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The relevance of immunoproteasome inhibition on pro-inflammatory processes in an *in-vitro* model of inclusion body myositis

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Background

Inclusion Body Myositis (IBM) is the most common sporadic inflammatory muscle disease in individuals aged 50 or above. The diagnosis of IBM is challenging, due to the lack of diagnostic data and its heterogenous clinical presentation. Currently, no treatment option is available (1,2).

The pathomechanisms of IBM are still unclear, but both inflammatory as well as myodegenerative mechanisms are involved. IBM is characterized by the presence of protein aggregations, also called inclusion bodies (2). The cytokine-inducible immunoproteasome with its subunits (β1i/LMP2/psmb9, β2i/MECL-1 and β5i/LMP7/psmb8) cleaves specific proteins and plays an important role in MHC-I mediated antigen presentation and the recruitment of CD8+ T-cells following inflammation and protein aggregation (3,4). The immunoproteasome inhibitor KZR-616 (Zetomipzomib) selectively targets immunoproteasome subunits β5i and β1i, while partially inhibiting β2i and the constitutive subunit β5. (5)

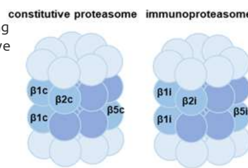


Fig. 1: Structure of the immunoproteasome (7).

Objective and Methods

Our goal in this project was to assess immunoproteasome activity and its inhibition by KZR-616 in the CCL-136 cell line and human primary myoblasts after incubation with cytokines IL-1β and/or IFN-γ for 48 hours and subsequent treatment with KZR-616.

Gene expression analysis of immunoproteasome subunits psmb8 and psmb9, cytokine IL-1β, and CXCL9 was quantified using qRT-PCR (expessed as fold change $2^{-\Delta\Delta ct}$). Immunoproteasome activity was assessed using the immunoproteasome activity assay, and formation of reactive oxygen species (ROS) was determined with a ROS assay. Fluorescence intensity was measured by TECAN Microplate Reader and fluorescence microscopy.

Results

The proinflammatory environment induced by inflammatory mediators IL-1β and IFN-γ is evident in the increased expression of IL-1β and CXCL9 as seen in Fig. 2. Cells stimulated with both IL-1β and IFN-γ showed the highest expression profiles. Cells treated with KZR-616 showed a lower activation and expression of the immunoproteasome subunits psmb8 and psmb9.

The concentration of KZR-616 seems to impact the strength of inhibition. Concentrations exceeding 1000 nM KZR-616 appear to be cytotoxic.

KZR-616 concentrations of 250 nM, 500 nM and 1000 nM induce a significant decrease in immunoproteasome activity and expression of the subunits psmb8 and psmb9.

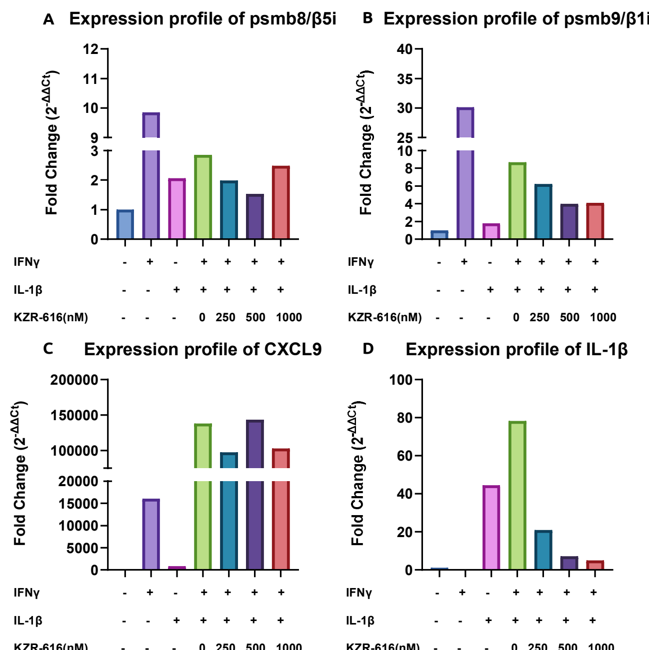
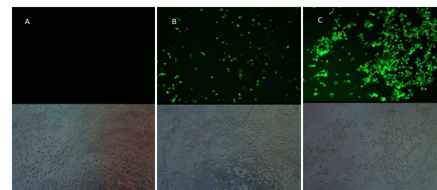


Fig. 2: Fold change of immunoproteasome subunits psmb8 (A), psmb9 (B), cytokine IL-1β (C), and chemokine CXCL9 (D) measured by qRT-PCR after stimulation with IL-1β and/or IFN-γ and after treatment with KZR-616 (N=1).



Images of fluorescence microscopy of CCL-136 with and without inducer. A) without CCL-136 (Medium); B) CCL-136 without Inducer; (C) CCL-136 with Inducer. Upper part of the images shows fluorescence in FLUO/FITC microscopy, the lower part phase contrast microscopy of the same frame (N=1).

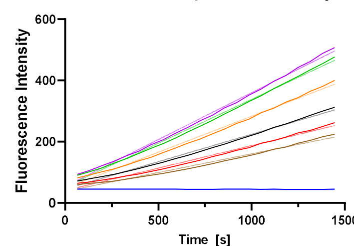
To analyse the formation of ROS, the emitted fluorescence of the ROS assay was detected by fluorescence microscopy. ROS production can be induced by using an appropriate ROS inducer.

The TECAN measurement apparently fails to provide trustworthy results in relation to the ROS assay.

AMC fluorescence was detected for the Ac-PAL-AMC, the preferred peptide substrate of the β1i/LMP2 immunoproteasome subunit.

Inflammatory mediators IL-1β and IFN-γ increased the immunoproteasome cleaving activity as seen in Fig. 3. Low concentrations of KZR-616 showed either no or only a minimal effect on the cleaving activity. KZR-616 concentrations of 250 nM, 500 nM and 1000 nM induces a significant decrease in immunoproteasome activity, for KZR 1000 nM even lower than the activity levels of unstimulated cells. Our results indicate an only partial inhibition of the subunits.

These findings hold true for all substrates in the activity assay, including Ac-ANW-AMC (β5i/LMP7 immunoproteasome subunit substrate) and Ac-KQL-AMC (β2i/MECL-1 immunoproteasome subunit and β2 constitutive proteasome subunit substrate).



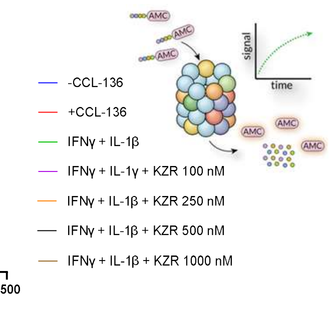


Fig. 3: AMC (7-Amino-4-methylcoumarin) fluorescence intensity relative to immunoproteasome cleaving activity of fluorogenic peptide substrate PAL (Pro-Ala-Leu) in the immunoproteasome activity assay measured by TECAN Microplate Reader. Comparison of unstimulated cells, cells stimulated with cytokines, and cells treated with KZR-616. A simple linear progression was performed. (N=1)

Fig. 4: Principle of the immunoproteasome activity assay (6).

Conclusion

The immunoproteasome appears to play a role in the proinflammatory pathomechanisms of IBM, contributing to protein degradation and accumulation. Our findings suggest that the immunoproteasome can be stimulated by IL-1β and IFN-γ, especially in combination.

KZR-616 seems to inhibit immunoproteasome activity, potentially leading to the downregulation of chronic inflammation and cell stress in muscle cells affected by IBM. KZR-616 could thus be an effective symptomatic treatment option for myopathies including IBM by promoting a more physiological cell environment and aiding in the restoration or preservation of muscle cell function. These findings support ongoing studies with lupus nephritis and systemic lupus erythematosus (5).

In summary:

→ **The immunoproteasome can be stimulated by IFN-γ and IL-1β**

→ **KZR-616 inhibits the immunoproteasome expression and its cleaving activity**

→ **KZR-616 leads to a downregulation of inflammation, making it a possible treatment option for IBM and other forms of myositis**

Outlook

The relevance of intracellular protein aggregation in IBM, due to an increased immunoproteasome activity and inflammation, still needs further investigation in ongoing experiments by AK Schmidt.

Further experiments will be performed to localize MHC-I and its expression after cytokine induction and treatment with KZR-616. The expression and localization of β1i and β5i subunits will be analyzed by immunocytochemistry and Western Blot.

Analysis of muscle cell death, cytotoxicity and immunocytochemical detection of β-amyloid aggregation will be performed to investigate protein aggregation and degradation and to correlate data with immunoproteasome functionality.

References

1. Gang Q, Bettencourt C, Machado P, Hanna MG, Houlden H. Sporadic inclusion body myositis: the genetic contributions to the pathogenesis. Orphanet Journal of Rare Diseases. 2014 Jun 19;9(1):88.
2. Keller CW, Schmidt J, Lünemann JD. Immune and myodegenerative pathomechanisms in inclusion body myositis. Ann Clin Transl Neurol. 2017 Jun;4(6):422–45.
3. Bhattarai S, Ghannam K, Krause S, Benveniste O, Marg A, De Bruin G, et al. The immunoproteasomes are key to regulate myokines and MHC class I expression in idiopathic inflammatory myopathies. Journal of Autoimmunity. 2016 Dec;75:118–29.
4. Ferrington DA, Gregerson DS. Immunoproteasomes. In: Progress in Molecular Biology and Translational Science [Internet]. Elsevier; 2012 [cited 2023 Oct 12]. p. 75–112. Available from: https://linkinghub.elsevier.com/retrieve/pii/B9780123978639000031
5. POS0695 KZR-616, A SELECTIVE IMMUNOPROTEASOME INHIBITOR FOR THE TREATMENT OF SYSTEMIC LUPUS ERYTHEMATOSUS: RESULTS FROM THE COMPLETED DOSE ESCALATION PHASE 1B PORTION OF THE MISSION STUDY | Annals of the Rheumatic Diseases [Internet]. [cited 2023 Dec 7]. Available from: https://ard.bmj.com/content/80/Suppl_1/595.2
6. Ac-Pro-Ala-Leu-AMC (PAL-AMC) [Internet]. [cited 2024 Jan 29]. Available from: https://southbaybio.com/products/ac-pro-ala-leu-amc.html
7. Basler, M., Li, J. & Groettrup, M. On the role of the immunoproteasome in transplant rejection. *Immunogenetics* **71**, 263–271 (2019). https://doi.org/10.1007/s00251-018-1084-0

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