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Research paper

Thieno[2,3-b]pyridine derivatives are potent anti-platelet drugs, inhibiting platelet activation, aggregation and showing synergy with aspirin

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ABSTRACT

Drugs which inhibit platelet function are commonly used to prevent blood clot formation in patients with Acute Coronary Syndromes (ACS) or those at risk of stroke. The thieno[3,2-c]pyridine class of therapeutic agents, of which clopidogrel is the most commonly used, target the $P2Y_{12}$ receptor, and are often used in combination with acetylsalicylic acid (ASA). Six thieno[2,3-b]pyridine were assessed for *in vitro* anti-platelet activity; all derivatives showed effects on both platelet activation and aggregation, and showed synergy with ASA. Some compounds demonstrated greater activity when compared to clopidogrel. These compounds, therefore, represent potential novel $P2Y_{12}$ inhibitors for improved treatment for patients.

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1. Introduction

Acute coronary syndromes (ACS) are life-threatening heart conditions ranging from chest pain (unstable angina) to myocardial infarction (MI). Typically in ACS, inappropriate platelet-rich thrombus formation occurs, eventually occluding blood flow, resulting in a lack of oxygen, heart damage and potential death [1,2]. Platelet hyperactivity is often a major contributory factor in the development of disorders such as ACS and also stroke due to acute arterial thrombosis [3]. MI and stroke are currently the two most common causes of morbidity in the developed world [4]. Thus, the ability to control platelet activity and reduce adverse arterial thrombus formation is a critical tool in modern clinical practice.

Platelets circulate within the blood in a resting state, but upon contact with a platelet agonist, will undergo various biochemical and physiological changes to become activated and begin to aggregate [5]. Adenosine diphosphate (ADP) acts to induce activation via binding with the $P2Y_1$ and $P2Y_{12}$ receptors, leading to platelet shape change, the release of α- and dense-storage granules, intracellular mobilization and an influx of Ca^{2+}. There are significantly less numbers of $P2Y_1$ present on the platelet membrane and these are involved in producing a platelet shape change but only generate a weak and transient aggregation [5]. In contrast, the $P2Y_{12}$ receptor is found in very large numbers on the platelet cell surface membrane. Although it was initially thought to be platelet specific, $P2Y_{12}$ has recently been shown to be present in low numbers on some other cell types such as leukocyte subtypes, microglia, vascular smooth muscle cells and in some cancer cell lines [6,7]. ADP is also contained in platelet dense granules whereby initial activation of the platelet results in release of the dense granules and hence stored ADP which acts to amplify the activation response, an elegant example of a positive feedback mechanism [4]. Platelet activation by alternative agonists such as collagen or thrombin also promote dense granule section and therefore ADP is again involved in amplification of platelet activation. Downstream of the initial $P2Y_{12}$ receptor activation, GPIIb/IIIa

receptor activation is induced, this results in further degranulation, thromboxane (TXA_2) production and prolonged platelet aggregation [8].

Due to the importance of $P2Y_{12}$ in platelet activation, $P2Y_{12}$ inhibitors have been developed which bind to the receptor, thereby blocking the binding of ADP. Indeed, a combination treatment of a $P2Y_{12}$ inhibitor and a COX-1 inhibitor, most commonly clopidogrel and aspirin (acetylsalicylic acid, (ASA)) results in reduced cardiac events in patients with ACS and patients having undergone percutaneous coronary intervention (stents) [9]. Prior to FDA (USA Food and Drug Administration) approval, the thieno[3,2-*c*]pyridine clopidogrel was shown to reduce the risk of death/MI and stroke in the CURE (Clopidogrel in Unstable angina to prevent Recurrent Events) and CREDO (Clopidogrel for the Reduction of Events During Observation) trials, and it was concluded that clopidogrel and ASA treatment had long-term benefits [9—12]. However, ASA and clopidogrel combination treatment is usually only recommended for a maximum of 12 months due to the potential for gut damage and bleeding. Furthermore, not all patients are appropriate for this treatment, as adequate liver function is needed to metabolise these drugs into their active forms [4,10]. Of those patients that are suitable for treatment, approximately 4%—30% will be classed as 'non-responders' [13]. Currently there are several proposed reasons for clopidogrel poor/no response which include genetics (CYP2C19*2 loss of function allele, $P2Y_{12}$ receptor gene polymorphism), drug interactions, (e.g. Paclitaxel, Statins, Calcium channel blockers), patient body mass index and co-morbidities such as diabetes, intestinal conditions and impaired renal function [8,14—20].

More recently a non-thienopyridine-based $P2Y_{12}$ inhibitor, ticagrelor has been approved for ACS patients. This is following results from the PLATO (Platelet Inhibition and Patient Outcomes) trial demonstrating that treatment of ACS patients with ticagrelor significantly reduced the rate of death from vascular causes, MI and stroke when compared with clopidogrel treatment [21]. However, paradoxically, this beneficial effect of ticagrelor was not observed within patients from the USA and Canada that had been enrolled in this trial [22]. It is proposed that this is due to differences in ASA maintenance doses used in this part of the world. Despite no increase in the rate of major bleeding overall in the ticagrelor arm of the PLATO trial, haemorrhagic side effects in non-CABG (coronary artery bypass graft) patients were greater. A further drawback of ticagrelor is the need for increased administration frequency due to its reversible nature, which in-turn is associated with increased cost.

Prasugrel is a newer thieno[3,2-*c*]pyridine with an improved efficacy but at the cost of an increased bleed risk, with the JUMBO-TIMI (Joint Utilization of Medications to Block Platelets Optimally — Thrombolysis in Myocardial Infarction) study finding significantly more bleeding events in patients taking prasugrel compared to patients taking clopidogrel [8,23]. However, in a randomised trial, it was found that prasugrel, was able to overcome the poor outcome for the CYP2C19*2 loss-of-function allele seen in some clopidogrel non-responders [18]. This suggests that refinement of this family of compound may be useful in these patients.

More recently, the synthesis of structurally related thieno[2,3-*b*] pyridines have been reported and found to have potent phospholipase C (PLC) inhibitory activity [24]. As PLC activity has been linked to blood coagulation [25,26], a representative group of these new thienopyridines were assessed for their anti-platelet activity (Fig. 1).

2. Results

2.1. Chemistry

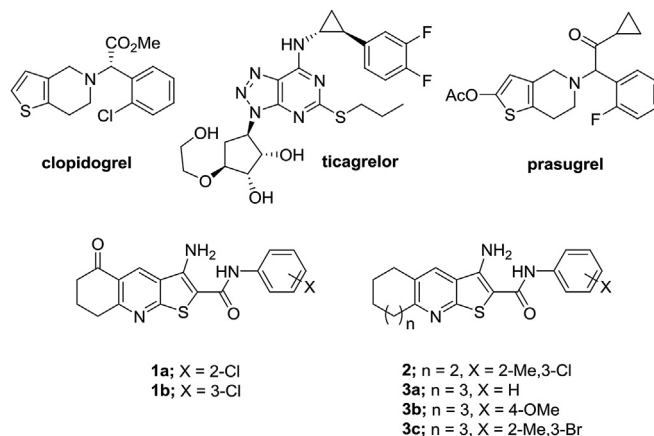A representative group of six thieno[2,3-*b*]pyridines (**1a-b, 2,**

Fig. 1. Thieno[2,3-*b*]pyridines assessed for anti-platelet activity and clinically used clopidogrel, prasugrel (both thieno[3,2-*c*]pyridines) and ticagrelor.

3a-c) were chosen, displaying various sized and functionalized cycloalkyl rings as well as various substitutions on the phenyl ring (Fig. 1). The preparation of the three cyclooctyl derivatives (**3a-c**) is shown below (Scheme 1). Cyclooctyl carbonitrile **4** was synthesized from cyclooctanone through a two-step procedure - formation of the corresponding hydroxymethylene salt which was then immediately heated at reflux with cyanothioacetamide and piperidinium acetate, followed by acidification with acetic acid to provide the bicyclic thiocarbonitrile **4**. Carbonitrile **4** was then condensed with substituted 2-halo-*N*-phenylacetamides **5a-c** to give the desired thieno[2,3-*b*]pyridines **3a-c**.

The preparation of thienopyridines **1a**, **1b** and **2** was achieved by repeating the same procedure starting from the appropriate cycloketone (1,3-cyclohexadione for **1a** and **1b**; cycloheptanone for **2**) to give the corresponding bicyclic thiocarbonitrile which was then reacted with the required 2-halo-*N*-phenylacetamides [27—29].

2.2. Biology

2.2.1. Thieno[2,3-b]pyridines inhibit ADP-stimulated platelet activation

The expression of CD62P (P-selectin) along with PAC1 binding as
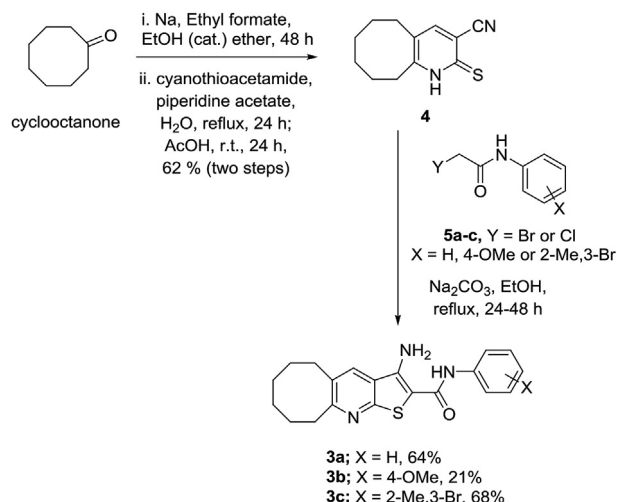
Scheme 1. Synthesis of cyclooctyl thieno[2,3-*b*]pyridine derivatives.

markers of platelet α-granule secretion and fibrinogen receptor activation, respectively, were analysed in PRP samples following treatment with the thienopyridine compounds. The platelet-rich plasma (PRP) was stimulated with ADP in order to induce platelet activation and hence expression of the two markers. Samples were treated with clopidogrel (active metabolite), thienopyridine **1**–**3** or vehicle control for 30 min prior to ADP stimulation. All thieno-pyridines **1**–**3** resulted in a significant decrease in CD62P expression when compared to ADP-stimulated controls (Fig. 1). When PAC1 binding was analysed, all six thienopyridines **1**–**3** resulted in inhibition, while clopidogrel was unable to produce the same effect. More interestingly, three of the six thienopyridines (**1a**, **1b** and **3a**) were able to inhibit the expression of CD62P and PAC1 binding to a greater degree than clopidogrel (Figs. 2 and 3). In the case of PAC1 binding, this was also true of **3c**, **3b** and **2**. Thienopyridine **3a** appeared to be the most superior compound at causing this effect. Taken together, these data show that the tested thieno[2,3-*b*]pyridines inhibited platelet activation in the presence of ADP and, under these conditions, were more effective than clopidogrel.

2.2.2. Thieno[2,3-b]pyridines inhibit ADP-stimulated platelet aggregation in PRP

After assessing platelet activation, light transmission aggregometry (LTA) was used to assess ADP-stimulated platelet aggregation in PRP following 30 min treatment with 10 μM of clopidogrel or the novel thienopyridines. This allowed the assessment of platelet function. Treatment with all six thienopyridines **1**–**3** resulted in a significant reduction in maximum aggregation when compared to vehicle-control treated PRP (Fig. 4). A paired *t*-test, to compare Maximum Aggregation (MaxA) values after thienopyridine treatment with that following clopidogrel treatment, revealed that **3b**, **1a**, and **3a** caused a significantly greater inhibition of aggregation ($p = 0.0009$, $p = 0.0124$ and $p = 0.0016$ respectively) (Fig. 4).

2.2.3. Thieno[2,3-b]pyridines inhibit collagen-stimulated platelet aggregation in whole blood

Although thienopyridines block ADP-induced aggregation, an effect on collagen-induced activation should also be observed as the secondary wave of platelet aggregation caused by dense-granule-derived ADP is inhibited. Indeed, the tested compounds inhibited collagen-induced aggregation of PRP, but to a lesser
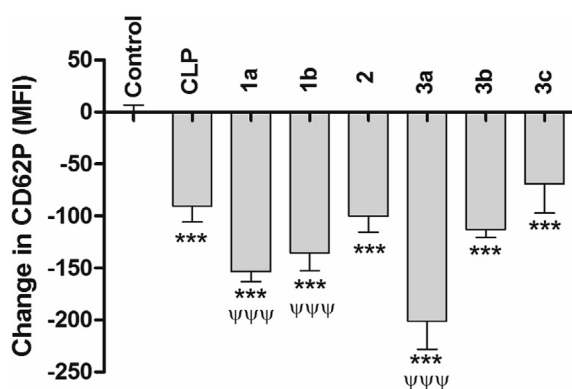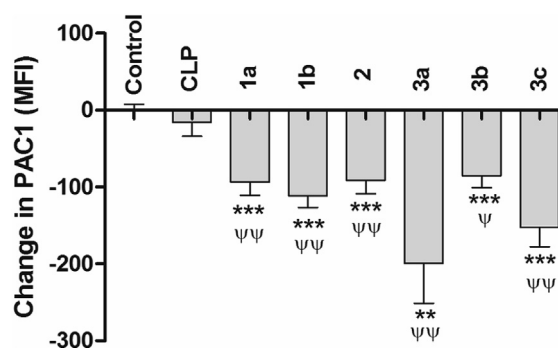

Fig. 3. Change in PAC1 binding in ADP-stimulated PRP following thienopyridine treatment was assessed by flow cytometry. Change in binding (i.e. inhibition) relative to control was determined. Data are presented as Mean ± SEM of four independent blood donors, where n = 2 for each donor. Statistical analysis was performed using the Student's t-test for paired data to determine differences from control and from clopidogrel (active metabolite, CLP)-treated samples. Significant differences from control (*** represents $p < 0.001$) and clopidogrel (ψ represents $p < 0.05$ and ψψ represents $p < 0.01$) are indicated.
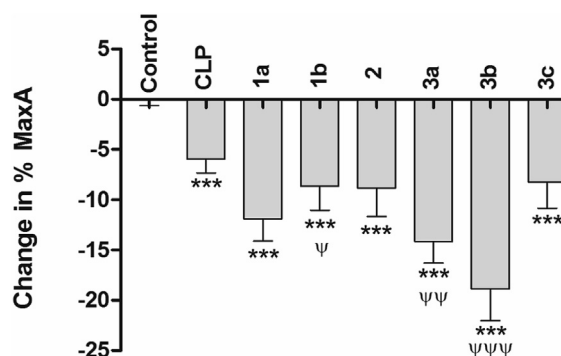

Fig. 4. Change in maximum aggregation (MaxA) in ADP-stimulated PRP following thienopyridine treatment was assessed by light-transmission aggregometry. Change in aggregation (i.e. inhibition) relative to control was determined. Data are presented as Mean ± SEM of seven independent blood donors, where n = 3 for each donor. Statistical analysis was performed using the Student's t-test for paired data to compare each drug-treated sample with the control and with clopidogrel (active metabolite, CLP)-treated samples. Significant differences from control (*** represents $p < 0.001$) and clopidogrel (ψ represents $p < 0.05$, ψψ represents $p < 0.01$ and ψψψ represents $p < 0.001$) are indicated.

degree than ADP-induced aggregation (Fig. 5). Interestingly, platelet aggregation in clopidogrel-treated samples was not significantly different from aggregation in the untreated samples, whilst the novel thienopyridines appeared more effective at inhibiting collagen-induced aggregation than clopidogrel, with the exceptions of **3c**.

2.2.4. Thieno[2,3-b]pyridines inhibit ADP-induced platelet-leukocyte aggregate formation

Following activation, platelets may adhere to local leukocytes (monocytes and neutrophils) via platelet CD62P binding with P-selectin glycoprotein ligand-1 (PSGL1) on the leukocyte surface. Platelet-leukocyte aggregates are considered a reliable marker of pro-thrombotic state [30]. Whole blood samples were pre-treated with clopidogrel, thienopyridine **1**–**3** or vehicle control for 30 min prior to ADP stimulation. Samples were double stained with the platelet marker CD42b and the leukocyte marker CD45 and analysed using flow cytometry. Platelets were identified in the whole blood sample (Fig. 6A) by expression of CD42b and gated (Fig. 6B). CD45-positive events within the platelet gate were

Fig. 2. Change in CD62P expression in ADP-stimulated PRP following thienopyridine treatment was assessed by flow cytometry. Change in expression (i.e. inhibition) relative to control was determined. Data are presented as Mean ± SEM of four independent blood donors, where n = 2 for each donor. Statistical analysis was performed using the Student's t-test for paired data to determine differences from control and from clopidogrel (active metabolite, CLP)-treated samples. Significant differences from control (*** represents $p < 0.001$) and clopidogrel (ψψψ represents $p < 0.001$) are indicated.
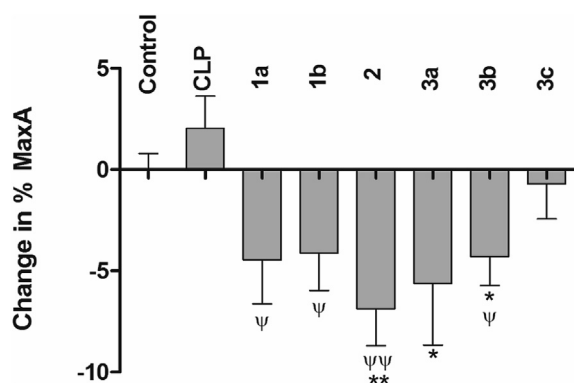
Fig. 5. Change in maximum aggregation (MaxA) in ADP-stimulated PRP following thienopyridine treatment was assessed by light-transmission aggregometry. Change in aggregation (i.e. inhibition) relative to control was determined. Data are presented as Mean $\pm$ SEM of seven independent blood donors, where n = 3 for each donor. Statistical analysis was performed using the Student's t-test for paired data to compare each drug-treated sample with the control and with clopidogrel (active metabolite, CLP)-treated samples. Significant differences from control (*** represents $p < 0.001$) and clopidogrel (ψ represents $p < 0.05$, $\psi\psi$ represents $p < 0.01$ and $\psi\psi\psi$ represents $p < 0.001$) are indicated.
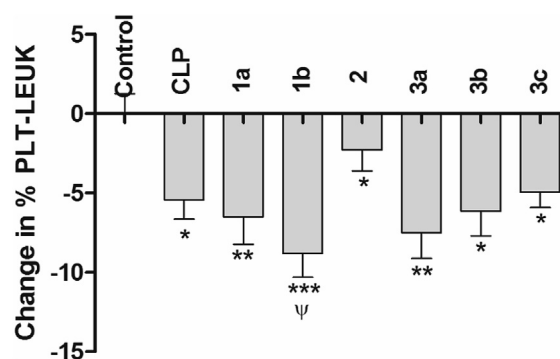

Fig. 7. Change in the percentage of platelet-leukocyte aggregates within the ADP-stimulated whole blood samples following thienopyridine treatment was assessed by flow cytometry. Change in CD42b+/CD45 + events (i.e. inhibition) relative to control was determined. Data are presented as Mean $\pm$ SEM of four independent blood donors, where n = 2 for each donor. Statistical analysis was performed using the Student's t-test for paired data to determine differences from control (* represents $p < 0.05$, ** represents $p < 0.01$, *** represents $p < 0.001$) and from clopidogrel (active metabolite, CLP)-treated samples (ψ represents $p < 0.05$).

identified (Fig. 6C). All novel thienopyridines **1**—**3**, resulted in a significant decrease in the percentage of platelet-leukocyte aggregates when compared to ADP-stimulated controls (Fig. 7). Statistical analyses to compare percentage of aggregates in the thienopyridine-treated samples with the clopidogrel-treated samples revealed a significant difference following treatment with **1b**.

2.2.5. Thieno[2,3-b]pyridines show synergy with aspirin

Management of ACS or stroke patients often involves the use of clopidogrel in combination with ASA based on a duel-hit hypothesis, whereby platelet function is inhibited via simultaneous inhibition of the P2Y$_{12}$ receptor and the COX-1 enzyme. We investigated the synergistic action of the novel thienopyridines **1**—**3** and ASA, using LTA to assess platelet function.

At the concentrations used in this study, all thienopyridines caused greater inhibition of platelet aggregation when compared with the inhibition caused by ASA alone (Fig. 8) (represented by *). This was also true of clopidogrel. When thienopyridine-combination treatments were compared with the respective thienopyridine only treatments, all showed synergy with ASA, with the exception of **3c** and **3b** (represented by ψ). However, these thienopyridines were shown to have a significant inhibitory effect

when used in isolation. An important facet of this work was to determine whether any of these novel thienopyridines **1**—**3** were more potent when used in combination with ASA, than the combination of clopidogrel and ASA. It was demonstrated that all compounds with the exception of **3c** showed significantly greater activity than clopidogrel when used in combination with ASA (represented by ϕ).

3. Discussion

Although platelet inhibitors such as clopidogrel and, more recently prasugrel and ticagrelor are currently used in clinical practice, the continued platelet hyperactivity in some patients taking these drugs highlights a need for continued refinement of this class of drugs [31]. The present study provides a significant contribution to the literature on P2Y$_{12}$ inhibitor therapy by reporting on the use of novel thieno[2,3-b]pyridine derivatives and their greater activity when compared to clopidogrel.

All six novel compounds significantly inhibited expression of both CD62P and PAC1 when compared to ADP-stimulated controls. When looking at the inhibition of PAC1, all the compounds had increased activity when compared with clopidogrel, while only **1a**, **1b** and **3a** showed greater activity in the inhibition of CD62P. Although both CD62P and PAC1 reflect platelet activation, CD62P is
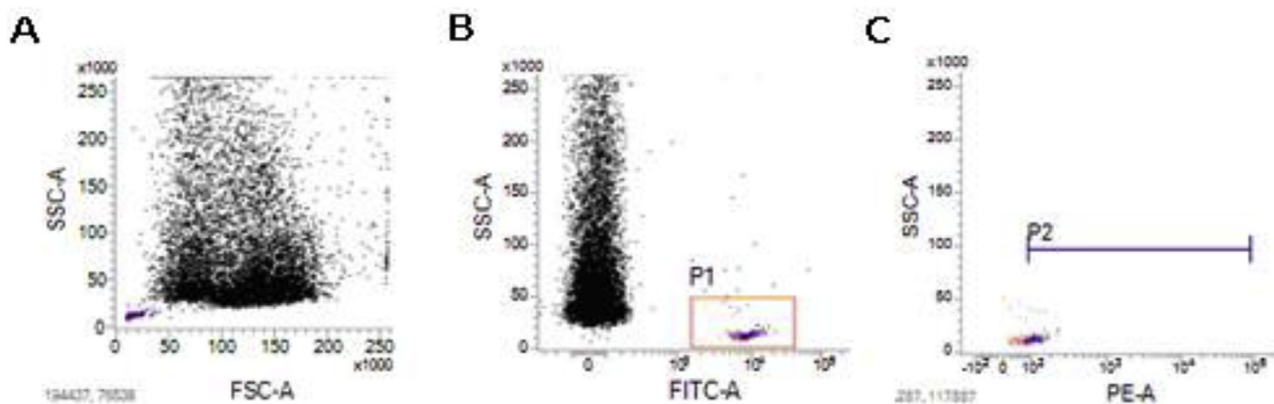

Fig. 6. (A) Whole blood was analysed by forward scatter (FSC) and side scatter (SSC). (B) The platelet population was identified by expression of CD42b and gated (P1). (C) CD45 positive events within the platelet gate (P1) were identified (P2).
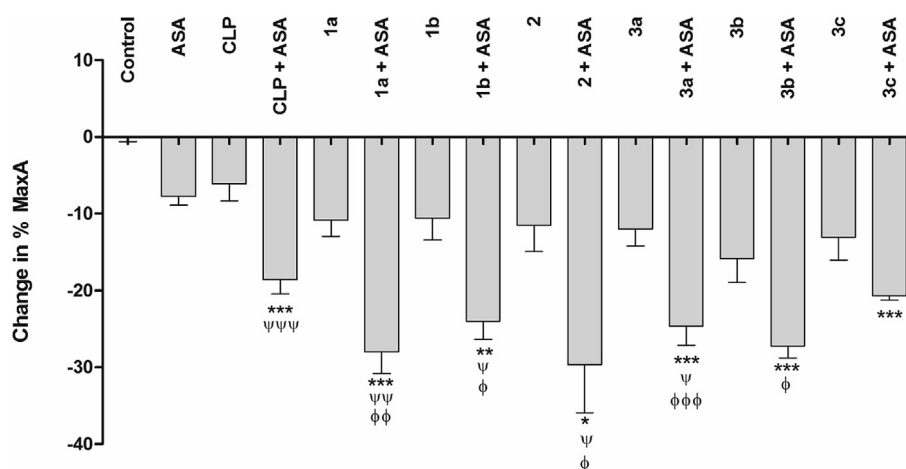
Fig. 8. Change in maximum aggregation (MaxA) in ADP-stimulated PRP following thienopyridine treatment in the presence or absence of ASA was assessed by LTA. Change in aggregation (i.e. inhibition) relative to control was determined. Data are presented as Mean $\pm$ SEM of seven independent blood donors, where n = 3 for each donor. Statistical analysis was performed using the Student's t-test for paired data to compare each drug-treated sample with the control and with clopidogrel (active metabolite, CLP)-treated. * denotes differences from ASA-only treated samples (* represents $p < 0.05$, ** represents $p < 0.01$ and *** represents $p < 0.001$). ψ denotes differences from thienopyridine-only treated samples (ψ represents $p < 0.05$, $\psi\psi$ represents $p < 0.01$, $\psi\psi\psi$ represents $p < 0.001$). ϕ denotes differences between combination treated samples and clopidogrel + ASA treated samples (ϕ represents $p < 0.05$, $\phi\phi$ represents $p < 0.01$ and $\phi\phi\phi$ represents $p < 0.001$).

expressed upon alpha-granule release [32], while PAC1 binds to activated GP IIb/IIIa (fibrinogen receptor) [33]. CD62P release and fibrinogen receptor activation are not necessarily simultaneous events during the process of platelet activation [34]. It is therefore important to analyse more than one marker of platelet activation when examining the effects of anti-platelet drugs. In the present study, clopidogrel had a very small effect on PAC1 binding, with all six novel thienopyridines **1**–**3** showing greater efficacy. This difference is likely to be highly important *in vivo*.

Aggregometry was used in this study to assess platelet function. All the thienopyridines were found to inhibit ADP-induced platelet aggregation, with **3b**, **1a** and **3a** showing greater efficacy than clopidogrel. Furthermore, collagen-induced aggregation was also hindered to some degree following derivative treatment, highlighting the importance of P2Y$_{12}$ in secondary activation from dense-granule-derived ADP. LTA is a gold standard measure of platelet function and is included in the majority of studies focusing on anti-platelet drugs [14,35–37]. However, strong platelet activation can occur without significant end-point aggregation and, activated platelets even with poor aggregation will still exert a systemic effect, increasing inflammation and stimulating further platelet activation. Therefore, it is critical to monitor both activation status and aggregation to assess the global effects of anti-platelet drugs.

Platelet-leukocyte aggregate formation has been shown to be increased in patients with ACS [38–40] and has been suggested as a monitoring tool for risk of MI in these patients [41]. Platelet-leukocyte aggregates have also been suggested to be a superior marker of platelet activation when compared with CD62P expression [42]. Our data shows that all tested thienopyridine derivatives **1**–**3**, as well as clopidogrel, were able to inhibit ADP-induced platelet-leukocyte aggregate formation. This is in agreement with similar studies that report decreases in platelet-leukocyte aggregate formation following clopidogrel, prasugrel or cangrelor treatment, both *in vitro* and *in vivo* [3,43–45].

Exploring synergy of the novel thienopyridine derivatives with ASA revealed that platelet aggregation was inhibited to a greater extent when any of the novel compounds were used in combination with ASA, when compared with ASA or derivative alone. The exception to this was **3b** which although appeared to show greater

efficacy when used as part of the combination treatment, statistical analysis proved this to be insignificant. More interestingly, when combined with ASA, all thienopyridines, with the exception of **3c**, showed a greater inhibitory effect than clopidogrel + ASA. These data support results which demonstrate a superior platelet-inhibitory effect of clopidogrel + ASA compared to either treatment alone [46,47]. Armstrong et al. studied the combination of prasugrel and ASA on platelet activation *in vitro* and reported that ASA did not significantly increase the inhibitory effect of prasugrel [35]. It was proposed that in the presence of strong P2Y$_{12}$ receptor blockade, ASA does not provide any benefit. However, our novel derivatives were extremely effective at P2Y$_{12}$ receptor blockade when used in isolation, and yet were enhanced by ASA. There is a lack of literature documenting the effects of prasugrel/cangrelor and ASA *in vivo*, but it appears that ASA-thienopyridine synergy is P2Y$_{12}$ inhibitor-specific.

It is clear that these thieno[2,3-*b*]pyridines have greater activity than the clinically used thieno[3,2-*c*]pyridines. Clinically used prasugrel, clopidogrel and cangrelor all affect platelet function to varying degrees in different patient groups [48–51]. Our derivatives follow this pattern and although this study has shown that all of them have the ability to inhibit activation and aggregation to some degree, some demonstrated greater activity than these clinical agents. Taken together, our data show that **1a** and **3a** are very effective platelet inhibitors. They are both more effective than clopidogrel at inhibiting the expression of CD62P and PAC1 in response to ADP stimulation. They are more effective at inhibiting platelet aggregation both alone and in combination with ASA. This global reduction in both platelet aggregation and activation highlights these molecules as worthy of further investigation to determine their potential as P2Y$_{12}$ inhibitors in the clinical setting.

When comparing the molecular structures of the tested thienopyridines the most consistently active compound, **1a**, has a strong similarity to clopidogrel with both containing a 2-chlorophenyl moiety linked to a larger heterocyclic group. This similar motif of a 2-substituted phenyl group is also found in the commonly used drug prasugrel. The active compound **3a** interestingly does not contain a 2-substituent on its phenyl ring. This suggests that alternative substitution patterns in these series of compounds can still lead to viably active compounds.

Of course, when considering any drug aimed at inhibiting platelet function, it is important to consider over-effectiveness, with risk of bleeding becoming an issue. Indeed, the superior activity of prasugrel over clopidogrel has also been associated with increased risk of bleeding in some studies [8,23,52]. It will be important to determine whether our derivatives are also associated with increased bleed risk *in vivo*.

4. Conclusion

In conclusion, the six thieno[2,3-*b*]pyridines derivatives tested all possessed anti-platelet activity, showing inhibitory effects on ADP-induced CD62P expression and PAC1 binding, platelet-leukocyte aggregate formation and aggregation. The study has involved *in vitro* testing on platelets obtained from healthy individuals. Testing the *in vitro* inhibitory effects of these derivatives on platelets obtained from patients who are candidates for clopidogrel/prasugrel treatment is necessary not only to assess activity in these patients, but also to assess whether these derivatives would be useful as alternative treatments for clopidogrel non-responders.

5. Materials and methods

5.1. Synthesis of compounds

5.1.1. General details

All reactions were carried out under a nitrogen atmosphere in dry, freshly distilled solvents unless otherwise noted. All NMR spectra were recorded on a Bruker Avance DRX 400 MHz spectrometer at ambient temperature. Chemical shifts are reported relative to the solvent peak of DMSO (δ 2.50 for ^{1}H and δ 39.5 for ^{13}C). ^{1}H NMR data is reported as position (δ), relative integral, multiplicity (s, singlet; d, doublet; t, triplet; m, multiplet; br, broad peak), coupling constant (J, Hz), and the assignment of the atom. ^{13}C NMR data are reported as position (δ) and assignment of the atom. All NMR assignments were performed using HSQC and HMBC experiments. High-resolution mass spectroscopy (HRMS) was carried out by electrospray ionisation (ESI) on a Brüker MicroTOF-Q mass spectrometer. Unless noted, chemical reagents were used as purchased. Acetamides **5a-c** were obtained using literature methods [29,53–55].

5.1.2. 2-Oxo-1,2,5,6,7,8,9,10-octahydrocycloocta[b]pyridine-3-carbonitrile 4

To sodium metal (1.15 g, 0.05 mol) in ether (250 mL) under an atmosphere of nitrogen at room temperature was added a solution of cyclooctanone (6.3 g, 0.05 mol) in ether (50 mL), dropwise. Ethanol (0.25 mL) was then added and the mixture stirred for 2 d. The mixture was then filtered under nitrogen and the solid washed with ether and then collected to provide the desired product (6.43 g, 73%) as a pale yellow solid. Salt (1.76 g, 10.0 mmol) was dissolved in H$_2$O (50 mL), followed by addition of cyanoacetamide (0.84 g, 10.0 mmol), and freshly prepared piperidinium acetate solution (9.5 mL). (The piperidinium acetate solution was prepared by mixing acetic acid (4.20 mL), water (10 mL), and piperidine (7.20 mL)). The mixture was heated at reflux overnight before being acidified with acetic acid (15 mL). The reaction mixture was allowed to cool to r.t. and stirred for a further 12 h before the residue was filtered off, washed with ice water and collected to give the *title compound* **4** (1.86 g, 85%) as a white solid which was used in the next reaction without further purification. m.p. > 230 °C. δ_H (400 MHz, (CD$_3$)$_2$SO) 1.35 (4H, br s, H-7 and H-8), 1.58 (2H, br s, H-6), 1.67 (2H, br s, H-9), 2.57–2.60 (2H, m, H-5), 2.82–2.85 (2H, m, H-10), 7.96 (1H, s, H-4), 13.97 (1H, br s, NH). The ^{1}H NMR data was

consistent with that previously reported [53].

5.1.3. General procedure A for synthesis of thieno[2,3-b]pyridine-2-carboxamides 3a-c

A mixture of 2-bromo- or 2-chloroacetamides **5a-c** (1 equiv), carbonitrile **4** (1 equiv) and anhydrous sodium carbonate (1.06 equiv) in absolute ethanol was stirred at reflux for 24–48 h. The mixture was cooled to room temperature and the solvent removed *in vacuo* to give the crude product which was recrystallized from methanol and washed with small amounts of water to give the *thieno[3,2-e]pyridine-2-carboxamides* **3a-c**.

5.1.3.1. 3-Amino-N-phenyl-5,6,7,8,9,10-hexahydrocycloocta[b]thieno [3,2-e]pyridine-2-carboxamide 3a.
The reaction was carried out according to general procedure A using 2-bromo-*N*-phenyl-acetamide **5a** (0.49 g, 2.3 mmol), carbonitrile **4** (0.5 g, 2.3 mmol) and anhydrous sodium carbonate (0.26 g, 2.4 mmol) in absolute ethanol (10 mL) for 20 h to give the *title product* **3a** (514 mg, 64%) as a pale green-yellow solid. m.p. >230 °C. ^{1}H NMR (400 MHz; DMSO-d_6) ^{1}H NMR (400 MHz; DMSO-d_6) 1.34 (4H, br s, H-7 and H-8), 1.71–1.74 (4H, br m, H-6 and H-9), 2.87 (2H, t, $J = 6.0$ Hz, H-5), 3.01 (2H, t, $J = 6.0$ Hz, H-10), 7.04–7.08 (1H, m, H-4'), 7.29–7.33 (4H, br s, NH$_2$ and H-3'), 7.69 (2H, d, $J = 8.0$ Hz, H-2'), 8.22 (1H, s, H-4), 9.34 (1H, br s, NH); ^{13}C NMR (100 MHz; DMSO-d_6) 25.4 and 25.5 (C-7 and C-8), 30.5 (C-9), 31.2 (C-5), 32.1 (C-6), 34.3 (C-10), 96.0 (C-2), 121.1 (C-2'), 123.3 (C-4'), 124.8 (C-3a), 128.3 (C-3'), 130.7 (C-4), 132.2 (C-4a), 139.0 (C-1'), 146.9 (C-3), 156.4 (C-11a), 162.7 (C-10a), 164.0 (C=O); IR: ν_{max}(ATR)/cm^{-1}: 3387, 3293, 2920, 2853, 1648, 1595, 1529, 1435, 1315, 1240, 1057, 748; m/z (ESI$^+$): 352 (MH$^+$, 100%); HRMS (ESI$^+$) found (MH$^+$): 352.1467 C$_{20}$H$_{22}$N$_3$OS requires 352.1478.

5.1.3.2. 3-Amino-N-(4'-methoxyphenyl)-5,6,7,8,9,10-hexahydrocycloocta[b]thieno[3,2-e]pyridine-2-carboxamide 3b.
The reaction was carried out according to general procedure A using 2-chloro-*N*-(4-methoxyphenyl)acetamide **5b** (0.23 g, 1.2 mmol), carbonitrile **4** (0.25 g, 1.2 mmol) and anhydrous sodium carbonate (0.15 g, 1.4 mmol) in absolute ethanol (5.0 mL) to give the *title product* **3b** (90 mg, 21%) as a beige solid. m.p. 215–217 °C. ^{1}H NMR (400 MHz; DMSO-d_6) 1.34 (4H, br s, H-7 and H-8), 1.66–1.79 (4H, br m, H-6 and H-9), 2.87 (2H, t, $J = 6.0$ Hz, H-5), 3.01 (2H, t, $J = 6.0$ Hz, H-10), 3.74 (3H, s, CH$_3$), 6.89 (2H, d, $J = 8.9$ Hz, H-3'), 7.22 (2H, br s, NH$_2$), 7.56 (2H, d, $J = 8.9$ Hz, H-2), 8.19 (1H, s, H-4), 9.24 (1H, br s, NH); ^{13}C NMR (100 MHz; DMSO-d_6) 25.4 and 25.5 (C-7 and C-8), 30.5 (C-9), 31.2 (C-5), 32.2 (C-6), 34.3 (C-10), 55.2 (CH$_3$), 96.2 (C-2), 113.6 (C-3'), 122.9 (C-2'), 124.9 (C-3a), 125.5 (C-4a), 130.7 (C-4), 132.2 (C-1'), 146.5 (C-3), 155.5 (C-4'), 156.3 (C-10a), 162.6 (C-11a), 163.9 (C=O); IR: ν_{max}(ATR)/cm^{-1}: 3426, 3326, 2932, 1593, 1509, 1497, 1267, 1247, 1032, 826; m/z (ESI$^+$): 404 (MNa$^+$, 100%), 382 (MH$^+$, 35%); HRMS (ESI$^+$) found (MNa$^+$): 404.1412 C$_{21}$H$_{23}$N$_3$NaO$_2$S requires 404.1403.

5.1.3.3. 3-Amino-N-(3'-bromo-2'-methylphenyl)-5,6,7,8,9,10-hexahydrocycloocta[b]thieno[3,2-e]pyridine-2-carboxamide 3c.
The reaction was carried out according to general procedure A using 2-bromo-*N*-(3-bromo-2-methylphenyl)acetamide **5c** (0.16 g, 0.53 mmol), carbonitrile **4** (0.115 g, 0.53 mmol) and anhydrous sodium carbonate (0.060 g, 0.56 mmol) in absolute ethanol (2 mL) for 48 h to give the *title product* **3c** (159 mg, 68%) as an off white solid. m.p. 230–232 °C. ^{1}H NMR (400 MHz; DMSO-d_6) 1.34 (4H, br s, H-7 and H-8), 1.71–1.73 (4H, br m, H-6 and H-9), 2.26 (3H, s, CH$_3$), 2.87 (2H, t, $J = 6.0$ Hz, H-5), 3.02 (2H, t, $J = 6.0$ Hz, H-10), 7.15–7.17 (3H, m, H-5' and NH$_2$), 7.31 (1H, d, $J = 8.0$ Hz, H-6'), 7.49 (1H, d, $J = 8.0$ Hz, H-4'), 8.20 (1H, s, H-4), 9.33 (1H, br s, NH); ^{13}C NMR (100 MHz; DMSO-d_6) 25.4 and 25.5 (C-7 and C-8), 30.5 (C-9), 31.2 (C-5), 32.2 (C-6), 34.3 (C-10), 96.1 (C-2), 124.5 (C-1'), 124.9 (C-3a),

126.8 (C-6′), 127.1 (C-5′), 129.7 (C-4′), 130.7 (C-4), 132.2 (C-4a), 134.1 (C-2′), 138.2 (C-3′), 146.5 (C-3), 156.4 (C-11a), 162.6 (C-10a), 164.2 (C=O); IR: v_{max}(ATR)/cm^{-1}: 3415, 3321, 2929, 2854, 1468, 1574, 1517, 1428, 1304, 1258, 1058, 773; m/z (ESI$^+$): 446 (^{81}BrMH$^+$, 100%), 444 (^{79}BrMH$^+$, 95%), 360 (20), 227 (50); HRMS (ESI$^+$) found (^{81}BrMH$^+$): 446.0713$C_{21}H_{23}^{81}BrN_3OS$ requires 446.0721. Found (^{79}BrMH$^+$): 444.0730$C_{21}H_{23}^{79}BrN_3OS$ requires 444.0740.

5.2. Biological methods

5.2.1. Ethics

The Manchester Metropolitan University ethics board granted approval for the study. Healthy volunteers were recruited for blood collection and gave written informed consent before donating a blood sample. The study was performed conforming to the Declaration of Helsinki.

Participants who had taken anti-platelet medication, anti-inflammatory medications, herbal medicines that may interfere with platelet function (Ginko Biolba, St John's Wort) or Selective Serotonin Reuptake Inhibitors in the past fortnight were excluded from the study. The study involved a total of eleven participants (n = 3 male, n = 8 female, age range 20–38). Samples from some participants (chosen at random) were used for analysis of multiple markers of platelet activation and function.

5.2.2. Sample collection and platelet-rich plasma purification

Blood was collected by venipuncture from the participants' antecubital vein into sodium citrated vacutainers. For experiments involving platelet-rich plasma (PRP), whole blood was centrifuged at 180 g for 15 min at 20 °C. Following centrifugation, the PRP was aspirated from the top of the vacutainers and placed into a fresh tube. The PRP was then diluted with an equal volume of Tyrode's buffer (NaCl (134 mM), KCl (2.9 mM), Na_2HPO_4 (0.34 mM), $NaHCO_3$ (12 mM), $MgCl_2$ (1 mM), HEPES (20 mM), Glucose (5 mM), adjusted to pH 7.4). For light-transmission aggregometry (LTA) experiments, platelet-poor plasma was also required and was isolated by centrifuging a 500 μL aliquot of diluted PRP at 5000 g for 5 min, and aspirating the supernatant from the platelet pellet.

5.2.3. Thienopyridine treatment

For experiments involving PRP, a 400 μL volume of diluted PRP was treated with 50 μL clopidogrel active metabolite (CLP in figures), thienopyridine (final concentrations 10 μM), or vehicle control (DMSO) for 30 min at 37 °C. In experiments involving whole blood, 500 μL whole blood was treated with 500 μL clopidogrel active metabolite, thienopyridine (final concentrations 10 μM) or vehicle control for 30 min at 37 °C. In ASA synergy experiments, 400 μL samples of diluted PRP were treated with 25 μL clopidogrel active metabolite or thienopyridine and 25 μL ASA (final concentration 30 μM) and incubated for 30 min at 37 °C. Following treatment diluted PRP samples were analysed using LTA. Thienopyridines were tested separately for their inherent cytotoxicity and it was discovered that they only exhibited toxicity at the higher 100 μM concentration, after treatment for 48–72 h.

5.2.4. Platelet activation analysis

A 90 μL volume of drug-treated PRP was activated using 10 μL ADP (final concentration 10 μM) (Labmedics, UK). After 5 min incubation at room temperature (RT), 10 μL PE-conjugated anti-human CD62P (BD Biosciences, UK) and 10 μL FITC-conjugated anti-human PAC1 (BD Biosciences, UK) was added and the samples were incubated for 10 min at 20 °C in the dark at RT. A 100 μL volume of 4% paraformaldehyde (Sigma, UK) was added to fix the samples before addition of 300 μL DPBS (Lonza, UK). Samples were analysed using a FACSVerse Flow cytometer (Becton Dickinson, UK) using FACSSuite software for analysis. Platelets were gated using FSC/SSC for size/granularity, and FITC and PE Mean Fluorescence Intensity (MFI) were recorded. Unstimulated controls (not treated with ADP) were also analysed to ensure a stimulation of CD62P and PAC1.

5.2.5. Light transmission aggregometry

The drug-treated PRP samples (450 μL volume) were analysed on a Chronolog 700 aggregometer using ADP (final concentration 10 μM) or collagen (final concentration 1 μg/mL) (Labmedics, UK) as the agonist. Maximum Aggregation (MaxA) was recorded over 5 min.

5.2.6. Platelet-leukocyte aggregate analysis

A 5 μL volume of the drug-treated whole blood was added to 55 μL Dulbecco's Phosphate Buffered Saline (DPBS) before activation with 10 μL ADP (final concentration 10 μM). After 5 min incubation at room temperature (RT), 10 μL PE-conjugated anti-human CD45 (BD Biosciences, UK) and 10 μL FITC-conjugated anti-human CD42b (BD Biosciences, UK) was added and the samples were incubated for 20 min at 20 °C in the dark. Finally, samples were diluted by addition of 2 mL DPBS before immediate analysis by flow cytometry. Platelets (CD42b positive events) were gated and further analysed for CD45 expression. Percentage of CD42b positive events expressing CD45 was recorded. Unstimulated controls (not treated with ADP) were also analysed to ensure low numbers of platelet-leukocyte aggregates under resting conditions.

5.2.7. Statistical analysis

Student t-tests for paired data were used to compare CD62P, PAC1 and MaxA values in drug-treated samples with that in control samples. * represents $p < 0.05$, ** represents $p < 0.01$, *** represents $p < 0.001$ when comparing with control.

ψ represents $p < 0.05$, ψψ represents $p < 0.01$, ψψψ represents $p < 0.001$ when comparing drug-treated samples with clopidogrel treated samples by paired t-test.

In ASA synergy experiments, t-tests for paired data were used to compare thienopyridine-only treated samples or ASA-treated samples with thienopyridine-ASA combination treated samples, and also each thienopyridine-ASA combination treated sample with clopidogrel-ASA treated samples. ϕ represents differences between combination treated samples and clopidogrel + ASA treated samples.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ejmech.2017.11.014.

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