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6-Nitrodopamine is Released by Washed Platelets and Blocks Dopaminergic and Serotonergic Potentiation of Platelet Aggregation

A 6-nitrodopamina é liberada das plaquetas e bloqueia a agregação plaquetária potencializado pela dopamina e serotonina.

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6-NITRODOPAMINE IS RELEASED BY WASHED PLATELETS AND BLOCKS DOPAMINERGIC AND SEROTONERGIC POTENTIATION OF PLATELET AGGREGATION

A 6-NITRODOPAMINA É LIBERADA DAS PLAQUETAS E BLOQUEIA A AGREGAÇÃO PLAQUETÁRIA POTENCIALIZADO PELA DOPAMINA E SEROTONINA.

Esta tese é apresentada à faculdade de ciências médicas da universidade estadual de campinas, como parte dos requisitos para obtenção do título de doutor em farmacologia.

This thesis is presented to the faculty of medical sciences at the state university of Campinas, as part of the requirements for obtainment of the title of doctor in pharmacology.

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RESUMO

Objetivos: a 6-nitrodopamina (6-ND) foi previamente identificada como uma substância endógena com propriedades antagônicas da dopamina, este estudo visa identificar seu papel na agregação plaquetária. Principais métodos: A cromatografia líquida acoplada à espectrometria de massa foi usada para quantificar os níveis de 6-nitrodopamina, cAMP e cGMP de amostras de plaquetas lavadas. A agregometria óptica foi usada para identificar o papel de 6-ND, Dopamina, antagonistas dopaminérgicos seletivos e agonistas dopaminérgicos seletivos na agregação estimulada por ADP e colágeno para identificar qualquer papel inibitório ou potencializador de 6-ND. As agregações foram realizadas em PRP humano e plaquetas lavadas. Resultados: As plaquetas liberam 6-ND após estimulação pela trombina. A pré-incubação de 6-ND em plaquetas antes da estimulação com trombina não afeta os níveis de cAMP ou cGMP. 6-ND não afeta a agregação de PRP induzida por ADP, colágeno ou epinefrina ou agregação de plaquetas lavadas induzida por trombina. 10 µM de dopamina é suficiente para potencializar a agregação induzida por ADP e colágeno em concentrações abaixo do limiar. 6-ND reduz a potenciação da dopamina e serotonina da agregação induzida por ADP e Colágeno. A análise de antagonistas dopaminérgicos conhecidos revelou que Haloperidol, PG01037 e SB277011A reduzem a potenciação da dopamina, mas não a potenciação do ADP basal e do colágeno. A cetanserina bloqueia a agregação potencializada pela serotonina, mas não pela dopamina. Sumanirol um agonista D2 seletivo, potencializou agregação que foi bloqueada por 6-ND e PG01037. Significado: Os dados sugerem que o 6-ND pode funcionar como um antagonista do receptor D2/3 e da serotonina. Dado que tanto a dopamina quanto o 6-ND são liberados pelo endotélio vascular, o 6-ND pode desempenhar um papel protetor contra trombose envolvendo disfunção vascular ou como antipsicótico.

Palavras-chave

Agregação plaquetária, catecolamina, dopamina, 6-nitrodopamina, antagonista da serotonina.

ABSTRACT

Aims: 6-nitrodopamine (6-ND) has been previously identified as an endogenous substance with dopamine antagonistic properties, this study aims to identify its role in platelet aggregation.

Main Methods: Liquid Chromatography couple to mass spectrometry was used to quantify 6-nitrodopamine, cAMP and cGMP levels from washed platelet samples. Optical aggregometry was used to identify the role of 6-ND, Dopamine, known receptor subtype selective dopaminergic antagonists and receptor subtype selective dopaminergic agonists have in ADP and Collagen stimulated aggregation to identify any inhibitory or potentiator role of 6-ND. Aggregations were carried out in human PRP and washed platelets.

Key findings: Platelets release 6-ND upon stimulation by thrombin. Preincubation of 6-ND in platelets before stimulation with thrombin, does not affect cAMP or cGMP levels. 6-ND does not affect ADP, Collagen or Epinephrine Induced PRP aggregation or Thrombin-induced washed platelet aggregation. 10 µM Dopamine is sufficient to potentiate ADP and Collagen induced aggregation at subthreshold concentrations. 6-ND reduces dopamine and serotonin potentiation of ADP and Collagen induced aggregation. Analysis of known dopaminergic antagonists revealed that Haloperidol, PG01037 and SB277011A reduce the potentiation by dopamine but not basal ADP and collagen potentiation. Ketanserin blocks serotonin-potentiated but not dopamine-potentiated aggregation. Sumanirole a selective D2 agonist, potentiated aggregation which was blocked by 6-ND and PG01037.

Significance: The data suggests that 6-ND may function as a D2/3 and serotonin receptor antagonist. Given that both dopamine and 6-ND are released by the vascular endothelium, 6-ND may play a protective role against thrombosis involving vascular dysfunction or as an anti-psychotic.

KEYWORDS

Platelet aggregation, catecholamine, dopamine, 6-nitrodopamine, serotonin, antagonist.

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SUMARIO

1. General Introduction

1.1 Platelet Aggregation

Many drugs and endogenous ligands have demonstrated to cause an effect on the induction or inhibition of platelet aggregation secondary to their known therapeutic or known functional actions [1].

Platelet aggregation works in collaboration with the coagulation cascade (thrombin) to cause the formation of a thrombus or a haemostatic plug [1][2]. Thrombus formation is a major complication of atherosclerotic diseases and thus platelet aggregation studies of different drugs are important in assessing the risk of haemostatic complications [1].

Platelet activation consists of a cascade of biochemical reactions. This is initiated by adhesion of platelets to disrupted subendothelial space through GPIa/IIa integrin receptors on the platelet surface via vonWillebrand factor (vWF) and exposed collagen in the sub-endothelium [2].

1.2 Platelet activation initiated by ADP and other agonists

Platelet activation can also be initiated without exposure to collagen but agonism of other receptors directly such as adenosine diphosphate (ADP) receptors (through P_{2Y1} and P_{2Y12}) and thrombin via protease activated receptors (PARs) [1][2][3].

'Inside-out' signalling occurs when agonists such as ADP cause the activation of a second integrin receptor GPIIb/IIIa (fibrinogen receptor) on the membrane via activation of an intracellular cascade [2][3]. There exists three known receptors of ADP on platelet membranes that mediate this process; P_{2X1}, P_{2Y1}, and P_{2Y12} [2][3][4].

P_{2X1} is an ligand gated ion channel linked to a calcium channel. Its role in aggregation is to potentiate aggregation via an increase in intracellular calcium in the platelet when calcium stores from the sarcoplasmic reticulum run low. P_{2Y1} and P_{2Y12} receptors are G-protein coupled receptors. P_{2Y1} is Gαq linked leading to the activation of phospholipase C, and the breakdown of PIP_2 into IP_3 and DAG. IP_3 acts on IP_3 receptors on the sarcoplasmic reticulum to mobilise calcium and increase intracellular concentrations of calcium. Calcium signalling is the major second messenger system within the platelet. Increased calcium concentrations cause the platelet shape change (filopodia development), increased fibrinogen receptor (GPIIb/IIIa) expression and activity and granule release of secondary mediators. Calcium can also activate other signalling systems such as the generation of thromboxane (TXA_2 – a potent platelet activator) by activating cPLA2 [4]. Many other platelet agonists also initiate aggregation via Gαq/Calcium signalling such as Thrombin generated in the coagulation cascade PARs) [2][4][5].

P_{2Y12} receptors are G-protein coupled receptors linked to the inhibition of adenylate cyclase (Gαi), thus inhibition leads to a reduction in the concentration

of cyclic adenosine monophosphate (cAMP) which in turn leads to the activation of the integrin receptor GPIIb/IIIa. cAMP concentrations inside platelets (and thus the P_{2Y12} receptor) determines if aggregation within the platelet is sustained or will reverse [3][4][6]. The same can be said of cGMP, in which studies have shown that activation of guanylate cyclase, GC, (by Nitric oxide (NO) or NO independent GC agonists) to increase cGMP levels also leads to an inhibition of aggregation (most likely via blockade of PDE3 breakdown of cAMP). Studies show that drugs which block GC activity have aggregating properties [6].

Granule release of secondary mediators is an important step in the aggregation process. Due to increases in Calcium and decreases in cAMP within the platelet, exocytosis occurs releasing mediators such as ADP, Serotonin – 5-HT, coagulation factors as well as others) from alpha and dense granules [1][2]. These then act on neighbouring platelets to cause more local aggregation. [2][3][4][5].

Outside-in signalling (the latter part of the aggregation process) also occurs in which the GPIIb/IIIa is activated directly by an agonist without an intracellular messaging cascade. For a fuller review of these signalling processes please see Li et al 2010 [3]. Aggregation concludes when the GPIIb/IIIa receptor then acts to bind fibrinogen which increases platelet-platelet adhesion, the expression of procoagulant factors and the formation of either a haemostatic plug or a pathological thrombus. [2][3].

Pharmacologically speaking, the current problem exists in that drug therapies to target platelet inhibition in cardiovascular disease does not distinguish pathology from physiology and as such, many adverse bleeding effects can occur with antiplatelet drugs [4].

1.3 Catecholamines in Platelet Aggregation

Endogenous catecholamines (such as noradrenaline, adrenaline and dopamine) are produced by cells of the sympathetic nervous system, the central nervous system and the adrenal medulla (adrenaline only). Catecholamines as well as relate compounds such as 5HT are known to influence platelet aggregation [7][8][9][10][11][12]. Noradrenaline and adrenaline are weak agonists that can potentiate and sustain aggregation by causing the release of vonWillebrand (vWF) factor [4][12][13], however they cannot initiate full platelet activation without the presence of another agonist such a ADP [12]

It is well established that α-2 adrenergic receptors are found on platelets to reduce cAMP concentrations, leading to a sustained aggregation response [4] and GIIb/IIIa activation [14]. Some studies have also shown that short acting β_2 agonists also increase the formation of clotting factors including vWF [13].

Literature shows that catecholamines have noticeable effects on platelet aggregation in the micromolar range (μM) [15][16] and thus are higher than found physiologically in circulation (pM (10^{-12})- nM (10^{-9}) [17][18][19], however

when considering that the endothelium produces catecholamines [20], the local concentration of catecholamines released from a portion of the endothelium will be much higher than compared with the entire circulation, potentially reaching concentrations that can potentiate aggregation.

In individuals with endothelial dysfunction and its related pathologies, platelet aggregation shows hyperactivity [21]. *In vitro* studies have shown that in hypoxic conditions, known to induce endothelial dysfunction, tyrosine hydroxylase expression is upregulated in multiple tissues including the endothelium, leading to increased dopamine production and release [22][23].

Increased catecholamine release due to endothelial dysfunction could lead to potentiated platelet activation and thus increased thrombotic risk. Platelets from patients with essential hypertension patients show increased α2 receptor responsiveness to epinephrine induced [24] and in platelets from patients who recently suffered heart attack, there are raised plasma concentrations of epinephrine and platelet α2 receptor density [25][26].

Dopamine has its own receptors present on platelets and is mostly reported to potentiate aggregation, but it still remains fully undetermined which receptor dopamine acts on [16][27][28][29][30].

D2-like receptors (consisting of D2, D3 and D4 receptors) are the main reported dopaminergic receptor on the platelet, with Gi-protein signalling, leading to a decrease in the activity of adenylate cyclase and cAMP production. Reduced cAMP levels are reported to increase platelet activation via inside out signalling [16]. D1-like receptors (D1 and 5) are Gs linked, which would lead to an increase in cAMP and thus be anti-aggregatory. Although expressed on the platelet, the role of D1-like receptors is yet to be confirmed [27][29].

6-Nitrodopamine (6-ND), used in nanotechnology for medical applications, is a member of a group of compounds known as nitro-catecholamines [31][32][33]. Nitro-catecholamines have previously been identified in rat brain tissue but their full physiological, pathological and pharmacological roles are yet to be evaluated [34].

6-ND differs in its structure from dopamine by containing an NO2 group on carbon six of the catechol-ring [32]. 6-ND is endogenously synthesised by endothelial cells [35]. In vitro, the synthesis of 6-ND occurs by the reaction of dopamine with nitrite or peroxynitrite radicals, which are produced under oxidative stress [36][37]. It is known in-vivo that these radicals can be converted to Nitric Oxide (NO) which acts as a potent vasodilator and inhibitor of platelet aggregation [38]. Until the present, 6-ND has not been investigated for any role in NO signalling.

6-ND among other 6-nitrocatecholamines may act as inhibitors of neuronal nitric oxide synthase (nNOS), making them potentially therapeutic in inhibiting NO induced neurotoxicity in Parkinson’s disease [39], however, when oxidated to

nitrosating products, they may themselves contribute to oxidative stress in neurodegenerative disease [37].

The action of 6-ND on platelets is unlikely to act to inhibit NOS since it's expression in the platelet is not confirmed [40]. 6-ND can inhibit monoamine oxidase B (MAO-B) and increase dopamine bioavailability in rat striatum [41]. Increased platelet MAO-B activity is thought to be a biomarker of vulnerability to dementia and other neurogenerative diseases [42], identifying therapeutic potential for 6-ND.

Our group has recently reported that 6-ND has activity as a dopaminergic d2 like receptor antagonist in human umbilical cord vessels, blocking smooth muscle contractions induced by dopamine but not other vasoconstrictors [35]

6-ND has also been recently reported to cause contractions of the rat vas deferens via a 6-ND receptor which is antagonised by tricyclic antidepressants. [43].

In this thesis, two publications are included investigating the role that 6-ND has in platelet function. The first publication details quantification of 6-ND release from platelets by LC-MS/MS, along with PRP and washed platelet - platelet aggregation assays to determine if 6-ND is a platelet activator like the other catecholamines or to see if it plays another role. The second publication details the effect of 6-ND on platelet cAMP and cGMP levels following thrombin stimulation of washed platelets.

2. Submission 1:

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6-Nitrodopamine is Released by Washed Platelets and Blocks Dopaminergic and Serotonergic Potentiation of Platelet Aggregation.

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6-Nitrodopamine is Released by Washed Platelets and Blocks Dopaminergic and Serotonergic Potentiation of Platelet Aggregation.

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ABSTRACT

Aims: 6-nitrodopamine (6-ND) has been previously identified as an endogenous substance with dopamine antagonistic properties, this study aims to identify its role in platelet aggregation.

Main Methods: Liquid Chromatography couple to mass spectrometry was used to quantify 6-nitrodopamine from washed platelet samples. Optical aggregometry was used to identify the role of 6-ND, dopamine, known receptor subtype selective dopaminergic agonists and antagonists in ADP, collagen, adrenaline and thrombin stimulated platelet aggregation. Aggregations were carried out in human platelet rich plasma (PRP) and washed platelets.

Key findings: Washed platelets release 6-ND upon stimulation by thrombin. 6-ND does not cause or inhibit platelet aggregation and does not affect ADP-, collagen- or adrenaline- induced PRP aggregation or thrombin-induced washed platelet aggregation. Dopamine (10 µM) potentiated ADP and Collagen induced aggregation at subthreshold concentrations. 6-ND (10-100 µM) reduced dopamine potentiation of ADP- and collagen- induced aggregation. The dopamine $D_{2\text{-like}}$ receptor antagonist haloperidol and the D_3 receptor antagonists PG01037 and SB277011A reduced the potentiation induced by dopamine but not the aggregation induced by l ADP or collagen. The selective D_2 agonist sumanirole (10-100 µM) potentiated ADP- and collagen-induced aggregation, which was blocked by 6-ND.

Significance: The data demonstrates that platelets release 6-ND and that 6-ND acts as a D_2 receptor antagonist. Given that both dopamine and 6-ND are released by the vascular endothelium, 6-ND modulates platelet reactivity and may play a protective role against thrombosis involving vascular dysfunction.

Keywords:

haloperidol, sumanirole, ADP, thrombin, collagen, adrenaline.

1. Introduction

6-Nitrodopamine (6-ND) is a novel catecholamine that is released by human umbilical cord vessels (HUCV) [1], rat isolated vas deferens [2] and by rat isolated heart [3]. The synthesis/release of 6-ND is coupled to nitric oxide (NO) synthesis, since it is inhibited by previous incubation of the tissues with the NO synthase inhibitor L-NAME. Indeed, in rats chronically treated with L-NAME, a 50% reduction in 6-nitronoradrealine extracted from rat brain was observed when compared to control animals [4]. Similar degree of inhibition was also observed in vas deferens, and hearts obtained from L-NAME chronically treated animals [2,3]. In the rat epididymal vas deferens, 6-ND has a neurogenic origin and acts in a specific receptor that is antagonized by tricyclic compounds [2] and by alpha1-adrenergic antagonists [5]. In the HUCV, 6-ND is released from the endothelium and acts as a dopamine D_2-like receptor antagonist [1].

Membranes from human blood platelets express high affinity, saturable and stereoselective binding sites for the $D_{1\text{-like}}$ receptor antagonist SCH-23390 [6], although it was later attributed to a labelling of a novel 5-hydroxytryptamine binding site [7]. The $D_{2\text{-like}}$ antagonist ^{3}H-spiroperidol binds human platelet membranes in a reversible, saturable and stereospecific manner [8]. The binding was specifically displaced by the $D_{2\text{-like}}$ antagonist haloperidol. In contrast to SCH-23390, the ^{3}H-spiroperdiol binding was not displaced by 5-hydroxytryptamine. Dopamine per se does not cause platelet aggregation directly [9,10] although at extremely high concentrations (2 mM) it enhances platelet aggregation [11]. Dopamine can increase the platelet sensitivity to other aggregating agents such as ADP, collagen and arachidonic acid [9].

In this study, LC-MS/MS was used to quantify 6-ND, dopamine, noradrenaline, and adrenaline release from thrombin-stimulated washed platelets. Optical aggregation was used in both PRP and washed platelets to determine the effect of 6-ND on platelet aggregation stimulated by adenosine diphosphate (ADP), collagen, adrenaline and thrombin. Further experiments were carried out to compare 6-ND interactions with dopamine and serotonin in potentiated aggregation. Further to this, known subtype selective dopaminergic agonists and antagonists were also characterised in dopamine potentiated aggregation to elucidate the mechanism by which 6-ND affects platelets aggregation.

Our results clearly demonstrate that 6-ND is released from human washed platelets following thrombin-induced aggregation. Furthermore, 6-ND inhibits dopamine and 5-hydroxytryptamine-induced platelet aggregation, and the mechanism of action is associated with D_2 receptor antagonism.

2. Method

2.1. Study Participants

Fifteen healthy volunteers from either sex over the age of eighteen, who were not taking any medication were invited to take part in the study. All participants were between the ages of 18-65 years old. The informed consent form was obtained from those who agreed to participate. The investigation followed the principles outlined in the Declaration of Helsinki and the protocol was approved by the Ethics Committee of the Faculty of Medical Sciences of the University of Campinas (FCM, UNICAMP, 3.092.338).

2.2. Reagents

Adenosine diphosphate (ADP), dopamine, adrenaline, serotonin, thrombin, ketanserin, clozapine, prostacyclin, SCH23390, PG01037, SB277011A, PD128907 and A412997 were acquired from Sigma-Aldrich Chemicals Co. (St Louis, Missouri, USA). Haloperidol was purchased from SM Empreendimentos Farmaceuticos LTDA (Brazil). Horm Collagen Reagent was acquired from Takeda (Osaka, Japan). 6-ND and the internal standard used for LC-MS/MS were purchased from Toronto Research Chemicals (Canada). Sumanirole was obtained from Tocris (Bristol, UK). Fenoldopam was obtained from Cayman Chemical Co (Michigan, USA). The deuterated dopamine, noradrenaline and adrenaline were obtained from CDN isotopes.

Reagents were prepared and diluted in physiological saline 0.9% NaCl, except for collagen which was diluted using the manufacturers supplied diluent. Serotonin, adrenaline and dopamine were prepared fresh on each day of the protocol to account for the rapid degradation properties they have. ADP and collagen stocks were prepared up to one week beforehand and stored at 4° Celsius. Other dopaminergic/serotonergic antagonists were stored according to manufacturer's instructions, diluted with saline to appropriate concentrations.

2.3. Preparation of PRP and Washed Platelets

40ml of whole blood were taken from human volunteers (n=5) in 9:1 volume with sodium citrate 3.2% anticoagulant (20ml) for the preparation of platelet rich plasma (PRP) or ACD-C for preparation of washed platelets (20ml).

Citrated PRP was prepared by centrifugation at 400g for 12 mins 25° Celsius then collected, the remaining blood was centrifuged again at 800g for 12 mins at 25°C to obtain PPP. PPP was used as a blank in aggregation to represent 100% aggregation. Platelet counts were performed in PRP but

not adjusted and were used in the study if the count was between 2-4 x 10^8 platelets/ml. PRP suspensions were used within 3 hours of preparation.

For washed platelets, blood anticoagulated with ACD-C was centrifuged at 400g for 12 minutes at 25° Celsius. The plasma was collected, 0.1uM prostacyclin was added and centrifuged again at 800g for 12 minutes at 25° Celsius to collect the platelet pellet. The pellet was carefully resuspended in warmed Krebs-Heinseleit's solution (KHS; without calcium) and 0.1uM prostacyclin once more. The centrifugation process was repeated once more at 800g and the resulting pellet was carefully resuspended in warmed KHS (without calcium and without Prostacyclin) and platelet count adjusted to 1.5x 10^8 platelets/ml. In washed platelet suspensions, calcium chloride was added just before drug incubation and aggregation. Washed platelet suspensions were used within 4 hours of preparation.

2.4 Quantification of Catecholamines in washed platelet supernatants

Quantification of 6-ND, Dopamine, Adrenaline and Noradrenaline in KHS was performed by LC-MS/MS [1,2,12]. Washed platelets were prepared as described above and 400uL of washed platelets were stimulated with 0.1IU/ml of thrombin for 5 minutes. Following this, the suspension was centrifuged at 4000g for 5 minutes and 1mM of Ascorbic acid added to each sample. The resulting supernatant was stored at -80 ºC until used for catecholamine determination by LC-MS-MS.

2.5 Optical Aggregometry

Platelet aggregation was performed with an optical/luminescence aggregometer (Chrono-log, Kordia Life Sciences, Leiden) at 37 °Celsius with 200 µL of PRP or washed platelet suspension placed in glass cuvettes containing a disposable stir bar for constant stirring. Platelet stimulation was carried out by adding adrenaline, ADP or collagen in the groups described below. In some groups, potentiating agents (serotonin or dopamine) were added to PRP, 30 seconds prior to the addition of the platelet agonist. 6-Nitrodopamine and dopaminergic antagonists were incubated in PRP or washed platelets 3 minutes prior to the platelet agonist. Negative controls were performed for each reagent separately before agonist stimulation/potentiation.

The maximal aggregation (%) was calculated using the Aggrolink Software (Chrono-log). PPP or Krebs solution (for washed platelets) was used as a control to provide a signal representing 100% aggregation. In the groups using 6-ND, the appropriate concentration was also added to the PPP or Krebs solution as the drug has a slight yellow colour that could interfere with

light transmittance values if not accounted for in the blank. Maximum aggregation (MA) was recorded.

2.6 Experimental Design

2.6.1 Evaluating 6-Nitrodopamine in Platelet Aggregation

Concentration response curves were ascertained for ADP, collagen and adrenaline separately in human PRP. This was repeated in washed platelets for thrombin at 0.1UI/ml. The protocol was then repeated for ADP and collagen in the presence of dopamine (1, 5 and 10uM) or Serotonin (10uM) to measure the potentiating ability of these compounds in platelet aggregation.

To ascertain if 6-ND has a direct effect on platelet aggregation, 6-ND at 1, 10 and 100uM was incubated with PRP for 30 seconds, 3 minutes and 45 minutes before stimulation with ADP, adrenaline, and collagen and in washed platelets for stimulation with thrombin.

The potential interaction between dopamine/serotonin and 6-ND was investigated next by preincubation of 6-ND of for 3 minutes followed by dopamine/serotonin addition and agonist stimulation (within 30 seconds of dopamine/low dose adrenaline addition). This was done for all 3 concentrations of 6-ND (1uM, 10uM and 100uM) to see if the effect of 6-ND was concentration dependent.

2.6.2 Characterising Dopaminergic Antagonists in Platelet Aggregation

Secondary to analysing the effect of 6-ND, the platelet dopamine receptor was also characterised using dopaminergic agonists and antagonists. Subtype selective dopaminergic agonists were prepared at 1, 10 or 100uM concentrations and incubated in PRP 30 seconds before stimulation with ADP, in the aim to identify the principal dopamine receptor responsible for dopaminergic interactions in the platelet. Selective antagonists for each dopamine receptor subtype were added to PRP and incubated for 3 minutes prior to ADP and dopamine stimulation (10uM dopamine 30 seconds preincubation in PRP). Negative controls were performed to show absence of effect of the antagonists on ADP and collagen stimulated aggregation.

2.6.3 Effect of Dopaminergic Agonists in platelet aggregation

Four selective dopaminergic receptor agonists (at 0, 1, 10 and 100uM) were incubated in PRP, 30 seconds prior to stimulation with ADP, in a similar design to dopamine above, to establish any potentiating/inhibitory activity each may have.

2.7 Statistical Analysis

Paired two tailed t-tests were used for statistical analysis to compare groups which were stimulated with the platelet agonists of adrenaline, ADP, or collagen. Values of $P<0.05$ were considered statistically significant.

3 Results

3.1 Release of 6-ND from thrombin-stimulated washed platelet

LC-MS/MS analysis revealed that 6-ND is endogenously released by thrombin stimulated washed platelets (0.59 ± 0.35 ng/mL; n=4). Adrenaline (21.18 ng/ml ± 19.8 ng/ml; n=4), and dopamine (1.96 ng/ml ± 1.72 ng/ml) were also released. Noradrenaline levels were below the limit of quantitation (0.1 ng/mL). The data is shown in figure 1.

3.2 6-ND does not stimulate, potentiate or inhibit platelet aggregation induced by adrenaline, ADP or collagen.

6-ND (up to 100 μM) did not induce platelet aggregation in neither PRP (n=5) nor washed platelets (data not shown). Furthermore 6-ND did not significantly affect adrenaline, ADP or collagen in PRP or thrombin induced aggregation in WP at any concentration tested both at 3 minute incubation or 45 minute incubation. (Figure 2, n=6 for adrenaline (Panel A), n=7 for ADP (panel B), n=4 for Collagen (Panel C), n=5 for thrombin-induced washed platelets (Panel D)).

3.3 6-ND inhibits dopamine- and collagen-induced potentiation of platelet aggregation

Dopamine (10 μM) potentiated platelet aggregation induced by both ADP (0.3 and 1 μM; figure 3, panel A) and collagen (0.1 and 0.3 μg/mL; figure 3, panel B). The potentiation induced by dopamine (10μM, 30 sec incubation) was reduced in PRP pre-incubated with 6-ND (1-100 μM) in a concentration-dependent manner (figure 3, panel C). Similar results were observed in collagen-induced platelet aggregation figure 3, panel D). A longer period of incubation (45 min) did not alter the effect of 6-ND (data not shown).

3.4 6-ND reduces the potentiation of platelet aggregation by serotonin.

Serotonin (10 μM) potentiated platelet aggregation induced by ADP (0.3 and 1 μM; Figure 4, panel A and B). 6-ND pre-incubated for either 3 min (panel A) or 45 min (panel B) concentration-dependently (1, 10 and 100 μM) reduced ADP induced platelet aggregation potentiated by Serotonin. The 5-HT_{2a} antagonist ketanserin (1 μM) inhibited 5-HT (panel A and B) but not dopamine/ADP induced aggregation (panel C).

3.5 Effect of Dopamine receptor antagonists.

PRP incubation (100 µM, 3 min) with the dopamine receptor antagonists SCH-23390 ($D_{1\text{-like}}$), L-741626 (D_2), SB277011A (D_3), PG01037 (D_3) and sonepiprazole (D_4) did not cause platelet aggregation (n=5, data not shown). Similar results were obtained with the D_2-like receptor antagonists haloperidol and clozapine (100 µM, n=5; data not shown). None of the dopamine antagonists had any effect on ADP and collagen induced aggregation (Figure 5, panel A).

Neither SCH-23390, L-741626 nor clozapine affected dopamine-induced potentiation of both ADP and collagen aggregation (data not shown). In contrast to clozapine, the $D_{2\text{-like}}$ antagonist haloperidol concentration-dependently (1-100 µM) inhibited dopamine-induced potentiation of both ADP and collagen aggregation (Figure 5, panel B). Both D_3 selective antagonists PG01037 (Figure 5, panel C) and SB277011A (panel D) also caused significant concentration-dependent inhibition of dopamine-induced potentiation of both ADP and collagen. The selective D_4- receptor antagonist sonepiprazole had no effect on dopamine-induced potentiation of both ADP and collagen aggregation (data not shown).

3.6 Sumanirole potentiates ADP induced Platelet Aggregation which is blocked by 6-ND and by D_3 receptor antagonist PG01037 and by D_3 selective agonist PD128907.

The D_2 selective agonist sumanirole (100 µM) potentiated both ADP (Figure 6, panel A) and collagen (Figure 6, panel B) induced aggregation. When preincubated with 6-ND (10 and 100 µM), the potentiation by 100 µM sumanirole of ADP (Figure 6, panel C) and of collagen (Figure 6, panel D) induced aggregation was blocked in a concentration-dependent manner. The D_3 antagonist, PG01037 (1,10 and 100 µM) also reduced the potentiation caused by sumanirole in ADP aggregation (figure 6, panel E)

Incubation of PRP with the D_3 selective agonist PD128907 (n=7) did not significantly potentiate nor inhibit platelet aggregation at 1 µM, 10 µM or 100 µM in ADP stimulated platelets (data not shown). When co-incubating sumanirole and PD128907 in PRP (30 seconds) before ADP stimulation (1µM), the potentiation achieved by sumanirole alone is reduced dose dependently by the presence of PD128907 (figure 7, Panel A). This is not seen with PD128907 when used with Dopamine (10µM) as the potentiating agent or just ADP (10µM) (figure 7, panel B).

When pre-incubating the D_2 selective antagonist, L741626, in PRP (3 minutes) before sumanirole/ADP stimulation (1µM), the potentiation achieved by sumanirole alone is not affected, the same said for dopamine/ADP stimulation (figure 7, Panel C).

4 Discussion

This is the first demonstration that 6-ND is released from human platelets. The fact that noradrenaline was not quantified but 6-ND, dopamine and adrenaline were, suggests that platelets are a store of these catecholamines rather than a font.

Although 6-ND does not affect platelet aggregation directly, it blocks dopamine and serotonin potentiation of agonist-induced aggregation, indicating a potential role as a modulator of platelet reactivity *in vivo*. Phentolamine (non-selective alpha antagonist), idazoxan (α_2 antagonist) and yohimbine (α_2 antagonist) all inhibit platelet aggregation potentiated by adrenaline [9,13,14,15]. Since 6-ND did not block adrenaline-induced aggregation, it does not act as an adrenergic α_2 antagonist. Similar results were observed in HUCV, where 6-ND selectively block dopamine-induced contractions of the vascular tissues, but it did not affect the contractions induced by noradrenaline or adrenaline [1].

It is unlikely that 6-ND activates intracellular systems that prevent platelet aggregation, such as stimulation of adenylate or guanylate cyclase [16], since the effect seems specific to dopamine and serotonin-induced potentiation. Indeed, 6-ND does not affect either cAMP or cGMP levels in human washed platelets stimulated by thrombin as quantified by LC-MS/MS [17]

Thus, the reduction of dopamine potentiated aggregation is due to antagonism of dopamine receptors in the platelets. Platelets express $D_{2\text{-like}}$ receptors, most richly, D_3 and D_5, as detected by Western blot analysis and immunocytochemical techniques using antibodies raised against dopamine D_1-D_5 receptor proteins [18,19]. The finding that dopamine-induced ADP and collagen platelet aggregation potentiation is blocked by the $D_{2\text{-like}}$ receptor antagonist haloperidol, indicates involvement of $D_{2\text{-like}}$ receptors. The lack of inhibition by clozapine, another $D_{2\text{-like}}$ receptor antagonist, could be explained by the difference in the K_i for D_2 receptors (0.7 nM and 157 nM, for haloperidol and clozapine, respectively [20,21,22].

The selective D_3 antagonist, PG01037, significantly blocked dopamine potentiation concentration dependently however only at concentrations above the ki values for both D_2 and D_3 receptors, possibly suggesting its antagonism may be non- selective, blocking D2 receptors (ki D_3 = 0.7nM, ki D_2 = 93nM) [23]. This is supported by the finding that that SB277011A, a third D3 subtype selective antagonist also only reduced dopamine potentiation at a higher concentration in ADP stimulated aggregation. This is further indicated by the finding that D_3 selective agonist PD128907 did not potentiate aggregation induced by ADP but blocked concentration dependently the potentiation of ADP-induced aggregation by sumanirole, a selective D_2 agonist. Since at concentrations higher than 1uM, PD128907 can also attach to D2, D_4 and other receptors [24]. It is possible that PD128907 could be acting as a weak D2-receptor antagonist at the concentration used. Synthetic agonists for dopamine

receptors may indeed have the affinity for the receptor but lack intrinsic activity to potentiate responses [25,26]. Clonidine has been noted to act similarly in platelets. As an α_2 agonist, it can subtly potentiate platelet aggregation by ADP but in fact blocks potentiation of platelet aggregation when adrenaline is combined as a potentiating agent [27, 28]. The failure of the selective D_4 antagonist sonepiprazole to block dopamine potentiated aggregation further support the concept of the selective involvement of the D_2 receptor.

L-741626 is a selective D_2 receptor antagonist [28]. Surprisingly it had no effect on platelet aggregation potentiated by dopamine nor sumanirole as shown in figure 7, contrary to a previous report [30]. There are in fact 2 two isoforms of the D_2 receptor, differing in 29 amino acids, D_{2L} (long form) and D_{2S} (short form). The difference lies in short chain of 29 amino acids. [31, 32]. Data is currently limited regarding agonist affinities and efficacies for these separate isoforms, with no molecules being identified as particularly exclusively selective for D_{2S} or D_{2L} [33] It is possible that dopamine and sumanirole, (and thus 6-ND) are binding to one isoform present in the platelet but that L-741626 antagonises the other isoform, which may not be present or may be non-functional in the platelet, explaining why it did not block D_2 potentiated aggregation. In Schedel et al [30], it was not measured the ability of L741626 to reduce maximum aggregation, but only its ability to reduce micro-aggregation in presence of dopamine, ADP and collagen adhesion plates together, whereas here, either ADP or collagen was used, not both at the same time and only with subthreshold concentrations [30].

Apart from its dopaminergic block, 6-ND also reduced serotoninergic potentiation of platelet aggregation, although much less potently compared with Ketanserin, a highly selective 5-HT_2 receptor antagonist [34]. Ketanserin did not block dopaminergic potentiation, confirming its specificity for the 5-HT_2 platelet receptor. Platelets only express one active serotonin receptor, 5-HT_2 [35]. Risperidone an atypical antipsychotic, also a dopaminergic and serotonin antagonist has been reported to block serotonin potentiation of platelet aggregation [36]

Conclusion

6-ND has no effect on platelet aggregation induced by ADP or Collagen but does reduce dopaminergic stimulation/potentiation of platelet aggregation at sub-threshold and threshold concentrations. Considering the concept that aggregation *in vivo* occurs due to synergism of many platelet agonists acting at threshold concentrations and that catecholamines such as dopamine are produced by the vascular endothelium, 6-ND is a potential drug target for patients with high circulating catecholamine concentrations and increased thrombotic risk. Due to its dual antagonism of serotonin and dopamine, it may also make a novel antipsychotic and should be tested for as such. Our data suggests that 6-ND most likely functions as a dopaminergic antagonist, with D_2

selectivity. Further study should be undertaken to elucidate its effects in other tissues and confirm its mechanism.

5 Addendum:

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7 Declaration of competing interest

No conflict of interest

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10 List of Figures:

Figure 1. Graph showing quantified concentrations of catecholamines by LC-MS/MS in Krebs solution of stimulated washed platelet supernatants. Epinephrine (21.18 ng/ml +- SEM19.8 ng/ml), 6-ND (0.6ng/ml +- SEM 0.35 ng/ml) and Dopamine (1.96 ng/ml +- SEM 1.72 ng/ml) were found to be liberated but not that of Noradrenaline. The lower limit of quantification was 0.1 ng/ml for all catecholamines. N=4.

Figure 2. Graph showing the effect of 6-ND incubation (3 minutes) on Maximum aggregation values after 5 minutes of platelet aggregation in PRP stimulated by adrenaline (panel A, n=6) ADP (panel B, n=7), Collagen (panel C, n=4) and in washed platelets stimulated by thrombin (panel D, n=6). 6-ND (1,10, or 100uM) had no effect on platelet aggregaton.

Figure 3. Graph showing maximum aggregation values of dopamine potentiation of platelet aggregation stimulated by ADP (panel A) and Collagen stimulation (panel B) in PRP. 10uM but not 1uM or 5uM Dopamine potentiated ADP induced aggregation at 0.3 and 1uM ADP stimulation (p= 0.023 and 0.004 resp, n=6) and at 0.1ug/ml and 0.3ug/ml in collagen stimulation (p= 0.041 and 0.0259, n=5). PRP was pre-incubated with 6-ND (1,10 or 100uM) for 3 minutes or 45 minutes and then stimulated with a combination of 10µM Dopamine (30 seconds incubation) and platelet agonist. 45-minute incubation showed similar results but are not shown. Panel C: 1µM of 6-ND reduced maximum potentiated aggregation significantly at 0.1 and 0.3uM ADP/10uM Dopamine stimulation (p=0.005 and 0.01 resp. n=14). 10uM of 6-ND reduced maximum potentiated aggregation significantly at 0.1 and 0.3uM ADP/10uM Dopamine

stimulation (p=0.005 and 0.00006 resp. n=14) 100µM of 6-ND almost abolished the potentiation caused by dopamine for 0.1 (p=0.0003, n=14) and 0.3uM (p=0.0004, n=14) . Panel D: Aggregation induced by 0.3µg/ml of Collagen was potentiated by 10uM Dopamine (p=0.0008 n=7) and then reduced in a dose dependent manner when 6-ND was also preincubated (1uM p=0.03 and 10uM p=0.002 compared to potentiation group, n=7). 100uM of 6-ND abolished the Dopamine potentiation at 0.3ug/ml without affecting agonist baseline response (p= 0.002 and 0.007 resp. n=7).

Figure 4. Graph showing maximum aggregation values for Serotonin potentiated plateelt aggregation. Preincubation of 6-ND both for 3 minutes (panel A) and 45 minutes (panel B) reduces serotonergic potentiation of platelet aggregation in a dose dependent manner, n=4. Serotonin (10uM) potentiated ADP (0.3uM) induced platelet aggregation (p=0.0055) and was reduced by 6-ND (10uM and 100uM) (p=0.0008 and p=0.0081) and Ketanserin (1uM) (p=0.0045). When stimulated by 1uM of ADP, serotonin again potentated aggregation (p=0,0218) and was reduced by 6-ND (100uM) (p=0.0089) For 45 minute incubations of 6-ND/Ketanserin, similar results were found. Serotonin (10uM) potentiated ADP (0.3uM) induced platelet aggregation (p=0.0107) and was reduced by 6-ND (100uM) (p=0.0038) and Ketanserin (1uM) (p=0.0135). When stimulated by 1uM of ADP, serotonin again potentated aggregation (p=0.0005) and was reduced by 6-ND (1uM and 100uM) (p=0.025 and p=0.0221). The 5HT2a antagonist Ketanserin was included as a control to show serotonergic block in the aggregation response. Panel C also shows how Ketanserin is serotonergic specific and did not block the potentiation caused by dopamine/ADP co-stimulation (n=4).

Figure 5. Graphs showing maximum platelet aggregation values of the effect of Selective Dopamine Antagonists on Platelet aggregation stimulated by ADP, Collagen and Dopamine. All dopaminergic antagonists used were tested alone without the presence of 10uM of Dopamine in ADP and collagen stimulated platelets, as a negative control. None showed any effect on aggregation in the absence of dopamine (panel A, n=6). 10uM Haloperidol reduced 10uM dopamine potentiation (p=0.0377) of 0.3uM ADP induced aggregation and 100uM Haloperidol abolished dopamine potentiation (p=0.0489) with100uM Haloperidol (n=5). For 1uM ADP stimulation, Dopamine potentiation (p=0.00003) was reduced dose dependently by 1, 10 and 100uM Haloperidol (p=0.0039, 0.00007 and 0.00001 resp., n=5). This trend was reproduced in Collagen stimulated aggregation, n=5. 10uM of dopamine potentiated aggregation responses to 0.1 and 0.3ug/ml of Collagen (p=0.0208 and 0.0129 resp.) and was reduced by 10uM of Haloperidol (p=0.0187 for 0.1ug/ml of Collagen) and 100uM of Haloperidol (p=0.0194 for 0.1ug/ml of Collagen and p=0.004 for 0.3ug/ml of Collagen). The D3-selective antagonist PG01037 dose dependently reduced dopamine potentiated ADP induced aggregation at 0.3uM, 1uM and 3uM of agonist stimulation (For 0.3ADP p=0.047, 0.0355, 0.0122 resp, 1uM ADP p= 0.0067, 0.0041 and 0.0014 resp and 3uM ADP p=0.0103, 0.0355

and 0.0188 resp, n=6). In Collagen stimulated aggregation, 10uM and 100uM PG01037 dose dependently reduced Dopamine potentiation at both 0.1ug/ml and 0.3ug/ml of Collagen stimulation (for 0.1ug/ml Collagen p= 0.0004 and 0.003 resp while for 0.3ug/ml of Collagen, p=0.0358 and 0.0401 n=6 resp). The D3 antagonist SB277011A also dose dependently reduced dopaminergic potentiation of platelet aggregation, shown in panel D. 100uM of SB277011A significantly reduced potentiation at all 0.3, 1 and 3uM of ADP (p= 0.0125, 0.0477 and 0.0411 resp, n=6, figure 5D). Although dopamine potentiation does appear to be reduced in collagen stimulation incubated with SB277011A, it was not found to be statistically significant.

Figure 6. Graphs showing maximum aggregation values of platelet aggregation potentiation by Sumanirole. Sumanirole (D2 agonist) at 100uM potentiated both ADP and Collagen induced aggregation (ADP 0.3uM p=0.0053, ADP 1uM p=0.0291, n=7, panel A. Collagen 0.1ug/ml p= 0.0204, collagen 0.3ug/ml p= 0.0322 n=6, panel B)

When preincubated with 6-ND (1, 10 and 100uM), the potentiation by 100uM Sumarinole of ADP induced aggregation was blocked concentration dependently (panel C) (ADP0.3uM/6-ND 10uM p 0.0009, ADP0.3uM/6-ND 100uM p=0.0022, ADP1uM/6-ND 100uM p= 0.0395 n=4). Potentation of 100uM Sumanirole was alsp reduced by 100uM 6-ND in 0.3 ug/ml of Collagen stimulation (Panel D, p= 0.0416, n=4). PG01037 at 1, 10 and 100uM was preincubated in PRP for 3 minutes and stimulated by a combination of 100uM of Sumarinole and ADP (0.1/0.3/1uM) (Panel E). 1,10 and 100uM of PG01037 dose dependently reduced the potentiation caused by Sumarinole at 0.3uM ADP stimulation (p=0.0034, 0.0198 and 0.0044 resp, n=4). 100uM of PG01037 also reduced Sumanirole potentiation at 1uM ADP stimulation (p=0.0381).

Figure 7. Graphs showing maximum aggregation values of PRP Platelet aggregation potentiated by the D2 agonist, Sumarinole. When preincubated with PD128907 (10 and 100uM, 30 seconds), the potentiation by 100uM Sumarinole of ADP induced aggregation was blocked dose dependently as (Panel A) (n=4, PD128907 10uM significantly blocked Sumarinole potentiation (10 and 100 uM), p= 0.0001 and p=0.0007 resp. 100uM PD128907 significantly reduced Sumarinole (100 uM) Potentiation, p =0.0195). Panel B shows that PD128907 did not effect Dopamine potentiated or ADP stimulated platelet aggregation, n=4. L741626, a highly selective D2 antagonist was preincubated for 3 minutes (10 and 100µM) before stimulation by Dopamine and ADP or Sumanirole and ADP. The presence of L741626 did not significantly alter the potentiated aggregation (Panel C, n=4)

Figure 1. Quantification by LC-MS/MS of Endogenous Catecholamines in Washed platelets stimulated by Thrombin, n=4

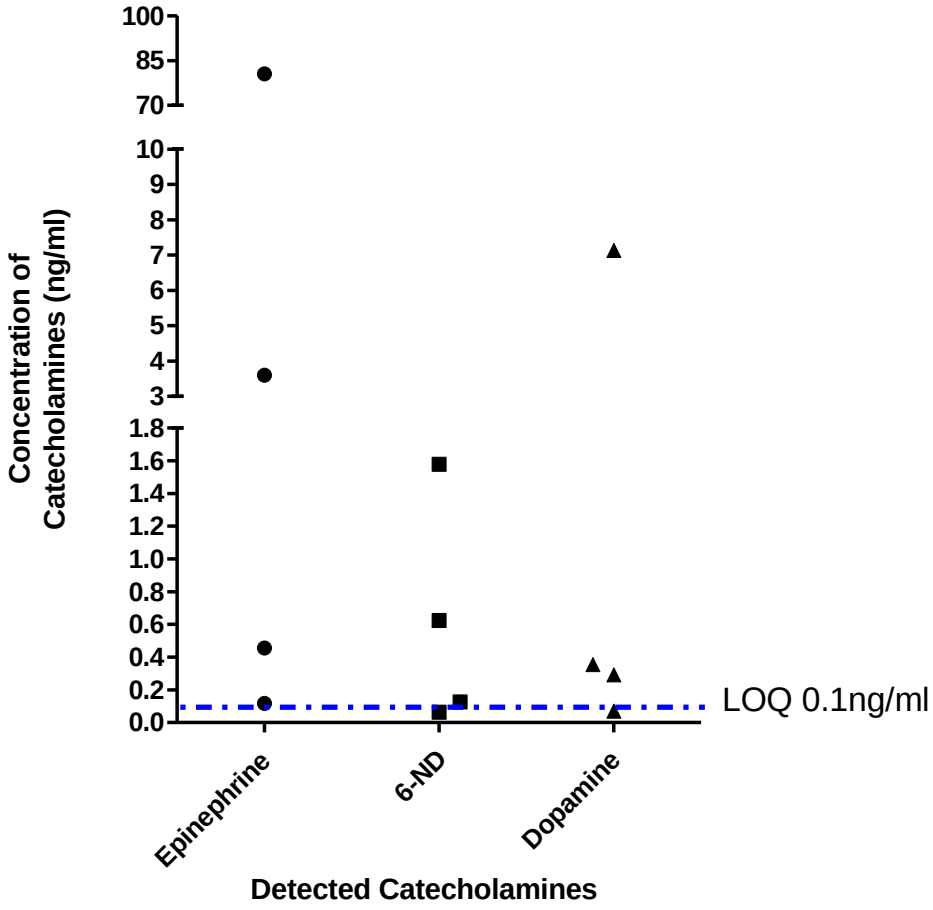
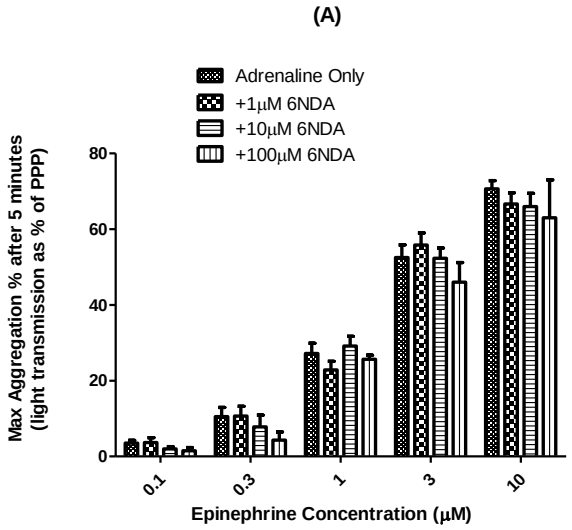
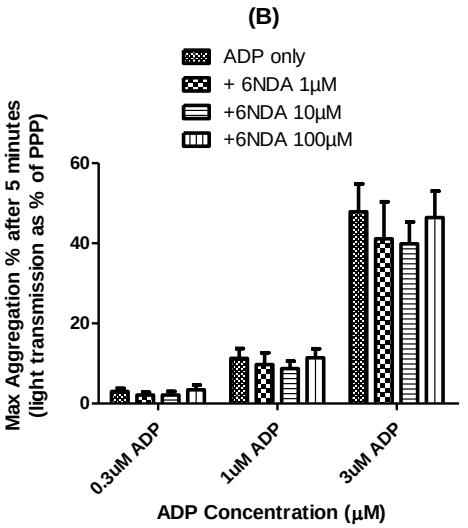


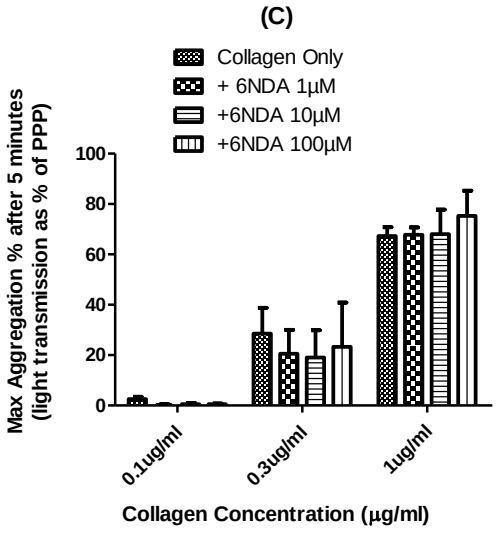
Figure 2. 6-ND Does Not Affect Adrenaline, ADP, Collagen or Thrombin Induced Aggregation.



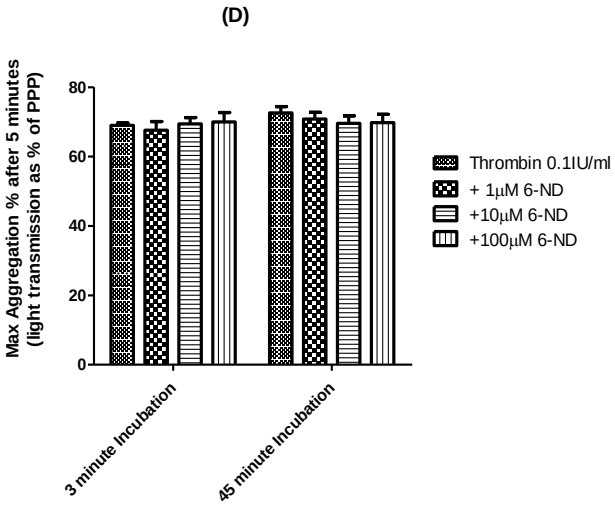
Paired T-test, n=5. Bars represent SEM



Paired T-test, n=7. Bars represent SEM



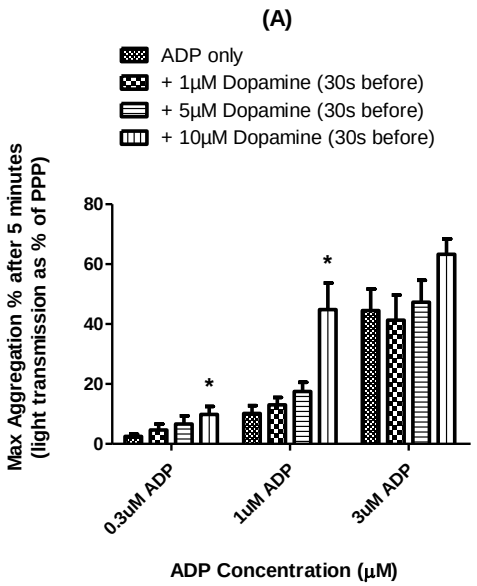
Paired T-test, n=4. Bars represent SEM



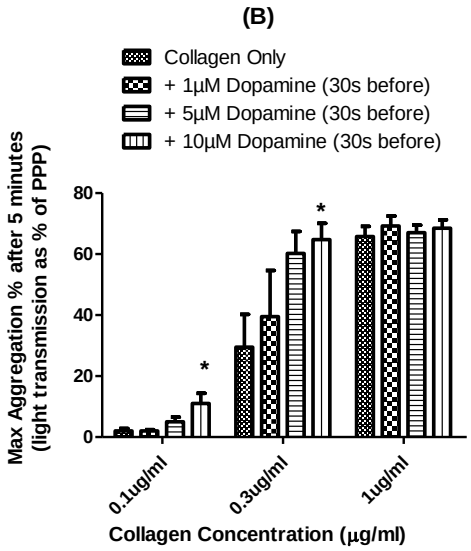
Thrombin Stimulation after 3 and 45 minutes 6-ND incubation.

Paired T-test, n=5. Bars represent SEM

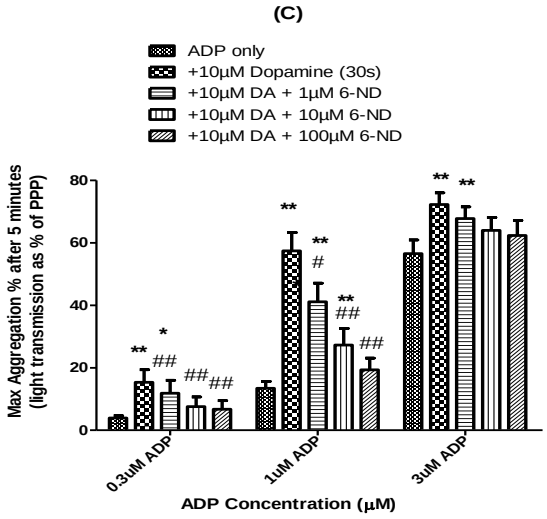
Figure 3. Dopamine potentiates ADP and collagen induced aggregation and is blocked by 6-Nitrodopamine



* signifies p=<0.05 in comparison to ADP only group

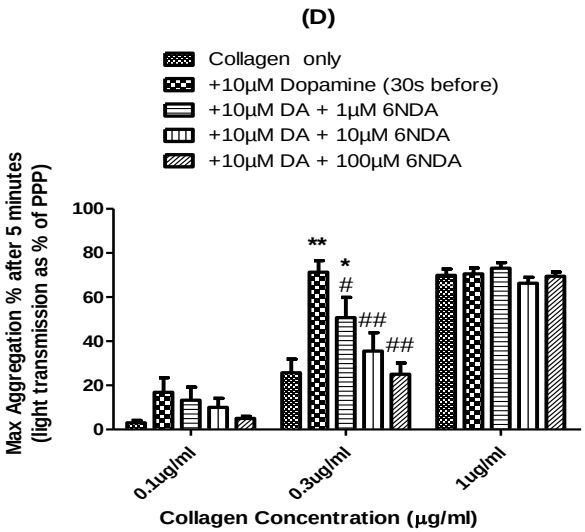


* signifies p=<0.05 in comparison to Collagen only group, Paired T-test, Bars represent SEM



* and ** signify p=<0.05 and <0.001 in comparision to ADP only group. Paired T test, n=14. Bars represent SEM

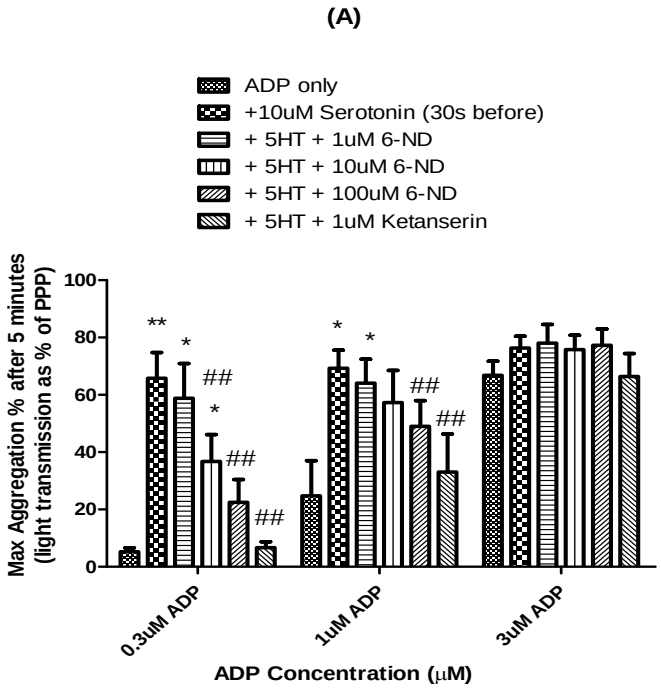
and ## signify p=<0.05 and <0.001 in comparison to +10µM Dopamine group. Paired T-test, n=14. Bars represent SEM



* and ** signify p=<0.05 and <0.001 in comparision to Collagen only group. Paired T test, n=7. Bars represent SEM

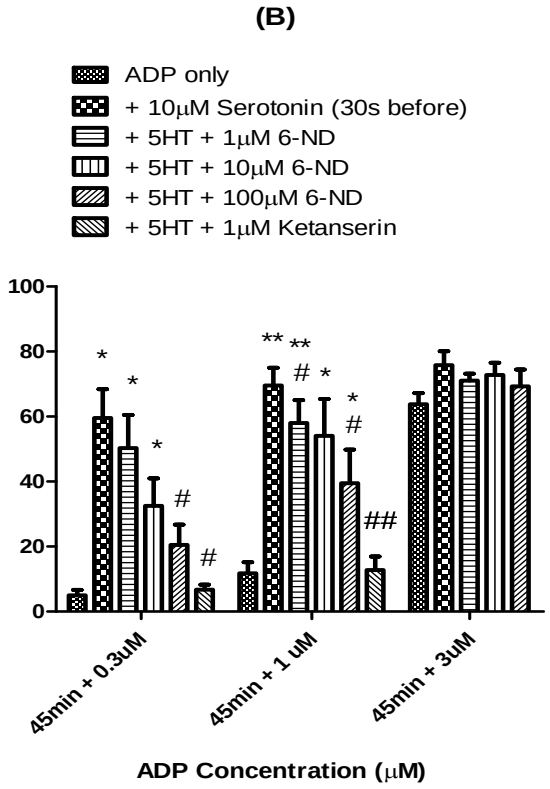
and ## signify p=<0.05 and <0.001 in comparison to +10µM Dopamine group. Paired T-test, n=7. Bars represent SEM

Figure 4. Serotonin Potentiation is blocked by 6-Nitrodopamine



* and ** signify p=<0.05 and <0.001 in comparision to ADP only group. Paired T test, n=4. Bars represent SEM

and ## signify p=<0.05 and <0.001 in comparison to +10μM Serotonin group. Paired T-test, n=4. Bars represent SEM



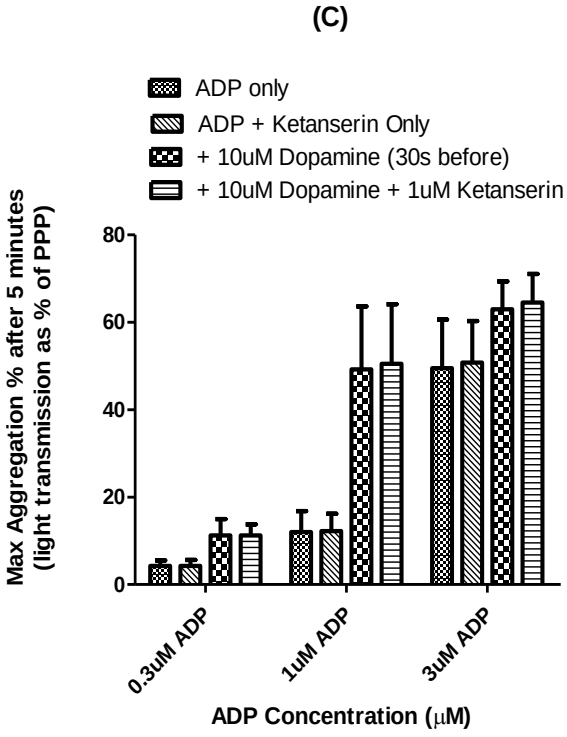
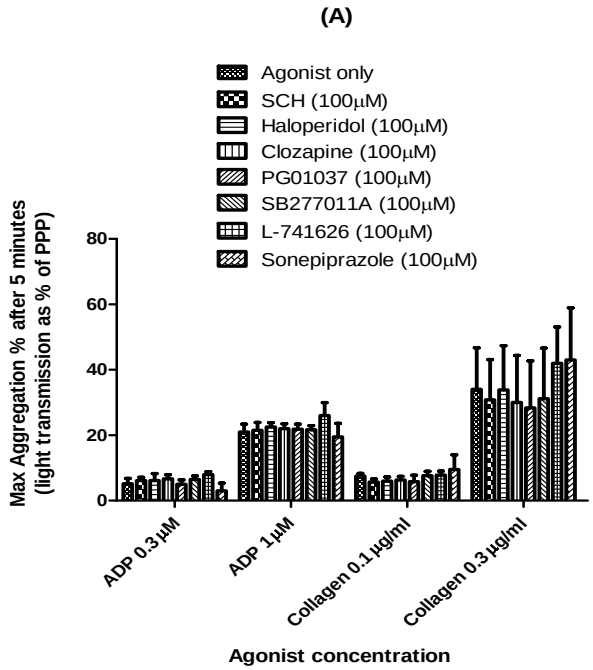
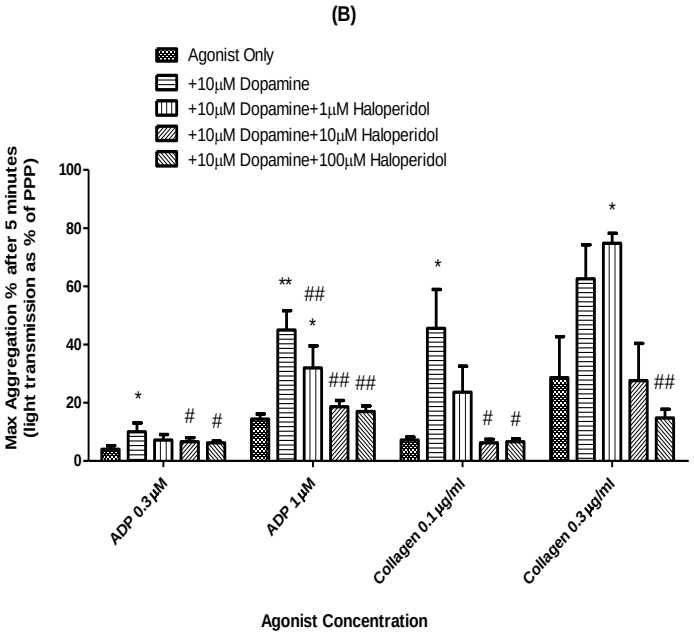
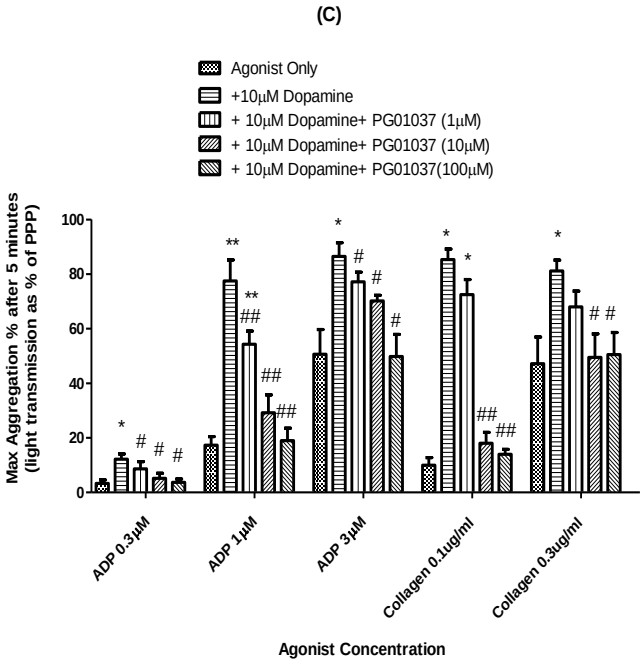
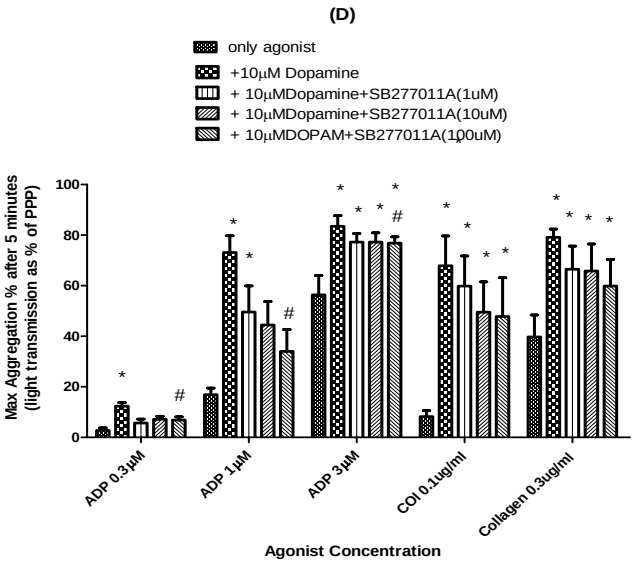


Figure 5. The Effect of Dopamine Selective Antagonists on Platelet Aggregation





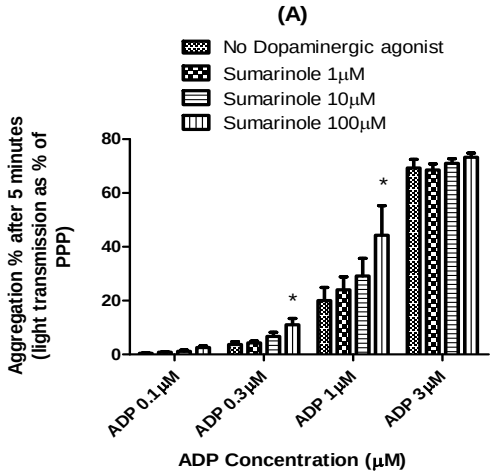




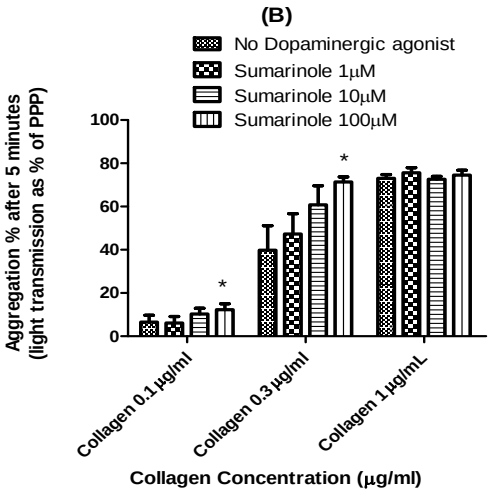
* signifies p=<0.05 in comparision to ADP only group. Paired T test, n=5. Bars represent SEM

signifies p=<0.05 in comparison to +10μM Dopamine group. Paired T-test, n=5. Bars represent SEM

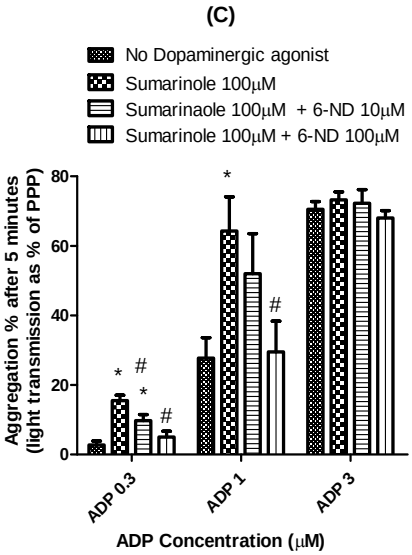
Figure 6. Sumarinole Weakly Potentiates Aggregation and is reduced by 6-ND and the D_3 selective antagonist, PG01037



* signifies p=<0.05 in comparision to ADP only group. Paired T test, n=7. Bars represent SEM

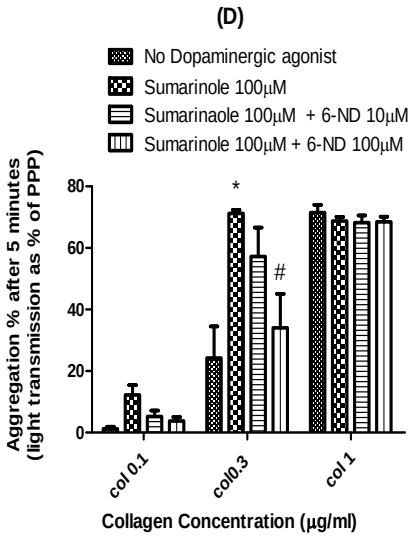


* signifies p=<0.05 in comparision to Collagen only group. Paired T test, n=6. Bars represent SEM



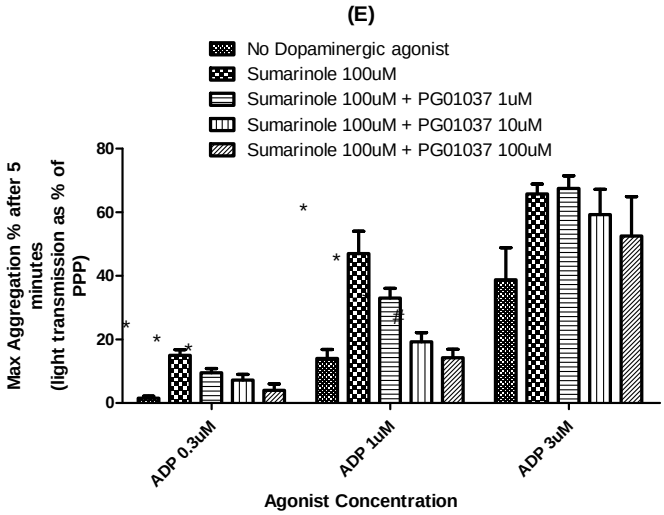
* p=<0.05 as compared to the ADP only group, Paired t-test. Bars represent SEM.

p=<0.05 as compared to the Sumarinole 100μM group, Paired t-test. Bars represent SEM



* p=<0.05 as compared to the Collagen only group, Paired t-test. Bars represent SEM.

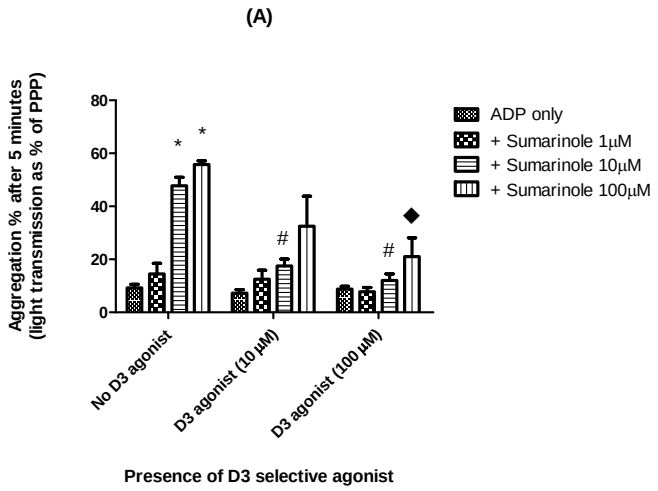
p=<0.05 as compared to the Sumanirole (100μM) group, Paired t-test. Bars represent SEM.



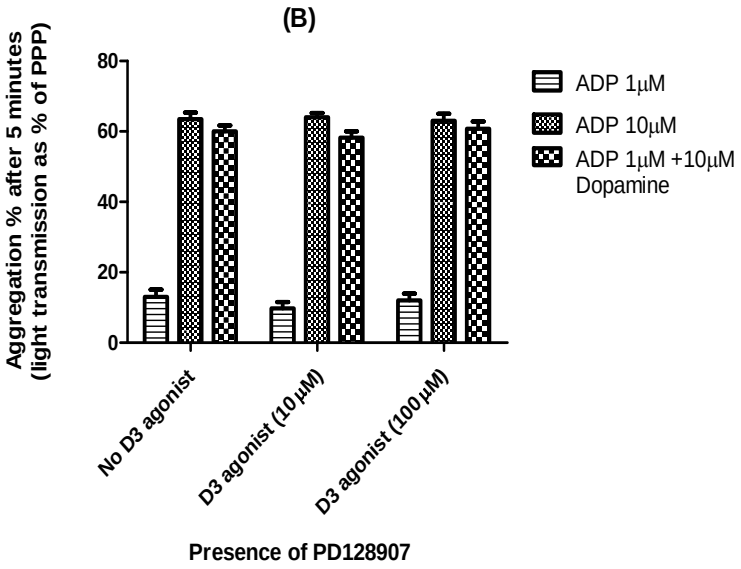
* p=<0.05 as compared to the ADP only group, Paired t-test. Bars represent SEM.

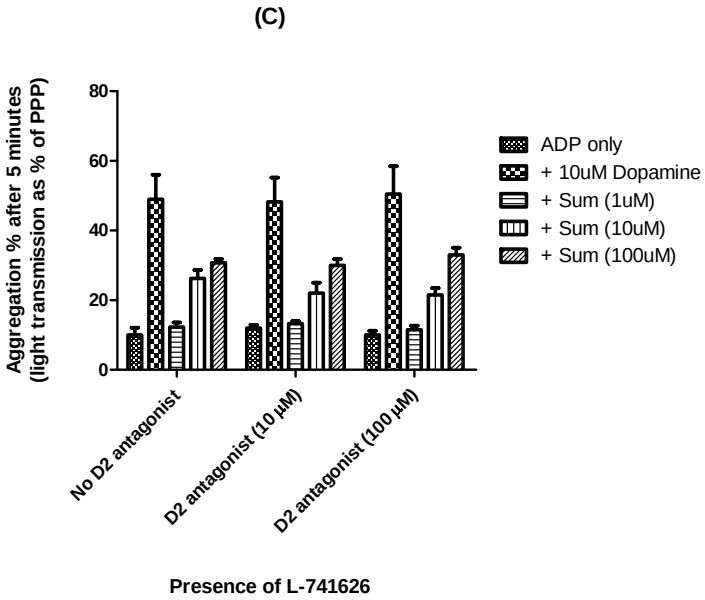
p=<0.05 as compared to the Sumarinole 100μM group, Paired t-test. Bars represent SEM

Figure 7. PD128907 Selectively Blocks Potentiation By Sumarinole but not L-741626 (D_2 antagonist)



* indicates p=<0.01 when compared to ADP control, no D3 agonist.
\# indicates p=<0.01 when compared to 10μM Sumarinole, no D3 agonist.
◆indicates p=<0.01 when compared to 100μM Sumarinole, no D3 agonist.





3. Submission 2

Journal of Chromatography B

Quantification of cyclic AMP and cyclic GMP levels in Krebs-Henseleit's solution by LC-MS/MS: application in washed platelet aggregation samples

--Manuscript Draft--

Manuscript Number:	CHROMB-D-22-00144
Article Type:	Full Length Article
Keywords:	cyclic nucleotides, LC-MS/MS, cAMP, cGMP, platelet aggregation, 6-nitrodopamine.
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	Weverton C Coelho-Silva, Masters in Pharmacology
	Rafael Campos
	Gilberto De Nucci
Abstract:	Cyclic Nucleotides are important in regulating platelet function. Increases in cAMP and cGMP inhibit platelet aggregation and are pharmacological targets for anti-platelet therapy. Here we report an improved method for determining cyclic nucleotide concentrations and for the first time in washed platelet supernatants by combining high-performance liquid chromatography and tandem mass spectrometry. Characteristic peaks of the substrates, cGMP or cAMP and their internal standards were identified in negative-ion electrospray ionization using multiple reaction monitoring. Compared with previously reported methods, the method presented here shows high precision with the lowest LLQ to date (10 pg/ml). The effect of a novel catecholamine, 6-Nitrodopamine, on cyclic nucleotide levels was quantified. Our results showed that this new method was fast, sensitive, and highly reproducible.
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07 February 2022

Dear Dr David S. Hage,

Please find attached the manuscript entitled "Quantification of cyclic AMP and cyclic GMP levels in Krebs-Henseleit's solution by LC-MS/MS: application in washed platelet aggregation samples" by Serpellone Nash *et al.*

We believe that the manuscript provides interesting results and methodology that advances the field of Liquid Chromatography in the biomedical setting. We hope you will find it acceptable for publication in the *Journal of Chromatography B.*

Yours sincerely,

Charles Serpellone Nash
Department of Pharmacology,
Faculty of Medical Sciences – UNICAMP
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And

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Quantification of cyclic AMP and cyclic GMP levels in Krebs-Henseleit's solution by LC-MS/MS: application in washed platelet aggregation samples

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Abstract

Cyclic Nucleotides are important in regulating platelet function. Increases in cAMP and cGMP inhibit platelet aggregation and are pharmacological targets for anti-platelet therapy. Here we report an improved method for determining cyclic nucleotide concentrations and for the first time in washed platelet supernatants by combining high-performance liquid chromatography and tandem mass spectrometry. Characteristic peaks of the substrates, cGMP or cAMP and their internal standards were identified in negative-ion electrospray ionization using multiple reaction monitoring. Compared with previously reported methods, the method presented here shows high precision with the lowest LLQ to date (10 pg/ml). The effect of a novel catecholamine, 6-Nitrodopamine, on cyclic nucleotide levels was quantified. Our results showed that this new method was fast, sensitive, and highly reproducible.

Keywords: cyclic nucleotides, LC-MS/MS, cAMP, cGMP, platelet aggregation, 6-nitrodopamine.

1. Introduction

Within platelet physiology, cyclic nucleotides are crucial mediators that play roles in modulating the aggregation response to thrombotic stimuli (Begonja et al 2013, Smolenski 2012). Phosphodiesterase inhibitors (PDEis) inhibit the breakdown of cAMP and cGMP. Platelets express PDE 2, 3 and 5, in which PDE 2 and 3 catalyse the breakdown of both cAMP and cGMP, whereas PDE5 catalyses the breakdown of cGMP specifically (Gresele et al 2011, Rondina and Weyrich 2013). An increase of cAMP in the platelet due to PDE2 and 3 inhibition blocks platelet aggregation due to an increase in PKA activation, VASP phosphorylation and reduced inside-out signalling. Inhibition of PDE5, by dipyridamole leads to increase cGMP which in turn competes with cAMP for PDE2 and 3, ultimately leading to an increased cAMP concentration as well (Noe et al 2010, Kherallah et al 2021).

A recently identified endogenous mediator, 6-nitrodopamine (6-ND) has not yet been assessed for its effects on cAMP and cGMP levels in platelet function. 6-ND has been previously characterised as a selective dopamine antagonist in the human umbilical cord vessels (Britto et al 2021)) and in dopamine potentiated platelet aggregation. (awaiting publication).

Enzyme-linked immunoassays are commonly used for quantifying nucleotides but can be time consuming and flawed. ELISA technique doesn't directly measure the concentration of a substance but indirectly detects the compound in question by immunoreactivity, leaving space for inaccuracies. In this work, we describe a novel and very sensitive validated method using liquid chromatography coupled to tandem mass spectrometry for dual quantification of cAMP and cGMP in Krebs-Henseleit's solution from washed platelet samples (Lorenzetti et al 2007, Oeckl et al 2012, Bähre and Kaever 2014, Jia et al 2014, Tsajokajev et al 2020). This method was applied to evaluate the effect of 6-ND on these nucleotides in platelets.

2. Methodology

2.1 Chemicals and solvents

Sodium nitroprusside (SNP), DMSO, Iloprost, Isobutylemethylxanthine (IBMX), thrombin, the nucleotides cGMP and cAMP, sodium 8-Bromo cGMP and 8-Bromo-cAMP were purchased from Sigma Chemical Co. (St. Louis, USA). Acetonitrile (HPLC grade) was obtained from Mallinckrodt (Mallinckrodt Chemicals, USA), formic acid, analytical grade was purchased from Merck (Rio de Janeiro, Brazil). Water was purified, using the Milli-Q or Elga UHQ systems, prior to use. 6-nitrodopamine was purchased from Toronto Research Chemicals (Toronto, Canada). All other reagents used were of commercially available grade.

2.2 Study Participants

4 healthy male volunteers over the age of eighteen, who were not taking any medication were invited to take part in the study. All participants were between the ages of 18-30 years old. The informed consent form was obtained from those who agreed to participate. The investigation followed the principles outlined in the Declaration of Helsinki and the protocol was approved by the Ethics Committee of the Faculty of Medical Sciences of the University of Campinas (FCM, UNICAMP, 3.092.338).

2.3 Preparation of human washed platelets

40ml of whole blood was taken from human volunteers (n=4) and anticoagulated in 9:1 volume with acid citrate dextrose (ACD) for preparation of washed platelets. The anticoagulated blood was centrifuged at 400g for 12 minutes at 25° Celsius. The plasma was collected, prostacyclin (0.1mM) was added and centrifuged again at 800g for 12 minutes at 25°C to collect the platelet pellet. The pellet was carefully resuspended in warmed (37°C) Krebs-Henseleit's solution (without calcium) and 0.1mM prostacyclin once more. The centrifugation process was repeated once more at 800g and the resulting pellet was carefully resuspended in warmed Krebs' solution (without calcium and without prostacyclin) and platelet count adjusted to 1.5x 10^8 platelets/ml. In washed platelet suspensions, calcium chloride was added just before stimulation with 0.1U/mL thrombin. Washed platelet suspensions were used within 1 hour of preparation.

2.4 Preparation of washed platelets for nucleotide quantification

400uL of washed platelets were incubated with IBMX (1mM) for 20 minutes at 37°C in cuvettes. Either saline or 6-ND (1, 10 or 100mM) was added to the cuvettes or Iloprost (for cAMP measurement) or SNP (for cGMP measurement) for 3 minutes with a magnetic bar for stirring. After 5 minutes incubation, 0.1IU/ml of thrombin was added to each cuvette and incubated for a further 5 minutes at 37°C. Following this, 400uL of cold HCl (100mM) was added the platelets and the suspension was centrifuged at 4000g for 5 minutes. The resulting supernatant was stored at -80C until analysis for cAMP or cGMP determination by LC-MS/MS.

2.5 Sample Extraction

Samples were extracted by solid phase extraction. To each sample, 1ml of Krebs-Henseleit's solution was added followed by 50uL of internal standards (2ng/mL of 8-

bromoadenosine and 8-bromoguanosine). The samples were homogenised for 10 seconds. The extraction cartridge (Strata -X33uM Polymeric Reversed Phase) was conditioned with 1 ml of methanol and then balanced by 2ml of deionized water with 0.2% acetic acid. The samples were injected into the cartridge, and the cartridge subsequently washed 3 times with deionized water with 0.2% acetic acid. The cyclic nucleotides were then eluted with 0.9mL Methanol/water (90/10 v/v) with 0.5% formic acid. The eluate was then evaporated under N_2 flow. The residue was dissolved and removed using 100uL of water with 0.4% acetic acid before being transferred to vials ready for injection. The extraction procedures described were also applied to the extraction of standard curve and quality controls.

2.6 Calibration Standards and Quality Control

Stock solutions (0.1mg/ml) of cAMP and cGMP were prepared in Krebs-Henseleit's solution. Calibration curves were prepared by adding the standards to blank Krebs-Henseleit's solution to yield final concentrations of 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1 and 2 ng/ml. The calibration curves were performed in duplicate for each day's assays. The quality control (QC) samples were prepared in blank plasma at the concentrations of 0.03, 0.15, 0.9 and 1.5 ng/mL, respectively. For each validation, seven replicates were analysed for each CQ level (three validations were performed). The spiked Krebs-Henseleit's samples (standards and QC) were extracted in each analytical batch along with the unknown samples.

2.7 LC–MS/MS analysis

The LC–MS/MS system consisted of LC ADVp Liquid Chromatograph Shimadzu System (Shimadzu Corporation, Japan) coupled to a LCMS 8060, Shimadzu Mass Spectrometer operating in electrospray negative-ionization mode. Samples were injected into the system by means of a SIL-30AC autoinjector, at a temperature of 8C. The chromatographic separation was performed at room temperature using a Sun-Fire™ C18 (5mM 21mm× 100 mm,) Waters® column and a flow rate of 0.4mL/min. Run time was 4 minutes. The mobile phase was 75% A (water containing 0.1% acetic acid) and 25% B (acetonitrile 90% with 0.4% acetic acid).

The mass spectrometer (LCMS 8060, Shimadzu, Japan) equipped with an electrospray source in the ESI negative polarity mode (ES^-) was configured for multiple reaction monitoring (MRM) to monitor the transitions 328.10 > 134.3 for cAMP, 407.90 > 214.15 for 8-bromo-cAMP, 344.20 > 150.25 for cGMP and 423.90 > 230.10 for 8-bromo-cGMP.

The source block temperature was set to 500 ◦C and the electrospray capillary voltage to 4.5 kV. The dwell time for each fragmentation pathway was 100 ms. Nitrogen was used as collision gas. Pressure of the collision gas (CAD) was 49.0 kPa. The injection volume was 3µL of each sample. The total run-time was 4 min. To optimize all MS parameters, a standard solution of the analytes and ISs were infused into the mass spectrometer. The optimized values of ion spray voltage, collision energy, and cone voltage were, respectively 2800 (V), 14 (eV), and 24 (V) for cAMP and 2800 (V), 16 (eV), and 24 (V) for cGMP. The MRM parameters can be seen in table 1. Data acquisition and analysis were performed using the software Masslynx 4.0 (Waters Corporation, Milford, MA, United States). Figure 1 shows the full scan spectra (upper trace) and the

product ion spectra (lower trace) obtained by the proposed fragmentation pathways for cAMP (A), 8-bromo-AMP (B), cGMP (C) and 8-bromo-GMP (D).

Table 1 Monitored ions, MRM, ESI, Negative ionization mode

Precursor ion		***Product ion***	***Time (msec)***	***DL. Bias (V)***	***Q array Bias (V)***	***Q1 Pre Bias (V)***	***Q3 Pre Bias (V***	***Collision Energy (CE)***
cAMP	328.1	134.30	100.0	0.0	0.0	-14.0	-13.0	-24.0
cGMP	344.2	150.25	100.0	0.0	0.0	-16.0	-15.0	-24.0
8-Br-cAMP	407.9	214.15	100.0	0.0	0.0	-18.0	-23.0	-26.0
8-Br-cGMP	423.9	230.10	100.0	0.0	0.0	-19.0	-10.0	-26.0

2.8 Method Validation

The method validation was carried out according to the United States Food and Drug Administration (FDA, 2001) bioanalytical method validation guidance and the Brazilian National Sanitary Surveillance Agency (Agência Nacional de Vigilância Sanitária [ANVISA], 2003).

2.9 Linearity, Precision and Accuracy

Calibration curves were prepared by assaying standard controls at eight concentrations of cAMP and cGMP (0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1 and 2 ng/mL). The linearity of each calibration curve was determined with the simplest regression equation describing the calibration curve, being $y = a + bx$ ($1/x^2$ weighted).

The precision and accuracy of assay was determined at least 3 distinct concentrations selected based on the literature and comparison with the previously established analytical values for similar studies. The following criteria were found to approve precision and intra-race accuracy: (i) for each concentration level, coefficient of variation (CV) that does not exceed 20% for LOQ and 15% for QC samples; (ii) mean value of the samples at each concentration level, within 85 – 115% of the actual value for QC and 80–120% for LOQ samples.

2.10 Statistical Analysis

All results were expressed as the mean ± standard error of the mean (SEM) of n experiments. All data were analysed using GraphPad Prism 6.0 and Microsoft Excel. Student's paired t-test was employed for comparing cAMP and cGMP concentrations

of each group with the vehicle group. For all analysis, differences between groups were considered significant when $p < 0.05$.

3. Results and Discussion

3.1. Optimisation of LC–MS/MS analytical method

The mass spectrometric settings for the MRM (Table 1) were optimised using the "Quantitative Optimisation" tool of the Analyst® software by continuous infusion of a standard solution containing a single analyte. The analysis time per sample for each nucleotide was 4 min (Fig. 2). To rule out interferences with molecules of similar structure and molecular weight, we analysed standard solutions of 8-bromoadenosine 3-5 cyclic monophosphate (MW: 408.2) and 8-bromoguanosine 3-5 cyclic monophosphate (MW:424). In all transitions measured, no signal was observed. The suitability of the internal standards was checked by analysing in Krebs' solution.

3.2. Precision, accuracy, linearity

Linearity was given in a range of 0.01-2 ng/mL with a correlation coefficient of r=0.9964 (cAMP) and r=0.9961 (cGMP). The limit of quantification (LOQ), as defined as accuracy and precision with ±20%, was 0.01 ng/mL for both cyclic nucleotides (Table 2).

Table 2 Limit of quantification validation

Limit of Quantification Validation	Mean (ng/ml)	S.D. (3 s.f.)	Accuracy (%)	CV (%)
cAMP (n=7)	0.01	0.000816	100	8.2
cGMP (n=7)	0.01	0.00139	94.3	14.8

The sensitivity demonstrated here is higher than that of immunological assays (sensitivity of 0.01 ng/ml) without the drawbacks of using radioactive labelling and additional acetylation steps during sample preparation. This enables the use of LC–MS/MS in the analysis of samples with only very low concentrations of cAMP and cGMP.

Intra- and inter-assay precision and accuracy were examined by the analysis of Krebs-Henseleit's solution spiked with different concentrations of cAMP and cGMP (Table 3). To determine intra-assay precision and accuracy, each sample was analysed 7 times. For inter-assay validation, the samples were analysed in three separate runs, each including its own standard curve. Based on these parameters, the lower limit of quantification (LLOQ) was 0.01 ng/mL for both cAMP and cGMP, according to internationally well accepted criteria (RSD and RE < 20%). In the study by Lorenzetti et al, a benchmark LLOQ was set at 0.25ng/ml, showing good accuracy and precision. Improved LLOQs have been reported including 0.164 ng/ml (Jia et al 2014) and

0.134ng/ml (Bähre and Kaever 2014), but the current study has the best LLOQ reported to date.

Table 3. Analysis of QC values for precision and accuracy

	cAMP					cGMP				
Identification of samples	CYLINDER	0.03 ng/ml	0.15 ng/ml	0.9 ng/ml	1.5 ng/ml	CYLINDER	0.03 ng/ml	0.15 ng/ml	0.9 ng/ml	1.5 ng/ml
Measured concentration	0.01	0.033	0.153	0.915	1.638	0.009	0.034	0.168	0.956	1.648
	0.009	0.031	0.15	1.015	1.579	0.009	0.029	0.156	1.008	1.593
	0.011	0.033	0.123	0.935	1.532	0.009	0.029	0.124	0.96	1.572
	0.01	0.032	0.152	0.949	1.449	0.011	0.028	0.178	0.966	1.543
	0.01	0.032	0.15	0.955	1.58	0.01	0.035	0.152	1.017	1.569
	0.011	0.034	0.123	0.925	1.547	0.007	0.028	0.12	0.997	1.567
	0.009	0.029	0.153	0.929	1.477	0.011	0.03	0.164	0.968	1.553
Intra-race average (in ng/mL)	**0.01**	**0.03**	**0.143**	**0.946**	**1.54**	**0.01**	**0.03**	**0.152**	**0.982**	**1.58**
Intra-race precision (CV %)	**8.2**	**5.1**	**9.8**	**3.5**	**4.2**	**14.8**	**9.5**	**14.5**	**2.5**	**2.2**
Intra-race accuracy (%)	**100.0**	**106.7**	**95.6**	**105.1**	**102.9**	**94.3**	**101.4**	**101.1**	**109.1**	**105.2**

This is the first LC-MS/MS method that can detect cAMP and cGMP levels in platelet supernatants. Other methods previously used to quantify cAMP and cGMP include ion exchange chromatography coupled to radioactive measurement using liquid scintillation counting (Jensen et al 2004) and ELISA (Zhang and Colman 2007). Liquid scintillation counting has waste disposal and safety issues, increasing the radioactivity of samples by up to 1000-fold. There may also be interference in the counting due to chemiluminescence and static electricity (National Diagnostics 2004). ELISA depends on the measurement of a secondary immunoreactive marker which can show incredibly high background interference if antigen blocking is not done sufficiently and runs risks of false positives and negatives (Sakamoto et al 2018).

Simultaneous quantification of nucleotides by LC-MS/MS was first carried by Lorenzetti et al (2007) differing from the current study in that positive ionisation was used instead of negative ionisation. Positive ionisation has been the most common method in to quantifying cAMP and cGMP, until recently, in which negative ionisation was reported for measurement of nucleotides in adenocarcinoma cell culture (Tsajokajev et al 2020). Negative ionisation is beneficial in the current study since the measured nucleotides contain negatively charged phosphate groups (Witters et al 1996). Another difference to be noted is that of injection volumes. Originally, a large injection volume was used of 40uL in the study by Lorenzetti et al, compared with the current volume of 3 uL used in this study which gives reduced peak spreading on the chromatograms (figure 2) (Boonan et al 2013).

3.3 Quantified Concentrations of cAMP and cGMP in washed platelet suspensions

Baseline levels of cAMP and cGMP upon thrombin stimulation were on average, 0.17 ng/ml (2 s.f., SE±0.036, n=4) and 0.0238 ng/ml (SE=±0.0096, n=4). Iloprost (1µM) and SNP (1µM) caused a significant ($p<0.05$) increase in cAMP (3.12 ng/ml, SE = ±2.164 n=4) and cGMP (0.0995 ng/ml, SE=±0.0321, n=4), respectively. 6-ND does not alter cAMP or cGMP levels in comparison to the negative control when stimulated by thrombin (figures 3 and 4 resp.)

Regarding the cGMP and cAMP quantification, 6-ND did not alter nucleotide levels in washed platelet samples. There are a variety of known drugs that modify platelet function by increasing the cAMP and cGMP signalling pathways, most notably PDE inhibitors (Grisele et al 2011). 6-ND has been shown to have antagonistic properties in the human umbilical cord in which it blocks the contractions caused by dopamine but not by other catecholamines (Britto et al 2021). It could be suggested that 6-ND was instead modulating contraction by interfering with cAMP signalling involved in the contraction but here we have shown this to be more so unlikely. Furthermore, 6-ND does not interfere with platelet aggregation stimulated by ADP, which involves Gi signalling and a decrease in cAMP (awaiting publication)

4. Conclusion:

The method presented here shows a high precision with low limit of quantification method of the nucleotides; cAMP and cGMP in Krebs solution taken from washed platelet samples. 6-ND, a novel catecholamine does not alter nucleotide levels in washed platelets.

5. Competing Interests:

All authors declare that they have no competing interests in this work.

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Figures:

Figure 1 Negative-ion spectra of cyclic AMP (A), 8-bromo-AMP (B), cyclic GMP (C) and 8-bromo-GMP. The spectra are recorded by selecting the pseudo molecular ion ([M + H]+) in the first quadrupole (Q1). After collision activation of the selected ions in the collision cell, the daughter ion spectra are recorded by scanning the last quadrupole (Q3).

Figure 2 LC-MS/MS Chromatograms for cAMP (A), 8-bromo-cAMP (B), cGMP (C) and 8-bromo-cGMP (D), 0.01ng/ml.

Figure 3. Quantification by LC-MS/MS of cyclic AMP from washed platelet supernatants stimulated by thrombin (0.1 IU/ml). IBMX (100 mM) was incubated for 20 minutes before Vehicle, iloprost (1 µM) or 6-ND (1, 10 or 100 µM) were preincubated in 200 uL of washed platelet suspensions before stimulation. After acidification, the samples centrifuged and supernatants were collected for cAMP quantification, n=4.

Figure 4. Quantification by LC-MS/MS of cyclic GMP from washed platelet supernatants stimulated by thrombin (0.1 IU/ml). IBMX (100 mM) was incubated for 20 minutes before Vehicle, SNP (1 µM) or 6-ND (1, 10 or 100 µM) were preincubated in 200 uL of washed platelet suspensions before stimulation. After acidification, the samples centrifuged and supernatants were collected for cGMP quantification, n=4.

Figure 1 Click here to access/download;Figure;Figure 1 - ionspray.pdf

Figure 1 Negative Ion Spectra for the cyclic nucleotides cAMP and cGMP along with internal standards

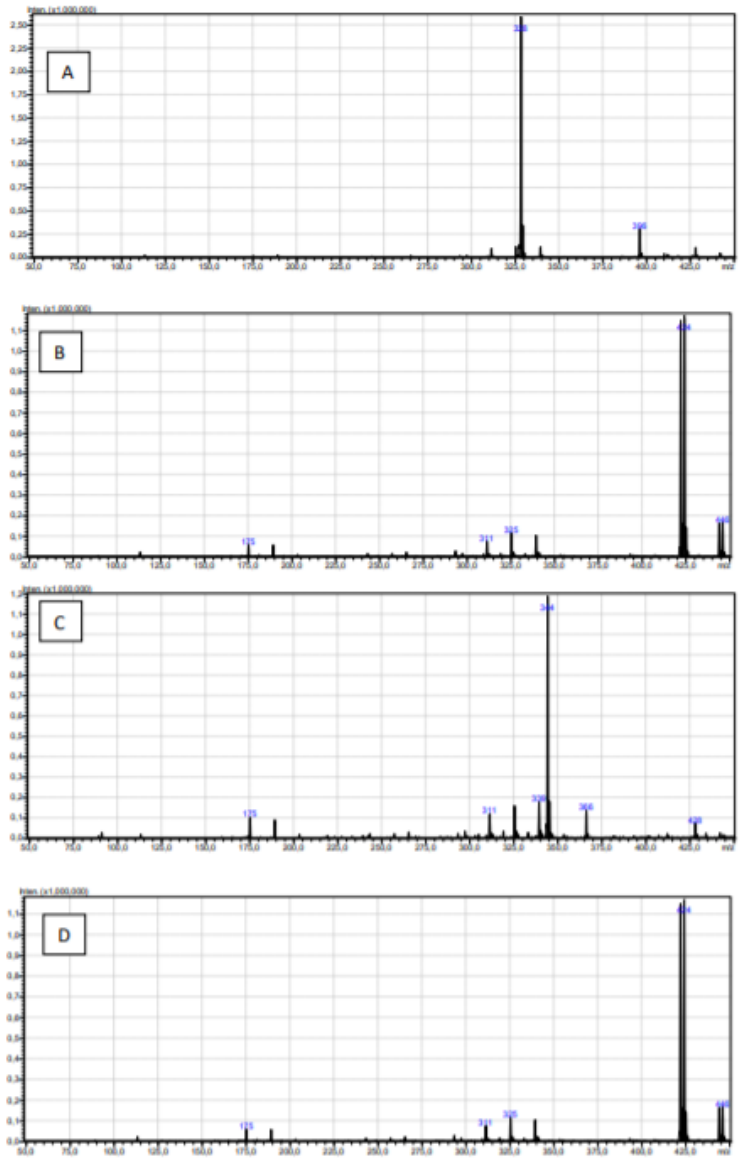


figure 2

Click here to access/download;Figure;Figure 2 chromotograms.pdf

Figure 2 LC-MS/MS Chromatograms for the cyclic nucleotides and their internal standards

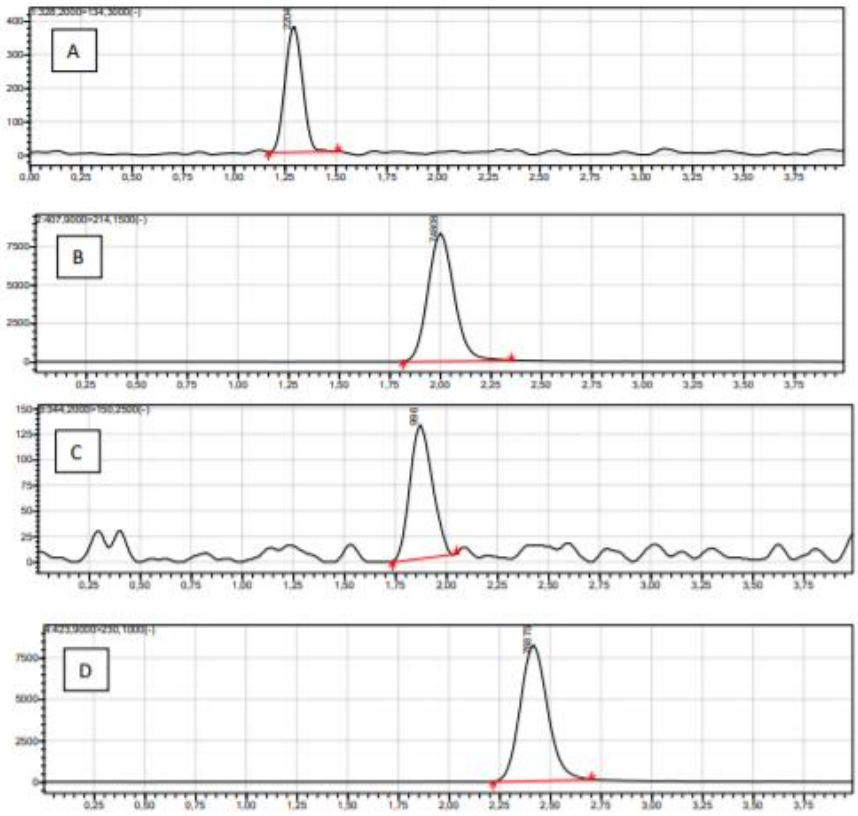
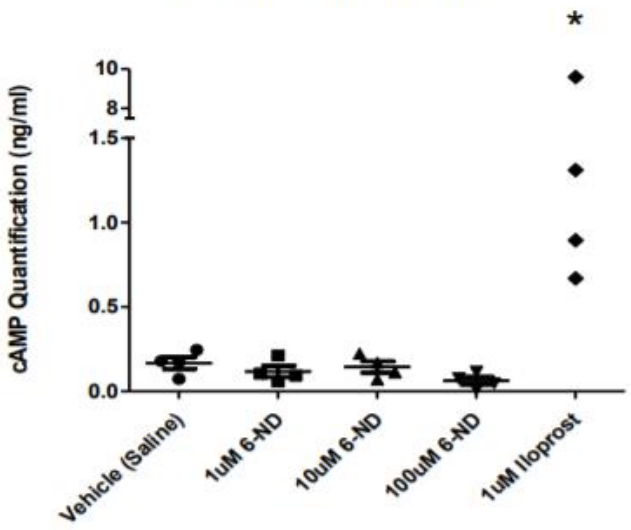


figure 3 Click here to access/download;Figure;Figures 3 - cAMP 6ND.pdf

Figure 3. LC-MS/MS Quantification of cAMP Produced by Washed Platelets Incubated with 6-ND, n=4

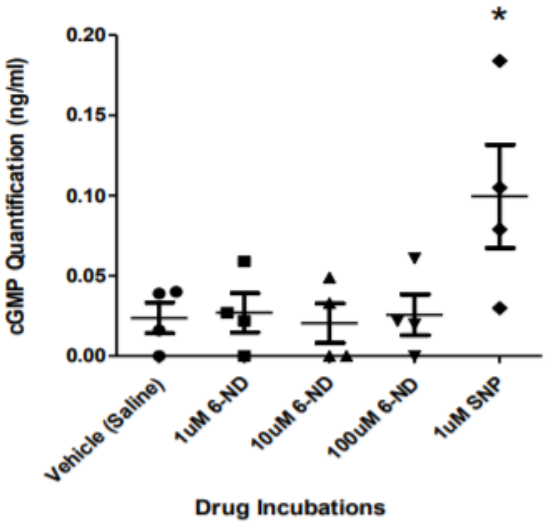


* represents a p-value of <0.05 in comparison to vehicle.

figure 4

Click here to access/download;Figure;Figures 4 - cGMP 6ND.pdf

Figure 4. LC-MS/MS Quantification of cGMP produced by Washed Platelets Incubated with 6-ND, n=4



* represents a p-value of <0.05 in comparison to vehicle.

4. General Discussion:

6-ND neither stimulates or inhibits platelet aggregation by ADP, Collagen, Thrombin or Adrenaline. Within platelet activation, one of the main signalling mechanisms involves the reduction of cAMP and deactivation of PKA. Following this, unphosphorylated VASP associates with actin filaments to facilitate the binding of fibrin to the fibrinogen receptor GIIb/IIIa, and potentiating aggregation between platelets, otherwise known as inside out signalling [44][45].

6-ND did not affect platelet aggregation by the principal agonists ADP or adrenaline, which utilise G-protein coupled receptors linked to the inhibition of adenylate cyclase,

ADP P2Y12 receptor and the adrenergic alpha 2 receptor are both coupled to Gi signalling which leads to a reduction in cAMP production and activation of PI3K [46][47] and 6-ND did not block ADP or Epinephrine induced aggregation, suggesting that 6-ND does not directly target Gi/cAMP signalling, nor that of P13K.

In support of this finding, we also report here that 6-ND does not affect cAMP in washed platelets stimulated by thrombin as quantified by LC-MS/MS. cGMP levels in platelets are also unchanged in platelets incubated with 6-ND and then stimulated by thrombin, suggesting it does not affect NO signalling pathways.

Considering that 6-ND also did not affect Collagen or Thrombin induced aggregation which are affected by cAMP and cGMP signalling also, it can be implied that 6-ND does not affect general platelet function.

What can be concluded is that 6-ND does have a role as dopamine and serotonin antagonist in reducing platelet potentiation by these agonists. Extensive characterisation of the dopamine receptor targeted by 6-ND in the platelet points to d2 like antagonism. Platelets have been previously reported to contain D3 receptors but not D2 receptors. However, our data here is inconclusive in distinguishing the two for the exact mechanism. Although a D2 selective agonist potentiated aggregation and was blocked by both a D3 selective antagonist and 6-ND, neither a D2 selective antagonist or D3 selective agonist had any effect in platelet aggregation. The problem most likely lies in the fact that catecholamines are weak agonists of platelets, and high concentrations are required to elicit platelet responses [29]. The D2 selective agonist only potentiated aggregation at a concentration much higher than that of the Ki values for both the D2 and the D3 receptor.[48][49]. Likewise the D3 antagonist was also only effective at blocking dopamine and sumarinole potentiation at concentration which captures both D2 and D3 receptors [50]. If the platelet dopamine receptor is indeed the d3 subtype, another mystery that needs further research is why the selective D3 agonist did not potentiate aggregation. It is possible that despite its high affinity [51] this agonist may have poor intrinsic activity for the d3 receptor in platelets, essentially making it a

dopamine d3 silent antagonist in the platelet. Synthetic adrenergic agonists have been previously reported to fail in potentiating platelet aggregation when compared to the endogenous catecholamines [52][53].

What is the elusive role of 6-ND?

If not a direct platelet mediator, what is the physiological function of 6-ND? From initial studies reported, the role of 6-ND seems to vary upon the tissue in which it acts.

In human umbilical cord vessels, 6-ND competitively antagonises muscle contraction stimulated by dopamine, but not by noradrenaline and adrenaline. Further to this 6-ND caused concentration dependent relaxation of pre-contracted endothelium intact tissues, in a similar manner to haloperidol, further suggesting that 6-ND antagonises a D2-like receptor. The possibility of D1 agonism was ruled out by controlling with the D1-like antagonist SCH-23390 which abolished the relaxations of HUCV caused by the D1 agonist fenolpadam but did not affect the relaxations caused by 6-ND [35]. This collaborates with the mechanisms proposed for platelets, as a dopamine D2 like antagonist.

Interestingly, contrary to our findings reported here, 6-ND has been reported to increase cAMP levels in neonatal rat ventricular myocytes, potently at very low concentrations even more so that other catecholamines [54]. 6-ND has also been shown to cause contractions of the rat vas deferens independent of other catecholamine signalling pathways [43]. These studies indicate a 6-ND receptor which potentially could be linked to PDE inhibition or adenylate cyclase activation. This would agree with our current findings if supposing that platelets don't express a functional version of this novel receptor.

Based on the results presented here, we suggest the main physiological role of 6-ND in platelets is to act as a store of this catecholamine.

How it could 6-ND be useful in platelet pharmacology?

Considering the concept that aggregation in-vivo occurs due to synergism of many platelet agonists acting at threshold concentrations and that catecholamines such as dopamine are produced by the vascular endothelium, 6-ND is a potential drug target for patients with high circulating catecholamine concentrations and increased thrombotic risk.

Plasma 5-HT levels are elevated in hypertension patients leading to increased cardiovascular and thrombotic risk [55].Due to its dual antagonism of serotonin and dopamine, it may also make a novel antipsychotic and should be tested for as such. Our data suggests that 6-ND most likely functions as a dopaminergic antagonist, probably with D2 selectivity. Further study should be undertaken to elucidate its effects in other tissues and confirm its mechanism.

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APPENDICES

Other works of significant contribution:

- Britto-Júnior J, Coelho-Silva WC, Murari GF, Serpellone Nash CE, Mónica FZ, Antunes E, De Nucci G. 6-Nitrodopamine is released by human umbilical cord vessels and modulates vascular reactivity. Life Sci. 2021 Jul 1;276:119425. doi: 10.1016/j.lfs.2021.119425. Epub 2021 Mar 26. PMID: 33781827. (a)

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