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 adequate therapies.

Recently in Gut, we described a novel mA-XPO1-NFXb pathway that is activated in patients with CD. Specifically, YTHDF1 mA 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.3 These findings opened the door to new therapeutic approaches directed to mA 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 models4 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, NFXb 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, NFXb 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, NFXb 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 propria4 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 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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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<0.09, *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.
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Competing interests None declared.

Patient consent for publication Not applicable.

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REFERENCES
SUPPLEMENTARY MATERIALS FOR
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SUPPLEMENTARY MATERIAL AND METHODS

Human patients and samples

All newly diagnosed adult CD patients had elevated TGA titers and displayed characteristic small intestinal histopathologic abnormalities, including villous atrophy, crypt hyperplasia and intraepithelial lymphocytosis. The samples were obtained after informed consent and using a protocol approved by the Donostia University Hospital. The study was approved by the Basque Country Clinical Research Ethics Board (CElm-E ref. PI2019133) and analyses were performed after informed consent was obtained from all subjects. All experiments were performed in accordance with relevant guidelines and regulations. Biopsy specimens from the distal duodenum of each patient were obtained during routine diagnosis endoscopy. None of the patients suffered from any other concomitant immunological disease.

In vitro experiments

Intestinal HCT-116 (#91091005) cell line was purchased from Sigma-Aldrich (Poole, UK) and cultured in DMEM media (Lonza, Basel, Switzerland, #12-604F) supplemented with 10 % FBS (Millipore, Burlington, MA, USA #S0115), 100 units/ml penicillin and 100 μg/ml streptomycin (Lonza, #17-602E).

100,000 HCT-116 cells were plated and incubated o/n at 37°C. Next day cells were incubated with a low-dose of 30 μg/mL PT-Gliadin (PTG) or left untreated (NT) and incubated for 48 h. Then treatment with PTG at a final concentration of 350 μg/mL with or without SAC based YTHDF1 inhibitors (50 μM for Y20 and 20 μM for Y22) was performed. After 24 h supernatants and cells were harvested for further RNA and protein analysis.
**In vivo experiments**

Wild Type C57BL/6 JRj (Janvier Labs) mice were used for PTG stimulations. Breeders were maintained on GFD (Altromin #C1074) for at least 4 weeks, and experiments were performed using their progeny. 4 weeks after birth, mice were fasted overnight and then 500 μg PTG only, 500 μg PTG + 72 μM Y20 or 500 μg PTG + 30 μM Y22 was administered by oral gavage together with 25 ng Cholera toxin (CT) (Enzo Life Sciences, #BML-G117-000) to facilitate intestinal permeability. 25 ng CT were used for oral gavage in control mice. Each group of mice was housed in different cages to avoid PTG exposure through feces in control mice. Gavage treatments were done once a week for 3 weeks (F3.E) and mice were sacrificed 3 hours after the last gavage. Duodenum was dissected, perfused with sterile PBS to remove intestinal content and then sectioned and lysed for subsequent RNA and protein extraction.

**Organ culture experiments**

3 biopsy specimens from the same individual were incubated at 37°C in RPMI media (Gibco, #21875-34) supplemented with 10 % FBS (Millipore, #S0115), 4 mM L-Glutamine (Gibco, #25030-081), 1 mM sodium pyruvate (Lonza, #13-115E), 20 mM Hepes (Lonza, #17-737E), 100 U/mL penicillin/streptomycin (Lonza, #17-602E) and 0.1 U/mL bovine insulin (Sigma-Aldrich, #I0516) alone or with the addition of 50 μM Y20 or 20 μM Y22 inhibitors. After 24h media was collected and biopsies were flash frozen for further RNA and protein expression analysis.
Supplementary Figure 1. YTHDF1 inhibitors ameliorate inflammation with no toxicity in vivo. A) IL8 murine homologues Cxcl5 and Cxcl1 and B) Il21 RNA levels were quantified by RT-qPCR using Rlp/p0 as endogenous control. n≥7 (*p<0.05 compared to control CT mice, according to one-tailed Student's t test; +p<0.09, #p<0.05, compared to PTG mice, according to one-tailed Student's t test). C-F) The effect of gluten gavage and YTHDF1 inhibitors in mice was measured. C) Diet consumption and D) mice weight quantified in different time-points, both before (day 6) and after gavage treatments. n≥7. Values represent means per group. E) Feces weight before first treatment (day 6) and after gavage treatment (day 15). n≥7 (#p<0.05 compared to PTG after treatment according to two-way ANOVA test). F) Summary table of immune cell infiltration in lamina propria, goblet cells in epithelial tissue and eosinophils in lamina propria counts from hematoxylin-eosin staining of small intestinal sections from CT, PTG, PTG+Y20 and PTG+Y22 mice. G) HCT-116 intestinal cells were left untreated (NT), treated with interferon gamma (IFNG) or IFNG and the two different YTHDF1 inhibitors (IFNG+Y20 and IFNG+Y22). IL8 RNA and protein levels were quantified by RT-qPCR using RPLP0 as endogenous control and ELISA,
respectively. n=2-3 (*p<0.05 compared to control NT, according to one-tailed Student's t test; +p<0.09, ##p<0.01 compared to IFNG according to two-tailed Student’s t test).