**Summer Student Project Report**

**Student**
Hannah Peters

**Title of Project**
"Exploring the role of BTK in human platelet function by selectively degrading BTK over TEC"

**Supervisors**
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**Lay Summary**

Platelets are small cells derived from megakaryocytes and function as regulators of haemostasis and thrombosis. However, inappropriate activation can lead to thrombosis and cardiovascular disease.

Platelets do not have a nucleus, meaning that genetic approaches cannot be used to investigate platelet function. Most current research relies on mouse models or pharmacological methods to investigate signalling pathways. However, these approaches face limitations, for example species differences.

Recently, new developments in targeted protein degradation have opened up a new approach to investigating platelet function. PROTACs (or PROteolysis TArgeting Chimeras) are small heterobifunctional molecules that harness the ubiquitin/proteasome system (UPS) to selectively degrade proteins in cells. This means that protein 'knockouts' can be achieved in cells without the need to manipulate the genome. In addition, due to the limited ability of platelets to resynthesise proteins, PROTAC-mediated degradation has huge potential to achieve sustained protein knockouts in platelets.

BTK (Bruton's tyrosine kinase) plays an important role in platelet function downstream of receptors such as collagen receptor glycoprotein VI (GPVI). BTK has been suggested as a novel target to lower the risk of inappropriate thrombus formation in at-risk patient groups. Previous studies to investigate the role of BTK in platelet function have relied on mouse models, but the development of PROTACs means that a BTK 'knock-out' can now be achieved in human platelets *in vitro*.

**What was the aim of your project?**

Recently, the Hers lab have demonstrated that environmental conditions can influence the selectivity of PROTAC-mediated degradation in platelets and have optimised conditions to allow complete degradation of BTK, without significant degradation of the closely related tyrosine kinase TEC. My project has focused on selectivity degrading BTK over TEC and investigating how BTK deletion impacts platelet function.

**How did you address the aim?**

I used a variety of biochemical assays and cell biology techniques to investigate the impact of BTK deletion on platelet function.

Western Blotting was first used to confirm that selective degradation of BTK over TEC can be achieved via PROTAC-mediated degradation under the optimised conditions recently established by the Hers lab. The PROTACs I tested included the multi-kinase TL-12-186 PROTAC degrader and the BTK specific DD-03-171 PROTAC degrader. These PROTACs are both Cereblon (CRBN) recruiting, since other E3 ligases such as von-Hippel Lindau (VHL) are not expressed in platelets. Western blotting was then used to assess the selectivity achieved with each of these PROTACs in different conditions.

Immunoblotting was also used to investigate the phosphorylation of proteins, evaluating how BTK deletion affects the phosphorylation of tyrosine kinases which lie downstream of BTK/TEC, such as PLCγ2, comparing this to the extent of phosphorylation observed in tyrosine kinases upstream of BTK/TEC, such as pSyk.

I also used a range of assays to evaluate platelet function and investigate how BTK degradation impairs platelet function and aggregation. These assays included light transmission aggregometry and plate aggregometry, using different agonists such as CRP (collagen-related peptide) and TRAP-6 to stimulate the platelets.

In addition, flow cytometry was used to monitor the expression of markers of platelet activation, such as P-selectin expression (a marker of α-granule secretion) and integrin $\alpha_{II}ß_3$ activation in response to stimulation with CRP and TRAP-6 agonists.

**What did you find out?**

1. PROTAC selectivity in platelets can be influenced by environmental factors

My project supported evidence that an effective BTK knock-out can be achieved in human platelets *in vitro* via PROTAC-mediated degradation applied under altered environmental conditions. The conditions to selectively degrade BTK over TEC and achieve a selective BTK knock-out in platelets involve using the BTK-specific DD-03-171 PROTAC degrader applied in altered buffer conditions. However further optimisation of this protocol will be needed going forward.

2. Phosphorylation in the GPVI signalling pathway is impacted by BTK degradation

Techniques such as immunoblotting were used to investigate the phosphorylation of proteins upstream and downstream of BTK/TEC and revealed that phosphorylation in the GPVI signalling pathway appears to be impacted by BTK specific degradation.

3. PROTAC-mediated BTK specific degradation affects signalling via the GPVI pathway, but not PAR-1

Flow cytometry was used to evaluate CRP- and TRAP-6-mediated P-selectin expression (a marker of α-granule secretion) and integrin $\alpha_{II}ß_3$ activation. Reduced markers of platelet

activation were observed in the selective BTK knock-out in response to CRP, which acts via the GPVI signalling pathway, but not TRAP-6, which acts via the PAR-1 receptor. This confirmed that PROTAC-mediated BTK specific degradation affects signalling via the GPVI pathway, but not PAR-1.

Assays such as Light Transmission Aggregometry also confirmed the finding that PROTAC-mediated BTK degradation impacts the GPVI signalling pathway, but not PAR-1. This was shown by the fact that significantly impaired platelet aggregation was observed in DD-03-171 treated platelets when stimulated with CRP, but not with TRAP-6.

4. BTK degradation significantly impairs levels of platelet activation and platelet aggregation when stimulated with CRP

Flow cytometry revealed that BTK specific degradation significantly reduces markers of platelet activation when platelets are stimulated with CRP, with only a minimal increase in P-selectin expression and levels of integrin $\alpha_{II}ß_3$ activation observed from basal levels. This suggests that the ability of the closely related tyrosine kinase TEC to regulate platelet activation in the absence of BTK is possibly lower than previously thought.

Light transmission aggregometry also revealed that BTK degradation significantly impairs platelet aggregation.

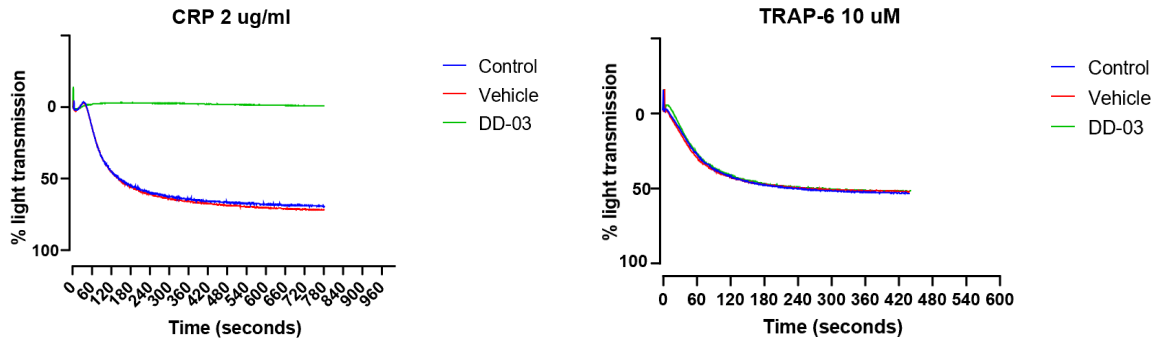


*Figure 1: Light transmission aggregometry confirmed the findings from Flow Cytometry that PROTAC-mediated BTK degradation impairs signalling via the GPVI pathway in response to CRP, but not via PAR-1 in response to TRAP-6. In addition, BTK degradation appears to significantly impair platelet aggregation when stimulated with CRP. The % light transmission for DD-03-171 treated platelets stimulated with CRP at 2 ug/ml was 1% compared to 60% for Vehicle, showing effective and sustained inhibition of platelet aggregation. In contrast, % light transmission for DD-03-171 treated platelets when stimulated with TRAP-6 at 10 uM was 56% compared to 61% for Vehicle.*

In conclusion, this studentship has helped develop conditions to achieve selective degradation of BTK over TEC in human platelets *in vitro* and has emphasised the huge potential of chemical degraders in platelet research. This project has also given insights into

role of BTK in platelet function, and crucially, also the ability of TEC to regulate platelet activation in the absence of BTK in platelets.

**What did you learn from participating in this project?**

This research summer studentship has given me an invaluable opportunity to experience leading research in pharmacology and the application of medicinal chemistry in platelet research at the University of Bristol. It has given me an invaluable opportunity to expand my practical skills and learn key cell biology techniques and biochemical assays.

I have become proficient in biochemical and cell biology techniques such as Western Blotting, SDS-PAGE, flow cytometry, as well as many key platelet biology techniques such as light transmission aggregometry and plate aggregometry. These are all key techniques that I would not have otherwise had the opportunity to develop within the scope of my Chemistry undergraduate degree.

This studentship has also taught me invaluable skills in terms of data analytics, statistical analysis and data presentation, in addition to experimental design and research planning skills.

**How has this project affected your long-term goals?**

This platelet society summer studentship has given me a valuable insight into research as a career. It has furthered my interest in the field of targeted protein degradation and has also encouraged me to consider progressing on to a PhD in the biomedical field after my current undergraduate degree programme.

I am now eager to gain more research experience in the future and learn more about the applications of and enormous potential of PROTACs both as a research tool and as a potential therapeutic in the clinic.

This project has also reaffirmed my interest in pursuing research at the interface of chemistry and biology, highlighting how novel advances in the areas of medicinal chemistry and chemical biology can be used to advance our understanding of human disease.