Effect of Bruton's tyrosine kinase inhibitors on platelet aggregation in patients with acute myocardial infarction

Maria Bhatti, Sarah Ayton, Olga Michail, Nicholas D. Gollop, Alisdair Ryding, Stuart Rushworth, Kristian Bowles, Tobias Geisler, Marcus Flather

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Title Page

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Author List:

Maria Bhatti[1], Sarah Ayton[1], Olga Michail[1], Nicholas D. Gollop[1], Alisdair Ryding[2], Stuart Rushworth[1], Kristian Bowles[1,2], Tobias Geisler[3], Marcus Flather[1,2]

[1]Norwich Medical School, University of East Anglia and Norfolk and Norwich University Hospital, Norwich, Norfolk, United Kingdom

[2]Norfolk and Norwich University Hospital, Colney Lane, Norwich, Norfolk, United Kingdom

[3]Department of Cardiology, University Hospital Tübingen, Tübingen, Germany.

Corresponding author: Professor Marcus Flather, m.flather@uea.ac.uk

Postal address: Norwich Medical School, University of East Anglia, Norwich Research Park, Norwich, Norfolk, United Kingdom, NR4 7TJ

Telephone number: +441603 592963

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Abstract

Aims: Despite widespread use of dual antiplatelet therapy in acute myocardial infarction, there remains a residual risk of morbidity and mortality. Bruton's Tyrosine Kinase inhibitors have been found to inhibit platelet aggregation through the Glycoprotein VI collagen-mediated pathway. The Bruton's Tyrosine Kinase inhibitor, Ibrutinib is used in the management of haematological malignancies and another Bruton's Tyrosine Kinase inhibitor, ONO-4059 (also known as tirabrutinib), is in clinical development. This is an observational study to evaluate the effects of Ibrutinib and ONO-4059 on platelet aggregation after acute myocardial infarction.

Methods and Results: Twenty patients with a confirmed diagnosis of acute myocardial infarction were enrolled and blood samples obtained within 48 hours of hospital admission. All patients were on dual antiplatelet therapy; aspirin plus a P2Y12 inhibitor (clopidogrel or ticagrelor). Blood samples were treated *ex vivo* with increasing concentrations of Ibrutinib (0, 0.5, 1, 2μM) and ONO-4059 (0, 0.2, 0.5, 1μM). Platelet aggregation was measured in response to collagen using a Multiplate analyser to estimate the area under the curve, with lower values indicating lower platelet aggregation. The median age was 63 years and 80% were male. The median area under the curve values for Ibrutinib concentrations 0 (control), 0.5, 1 and 2mmol/l were 18.5, 8 (P=0.0004), 4.5 (P<0.0001) and 2 (P<0.0001) units and for ONO-4059 concentrations 0 (control), 0.2, 0.5 and1μM, median area under the curve values were 13, 12 (P=0.7), 6.5 (P=0.0001) and 5.5 (P=0.0004 compared to control).

Conclusion: The Bruton's Tyrosine Kinase inhibitors, Ibrutinib and ONO-4059, show further inhibition of platelet aggregation in blood samples from patients with acute myocardial infarction, receiving dual antiplatelet therapy in a dose dependent

manner. These results provide a rationale for Bruton's Tyrosine Kinase inhibitors to be tested as a potential new antiplatelet strategy for acute myocardial infarction.

Keywords: Ibrutinib; Acute myocardial infarction; Bruton's Tyrosine Kinase Inhibitor; Tyrosine Kinases; platelet aggregation

1. Introduction

Dual antiplatelet therapy, with aspirin and a P2Y12 inhibitor (commonly, clopidogrel or ticagrelor), is a cornerstone of the long-term management of acute myocardial infarction (MI). [1-3] Despite its widespread use, there is a 15% risk of death or recurrent events at 12 months, and 8% of acute MI patients are readmitted within one month of an acute episode. [4] This suggests there is scope for development of anti-platelet therapies which act via alternative pathways.

Ibrutinib is a Bruton's tyrosine kinase (BTK) inhibitor, which is licensed for the treatment of chronic lymphocytic leukaemia (CLL), Mantle Cell Lymphoma (MCL) and lymphocytic lymphoma and exerts its therapeutic effect by disrupting malignant B-cell function and survival. [5-8] With long-term follow up, more than half of patients on Ibrutinib experienced a bleeding event [9,10] and the pooled relative risk of the occurrence of any bleeding with Ibrutinib is 2.72. [11] The majority of bleeds are cutaneous in nature and there is a low occurrence of major bleeding. [8] *In-vitro* and *ex-vivo* studies have found Ibrutinib significantly inhibits platelet aggregation in response to collagen. [12-14] Ibrutinib irreversibly inhibits BTK and Tec, both of which are non-receptor tyrosine kinases involved in platelet aggregation, via the Glycoprotein VI (GPVI) collagen mediated pathway. [15,16] This pathway is not currently targeted in the management of acute MI.

Several other BTK inhibitors are in clinical development stages. [17] ONO-4059 (also known as tirabrutinib) is an irreversible selective inhibitor of BTK, which in a Phase I trial of 90 patients with relapsed/refractory B-cell malignancies had a lower incidence of associated toxicities compared to Ibrutinib. [18,19]

We hypothesise that the BTK inhibitors, Ibrutinib and ONO-4059, could have additional therapeutic antiplatelet activity in patients with acute MI. This study aimed to assess the *ex-vivo* effects of Ibrutinib and ONO-4059 on platelet aggregation in blood samples obtained from patients with acute MI treated with dual antiplatelet therapy.

2. Method

2.1. Study Design

Single-centre, observational, prospective study approved by a national accredited Research Ethics Committee and conducted according to the ethical guidelines of the Declaration of Helsinki and Good Clinical Practice. All patients provided written informed consent prior to participation.

2.2. Subject Selection and Recruitment

Patients with acute MI were recruited between March and July 2016. Inclusion criteria were individuals aged >18 years with a clinical diagnosis of acute MI with elevated serum troponin, with or without electrocardiogram (ECG) changes of myocardial ischaemia (+/-ST elevation). Exclusion criteria were participation in another research study and difficult venepuncture.

2.3. Blood sample collection and analysis

A single venous blood sample was collected in a Hirudin vacutainer (Roche, UK) between 24 and 48 hours of hospital admission. Blood samples were immediately analysed. Blood samples were treated with 0.5, 1 and 2μM of Ibrutinib and with 0.2,

0.5, 1µM of ONO-4059. A control sample (0µM) was also tested alongside each inhibitor. The concentrations used were determined through preliminary investigation carried out in healthy volunteers. Accuracy of platelet aggregation is subject to time delays between collection and analysis, therefore as only 4 samples could be analysed at one time, a 1-hour incubation period was used for Ibrutinib and 30 minutes for ONO-4059. All concentrations of Ibrutinib were analysed simultaneously for each patient, as were all concentrations of ONO-4059. Ibrutinib and ONO-4059 were purchased from Selleck Chemicals (USA). They were dissolved in dimethyl sulfoxide (DMSO) and diluted in phosphate buffered saline (PBS). Platelet aggregation in response to 20µl of collagen (Roche, UK), which equates to a final concentration of 3.2 µg/mL, was measured using a multiplate analyser (Roche, UK) in accordance with manufacturer's guidelines. As platelets bind to the multiplate sensors, there is an increase of impedance and this is transformed into an Aggregation Unit. The aggregation unit is then plotted against time. The overall platelet activity is measured by calculating the area under the curve (AUC).

2.4. Statistical analysis

This was an observational pilot study, so no formal sample size calculations were undertaken. Baseline characteristics are reported as percentages of the study population. Normally distributed baseline characteristics are reported as means with standard deviations. Non-normally distributed baseline characteristics are reported as medians with interquartile ranges. For each concentration of Ibrutinib and ONO-4059 the median AUC with interquartile ranges is given. Lower AUC values indicate less platelet aggregation. Comparisons between control (0µM) and samples treated with Ibrutinib and ONO-4059 were done with Wilcoxon signed rank test as the data

were not normally distributed. A *p*-value of $p<0.05$ was considered to be statistically significant.

3. Results

22 participants were enrolled and two participants were excluded due to non-MI diagnosis at discharge. Of the 20 participants included in the final analysis, 6 patients did not have blood samples treated with ONO-4059, as early in the study the doses were adjusted for this inhibitor.

Baseline characteristics are shown in Table 1. The mean age of participants was 63.2± 11.4 years and 80% of participants were male. All patients received dual antiplatelet therapy, with aspirin (300mg loading dose and 75mg maintenance) and a P2Y12 inhibitor (30% received clopidogrel and 75% ticagrelor; three patients received both Clopidogrel and Ticagrelor whilst in hospital; Clopidogrel was given as a loading dose and subsequently switched to Ticagrelor whilst in hospital). Participants also received a beta-blocker, angiotensin-converting enzyme inhibitor/angiotensin II receptor blocker (ACEi/ARB) and a statin. Three patients received GPIIIa/IIb inhibitors during admission.

Table 1: Baseline Characteristics

Characteristic	**n (%) (n=20)**
Age (years, mean ± SD)	63.2 ± 11.4
Male	16 (80)
Current smoker	5 (25)

Family history of CAD (n=19)	12 (63)
Diabetes	1 (5)
Hypertension	7 (35)
Hypercholesterolaemia	9 (45)
History of CAD	5 (25)
NSTEMI	14 (70)
STEMI	6 (30)
Inpatient coronary angiography	18 (90)
Site of infarct	
Anterior	5 (25)
Inferior	5 (25)
Anterolateral	2 (10)
Indeterminate	5 (25)
Inferior Posterior	1 (5)
Lateral	2 (10)
Medication given during hospitalisation	
Aspirin	20 (100)
Clopidogrel	6 (30)
Ticagrelor	15 (75)
Beta-blocker	15 (75)
ACEi	16 (80)
ARB	2 (10)
Diuretics	1 (5)
Aldosterone	1 (5)

Glycoprotein IIb/IIIa inhibitors	3 (15)
Statin	19 (95)
Peak Troponin I level (ng/L) **median (IQR)**	1101.5 (275 – 7112.75)
Platelet count (10^9/L) **median (IQR)**	242 (199.5 - 287.25)

ACEi = angiotensin coverting enzyme inhibitor, ARB = angiotensin receptor blocker, CAD = coronary artery disease, IQR = interquartile range, NSTEMI = Non ST-elevation myocardial infarction, SD = standard deviation, STEMI = ST-elevation myocardial infarction

For Ibrutinib concentrations of 0, 0.5, 1, 2µM, the median (IQR) AUC for platelet aggregation were 18.5 (7 – 29.25), 8 (2.5 – 18.75), 4.5 (1 – 15.75) and 2 (0 – 10.5) (P value of 0.0004, <0.0001 and <0.0001 respectively compared to control: Figure 1). For ONO-4059 concentrations of 0, 0.2, 0.5, 1µM, the mean (IQR) platelet aggregation was 13 (7 – 23.5), 12 (8.75 – 22.25), 6.5 (2.75 – 14.25), 5.5 (2.5 – 9.25) (p values 0.7, 0.0001 and 0.0004 respectively compared to control: Figure 2).

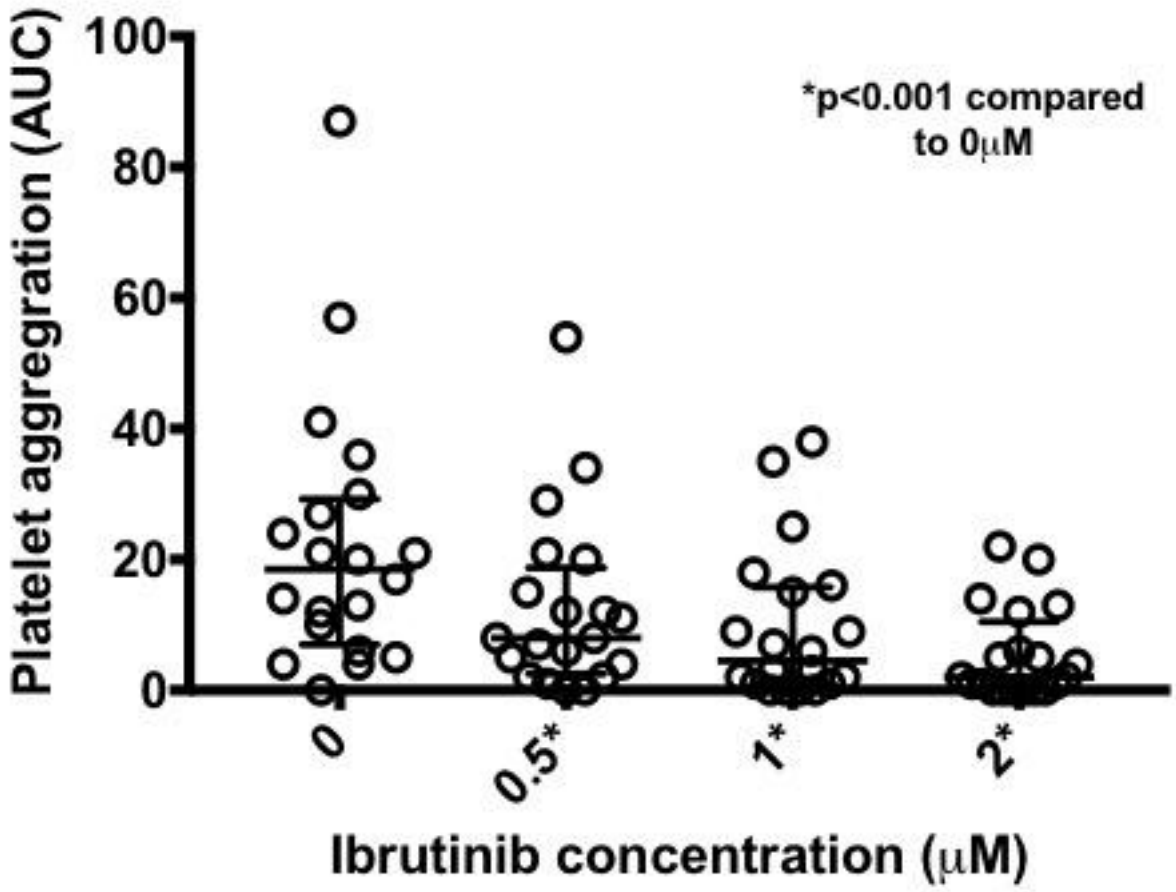

Figure 1: Platelet aggregation was measured using a Multiplate analyser (collagen agonist concentration 3.2 µg/mL) with ex-vivo addition of increasing doses of Ibrutinib in blood samples from patients with acute myocardial infarction treated with dual anti-platelet therapy (n=20) Each circle represents individual data points, with horizontal lines representing median values, with lower and upper quartiles. Wilcoxon signed rank test was carried out to compare 0µM (control) with 0.5, 0.1 and 0.2µM of Ibritunib.

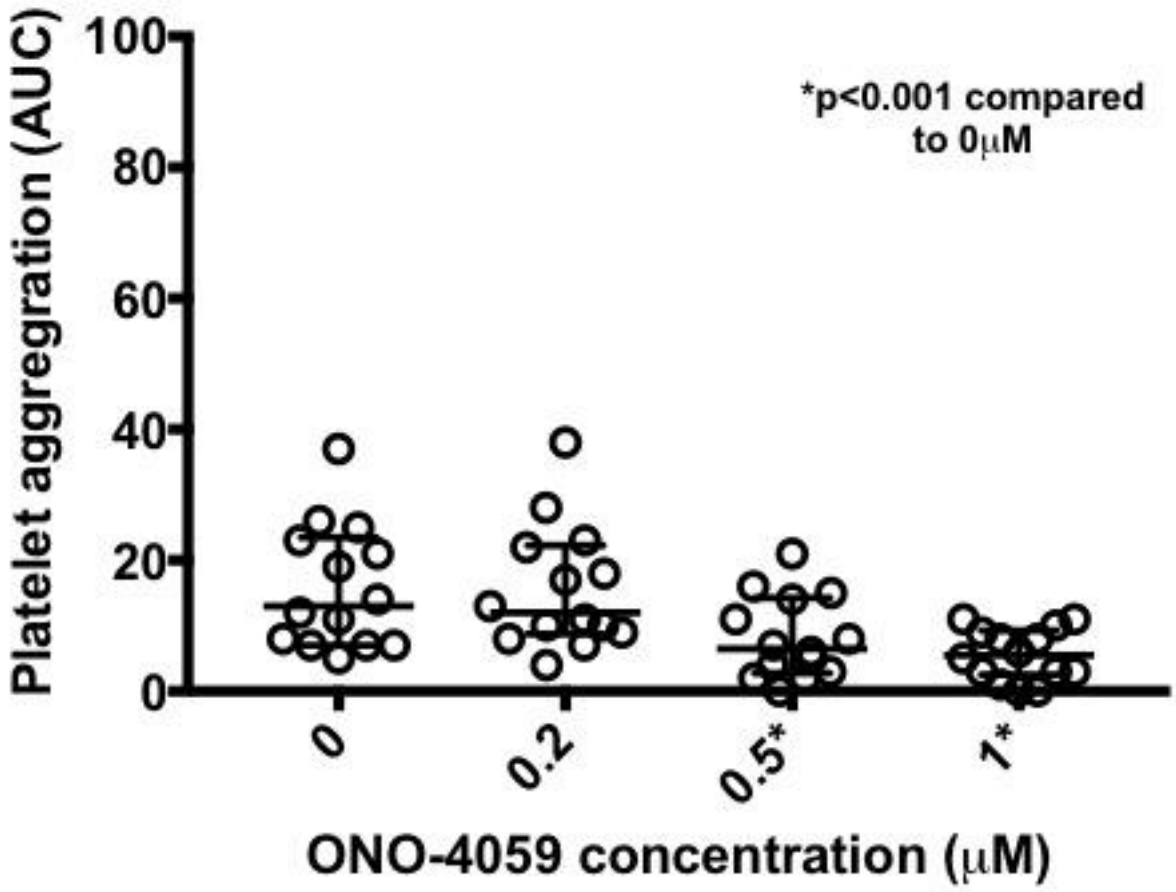

Figure 2: Platelet aggregation was measured using a Multiplate analyser (collagen agonist concentration 3.2 µg/mL) with ex-vivo addition of increasing doses of ONO-4059 in blood samples from patients with acute myocardial infarction treated with dual anti-platelet therapy (n=14) Each circle represents individual data points, with horizontal lines representing median values, with lower and upper quartiles. Wilcoxon signed rank test was carried out to compare 0µM (control) with 0.2, 0.5 and 1µM of ONO-4059.

4. Discussion

The BTK inhibitors Ibrutinib and ONO-4059 provide dose dependent inhibition of platelet aggregation in *ex-vivo* samples from patients with acute MI already treated with dual antiplatelet therapy.

Rushworth et al. [14] analysed platelet aggregation in blood samples obtained from patients with CLL/MCL treated with Ibrutinib to investigate the *in-vitro* effect of Ibrutinib on platelet function. Platelet aggregation in response to collagen was significantly inhibited while platelet aggregation in response to ADP was also inhibited, but to a lesser extent. Platelet aggregation in response to Arachidonic Acid and Thrombin Related-activated Peptide (TRAP) was not inhibited. Levade et al. [12] incubated healthy donor blood with Ibrutinib and demonstrated that Ibrutinib inhibits collagen induced platelet aggregation in a dose dependent manner with higher doses being associated with lower maximum platelet aggregation. Kamel et al. [13] and Levade et al. [12] demonstrated that platelet aggregation in response to collagen was inhibited in patients receiving Ibrutinib for CLL/MCL, but not in response to ADP, Arachidonic acid, adrenaline, ristocetin, and U46619 (thromboxane A2). Patients with CLL/MCL with bleeding complications on Ibrutinib were found to have significantly lower platelet aggregation compared to those without evidence of bleeding. [13] More recently, Nicolson and colleagues report that inhibition of BTK kinase actually causes only partial inhibition of GPVI signalling in platelets and they provide evidence that BTK supports GPVI signalling by functioning as an adapter protein as well as a kinase. [20] Taken together, these findings support the hypothesised role of BTK in GPVI collagen mediated pathway in platelets and our

findings provide further evidence of this in patients with acute MI receiving dual antiplatelet therapy.

In the study by Kamel et al., the majority of patients received 420mg of Ibrutinib daily. [13] Pharmacokinetic and pharmacodynamics studies suggest *in-vivo* plasma concentration of 0.4μM is achieved with a once daily dose of 560mg; [21, 22] a 420mg dose of Ibrutinib is likely to have a lower plasma concentration. Our results show inhibition of platelet aggregation with Ibrutinib at a concentration of 0.5μM which could be achieved with current therapeutic doses. The exact doses to be tested in potential future studies in acute MI remain to be determined.

Ibrutinib acts via BTK and Tec expressed in platelets, both of which are non-receptor tyrosine kinases belonging to the Tec family kinases (TFK). [15, 16] Inhibition of BTK and Tec interferes with the downstream signalling of the GPVI collagen mediated pathway. Platelet adhesion, an initial step in thrombus formation, occurs when GPVI binds to exposed collagen. This leads to PI3K dependent phosphorylation of BTK and Tec. These then in turn activate PLCy2. [15, 23] Ibrutinib treated platelets have reduced phosphorylation of PLCy2 and autophosphorylation of BTK. [12] Ibrutinib therapy also affects other pathways involved with platelet aggregation, including platelet activation by von Willebrand factor (vWF), reducing platelet adhesion onto vWF with high shear rate. [12] Additionally, BTK is essential in GPIb dependent thrombus formation [24] and is involved in activation of platelets via C-type lectin-like receptor-2 (CLEC-2). [25] BTK and Tec also appear to be involved in the later stages of thrombus formation via the fibrinogen receptor integrin αIIbβ3, which leads to phosphorylation of the two tyrosine kinases. [26,27]

Animal models of GPVI-deficiency have demonstrated potential protection against platelet activation, adhesion and thrombus formation at sites of vascular injury. [28-30] Increased GPVI expression has been linked to patients with acute MI, which may suggest GPVI inhibition could be beneficial. [31] Studies targeting GPVI are underway in stable coronary artery disease. [32,33]

Collagen exposed on atherosclerotic plaques is morphologically different to collagen within physiological tissue. [34,35] Busygina et al. explored the *in-vitro* and *ex-vivo* effects of Ibrutinib and ONO-4059 on platelet aggregation in response to atherosclerotic plaque material and native collagen fibres, in static and high shear arterial flow conditions. Both BTK inhibitors inhibited platelet aggregation in response to plaque derived collagen and native collagen under static conditions, however with high shear arterial flow platelet aggregation was impaired with plaque material, but not with native collagen fibres. [36] This suggests that Ibrutinib and ONO-4059 may be advantageous in atherosclerotic disease by inhibiting thrombus formation on atherosclerotic plaques while sparing physiological platelet function.

4.1. Limitations

This was an exploratory *ex-vivo* study with a small sample size, therefore the results cannot be extrapolated to the use of these agents in a clinical setting. This study cannot provide any reliable information on safety aspects of BTK inhibitors, including risk of bleeding, (or other reported side effects such as thrombocytopenia, neutropenia and increased risk of infection) given in the context of acute MI where other antithrombotic treatments are also given. All these potential side effects would need to be carefully assessed before BTK inhibitors could be used as a treatment

acute MI. In this study we only measured platelet aggregation in response to collagen, whereas BTK inhibitors may have other mechanisms of inhibition particularly in the context of acute MI and routinely administered antiplatelet therapy, which could be explored in future studies. Measurement of platelet aggregation is subject to a number of influences that need to be carefully controlled and results can be affected by factors such as smoking on the day of testing, shaking or mixing of blood and the time between obtaining sample and analysis. Samples were transported and analysed as soon as possible and the estimated average time from obtaining blood samples to analysis was 70 minutes. This was in keeping with recommendations for collection, transport and analysis for measurement of platelet aggregation. [37]

4.2. Conclusion

We have demonstrated that the BTK inhibitors Ibrutinib and ONO-4059 show dose dependent inhibition of platelet aggregation, at physiologically relevant concentrations, in blood samples taken from patients with acute MI on dual antiplatelet therapy. These preliminary observations support further exploration of BTK inhibitors as potential therapeutic antiplatelet agents for coronary artery disease through the GPVI collagen pathway, which is not utilised in current anti-platelet regimens.

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7. Declarations of Interest: none

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Highlights

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Effect of Bruton's Tyrosine Kinase inhibitors on platelet aggregation in patients with Acute Myocardial Infarction

- Bruton’s tyrosine kinase inhibitors affect the glycoprotein VI collagen pathway
- They add further platelet inhibition in blood samples after myocardial infarction
- This provides a rationale for clinical testing as potential new antiplatelet agents