Investigating the trimming function of Endoplasmic Reticulum Aminopeptidase 1

Centre for Cancer Immunology— Cancer Sciences, University of Southampton

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The **Centre for Cancer Immunology** was opened in **Spring 2018**, following a charity drive raising over **£ 27 Million for its construction**. The UK's first building exclusively for the study of the immune system and its response to cancer—the CCI is a **cutting edge research lab** on the frontier of this rapidly growing field of medical research.

Introduction

For the last 7 years the Cancer Sciences department of the University of Southampton has been hosting at least one student from the University of Bath every year. Starting September 2019 I was able to take on this role.

The Centre sometimes offers for students to work directly with a PhD or Postdoc researcher on their current project, or to take charge of a project of their own.

The focus of Professor Tim Elliott's lab group is the **protein ERAP1—a zinc metalloprotease found in the endoplasmic reticulum of all human cells.**

ERAP1 is a key element in viral infections, auto-immunity and even the immune response to cancer—yet its exact mechanism is still unknown. My project was to probe into the characteristics of this protein to glean more about how it functions.

ERAP1 and the immune system

The adaptive immune system detects virus-infected or cancerous cells through the monitoring of short peptides presented at the surface of cells on so-called MHC class I molecules. These MHC I molecules best present peptides of a specific length—so the function of ERAP1 is to trim peptides from poorly folded or degraded proteins for suitable presentation to the immune system. Fragments from viral or mutated proteins can be recognised by circulating lymphocytes; inducing an immune response.

ERAP1 is of great interest currently as its activity varies between individuals. Over or under activity of ERAP1 can result in changes to the immune response as a certain important peptide might be destroyed, or not shortened enough for presentation.

Survival rates of certain cancers, such as HPV-induced head and neck cancer, have even been linked to the present gene for ERAP1. This shows that certain variations can better produce immune active peptides.

Alternatively, there is evidence that some tumours may avoid detection by the immune system through mutation to ERAP1, as dramatic increases in enzyme activity can destroy known epitopes, allowing a cancer to spread unchecked.

...And so we are interested in learning more about how ERAP1 functions, as well as in producing potent, yet safe inhibitors of this enzyme in order to improve the survival rates for cancer patients.

Research Objectives

- **Test the effect of various N-terminal extensions on the function of ERAP1, to attempt to determine the specificity and "Reading frame" of its active site.**

ERAP1 trims from the N-terminus of peptides, and is capable of binding peptides of up to 15 amino acids length. We know that ERAP1 has different activities towards different amino acids; trimming some extremely well, whilst having almost no effect on others. We wanted to test "combination" extensions, such as to see the activity of X-peptide related to XX-peptide, and compare XY– extensions with YX-.

- **Test six novel inhibitors for effect on ERAP1 activity, as well their toxicity**

Another lab was creating new inhibitors, computationally determined to bind to the active site of ERAP1. They sent five such inhibitors to us for blind testing of their effect—with one extra "dummy" compound to ensure validity. We wanted to determine if any were of useful therapeutic activity—as well as their safety for human cells.

- **Test the activity of a mutant form of ERAP1 (Δ12) with the first twelve amino acids removed, to determine their importance to the structure.**

Mice and Humans both have a form of ERAP1, with slight differences in the gene. One such difference is that human ERAP1 encodes 12 extra amino acids—we do not yet know if this has a function, or if it is merely an artifact of evolution that is not harmful

Conclusions

Due to COVID-19 my placement was ended early, so the Δ12 mutant experiment and a return to the N-terminal extensions were cancelled.

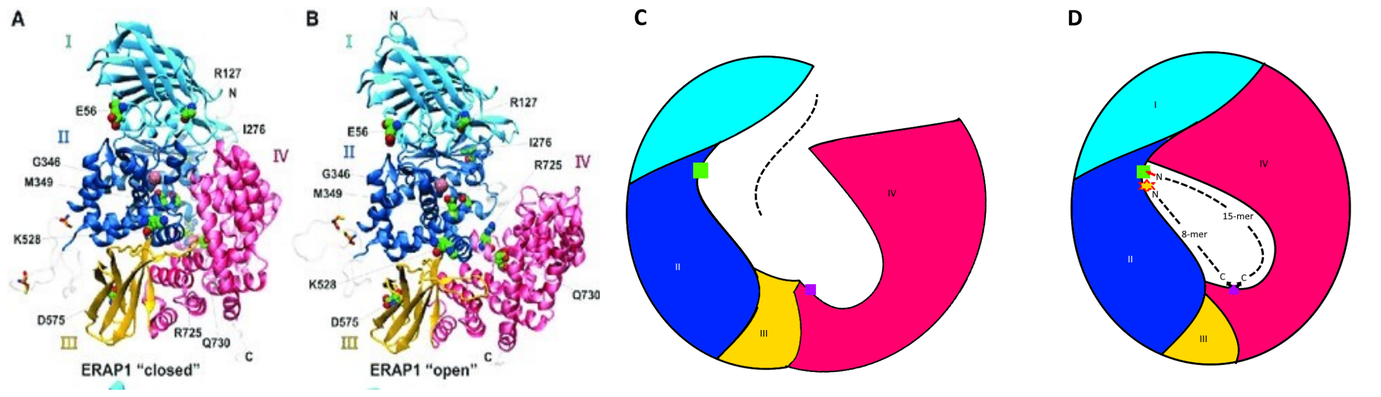

Figure 1. Ribbon representation of ERAP1, **Tension-based "molecular-ruler" mechanism.**

A and B: Ribbon representation of ERAP1— Papakyriakou, Athanasios & Stratikos, Efstratios. (2017). The Role of Conformational Dynamics in Antigen Trimming by Intracellular Aminopeptidases. Frontiers in Immunology. 8. 10.3389/fimmu.2017.00946. **Available via licence: Creative Commons Attribution 4.0 International.**

C and D: Potential mechanism for length specificity—ERAP1 has a much larger internal cavity than other known zinc metalloproteases, and has known specificity for producing peptides of 8-9 amino acids length (8-9mer). Domain I (cyan) encloses the top of the active site, Domain II (blue) is catalytic, containing the Zinc ion active site, Domain III (yellow) is believed to act as a "hinge" allowing ERAP1 to adopt two distinct conformational states— an "open" weakly-active state, and a "closed" state in which free-peptide trimming is believed to occur. Domain IV (Red) is regulatory in nature and contains the C-Terminus binding site (Purple square). The active site (Green square) Is believed to be flexible.

The above proposed mechanism follows that the flexibility of the active site enables the N-terminus of the peptide to be "dragged" into a conformation favourable for trimming much as paper is pulled into a shredder. Extended peptides bulge into the cavity to fit within the closed ERAP1 structure so can be freely trimmed, though once the peptide is shorter there is tension between the action of the active site and the binding between the C-terminus and Domain IV. Depending on the difference in binding strength the N-terminus may be released and the correct length peptide leaves the enzyme, or the C-Terminus may be released and the peptide over-trimmed or even destroyed outright.

As such, allotypes (variant protein forms derived from genetic differences) of ERAP1 known to over-trim peptides may reduce the binding strength of common peptides to domain IV, whereas variants that under-trim may have a weaker "pull" at the active site. Alternatively changes to the shape of the cavity or allosteric binding to a peptide may increase or decrease the tension upon either side earlier or later in trimming.

Methods and Results

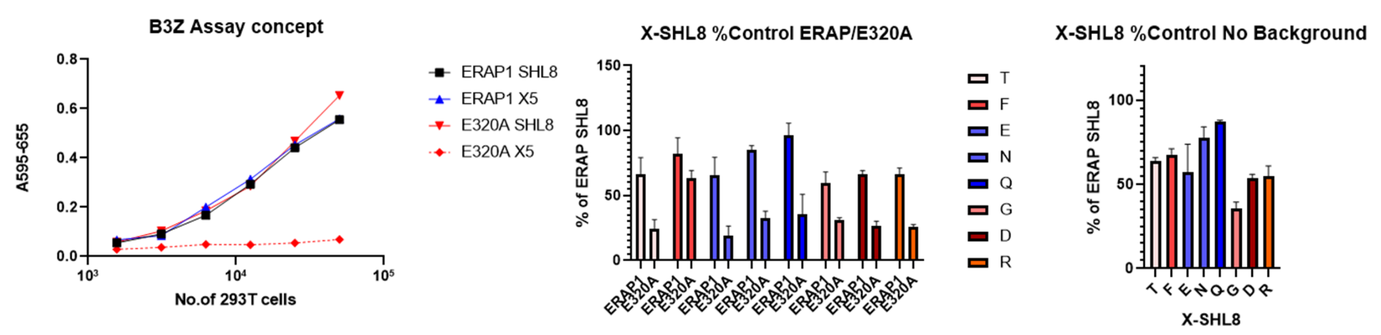

Figure 2. N-terminal extension testing gives high background readings with current method.

ERAP1 knockout HEK 293T cells were transfected using Fugene-6 reagent with either human ERAP1 gene, or the loss of function variant E320A; as well as H-2Kb (MHC I molecule) and the gene for peptide ES-X-SIINFEHL, where ES is the adenovirus E3/gp19k signal peptide sequence (ES) 5'-MRYMILGLLALAAVCSAA-3' (For targeting of the peptide to the ER) and X is the N-terminal extension. X5 is the extension AIVMK, known to be trimmed well by ERAP1—but untouched by E320A.

Using preliminary trimming data for single peptide extensions, a sample was chosen based on the predictive software SignalP 5.0, to determine the form such an extension would take on entering the ER. We needed efficiently trimmed and poorly trimmed extensions with low background, so that we could get good data from combinations of such extensions as stated earlier.

The transfected 293T cells were titrated with *LacZ* inductible B3Z T-cells, which are sensitive to H-2Kb bound SIINFEHL peptide. T cell receptor binding resulted in expression of LacZ allowing determination of ERAP1 activity by the cell surface expression of peptide-bound MHC I, and thus the number of activated B3Z cells. Chlorophenol red-β-D-galactopyranoside (Sigma) colorimetric assay, or CPRG, was used to determine the activation of B3Z cells, taking absorbance at 595-655nm. The background on the sample of amino acid extensions were considered too high so the project was put on hold.

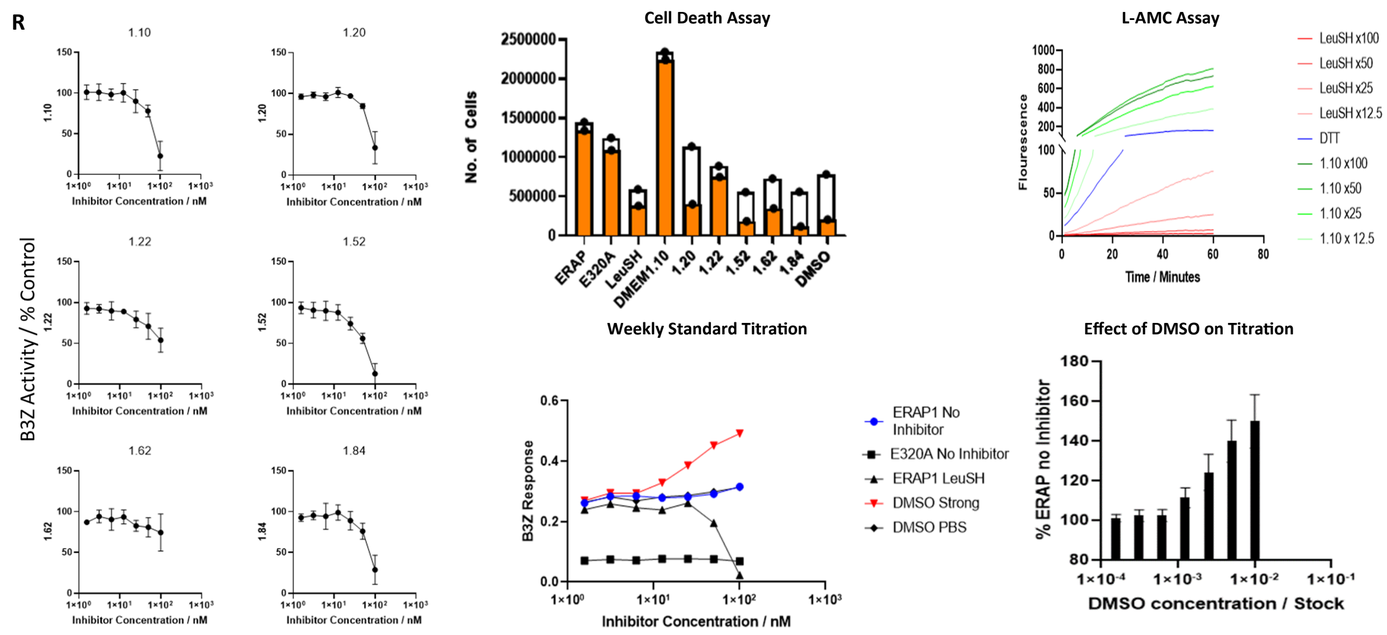

Figure 3. The inhibitor Leucinethiol has higher efficacy than the novel compounds for modulating ERAP1.

Inhibitor testing used a modified form of the N-terminal extension assay, using only peptide X5-SIINFEHL with ERAP1 and E320A as the positive and negative controls. Instead of titrating number of transfected cells against B3Z, each well had the same number of cells and the added inhibitor concentration was varied. Initial titrations showed an odd increase in ERAP1 activity, which was later deemed to be a side-effect of the solvent the inhibitors came in—DMSO. Subtracting the effect of DMSO gave the expected curves **(R)**, though these curves we deemed to be an effect of cell death rather than inhibition. Inhibitor "1.62" was deemed to be the dummy compound as it showed significantly lesser effect on the cells. A flourimetric assay of ERAP1 trimming of L-AMC was carried out with the inhibitors, with the known inhibitor Leucinethiol as a reference. The inhibitors were thus shown to be inactive in vitro as well as in vivo.

The CCI was temporarily closed with the lockdown back in March, and has followed a slow reopening procedure since—following the guidelines of the University of Southampton. As such since the two research paths I had followed had ended, I instead returned to studying the literature. The lab has numerous organised guest lectures, research and journal clubs, as well as weekly lab meetings for each lab group at the CCI. These activities and literature examination led to the hypothesis that the ERAP1 length specificity is due to the mechanism similar to the one shown above.

Potential experiments to test a "tension" based hypothesis might include:

- X-Ray Crystallography of ERAP1 to determine the cause of the change between the "Open" and "Closed" ERAP1 conformations, using compounds that would simulate binding of a peptide to either the active site or domain IV.
- Mass spectrometry of in vitro ERAP1 activity assays, to compare the length of peptides produced while the domain IV binding site of ERAP1 is blocked with an assay with normal length specificity.