Inhibition of YTHDF1 by salvianolic acid overcomes gluten-induced intestinal inflammation

Coeliac disease (CD) is a chronic inflammatory and autoimmune disorder, primarily affecting the small intestine, developed in genetically susceptible individuals upon gluten ingestion. The only effective treatment so far is a lifelong, strict gluten-free diet. However, difficulties to follow dietary compliance can lead to complications, highlighting the unmet need for adjuvant therapies.

Recently in Gut, we described a novel m^{6}A-XPO1-NFκB pathway that is activated in patients with CD. Specifically, YTHDF1 m^{6}A reader was found to selectively bind the 5' UTR of *XPO1* mRNA and induce its translation, increasing XPO1-mediated inflammation in intestinal cells both *in vitro* and *in vivo*.[1] These findings opened the door to new therapeutic approaches directed to m^{6}A machinery proteins, already in use for the treatment of other disorders.[2]

Interestingly, novel studies have described salvianolic acid (SAC) as a selective inhibitor of YTHDF1, which can rescue Fragile X syndrome linked defects in neural progenitor cells.[3] In this study, we used our previously developed *in vitro* and *in vivo* gluten exposure models[1] in order to test whether two forms of SAC (termed Y20 and Y22) could be used to ameliorate intestinal inflammation. Our *in vitro* data show a reduction of the pepsin-trypsin digested gliadin (PTG)-induced inflammation, represented by the enhanced XPO1, NFκB and *IL8*, at both RNA and protein levels, in cells treated with YTHDF1 inhibitors (figure 1A,B). Additionally, mice treated with PTG and SAC presented lower levels of *Xpo1*, NFκB and *Mip2a*, *Cxcl5* and *Cxcl1* cytokines (homologues for human *IL8*) than those exposed only to PTG, which showed induced intestinal inflammation (figure 1C–E, online supplemental figure 1A). Small intestinal epithelium morphometric and histologic quantification of intestinal response to PTG was also addressed (figure 1F). While PTG treatment showed a significant decrease of the villus height to crypt depth ratio (figure 1 F and G), a slight recovery can be observed in Y20 treated mice group when compared with PTG treated mice (figure 1G), suggesting that this inhibitor could help protecting intestinal disruption during inflammation. Th1 response related cytokines, *Ifng* and *Il21*, and the CD45+ intraepithelial lymphocyte-specific gene expression was also augmented in PTG treated mice but was reduced in SAC treated mice (figure 1H, online supplemental figure 1B); suggesting a decrease in the coeliac characteristic immune cell infiltration after SAC treatment. Moreover, in the intestinal biopsy *ex vivo* model from newly diagnosed CD patients, a reduction of XPO1, NFκB and *IL8* was observed when incubated with the inhibitors (figure 1I,J). All these *in vitro*, *in vivo* and *ex vivo* results show that SAC based selective YTHDF1 inhibitors can help ameliorate gluten-induced intestinal inflammation.

In addition, we were able to show that both SAC forms do not show toxicity in our *in vivo* model. No significant changes were observed between control and treated mice regarding their size and weight. Moreover, no gastrointestinal effects could be detected in terms of diet consumption or faeces weight in treated mice groups (online supplemental figure 1C-E). In addition to the lymphocyte markers (figure 1H, online supplemental figure 1B), we could also confirm that our *in vivo* PTG stimulation activates an inflammatory response by the increased number of goblet cells present in the epithelial cells as well as eosinophil counts in the lamina propia[4 5] (online supplemental figure 1F). In mice treated with PTG and either YTHDF1 inhibitor, these counts were reverted to control values, pointing again that these SAC molecules can, at least partially, protect from intestinal inflammation (online supplemental figure 1F). Interestingly, in another intestinal inflammatory scenario (on IFNG

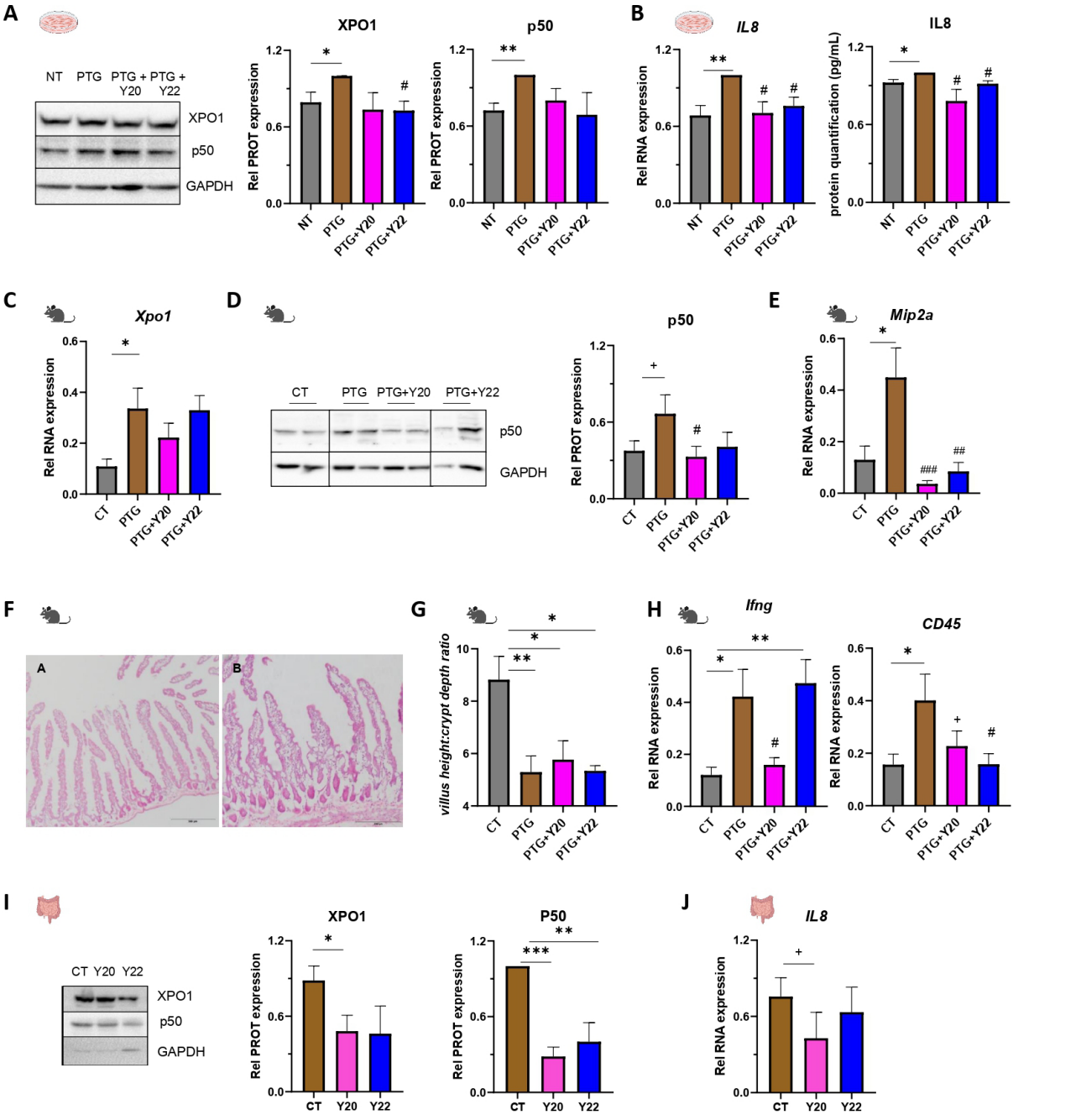

Figure 1 YTHDF1 inhibitors reduce gluten-induced intestinal inflammation. (A–B) HCT-116 intestinal cell line was left untreated (NT), treated with PTG or PTG and two different YTHDF1 inhibitors (PTG+Y20 and PTG+Y22). (A) XPO1 and p50 protein levels were quantified by western blot with GAPDH as loading control. (B) *IL8* RNA and protein levels were quantified by RT-qPCR using *RPLP0* as endogenous control and ELISA, respectively. n=4 (*p<0.05, **p<0.01 compared with control (NT), according to two-tailed Student's t-test; #p<0.05 compared with PTG according to two-tailed Student's t-test). (C–H) C57BL/6 mice on gluten-free diet were gavaged with PTG and cholera toxin (PTG) or together with a YTHDF1 inhibitor (PTG+Y20 and PTG+Y22) during 3 weeks, once a week. Control mice received only cholera toxin (CT). (C) *Xpo1* RNA levels were quantified by RT-qPCR using *Rplp0* as endogenous control. n≥7 (*p<0.05 according to one-tailed Student's t-test). (D) p50 protein levels were quantified by western blot using GAPDH as loading control. n≥3. (+p<0.09 compared with control CT mice, according to one-tailed Student's t-test; #p<0.05 compared with PTG mice, according to one-tailed Student's t-test). (E) *IL8* murine homolog *Mip2a* RNA levels were quantified by RT-qPCR using *Rplp0* as endogenous control. n≥7 (*p<0.05 compared with control CT mice, according to one-tailed Student's t-test; ##p<0.01, ###p<0.001 compared with PTG mice, according to one-tailed Student's t-test). (F) H&E staining of small intestinal sections from CT (A) and PTG treated mice (B). G) Villus height to crypt depth ratio to evaluate effects of gluten and YTHDF1 inhibitors on small intestinal epithelium morphometrics and for the histologic quantification of intestinal responses to disease process. n≥4 (*p<0.05, **p<0.01 according to one-tailed Student's t-test). (H) *Ifng* and *CD45* RNA levels were quantified by RT-qPCR using *Rplp0* as endogenous control. n≥7 (*p<0.05, **p<0.01 compared with control CT mice, according to one-tailed Student's t-test; +p<0.9, #p<0.05 compared with PTG mice, according to one-tailed student's t-test). (I–J) human intestinal biopsies from patients with CD at diagnosis were incubated with or without YTHDF1 inhibitors Y20 and Y22 for 24 hours. (I) XPO1 and p50 protein levels were quantified by western blot with GAPDH as loading control. (J) *IL8* RNA levels were quantified by RT-qPCR using *RPLP0* as endogenous control. n=5 (+p<0.09, *p<0.05, **p<0.01, ***p<0.001 compared with control (CT), according to one-tailed Student's t-test). All values are mean±SEM.

stimulation), both inhibitors also showed the ability to reduce inflammatory *IL8* chemokine levels (online supplemental figure 1G), showing that these small molecules could also be useful in other gluten-independent intestinal inflammatory conditions.

To sum up, here, we present two different selective YTHDF1 inhibitors that have the ability to reduce gluten-induced inflammation in intestinal cells without apparent side effects *in vivo*. Although further exploration of other conventional CD pathways is still needed, this study paves the way for the development of promising therapeutic strategies for intestinal inflammatory disorders as CD.

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Funding This study was supported by the Spanish Ministry of Science, Universities and Innovation (Grants PGC2018-097573-A-I00 to AC-R, and PID2020-114953RB-C21 to LH and DS cofunded by the European Regional Development Fund (ERDF)), the Biomedical Research Centre in Pathophysiology of Obesity and Nutrition (CIBEROBN) (Grant CB06/03/0001 to LH), the Merck Health Foundation (to LH), and the Government of Catalonia (2021SGR00367 to LH). CL was funded by National Administration of Traditional Chinese Medicine (ZYYCXTD-202004). CH is a Howard Hughes Medical Institute Investigator and has been funded by the National Institute of Health HG008935. AO-G was funded by postdoctoral fellowships from the University of the Basque Country (ESPDOC21/56). HR-M was funded by predoctoral grant from the Spanish Ministry of Science, Universities and Innovation (PRE2019-089350). Ciberehd is funded by the Instituto de Salud Carlos III.

Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and this study was approved by the Basque Country Ethics Committee CEIm-E with reference number PI2019133. Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/gutjnl-2023-330459).

AO-G and HR-M are joint first authors.

To cite Olazagoitia-Garmendia A, Rojas-Márquez H, Romero MdM, *et al*. *Gut* 2024;**73**:1590–1592.

Received 9 June 2023
Accepted 11 October 2023
Published Online First 31 October 2023

Gut 2024;**73**:1590–1592. doi:10.1136/gutjnl-2023-330459

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