POSTER 1114

Potent AhR Agonist EQ504 Facilitates Gut Epithelial Repair in Inflammatory Disease Models

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Introduction

- **The intestinal epithelial barrier** is a dynamic, single-cell layer lining the gut that plays a vital role in maintaining health and homeostasis. The epithelial layer **blocks microbes and toxins** while **allowing** the **selective absorption of nutrients and water.**
- The barrier is reinforced by tight junctions that regulate permeability and contributes to intestinal homeostasis by **secreting mucins**, **antimicrobial peptides, and immune mediators**.
- During disorders such as ulcerative colitis (UC), the barrier is compromised.
- The **Aryl Hydrocarbon Receptor (AhR)** is a ligand-activated transcription factor that senses environmental, dietary, and microbial signals to regulate gene expression involved in detoxification, immune responses, and gut homeostasis. The most well-known gene activated by the AhR pathway is the CYP1A1[6].
- Indirubin is a naturally occurring compound, constituent of Indigo Naturalis. It acts as an AhR agonist, and it has been demonstrated to be beneficial in refractory ulcerative colitis (UC), highlighting the potential of targeting the AhR pathway in UC patients [3,5].

- **In the gut, activation of AhR plays a key protective role:**
  1. Induces production of key cytokines and cytokine receptors (***IL10, IL10R, IL22R***) that promote an anti-inflammatory response within the intestine[1].
  2. Maintains the Gut Epithelial Barrier through **modulation of MUCINS** and **tight junction** proteins (*ZO-1, OCLNs, CLDNs*)[2,4].
  3. Promotes epithelial **tissue turnover and repair**[1].

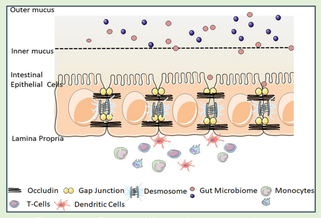


Figure 1. Gut epithelial organization

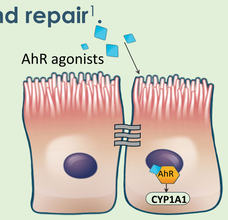


Figure 2. Binding of ligands to the AhR protein induces nuclear translocation and transcription of genes, including genes coding for CYPs enzymes

- **EQ504** is a novel small molecule agonist of AhR that was derived from a naturally occurring AhR ligand

We aim to demonstrate the efficacy of EQ504 in promoting healing of gut epithelial cells from inflammation and damage, highlighting its potential therapeutic value for patients with inflammatory mucosal diseases.

Methods

- **Gene and protein expression analysis**

• Caco2 cells were grown in 12- or 96- well plates for 7 days. Cells were then treated with a titration of the AhR agonists EQ504 and Indirubin, or Vehicle. After 24h, cells were collected and processed for RNA isolation. Gene expression was analyzed by RT-qPCR. For protein analysis, at day 7 the cells were pre-treated with EQ504 or controls for 1hour, challenged with cytokines for 2h and analyzed by WB.

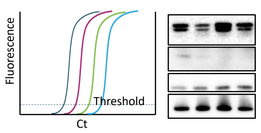


Figure 3. (left) Reverse Transcription quantitative PCR (RT-qPCR) and (right) Western Blotting (WB) readout

- **Epithelial Barrier function analysis by Transepithelial Electrical Resistance (TEER)**

• Caco2 cells were plated on 12mm- 0.4µm Polycarbonate membrane inserts and the EVOM instrument was used to record the TEER.
• The barrier was considered formed when the TEER values were above 800Ω.

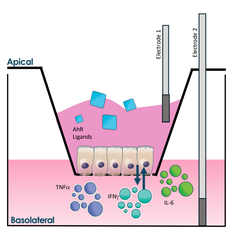


Figure 4. schematic of TEER assay

- **Epithelial healing and regeneration by in vitro scratch assay**

• T84 cells were plated on 24–well TC treated plates until confluence. Once at confluent, the cell monolayer was scratched using a 200µl tip, and the cells ere treated with EQ504, Indirubin or Vehicle. New agonists were added every 3 days, and the cells were imaged and collected at different time-points for gene expression analysis by RT-qPCR.

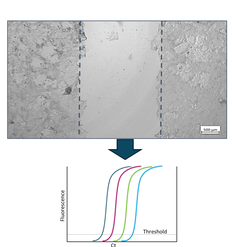


Figure 5. Scratch imaging and qPCR readout

Results

EQ504 is a strong inducer of CYP1A1 in gut epithelial cells

- AhR is highly expressed in Caco2 cells, and its total protein level does not change upon short treatment with cytokines.
- Treatment of Caco2 cells with EQ504 or Indirubin led to upregulation of *CYP1A1*, confirming target engagement by these compounds.
- Comparison of EQ504 and Indirubin showed that EQ504 is more potent in engaging with the AhR target and inducing *CYP1A1* gene expression.

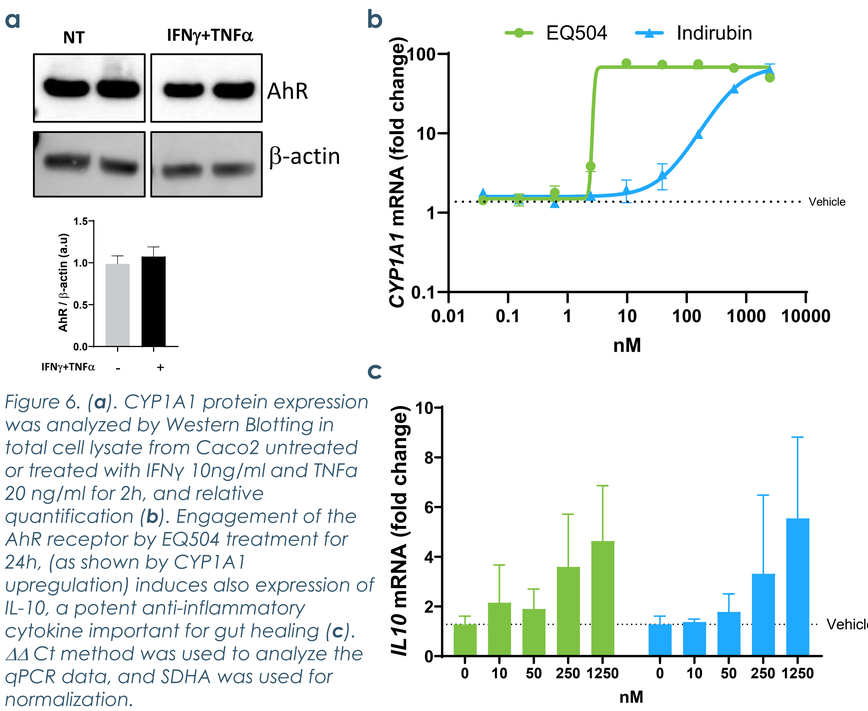


*Figure 6. (**a**). CYP1A1 protein expression was analyzed by Western Blotting in total cell lysate from Caco2 untreated or treated with IFNγ 10ng/ml and TNFa 20 ng/ml for 2h, and relative quantification (**b**). Engagement of the AhR receptor by EQ504 treatment for 24h, (as shown by CYP1A1 upregulation) induces also expression of IL-10, a potent anti-inflammatory cytokine important for gut healing (**c**). ΔΔ Ct method was used to analyze the qPCR data, and SDHA was used for normalization.*

EQ504 promotes scratch repairs in T84 gut epithelial cell line

- A scratch assay was used to study the effect of AhR activation on gut repair.
- EQ504 showed better potency than Indirubin in inducing recovery of the epithelial monolayer.
- RT-qPCR analysis during the repair period, showed increased expression of *IL22RA* in cells treated with EQ504, and downregulation *CLDN2*, a tight junction protein that creates cation channels and impacts the barrier function of the gut[4].

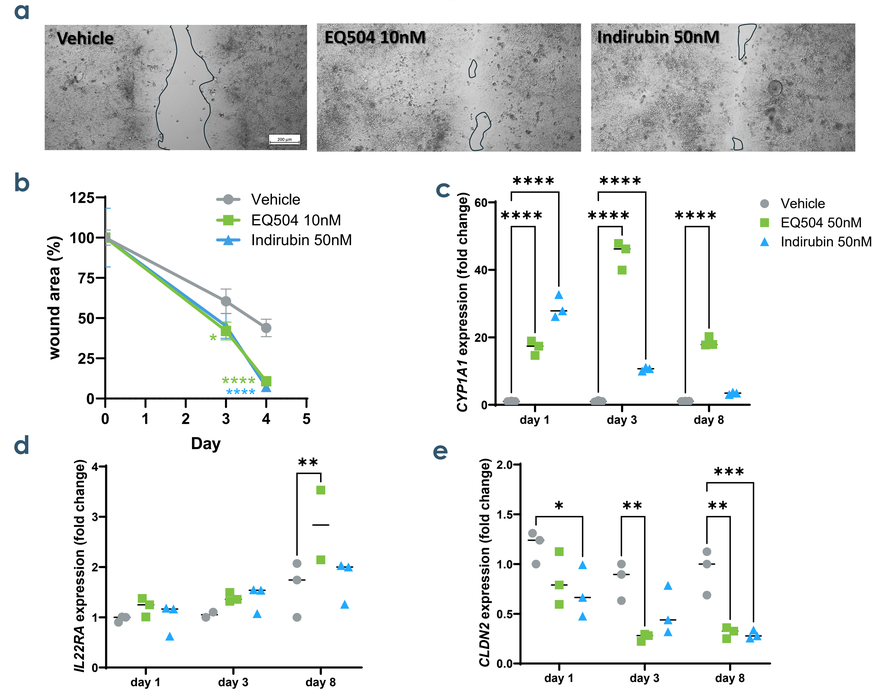


*Figure 7. (**a**) T84 monolayer scratched and imaged over time. (**b**) Wound area quantification was analyzed using the Fiji "Wound Healing size tool master" macro and normalized on the initial wound area (day0). ****p<0.0001, *p<0.05 vs D0 by Two-way ANOVA. **c-e**. CYP1A1 (**c**), IL22RA (**d**) and CLDN2 (**e**) gene expression in T84 cells scratched and treated with EQ504, Indirubin or Vehicle for the indicated time. ΔΔ Ct method was used to analyze the RT-qPCR data, and SDHA was used for normalization. ****p<0.0001 ,***p<0.001, **p<0.01, *p<0.05 vs Vehicle by Two-way ANOVA.*

EQ504 preserved barrier function during inflammatory conditions

• TEER assay was used to assess recovery of epithelial tight junctions upon cytokine-induced stress. The barrier function was preserved over time with EQ504 treatment.

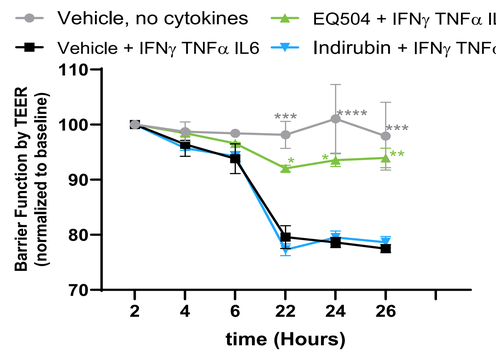


*Figure 8. CACO2 monolayer challenged with IFNγ 10ng/ml, TNFa 20 ng/ml and IL6 10ng/ml to assess recovery of epithelial tight junctions in the presence of 100nM EQ504 or Indirubin. ****p<0.0001 ,***p<0.001, **p<0.01, *p<0.05 vs Vehicle+ IFNγ, TNFα, IL6 by Two-way ANOVA*

• ERK was phosphorylated in EQ504 treated cells, suggesting preservation of cell survival upon cytokines treatment. Phospho-NF-kB (p65) was downregulated and IKB (NF-kB inhibitor) expression upregulated by EQ504.

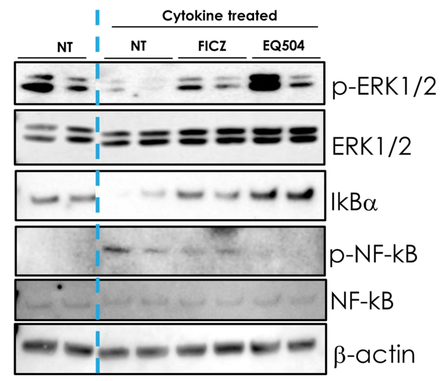


Figure 9 .Caco2 monolayer challenged with IFNγ 10ng/ml and TNFa 20 ng/ml for 2h to induce inflammatory pathway activation, in the presence of 50nM EQ504 or FICZ (AhR agonist used as positive control in the study). Western blotting analysis of total protein lysate from Caco2 cells treated as indicated.

• Gene expression analysis showed upregulation of CLAUDIN2 upon cytokines treatment, EQ504 and indirubin treatment showed dose-dependent reduction of *CLDN2* during inflammation.

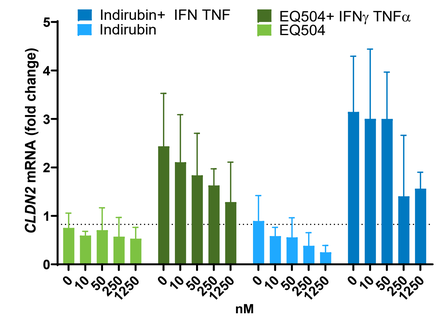
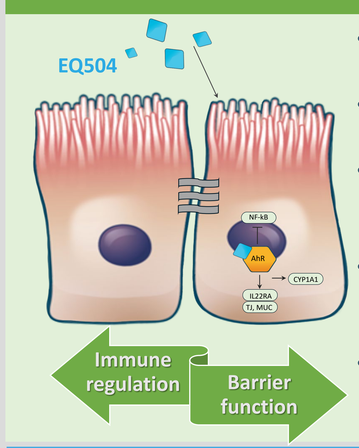


Figure 10. RT-qPCR analysis of CLAUDIN 2 gene expression in Caco2 monolayer challenged with IFNγ 10ng/ml and TNFa 20 ng/ml for 24h to induce inflammatory pathway activation, with or without a titration of EQ504 or Indirubin. ΔΔ Ct method was used to analyze the RT-qPCR data, and SDHA was used for normalization.

Conclusions



- **EQ504 is as strong inducer of the AhR pathway in intestinal epithelial cells.**
- **EQ504 preserves the barrier function *in vitro*.**
- **EQ504 prevents upregulation of CLAUDIN2 (leaky gut claudin) induced by stress like cytokines or injury.**
- **EQ504 promotes healing of intestinal epithelial cells by promoting IL22RA and IL10 expression.**
- **EQ504 promotes survival pathways and prevents cytokine-induced inflammatory signaling *in vitro*.**

Acknowledgments

We would like to acknowledge the whole Equillium research team for constant feedbacks and suggestions on the project.

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Disclosures

This study was funded by Equillium, Inc.
Valeria Marrocco, Dalena Chu and Cherie Ng are currently employees of Equillium.
Valeria Marrocco and Cherie Ng are stockholders of Equillium.
Stephen Connelly is currently an employee, stockholder, and officer of Equillium.
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Presented at the American Association of Immunology, Hawaii 2025