δ. Incubation with PPARβ/δ ligands showed inhibition of platelet aggregation and mobilization of intracellular calcium following stimulation by several platelet agonists. PPARβ/δ can also be activated by the prostaglandin PGF2α, and therefore some of the inhibitory effects of PGF2α on platelet activity could also be mediated through PPARβ/δ in addition to the prostaglandin IP receptor but this has yet to be tested. Similar to PPARα, treatment of platelets with synthetic ligands of PPARβ/δ have been shown to cause an increase in intracellular cAMP levels and PKCa has been identified as a potential binding partner of the receptor indicating a plausible mechanism by which PPARβ/δ regulates platelet reactivity (Figure 2). PPARβ/δ ligands have been shown to decrease plaque formation and attenuate the progression of atherosclerosis. As platelets play a key role in the initiation and progression of atherosclerosis, anti-platelet effects of PPARβ/δ ligands may partly explain such observed reduction in the development of atherosclerosis.

4.3.3 PPARγ

PPARγ is the most widely studied of the PPAR family in platelets. This is mainly because of its direct involvement with numerous cardiovascular diseases, such as diabetes mellitus, atherosclerosis, and thrombosis. Synthetic ligands of PPARγ, the thiazolidinediones (pioglitazone, rosiglitazone, etc.), are currently in use for the treatment of type 2 diabetes and have been observed clinically to have cardioprotective properties. The anti-platelet activity of PPARγ ligands may provide a mechanistic basis that in part underlies these observations. For example, a clinical study conducted on patients suffering from coronary heart disease and taking rosiglitazone reported its long-term antiplatelet protective properties. The anti-platelet activity of PPARγ ligands has been identified as a potential binding partner of the receptor suggesting a plausible mechanism by which PPARγ regulates platelet reactivity (Figure 2). PPARγ ligands have been shown to decrease plaque formation and attenuate the progression of atherosclerosis. As platelets play a key role in the initiation and progression of atherosclerosis, anti-platelet effects of PPARγ ligands may partly explain such observed reduction in the development of atherosclerosis.

4.4 Retinoic acid receptor

RARs play a critical role in numerous biological processes, including development, reproduction, immunity, organogenesis, and homeostasis. Three forms of RAR exist—RARα, RARβ, and RARγ. Of these, RARα is ubiquitously distributed and has been reported to be robustly expressed in human platelets, whilst the other two isoforms have tissue-specific distribution. RARs are activated by retinoids which are metabolites of vitamin A and several synthetic ligands also exist. In platelets, RARs has been observed to directly interact with actin-related protein-2/3 complex (Arp2/3) subunit 5 (Arp2/3/5) which is required for the regulation of platelet cytoskeletal processes. Treatment of platelets with the endogenous RAR ligand all-trans-retinoic acid (aTRA) disrupts the RARs–Arp2/3 interactions resulting in an inhibition of both cytoskeletal rearrangements and platelet spreading (Figure 3). Recent developments have reported that RARα is capable of regulating protein synthesis (including microtubule-associated protein-1 light chain 3 beta 2) in human platelets by binding to a subset of mRNAs and blocking translation. Schwetz et al. found that platelets treated with RAR ligand aTRA for several hours displayed significantly altered levels of protein synthesis compared to controls.

4.5 Retinoid X receptor

RXR is regarded as one of the most important receptors in this superfamily. Most likely due to its ability to interact with almost a quarter of the known human NRs (PPARs, LXR, FXR, PXR, etc.) and form heterodimers, although, the presence of RXR homodimers has also been reported. RXR is well characterized and is involved in the regulation of some of the most vital and fundamental biological processes including cell proliferation, differentiation and death, haematopoiesis, metabolism (glucose, fatty acid, and cholesterol), and pattern formation during embryogenesis. Human platelets have been shown to express RXRα and RXRβ (but the presence or absence of RXRγ has not been established) and are known to form heterodimers with PPARα, PPARγ, and LXR in platelets. Treatment of platelets with the endogenous ligand of RXR, 9-cis-retinoic acid or the synthetic ligand, methoprene acid, results in inhibition of platelet function stimulated by Gq-coupled GPCRs—ADP, U46619 or thrombin and also, GPVI-mediated platelet activation via stimulation by collagen or CRP-XL. Regulation of GPCR-mediated platelet activation by RXR has been associated with its binding to Gqα in a ligand-dependent manner that inhibits Gq-induced Rac activation and intracellular Ca2+ mobilization. Exposure to RXR ligands has also been shown to reduce integrin αIIbβ3 outside-in signalling and cytoskeletal rearrangements. The negative regulation of several platelet activation pathways and processes results in robust inhibition of thrombosis and haemostasis in vivo. As seen with several other NRs, treatment with RXR ligands has also been shown to up-regulate PKA activity and VASP S157 phosphorylation via a process that is dependent on cAMP and also involves NFκB (Figure 3). This suggests that RXR ligands inhibit platelet function using several different
inhibitory mechanisms which is likely to reflect its ability to form hetero-
dimers with several different NRs in platelets.43

4.6 Vitamin D receptor
The VDR is another ligand-activated transcription factor that mediates
the actions of vitamin D and its metabolites. VDR is also known to form
a heterodimer with the RXR and regulate calcium homeostasis, cell
growth and differentiation, detoxification of xenobiotics, and modulation
of adaptive and innate immunity.70 Although anticoagulant effects of vita-
mamin D have been reported and VDR signalling has been characterized in
monocytes and vascular cells, the role for the VDR in platelet function
remains unknown. Human platelets have been found to express the
VDR. Biochemical fractionation studies along with immuno-electron
microscopy analysis identified the VDR to be localized in the soluble and
mitochondrial compartment.44 Although little is known about the role
for vitamin D and the VDR in platelet function, a patient study identified
a strong association between low vitamin D plasma levels and a high
mean platelet volume, a marker of platelet hyperactivity.45

5. Future perspectives

5.1 Could NRs offer anti-platelet therapeutic targets?
The role of platelets in controlling haemostasis and initiating thrombosis
is well known. This makes them important therapeutic targets for the
treatment of cardiovascular diseases, particularly atherothrombosis.71
Despite significant advances in the development of antithrombotic thera-
peutics, they are associated with increased bleeding risk and their efficacy
is often compromised in patients suffering from several conditions, such
as hypertension and diabetes.71,72,73 Therefore, more refined and effective
therapeutics that ensure a balance between the treatment of throm-
bosis and related complications is needed.

A key step forward would be using our current knowledge of the
molecular mechanisms governing platelet functions as the basis for the
development of more effective and safer anti-platelet therapies. Both
natural and synthetic ligands of NRs have been shown to exhibit non-
genomic effects to alter platelet function through a variety of mecha-
nisms, several of which appear to be shared by different NR family mem-
bers.21,74 For instance, RXR, FXR, PPARα, PPARβ, and PPARγ receptors
have been shown to be involved in the regulation of platelet inhibitory
signalling pathways by either increasing cAMP or cGMP levels or directly
modulating PKA/PKG activity. LXR and PPARγ can negatively regulate
signalling downstream of the collagen receptor via interactions with dif-
ferent components of the GPVI signalling cascade. Given the potential of
heterodimeric receptor interactions between RXR and other NRs, the
idea of cross-talk becomes even more pronounced.43

5.2 Important considerations
Development of NRs as anti-platelet therapeutic targets requires a few
important considerations. First, the majority of the studies conducted so
far focus on understanding the acute effects of NR ligands on platelet
function; therefore, prior to further development it would be important
to study the implications of chronic exposure of platelets to NR ligands.
As NRs can regulate the expression of multiple genes, in various cell
types it is therefore highly likely that chronic exposure to NR ligands
could lead to systemic effects that might indirectly affect platelet
function.75

Figure 3 Inhibition of platelet function by RAR and RXR ligands is mediated through Arp2/3- and Gq-induced Rac activation and up-regulation of PKA
activity, respectively. RAR ligand atRA disrupts the RARα–Arp2/3 interaction resulting in an inhibition of cytoskeletal rearrangements and platelet spreading.
RXR ligands, 9cRA and methoprene acid inhibit platelet activation to a range of platelet agonists that include GPCR agonists (ADP, U46619 or thrombin)
and GPVI agonists (collagen or CRP-XL). Interaction of RXR with Gq and subsequent negative regulation of Rac activation is one of the probable explana-
tions for the reduction in GPCR-mediated platelet activation. These ligands have also been shown to up-regulate PKA activity in a cAMP- and NFκB-dependent
manner providing a more generalized mechanism of inhibition.
has the ability to activate both PPARs and RXRs.83 Guggulsterone is regarded as an ER agonist but an antagonist to FXR, GR, and AR,84 although it does not appear to function as a non-genomic FXR antagonist in platelets.85 This makes the selective targeting of the NRs even more challenging and as such identification of ligands that function in a receptor- and gene-specific manner is important. Future work will be required to establish how and when NR heterodimers regulate platelet activity, and for each NR to establish its role in normal physiological processes.

### 6. Conclusions

Platelets are known to act as direct contributors towards the progression of CVDs such as atherosclerosis,86 and their activity becomes considerably enhanced in cases of hyperlipidaemia,87 obesity,88 diabetes mellitus,89 or hypertension.90 Many NRs have been found to be expressed in human platelets, including AR, ER, GR, FXR, LXR, PPARs, RAR, RXR, and VDR, and agonists for several of these receptors have been shown to elicit anti-platelet effects by a variety of mechanisms. NRs including PPARs, LXR, and FXR ligands have all been reported to have anti-atherosclerotic effects,91,92 coupling this with their anti-platelet effects; there exists the possibility of a potentially new paradigm of treatment that can target a range of pathophysiological conditions whilst also offering platelet-targeted anti-thrombotic activity. Of FDA-approved drugs, 13% function by targeting NRs for the treatment of numerous pathological conditions93 (Table 3), and as a result, the effects on platelets might be a likely consequence associated with the administration of these drugs. It is important for the future development and use of NR agonists that their acute and long-term effects on platelet function are fully understood.

### Authors’ contributions

AJU, G.D.F, and J.M.G wrote the review. AJU and G.D.F contributed equally.

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